INFLUENCE OF FORAGE LEVEL AND CORN GRAIN PROCESSING ON WHOLE-BODY UREA KINETICS, AND SEROSAL-TO-MUCOSAL UREA FLUX AND EXPRESSION OF UREA TRANSPORTERS AND AQUAPORINS IN THE OVINE RUMEN, DUODENUM, AND CECUM

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

in Partial Fulfillment of the Requirements

For the Degree of Master of Science

in the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon, SK, Canada

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Spring 2018

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ABSTRACT

The process of urea recycling to the gastro-intestinal tract (GIT) is an evolutionary adaptation that allows ruminants to maintain a positive nitrogen (N) balance, particularly when faced with a dietary protein deficit. Recycled urea-N can enter all GIT compartments; however, the rumen is where most of the anabolic usage occurs as it provides a N source for microbial protein synthesis, thus providing amino acids to the host animal when microbial protein is digested at the small intestine. The objective of this thesis research was to determine the effects of forage level and corn grain processing on N utilization, primarily focusing on dietary effects on whole-body urea kinetics and apparent N balance, and ex vivo serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) across ruminal, duodenal, and cecal epithelia. Additionally, to better understand the mechanisms associated with whole-body N utilization across the aforementioned GIT regions, dietary effects on regional fermentation characteristics and messenger ribonucleic acid (mRNA) expression of urea transporters (UT) and aquaporins (AQP) were also assessed. Sheep were used as an experimental model for ruminants. Thirty-two wether lambs (37.2 ± 1.7 kg initial body weight [BW]) were blocked by BW into groups of 4 and assigned to 1 of 4 diets (n = 8) in a 2 x 2 factorial design. Dietary factors were forage level (30% [LF] vs. 70% [HF]; DM basis) and corn grain processing (whole-shelled [WS] vs. steam-flaked [SF]). Provision of WS or SF corn was expected to alter the location of starch digestion in the ruminant GIT, with SF corn expected to shift starch digestion to the rumen, and WS corn expected to shift starch digestion to the intestine and hindgut. Four blocks of lambs (n = 4) were used for the in vivo metabolism trial to determine N balance and whole-body urea kinetics. Whole-body urea kinetics were determined using 4-d double-labelled urea ([$^{15}N^{15}N$]-urea) isotopic infusions, with concurrent total collections of urine and feces to determine isotopic enrichments and N balance. After 23 d of dietary adaptation, all lambs were killed (one per day for logistical reasons) on the morning of d 24 and ruminal, duodenal, and cecal epithelia were collected to determine $J_{sm\text{-}urea}$ (using the Ussing chamber model) and mRNA expression for UT and AQP. Lambs fed LF had greater DM (1.20 vs. 0.86 kg/d; $P < 0.01$) and N (20.1 vs 15.0 g/d; $P < 0.01$) intakes than those fed HF. Lambs fed SF corn had greater DM (1.20 vs. 0.86 kg/d; $P < 0.01$) and N (20.6 vs. 14.5 g/d; $P < 0.01$) intakes than those fed WS. When expressed as a percent of N intake, total N excretion was greater in lambs fed HF diets compared to those fed LF diets (103 vs. 63.0%; $P < 0.01$). Also, total N excretion (as a percent of N intake) was greater in lambs fed WS corn compared to those fed SF corn (93.6 vs. 72.1%; $P = 0.02$).

Apparent N balance, expressed as a percent of N intake, was greater in lambs fed LF diets compared to those fed HF diets (37.0 vs. -2.55%; $P < 0.01$). Similarly, lambs fed SF corn also exhibited a greater apparent N balance (28.0 vs. 6.50; $P = 0.02$) compared to lambs fed WS corn, when expressed as a percent of N intake. Endogenous urea production (UER) tended to be greater in lambs fed HF diets (17.6 vs. 14.3 g/d; $P < 0.10$) compared to those fed LF diets. Moreover, lambs fed HF diets also had greater urinary urea-N loss (as a proportion of UER) (0.38 vs. 0.22; $P < 0.01$), reduced amounts of urea-N returning to the GIT (as a proportion of UER) (0.62 vs. 0.78; $P < 0.01$), and overall, a reduced amount of urea-N allocated towards anabolism (as a proportion of urea-N GIT entry; GER) (0.12 vs. 0.26; $P < 0.01$) compared to those fed LF diets. Ruminal pH was similar in lambs fed SF and WS with HF diets, but it was lower in lambs fed SF compared to WS with LF diets (interaction, $P = 0.01$). Lambs fed LF had a lower cecal pH compared to those fed HF ($P < 0.01$). Ruminal $J_{sm\text{-}urea}$ was unaffected by diet. Duodenal $J_{sm\text{-}urea}$ was greater in lambs fed HF compared to LF (77.5 vs. 57.2 nmol/($cm^2 \times h$); $P < 0.01$). There were positive correlations between $J_{sm\text{-}urea}$ and serosal-to-mucosal mannitol flux ($J_{sm\text{-}mannitol}$) in duodenal ($r = 0.88$; $P < 0.01$) and cecal ($r = 0.93$; $P < 0.01$) epithelia. Lambs fed LF diets had increased mRNA expression of AQP-3 (1.21 vs. 0.90; $P = 0.03$) in ruminal epithelia and tended to have greater mRNA expression of AQP-3 (1.27 vs. 0.99; $P < 0.10$) in duodenal epithelia compared to lambs fed HF diets. Expression of UT-B mRNA in ruminal, duodenal, and cecal epithelia was largely unaffected by dietary treatment, except that cecal UT-B expression tended to be greater in lambs fed HF diets (0.95 vs. 0.71; $P < 0.10$) compared to lambs fed LF diets. The results presented in this thesis suggest that the provision of highly digestible diets improves N retention for anabolic usage and shifts urea-N excretion from the urine to the feces by enhancing urea recycling to the GIT. This shift in urea excretion can result in more stable forms of N losses, yielding an environmental benefit. Provision of LF diets also increased expression of AQP-3 in the ruminal and duodenal epithelia, providing insight into the molecular mechanisms associated with $J_{sm\text{-}urea}$ in both ruminal and post-ruminal regions.

ACKNOWLEDGEMENTS

To start the acknowledgements of my thesis, I would like to extend my sincere gratitude and thanks to my thesis advisor, Dr. Timothy Mutsvangwa. Tim, your encouragement to pursue a M.Sc. degree has provided me with an invaluable experience in academia, one that has given me many life-long skills. Without your guidance, patience, constructive criticism, and sense of humour, this project would not have been possible. Thank you for giving me the opportunity to make the most of my graduate degree.

I would also like to send a special thanks to Dr. Greg Penner, who contributed a great amount of time, effort, and resources towards my Ussing chamber experiment. By welcoming me as an honorary Team Rumen member, running my trial over the summer of 2016 went off without a hitch, and for that I am grateful. To my other committee members: Dr. John McKinnon, Dr. Bernard Laarveld, and Dr. Fiona Buchanan - thank you for your guidance, advice, and suggestions throughout my program. To Dr. Darren Korber – thank you for serving as my external examiner.

To my wonderful colleagues and friends: Eranga DeSeram, Saman Abeysekara, Gill Gratton, Jolet Köhler, Tonderai Chambwe, Audrey Makurumure, Danielle Skibinsky, Samantha Lundquist, and Sarah Thomas - you have all shown me what teamwork is really about, and without your steadfast work ethic and willingness to help, I don't think I would have seen my project through to the end. You are all incredible individuals. As well, a special shout-out to Koryn Hare, for being an amazing friend and barn mate – I wouldn't have survived this degree without you!

I would like to extend my gratitude to Natalia Rudnitskaya, for her assistance with laboratory training; to Coral Kent-Dennis, Kasia Burakowska, Jing Zhang (University of Guelph), and Dr. Mika Asai-Coakwell, for their help with gene expression analysis; and to Myles Stocki, for conducting my isotopic analysis. I would also like to recognize the staff at the LRB, Ken MacLeod and Dave Reimer, for their assistance in animal care during my trial, and Dr. Kamal Gabadage at the WCVM for provision of veterinary care to my lambs.

Last but not least, a huge thank-you to my parents, John & Erzsébet Scott, for their love and support, and for providing me with every opportunity to pursue my love of animals; to Matthew & Deanna Laity, for inspiring my love of the dairy industry; and to my wonderful partner, Colby Klein, who has helped me smile and laugh even through the toughest of times. You have all provided so much encouragement and love throughout this crazy journey!

I feel fortunate to have had this opportunity at the University of Saskatchewan. Saskatoon will *always* hold a special place in my heart, and I want to thank everyone who made my graduate program here the memorable one that it was.

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LIST OF ABBREVIATIONS

$[^{14}N^{15}N]$-urea = single-labelled urea

$[^{15}N^{15}N]$-urea = double-labelled urea

A:P = acetate-to-propionate

AA = amino acid

ADF = acid detergent fiber

AQP = aquaporin

AQP-10 = aquaporin-10

AQP-3 = aquaporin-3

AQP-7 = aquaporin-7

ATP = adenosine triphosphate

BUN = blood urea-N

BW = body weight

cDNA = complementary deoxyribonucleic acid

CO_2 = carbon dioxide

CP = crude protein

CPS1 = carbamoyl phosphate synthetase I

C_t = cycle threshold

ddH2O = double-distilled water

DM = dry matter

DMI = dry matter intake

DNA = deoxyribonucleic acid

EE = ether extract

F:C = forage-to-concentrate

GAPDH = glyceraldehyde-3 phosphate dehydrogenase

GC = gas chromatography

GER = gastro-intestinal entry rate of urea-N

GIT = gastro-intestinal tract

GPR = G-protein coupled receptors

GPR4 = G-protein coupled receptor 4

GPR41 = G-protein coupled receptor 41

GPR43 = G-protein coupled receptor 43

G_t = tissue conductance

HCO_3^- = bicarbonate

HF = high forage

HPRT1 = hypoxanthine-guanine phosphoribosyltransferase

IRMS = isotope ratio-mass spectrometry

I_{sc} = short-circuit current

$J_{sm\text{-}mannitol}$ = serosal-to-mucosal mannitol flux

$J_{sm\text{-}urea}$ = serosal-to-mucosal urea flux

LF = low forage

LRB = livestock research building

MDV = mesenteric-drained viscera

mRNA = messenger ribonucleic acid

N = nitrogen

N_2O = nitrous oxide

NDF = neutral detergent fiber

NFC = non-fiber carbohydrates

NH_3 = ammonia

NH_3-N = ammonia nitrogen

NH_4^+ = ammonium

$NiCl_2$ = nickel chloride

NO = nitric oxide

NO_2^- = nitrite

NO_3^- = nitrate

NPN = non-protein nitrogen

OM = organic matter

PDV = portal-drained viscera

PUN = plasma urea-N

qPCR = quantitative polymerase chain reaction

RDP = ruminally-degradable protein

RDS = ruminally-degradable starch

RFC = ruminally-fermentable carbohydrates

Rn = reporter signal

RNA = ribonucleic acid

ROC = urea-N return to ornithine cycle

RPLP0 = 60S acidic ribosomal protein P0

R_t = tissue resistance

R_{te} = transepithelial tissue resistance

RUP = ruminally-undegradable protein

SCFA = short-chain fatty acid

SF = steam-flaked

TMR = total mixed ration

UER = endogenous urea-N production

UFE = fecal urea-N excretion

UT = urea transporter

UT-A = urea transporter-A

UT-B = urea transporter-B

UUA = urea-N utilized for anabolism

UUE = urinary urea-N excretion

V-A = venous-arterio

V_{te} = transepithelial voltage

WS = whole-shelled

1.0 GENERAL INTRODUCTION

Urea recycling in ruminants is an evolutionary adaptation that allows the animal to remain in a positive nitrogen (N) balance when faced with a dietary protein deficiency. Urea-N that is secreted from the bloodstream into the rumen provides N for microbial growth, thus contributing amino acids to the host animal when ruminal microorganisms flow out of the rumen and are then digested in the small intestine (Lapierre and Lobley, 2001). Perusal of the literature indicates that, under a wide range of dietary conditions, endogenously produced urea can account for 87.0 to 141.7% of digestible N intake (Lapierre and Lobley, 2001; Lobley et al., 2000). If there was no mechanism to recover some of this endogenously-produced urea and it was all lost through urinary excretion, then ruminants would have difficulty maintaining a positive N balance under a wide variety of dietary conditions. Recycling of urea to the gastro-intestinal tract (GIT) is a salvage mechanism for urea, and published studies indicate that 61.0 to 79.0% of endogenously-produced urea is recycled to the GIT (Lobley et al., 2000; Archibeque et al., 2001). Urea-N that is lost through the urine can have negative environmental consequences. Urinary urea-N is rapidly degraded to ammonia (deionized, NH_3; ionized [ammonium], NH_4^+) by urease enzymes that are ubiquitous in the environment, with the resulting NH_3 being transformed by nitrifying and denitrifying microorganisms (Dijkstra et al., 2011). Potential products from the metabolism of NH_3 by these microorganisms include nitrite (NO_2^-), nitrate (NO_3^-), nitric oxide (NO), and nitrous oxide (N_2O) (Dijkstra et al., 2011). Though some of these compounds may be beneficial for plant uptake and growth (NH_4^+, NO_3^-), many of them contribute to chemical leaching into groundwater (NO_2^-, NO_3^-) and to greenhouse gaseous losses into the atmosphere (N_2O, NO, N_2). In contrast to the urine, most N losses in the feces are in the form of organically-bound N compounds, such as undigested feed protein, microbial protein, and endogenous losses. Because of this, mineralization processes that free organic-N from fecal material and convert it to inorganic NH_4^+ help in providing a stable form of N that is available for plant uptake. Knowing the impacts that excreted urea-N has on the environment, a thorough understanding of the urea salvage mechanism in ruminants is critical to promote not only efficient production, but also to maintain sustainable agricultural ecosystems.

From an anabolic perspective, endogenously produced urea is of most benefit to the ruminant when it is recycled to the rumen, either through the saliva or the blood. Epimural bacteria

that are closely attached to the ruminal wall will rapidly hydrolyze urea as it is transferred from the bloodstream into the rumen, releasing free NH_3 (also referred to as ammonia nitrogen, NH_3-N), and carbon dioxide (CO_2). Free NH_3 in the rumen can then be utilized by the microbial population for synthesis of microbial protein, which will later contribute to the ruminant's metabolizable protein supply during post-ruminal digestion and absorption (Reynolds and Kristensen, 2008). Aside from the rumen, it is also known that endogenous urea can be recycled to the various post-ruminal regions of the GIT, including the intestines and hindgut. Much like the rumen, ureolytic bacteria present in these regions will hydrolyze urea to release free NH_3, which can be sequestered by the microbial communities; however, most of the microbial protein produced will not be of anabolic benefit to the animal (Lapierre and Lobley, 2001). Instead, urea-N recycled to post-ruminal regions is typically excreted in the feces as organic microbial-N (Thornton et al., 1970), which, as mentioned previously, is far less detrimental to the environment compared to urea-N losses in the urine. For both entry into ruminal and post-ruminal sites, dietary factors play a critical role in governing the magnitude of endogenous urea-N entry into the GIT, including dietary intake (Black and Griffiths, 1975; Sarraseca et al., 1998), fermentable carbohydrate content (Oncuer et al., 1990; Walpole et al., 2015), and ruminally-degradable protein (RDP) content (Siddons et al., 1985; Davies et al., 2013). Though many of these factors have been studied extensively in the rumen, less research has been dedicated to the post-ruminal regions of the GIT.

The mechanisms of movement by which urea is transferred across the GIT epithelium from the serosal to the mucosal side has long been acknowledged to take place via passive diffusion (Houpt and Houpt, 1968). Having already identified the role carrier-mediated transport plays in the movement of urea within the renal system (Sands et al., 1997; Rojek et al., 2008; Starke et al., 2012), researchers began to discover and assess the role that facilitative transport has in the movement of urea into the GIT. It is currently known that urea transporter-B (UT-B) and various isoforms of aquaporins (AQP) are associated with carrier-mediated transport of urea across the ruminal epithelium (Walpole et al., 2015). Interestingly, these transporters have shown that their function can be manipulated by certain dietary parameters, namely fermentative end-products, and changes in pH. Utilizing the ex vivo Ussing chamber method, Abdoun et al. (2010) found that the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) across ruminal epithelium was increased when the tissue was exposed to short-chain fatty acids (SCFA), CO_2 and a pH of 6.4. In a later study, Lu et al. (2015)

assessed the influence of SCFA and reduced pH levels on UT messenger ribonucleic acid (mRNA) expression and protein abundance in cultured goat ruminal epithelial cells and found that both mRNA expression and protein abundance of UT-B and G-protein coupled receptors (GPR) increased when exposed to fermentative end-products and reduced pH. Aquaporins have been shown to respond to in vivo dietary treatments when Walpole et al. (2015) fed Holstein steer calves a forage-based control diet vs. a 50:50 forage-to-concentrate (F:C) diet. The study reported that as the amount of time the animals were fed the 50:50 diet increased, so did their mRNA expression of individual isoforms of AQP (Walpole et al., 2015). These data provide strong evidence that urea recycling through facilitative transporters can be altered by dietary energy levels and end-products associated with fermentative substrates.

In terms of hindgut expression, Ludden et al. (2009) confirmed the expression of UT-B in the cecum and colon of sheep; however, the current literature does not report any studies that have confirmed the expression of AQP mRNA in post-ruminal regions. Moreover, despite earlier studies having assessed the effects of hindgut infusions of fermentative substrate on urea recycling and N excretion (Ørskov et al., 1970; Thornton et al., 1970; Oncuer et al., 1990), no studies in the literature appear to have assessed the direct dietary effects of forage level and corn grain processing on whole-body urea kinetics, ex vivo $J_{sm\text{-}urea}$, and UT and AQP expression throughout the ruminant GIT.

Knowing that urea recycling to the hindgut plays a major role in influencing the form of urea-N lost to the environment, and that fermentative end-products influence facilitative transporters responsible for the movement of that urea, it is of the utmost importance to uncover and understand the facilitative transport mechanisms associated with the movement of urea across the GIT epithelia. Therefore, this thesis will provide new, basic research on the effects of forage level and corn grain processing on whole-body urea kinetics, ex vivo urea flux, and expression of UT and AQP throughout the ovine GIT.

2.0 LITERATURE REVIEW

2.1 Nitrogen Metabolism

Nitrogen metabolism in ruminants is a fine-tuned, complex process that includes N sources from dietary, recycled, and endogenous supply (Bach et al., 2005). Nitrogen sourced from dietary protein can be both RDP and ruminally-undegradable protein (RUP), with the former also including sources of non-protein N (NPN) (Bach et al., 2005). Most N metabolism is governed by the proteolytic activity that takes place by the microbiota present within the rumen, producing small peptides, amino acids (AA), NH_3, and microbial protein (Storm and Ørskov, 1983). The ruminant's ability to utilize N through microbial sequestration stems from its unique digestive physiology, in particular, the aforementioned rumen.

2.1.1 Ruminant Digestive Physiology

2.1.1.1 The Forestomach

The ruminant's ability to utilize complex carbohydrates and N is due to pre-gastric fermentation that takes place in the rumen, which is the largest compartment of the ruminant forestomach that lies anterior to the small intestine (Stewart and Smith, 2005). Due to its anaerobic environment, the rumen is a host to a multitude of dense microbial communities, including bacteria, protozoa, fungi, archaea, and bacteriophages (Sherwood et al., 2013; Mountfort, 1987). Many of these microbial communities provide metabolizable substrates for the ruminant in exchange for its host environment, illustrating a symbiotic relationship. The microbial populations in the rumen specialize in the breakdown of complex carbohydrates (hemicellulose, cellulose, lignin) into absorbable SCFA that are the major source of energy for the host animal (Stewart and Smith, 2005; Flint et al., 2008). The breakdown of these complex carbohydrates is performed by epimural, fluid-associated, and feed-associated bacteria, with about 75% of carbohydrate digestion attributed to feed-associated bacteria (Sherwood et al., 2013). Acetate, propionate, and butyrate are the three major SCFA produced within the rumen, and can be directly utilized as an energy source by absorption through the ruminal epithelium and subsequent entry into the Kreb's cycle as acetyl-CoA. Propionate acts as a precursor to glucose synthesis, providing upwards of 70% of the ruminant's glucose and glycogen supply (Sherwood et al., 2013). Through production of SCFA by anaerobic fermentation, adenosine triphosphate (ATP) is produced, supplying an energy source that facilitates microbial sequestration and utilization of N (both dietary and endogenous sources)

(Kennedy and Milligan, 1980; Huntington, 1989). The utilization of N in the rumen provides microbial protein to the ruminant and is a major contributor of AA supply to the small intestine. Depending on dietary conditions, microbial protein can account for 50 to 80% of absorbable protein entering the duodenum, providing a significant contribution to metabolizable protein requirements (Storm and Ørskov, 1983).

The two other components of the pre-gastric forestomach include the reticulum and the omasum. The function of the reticulum closely resembles that of the rumen, in that it is a anaerobic compartment responsible for hosting microbial communities, digestion and fermentation of nutritive substrates, and absorption (Sherwood et al., 2013). The omasum receives digesta from the reticulum via the omasal orifice, and functions to absorb water and nutrients, as well as to transfer digesta into the abomasum via the omasoabomasal orifice (Sherwood et al., 2013). The omasum's specialized function of absorption can be attributed to its compartmentalized nature; various laminae lined with small papillae increase surface area of the epithelium, allowing for significant absorptive capacity (Church, 1988).

2.1.1.2 The Abomasum and Small Intestine

Proceeding the forestomach in ruminants, the GIT structure and function are similar to that of a monogastric animal. The abomasum is the true, glandular-type stomach responsible for acid hydrolysis and digestion of dietary protein and ruminal microbes via secretion of gastric juices, and for transferring digesta from the abomasum into the small intestine (Sherwood et al., 2013). The small intestine can be broken down into three main segments: the duodenum, the jejunum, and ileum. The main function associated to these regions are digestion and absorption. Digestion of proteins, carbohydrates, and lipids is facilitated by secretions from the exocrine pancreas and liver (Sherwood et al., 2013). Then, absorption of the resulting AA, monosaccharides, and triglycerides is enabled by the presence of folds, villi, microvilli, and specialized transport mechanisms along the intestinal epithelium (Sherwood et al., 2013).

2.1.1.3 The Hindgut

The cecum and colon, collectively referred to as the hindgut, is the region of the GIT in ruminants that lies aboral to the four-chambered stomach. The cecum is a blind sac located at the junction between the last segment of the small intestine (ileum) and the beginning of the large intestine (Sherwood et al., 2013). In short, the primary function of the cecum is fermentation of

carbohydrates that escaped gastric digestion (Gressley et al., 2011). In ruminants, a distinct anatomical marker dividing the cecum and colon is lacking. Because of this, similar fermentative profiles of acetate, propionate, and butyrate exist between the two regions (Elsden et al., 1946; McNeil, 1988). Like the rumen, the cecum plays a role in the absorption of these SCFA, particularly when cecal pH is reduced (Myers et al., 1967); however, the cecum has a reduced ability to regulate and maintain digesta pH due to differences in its physiological buffering capacity compared to the rumen (Gressley et al., 2011). These differences include lack of salivary bicarbonate (HCO_3^-) influx, and lack of protozoal populations (which, in the rumen, reduce the rate of fermentation by sequester fermentable carbohydrates) (Gressley et al., 2011). Additionally, differences in gut epithelia type (the cecum lacks the protective stratum corneum of the rumen) may predispose cecal epithelium to damage induced by the presence of organic acids (Gressley et al., 2011). With the reduced ability to regulate digesta pH and a lack of protective barrier, the cecum can be deemed as having a sensitive mucosal layer compared to its forestomach counterparts (Gressley et al., 2011).

The colon takes up the majority of an animal's large intestine and, in ruminants, is an elongated, coiled structure (Sherwood et al., 2013). The main function of the colon is to extract water and minerals from colonic contents, leaving feces to be excreted. Additionally, the colon also serves as the last fermentative region of the ruminant GIT, producing SCFA that are available for absorption to the animal, and that assist in reducing the growth of pH-sensitive pathogenic bacterial populations (Sherwood et al., 2013).

Within the hindgut, microbial communities make use of available N to synthesize microbial protein just as they do in the rumen; however, due to a lack of mechanism for the microbial protein to be digested, the majority will be excreted in the feces (Lapierre and Lobley, 2001). Because of this loss, microbial protein synthesized in the hindgut has negligible anabolic value to the ruminant (Lapierre and Lobley, 2001). A more extensive discussion of this area is provided in Section 2.2.1.2.

2.1.2 Partitioning of Dietary Nitrogen Sources in the Rumen

Nitrogen provided to the ruminant is classified as being from either dietary, recycled, or endogenous sources (Bach et al., 2005). Dietary protein is categorized into RDP and RUP, with RDP consisting of both NPN (e.g., recycled urea-N) as well as true dietary protein (Bach et al.,

2005). Through proteolytic microbial activity, RDP is broken down into peptides, AA, and free NH_3 (see section 2.1.3.1), which are the precursors for microbial protein synthesis. Non-protein nitrogen consists of N sources from dietary NO_3^-, exogenous and endogenous urea, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), NH_3-N, and AA, with the N harvested from AA and NH_3-N contributing towards microbial growth and protein synthesis (Bach et al., 2005). Recycled N sources are derived from endogenous urea produced by the liver via the ornithine cycle, which can enter the ruminant GIT by means of saliva or by movement across the ruminal epithelium from the blood (Reynolds and Kristensen, 2008; Lapierre and Lobley, 2001). When urea is transported across the ruminal epithelium, epimural ureolytic bacteria associated with the rumen wall degrade urea via urease activity, thus releasing free NH_3-N that is available to the microbes for microbial protein synthesis (Reynolds and Kristensen, 2008). Similarly, urea introduced into the rumen via saliva will also face microbial hydrolysis (Abdoun et al., 2006). Additionally, endogenous N (derived from protein secretions and epithelial sloughing) can provide N inflow into the GIT (Lapierre and Lobley, 2001). Of course, the relative contribution of endogenous N to microbial protein synthesis or to the metabolizable protein requirement depends on where in the GIT the endogenous N is sourced from. For example, small contributions of endogenous N flow into the duodenum from the forestomachs (Siddons et al., 1982); however, 50-75% of protein reaching the terminal ileum can be traced back to endogenous duodenal contributions (Van Bruchem et al., 1989; Van Bruchem et al., 1997), from sources including pancreatic digestive juices, bile, mucus glycoproteins, lymph, and intestinal mucosal slough.

2.1.3 Degradation and Utilization of Nitrogen in the Rumen

2.1.3.1 Mechanisms of Rumen Protein Degradation

Nitrogen metabolism in the rumen is performed by the microbial communities present, which exhibit a symbiotic relationship with their host by, among other things, provision of microbial protein to the animal (Bach et al., 2005). Protein metabolism begins by the proteolytic microbiota attaching to the free-floating feed particles within the rumen, as shown in Figure 2.1. After attachment, extracellular microbial proteases act on the protein fraction of the feed particle to release products including peptides and AA (Brock et al., 1982; Bach et al., 2005). Due to the complex nature of varying bonds within a protein molecule, synergistic action of several proteases is often required to completely degrade the protein molecule to peptides and AA (Wallace at al.,

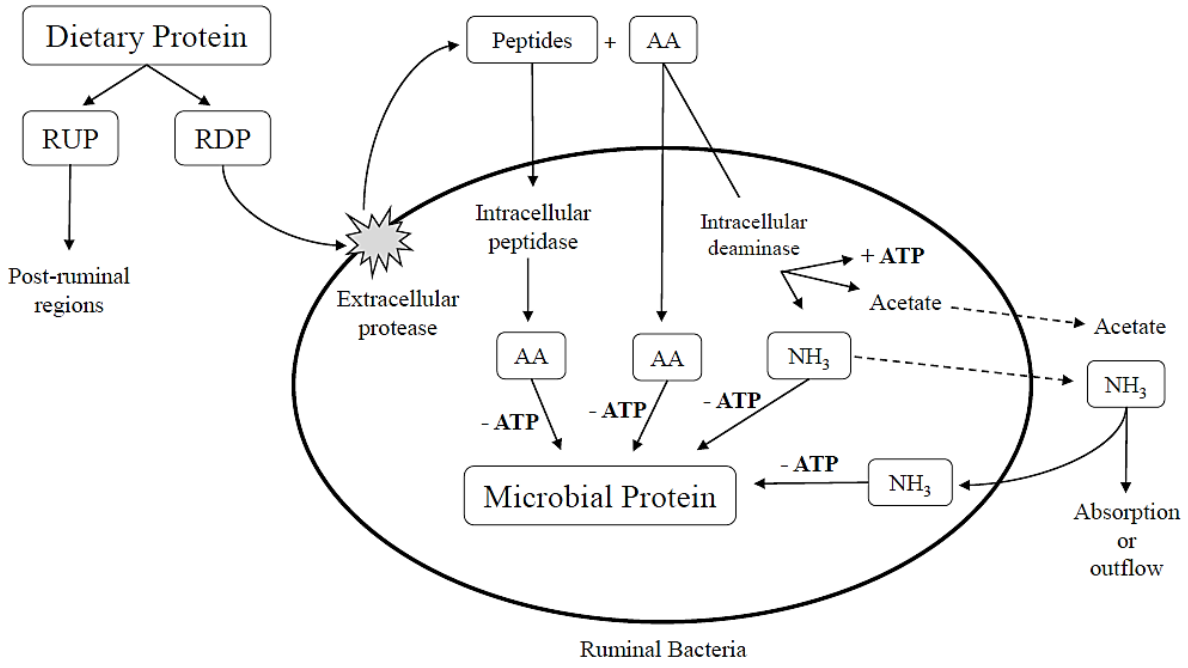

Figure 2.1. Illustration of the mechanism of proteolytic action by ruminal bacteria. Adapted from Bach et al. (2005).

1997). The peptides and AA resulting from protein degradation are absorbed into the microbial cells, where they are subjected to various fates (Bach et al., 2005). The fates of these absorbed peptides and AA are dependent on energy availability from ruminally-fermentable carbohydrates (RFC). If energy availability for microbial growth is not limiting, absorbed AA and peptides can be directly incorporated into microbial protein (Bach et al., 2005). However, if energy availability is limiting, absorbed AA and peptides are further degraded by microbial peptidases into NH_3 which, in turn, becomes the major source of N for microbial protein synthesis (Bach et al., 2005).

Aside from bacteria, protozoa also play a role in N metabolism in the rumen. Protozoa are predatory in nature, engulfing not only large molecules such as proteins and carbohydrates, but also ruminal microbes (Van Soest, 1994; Bach et al., 2005). Despite their digestion of proteins and ruminal bacteria, protozoa are responsible for only a small fraction of microbial protein flow into the small intestine. Punia et al. (1992) conducted a study assessing the flow of protozoal N to the duodenum of sheep and cattle, and found that despite protozoal N contributing upwards of 48% of total N in the rumen, only 11-20% of total N flow from the abomasum into the duodenum was of protozoal origin. This can be attributed to factors such as increased residence time in the rumen and longer generation intervals (Jouany, 1996; Bach et al., 2005). Additionally, with their high level of proteolytic activity and inability to utilize NH_3-N for protein synthesis, protozoa greatly elevate free ruminal NH_3-N concentrations (Jouany, 1996). Because the absorption of NH_3-N from the rumen into the portal blood is a concentration-dependent process (Rémond et al., 1993; Abdoun et al., 2006), the elevated ruminal NH_3-N concentration in the presence of protozoa increase NH_3 absorption into portal blood, thus resulting in significant wastage of N via renal excretion of urea.

2.1.3.2 Utilization of Nitrogen for Microbial Protein Synthesis

As mentioned in the previous section, synthesis of microbial protein from N sources is an energy-dependent process (Bach et al., 2005). Typically, this energy is obtained through the fermentation of dietary fermentable carbohydrates, but can also be obtained through the fermentation of branched-chain AA. Branched-chain AA introduced into the rumen can potentially be fermented into branched-chain SCFA, in particular, isobutyric and isovaleric acids, which are essential for the growth of ruminal microbes (Bach et al., 2005; Allison and Bryant, 1963). Additionally, branched-chain AA are a preferred substrate for the growth of cellulolytic bacterial populations in the rumen (Allison et al., 1962). As reviewed by Bach et al. (2005), fermentation

of these branched-chain AA into branched-chain SCFA provide ATP for cellulolytic bacteria, resulting in increased fiber digestion, SCFA and energy production, and microbial protein synthesis. Moreover, the provision of branched-chain AA also provides a direct source of AA to be incorporated into microbial protein synthesis. When assessing the sources of N utilized by microbes to synthesize microbial protein, it has been reported that up to 66% of microbial protein synthesis is derived from N sourced from AA, with the remainder being sourced from NH_3-N (Russell et al., 1983).

Aside from energy and N sources, other factors will influence microbial N utilization within the rumen, including ruminal pH and dilution rates (Bach et al., 2005). Ruminal pH is altered predominantly by the fermentation of organic matter (OM), with highly fermentable diets reducing the pH of the rumen after feeding by release of protons, potentially resulting in acidosis (pH ranges of 5.5 – 5.8 for sub-acute ruminal acidosis, and 5.0 – 5.2 for acute ruminal acidosis) (Aschenbach et al., 2011; Penner et al., 2007). Insults in pH within these ranges have the potential to alter or reduce the growth of certain microbial populations within the rumen, resulting in changes to the type and amount of fermentative end-products produced, as well as microbial N utilization (Aschenbach et al., 2011). As described in a review paper by Bach et al. (2005), dilution rates within the rumen are attributed to factors such as feed intake (Merchen et al., 1986), F:C ratio (Rode and Satter, 1988), and particle size of the diet (Woodford and Murphy, 1988), and have an important role in influencing ruminal fermentation characteristics and microbial growth (Russell et al., 1992). Typically, increases in dilution rate are indicative of high passage rates, which reduces the amount of time that microbes have access to digestive substrate. As a result, microbial populations will have a reduced ability to produce SCFA and energy, leading to a consequential decrease in sequestration of N and microbial growth (Okine et al., 1994).

Lastly, nutrient synchrony strategies have been studied to determine microbial efficiency of utilizing N when presented with a synchronous fermentable energy supply. Energy and protein availability can be manipulated in the rumen by altering their dietary amounts and types (Lapierre and Lobley, 2001). In theory, the more synchronous the supply and rate of degradation of RDP and RFC, the more efficiently the microbial community is able to sequester N for the purpose of microbial protein synthesis (Cabrita et al., 2006). For example, Davies et al. (2013) found that

provision of RDP in a low crude protein (CP) diet in combination with RFC improved efficiency of microbial N production, resulting in increased microbial N supply to the duodenum.

2.1.4 Ammonia Absorption and the Ornithine Cycle

As previously discussed, ruminal NH_3 is derived from proteolytic degradation of dietary protein, endogenous N, as well as recycled N (Bach et al., 2005). This NH_3 acts as a N source for synthesis of microbial protein; however, not all NH_3 will be sequestered. Ammonia that is not incorporated into microbial protein leaves the rumen through absorption across the ruminal epithelium (42 – 66% of irreversible NH_3 loss out of the rumen) or by flowing with the fluid phase of ruminal digesta into the omasum (8% of irreversible NH_3 loss out of the rumen) (Siddons et al., 1985).

2.1.4.1 Factors Influencing Ruminal Ammonia Concentration and Absorption of Ammonia from the Rumen

Absorption of NH_3 from the rumen is a concentration-dependent process, completed by NH_3 passing through the ruminal epithelium via passive diffusion down its concentration gradient (Tan and Murphy, 2004). This process relies heavily on the concentration of NH_3 in the rumen: as levels of ruminal NH_3 increase, so does the net flux of NH_3 into the portal blood (Tan and Murphy, 2004). Because of this, it is important to consider the influence of the diet on NH_3 production in the rumen. Residence time in the rumen, amount of dietary protein fed, as well as protein solubility all play important roles in determining the amount of NH_3 present in the rumen (Tamminga, 1983). When feeding diets with varying CP concentrations in an oscillating manner in lambs, Doranalli et al. (2011) observed greater ruminal NH_3 concentrations when high CP diets were fed compared to low CP diets (5.94 vs. 3.65 mmol/L). Similarly, Marini and Van Amburgh (2003) detected a quadratic increase in ruminal NH_3 concentration in Holstein heifers fed diets containing 1.45, 1.89, 2.50, 2.97, and 3.40% N. Whilst assessing protein solubility, Davies et al. (2013) found that ruminal NH_3 concentrations were greater in beef heifers fed high RDP compared to those fed low RDP. Moreover, provision of rapidly fermentable carbohydrates will also alter ruminal NH_3 concentrations by means of microbial sequestration, as discussed in section 2.1.3.2. Knowing that microbial protein synthesis is an energy-dependent process, NH_3 absorption can be influenced by both the quantity, as well as the degradability of dietary carbohydrates (Reynolds and Kristensen, 2008). Bailey et al. (2012) provided supplemental energy to beef cattle by dosing 600 g of glucose

intraruminally once daily to determine the effects of non-structural carbohydrates on N utilization. As expected, the provision of additional energy in the rumen reduced NH_3 concentrations, supporting the notion of increased sequestration and utilization of N for microbial protein synthesis when more RFC is available.

2.1.4.2 Mechanisms of Ammonia Absorption

Depending on dietary conditions, significant amounts of NH_3 are absorbed across the ruminal epithelium and enter the portal blood (Figure 2.2). Ammonia in the rumen is present as both ionized (NH_4^+) and deionized (NH_3) forms (Reynolds and Kristensen, 2008). Whichever form predominates, however, relies on the surrounding pH (Abdoun et al., 2006; Reynolds and Kristensen, 2008). Different mechanisms are responsible for the transport of NH_3 across the ruminal epithelium depending on whether NH_3 is protonated or non-protonated. In its neutral state, deionized NH_3 can be absorbed by means of simple diffusion due to its lipophilic nature, whereas its ionized counterpart, NH_4^+ (lipophobic), relies exclusively on potassium channels (Bödeker and Kemkowski, 1996; Reynolds and Kristensen, 2008). It has been reported that between pH values of 6 and 7, almost all NH_3 is present in its ionized form (NH_4^+) (Abdoun et al., 2006). Because normal ruminal pH is typically below this given range on a wide variety of dietary conditions, the majority of NH_3 present in the rumen will also be in the ionized NH_4^+ form (Huntington and Archibeque, 1999; Abdoun et al., 2006; Reynolds and Kristensen, 2008). Moreover, Bödeker et al. (1992a) proposed that the form of NH_3 transported across the ruminal epithelium did not just depend on ruminal pH, but also the proton concentration above the luminal membrane of the mucosa. The studies conducted by Bödeker et al. (1992a, 1992b) suggested that availability of protons for the formation of NH_4^+ could be reduced by the hydrogen reacting with secreted HCO_3^- (i.e., $H^+ + HCO_3^- \leftrightarrow H_2O + CO_2$) or by reaction with de-protonated SCFA (i.e., $H^+ + SCFA^- \leftrightarrow$ HSCFA), resulting in an increase in deionized NH_3, thus facilitating a greater absorption of ammonia as NH_3 (Bödeker et al., 1992a; Bödeker et al., 1992b). Once inside the epithelial cell, deionized NH_3 has the potential to be protonated to NH_4^+ via the dissociation of HCO_3^- and HSCFA, thereby maintaining the NH_3 gradient across the ruminal epithelium, further stimulating absorption of deionized NH_3 (Bödeker et al., 1992a; Bödeker et al., 1992b; Abdoun et al., 2006). The mechanism for transport of NH_3 and NH_4^+ across the basolateral membrane is unknown, and requires further investigation (Abdoun et al., 2006).

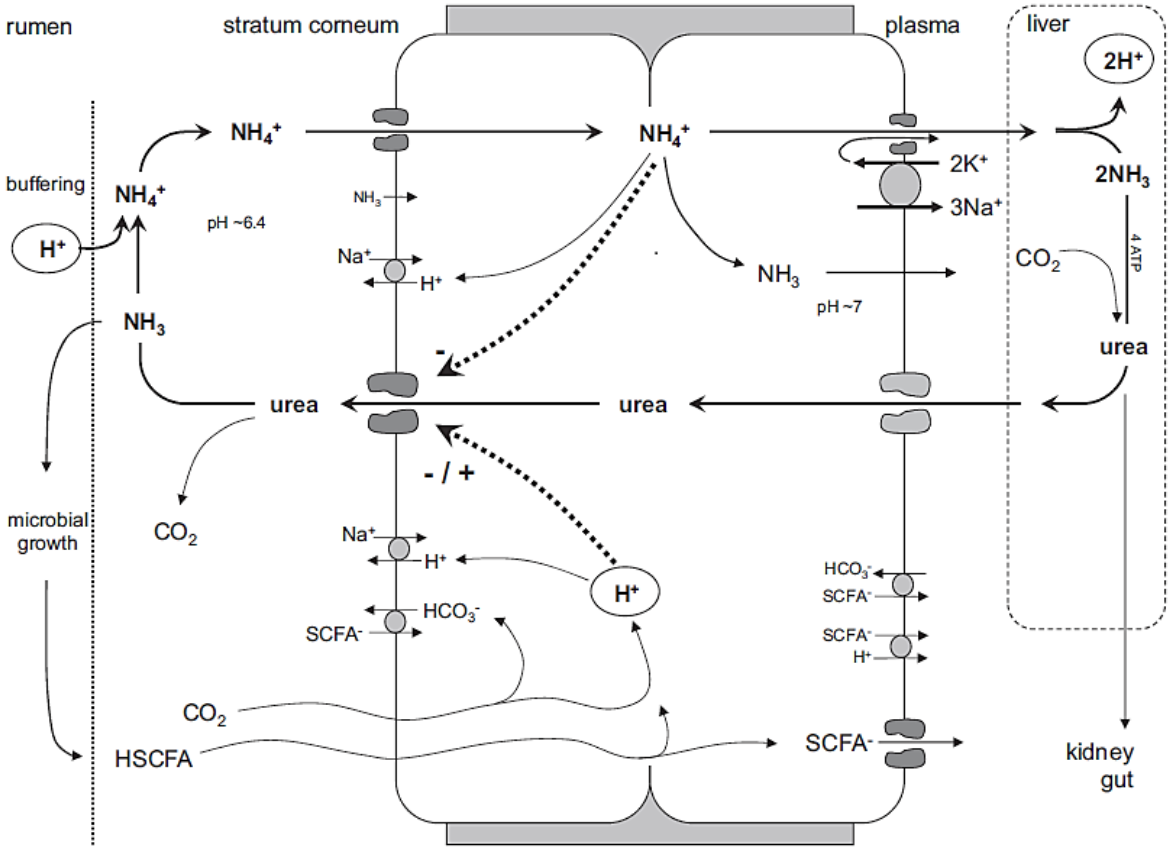

Figure 2.2. Illustration of the pathways for ammonia (NH_3/NH_4^+) and urea transport across the ruminal epithelium, and the interaction with short-chain fatty acids (SCFA) and bicarbonate (HCO_3^-). Sourced from Lu et al. (2014) with permission from the publisher.

2.1.4.3 The Ornithine Cycle

In order to prevent NH_3 toxicity, NH_3 absorbed from the GIT is transported via portal blood to the liver for detoxification into urea through the ornithine cycle (also referred to as the urea cycle, or ureagenesis; see Figure 2.3) (Tan and Murphy, 2004). In the first reaction of the ornithine cycle, NH_3 associates with HCO_3^- to form carbamoyl phosphate ($2ATP + NH_3 + HCO_3^- \rightarrow$ carbamoyl phosphate $+ 2ADP + P_i + H^+$) via carbamoyl phosphate synthetase I (CPS1), which takes place in the mitochondria of the hepatocyte (Visek, 1979; Meijer et al., 1990). This first reaction is considered the rate-limiting step of urea synthesis (Meijer et al., 1990). From here, the ornithine cycle consists of 4 enzymatic reactions required to convert toxic NH_3 into non-toxic urea, involving ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinase, and arginase (Visek, 1979). Once synthesized, urea may be directed towards urinary excretion via the kidneys or recycled to the GIT via blood or salivary routes (Lapierre and Lobley, 2001; Reynolds and Kristensen, 2008). Any NH_3 that escapes ureagenesis in periportal hepatocytes is used for the amidation of glutamate to form glutamine in perivenous hepatocytes, a reaction that is catalyzed by the enzyme glutamine synthetase (Häussinger et al., 1992). Glutamine synthesis in perivenous hepatocytes is a "high affinity, low capacity" system that ensures that toxic NH_3 does not escape into peripheral circulation (Häussinger et al., 1992). Glutamine is released into the bloodstream and, on subsequent passage of blood through the liver, it releases the NH_3 (through a reaction catalyzed by glutaminase) for use as a substrate in urea synthesis in periportal hepatocytes (Häussinger et al., 1992).

Like any metabolic pathway, the ornithine cycle has both short-term and long-term regulation to control its function. One of the most important short-term regulatory mechanisms controlling the ornithine cycle is the presence of mitochondrial N-acetylglutamate (Meijer et al., 1990; Tymockzo et al., 2011). N-acetylglutamate is synthesized from acetyl-CoA and glutamate, providing a biological indication that there is existing NH_3 to be detoxified (Tymockzo et al., 2011). The greater the concentration of N-acetylglutamate within the mitochondria, the more CPS1 is activated, producing increased levels of carbamoyl phosphate destined for ureagenesis (Meijer et al., 1990). In addition to this regulatory mechanism, availability of ornithine also plays a key role in governing the ornithine cycle in two ways: (1) ornithine is the required substrate for ornithine transcarbamylase (facilitating the second step of the ornithine cycle) and; (2) ornithine upregulates the action of CPS1 (Krebs et al., 1973).

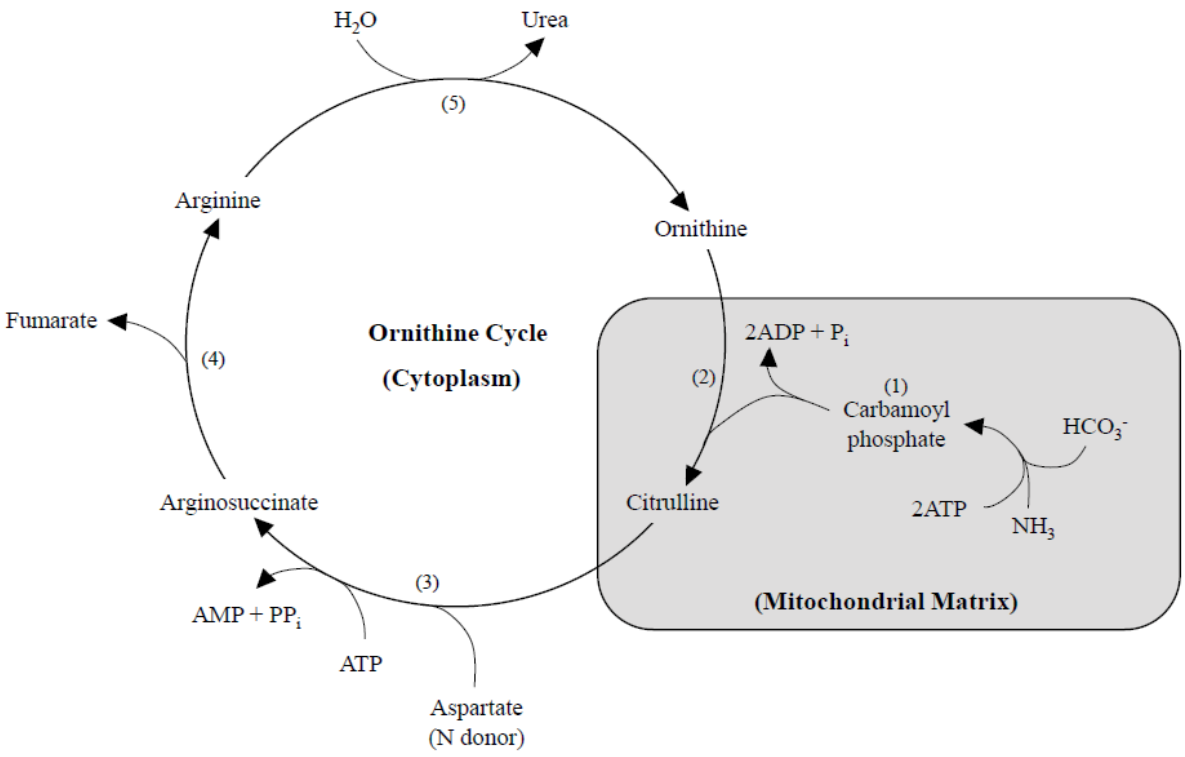

Figure 2.3. Illustration of the Ornithine Cycle (also referred to as the Urea Cycle or Ureagenesis). Adapted from Visek (1979) and Tymoczko et al. (2011). Enzymatic reactions are as follows: (1) carbamoyl phosphate synthetase I; (2) ornithine transcarbamylase; (3) arginosuccinate synthetase; (4) arginosuccinase; and (5) arginase.

In terms of long-term regulation, Meijer et al. (1990) explains that chronic alterations to AA catabolism in the body (e.g., consumption of diets high in protein [Freedland and Sodikoff, 1962], or during periods of starvation [Schimke, 1962]), as well as the presence of various hormones (e.g., glucagon, insulin, glucocorticoids; Morris, 2002) will have a direct effect on the presence and function of ornithine cycle enzymes.

2.1.5 Mechanisms of Renal Urea Excretion

In vertebrates, the kidneys are responsible for the concentration of urine as an excretory product, and in doing so, function to regulate organic solutes (e.g., ions involved in physiological acid-base balance), plasma volume, osmotic balance, as well as concentrate waste products with the intent of elimination (Sherwood et al., 2013). The functional unit of the kidney is the nephron, consisting of tubules and associated vasculature that complement the structure and function of one another (Sherwood et al., 2013). The first structure of the nephron encountered by the afferent arteriole (unfiltered blood) is the glomerulus, which is responsible for filtering protein-free filtrate into Bowman's capsule. From here, the filtrate undergoes a series of both controlled and uncontrolled reabsorption and secretion of select substances in order to produce urine of various concentrations (Sherwood et al., 2013). Due to the increasing concentration gradient between the nephron tubules and peritubular capillaries, urea is passively reabsorbed (paracellularly) throughout the proximal tubule, salvaging as much as 40% for recirculation to the body (Sherwood et al., 2013). Interestingly, urea permeability changes throughout the length of the nephron, at times relying more heavily on the presence of urea transporters (UT) to facilitate movement of the molecule. Urea transporters are carrier-mediated transporters expressed in kidney tissue that facilitate the concentration of urinary urea, as well as its recycling both within renal tissues and to the blood stream (Knepper and Françoise, 1987; Stewart et al., 2005; Sherwood et al., 2013). Many different variants of UT have been discovered in kidney tissue, including UT-A1, -A2, UT-B1, and -B2 (Sands et al., 1997). More information regarding urea recycling and transporters will be provided in the next section. Ultimately, urea that remains within the interstitial fluid of the renal medulla will be concentrated back into the tubular nephron (with final concentrations dependent on hormonal influence and homeostatic mechanisms), proceed to the ureters, bladder, and finally excreted through the external urethral orifice (Sherwood et al., 2013).

2.2 Urea Recycling

Urea-N salvaging is a distinct evolutionary adaptation that ruminants evolved to maintain a positive nitrogen balance in the face of dietary protein deficiency, or during asynchronous supply of energy and protein (Reynolds and Kristensen, 2008). This mechanism takes place through the recycling of blood urea nitrogen (BUN) to the lumen of the GIT, occurring predominantly at the reticulo-rumen epithelia (Lapierre and Lobley, 2001), but also occurring through the saliva, intestines, and hindgut (Lapierre and Lobley, 2001; Siddons et al., 1985). Available research indicates that urea-N recycled back to the GIT can range from 29 to 99% of endogenous urea output (Theurer et al., 2002; Marini and Van Amburgh, 2003; Wickersham et al., 2008). Approximately 3 to 10% of recycled urea is lost in feces, 26 to 50% is reabsorbed as NH_3, and the remaining 35 to 55% is used for anabolic purposes (Lapierre and Lobley, 2001). Factors such as diet composition, level of feed intake, ruminal environmental conditions, and productive state of the animal will influence the relative partitioning of recycled urea towards fecal loss, reabsorption as NH_3, or anabolic use (Huntington and Archibeque, 1999). By salvaging urea from its fate of excretion, urea is recycled to the GIT and degraded back into NH_3 by microbial urease activity, thus making it an important source of N for microbial protein synthesis (Reynolds and Kristensen, 2008). According to Virtanen (1966), the utilization of urea for microbial protein synthesis not only allows ruminants to conserve N and survive in less-than-optimum dietary conditions, but also continue to produce milk and meat at desirable levels. With the importance of urea recycling from an animal health, production, and economic standpoint, it is imperative to better understand the underlying mechanisms associated with urea recycling to further improve N efficiency in the ruminant.

2.2.1 Urea Entry into the GIT

2.2.1.1 Rumen

Urea recycling is of most nutritional benefit to the ruminant when it is recycled to the rumen where it can be used as a source of N for microbial protein synthesis, thus contributing AA to the host animal when ruminal microorganisms flow out of the rumen and are digested at the small intestine. As reviewed by Lapierre and Lobley (2001), urea-N is contributed to the forestomachs from both saliva and the blood, with values ranging from 15 to 100% (saliva) and 27 to 54% (blood) of total urea-N entry to the GIT. The amount of endogenous urea-N transferred to the rumen is dependent on multiple factors, including dietary N intake, protein degradability,

energy density, carbohydrate fermentability, particle size and salivary production, ruminal NH_3 concentrations, and blood urea concentrations (Kennedy and Milligan, 1980; Huntington, 1989; Reynolds and Kristensen, 2008). Select factors will be discussed in greater detail in section 2.2.2.

In order to determine the quantitative transfer of urea-N from the saliva to the rumen, the difference between splanchnic urea-N flux and urinary urea-N output is calculated using the data obtained from the venous-arterio (V-A) difference technique that involves strategic placement of catheters in the splanchnic vasculature (Huntington, 1989; Theurer et al., 2002). Past studies have assessed dietary impact on salivary urea-N contributions to the rumen, and have proposed that factors such as the level of feed intake and F:C ratio will influence salivary contribution to urea-N entering the rumen (Kennedy and Milligan, 1980; Huntington, 1989). For example, Huntington (1989) found that steers fed high concentrate diets had decreased salivary transfer of urea-N to the rumen compared to those fed high forage diets. Additionally, though not significant, Theurer et al. (2002) observed a grain processing effect wherein steers fed steam-flaked sorghum had reduced secretion of salivary urea-N to the rumen compared to those animals fed dry-rolled sorghum (2.0 vs. 13.3 g/d urea-N flux). The results of these studies can be presumably attributed to the reduction in mastication and, subsequently, reduced rumination that is observed in ruminants fed low forage, more heavily processed diets.

The primary "driver" for urea transfer across the ruminal epithelium from the blood is the naturally occurring diffusional gradient between the serosal and mucosal side of the rumen (Houpt and Houpt, 1968). This gradient is established and maintained by the intraruminal hydrolysis of urea entering the rumen by the action of bacterial urease, as well as the overall permeability of the epithelium (i.e. barrier function) (Houpt and Houpt, 1968). Hormones and second messengers are also thought to have an influence on urea flux via altering permeability of the ruminal epithelium, in particular, vasopressin (Houpt, 1970) and gastrin (Harrop and Phillipson, 1970; Rémond et al., 1993), though further studies are required to validate their respective mechanisms. Aside from diffusional transport, ruminal transfer of urea also relies on carrier-mediated transport mechanisms, namely UT (Marini and Van Amburgh, 2003) and AQP (Walpole et al., 2015). These transporters and their mechanisms will be discussed in more detail in a later section.

Aside from the rumen, it is known that endogenous urea-N produced by the liver does enter the hindgut of ruminants. By usage of the V-A method, measurements of urea transfer across the

splanchnic tissues can be obtained in order to differentiate endogenous urea-N contribution to the rumen vs. post-ruminal sites (Huntington, 1989; Seal and Parker, 1996). Studies by Huntington (1989) and Seal and Parker (1996) successfully measured urea transfer across the portal-drained viscera (PDV; ruminal and post-ruminal regions) and mesenteric-drained viscera (MDV; post-ruminal regions), but did not differentiate the regions within the post-ruminal sites. Recent literature on comparative urea-N entry to ruminal vs. differentiated post-ruminal sites is sparse.

2.2.1.2 Post-Ruminal Sites

Urea recycling in the hindgut of monogastric species has been studied throughout the literature (Mosenthin et al., 1992a; Mosenthin et al., 1992b; Collins et al., 2010). In a study conducted by Mosenthin et al. (1992b), the group aimed to validate the occurrence of endogenous urea secretion into the hindgut of gilts. They found that gilts infused with starch through ileal T-cannulas exhibited greater fecal N excretion (in the form of bacterial N) compared to those infused with water (Mosenthin et al., 1992b). The study concluded that the microbial N was sourced from ileal digesta, however, as opposed to luminally secreted endogenous urea-N synthesized by the liver. In contrast, Collins et al. (2010) studied urea flux across human ascending and descending colonic tissue using Ussing chambers and found both regions of the colon to be permeable to urea (by means of absorption and secretion). Collectively, both studies provide valuable data regarding endogenous urea transfer to the GIT, leading animal scientists to speculate the importance of urea recycling to the hindgut in ruminants.

In ruminants, it is currently known that endogenous urea-N can be secreted into the various compartments of the hindgut, including the lattermost region, the colon (Ritzhaupt et al., 1997). Urea recycling at post-ruminal sites of the GIT can account for up to 70% of urea-N entry into the GIT, although the majority will not go towards anabolic usage for the animal (Lapierre and Lobley, 2001). Microbial populations present within post-ruminal sites (i.e. intestine, cecum, colon) will sequester NH_3-N arising from the degradation of recycled urea-N and use it as a source of N for microbial protein synthesis (Lapierre and Lobley, 2001). Microbial protein that is produced in these post-ruminal regions, however, is excreted in feces as this is past gastric digestion and there is no mechanism to retrieve microbial AA (Siddons et al., 1985; Lapierre and Lobley, 2001). Although urea that is recycled to post-ruminal sites might not contribute to the metabolizable protein needs of the ruminant, it can have a major influence on the environmental impact of

intensive livestock operations. This is because urea-N that is recycled to the hindgut will primarily be excreted in feces as microbial N rather than as urinary urea-N, which can be lost into the environment through volatilization. Importantly, various dietary factors will influence the magnitude of endogenous urea-N entry into the hindgut, including dietary intake (Sarraseca et al., 1998), fermentable energy sources (Thornton et al., 1970; Oncuer et al., 1990), and protein content (Siddons et al., 1985). In terms of partitioning where urea-N enters the hindgut, Varady et al. (1979) found that the small intestine accounted for the majority of endogenous urea-N entry into post-ruminal tissues in sheep fed a hay and barley-based diet. Though conducted in monogastrics, a study by Mosenthin et al. (1992a) also determined that the small intestine was the location for the majority of endogenous urea-N entry into post-stomach regions in pigs, which was later confirmed by Columbus et al. (2014).

Transfer of urea to post-ruminal sites of the ruminant GIT is commonly accepted to take place through diffusional transport; however, recent research in ruminant nutrition has revealed the presence of carrier-mediated UT in the hindgut of sheep (Marini et al., 2004; Ludden et al., 2009). By validating the presence and functional role of these transporters, a greater insight will be gained into the role that the hindgut plays in endogenous urea-N entry.

2.2.1.3 Mechanisms of Urea Transport from the Blood to the GIT

2.2.1.3.1 Simple Diffusion

It is known that one of the major mechanisms for the transfer of endogenous urea-N from the blood into the rumen is passive diffusion (Houpt and Houpt, 1968). This, in part, is because of the chemical nature of urea, being a small, uncharged polar compound. Diffusional transport requires the presence of a concentration gradient and, in ruminants, that gradient is established by the activities of the microbial populations present in the GIT. In the rumen, for example, passive diffusion of urea across the epithelium is facilitated by urease activity of bacteria that are associated with the rumen wall (Rémond et al., 1993). Ureolytic action degrades urea into free NH_3 and CO_2, keeping a favorable gradient for urea to cross the ruminal epithelium. Because the amount of urea-N transported into the GIT by diffusion is highly variable, researchers have started to study the presence and function of carrier-mediated transport mechanisms associated with the movement of urea (Ritzhaupt et al., 1997; Abdoun et al., 2006; Ludden et al., 2009; Walpole et al., 2015).

2.2.1.3.2 Carrier-Mediated Transport

It is currently known that the transport of urea also depends on carrier-mediated UT – primarily UT-A (SLC14a2 gene) and UT-B (SLC14a1 gene) (Stewart et al., 2005) – that are expressed in the kidney and ruminal epithelium, respectively (Stewart et al., 2005; Ritzhaupt et al., 1997). An early study by Ritzhaupt et al. (1997) determined that urea transport across ruminal tissue was facilitated by carrier-mediated transport when the addition of 0.75 mmol/L of phloretin, a known inhibitor of UT function, reduced mucosal-to-serosal flux rates of ^{14}C-urea. These findings have since been supported by various published studies (Stewart and Smith, 2005; Doranalli et al., 2011; Walpole et al., 2015) assessing the functional roles of UT in ruminal tissue using Ussing chamber methodology, as well as presence of the transporters as assessed by protein abundance (Stewart et al., 2005; Ludden et al., 2009; Røjen et al., 2011) and quantitative polymerase chain reaction (qPCR) (Ludden et al., 2009; Doranalli and Mutsvangwa, 2010; Røjen et al., 2011; Lu et al., 2015; Walpole et al., 2015). Knowing that urea recycling occurs in the hindgut in both monogastrics and humans (Stewart and Smith, 2005), further research has begun to study the expression of these transporters localized to the hindgut. Collins et al. (2010) validated the presence of UT-B in human colonocytes and found that urea recycling via UT-B at this site contributed to the maintenance of normal colonic bacteria. Similarly, Ludden et al. (2009) detected the presence of UT-B in the cecum, large colon, and spiral colon of protein-supplemented sheep via immunoblotting techniques. These findings provide evidence that the expression of UT-B is not limited to the rumen.

Utilizing ex vivo Ussing chamber methodology, various laboratories have studied the effects of pH, fermentative end-products, and NH_3 on UT function and expression. Abdoun et al. (2010) and Lu et al. (2014) completed a series of experiments investigating the effects of SCFA and CO_2 or ruminal environment (ruminal pH) on ruminal epithelium permeability to urea and UT-B expression. Both studies confirmed that production of SCFA and CO_2 from RFC was accompanied by a decrease in pH (Abdoun et al., 2010; Lu et al., 2014), with Abdoun et al. (2010) noting a 4-fold increase in $J_{sm\text{-}urea}$ at a pH of 6.4 compared to 7.4 (see section 2.2.2.2.2 for further discussion). Moreover, the addition of phloretin reduced $J_{sm\text{-}urea}$ by 50%, validating the functional role of UT-B in urea transfer across the ruminal epithelium (Abdoun et al., 2010). Lu et al. (2015) also assessed the effects of SCFA and acidic pH on UT protein expression in ruminal epithelial cell cultures. Additionally, the presence of GPR4 (GPR41 and GPR43), which are speculated to

be sensitive to the presence of SCFA and other luminal conditions, were also evaluated (Lu et al., 2015). The G-proteins GPR41 and GPR43 (also known as free fatty acid receptors, FFAR3 and FFAR2) are thought to be responsible for mediating the regulatory effects that SCFA have on ruminal epithelial development (Wang et al., 2009). The study conducted by Lu et al. (2015) revealed that cultured ruminal epithelial cells from goats fed high levels of non-fiber carbohydrates exhibited greater levels of GP41 than those fed low levels of non-fiber carbohydrates, and that the expression of GPR41 was positively correlated with that of UT-B (Lu et al., 2015). Based on these results, Lu et al. (2015) hypothesized that these G-coupled proteins were key players in signalling the induction of UT-B expression, and that they were influenced by fermentative end-products. Similarly, both studies by Lu et al. (2014, 2015) noted the inhibitory effects of NH_3 on urea transport and corresponding UT-B gene expression, particularly at pH values of 6.4. Though the mechanism is not fully understood, it is speculated that when intracellular concentrations of NH_4^+ increase, there is direct competition with urea for access to the UT, thereby decreasing $J_{sm\text{-}urea}$ (Lu et al., 2014).

The functional role of UT in urea transfer across the ruminal epithelium has now been established using inhibitors such as phloretin in Ussing chamber models (Abdoun et al., 2010; Doranalli et al., 2011); however, these studies have also demonstrated that the addition of phloretin does not completely inhibit urea transfer, suggesting that other urea transport mechanisms could also be involved. A potential candidate for alternative urea transport mechanisms are AQP, which are trans-membrane proteins that are primarily associated with the movement of water in various tissues (Ma and Verkman, 1999). A sub-group of these membrane-spanning proteins, referred to as aquaglyceroporins (primarily AQP-3, -7, -9, and -10), have been found in various locations of the GIT in non-ruminants and are known to be permeable to urea (Rojek et al., 2008). In ruminants, there has also been evidence supporting the presence of AQP in the GIT. Røjen et al. (2011) conducted a study to test the effects of dietary N concentration on UT-B and AQP expression in bovine ruminal papillae. The study confirmed the presence of UT-B and AQP-3, -7, and -8 in the rumen; however, contrary to expectations, the expression of AQP-3, -7 and -10 did not increase when exposed to low dietary N concentrations (Røjen et al., 2011). Expression of UT-B was reduced in animals fed low dietary N concentrations (12.9 % CP), whereas expression of AQP were greater in cows fed high levels of dietary N (17.1% CP) (Røjen et al., 2011). These findings led Røjen et al. (2011) to conclude that increased ruminal epithelial urea permeability commonly

observed in cattle fed low N diets was not due to the function of AQP, and moreover, that regulation of AQP expression could be affected by factors other than dietary N content. Walpole et al. (2015) conducted the first study to examine the functional role of AQP in ruminal urea transfer. The study's objectives were to determine the functional roles of AQP in $J_{sm\text{-}urea}$ across the ruminal epithelium, and to determine the effects of dietary fermentable carbohydrates on expression of AQP. Twenty-five Holstein steer calves were assigned to either a 91.5% forage-based control diet, or a 50:50 F:C ratio diet (41.5% barley grain). The two diets were fed for either 3, 7, 14, or 21 d in order to determine a dietary adaptation response. To assess the functional role of AQP in $J_{sm\text{-}urea}$ using Ussing chambers, 1 mmol/L of nickel chloride ($NiCl_2$) was added to inhibit AQP-mediated urea transport. In this experiment, Walpole et al. (2015) found that AQP played a definitive role in the movement of urea from the serosal side of the ruminal epithelium to the mucosal side. This was validated by the addition of $NiCl_2$ reducing $J_{sm\text{-}urea}$ by 23% (Walpole et al., 2015). Walpole et al. (2015) also found that gene transcript abundance of AQP-3 in ruminal epithelium was positively correlated to the level of RFC present in the diet. The study also observed a positive relationship in the expression of AQP-3 and the amount of time the 50:50 F:C diets were fed to growing steers (Walpole et al., 2015). From these findings, it can be concluded that diet plays an important role in regulating urea transport mechanisms through AQP.

2.2.2 Regulation of Urea Recycling

As previously mentioned, urea recycling is influenced by many factors, including dietary (e.g., intake, protein degradability, carbohydrate fermentability) and physiological (e.g. fermentative end-products, NH_3 concentration, endogenous urea production) factors alike. In terms of dietary factors, many studies have assessed in vivo dietary effects on N recycling (Walpole et al., 2015; Davies et al., 2013; Doranalli et al., 2011; Marini et al., 2004). Currently, the results strongly support that N and energy are the two most important dietary factors governing the amount of urea salvaging that takes place within the ruminant animal (Reynolds and Kristensen, 2008).

2.2.2.1 Dietary Protein and Nitrogen Supply

Dietary N levels, as well as intake, have an important role in governing a ruminant's ability to recycle endogenous urea to the GIT. Marini and Van Amburgh (2003) conducted a study assessing dietary N levels on urea recycling in Holstein heifers. Diets fed were isocaloric, and

contained N levels of 1.44, 1.89, 2.50, and 2.97% (dry matter [DM] basis), and were fed at 1.8 times maintenance intake. Results from the study indicated that dietary N levels altered the partitioning of the various fates of endogenous urea-N produced. For example, increasing dietary N levels increased both endogenous urea-N production as well as urinary urea-N excretion, but decreased the amount of fecal N excretion (Marini and Van Amburgh, 2003). Because of this, urea-N utilized for anabolism (as a percentage of endogenous urea-N returned to the GIT) remained relatively constant despite intake increasing with dietary N content (Marini and Van Amburgh, 2003). In contrast, animals fed the low N diets had less endogenous urea-N return to the ornithine cycle (as percentage of endogenous urea-N returned to the GIT) providing them with greater anabolic benefit. These findings support the claimed benefits that urea recycling has on ruminants when faced with a protein deficiency. To assess the effects of oscillating dietary CP levels on urea recycling, Doranalli et al. (2011) provided oscillating levels of dietary CP to 9 wether lambs per treatment. Dietary CP content starting at 103 g CP/kg DM and increasing to 161 g CP/kg DM were deemed OSC-HIGH diets, whereas dietary CP content starting at 161 g CP/kg DM and decreasing down to 103 g CP/kg DM were deemed OSC-LOW. Doranalli et al. (2011) observed a reduction in ruminal NH_3-N concentrations for wether lambs fed OSC-LOW, as well as a corresponding increase in $J_{sm\text{-}urea}$ across ruminal epithelium in lambs fed OSC-LOW diets. Additionally, provision of increasing levels of RDP (0, 59, 118, and 177 g of N/kg body weight [BW] via ruminal casein dose) resulted in linear increases in endogenous urea production, as well as a decrease in urea entry into the GIT (Wickersham et al., 2008). This decrease is attributed to the increased amounts of NH_3 released due to the proteolytic activity of ruminal bacteria on RDP. The NH_3 released is known to decrease urease activity in the rumen (Cheng and Wallace, 1979), resulting in a loss of the diffusional gradient favourable to serosal-to-mucosal transfer of urea (Marini et al., 2004; Abdoun et al., 2006; Lu et al., 2014). When assessing the effects of ruminal NH_3 levels on UT-B, Marini et al. (2004) found that prevalence of UT-B (98 and 40 - 45 kD bands) decreased in ruminal tissue as the amount of N/kg DM in the diet increased. This finding could provide an additional explanation as to how the presence of NH_3 acts on the inhibition of $J_{sm\text{-}urea}$. Collectively, the data described illustrates the effects of dietary protein intake, level, and degradability on urea recycling.

In summary, it has been confirmed that in times of protein deficit, urea recycling will aid in improving N retention via return to the GIT, and provide an available N source for the ruminal

microbial population to synthesize microbial protein, thus enhancing the efficiency of nutrient utilization (Lapierre and Lobley, 2001; Doranalli et al., 2011). With less protein being provided in the diet, the NH_3 concentration in the rumen decreases, allowing urea to flow down its concentration gradient from the BUN pool to the rumen.

2.2.2.2 Fermentable Energy Sources and Fermentative End-Products

The effects of fermentable energy sources and fermentative end-products on urea recycling have been an area of increasing interest. Proven in peer-reviewed studies, the provision of RFC to ruminants improves microbial NH_3 sequestration by supplying an energy source (Kennedy and Milligan, 1980; Huntington, 1989), thus reducing the ruminal NH_3 concentration. Reducing ruminal NH_3 concentration removes the inhibitory action NH_3 elicits on urease functions (Cheng and Wallace, 1979), thus improving N retention by maintaining a favourable diffusional gradient for urea, enhancing its transfer to the rumen. Increasing energy availability in the rumen to elicit the aforementioned response can be achieved through means of grain processing, presence of SCFA and CO_2, and reduction in ruminal pH.

2.2.2.2.1 Effect of Grain Processing

Grain processing is an effective method to increase rapidly fermentable carbohydrate content available to bacteria in the rumen. Typically, the greater the degree of grain processing, the higher the starch digestibility, resulting in the rumen becoming a favorable site for urea transfer (vs. post-ruminal regions) (Huntington, 1989). Common types of cereal grain processing methods utilized in the feed industry include grinding, cracking, dry-rolling, roasting, reconstituting, and steam-flaking (Theurer, 1986). To study the effect of grain processing on urea recycling and N balance, Doranalli and Mutsvangwa (2007) fed four dietary treatments in a 2 × 2 factorial design to four Suffolk ram lambs. Dietary factors were provision of dry-rolled barley or pelleted barley as a fermentable energy source, combined with dietary levels of RDP provided at 60 vs. 70% of total dietary CP content. Diets were composed of 80% concentrate mixture and 20% barley silage (DM). The study found that feeding dry-rolled barley resulted in greater N digestibility compared to feeding pelleted barley; however, the amounts of endogenous urea-N produced and urea-N returned to the GIT were not different (Doranalli et al., 2007). Interestingly, the high concentrate diets fed by Doranalli and Mutsvangwa (2007) resulted in substantial amounts of endogenous urea produced being recycled to the GIT. In a similar study, Davies et al. (2013) fed four duodenally-

cannulated beef heifers diets varying in ruminally-degradable starch (RDS) content (whole-shelled corn vs. steam-rolled corn), and determined that animals fed higher levels of RDS in conjunction with increased RDP levels exhibited greater amounts of urea recycling to the GIT. These results confirm the proposed relationship between RFC and urea recycling mentioned above, with the findings of other studies (Huntington, 1989; Theurer et al., 2002; Walpole et al., 2015) supporting this notion.

2.2.2.2.2 Effects of SCFA, CO_2, and pH

When starch is fermented in the rumen, SCFA and CO_2 are produced as end-products by the bacterial community due to amylolytic, cellulolytic, and hemicellulolytic degradation (Ørskov, 1986). As the production of SCFA increases within the rumen, there is a corresponding reduction in ruminal pH (Owens et al., 1998). Production of these SCFA provide energy (as ATP) for the microbial community, facilitating the sequestration of NH_3 to synthesize microbial protein. Both in vivo and in vitro, it has been shown that the presence of SCFA can effect urea transfer into the rumen (Rémond et al., 1993; Abdoun et al., 2010; Bailey et al., 2012), with butyrate receiving attention for its reported stimulatory effects on urea recycling (Norton et al., 1982; Simmons et al., 2009). In a more recent study conducted by Agarwal et al. (2015), the effects of rumen butyrate concentrations on urea recycling in sheep failed to affect N utilization and retention in sheep, leaving conclusions of the previous studies (Norton et al., 1982; Simmons et al., 2009) in question. Additionally, altering levels of CO_2 (which is released as a by-product of the various fermentative pathways of pyruvate) in the rumen has also been shown to influence urea transfer to the rumen (Rémond et al., 1993; Abdoun et al., 2010). Because endogenous urea-N recycled back to the GIT is a N source for microbial protein synthesis, understanding how fermentative end-products regulate its transfer across the ruminal epithelium is critical. Bailey et al. (2012) studied the effects of supplemental energy to beef cattle in the form of 600 g glucose or 480 g SCFA infused ruminally combined with 120 or 240 g casein, and its effects on urea kinetics. The group observed that cattle provided with glucose infusions had an increase in endogenous urea-N entry into the GIT, as well as increased microbial uptake of recycled urea-N compared to those animals dosed with SCFA (Bailey et al., 2012). To study the effects of fermentative end-products on urea flux across the ruminal epithelium ex vivo, Abdoun et al. (2010) conducted a series of Ussing chamber experiments to assess the effects of SCFA, CO_2, and pH on urea transfer across the ruminal epithelium. The study found that at a luminal pH of 6.4 in combination with SCFA (40 mmol/L),

$J_{sm\text{-}urea}$ was increased from 18.8 ± 1.5 nmol/(cm^2 × h) (pH 7.4, no SCFA) to 82.4 ± 12.1 nmol/(cm^2 × h), and when combined with CO_2 (10%), increased $J_{sm\text{-}urea}$ from 39.2 ± 12.0 nmol/(cm^2 × h) (pH 7.4 with 10% CO_2) to 127.2 ± 5.5 nmol/(cm^2 × h). A few years later, Lu et al. (2014) also assessed the effects of SCFA and pH on urea-N transfer across the ruminal epithelium, and found that when luminal pH was 6.4, $J_{sm\text{-}urea}$ increased linearly with increasing SCFA concentrations (0, 40, and 80 mmol/L). The findings from these studies provide evidence that fermentative end-products play a regulatory role in the permeability of ruminal epithelia to urea. Furthermore, the study by Lu et al. (2014) also successfully sequenced UT-B in the ruminal epithelium of sheep, but did not study the regulatory effects of SCFA or pH on its expression; however, the group speculated that the increase in urea flux witnessed could be attributed to stimulatory effects of pH, SCFA, and CO_2 on UT-B expression and activity. In a follow-up study, Lu et al. (2015) also reported an up-regulatory effect of GPR4, GPR41, and UT-B in ruminal tissue of goats fed high levels of non-fiber carbohydrates (NFC), isonitrogenous diets compared to those fed low NFC, isonitrogenous diets. Interestingly, ruminal concentrations of NH_3-N were greater in the high NFC group, although serum urea concentrations were unchanged between the treatment groups. These findings led Lu et al. (2015) to conclude that SCFA and pH played a key role in the dietary regulation of UT-B expression (via GPR4 and GPR41), and that this mechanism may have priority over plasma urea concentrations in governing urea flux across the ruminal epithelium. Interestingly, Abdoun et al. (2010) had previously proposed that potential regulation of UT-B by fermentative end-products could be attributed to intracellular proton accumulation from transport of SCFA, altering the permeability of the ruminal epithelium to carrier-mediated urea transport. More research is needed to uncover the underlying mechanisms of pH, SCFA, and CO_2 on urea transport.

It is known that starch fermentation occurs in the hindgut, namely the large intestine and the cecum, and that fermentative profiles can be quite similar to that of the rumen (Ørskov, 1986). Some research has assessed the effects of fermentation in the hindgut of ruminants on urea recycling. Thornton et al. (1970) fit four wether lambs with ileal re-entrant cannulas in order to study the effects of glucose infusions into the hindgut on transfer and losses of endogenous N. The group found that fecal N output per day was greater as the percent of glucose in the infusate increased (4.22 vs. 5.20 g N/d), leading them to conclude that under favorable fermentative conditions in the hindgut, urea-N will be preferentially recycled to post-ruminal regions and utilized for microbial protein synthesis (Thornton et al., 1970). In support of these findings, Oncuer

et al. (1990) also observed that as terminal ileum infusate fermentability increased (by provision of starch, glucose, or the two combined), fecal N losses increased, further implying that provision of fermentable substrate in the hindgut facilitates microbial protein synthesis by providing an energy source for NH_3 sequestration (Lapierre and Lobley, 2001). Knowing that fermentative end-products in the rumen and hindgut influence urea transfer across the GIT epithelium, and that there is a proposed mechanism in place for these end-products to alter UT and AQP function in the rumen, this study sought to determine if the same relationships can be seen within the post-ruminal regions of ruminants.

2.3 Methods for Measuring Urea Transfer from the Bloodstream to the GIT

2.3.1 In Vivo Whole-Body Urea Kinetics (Lobley Model)

The beginnings of the whole-body urea kinetics model started in the 1950s, when Walser and his colleagues set out to determine mathematical models that would describe the monomolecular reactions between $N^{14}N^{14} + N^{15}N^{15} \rightleftharpoons 2N^{14}N^{15}$ under ordinary, non-equilibrium circumstances (Walser et al., 1954). Soon after these initial studies, experiments were put into place wherein systemic infusions of double-labelled urea ($[^{15}N^{15}N]$-urea) were provided to humans, and the emerging concentrations of formed single-labelled urea ($[^{14}N^{15}N]$-urea) were observed (Walser, 1968; Jackson et al., 1984; Jackson et al., 1993). These studies assessed rates of excretion of both labelled and unlabelled urea-N, and from there, determined the rate of urea production based on regular protein intakes for men (Jackson et al., 1984; Jackson et al., 1993).

Due to the physiological significance of urea recycling in ruminants, the whole-body urea kinetics model was an attractive scientific methodology to determine the multiple entry rates of urea-N to the GIT, return to ornithine cycle, as well as losses and anabolic retention (Sarraseca et al., 1998). Sarraseca et al. (1998) was the first group to apply the whole-body urea kinetics model to ruminants, feeding sheep 560, 1,110, and 1,670 mg N/kg$^{0.75}$ (25, 51, and 76 g DM/kg$^{0.75}$, respectively) for a total of 8 d. On the last 4 d of the experimental period, N balance was determined by total collections of feces and urine, with jugular infusions of $[^{15}N^{15}N]$-urea taking place from d 3 to 4. The study found that endogenous urea-N production increased with intake, as did urea-N entry into the GIT. The proportion of recycled urea-N to the GIT relative to the amounts returned to the ornithine cycle remained unchanged across dietary treatments, leading the group to speculate that the remainder was allocated towards anabolic usage (Sarraseca et al., 1998). With peaked

interests from this study, Lobley et al. (2000) conducted a study on 4 wether sheep fed 1 of 2 dietary treatments: (1) mixed concentrate-forage diet (CF; total 21.5 g N/kg DM); and (2) 1:1 mixture of grass pellets and chopped hay (HG; total 31.5 g N/kg DM), fed at 1000 g/d as fed. The methodology of obtaining urea kinetics data was almost identical to that of Sarraseca et al. (1998); however, Lobley et al. (2000) conducted the infusions over the entire 4-d period to increase [$^{15}N^{15}N$]-urea enrichments with the objective of reaching a plateau. From here, Lobley et al. (2000) developed a series of equations that better quantified "whole-body" fractional transfers and fates of urea-N to be used in the final analysis. These quantifications included: endogenous urea production (UER), urea-N entry into the GIT (GER), urea-N return to the ornithine cycle (ROC), urea-N loss to feces (UFE), urea-N loss in the urine (UUE), and urea-N left for anabolism (UUA), which is calculated by subtracting ROC and UFE from GER (Lobley et al., 2000). In addition, Lobley et al. (2000) also determined fractional transfers of: UER to UUE, UER to the GIT, GER to ROC, GER to the feces, and GER to UUA. The refinements implemented in the previously mentioned study provided a more complete picture of urea-N transfer throughout the body, particularly in the quantifications of the anabolic fractions. Additionally, Lobley et al. (2000) gathered invaluable information regarding time frames necessary for plateaus to be reached with both [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea, allowing the mathematical models to accurately quantify these two molecules and reduce the need for invasive splanchnic-tissue studies (Lobley et al., 2000). Figure 2.4 illustrates the refined [$^{15}N^{15}N$]-urea isotopic infusion model developed by Lobley et al. (2000).

2.3.1.1 Strengths and Limitations of the Lobley Model

The whole-body urea kinetics model used by Lobley et al. (2000) is accurate in its predictions of the fates of urea-N in the ruminant GIT. Additionally, if ruminal sampling is feasible, the model can include information pertaining to blood and salivary urea contributions to ruminal NH_3 by comparing ^{15}N enrichments of urea (Koenig et al., 2000; Lapierre and Lobley, 2001). Moreover, if [^{15}N]-NH_3 ruminal infusions are done on separate animals at the same time as intravenous infusions, data can be combined to provide estimates of intraruminal N recycling given the acquisition of a steady-state N pool (Lapierre and Lobley, 2001). The whole-body urea kinetics model is also far less invasive than its alternative, the V-A method, and is therefore used widely amongst research groups that are investigating regulatory mechanisms of urea recycling in ruminants.

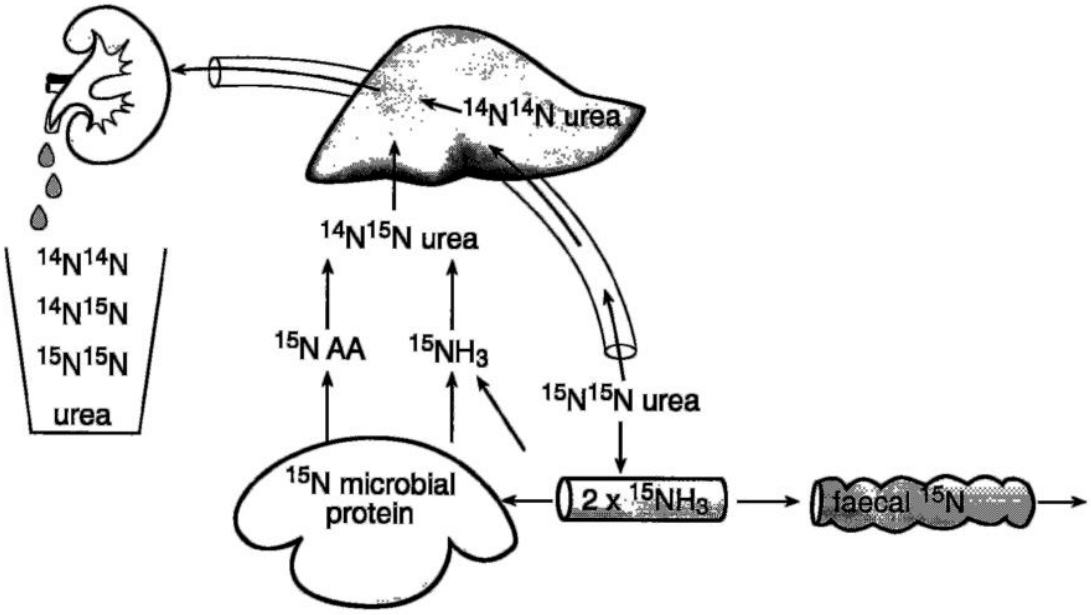

Figure 2.4. Illustration of the refined [$^{15}N^{15}N$]-urea isotopic infusion model (in ruminants) developed by Lobley et al. (2000). To quantify the fates of urea throughout the gastro-intestinal tract (GIT), [$^{15}N^{15}N$]-urea is infused into the jugular vein, where it is subject to entry into the GIT. When [$^{15}N^{15}N$]-urea enters the GIT, it is rapidly hydrolyzed by ureolytic bacteria, releasing two free $^{15}NH_3$ molecules. Free $^{15}NH_3$ can be sequestered by bacteria to synthesize microbial protein (later absorbed as ^{15}AA or excreted in the feces as ^{15}N), or subject to reabsorption across the GIT epithelia into the portal blood. In the liver, indiscriminate incorporation of labelled and unlabelled N into hepatic ureagenesis results in the formation of [$^{14}N^{14}N$]-, [$^{14}N^{15}N$]-, and [$^{15}N^{15}N$]-urea, which can be recycled back to the GIT, or lost in the urine. Sourced from Lapierre and Lobley (2001) with permission from the publisher.

Limitations to the model include time investments to reach steady-state conditions, ambiguity regarding site of gut entry, calculations subject to cumulative errors, and the inability to run simultaneous jugular and ruminal infusions (Lapierre and Lobley, 2001). Lobley et al. (2000) determined that infusions are most beneficial if conducted for at least 4 d to obtain plateau enrichments, with the collection and measurement period spanning a minimum of 48 h (Lapierre and Lobley, 2001). Moreover, steady-state conditions of N and/or urea pool sizes must be attained prior to the start of infusions and maintained to the end of the experiment, which can be difficult to sustain (Lapierre and Lobley, 2001). The whole-body urea kinetics model is also unable to provide information regarding the site of urea entry in the GIT, as the entire GIT is treated as a single compartment, whereas models such as the V-A difference method have the ability to differentiate between GIT regions depending on catheter placement (Lapierre and Lobley, 2001). Also, because the anabolic values obtained through the model are based on "differences between total urea-N entry and the amounts [of urea] converted to…catabolic fates," the estimates are subject to cumulative calculation errors (Lapierre and Lobley, 2001). Lastly, though extension of the model is possible if one has direct ruminal access, ruminal infusions of [^{15}N]-NH_3 cannot be simultaneously run with jugular [^{15}N^{15}N]-urea infusions due to conflicts of labelling nitrogenous compounds, such as NH_3 and AA (Lapierre and Lobley, 2001).

2.3.2 Ex Vivo Ussing Chamber Model

2.3.2.1 History, Description, and Methodology of the Ussing Chamber

The Ussing chamber model was first developed by Hans Ussing in 1951 during his studies investigating sodium movement in frog skin (Ussing and Zerahn, 1951). Since then, it has become one of the most useful experimental models for the study of epithelial transport of ions, nutrients, and medicinal drugs, thus providing major insights into the molecular mechanisms associated with transepithelial transport (Clarke, 2009). Ussing chambers come in various shapes and sizes, but ultimately, are categorized into 1 of 2 types: (1) the circulating chamber; or (2) the continuously perfused chamber (Li et al., 2004). In a review paper by Li et al. (2004), the 2 different Ussing chambers are described as follows. The circulating chamber (Figure 2.5) is a unit consisting of 2 half-chambers fitted to two glass columns, with a U-shaped circulatory tubing system within each column that is filled with experimental buffer. To attain desired experimental conditions, the solution within the chambers can be thermoregulated via water-jacket reservoirs, and are gassed with gases such as O_2, CO_2, or N_2 in order to mix the experimental solution. The 2 halves of the

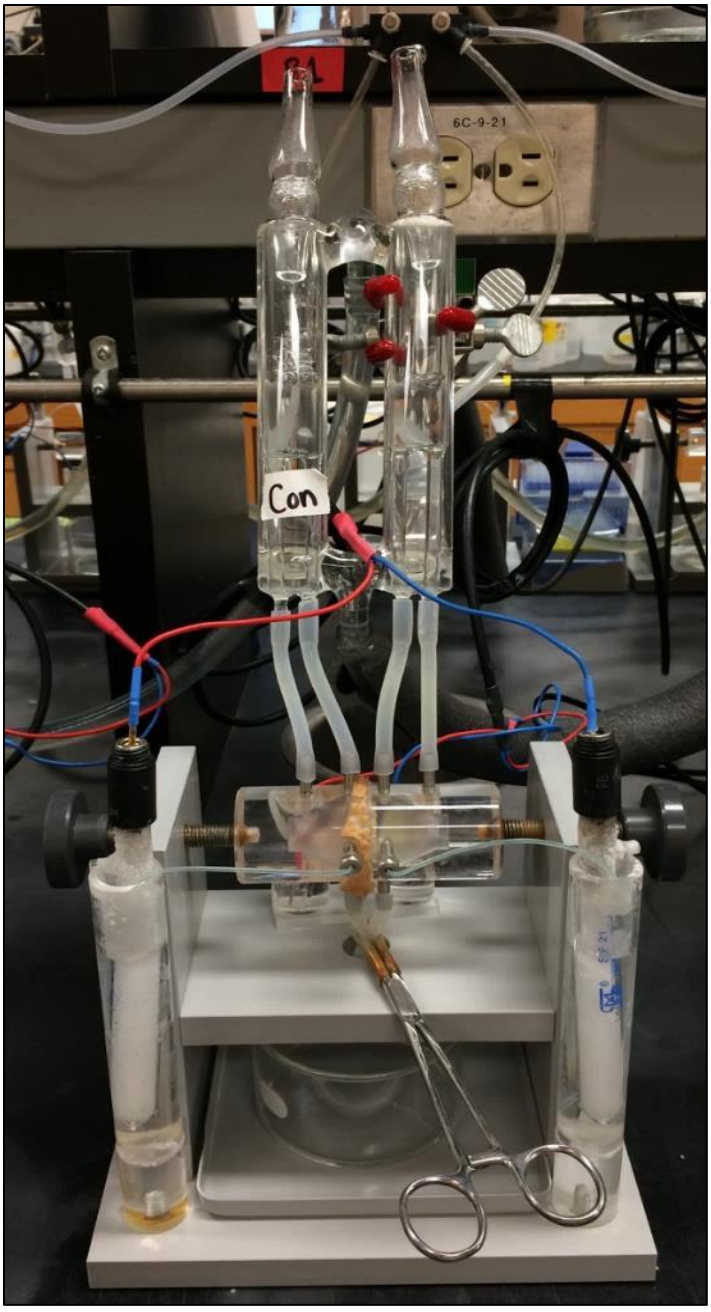

Figure 2.5. Diagram of the circulating Ussing chamber.

Ussing chamber are specifically designed to minimize fluctuations in hydrostatic pressure to reduce damage to the epithelial sample. The continuously perfused chamber is similar in that like the circulating chamber, it too consists of 2 halves in which tissue is mounted. It differs, however, in that the experimental solutions bathing the tissue are continuously delivered to the chamber from reservoirs held 20 to 50 cm above the chamber. Unlike the circulating chamber that relies on dimensions of the tubes to regulate flow rate, the continuously perfused chamber relies on a valve system to control flow and hydrostatic pressure. The continuously perfused chamber is not commercially available, making the circulating chamber most common in laboratories.

Regardless of the model used, Ussing chambers not only provide information regarding epithelial transport, but also critical electrophysiological measurements pertaining to tissue resistance (R_t or its reciprocal, G_t) and short-circuit current (I_{sc}) (Li et al., 2004; Clarke, 2009). Tissue resistance is, essentially, a value indicating tissue integrity based on permeability and functional tight junctions. It is calculated by Ohm's law, as: $R_t = \Delta V / \Delta I$, where R_t is tissue resistance, V is voltage, and I is electric current. The R_t value is obtained by voltage clamping; a series of short-current pulses injected into the system via a resistor, measured as voltage deflection by a voltmeter (Li et al., 2004). Commonly, R_t values are interpreted through tissue conductance (G_t) values, which are the reciprocal of R_t (Clarke, 2009). Interpretation of R_t and G_t data provides excellent insight into paracellular pathways – typically, greater G_t (or lower R_t) values are indicative of leaky epithelium with compromised tight junctions, whereas lower G_t (or higher R_t) values are indicative of intact, undamaged epithelial tissue. As described by Li et al. (2004), I_{sc} is "defined as the charge flow per time when the tissue is short-circuited," meaning that transepithelial voltage (V_{te}) of the tissue (the electrical current generated across an epithelium due to transport of ions, namely driven by Na^+/K^+ ATPase) is short-circuited to maintain a V_{te} of 0 mV such as to eliminate an electrical gradient across the epithelium and minimize the movement of ions. To induce short-circuit conditions, a current is applied throughout the system, and is consistently adjusted and measured by a feedback amplifier. In circumstances where epithelial tissue fails under a voltage clamp at 0 mV, the system can run "open," wherein V_{te} and transepithelial resistance (R_{te}) are known and can calculate an "open" or "equivalent" I_{sc} value: $I_{sc} = V_{te} / R_{te}$ (Li et al., 2004). It is important when conducting an Ussing chamber experiment to provide a stabilization period ranging from 10 to 40 min after mounting experimental tissue to allow oscillating V_{te}, I_{sc}, and R_{te} to normalize (Li et al., 2004; Doranalli et al., 2011).

The structure of the Ussing chamber facilitates its experimental function. Mounted between the 2 halves of the chamber, the epithelial sample separates the "mucosal" (apical, luminal) and "serosal" (basolateral) sides of the chamber, with the corresponding membrane of the epithelia matching its designated half (Clarke, 2009). Temperature and pH-controlled, gassed experimental buffers are designed to match in vivo physiological conditions of the respective sides of the tissue to facilitate accurate data collection. For measurements of solute movement and determination of tissue permeability, experimental designs commonly incorporate the usage of both a target molecule, as well as a hydrophilic molecule known to cross epithelial tissue by paracellular (diffusional) transport (Clarke, 2009). Examples of commonly used paracellular molecules are mannitol and inulin. Both radiolabelled and non-radiolabelled versions of the solutes are added to one or both sides of the chamber, with the provision of an equilibration period in order to achieve a steady-state flux within the system prior to obtaining isotopic flux measurements (Clarke, 2009). Isotopic flux measurements can be conducted by monitoring substrate movement (i.e. flux) from the mucosal to the serosal side, or from the serosal to the mucosal side. In order to obtain information regarding mechanisms of epithelial transport, the use of pharmacological or pathological drugs and/or inhibitors can be added to one side of the ussing chamber to elicit a physiological change in substrate transport (Kirat and Kato, 2006; Doranalli et al., 2011; Lu et al., 2014; Walpole et al., 2015). In regard to urea transport, for example, the inhibitors phloretin (Ritzhaupt et al., 1997; Stewart et al., 2005; Doranalli et al., 2011; Walpole et al., 2015) and $NiCl_2$ (Walpole et al., 2015) have been used to study the function roles of UT and AQP ex vivo in ruminal epithelium. The addition of these inhibitors effectively alters urea transfer across the ruminal epithelium, thereby providing insight on the mechanisms that govern its transport. Additionally, altering the environment of the physiological buffers so as to impose a challenge to the tissue can also provide valuable information regarding tissue function. An example of this is when Penner et al. (2010) imposed either an acidic (pH 5.2, 293 mOsm/L) or osmotic (pH 6.1, 450 mOsm/L) challenge to ovine ruminal epithelia to determine the effects of subacute ruminal acidosis on epithelial barrier function.

2.3.2.2 Tissue Preparation

When preparing tissue for an Ussing chamber experiment, extreme caution must be taken to maintain the integrity of the tissue. Firstly, tissue collected for experimentation must be obtained from a euthanized animal immediately and kept in physiological conditions during transport as

much as feasible. For preparation of GIT tissue, the underlying seromuscular layer must be carefully removed prior to mounting the tissue. The removal of the seromuscular layer aids in mitigating the diffusional barrier present on the epithelial tissue to various isotopes, nutrients, and oxygen, as well as reduce the presence of neuromuscular contractions that may arise with exposure to changes in I_{sc} (Clarke, 2009). Once tissue stripping is complete, a piece of epithelium, cut to fit the exposed portion of the half-chamber, is mounted on pins to hold the tissue in place. Usage of rubber washers or parafilm liners on either side of the epithelium is optional, but has been shown to mitigate epithelial edge damage (Clarke, 2009). Once the tissue is finally mounted, the two halves of the chamber are assembled and held in place by pressure clamps.

2.3.2.3 Strengths and Limitations of the Ussing Chamber Model

The Ussing chamber model has provided great contributions to the scientific knowledge of transepithelial transport processes and continues to be relevant in many physiology laboratories to date. Some of the major strengths of the Ussing chamber model include the ability to: (1) study a vast array of tissue types (e.g. reproductive tract, gastrointestinal tract, organ tissues); (2) maintain viable tissue in a closed, controlled system for an extended period of time; (3) expose the tissues to a wide variety of treatments with technical replicates; and (4) obtain precise measurements of molecular transport under mimicked in vivo conditions. The Ussing chamber method enables an ex vivo/in vitro approach in quantifying in vivo phenomenon over a diverse spectrum of subject material, including but not limited to gut barrier health (Benjamin et al., 2000; Penner et al., 2010; Wilson et al., 2012), the effects of pharmacological substances and drug absorbencies (Lampen et al., 1996; Lennernäs et al., 1997), and tissue permeability for substrate transport (Schröder et al., 1997; Abdoun et al., 2010; Walpole et al., 2015).

However, the model is not without its weaknesses. Firstly, due to time sensitivity and the fragile nature of live tissues, viability and functionality of the tissue being used in an Ussing chamber experiment is frequently questioned (Clarke, 2009). Despite real-time electrical measurements taking place to provide an insight into tissue viability, changes in G_t values can be challenging to discern (Clarke, 2009). Additionally, during periods where tissues cannot be clamped at ~ 0 mV to regulate unwanted ion movement, they may be run on an “open” circuit (Li et al., 2004). Transport, flux, or uptake data needs to be interpreted cautiously in these instances, as not all ion movement has been controlled in the system. Secondly, maintaining a tissue ex vivo

removes in vivo physiological effects, including nutrient supply and waste removal, and hormonal and paracrine signalling, thus further limiting the extent of data interpretation (Clarke, 2009). Thirdly, the materials making up the Ussing chamber are designed for reuse and are therefore subject to contamination between experimental studies. In particular, soft plastics used for circulation tubing between the chambers and the columns are particularly prone to contamination by compounds dissolved in dimethyl sulfoxide or ethanol, as are the solidified agar bridges (Clarke, 2009). To avoid this issue, thorough cleaning regimens and protocols are imperative in the laboratory. Lastly, when studying the effects of drugs on tissues ex vivo, there are many factors unique to the Ussing chamber that must be accounted for: (1) different tissues vary in diffusional barriers (e.g. intestinal tissue limits diffusion through goblet cell mucosal secretions on the apical surface, and by presence of collagen and fibroblasts on the basolateral submucosal membrane); (2) accounting for these diffusional barriers, equilibration time and drug concentration may not be the same for all tissue types utilized in any given study; and (3) composition of experimental buffers can have an important influence on a drug's mode of action (e.g. CO_2/HCO_3^--containing buffers influence drug action by altering the buffering capacity of both the closed system as well as the epithelial cells of the tissue), in addition to a lack of in vivo stimuli (e.g. hormones) (Clarke, 2009). Despite the weaknesses listed, it is important to note that constant advancements are being made in Ussing chamber studies, and through careful data interpretation and experience, it remains an excellent model to delve into the understandings of life sciences. For these reasons, the Ussing chamber model was deemed a beneficial apparatus to use in this study to obtain valuable total flux data across the ovine GIT.

2.3.3 Considerations to the Methodologies

To account for the strengths and weaknesses of the whole-body urea kinetics and Ussing chamber models, it was decided to use both the in vivo and ex vivo methodologies in combination for this study. The in vivo isotope infusions provide information regarding whole-body urea-N utilization and kinetics; however, it fails to represent the various compartments of the GIT. Therefore, samples from the rumen, duodenum, and cecum were collected at the time of slaughter to assess ex vivo $J_{sm\text{-}urea}$ across the individual regions, as well as interpret any dietary treatment effects on urea flux.

2.4 Summary

Most of the current literature has focused on urea recycling to the rumen, as it is most beneficial to the ruminant from an anabolic and production standpoint; however, it is known that urea recycling also occurs in the hindgut regions (Stewart and Smith, 2005; Ludden et al., 2009; Collins et al., 2010). The serosal-to-mucosal movement of urea-N across the GIT epithelium has been confirmed to take place via passive diffusion and by carrier-mediated transport mechanisms, namely UT (Stewart et al., 2005; Ludden et al., 2009; Walpole et al., 2015) and AQP (Walpole et al., 2015). Moreover, it is known that dietary starch content and fermentative end-products have an influence on both $J_{sm\text{-}urea}$ and the expression of UT and AQP in the rumen (Abdoun et al., 2010; Lu et al., 2015; Walpole et al., 2015). Currently, comparative studies assessing dietary influences on ruminal vs. post-ruminal urea transfer are sparse; therefore, it would be of great interest to evaluate the impact of forage level and corn grain processing on ruminal and post-ruminal urea recycling, and the influence on the expression of UT and AQP in the rumen, duodenum, and cecum. Combining this knowledge with in vivo N balance and whole-body urea kinetics data will facilitate a deeper understanding of urea recycling in ruminants.

2.5 Hypothesis

The hypothesis of this experiment was that altering the F:C ratio and the extent of corn grain processing would influence urea-N recycling to ruminal and post-ruminal regions of the GIT, and $J_{sm\text{-}urea}$ and the expression of UT and AQP in ovine ruminal, duodenal, and cecal epithelia.

2.6 Objectives

The objectives of this experiment were: (1) to determine the influence of forage level and corn grain processing on whole-body urea-N kinetics, and $J_{sm\text{-}urea}$ and the expression of UT and AQP in the ovine ruminal, duodenal, and cecal epithelia; and (2) to determine the influence of forage level and corn grain processing on whole-body N balance and fermentation characteristics in the ovine rumen, duodenum, and cecum.

3.0 MATERIALS AND METHODS

3.1 Animals, Experimental Design, and Dietary Treatments

Thirty-two wether lambs (37.2 ± 1.7 kg initial BW upon arrival) were used in this study. Animals were housed at the University of Saskatchewan Livestock Research Building (LRB), and were cared for in accordance with the guidelines of the Canadian Council of Animal Care (2009). All procedures conducted in this experiment were approved by the University of Saskatchewan Animal Research Ethics Board (Protocol No. 20040048). Upon arrival at the LRB, lambs were weighed and blocked by BW into groups of 4 (for a total of 8 blocks) and randomly assigned within block to 1 of 4 dietary treatments (n = 8). Experimental diets were arranged into a 2 × 2 factorial design, with dietary factors being: (1) dietary forage content i.e., low (30% [LF]) vs. high (70% [HF]) forage; and (2) method of corn grain processing i.e., whole-shelled [WS] vs. steam-flaked [SF]). Ingredient and chemical composition of the experimental diets are presented in Table 3.1. Dietary treatments were formulated to be isonitrogenous (10.7 ± 0.2% CP) so as to remove any influence on urea recycling associated with varying CP content. To investigate the effects of grain processing, corn was selected as the cereal grain to be included in the diets based on its high starch content (68.0 to 75.4% starch, DM basis) (Theurer, 1986; Crocker et al., 1998; Zinn et al., 2002). Feeding either WS or SF corn was intended to manipulate the site of starch digestion, with more starch being fermented in the rumen when SF corn was fed compared to WS corn (Lee et al., 1982; Huntington, 1997; Crocker et al., 1998). Additionally, in a study conducted by Crocker et al. (1998), starch reactivity (i.e., the amount of starch degraded to glucose over a given time period when incubated with amyloglucosidase) was 28.5% units greater with SF corn compared to WS corn. Based on this evidence from previous studies investigating the effects of processing on starch digestion, it was decided that provision of either SF or WS corn would be used to manipulate the site of starch digestion between ruminal and post-ruminal sites. In support of this decision, diet fermentability predictions obtained from the Nutritional Dynamic System (NDS; CNCPS v6.5) estimated that rumen escape starch for the WS-containing (HFWS, LFWS) and SF-containing (HFSF, LFSF) diets were 14.0 and 4.96% of DM, respectively. Corn grain processing was conducted at the Canadian Feed Research Centre (North Battleford, SK). In brief, WS corn was held in a steam chest for 20 min at 100°C, followed by processing through a AT Ferrell 18×39 dual drive flaking mill (A.T. Ferrell Co., Bluffton, IN) with continuous steam injection. Processing index for the produced SF corn was reported at ~78%. Feeding of experimental diets took place at

Table 3.1. Ingredients and chemical composition of experimental diets containing low (LF) and high (HF) forage levels in combination with whole-shelled (WS) or steam-flaked (SF) corn.

Item	Experimental diets[1]			
	LF		HF	
	WS	SF	WS	SF
Ingredients, % of DM				
Barley green feed	26.4	26.4	70.7	70.7
Corn, WS	64.6	-	22.0	-
Corn, SF	-	64.6	-	22.0
Pelleted ingredients				
Barley, ground	0.66	0.66	0.66	0.66
Limestone, ground	1.35	1.35	1.01	1.01
Salt, white	0.47	0.47	0.47	0.47
Canola meal, solvent-extracted	5.83	5.83	5.15	5.15
Commercial micro-premix[2]	0.05	0.05	0.05	0.05
Bovatec[3]	0.02	0.02	0.02	0.02
Dicalcium phosphate	0.67	0.67	-	-
Chemical composition				
DM, %	91.6 ± 0.6	90.4 ± 1.2	90.2 ± 1.4	90.3 ± 0.9
ADF, % of DM	12.1 ± 0.4	12.5 ± 0.3	26.2 ± 1.3	26.8 ± 0.2
NDF, % of DM	22.2 ± 1.0	22.0 ± 0.9	40.6 ± 2.3	40.7 ± 1.9
NFC[4], % of DM	59.5 ± 1.2	60.0 ± 1.7	39.9 ± 2.5	40.1 ± 3.2
Starch, % of DM	47.1 ± 0.7	48.0 ± 1.3	26.4 ± 0.8	26.6 ± 0.4
CP[5], % of DM	10.5 ± 0.3	10.7 ± 0.7	10.7 ± 0.7	11.0 ± 1.4
Soluble protein, % of DM	1.95 ± 0.3	1.55 ± 0.3	2.78 ± 0.4	2.58 ± 0.7
EE, % of DM	2.93 ± 0.1	2.52 ± 0.2	2.41 ± 0.1	1.95 ± 0.1
Ca, % of DM	0.74 ± 0.03	0.84 ± 0.1	0.70 ± 0.1	0.74 ± 0.1
P, % of DM	0.43 ± 0.02	0.43 ± 0.1	0.30 ± 0.01	0.29 ± 0.03
NE_g[6], Mcal/kg of DM	1.20 ± 0.01	1.16 ± 0.03	0.88 ± 0.05	0.88 ± 0.02

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]Commercial micro-premix contained (per kg of premix, DM basis): 0.67% P, 0.13% Cl, 0.33% Mg, 1.21% K, 0.04% NaCl, 0.08% Na, 5.18% Ca, 65.49 mg Co, 11.06 mg Cu, 20.30 mg F, 56.02 mg I, 143.35 mg Fe, 3,505.89 mg Mn, 1,512.59 mg Zn, 55.95 mg I, 11.60 mg Se, 3,429.97 mg chelated Mn, 1,430.99 mg chelated Zn, 11.60 mg chelated Se, 58.38 mg biotin, 4,199.77 IU vitamin E, 488.60 KIU vitamin A, 54.37 KIU vitamin D3. Supplied by Masterfeeds, Taber, AB.
[3]Bovatec 200,000 mg/kg (ionophore, lasalocid sodium) included in the diet as a rumen modifier; supplied by Masterfeeds, Taber, AB. Manufactured by Zoetis Canada Inc.
[4]Non-fiber carbohydrates = 100 – (% NDF + % CP + % EE + % Ash).
[5]Diets were formulated to be isonitrogenous.
[6]Net energy of gain.

0700 and 1700 h daily. Diets were provided as a total mixed ration (TMR) and fed to allow for ≤ 5% refusals. Water was provided free choice.

Initially, all lambs were placed in a common outdoor pen for a minimum of 2 weeks to facilitate habituation, where they were fed ad libitum grass hay along with daily provision of WS corn mixed with a supplemental pellet (the amounts of WS corn and pellet fed were determined based on predicted daily intakes). The composition of the pellet is presented in Table 3.1. At the onset of the experiment, lambs were brought into the metabolism wing of the LRB in their respective blocks and housed in individual floor pens. Each lamb was given 23 d of dietary adaptation to their assigned dietary treatment and was then killed at approximately 1000 h on d 24 to facilitate collection of tissues for ex vivo experimentation. Additionally, 4 blocks of animals were randomly selected to conduct in vivo measurements of whole-body urea kinetics (see below). For logistical reasons, only one animal could be killed per day to obtain tissues for ex vivo measurements. Therefore, it was necessary to stagger the initiation of feeding experimental diets to individual animals within each block such that each animal was exposed to the experimental diet for 23 d before it was killed. Within each block, the order of initiation of animals on experimental diets was random.

3.2 Sample Collection

3.2.1 Feeding and Feed Sampling

Individual feed ingredients as well as TMR samples were collected bi-weekly over the duration of the experiment to determine their DM and chemical composition. Amounts of TMR offered and orts were recorded daily over the duration of the experiment, with records from the last 7 d of the 23-d experimental period used to determine dry matter intake (DMI). For animals utilized in the in vivo metabolism trial (see below), orts were collected over the 4-d period (i.e., d 19 to 23) so as to determine actual nutrient intake for digestibility calculations. All feed and orts samples collected were kept frozen at -20°C pending processing and analysis upon completion of the trial.

3.2.2 In Vivo Metabolism Trial

Four blocks of animals were randomly selected from the 8 blocks and used for in vivo measurements of whole-body urea kinetics and N balance. The in vivo metabolism trial was conducted for 4 d (d 19 to d 23) for each animal at its designated time-point during the experiment.

Animals were housed in metabolism crates to facilitate total collections of feces and urine. Animals were placed in metabolism crates on d 18 to allow for 24 h of acclimatization before the initiation of total fecal and urine collections on d 19. On d 18, animals were fitted with temporary jugular catheters (0.86-mm i.d. × 1.32-mm o.d.; Scientific Commodities Inc., Lake Havasu City, AZ) in the left and right jugular veins, to allow isotopic infusions. Prior to the initiation of jugular isotopic infusions on d 19, background samples of feces and urine were collected to determine ^{15}N (feces), and [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea (urine) natural abundance. Thereafter, intra-jugular infusions of [$^{15}N^{15}N$]-urea were conducted for 4 d (d 19 to 23) using a peristaltic pump to determine urea-N kinetics as described by Lobley et al. (2000). The [$^{15}N^{15}N$]-urea (99.8 atom % ^{15}N; Cambridge Isotope Laboratories, Andover, MA) intrajugular infusate was prepared in 0.9% sterile saline solution, with infusions beginning at approximately 0800 h on d 19 until 0800 h on d 23, with approximately 450 mL infused daily. The amounts of [$^{15}N^{15}N$]-urea infused per day (range = 0.036 to 0.146 g/d; mean = 0.083 g/d) were calculated based on mean daily N intakes during the last 4 d before the initiation of isotopic infusions, such that urinary [$^{15}N^{15}N$]-urea enrichments of 0.15 atom percent excess were achieved.

Fecal collections were conducted using fecal bags, with collections done once daily at 0730 h. Fecal bags were fitted the day prior to the start of collection (d 18) to allow for acclimatization. Feces collected were thoroughly mixed, weighed and a representative sample (25% of total output) was taken and stored at -20°C pending chemical analysis. Simultaneously, urine was collected in plastic containers placed below the individual metabolic crates. These containers contained 40 mL of 37% HCl to reduce urine pH to < 3 in order to prevent the loss of NH_3 and prevent bacterial growth. Urine collected was weighed and recorded once daily (0730 h), then a representative 50-mL sub-sample was taken and stored at -20°C pending analysis for urea-N concentration, and [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea enrichment. The remaining urine was collected, composited over the 4-d in vivo period, and stored at -20°C for later determination of total N content. After termination of total collections at approximately 0730 h on d 23, animals were returned to their respective floor pens prior to their kill date on d 24 of the experimental period (described below).

3.2.3 Digesta and Gastro-Intestinal Tissue Sampling

Prior to killing, venous blood samples were collected into heparin-coated vacutainers (BD; Franklin Lakes, NJ) by jugular venipuncture at 3 h after the morning feeding (~1000 h) on d 24

(i.e., after 23 d on experimental diets). Plasma samples were refrigerated at 4°C until they were centrifuged at 3,000 x *g* for 15 min at 4°C to obtain plasma. Plasma samples were then stored at -20°C pending chemical analysis for urea-N and glucose concentrations. Following blood sampling, animals were killed by captive bolt stunning, pithing, and exsanguination. The time of killing (i.e., 3 h after the morning feeding) was chosen to allow sufficient time for microbial fermentative activities to occur in the GIT after feeding (Doranalli et al., 2011; Walpole et al., 2015). Immediately after killing, the abdominal cavity was opened, and the entire GIT was removed. Thereafter, whole digesta contents were collected from the rumen, duodenum, and cecum. Digesta was temporarily stored in clean plastic containers. Following thorough mixing of whole ruminal digesta, a representative sub-sample was strained through 2 layers of cheesecloth to obtain ruminal fluid. Ruminal fluid pH was then measured using a portable pH meter (Accumet AP110 Portable pH Meter; Fisher Scientific, Ottawa, ON). Thereafter, two 10-mL sub-samples were collected and preserved with the addition of either 2 mL of 1% sulfuric acid (for NH_3-N analysis) or 2 mL of 25% metaphosphoric acid (for SCFA analysis). Preserved ruminal fluid samples were stored at -20°C pending chemical analysis. Whole duodenal and cecal digesta were thoroughly mixed before sub-samples were collected, weighed, and then mixed with equal weights of double-distilled water (ddH_2O). The addition of ddH_2O to duodenal and cecal digesta was to reduce the viscosity of the sample, thus facilitating ease of handling during chemical analysis. Thereafter, duodenal and cecal digesta pH was measured using a portable pH meter. Duodenal and cecal digesta sub-samples were then preserved with 2 mL of 1% sulfuric acid or 25% metaphosphoric acid and stored at -20°C pending NH_3 and SCFA analysis, respectively.

Simultaneous to digesta collection, tissue samples were obtained from the caudal-ventral sac of the rumen (Doranalli et al., 2011), proximal duodenum (Marini et al., 2004), and distal cecum (Ludden et al., 2009; Haenen et al., 2013). Tissue samples were washed in an O_2-saturated transport buffer that was pre-heated to 38°C and was maintained at pH 7.4. The saturation of the transport buffer with O_2 was achieved by continuous gassing with carbogen (95% O_2, 5% CO_2 mixture). After washing of tissue samples, the underlying muscular layer was removed gently from the mucosa (for ruminal tissue) before the tissues were transported immediately (within 15 min of killing) to the laboratory in the transport buffer for ex vivo measurements. In addition to tissue samples that were obtained for ex vivo measurements, additional mucosal samples were obtained for later analysis of mRNA expression of UT-B, and AQP-3, and -7. Tissue samples were collected

from the caudal-ventral sac, proximal duodenum (within the 1st 10 cm), and blind sac of the cecum using sterile forceps and tweezers. Samples of ruminal papillae were obtained by clipping, whereas proximal duodenum and cecum samples were acquired by scraping the mucosa from the epithelium using a sterile glass slide. All samples were cleaned in sterile phosphate-buffered saline (PBS; pH 7.4, 4°C), transferred into cryovials, snap-frozen in liquid N_2, and then stored at -80°C pending analysis for gene expression.

3.2.4 Ex Vivo Ussing Chamber Experiment

Upon arrival at the laboratory, duodenal and cecal tissue samples had the underlying muscular layer removed gently to avoid damage of the mucosal layer. During removal of the muscular layer, duodenal and cecal tissue samples were bathed in O_2-saturated, pre-heated (38°C) transport buffer to maintain tissue viability. Thereafter, stripped ruminal, duodenal, and cecal tissue samples were cut into strips, and mounted between two halves of an Ussing chamber, with an exposed surface area of approximately 3.14 cm^2 (for ruminal tissue) or 1.0 cm^2 (for duodenal and cecal tissue). Once mounted, tissue samples were bathed in incubation buffer solutions (15 mL for both mucosal and serosal sides) that were maintained at 38°C. A total of 24 Ussing chambers were used, allocating 8 chambers per GIT region. The chemical compositions of the mucosal and serosal buffers are presented in Table 3.2. For all tissues, the pH of the incubation buffer on the serosal side was maintained at 7.4 to mimic physiological blood pH. For ruminal tissue, the pH of the incubation buffer on the mucosal side was maintained at 6.4. This pH was based on a study by Abdoun et al. (2010) who observed that maximal $J_{sm\text{-}urea}$ in ruminal epithelial tissue mounted in Ussing chambers was achieved at a mucosal buffer pH of 6.4. For duodenal tissue, the pH of the incubation buffer on the mucosal side was maintained at 7.4 (Schröder et al., 1995), whereas for cecal tissue the incubation buffer was maintained at a pH of 6.4 to mimic in vivo physiological conditions (Kirat and Kato, 2006). Also to mimic in vivo physiological conditions, mucosal buffers for the duodenal and cecal tissues contained glucose and SCFA, respectively, as energy sources. The pH of incubation buffers was adjusted by the addition of 1 mmol/L NaOH or 37% HCl. Both serosal and mucosal incubation buffers were mixed by gassing with carbogen (95% O_2, 5% CO_2 mixture), with temperature maintained at 38°C by water-jacket reservoirs. All tissues were incubated under short-circuit conditions using a computer-controlled voltage clamp (Dipl.-Ing. K. Mussler, Scientific Instruments, Aachen, Germany), with G_t and I_{sc}

Table 3.2. Chemical composition for transport, incubation 1, and incubation 2 buffers used in the Ussing chamber experiment.

	Experimental buffers[1]		
Chemical, mmol/L	Transport	Incubation 1	Incubation 2
$CaCl_2$	1.00	1.00	1.00
$MgCl_2$	1.00	1.00	1.00
Na_2HPO_4	2.00	2.00	2.00
NaH_2PO_4	1.00	1.00	1.00
Phenyl-phosphorodiamidate	1.00	1.00	1.00
Butyric acid	5.00	5.00	0.00
NaCl	60.0	60.0	60.0
KCl	5.00	5.00	5.00
Glucose	10.0	10.0	10.0
Na-acetate	25.0	25.0	2.50
Na-propionate	10.0	10.0	1.00
Na-butyrate	0.00	0.00	0.50
Na-gluconate	15.0	15.0	46.0
$NaHCO_3$	25.0	25.0	25.0
HEPES	0.00	0.00	5.00
Antibiotics, mg/L			
Penicillin G sodium salt	0.00	60.0	60.0
Kanamycin sulfate	0.00	100.0	100.0
Fluorocytosine	0.00	50.0	50.0
Buffer characteristics			
Osmolality, mOsmol/L	291 ± 2.37	291 ± 2.37	284 ± 0.60
Temperature, °C	38	38	38
pH	7.4	6.4	7.4
Gas	Carbogen[2]	Carbogen	Carbogen

[1]pH adjustments were made using either 1 mmol/L NaOH or 37% HCl and were based on physiological conditions. Transport buffer was used to transport tissues from the slaughter site to the laboratory, and for subsequent tissue handling. Incubation 1 buffer was used to bathe ruminal and cecal epithelia on the mucosal side, whereas Incubation 2 buffer was used to bathe duodenal epithelia on the mucosal side, as well as ruminal, duodenal, and cecal epithelia on the serosal side.
[2]Carbogen gas (95% O_2, 5% CO_2 mixture).

measured every 6 sec. Tissues were allocated 20 min for stabilization of electrophysiological measurements prior to the start of the experiment.

After the 20-min electrophysiology stabilization period, 74 KBq/15-mL column of ^{14}C-urea (Perkin-Elmer, Waltham, MA) and 148 KBq/15-mL column of ^{3}H-mannitol (Perkin-Elmer, Waltham, MA) were added to the serosal sides of the tissue samples to determine serosal-to-mucosal fluxes of urea and mannitol ($J_{sm\text{-}mannitol}$). Both ^{14}C-urea and ^{3}H-mannitol were mixed with unlabelled urea and mannitol to achieve final concentrations of 1 mmol/L. The addition of ^{14}C-urea to only the serosal side elicits a concentration gradient from the serosal-to-mucosal side, thus mimicking physiological conditions in which urea is not normally found in ruminal fluid (Doranalli et al., 2011). The addition of ^{3}H-mannitol allowed for the assessment of paracellular tissue permeability (i.e. diffusional transport). A 45-min isotope equilibration period was allotted prior to obtaining flux measurements. After isotope equilibration, $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were measured over a 30-min flux period. To facilitate measurements of $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, samples of incubation buffer (100 μL from the serosal side and 500 μL from the mucosal side) were collected at the beginning and end of the 30-min flux period. To maintain the volume of buffer on the mucosal side after the first sampling, 500 μL of fresh mucosal buffer was added. Samples were placed in scintillation vials (Perkin-Elmer, Waltham, MA), mixed with 4 mL of liquid scintillation cocktail, and were analyzed in a liquid scintillation counter (Tricarb 2910, Perkin-Elmer, Waltham, MA) on the same day, following the experiment.

3.2.5 Gene Expression and Real-Time Quantitative PCR

Ruminal, duodenal, and cecal samples that were stored at -80°C after collection at the time of the kill were ground and homogenized using pre-frozen (-80°C) mortars and pestles. During grinding, all samples and materials were kept in contact with dry ice and liquid N_2 to prevent thawing. Once ground, RNA extraction was conducted using TRIzol™ reagent in accordance with the TRIzol™ Reagent Extraction Protocol (Invitrogen, Burlington, ON). Quantitative assessment of RNA was completed using a Nanodrop 2000c Spectrophotometer (Thermo Scientific Inc., Waltham, MA), with a basic contaminant assessment conducted using the absorbance ratios of 260:280 nm and 260:230 nm. After RNA concentration was determined, RNA samples were analyzed on a 1.2% denaturing agarose gel to assess RNA integrity and to check for genomic DNA contamination. Primers (Table 3.3; primers were designed using NCBI-Primer3 and BLAST) and

Table 3.3. Gene name, NCBI accession number, forward and reverse sequences, amplicon size, efficiency, and function of primers for real-time quantitative polymerase chain reaction (qPCR).

Item[1]	Accession Number[2]	Primer Sequence	Amplicon Size[3]	Efficiency (%)	Function
GAPDH	NM_001190390.1	F[4]: AAGCTCATTTCCTGGTACGACA	200	101	Reference
		R[5]: GAAATGTATGGAGGTCGGGAGA			
HPRT1	XM_015105023.1	F: TATGGACAGGACCGAACGAC	121	101	Reference
		R: GATGTAATCCAACAGGTCGGC			
RPLP0	XM_004017413.2	F: GAAATCCTGAGCGATGTGCAG	187	95	Reference
		R: AGCGGGAATGCAGAGTTTCC			
UT-B	NM_001163054.1	F: GTGGTGTTTGTCAGCAACCC	146	94	Target
		R: ATGGCAGACCTGTCCTGATTG			
AQP-3	XM_015093074.1	F: CTATGTGCTTCCTGGCTCGT	121	95	Target
		R: GCCCAGATCGCATCGTAATA			
AQP-7	XM_012118379.2	F: CGCATGTCCTGGAAGAAGTT	96	100	Target
		R: AGCGGTGTAGAAGAGGCTGT			

[1]GAPDH = Glyceraldehyde-3 phosphate dehydrogenase; HPRT1 = Hypoxanthine-guanine phosphoribosyltransferase; RPLP0 = 60S acidic ribosomal protein P0; UT-B = Urea transporter-B (SLC14A1); AQP-3 = Aquaporin-3; AQP-7 = Aquaporin-7.
[2]NCBI accession number (National Center for Biotechnology Information).
[3]Amplicon size represented in base pairs.
[4]F = Forward primer sequence.
[5]R = Reverse primer sequence.

prepared RNA samples were then shipped to the Advanced Analysis Centre (Genomics Facility) at the University of Guelph (Guelph, ON) for primer efficiency analysis, further RNA integrity assessment (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany), and qPCR analysis. Real-time qPCR was conducted using the StepOnePlus™ Real-Time SYBR PCR System (Thermo Scientific Inc., Waltham, MA).

In preparation for qPCR analysis, complementary DNA (cDNA) was made using 800 ng of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Scientific Inc., Waltham, MA). Thereafter, 10 uL of 2X PerfeCTa SYBR Green FastMix (Quantabio Inc., Beverly, MA) was combined with 0.8 uL of the desired forward and reverse primers (5 uM), 5.0 uL of cDNA template, and 4.2 uL of nuclease-free H_2O for a total volume of 20 uL/reaction. Each qPCR cycle included a 30-second pre-incubation period at 95°C for activation of polymerase, followed by a 3-second denaturing cycle at 95°C, and a 30-second annealing and extension period at 60°C for a total of 40 cycles. After each qPCR run, a melting curve analysis was performed to confirm the purity of the obtained PCR products, which was indicated by a single peak. Each plate was designed using the gene maximization method, with all samples run in duplicate for each GIT region. A no-template control was analyzed to determine the presence of any contaminants and/or primer-dimer formation within the qPCR reactions (without the cDNA template), which could potentially lead to false positive results. Lastly, an inter-plate calibrator was designed by pooling aliquots from all rumen samples and analyzing for Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and 60S acidic ribosomal protein P0 (RPLP0) in duplicate to account for run-to-run variation on each plate. For each run, cycle threshold (C_t; the number of cycles required for amplification fluorescence to exceed that of background fluorescence) was determined using a normalized reporter signal (Rn; emission intensity of the reporter signal) set to a ΔRn of 0.5 (ΔRn = Rn cycle [reaction with template] – Rn baseline [reaction without template]). Obtaining a C_t value at ΔRn of 0.5 places the reading within the exponential phase of the reaction (Bustin, 2000). All C_t values obtained for target genes were normalized by subtracting the geometric mean of the C_t values obtained from three selected housekeeping genes (GAPDH, RPLP0, and Hypoxanthine-guanine phosphoribosyltransferase [HPRT1]) to obtain ΔC_t (Vandesompele et al., 2002). It should be noted that all three housekeeping genes were used for normalization of ruminal and cecal tissue samples; however, RPLP0 was excluded from duodenal tissue due to significant effects of dietary treatment. Next, fold-change

was calculated using the $2^{-\Delta\Delta Ct}$ method by designating one of the dietary treatments as a control (HFWS). Then, the $2^{-\Delta\Delta Ct}$ value for each target gene was divided by the arithmetic mean of the $2^{-\Delta\Delta Ct}$ values for HFWS in order to obtain a fold-change ratio of mRNA expression relative to the control diet, which was set to one.

3.3 Sample Analysis

Samples of TMR, orts, and feces were dried to a constant weight at 60°C in a forced-air oven over 3 d (TMR and ort samples) or 7 d (fecal samples) to determine DM (AOAC, 1990; method 930.15). Samples of TMR and orts were then ground through a 1-mm screen using the Christy-Norris mill (Christy and Norris Ltd., Chelmsford, England), whereas fecal samples were ground through a 1-mm screen using the Retsch ZM100 ultra centrifuge mill (Retsch-Allee, Haan, Germany). Ground orts and fecal samples were pooled for each animal proportionally, with sub-samples submitted to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis of chemical composition. Prior to pooling fecal samples, background and daily fecal sub-samples for each animal on the in vivo metabolism trial were kept for later analysis of ^{15}N enrichment. Samples of TMR, orts, and feces were analyzed for ash (AOAC, 2000; method 942.05), acid detergent fiber (ADF) (AOAC, 2000; method 973.18), neutral detergent fiber (NDF) (Van Soest et al., 1991), starch (Hall, 2009), CP (AOAC, 2000; method 990.3), soluble protein (Krishnamoorthy et al., 1982), ether extract (EE) (AOAC, 2000; method 2003.05), and minerals (Ca and P) (AOAC, 2000; method 985.01).

Composited urine samples were thawed overnight in the refrigerator at 4°C and analyzed for N using the macro-Kjeldahl procedure (AOAC, 1990; method 976.05). Background and daily urine samples were thawed overnight in the refrigerator at 4°C in preparation for urinary urea-N analysis. Samples were diluted prior to analysis such that the absorbance (at 600 nm) reading fell within the standard curve for accurate results. Similarly, plasma samples were thawed overnight in the refrigerator at 4°C prior to plasma urea-N (PUN) analysis. Urinary and plasma urea-N concentrations were determined according to procedures described by Fawcett and Scott (1960). Additionally, plasma samples were also analyzed for glucose concentration using the glucose oxidase/peroxidase enzyme (No. P7119; Sigma, St. Louis, MO) and diansidine dihydrochloride (No. F5803; Sigma, St. Louis, MO) assay. Absorbance was recorded at 450 nm using a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

Following urea-N analysis, background and daily urine samples were analyzed for urinary [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea enrichment as described by (Lobley et al., 2000). Briefly, urea-N was isolated by applying a pre-determined volume of urine that contained 1.5 mg of urea-N through a pre-packed ion exchange column (Poly-Prep® Columns, AG® 50W-X8, hydrogen form #7316213; Biorad, Richmond, CA) as described by Archibeque et al. (2001). Previous studies have found that 1.5 mg of urea-N allows for a sufficient amount of sample to properly undergo isotope ratio-mass spectrometry (IRMS) while minimizing non-monomolecular reactions during in the degradation of urea (Sarraseca et al., 1998; Archibeque et al., 2001). After the urine had completely drained through the column, 7 mL of ddH_2O was applied, with the eluant being discarded. Thereafter, urea was eluted by applying 20 mL of ddH_2O through the column with the eluant being collected into test tubes. The 20-mL eluant samples were then air-dried at 60°C overnight. Next, the eluted urea was transferred into 17- x 60-mm borosilicate tubes by rinsing with 1 mL of ddH_2O three times. Samples were then freeze-dried over a period of 72 hours, and sent for analysis of [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea enrichment at the N-15 Analysis Laboratory, University of Illinois (Urbana-Champaign, IL). The analysis of [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea enrichment was conducted using IRMS as described by Lobley et al. (2000). The IRMS assay conducted produced mass/charge (m/z) values of 28, 29, and 30 for [$^{14}N^{14}N$]-, [$^{14}N^{15}N$]- and [$^{15}N^{15}N$]-urea, respectively. Also, standards prepared using [$^{15}N^{15}N$]-urea (99.8% ^{15}N) and [$^{14}N^{14}N$]-urea (natural abundance; 0.368% ^{15}N) were used to correct values obtained for [$^{14}N^{15}N$]-urea to account for non-monomolecular reactions (Lobley et al., 2000).

In preparation of fecal ^{15}N enrichment analysis, ground background and daily fecal samples from each lamb on the in vivo trial were pulverized into a fine powder using a ball mill grinder. Thereafter, ground samples (~2 mg) were weighed into 6- x 4-mm tin capsules (Elemental Microanalysis Limited, Okehampton, UK) and subsequently positioned into a 96-well plate. Fecal ^{15}N enrichment was then determined by combustion to N_2 gas in a Costech ECS4010 elemental analyzer (Costech Analytical, Valencia, CA) and continuous flow IRMS (Delta V Advantage mass spectrometer, Thermo Scientific Inc., Bremen, Germany) as described by Lobley et al. (2000).

To determine digesta NH_3-N concentrations, ruminal, duodenal, and cecal digesta samples (preserved with sulfuric acid) were thawed overnight in the refrigerator at 4°C. Once thawed, the digesta samples were gently mixed and transferred into new 15-mL centrifuge tubes, and then

centrifuged at 1,000 × *g* for 10 min at 4°C. Next, ~1.5 mL of supernatant was transferred from the 15-mL centrifuge tube into a 2-mL micro-centrifuge tube. Thereafter, 20 µL of sample was utilized for ruminal NH_3-N analysis, whereas for duodenal and cecal NH_3-N analysis, 40-µL of sample was analyzed (to account for the additional dilution with ddH_2O at the time of collection). Ammonia-N concentration was determined using the phenol-hypochlorite method as described by Fawcett and Scott (1960). To quantify SCFA concentrations, ruminal, duodenal, and cecal digesta samples (preserved with metaphosphoric acid) were thawed overnight in the refrigerator at 4°C. After thawing, the digesta samples were gently mixed and transferred into new 15-mL centrifuge tubes, and then centrifuged at 12,000 × *g* for 10 min at 4°C. For duodenal and cecal digesta, the supernatant obtained was placed into a new 15-mL centrifuge tube and centrifuged again at 12,000 × *g* for 10 min at 4°C to ensure adequate separation of solid particulate from the liquid phase. After centrifugation, ~1.75 mL of supernatant from ruminal, duodenal, and cecal samples was transferred into a 2-mL micro-centrifuge tube and then centrifuged at 16,000 × *g* for 10 min at 4°C. Supernatants obtained from duodenal and cecal samples were then filtered through sterile 0.45-µm syringe filters (13-mm diameter, polyvinylidene fluoride, Fisher Scientific, Ottawa, ON) into gas chromatography (GC) vials. Thereafter, ruminal, duodenal and cecal samples were mixed with 0.2-mL of internal standard (isocaproic acid) and stored at 4°C pending SCFA analysis using GC according to the method described by Khorasani et al. (1996). Individual SCFA were separated using an Agilent GC machine (Agilent 6890 Series, 254 Agilent Technologies, Waldbronn, Germany) with a column length of 30.0 m (255 model 7HM-G009-11, Zebron, Phenomenex, Torrance, CA).

3.4 Calculations and Statistical Analysis

Dry matter intake was determined for all animals on trial (n = 32) based on the last 7 d of the experimental period and was calculated as: [TMR offered (kg/d) × TMR %DM] – [TMR refused (kg/d) × TMR %DM]. Additionally, for those animals on the 4-d in vivo metabolism trial (n = 16), DMI was calculated as: [TMR offered (kg/d) × TMR %DM] – [TMR refused (kg/d) × orts %DM]. Daily nutrient intake was calculated as: [DM offered (kg/d) × %nutrient in TMR] – [DM refused (kg/d) × %nutrient in orts], and apparent nutrient digestibility (as a percent) was calculated as: [nutrient intake (kg/d) – fecal nutrient output (kg/d)] ÷ [nutrient intake (kg/d)] × 100.

Apparent N balance was calculated as: [average N intake (g/d)] – [total N excretion (g/d)], with total N excretion accounting for both urinary and fecal N losses (g/d). Apparent N balance, as a percent of N intake, was calculated as: [apparent N balance (g/d)] ÷ [N intake (g/d)] × 100.

Whole-body urea kinetics data was calculated using urinary [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea, and fecal ^{15}N enrichments as described by Lobley et al. (2000). The model provides quantitative information regarding transfers of urea-N throughout the body. The model accounts for urea-N synthesized in the liver (endogenous urea production, UER), which will either return to the GIT (gastro-intestinal entry rate, GER) or is lost in the urine (urinary urea-N excretion, UUE). The urea-N returned to the GIT will be subject to degradation to NH_3 via ureolytic bacteria, with some of the NH_3 being reabsorbed into the portal blood and returned to the liver (urea-N returned to the ornithine cycle, ROC). Ammonia not reabsorbed will be lost through fecal excretion (fecal urea-N excretion, UFE), with the remainder assumed to contribute to anabolic usage (urea-N utilized for anabolism, UUA) (Lobley et al. 2000).

Data were analyzed as a randomized complete block design with a 2 × 2 factorial design using the mixed model procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). The statistical model used was: $Y = \mu + F + G + (F \times G) + B + e$, where Y is the dependent variable, μ is the mean, F is the effect of forage level, G is the effect of corn grain processing, $F \times G$ is the interaction between forage level and corn grain processing, B is the block (random) effect, and e is the error term. To ensure normality, both block and error were checked for normal distribution using Shapiro-Wilke values and visual distribution plot analysis, with studentized residual values used to detect and remove outlier data from any given dataset. Mean comparison were conducted using the Bonferonni method. Significance was declared when $P < 0.05$, and tendencies when $0.05 \leq P \leq 0.10$. Correlations between region-specific NH_3-N concentrations and $J_{sm\text{-}urea}$, PUN concentrations and $J_{sm\text{-}urea}$, G_t and $J_{sm\text{-}urea}$, and $J_{sm\text{-}mannitol}$ and $J_{sm\text{-}urea}$ were determined using the correlation procedure in SAS (Version 9.4, SAS Institute Inc., Cary, NC). Data sets were checked for normal distribution using Shapiro-Wilke values. Pearson correlation was used when both sets of data were normally distributed, and spearman correlation was used if at least one of the data sets were not normally distributed.

For analysis of qPCR mRNA transcript abundance, the results are expressed as fold-change relative to the HFWS dietary treatment, which was designated as the control treatment. The choice

of HFWS as the control treatment was based on 2 factors: (1) it was expected that this diet would result in the greatest ruminal NH_3-N concentration relative to the other diets due to its high soluble protein content; and (2) it was also expected that due to the unprocessed nature of WS corn, ruminally-fermentable starch content would be lower compared to SF corn, resulting in reduced ruminal SCFA concentrations relative to the other diets. Elevated ruminal NH_3-N concentrations have been reported to inhibit urea-N flux across ruminal epithelium (Lu et al., 2014, 2015). Also, urea-N flux across the ruminal epithelium has been reported to be decreased when SCFA concentrations are reduced (Abdoun et al., 2010). Collectively, therefore, the greater ruminal NH_3-N concentrations and decreased ruminal SCFA concentrations with the HFWS relative to the other diets were expected to result in the lowest rates of urea-N transfer into the rumen.

4.0 RESULTS

4.1 Dietary Characteristics

Ingredient and chemical composition of the dietary treatments are shown in Table 3.1. As per dietary formulation, experimental diets were isonitrogenous (mean CP content of 10.7 ± 0.2%). Also, the soluble protein content of the HF diets was greater than that of the LF diets. As expected, the LF diets had greater levels of starch (47.6% vs. 26.5%) and NFC (59.8% vs. 40.0%), and lower levels of ADF (12.3 vs. 26.5%) and NDF (22.1 vs. 40.7%) compared to the HF diets.

4.2 Final Body Weights, Dry Matter Intake and Apparent Nitrogen Balance

Mean BW (± S.D.) at the end of the experiment were 39.3 ± 5.2, 40.1 ± 3.3, 43.2 ± 4.0 and 44.3 ± 3.5 kg BW for HFWS, HFSF, LFWS and LFSF dietary treatments, respectively. For all 32 animals (i.e., n = 8) that were used in the study, DMI was greater in lambs fed SF compared to those fed WS (1.24 vs. 1.04 kg/d; P = 0.03; Figure 4.1). Lambs fed LF diets tended to have greater DMI (1.22 vs. 1.06 kg/d; P = 0.07) than those fed HF diets. For animals that were used in the in vivo metabolism trial (i.e., n = 4), similar trends were observed for the effects of forage level and method of grain processing on DMI (Table 4.1). Lambs fed LF diets had greater DMI (1.20 vs. 0.86 kg/d; P < 0.01) compared to lambs fed HF diets. Lambs fed SF corn also exhibited greater DMI (1.20 vs. 0.86 kg/d; P < 0.01) compared to those fed WS corn.

Nitrogen intake was greater in lambs fed LF diets compared to those fed HF diets (20.1 vs. 15.0 g/d; P < 0.01). Nitrogen intake was greater in lambs fed SF corn compared to those fed WS

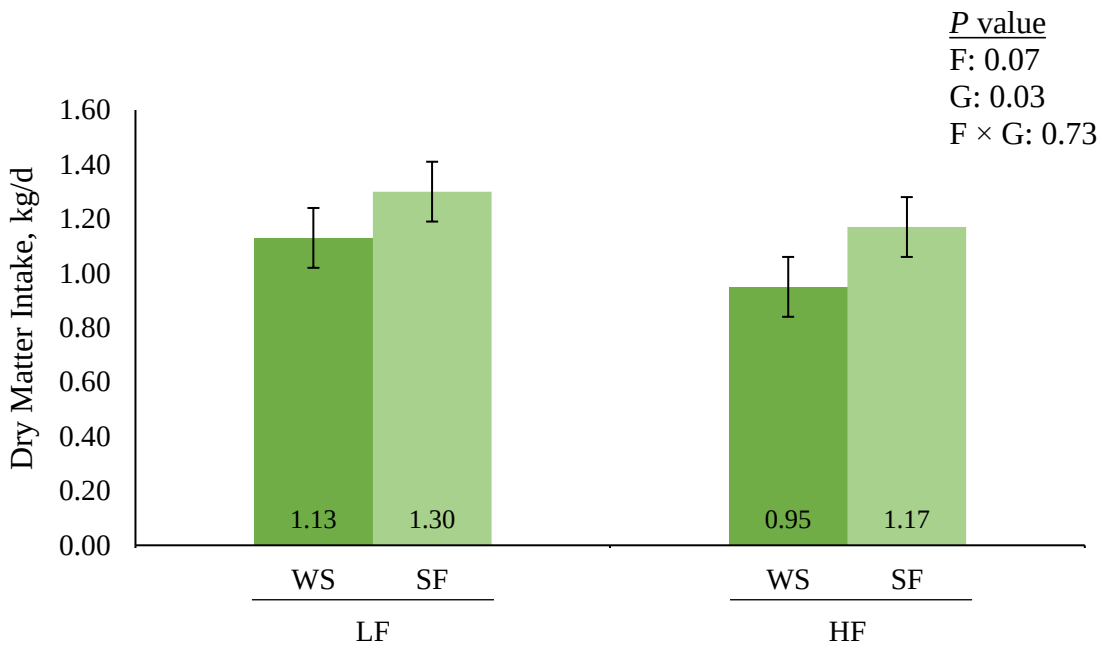

Figure 4.1. The effects of forage level (F) and grain processing (G) on dry matter intake (DMI) based on intakes calculated from the last 7 d of the experimental period (n = 8). Experimental diets were arranged into a 2 × 2 factorial design, with dietary factors being: (1) dietary forage content i.e., low (30% [LF]) vs. high (70% [HF]) forage; and (2) method of corn grain processing i.e., whole-shelled [WS] vs. steam-flaked [SF]). SEM = 0.11.

Table 4.1. The effects of forage level and grain processing on dry matter intake (DMI) and nitrogen utilization (n = 4).

	Experimental diets[1]							
	LF		HF			*P* value[2]		
Item	WS	SF	WS	SF	SEM	F	G	F × G
DMI, kg/d	1.08	1.31	0.63	1.08	0.14	<0.01	<0.01	0.14
N intake, g/d	18.2	22.0	10.8	19.2	2.39	<0.01	<0.01	0.10
Urinary excretion								
Total, g/d	891	1101	2877	982	532	0.10	0.14	0.07
Total N, g/d	6.22^{bc}	4.84^{c}	7.59^{ab}	9.00^{a}	0.65	<0.01	0.99	0.03
Total N, % of N intake	36.7	22.9	80.2	49.4	9.96	<0.01	0.01	0.28
Urea-N, g/d	3.84	2.25	6.20	6.70	0.69	<0.01	0.32	0.07
Urea-N, % of urinary N	58.8	45.3	80.7	74.4	5.70	<0.01	0.06	0.46
Fecal excretion								
DM, g/d	223	327	208	361	42.0	0.64	<0.01	0.26
N, g/d	5.94	7.48	4.11	7.28	0.99	0.05	<0.01	0.11
N, % of N intake	32.4	34.0	37.7	37.8	2.40	0.06	0.70	0.72
Total N excretion								
g/d	12.2^{b}	12.3^{b}	11.7^{b}	16.3^{a}	1.17	0.02	<0.01	<0.01
% of N intake	69.1	56.9	118	87.2	9.93	<0.01	0.02	0.26
Apparent N balance								
g/d	5.99	9.72	- 0.91	2.93	1.88	<0.01	0.02	0.97
% of N intake	30.9	43.1	- 17.9	12.8	9.93	<0.01	0.02	0.26

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.

corn (20.6 vs 14.5 g/d, $P < 0.01$). In lambs fed WS corn, dietary forage level had no effect on urinary N excretion (g/d), whereas in lambs fed SF corn, feeding HF diets increased urinary N excretion compared to feeding LF diets (interaction; $P = 0.03$). As a percent of N intake, total urinary N excretion was greater in animals fed HF compared to those fed LF diets (64.8 vs. 29.8%; $P < 0.01$). As a percent of N intake, lambs fed WS corn exhibited greater urinary N excretion compared to those fed SF (58.5 vs. 36.2%; $P = 0.01$). Urinary urea-N loss (g/d) was lower in animals fed LF diets compared to those fed HF diets (3.05 vs. 6.45 g/d; $P < 0.01$). As a percent of urinary N excretion, urea-N loss was greater in lambs fed HF diets compared to those fed LF diets (77.6 vs. 52.1%; $P < 0.01$). Lambs fed WS corn tended to exhibit greater amounts of urea-N lost in the urine (as a percent of urinary N) compared to those fed SF corn (69.8 vs. 59.9%; $P = 0.06$). Fecal N excretion tended to be greater in lambs fed LF compared to those fed HF diets (6.71 vs. 5.70 g/d; $P = 0.05$). Lambs fed SF corn had greater fecal N excretion compared to those fed WS corn (7.38 vs. 5.03 g/d; $P < 0.01$). As a percent of N intake, lambs fed HF diets tended to have greater fecal N excretion compared to those fed LF diets (37.8 vs. 33.2%; $P = 0.06$). Total N excretion (g/d) was greater in lambs fed the HF diet with SF corn compared to those fed the HF diet with WS corn, whereas lambs fed the LF diet with either WS or SF corn had similar total N excretion (interaction; $P < 0.01$). As a percent of N intake, lambs fed HF diets had greater total N excretion compared to those fed LF diets (102.6 vs. 63.0%; $P < 0.01$). Lambs fed WS corn also exhibited greater total N excretion (as a percent of N intake) compared to those fed SF corn (93.6 vs. 72.1%; $P = 0.02$). Apparent N balance (g/d) was lower in lambs fed HF diets compared to those fed LF diets (1.01 vs. 7.86 g/d; $P < 0.01$). Similarly, lambs consuming WS corn had a lower apparent N balance (g/d) compared to those fed SF corn (2.54 vs. 6.33 g/d; $P = 0.02$). Apparent N balance (as a percent of N intake) was greater in lambs fed LF compared to those fed HF diets (37.0 vs. -2.55%; $P < 0.01$). Apparent N balance (as a percent of N intake) was greater in lambs fed SF corn compared to those fed WS corn (28.0 vs. 6.50%; $P = 0.02$).

4.3 Whole-Body Urea Kinetics

Lambs fed HF diets with SF corn tended to have greater UER compared to those fed HF diets with WS corn, whereas lambs fed LF diets with WS or SF corn had similar UER (interaction; $P = 0.06$; Table 4.2). Gastro-intestinal urea-N entry rate tended to be greater in lambs fed SF corn compared to those fed WS corn (12.7 vs. 9.73 g/d; $P = 0.10$). Dietary forage level had no effect on GER ($P = 0.98$). Lambs fed the HF diet with SF corn tended to have greater ROC compared to

Table 4.2. The effects of forage level and grain processing on whole-body urea-N recycling kinetics (n = 4).

	Experimental diets[1]							
	LF		HF			*P* value[2]		
Item	WS	SF	WS	SF	SEM	F	G	F × G
Urea-N kinetics[3], g/d								
UER	15.0	13.5	14.4	20.8	2.37	0.09	0.21	0.06
GER	11.2	11.3	8.25	14.1	2.14	0.98	0.10	0.11
ROC	8.07	7.48	7.37	11.5	1.68	0.23	0.20	0.10
UFE	0.57	0.76	0.15	0.50	0.12	<0.01	<0.01	0.35
UUE	3.84	2.25	6.20	6.70	0.69	<0.01	0.32	0.07
UUA	2.55	3.03	0.73	2.11	0.59	0.01	0.07	0.35
Fractional Urea-N transfers								
UER to urine	0.26	0.18	0.44	0.32	0.05	<0.01	0.02	0.65
UER to GIT	0.74	0.82	0.56	0.68	0.05	<0.01	0.02	0.65
GER to ROC	0.72	0.63	0.91	0.82	0.06	<0.01	0.08	1.00
GER to feces	0.05	0.08	0.02	0.03	0.01	<0.01	0.04	0.60
GER to UUA	0.23	0.29	0.08	0.15	0.05	<0.01	0.12	0.90

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.
[3]UER = endogenous urea-N production; GER = gastro-intestinal entry rate; ROC = return to ornithine cycle; UFE = fecal urea-N excretion; UUE = urinary urea-N excretion; UUA = urea-N utilized for anabolism.

those fed the HF diet with WS corn, whereas lambs fed the LF diet with WS or SF corn had similar ROC (interaction; $P = 0.10$). Lambs fed LF diets had greater UFE (0.67 vs. 0.33 g/d; $P < 0.01$), but had lower UUE (3.05 vs. 6.45 g/d; $P < 0.01$) compared to those lambs fed HF diets. Lambs fed SF corn exhibited greater UFE compared to those fed WS corn (0.63 vs. 0.36 g/d; $P < 0.01$). Lambs fed the LF diet with WS corn tended to have greater UUE compared to those fed the LF diet with SF corn, whereas lambs fed HF diets with WS or SF corn tended to have similar UUE (interaction; $P = 0.07$). For lambs fed LF, UUA was greater when compared to lambs fed HF diets (2.79 vs. 1.42 g/d; $P = 0.01$). Lambs fed SF corn tended to have greater UUA compared to those fed WS corn (2.57 vs. 1.64 g/d; $P = 0.07$). For fractional urea-N transfers, lambs fed HF had greater UER to urine (0.38 vs. 0.22) and GER to ROC (0.87 vs. 0.68), but had lower UER to GIT (0.62 vs. 0.78), GER to feces (0.03 vs. 0.07) and GER to UUA (0.12 vs. 0.26) compared to lambs fed LF diets ($P < 0.01$). Lambs fed SF corn had greater UER to GIT (0.75 vs. 0.65; $P = 0.02$) and GER to feces (0.06 vs. 0.04; $P = 0.04$), but had lower UER to urine (0.25 vs. 0.35; $P = 0.02$) compared to lambs fed WS corn. Additionally, lambs fed WS corn tended to have greater GER to ROC compared to those fed SF corn (0.82 vs. 0.73; $P = 0.08$).

4.4 Apparent Total-Tract Digestibility

Total-tract digestibilities of DM (77.4 vs. 67.1%), OM (79.3 vs. 69.4%), EE (87.2 vs. 72.5%), and starch (99.5 vs. 96.9%) were greater in lambs fed LF diets compared to those fed HF diets ($P < 0.01$; Table 4.3). Total-tract digestibility of ADF was lower (32.1 vs. 43.6%; $P = 0.04$), whereas that of NDF (36.3 vs. 47.9%; $P = 0.06$) tended to be lower in lambs fed LF compared to those fed HF diets. Lambs fed LF diets tended to have greater CP digestibility compared to those lambs fed HF diets (66.8 vs. 62.3%; $P = 0.07$). Lambs fed WS corn had greater total-tract digestibility of EE than those fed SF corn (82.4 vs. 77.3%; $P < 0.01$); however, total-tract digestibilities of DM, OM, CP, NDF, ADF, and starch were unaffected by grain processing.

4.5 Ruminal, Duodenal, and Cecal pH and Fermentation Characteristics

Ruminal pH was similar in lambs fed SF and WS with HF diets, but it was lower in lambs fed SF compared to WS with LF diets (interaction, $P = 0.01$; Table 4.4). Duodenal pH was unaffected by dietary treatment; however, cecal pH was lower in lambs fed LF diets compared to those fed HF diets (6.26 vs. 6.96; $P < 0.01$).

Table 4.3. The effects of forage level and grain processing on apparent total-tract nutrient digestibility (n = 4).

	Experimental diets[1]							
	LF		HF			*P* value[2]		
Item	WS	SF	WS	SF	SEM	F	G	F × G
Nutrient digestibility, %								
DM	79.7	75.0	67.6	66.5	1.81	<0.01	0.13	0.33
OM[3]	81.5	77.1	70.0	68.8	1.67	<0.01	0.13	0.36
CP	67.6	66.0	62.3	62.2	2.40	0.07	0.71	0.72
EE	88.8	85.6	75.9	69.0	1.37	<0.01	<0.01	0.18
NDF	39.1	33.4	49.3	46.5	5.76	0.06	0.45	0.79
ADF	31.7	32.4	44.1	43.0	5.51	0.04	0.97	0.86
Starch	99.3	99.7	96.6	97.1	0.69	<0.01	0.55	0.91

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.
[3]OM = 100-ash.

Table 4.4. The effects of forage level and grain processing on ruminal, duodenal, and cecal pH, and NH_3-N and short-chain fatty acid (SCFA) concentrations (n = 8).

	Experimental diets[1]							
	LF		HF			*P* value[2]		
Item	WS	SF	WS	SF	SEM	F	G	F × G
Rumen								
pH	5.90[a]	5.40[b]	6.05[a]	6.08[a]	0.11	<0.01	0.02	0.01
NH_3-N, mg/dL	4.68	0.92	13.4	9.97	1.23	<0.01	<0.01	0.88
SCFA, mmol/L								
Acetate	52.3	61.5	60.4	65.1	4.88	0.14	0.08	0.56
Propionate	22.3	35.4	15.4	19.0	3.21	<0.01	0.01	0.15
Isobutyrate	0.72[a]	0.20[b]	0.72[a]	0.72[a]	0.06	<0.01	<0.01	<0.01
Butyrate	14.0	14.7	12.3	14.6	1.48	0.52	0.29	0.56
Isovalerate	1.90[a]	0.40[b]	0.82[b]	0.85[b]	0.23	0.18	<0.01	<0.01
Valerate	1.49	2.38	0.85	0.84	0.34	<0.01	0.18	0.17
Total SCFA	92.7	114.6	90.5	101.1	7.14	0.21	0.01	0.36
A:P[3]	2.66	1.93	4.01	3.46	0.27	<0.01	0.03	0.74
Duodenum								
pH	4.70	4.48	4.78	4.95	0.30	0.37	0.94	0.51
NH_3-N, mg/dL	11.4	10.0	12.1	11.5	1.05	0.27	0.33	0.73
SCFA, mmol/L								
Acetate	3.47	4.67	2.96	3.28	0.93	0.32	0.43	0.64
Propionate	0.10	0.51	N.D.	N.D.	0.16	0.06	0.20	0.20
Isobutyrate	N.D.	N.D.	N.D.	N.D.	-	-	-	-
Butyrate	0.06	0.09	N.D.	N.D.	0.06	0.18	0.78	0.78
Isovalerate	N.D.	N.D.	N.D.	N.D.	-	-	-	-
Valerate	N.D.	N.D.	N.D.	N.D.	-	-	-	-
Total SCFA	3.63	5.27	2.96	3.28	1.07	0.23	0.37	0.54
Cecum								
pH	6.13	6.39	6.89	7.02	0.14	<0.01	0.13	0.63
NH_3-N, mg/dL	7.92	9.32	13.8	14.5	1.69	<0.01	0.47	0.83
SCFA, mmol/L								
Acetate	60.0	73.6	43.6	46.4	4.13	<0.01	0.06	0.20
Propionate	14.4	16.3	8.37	9.15	1.14	<0.01	0.26	0.64
Isobutyrate	0.47	0.43	0.72	0.71	0.10	0.01	0.80	0.89
Butyrate	9.63	6.26	3.33	2.60	1.11	<0.01	0.08	0.24
Isovalerate	0.50	0.29	0.69	0.65	0.10	0.01	0.22	0.40
Valerate	1.06	1.20	0.73	0.71	0.15	0.01	0.69	0.59
Total SCFA	86.1	98.1	57.5	60.2	5.76	<0.01	0.21	0.43
A:P	4.27	4.65	5.25	5.17	0.24	<0.01	0.54	0.35

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.
[3]Acetate-to-propionate ratio.

Ruminal NH_3-N concentrations were lower in lambs fed LF diets compared to those fed HF diets (2.80 vs. 11.7 mg/dL; $P < 0.01$). Lambs fed SF corn exhibited lower ruminal NH_3-N concentrations compared to those fed WS corn (5.45 vs. 9.04 mg/dL; $P < 0.01$). Duodenal digesta NH_3-N concentrations were unaffected by dietary treatment. Cecal NH_3-N concentrations were lower in lambs fed LF diets compared to those fed HF diets (8.62 vs. 14.2 mg/dL; $P < 0.01$).

Lambs fed LF diets had greater ruminal concentrations of propionate (28.9 vs. 17.2 mmol/L) and valerate (1.94 vs. 0.85 mmol/L) but exhibited a lower acetate-to-propionate (A:P) ratio (2.30 vs. 3.74) compared to those fed HF diets ($P < 0.01$). Ruminal concentrations of acetate, butyrate, and total SCFA were unaffected by dietary forage level. In animals fed SF corn, ruminal acetate concentration tended to be greater (63.3 vs. 56.4 mmol/L; $P = 0.08$), whereas propionate (27.2 vs. 18.9 mmol/L) and total SCFA (108 vs. 91.6) concentrations were greater than those in animals fed WS corn ($P = 0.01$); however, lambs fed SF corn had a lower A:P ratio (2.70 vs. 3.34; $P = 0.03$) compared to those fed WS corn. With LF diets, lambs fed WS corn exhibited greater ruminal concentrations of isobutyrate and isovalerate compared to those fed SF corn, whereas lambs fed WS and SF corn with HF diets had similar concentrations of these SCFA (interaction, $P < 0.01$). Dietary treatment did not affect duodenal SCFA profiles, except that lambs fed LF diets tended to have greater propionate concentrations compared to those fed HF diets ($P = 0.06$). Cecal concentrations of acetate (66.8 vs. 45.0 mmol/L), propionate (15.4 vs. 8.76 mmol/L), butyrate (7.95 vs. 2.97 mmol/L), valerate (1.13 vs. 0.72 mmol/L), and total SCFA concentration (92.1 vs. 58.9 mmol/L) were greater in lambs fed LF diets compared to those fed HF diets ($P \leq 0.01$). Lambs fed HF diets exhibited greater cecal concentrations of isobutyrate (0.72 vs. 0.45 mmol/L) and isovalerate (0.67 vs. 0.40 mmol/L) compared to those fed LF ($P = 0.01$). Lambs fed HF diets also exhibited an increased A:P ratio (5.21 vs. 4.46) compared to those fed LF diets ($P < 0.01$). Method of grain processing largely had no effects on cecal SCFA concentrations, except that cecal concentration of acetate tended to be greater in lambs fed SF corn compared to those fed WS corn (60.0 vs. 51.8 mmol/L; $P = 0.06$). Additionally, lambs fed WS corn tended to have greater butyrate concentration compared to those fed SF corn (6.48 vs. 4.43 mmol/L; $P = 0.08$).

4.6 Concentrations of Plasma Metabolites

Lambs fed LF diets had greater plasma glucose concentrations compared to those fed HF diets (75.6 vs. 68.3 mg/dL; $P < 0.01$; Table 4.5). Additionally, lambs that were fed SF corn had

Table 4.5. The effects of forage level and grain processing on plasma metabolites (n = 8).

	Experimental diets[1]							
	LF		HF			*P* value[2]		
Item	WS	SF	WS	SF	SEM	F	G	F × G
Plasma metabolites, mg/dL								
Glucose	72.2	78.9	66.2	70.3	2.36	<0.01	0.03	0.58
Urea-N	10.0	9.48	12.8	12.3	1.04	<0.01	0.53	0.98

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.

greater plasma glucose concentrations compared to those fed WS corn (74.6 vs. 69.2 mg/dL; P = 0.03). Plasma urea-N concentrations were affected by forage level, with lambs fed HF diets having greater PUN concentrations compared to those fed LF diets (9.74 vs. 12.6 mg/dL; $P < 0.01$).

4.7 Total Fluxes, Tissue Conductance, and Short-Circuit Current Measurements in Ruminal, Duodenal, and Cecal Epithelia

Dietary treatment had no observable effect on ruminal and cecal $J_{sm\text{-}urea}$; however, lambs fed HF diets had greater duodenal $J_{sm\text{-}urea}$ compared to those fed LF diets (77.6 vs. 57.2 nmol/(cm^2 × h); $P < 0.01$; Table 4.6). Similarly, ruminal and cecal $J_{sm\text{-}mannitol}$ were unaffected by dietary treatment; however, duodenal $J_{sm\text{-}mannitol}$ was greater in lambs fed HF compared to those fed LF diets (36.3 vs. 27.2 nmol/(cm^2 × h); $P < 0.01$). Lambs fed LF had greater ruminal G_t compared to those lambs fed HF (6.40 vs. 3.98 mS/cm^2; $P < 0.01$); however, LF reduced ruminal I_{sc} when compared to HF (0.33 vs. 1.01 μEq/(cm^2 × h); $P < 0.01$). Dietary treatment had no effect on duodenal G_t or I_{sc}. Feeding HF with SF compared to WS tended to increase cecal G_t, whereas G_t between LF combined with WS or SF were similar (interaction; P = 0.05). Various correlations were run to determine the relationship between different variables to $J_{sm\text{-}urea}$. Ruminal NH_3-N concentration had a weak negative correlation to $J_{sm\text{-}urea}$ (r = -0.30; P = 0.11), whereas duodenal NH_3-N concentration exhibited a weak positive correlation to $J_{sm\text{-}urea}$ (r = 0.33; P = 0.07), and cecal NH_3-N concentration exhibited no correlation to $J_{sm\text{-}urea}$ (r = 0.03; P = 0.89) (Figure 4.2). Plasma urea-N concentration was negatively correlated to both ruminal $J_{sm\text{-}urea}$ (r = -0.41; P = 0.02), as well as cecal $J_{sm\text{-}urea}$ (r = -0.15; P = 0.41); however, a positive correlation was observed between duodenal $J_{sm\text{-}urea}$ and PUN concentration (r = 0.28; P = 0.12) (Figure 4.3). For ruminal tissue, G_t had no correlation to $J_{sm\text{-}urea}$ (r = 0.09; P = 0.62), but G_t and $J_{sm\text{-}urea}$ were positively correlated for both duodenal (r = 0.35; P = 0.06) and cecal (r = 0.37; P = 0.05) tissues (Figure 4.4). Ruminal $J_{sm\text{-}mannitol}$ had no correlation to $J_{sm\text{-}urea}$ (r = 0.04; P = 0.84) (Figure 4.5). Conversely, duodenal $J_{sm\text{-}mannitol}$ had a strong positive correlation to $J_{sm\text{-}urea}$ (r = 0.88; $P < 0.01$), and cecal $J_{sm\text{-}mannitol}$ also exhibited a strong positive correlation to $J_{sm\text{-}urea}$ (r = 0.93; $P < 0.01$).

4.8 Abundance of UT and AQP mRNA in Ruminal, Duodenal, and Cecal Epithelia

Within the ruminal epithelia, the fold-change of mRNA expression of AQP-3 was greater in lambs fed LF diets than those fed HF diets (1.21 vs. 0.90; P = 0.03; Table 4.7). Ruminal mRNA abundance for UT-B and AQP-7 were unaffected by dietary treatment. For duodenal tissue, fold-

Table 4.6. The effects of forage level and grain processing on serosal-to-mucosal urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) flux, tissue conductance (G_t), and short-circuit current (I_{sc}) of ruminal, duodenal, and cecal epithelia (n = 8).

	Experimental diets[1]							
	LF		HF			*P* value[2]		
Item	WS	SF	WS	SF	SEM	F	G	F × G
Rumen								
$J_{sm\text{-}urea}$, nmol/(cm^2 × h)	135.3	129.4	122.9	132.5	18.4	0.79	0.91	0.65
$J_{sm\text{-}mannitol}$, nmol/(cm^2 × h)	8.81	9.64	8.15	8.01	1.20	0.09	0.60	0.46
G_t, mS/cm^2	6.26	6.54	3.67	4.29	0.49	<0.01	0.36	0.72
I_{sc}, µEq/(cm^2 × h)	0.12	0.53	1.09	0.93	0.18	<0.01	0.42	0.08
Duodenum								
$J_{sm\text{-}urea}$, nmol/(cm^2 × h)	54.1	60.3	77.8	77.3	6.52	<0.01	0.67	0.61
$J_{sm\text{-}mannitol}$, nmol/(cm^2 × h)	27.1	27.3	40.0	32.5	3.17	<0.01	0.26	0.23
G_t, mS/cm^2	21.5	22.4	21.4	25.2	2.17	0.52	0.27	0.50
I_{sc}, µEq/(cm^2 × h)	0.38	0.60	0.39	0.28	0.15	0.16	0.65	0.14
Cecum								
$J_{sm\text{-}urea}$, nmol/(cm^2 × h)	66.4	55.8	58.9	74.7	6.92	0.42	0.71	0.07
$J_{sm\text{-}mannitol}$, nmol/(cm^2 × h)	30.8	28.8	28.3	38.8	3.79	0.33	0.27	0.11
G_t, mS/cm^2	23.0	21.4	20.8	29.6	2.66	0.25	0.17	0.05
I_{sc}, µEq/(cm^2 × h)	1.30	1.62	1.73	1.91	0.30	0.22	0.40	0.81

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.

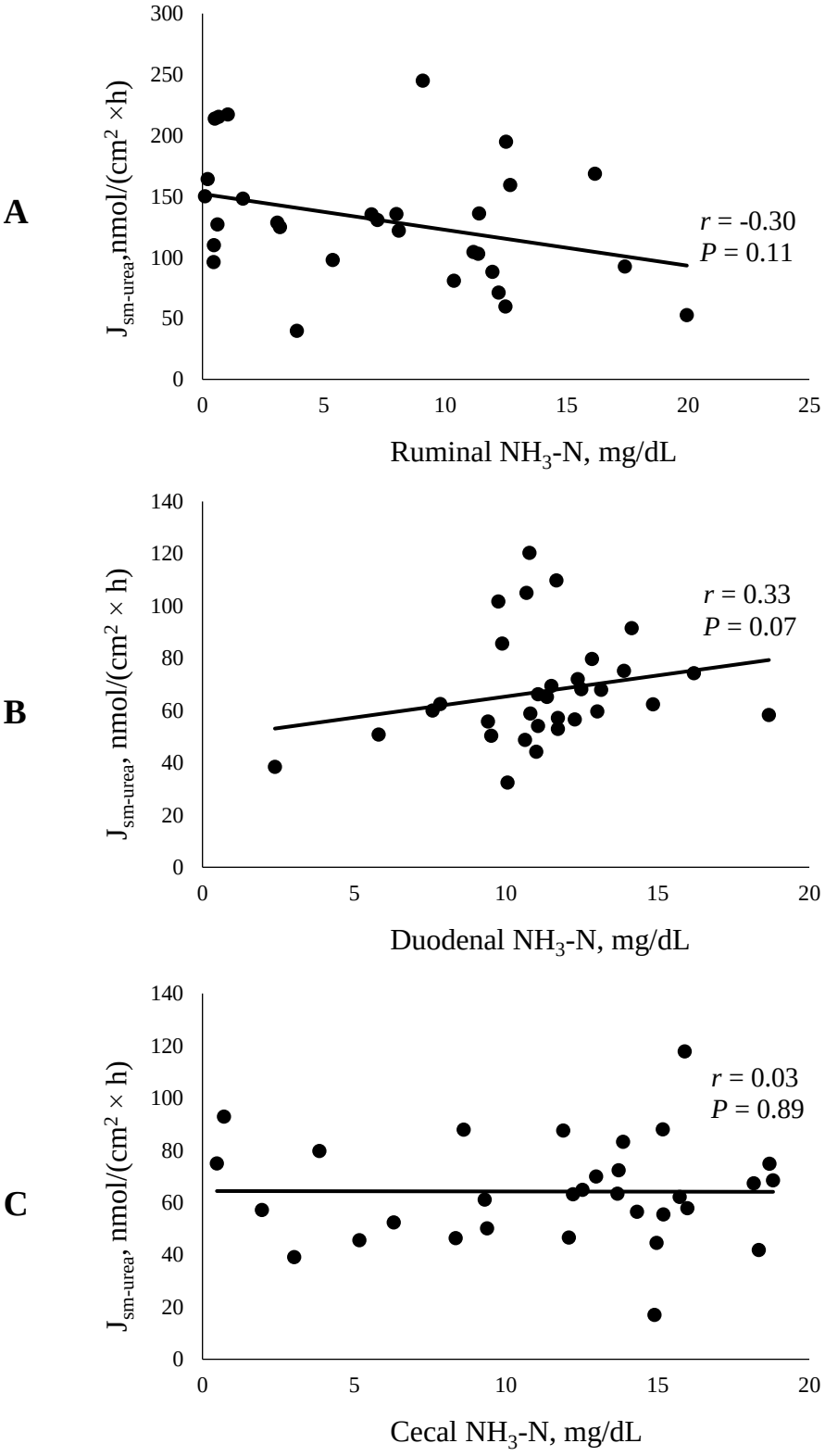

Figure 4.2. Correlations between ruminal (A), duodenal (B), and cecal (C) ammonia (NH_3-N) concentrations and ruminal, duodenal, and cecal serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$).

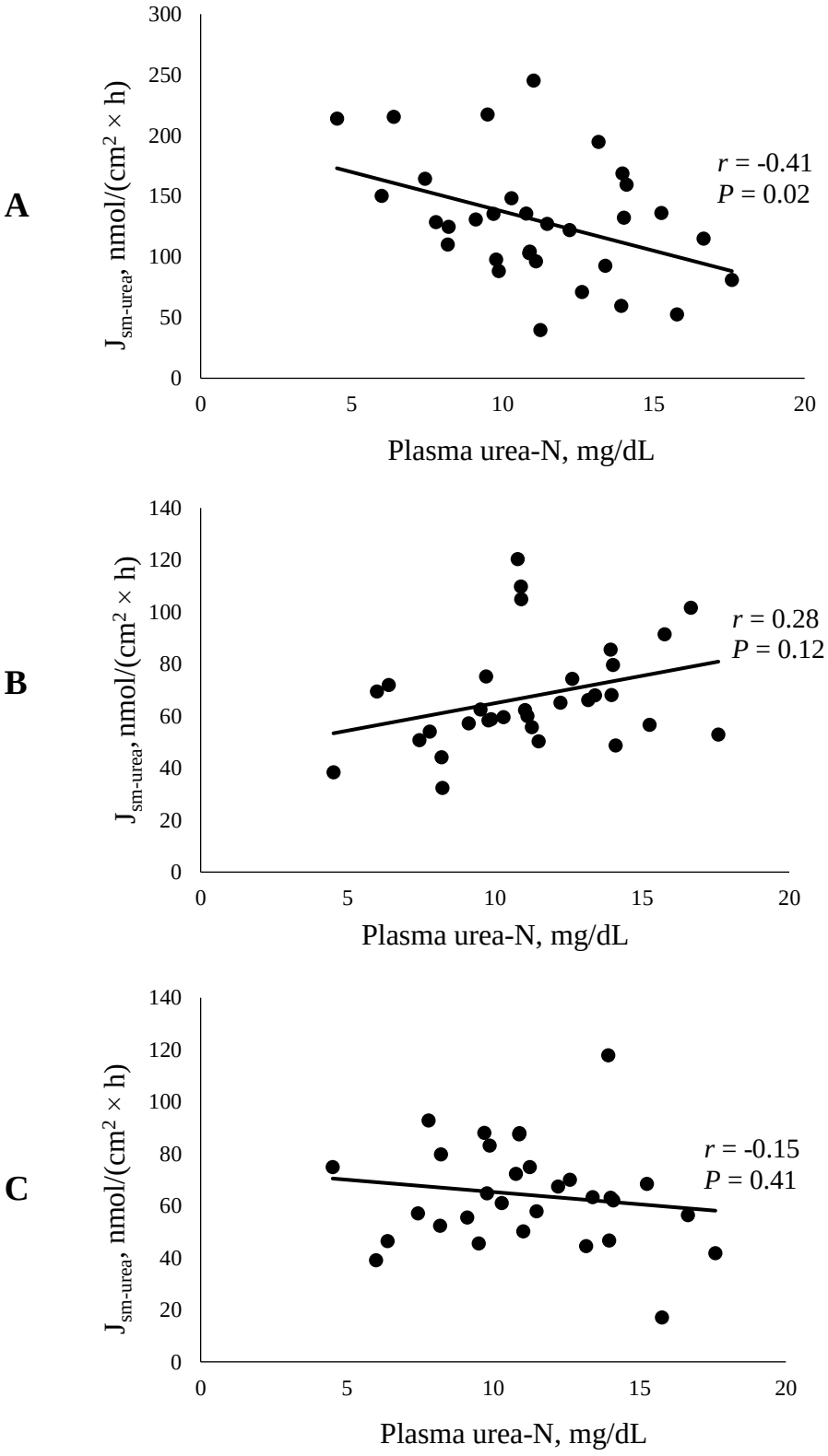

Figure 4.3. Correlations between plasma urea-N (PUN) concentrations and ruminal (A), duodenal (B), and cecal (C) serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$).

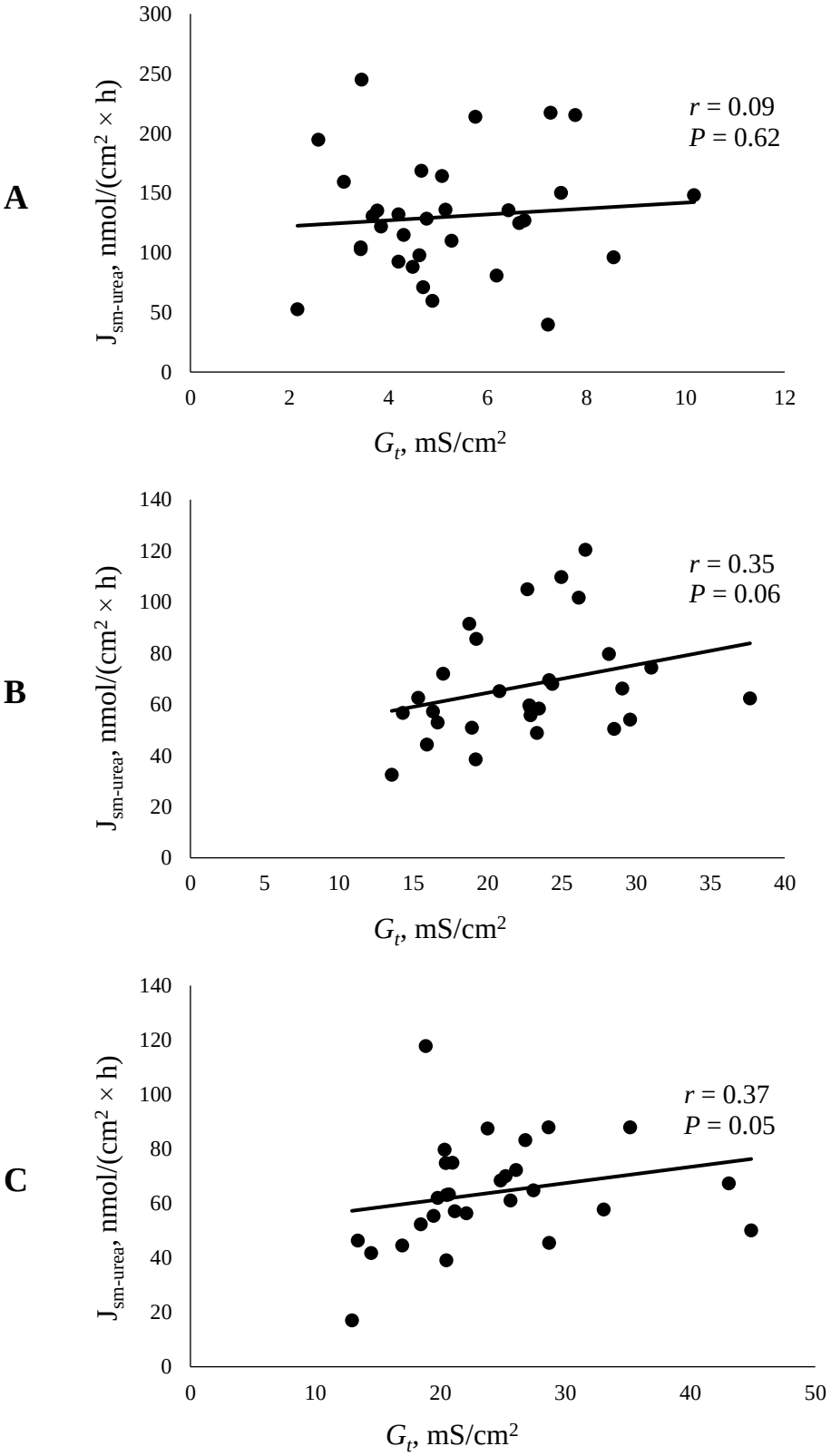

Figure 4.4. Correlations between ruminal (A), duodenal (B), and cecal (C) G_t and ruminal, duodenal, and cecal serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$).

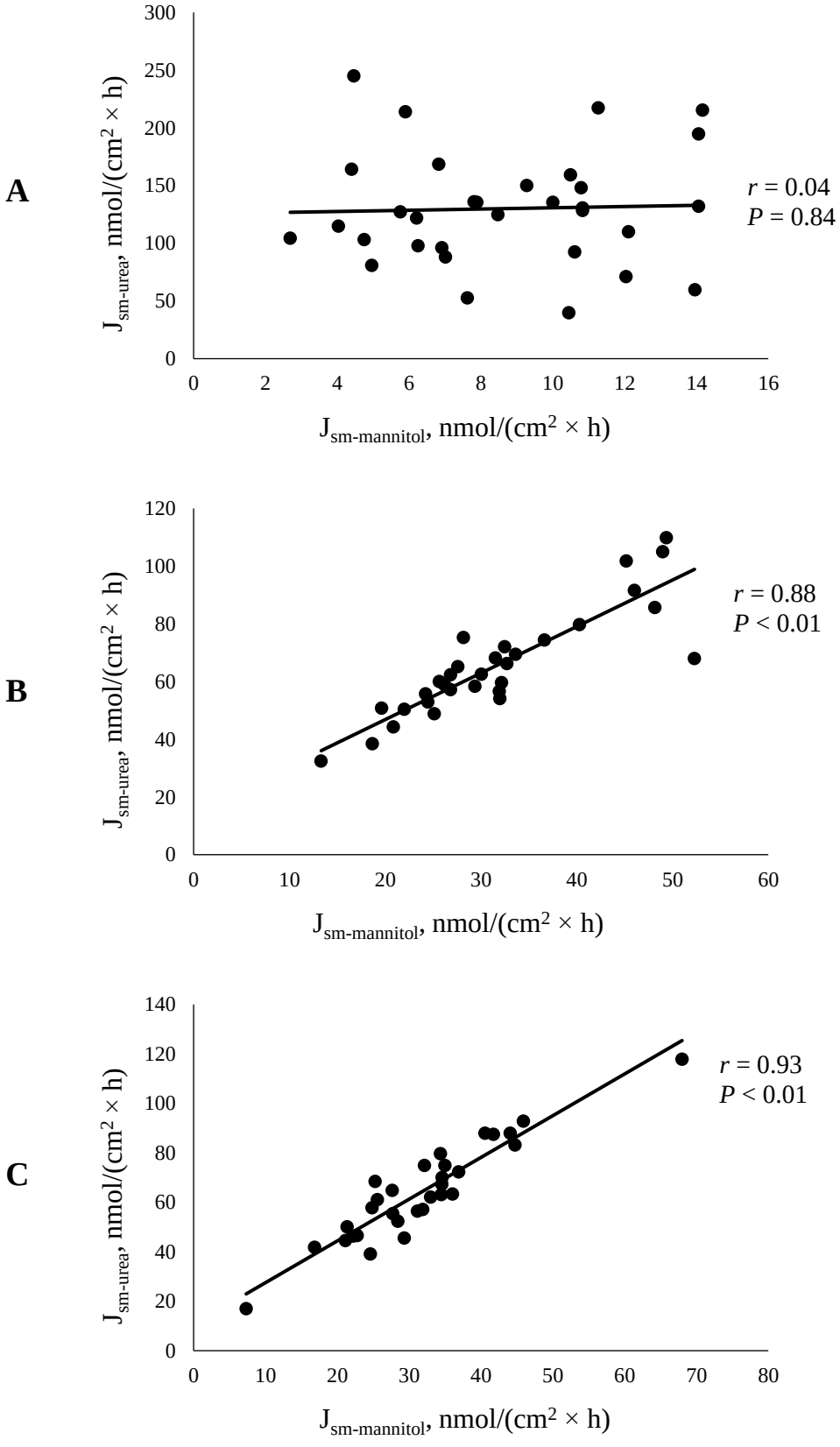

Figure 4.5. Correlations between ruminal (A), duodenal (B), and cecal (C) serosal-to-mucosal mannitol flux ($J_{sm\text{-}mannitol}$) and ruminal, duodenal, and cecal serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$).

Table 4.7. The effects of forage level and grain processing on mRNA abundance (presented as fold-change relative to the designated control diet) of UT-B, AQP-3, and AQP-7 in ruminal, duodenal, and cecal epithelia (n = 8).

	Experimental diets[1]							
	LF		HF[3]			*P* value[2, 4]		
Item[5]	WS	SF	WS	SF	SEM	F	G	F × G
Rumen								
UT-B	1.28	1.21	1.00	0.91	0.19	0.32	0.79	0.85
AQP-3	1.05	1.37	1.00	0.79	0.16	0.03	0.79	0.11
AQP-7	1.85	1.90	1.03	1.00	0.86	0.57	0.35	0.65
Duodenum								
UT-B	1.35	0.68	1.00	0.81	0.26	0.74	0.46	0.36
AQP-3	1.16	1.37	1.00	0.97	0.15	0.06	0.73	0.41
AQP-7	0.85	0.65	1.00	0.65	0.19	0.22	0.08	0.96
Cecum								
UT-B	0.79	0.63	1.00	0.90	0.14	0.07	0.33	0.70
AQP-3	0.85	1.00	1.00	0.97	0.14	0.61	0.68	0.31
AQP-7	1.36	0.70	1.11	1.35	0.37	0.71	0.36	0.57

[1]LF = low forage; HF = high forage; WS = whole-shelled corn; and SF = steam-flaked corn.
[2]F = effect of forage level; G = effect of grain processing; and F × G = interaction of forage level and grain processing.
[3]HFWS designated as control diet to calculate fold change.
[4]For statistical analysis, raw data was transformed using a log10 transformation to meet normal distribution requirements of the statistical model.
[5]mRNA abundance for UT-A and AQP-10 is not reported due to low primer efficiency.

change of AQP-3 tended to be greater in lambs fed LF diets compared to lambs fed HF diets (1.27 vs. 0.99; $P < 0.10$). In terms of grain processing, lambs fed WS corn tended to have greater duodenal expression of AQP-7 compared to lambs fed SF corn (0.93 vs. 0.65; $P < 0.10$). Fold changes for UT-B in duodenal epithelia were unaffected by dietary treatment. In cecal epithelia, UT-B fold-change tended to be greater in lambs fed HF diets compared to those fed LF diets (0.95 vs. 0.71; $P < 0.10$), whereas fold-changes for AQP-3 and AQP-7 were unaffected by dietary treatment. The current study also aimed to investigate expression of UT-A and AQP-10; however, due to low primer efficiency and expression within the studied regions, the data was removed from this thesis.

5.0 DISCUSSION

5.1 Effects of Dietary Treatment on Dry Matter Intake and Apparent Total-Tract Digestibility

Dry matter intake was affected by dietary treatment in the present study, with lambs fed LF diets exhibiting a greater DMI compared to those fed HF diets. It has been previously reported in the literature that fiber content of the diet (i.e., NDF) plays a major role in influencing voluntary feed intake in ruminants (Jung and Allen, 1995; Allen, 1996; Beauchemin, 1996). As dietary NDF content increases, voluntary intake is reduced because the fibrous fractions of the diet contribute to greater ruminal fill and distension, thereby reducing the rate of digesta passage (Jung and Allen, 1995; Beauchemin, 1996; Allen and Piantoni, 2013). Ruminal fill and distension also contribute to physical regulation of feed intake, wherein tension-sensitive mechanoreceptors located in the reticulorumen will generate an action potential when exposed to pressure, resulting in altered feeding behavior driven by the satiety centers in the brain (Leek, 1986; Allen and Piantoni, 2013). In the present study, HF diets contained 84.2% more NDF content compared to LF diets, thereby suggesting that the greater NDF content in HF diets could have reduced DMI due to ruminal fill. Also, it was observed that lambs fed SF corn had greater DMI compared to those fed WS corn. Perusal of the literature indicates that the effects of grain processing on DMI in ruminants are equivocal. In some studies, the provision of more extensively processed grains did not appear to influence DMI (Joy et al., 1997; Crocker et al., 1998; Yang et al., 2001; Doranalli and Mutsvangwa, 2007), whereas other studies have reported reduced DMI in ruminants fed more extensively processed grains (Stock et al., 1995; Owens et al., 1997). The reduction in DMI with

more extensive grain processing has been attributed to induction of ruminal acidosis due to the excessive production and accumulation of SCFA in the rumen (Stock et al., 1995; Owens et al., 1997). More recently, it has been proposed that the increased availability to the liver of oxidative substrates such as SCFA (e.g., propionate) arising from portal uptake when more extensively processed grains are fed generates ATP, resulting in reduced vagal afferent "firing" and, subsequently, inhibition of "feeding centers" in the hypothalamus (Allen et al., 2009). This is referred to as the hepatic oxidation theory (Allen et al., 2009). According to Allen (2000) and Allen et al., (2009), the increase in propionate production and absorption in the rumen of animals fed extensively processed grains provides a major oxidative substrate to the ruminant liver and may be responsible for hypophagic effects. In the present study, ruminal concentration of propionate was greater in lambs fed SF corn compared to those fed WS corn. Because the absorption of propionate into the portal blood is a concentration-dependent process, the greater supply of propionate to the liver in lambs fed SF corn compared to those fed WS corn would have been expected to suppress DMI; however, contrary to these expectations, results from the present study show that lambs fed SF corn had greater DMI than those fed WS corn. The reasons for this discrepancy are unclear.

In the current study, most of the dietary effects observed on apparent total-tract digestibility were due to F:C ratio rather than grain processing. Overall OM digestibility (including DM, CP, EE, and starch) was increased when LF diets were fed to lambs compared to HF diets. This finding is in agreement with the increase in digestibility that is typically associated with low-fiber, high-concentrate diets (Yang et al., 2001). Similar findings in sheep have been reported throughout the literature. Merchen et al. (1986) and Colucci et al. (1989) found that overall OM digestibility improved in sheep fed forage levels at 25% and 30% of dietary DM, respectively, compared to sheep fed forage levels of 75% and 80% of dietary DM, respectively. In dairy cattle, Yang et al. (2001) reported that providing a lower forage level (35% vs. 55% DM) also resulted in improved total-tract OM digestibility. Because concentrate-based diets are lower in fiber content (i.e., NDF constituents – hemicellulose, cellulose and lignin), the inhibitory effects of lignin on enzymatic hydrolysis of cell-wall polysaccharides are greatly reduced, thereby increasing the fermentability of dietary substrate (Jung and Allen, 1995). As fermentability of the diet increases, an increase in total-tract digestibility is expected.

Neutral detergent fiber digestibility tended to be greater, whereas ADF digestibility was greater in lambs fed HF diets compared to those fed LF diets. A similar finding was reported by (Yang et al., 2001), where ADF digestibility was improved in dairy cattle as the amount of forage in the diet was increased. Although the current study measured apparent total-tract digestibility, it is known that the majority of fiber digestion takes place within the rumen due to the activities of the fibrolytic bacterial population (Varga and Kolver, 1997), with the major fiber-digesting bacteria being *Fibrobacter succinogenes, Ruminococcus flavefaciens,* and *Ruminococcus albus* (Cheng et al., 1991). The ruminal ecosystem is made up of diverse microorganisms, and ruminal environmental conditions such as pH and NH_3-N concentration have a major influence on the profile of microorganisms that predominate. Compared to their amylolytic counterparts, fibrolytic bacteria are very sensitive to ruminal pH, and they thrive in a more alkaline pH environment with reduced proliferation observed at ruminal pH ranging between 5.0 to 6.0 (Hoover, 1986). When the growth of fibrolytic bacteria is inhibited in more acidic conditions, ruminal fiber digestion is decreased (Hoover, 1986). Moreover, fibrolytic bacteria have lower maintenance requirements, relying mostly on NH_3-N as their N source for growth as opposed to preformed AA or peptides (Russell et al., 1992). Although bacterial profiles were not determined in this study, mean ruminal pH in lambs fed HF diets was 6.07 whereas it was 5.65 in lambs fed LF diets. In addition, greater ruminal concentrations of NH_3-N were observed in lambs fed HF diets compared to those fed LF diets. Taken together, it can be surmised that the more alkaline ruminal conditions and greater ruminal concentrations of NH_3-N favoured the proliferation of fibrolytic bacteria, thereby potentially explaining the increase in fiber digestibility that was observed in lambs fed HF diets.

It is well-known that grain processing improves nutrient digestibility in ruminants (Lee et al., 1982; Theurer, 1986; Crocker et al., 1998). In corn, steam-flaking greatly enhances digestibility by disruption of the starch-encapsulating protein matrix, and by gelatinization of the starch granules (Zinn et al., 2002), thereby increasing surface area for enzymatic degradation. In the current study, it was expected that the provision of SF corn would increase total-tract nutrient digestibility; however, minimal effects of grain processing were observed. Ruminal pH was lower and total SCFA concentration was greater in lambs fed SF corn compared to those fed WS corn, which could be taken as indirect evidence that feeding SF corn increased the extent of ruminal digestion when compared to feeding WS corn. However, total-tract digestibility was largely unaffected by grain processing. If it is assumed that the extent of ruminal digestion was greater

with SF corn compared to WS corn, then compensatory post-ruminal digestion occurred in lambs fed WS corn such that total-tract digestibilities were similar. Ether extract digestibility was increased by 6.53% in lambs fed WS corn compared to those fed SF corn. Reasons for increased EE digestibility associated with feeding unprocessed corn are not clear; although it has been reviewed in the literature that grain processing methods typically do not enhance digestibility to the same extent in small ruminants as in cattle (Ørskov, 1976; Theurer, 1986). This is thought to be attributed to increases in rumination and mastication associated with feeding small ruminants whole grains (Ørskov et al., 1974).

5.2 Effects of Dietary Treatment on Fermentation Characteristics in the Rumen, Duodenum, and Cecum

Interactive dietary effects on ruminal pH were observed, whereby ruminal pH was similar in lambs fed WS and SF corn in combination with HF diets, but for LF diets ruminal pH was 8.47% lower in lambs fed SF compared to those fed WS corn. The reasons for this interaction are unclear but could be related to the differences in forage content between LF and HF diets. Because SF corn is more ruminally-fermentable compared to WS corn (Lee et al., 1982), it was expected that feeding SF corn would depress ruminal pH when compared to feeding WS corn; however, this was not the case for animals fed diets containing SF and WS corn with HF. It is plausible that the greater forage content with HF diets could have stimulated greater chewing and rumination activities in lambs fed both SF and WS, thus leading to more saliva production. Saliva is rich in HCO_3^- and phosphates, which are important buffering agents that assist in the regulation of ruminal pH (Van Soest, 1994). Although saliva production was not measured in the present study, others (Maekawa et al., 2002; Beauchemin et al., 2008) have reported increased rumination and chewing activities, and saliva production in ruminants fed high forage diets. Thus, we can surmise that sheep fed HF diets in this study produced more saliva which, in turn, could have provided a greater buffering capacity thus possibly attenuating any negative effects of feeding SF corn on ruminal pH.

Ruminal concentrations of acetate and butyrate were unaffected by dietary treatment; however, propionate levels were higher in lambs that were fed LF diets compared to those fed HF diets. Similar findings were reported by Yang et al. (2001), who studied the effects of F:C ratio and extent of barley grain processing on rumen fermentation characteristics in dairy cattle. The

study reported that animals fed a 35:65 F:C ratio diet had higher propionate concentrations in the rumen compared to those animals fed a 55:45 F:C ratio diet (Yang et al., 2001). In the same study, Yang et al. (2001) also reported slight elevations in ruminal propionate concentrations in cattle fed flat steam-rolled as opposed to coarse steam-rolled barley. Similar effects of grain processing on ruminal propionate concentrations were also observed in the current study: lambs fed SF corn had greater propionate concentrations compared to lambs fed WS corn. Lastly, total SCFA concentrations in the rumen were increased by 16.3 mmol/L in lambs fed SF corn compared to those lambs fed WS corn in the current study. This finding can be attributed to the increase in RFC when feeding SF corn vs. WS corn.

No dietary effects were observed on duodenal pH; however, pH values ranged from 4.48 to 4.95 across dietary treatments. Published studies reporting duodenal pH in ruminants are scarce. In Holstein steers fed a diet containing a 50:50 F:C ratio, Pederzolli et al. (2018) found that duodenal pH ranged from 4.99 to 5.28, which are higher than the pH values reported in the current study. This difference in pH range can be attributed to the dietary treatment differences, namely the F:C ratio. When compared to ruminal concentrations, duodenal concentrations of the three major (i.e., acetate, propionate, and butyrate) and total SCFA were much lower. This is not surprising as the duodenum is not considered to be a fermentative region (Sherwood et al., 2013), so the production of SCFA is minimal and total SCFA concentration is not the major "driver" of duodenal pH. As digesta is transferred from the rumen to the proximal duodenum, it passes through the abomasum which typically has a pH ranging from 2.77 to 3.27 (Constable et al., 2005). In the present study, the acidic abomasum is likely to be the major factor influencing the low pH that was observed in duodenal digesta. As already indicated, duodenal concentrations of SCFA were negligible and were not affected by dietary treatment. Pederzolli et al. (2018) also reported trace amounts of SCFA in duodenal digesta of Holstein steers.

Cecal pH decreased by 10.1% when lambs were fed LF diets compared to HF diets. Coupled with this finding, total SCFA concentration in the cecum was increased in sheep fed LF diets compared to those fed HF diets. It can be surmised that the provision of greater levels of dietary concentrate (i.e., by feeding LF diets) resulted in increased fermentative activity in the cecum, resulting in greater total SCFA concentrations. The greater total SCFA concentrations in sheep fed LF compared to HF diets are likely the major "driver" of the observed differences in

cecal pH. Cecal concentrations of acetate, propionate, and butyrate were greater in sheep fed LF compared to HF diets. Similar findings were reported by Siciliano-Jones and Murphy (1989), who observed increased concentrations of acetate, propionate, butyrate, lactate, and total SCFA in the cecum of Holstein steers as dietary provision of concentrate increased. Although SCFA production is the major determinant for decreasing cecal pH, in general, the hindgut's reduced capability of maintaining digesta pH during fermentation and SCFA production should also be considered. Though HCO_3^- is known to pass through the hindgut epithelium as it does across ruminal epithelium, the hindgut does not receive additional HCO_3^- supply through saliva production (Gressley et al., 2011). This, in turn, makes the hindgut region more susceptible to fluctuations in pH with the provision of fermentable diets.

In the present study, corn grain processing had no effect on cecal pH or had only minimal effects on cecal SCFA concentrations. Because WS corn is less fermentable in the rumen compared to SF corn, it was anticipated that feeding WS could shift the site of starch digestion from the rumen to post-ruminal sites. However, the lack of effect of corn grain processing on fermentation characteristics in the cecum imply that feeding WS corn did not result in more corn being fermented in the cecum. Ørskov et al. (1974) found that apparent total-tract digestibility of processed barley (pelleted) in lambs was not greatly affected compared to feeding the whole barley grains. In that study, Ørskov et al. (1974) also recorded rumination time and number of regurgitations. Though time spent eating was almost identical (mean of 158 min/24 h), lambs fed whole barley ruminated an additional 186 min/24 h and regurgitated an additional 258 times/24 h compared to lambs fed pelleted barley. These observations suggest that, when fed whole compared to processed cereal grains, sheep spend more time ruminating and this could limit the amount of whole grains reaching post-ruminal sites.

Ruminal NH_3-N concentrations were reduced by 8.90 mg/dL when LF diets were fed compared to HF diets. It is well-established that ruminal NH_3-N concentrations are influenced by a multitude of factors, including N intake, and dietary contents of RDP and RFC. Typically, ruminal NH_3-N concentration increases in tandem with N intake (Doranalli et al., 2011; Davies et al., 2013); however, results from the present study indicate that, although N intake was 34.0% greater in lambs fed LF compared to those fed HF diets, lambs on LF diets had lower ruminal NH_3-N concentrations. The greater ruminal NH_3-N concentrations with HF diets could be partly

attributed to increased provision of dietary RDP and reduced dietary RFC. Although dietary RDP content was not measured in the current study, predicted values obtained from NDS (CNCPS v6.5) based on dietary ingredient composition indicated that RDP contents for the LF and HF diets were 5.34 and 6.24% (as % of dietary DM), respectively, thus suggesting that provision of RDP in lambs fed LF diets could have been reduced compared to lambs offered HF diets. Moreover, chemical analysis of experimental diets indicated that soluble protein levels (as a % of CP) were 8.3% units lower in LF diets compared to HF diets. Taken together, the lower dietary RDP content coupled with a lower soluble protein content for LF diets compared to HF diets could have resulted in reduced substrate availability for ruminal proteolytic bacteria, thus potentially reducing ruminal NH_3-N concentrations (Bach et al., 2005). Available evidence indicates that increasing ruminal energy availability by feeding more dietary concentrates (e.g., RFC) is associated with reduced ruminal NH_3-N concentrations, primarily because of a more efficient sequestration of ruminal NH_3-N for microbial growth (Seal et al., 1992; Yang et al., 2001; Davies et al., 2013). Thus, in the present study, providing more RFC by feeding more concentrate (i.e., LF diets) could have reduced ruminal NH_3-N concentration through this mechanism. In support of this assertion, lambs fed LF diets had a lower ruminal pH and numerically greater ruminal concentrations of total SCFA, thus suggesting a greater extent of ruminal carbohydrate fermentation when compared to lambs fed HF diets.

In addition to LF diets influencing ruminal NH_3-N levels, lambs fed SF corn also exhibited lower levels of ruminal NH_3-N compared to lambs fed WS corn. As discussed above, it has been reported throughout the literature that increasing provision of dietary concentrate and RFC availability improves NH_3-N sequestration in the rumen (Kennedy and Milligan, 1980; Lee et al., 1982; Huntington, 1989; Davies et al., 2013). In the present study, total SCFA concentrations were increased in lambs were fed SF corn as opposed to those fed WS corn, thus suggesting a greater ruminal energy availability with SF diets. This greater ruminal energy availability allowed improved sequestration of NH_3-N into microbial protein, thereby decreasing ruminal NH_3-N levels. In agreement with the present study, Davies et al. (2013) reported that greater provision of RFC (by means of grain processing) in the diets of beef heifers resulted in a decrease in ruminal NH_3-N concentration, coupled with a numerical increase in total SCFA production.

According to Tan and Murphy (2004), concentrations of NH_3-N in the digesta of post-ruminal regions are substantial, with NH_3-N being sourced primarily from ruminal outflow, and ureolytic catabolism of recycled endogenous urea. In the current study, duodenal concentrations of NH_3-N were not different between dietary treatments, but ranged from 10.0 to 12.1 mg/dL. Studies presenting information on duodenal digesta NH_3-N concentrations are limited; however, Plaizier et al. (2014) reported jejunal digesta NH_3-N concentrations ranging from 7.34 to 11.90 mg/dL in yearling Holstein calves fed concentrate-based diets for varying lengths of time, and these data are in the same range as data observed in the present study.

Decreasing dietary forage level resulted in a reduction of cecal NH_3-N concentration by 39.3%. Although the extent of carbohydrate digestion was not determined within the different compartments of the GIT, it was anticipated that there would be a greater flow of undigested dietary carbohydrates reaching the lower GIT with LF diets compared to HF diets. If this was the case, then that would provide more substrate for microbial fermentation in the lower GIT. Indirect evidence in support of greater fermentative activity in the lower GIT in lambs fed LF diets is provided by the fermentation characteristics that were observed in the cecum, showing reduced cecal pH as well as increased total SCFA concentrations with LF compared to HF diets. With greater cecal fermentation, the increased energy availability to the microbial population could potentially result in reduced cecal NH_3-N concentrations as the microorganisms would be more efficient at sequestering NH_3-N. Similar findings were reported by Ørskov et al. (1970), who reported that cecal starch infusions in sheep resulted in an 83% increase in cecal microflora counts as well as a 3.2-g increase in fecal N excretion compared to sheep not receiving cecal starch infusions. Such findings support the notion of bacterial sequestration of free NH_3-N in the cecum during periods of enhanced fermentation, leading to a reduction in free cecal NH_3 (Tan and Murphy, 2004).

5.3 Effects of Dietary Treatment on Plasma Glucose and Urea-N Concentrations

Concentrations of plasma glucose were elevated in lambs fed LF diets compared to those fed HF diets. This finding is in agreement with previous studies assessing the effects of dietary forage level on plasma glucose concentrations in ruminants (Evans et al., 1975; Evans and Buchanan-Smith, 1975; Seal et al., 1992). Ruminants perpetually depend on hepatic gluconeogenesis to meet their glucose needs, with propionate that is derived from microbial

fermentation of dietary carbohydrates being the major precursor for glucose synthesis (Danfaer et al., 1995). In the present study, feeding LF diets elevated propionate concentrations in the rumen and cecum. As propionate absorption into portal blood is concentration-dependent (Aschenbach et al., 2011), greater propionate concentration in the rumen and cecum could potentially increase substrate availability for hepatic gluconeogenesis which, in turn, would elevate plasma glucose concentrations in lambs fed LF diets. Similarly, lambs fed SF corn compared to those fed WS corn also exhibited increased concentrations of plasma glucose. Ørskov et al. (1974) conducted an experiment assessing the effects of grain type and extent of processing on ruminal fermentation characteristics. In all grain types, provision of pelleted cereal grains increased propionate levels in the rumen compared to feeding whole cereal grains. The increase in ruminal propionate production observed in the present study with lambs fed SF corn is in agreement with the findings of Ørskov et al. (1974), and provide an explanation for the reported increase in plasma glucose concentrations.

Plasma urea-N concentration increased by 29.4% when lambs were fed HF diets compared to LF diets. The major source of N for hepatic ureagenesis is NH_3 derived from the GIT, primarily the rumen and, to a smaller extent, the cecum (Hoover, 1978). In the present study, both ruminal and cecal NH_3 concentrations were greater in lambs fed HF diets compared to those fed LF diets. Because the absorption of NH_3 into the portal blood is a concentration-dependent process, we can surmise that, when feeding HF diets, portal uptake of NH_3 was increased compared to LF diets. This would result in increased provision of substrate for hepatic ureagenesis. Supporting evidence for increased hepatic ureagenesis for lambs fed HF diets is provided by the numerical increase in UER compared to those fed LF diets, as determined by the [$^{15}N^{15}N$]-urea isotopic technique (Lobley et al., 2000). Other studies have also reported increased PUN concentrations coupled with elevated ruminal NH_3 concentrations (Doranalli and Mutsvangwa, 2010; Bailey et al., 2012).

5.4 Effects of Dietary Treatment on Apparent Nitrogen Utilization

In the current study, dietary N intake was 34.0% greater in lambs fed LF diets compared to those fed HF diets. Similarly, N intake in lambs fed SF corn was 42.1% greater than in lambs fed WS corn. Because diets were formulated to be isonitrogenous (and chemical analysis indicated that there were only small differences in dietary CP contents, with a range of 10.5 to 11.0% CP), the differences in N intake are reflective of the differences that were observed in DMI.

Data on the major variables that are related to N utilization (i.e., urinary and fecal N excretion, total N excretion, and apparent N balance) revealed that, on a quantitative basis (i.e., g/d), dietary F:C ratio and grain processing had major effects on N utilization; however, it should be noted that N intakes were also influenced by dietary treatments, so direct comparisons of these quantitative data relating to N utilization are not very meaningful as they would be affected by the level of N intake. For this reason, the focus of this discussion will be on the major variables of N utilization when expressed as a proportion of N intake, which in this case are more meaningful comparisons. Total N loss in the urine (as a percentage of N intake) was 35% units greater in lambs fed HF diets compared to those fed LF diets. Typically, as N intake increases, there is a corresponding increase in the amount of urinary N excreted when expressed as a proportion of N intake (Marini et al., 2004). The major form of urinary N excretion was urea-N, which represented 77.6 and 52.1% of total urinary N excretion in lambs fed HF and LF diets, respectively. The differences in urinary N excretion, therefore, largely arise from differences in urea-N excretion. Urinary urea-N arises from hepatic ureagenesis, with ruminally-derived NH_3 being the major source of N (Huntington and Archibeque, 1999). It is well established that the absorption of NH_3 from the rumen into the portal blood is a concentration-dependent process (Tan and Murphy, 2004); although ruminal absorption of NH_3 into portal blood was not measured in the present study, it can be surmised that lambs fed HF diets likely exhibited greater NH_3 uptakes because they had greater ruminal NH_3 concentrations when compared to those fed LF diets. This would have provided more substrate for hepatic ureagenesis in lambs fed HF diets, thus the greater excretion of urea-N in urine when compared to lambs fed LF diets. Other studies, such as that conducted by Lobley et al. (2000), have also reported numerical increases in urinary urea-N (as a percent of total urinary N) in sheep fed a 50:50 F:C diet compared to a 50:50 grass pellet and loose hay diet. In terms of grain processing effects on urinary N excretion, lambs fed WS corn had greater total urinary N excretion when expressed as a percentage of N intake compared to lambs fed SF corn. In addition, similar trends were observed for urea-N excretion as a percentage of total urinary N. Therefore, as discussed previously, the observed difference in total urinary N excretion can, in part, be attributed to the differences observed in urinary urea-N excretion. Lambs fed WS corn exhibited increased ruminal NH_3 concentrations compared to lambs fed SF corn. Based on the preceding discussion regarding the influence of forage level on urinary N excretion, similar mechanisms involving ruminally-derived NH_3 would be responsible for the differences in urinary

N excretion and urinary urea-N excretion (as a percentage of N intake) between lambs fed WS and SF corn. Similar studies in sheep (Doranalli and Mutsvangwa, 2007) and cattle (Gozho et al., 2008; Hales et al., 2012; Davies et al., 2013) have assessed the effects of grain processing on urinary N losses; however, the reported results do not show the same relationship observed in the present study. Matras et al. (1991) also assessed the effects of grain source and processing on N utilization, and found that although urinary N excretion (g/d) was greatest in lambs fed whole barley grain, urinary N excretion (g/d) was greater in lambs fed rolled sorghum compared to steam-flaked sorghum. Although urinary losses did vary between treatment groups, overall N balance (in g/d, and as a percentage of N intake) was not affected (Matras et al., 1991).

Evidence available in the literature indicates that greater N intakes will often result in an increase in urinary N excretion, whereas fecal N losses largely remain constant (Marini et al., 2004; Davies et al., 2013). In the current study, when expressed as a proportion of N intake, fecal N excretion exhibited a narrow range across dietary treatments, ranging from 32.4 to 37.8%, with a tendency for lambs fed HF diets to exhibit a 4.6% unit increase in fecal N excretion compared to those fed LF diets. This finding suggests that the 63.5% increase in total N excretion (calculated by the sum of total N losses in both the urine and the feces), expressed as a percentage of N intake, between HF and LF fed lambs can be attributed primarily to differences in urinary N losses. Similarly, lambs fed WS corn also had greater total N excretion (as a percentage of N intake) when compared to lambs fed SF corn. As mentioned above, the disparity between grain processing treatments on total N excretion can be attributed to the difference in urinary N loss; lambs fed WS corn had a 61.6% increase in total urinary N loss (as a percent of N intake) compared to lambs fed SF corn. Other studies assessing N balance in sheep have also reported greater variation in urinary N output compared to fecal N output, thereby increasing total N excretion (Marini et al., 2004; Doranalli et al., 2011; Agarwal et al., 2015), though of course these findings are dependent on dietary influence.

Apparent N balance is calculated as the difference between N intake and total N losses (through both the feces and the urine) and provides a “snapshot” of the N content within the body that can be allocated towards anabolism and production. As recommended by the NRC (1985), CP requirements for growing lambs weighing either 30 or 40 kg is between 14.7 to 11.6% CP (DM basis), respectively. In the current study, CP content ranged from 10.5 to 11.0% CP (DM basis),

indicating that these diets were deficient in CP by NRC (1985) standards (for the objectives of this study, low CP levels were desired so as to maximize urea recycling). In the present study, lambs fed LF diets had a greater apparent N balance, when expressed as a percentage of N intake, compared to lambs fed HF diets. Additionally, lambs fed SF corn also exhibited an increase in apparent N balance, when expressed as a percentage of N intake. The differences in N balance between the two groups can be attributed to the increased N loss through urinary excretion between the treatment groups, as discussed previously. By the ability to retain more N through urea recycling (for both lambs fed LF, as well as lambs fed SF), these animals were able to remain in a positive N balance despite being faced with a protein deficit. Though we can assume that the N retained by these animals would go towards anabolic usage, average daily gain was not recorded in the present study as growth performance was not an objective. This limits our ability to suggest potential rates of daily protein accretion within the lambs.

5.5 Effects of Dietary Treatment on Whole-Body Urea Kinetics, Regional $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, and mRNA Abundance of UT and AQP

One of the major objectives of the present study was to assess the effects of forage level and grain processing (as a strategy to manipulate the site of starch fermentation) on whole-body urea kinetics. It is well-established that urea secretion into the GIT, particularly the rumen, is an important evolutionary adaptation that allows ruminants to maintain a positive N balance when dietary N supply might be limited (Lobley et al., 2000; Marini and Van Amburgh, 2003). When N intake is limiting, urea that is recycled to the rumen becomes an important source of N for microbial protein production, thus supplying essential amino acids to the host animal when microbes flowing out of the rumen are subsequently digested in the small intestine (Fuller and Reeds, 1998). Thus, it is important to improve our understanding of the mechanisms that regulate urea recycling so that strategies can be developed to enhance urea secretion into the GIT. In the present study, endogenous urea production (i.e., UER) ranged from 13.5 to 20.8 g/d across dietary treatments. These data are in agreement with published studies (11.5 to 22.4 g/d, Sarraseca et al., 1998; 2.40 to 19.2 g/d, Marini et al., 2004) that have reported UER in sheep at similar N intakes as in the present study. The rate of endogenous urea production is dependent on many factors, such as the level of dietary N intake (Marini and Van Amburgh, 2003) and ruminal NH_3 concentration (Doranalli and Mutsvangwa, 2010). Although only a tendency, UER in lambs fed HF diets were 23.1% greater compared to lambs fed LF diets. This response could partly be attributed to the

observed differences in ruminal NH_3 concentration, as lambs fed HF diets exhibited greater (by +8.90 mg/dL) ruminal NH_3 concentrations compared to those fed LF diets. As discussed elsewhere in this thesis, the absorption of NH_3 into the portal blood is a concentration-dependent process, so it is plausible that lambs fed HF diets absorbed more NH_3 from the rumen than those fed LF diets, thus more substrate was available for hepatic ureagenesis. Because ruminally-derived NH_3 is a major source of N for hepatic ureagenesis (Reynolds and Huntington, 1988; Huntington, 1989; Theurer et al., 2002), it is not surprising that other studies (Wickersham et al., 2008; Davies et al., 2013) have also reported greater UER with elevated ruminal NH_3 concentrations. The endogenous urea produced in the liver can then be released into the bloodstream, and the greater PUN concentrations in lambs fed HF diets compared to those fed LF diets confirm the greater rates of endogenous urea production with HF diets mentioned above. Presumably, if the site of starch fermentation was manipulated by the degree of corn grain processing, this had no influence on UER even though ruminal NH_3 concentrations were greater in lambs fed WS corn compared to those fed SF corn. The reasons for this discrepancy when compared to the responses with forage level are unclear. However, it should be noted that the difference in ruminal NH_3 concentration between lambs fed WS and SF corn (i.e., 3.59 mg/dL) was much smaller than that between lambs fed HF and LF diets (i.e., 8.90 mg/dL); thus, it can be surmised that there might not have been differences in substrate supply for hepatic ureagenesis (i.e., ruminally-derived NH_3) between lambs fed WS and SF corn. Indirect evidence for this assertion is provided by the observation that PUN concentrations were not altered by grain processing.

The difference between UER and UUE provides an estimate of GER, which is the amount of endogenously produced urea-N that is recycled to the GIT. Essentially, GER represents the evolutionary mechanism ruminants have acquired in order to increase N retention when faced with a protein deficit. Although it is known that urea is secreted to both ruminal and post-ruminal sites, the magnitude of entry is greatest at the rumen, and is of the greatest anabolic benefit to the animal (Lapierre and Lobley, 2001). Many physiological factors play a role in the regulation of GER, including GIT NH_3-N concentrations (Lu et al., 2014), SCFA and reduced pH (Abdoun et al., 2010; Lu et al., 2015), and PUN concentrations (Sunny et al., 2007; Muscher et al., 2010). In the current study, GER was not affected by dietary treatment; however, the fractional transfer of UER to GIT (i.e., the GER:UER ratio) was 25.8% greater in lambs fed LF diets compared to those fed HF diets. The greater GER:UER ratio that was observed in lambs fed LF diets compared to those

fed HF diets could be partly attributed to the provision of more RFC when feeding LF diets. The dietary provision of more RFC by decreasing dietary forage content has been demonstrated to increase urea secretion into the rumen (Huntington, 1989). Because the sequestration of ruminal NH_3 into microbial protein is dependent on energy availability (Bach et al., 2005), providing more RFC is typically associated with a decrease in ruminal NH_3 concentration (Seal et al., 1992; Davies et al., 2013). In fact, in the present study, ruminal NH_3 concentrations were lower in lambs fed LF diets compared to those fed HF diets. It has been shown in various ex vivo studies (Abdoun et al., 2006; Lu et al., 2014) that increasing luminal NH_3 concentration has inhibitory effects on $J_{sm\text{-}urea}$ across ruminal epithelia, so the lower ruminal NH_3 concentrations in lambs fed LF diets compared to those fed HF diets could partly explain the greater GER:UER ratio that was observed with LF diets. It has been reported in the literature that urea-N transfer into the GIT is positively correlated with PUN concentrations (Reynolds and Huntington, 1988; Sunny et al., 2007). However, in the current study, lambs fed LF diets had lower PUN concentrations compared to those fed HF diets, so the greater GER:UER ratio in lambs fed LF diets compared to those fed HF diets is incongruent with the reported positive correlation between urea-N transfer to the GIT and PUN concentration. Lapierre and Lobley (2001) suggested that the positive correlation between urea-N transfer to the GIT and PUN concentrations might not apply in all feeding situations. For example, when PUN concentration was elevated in sheep by using acute mesenteric vein infusion of amino acids, only a weak correlation between urea-N flux across the PDV and PUN concentration was observed (r^2 = 0.21) (Lobley et al., 1998; Lapierre and Lobley, 2001). According to Lapierre and Lobley (2001), weak correlations (range of r^2 values = 0.01 to 0.19) were reported between urea-N flux across the PDV and PUN concentration in beef and dairy cattle under a wide range of dietary conditions. In the current study, although PUN concentrations were greater in lambs fed HF diets compared to those fed LF diets, it is plausible that the greater ruminal NH_3-N concentrations in lambs fed HF diets could have inhibited urea-N entry into the rumen as has been reported by others (Abdoun et al., 2006; Lu et al., 2014), thus negating any stimulatory effects of elevated PUN concentration on urea-N transfer to the GIT.

In the current study, there was a 15.4% increase in GER:UER in lambs fed SF corn as opposed to WS corn. Other studies have reported similar findings with the dietary provision of more extensively processed grains. For instance, Delgado-Elorduy et al. (2002) reported a 143.1% increase in urea-N recycled to the GIT in dairy cattle fed steam-flaked corn as opposed to those

fed steam-rolled corn. Similarly, Theurer et al. (2002) also reported increases in urea-N flux to ruminal tissues, as well as total PDV, in steers fed steam-flaked sorghum as opposed to those fed dry-rolled sorghum. Because extensive grain processing is a method utilized to increase dietary RFC, provision of SF corn likely increased the GER:UER ratio by mechanisms similar to those previously discussed for forage level. For example, ruminal NH_3 concentrations were lower in lambs fed SF corn compared to lambs fed WS corn, suggesting that provision of SF corn increased energy availability in the rumen, facilitating microbial NH_3 sequestration, thereby mitigating the inhibitory effects of free NH_3 on ruminal urea secretion into the GIT. In addition to this, it has been reported that provision of high dietary levels of RFC may increase the expression of UT-B in the ruminal epithelia of goats compared to feeding diets low in RFC (Lu et al., 2015). Although not significant, the current study showed a numerical increase in the mRNA expression of UT-B in the ruminal epithelia of lambs fed LF diets compared to those fed HF diets, which may facilitate the transfer of UER to the GIT. This conclusion is to be made with caution, however, as there was no dietary effect on ruminal $J_{sm\text{-}urea}$ in the current study.

When urea is recycled back to the GIT, it is subject to many fates, including usage for anabolism, reabsorption and ureagenesis, and excretion in the feces. Of these fates, the first is the most crucial for the ruminant, as it contributes to the animal's maintenance and growth requirements. Calculated by difference (UUA = GER - [UFE + ROC]), UUA represents endogenous urea that is retained for the animal's anabolic usage. As described by Lobley et al. (2000), the isotopic infusion model assumes that the major component of UUA is the sequestration of free NH_3 into microbial protein in the rumen (which is subsequently digested in the small intestine and contributes to the metabolizable protein requirement). However, liberated NH_3 in the GIT sourced from recycled endogenous urea-N can also be reabsorbed across the GIT epithelia and contribute to amination and transamination reactions within various tissues (Lobley et al., 2000). Of the two reactions, transamination reactions involve only the exchange of an amino group rather than contributing to the formation of an AA; therefore, it is possible that UUA values may be overestimated in the isotopic infusion model (Lobley et al., 2000). In the present study, lambs fed LF diets had 96.5% increase in UUA, and a 116.7% increase in UUA as a proportion of GER (i.e., the UUA:GER ratio) compared to lambs fed HF diets. In addition, lambs fed SF corn tended to exhibit an increase in UUA (g/d) over lambs fed WS corn. Because the major anabolic use of recycled urea-N is via its contribution of a N (as NH_3) source for microbial protein synthesis,

which is an energy-dependent process, the extent to which recycled urea-N can be used for this purpose largely depends on the availability of RFC (Joy et al., 1997; Crocker et al., 1998; Bach et al., 2005). In the present study, the provision of more RFC via feeding more concentrate (i.e., feeding LF diets) or more extensively processed grain (i.e., SF corn) resulted in greater anabolic use of recycled urea-N. Although the study did not use the [$^{15}N^{15}N$]-urea isotopic technique, Delgado-Elorduy et al. (2002) used the V-A method to determine the effects of feeding steam-rolled or SF corn on splanchnic N metabolism in lactating dairy cows. The study reported that cows fed SF corn had a tendency for increased urea-N flux (g/d) into the PDV compared to cows fed steam-rolled corn. Joy et al. (1997) and Crocker et al. (1998) both studied the effects of corn grain processing on N passage throughout the GIT of lactating dairy cows, and reported that as dietary inclusion of SF corn increased, non-NH_3-N (as a percent of N intake) flow to the duodenum was greater (Crocker et al., 1998), and was numerically greater (Joy et al., 1997) compared to cows fed dry-rolled corn. Moreover, Joy et al. (1997) also found that provision of SF corn resulted in increased flow of microbial N (as a percent of non-NH_3-N) to the duodenum compared to cows fed dry-rolled corn. When interpreted together, the data collected by these studies might suggest that increased provision of RFC improves urea-N recycling to the GIT, and subsequent sequestration of endogenously-derived NH_3 for anabolic usage. Other studies have assessed the influence of dietary concentrate level (Lobley et al., 2000) and grain processing (Doranalli and Mutsvangwa, 2007; Davies et al., 2013) on whole-body urea kinetics using the [$^{15}N^{15}N$]-urea isotopic technique; however, they did not observe dietary effects on UUA (g/d, or as a proportion of GER). A potential reason for this could be attributed to the limitations of estimating UUA in the isotopic infusion model: (1) the model lacks the ability to differentiate between the anabolic routes of labelled N (as previously mentioned), thus potentially assuming a larger contribution to microbial protein production; and (2) because UUA is calculated by difference, UUA values obtained in the model are subject to cumulative calculation errors. Unfortunately, these limitations can lead to overestimates of UUA, providing potential challenges in elucidating dietary effects.

In the present study, no dietary effects were observed on total ROC values (g/d); however, as a proportion of GER (i.e., the ROC:GER ratio), lambs fed HF diets had a 0.19 unit increase in urea-N that re-entered the ornithine cycle compared to lambs fed the more fermentable LF diet. This finding was expected given the large difference in fermentable substrate between the HF and LF diets. In the rumen, numerical increases in total SCFA production along with a reduction in pH

in lambs fed LF diets compared to HF diets is indicative of increased energy availability for microbial protein synthesis. Moreover, ruminal NH_3 concentrations were lower in the LF group than the HF group, suggesting improved NH_3 sequestration by the microbial community. In the farther fermentative region, the cecum, similar findings were also found: lambs fed LF diets had increased total SCFA concentrations, reduced regional pH, and reduced cecal NH_3 concentrations compared to lambs fed the HF diet, once more suggesting improved microbial sequestration of NH_3 sourced from recycled endogenous urea. These findings would suggest that lambs fed LF diets had an improved ability to sequester NH_3-N derived from the degradation of endogenous urea over their HF fed counterparts, resulting in less NH_3 subject to absorption across the ruminal epithelia and re-transformation into urea by the liver.

As mentioned above, NH_3 obtained from the hydrolysis of endogenous urea may be subject to excretion through the feces (Lobley et al., 2000). The literature reports that 3 to 20% of GER is excreted in the feces of cattle (Marini and Van Amburgh, 2003; Davies et al., 2013), and 1.5 to 7.1% in sheep (Doranalli and Mutsvangwa, 2007; Lobley et al., 2000), with the results varying widely due to the influence of diet. For example, it is known that energy provision within the hindgut has a major influence on the proportion of urea-N lost in the feces, particularly in sheep (Thornton et al., 1970; Oncuer et al., 1990). In the current study, lambs fed LF diets exhibited a 103% increase in UFE (g/d) compared to lambs fed HF diets. Moreover, as a proportion of GER (i.e., the UFE:GER ratio), lambs fed LF diets had a 133% increase in the amount of UFE compared to lambs fed HF diets. It has been previously reported by Thornton et al. (1970) that provision of glucose to the terminal ileum of sheep resulted in an increase in the total amount of fecal N compared to sheep infused with water. The group suggested that this increase in fecal N excretion could be attributed to endogenous urea-N being transferred from the blood to the hindgut, providing a N source to the microbial population, resulting in microbial protein N being excreted in the feces (Thornton et al., 1970). In addition to this finding, the group also reported a decline in the amount of urinary urea-N as excretion of fecal N increased, allowing them to conclude that under favorable fermentative conditions in the hindgut of sheep, endogenous urea-N will be preferentially recycled to the latter regions of the GIT as opposed being excreted in the urine (Thornton et al., 1970). Similar findings were reported in the current study, wherein lambs fed LF diets exhibited a 52.7% decrease in UUE, and a 42.1% decrease in UUE as a proportion of UER (i.e., the UUE:UER ratio). Knowing that the LF diets in the present study provided greater amounts

of fermentable substrate to the hindgut (see previous paragraph for supporting evidence), we can surmise that energy availability and ATP yield from OM digestion in the hindgut of lambs fed LF diets supported microbial protein synthesis, thereby reducing cecal NH_3 concentrations, and promoting endogenous urea transfer to the hindgut. In lambs fed SF corn, UFE was 0.27 g/d greater than lambs fed WS corn. As a proportion of GER, lambs fed SF corn also had a 50.0% increase in UFE:GER compared to lambs fed WS corn. Doranalli and Mutsvangwa (2007) reported a grain processing effect on UFE in growing lambs; lambs fed dry-rolled barley tended to have greater UFE:GER compared to those fed pelleted barley. Other studies have also assessed the influence of grain processing on UFE (Gozho et al., 2008; Davies et al., 2013), with no treatment effects observed. It is known, however, that processing of cereal grains does increase energy availability and starch digestibility (Theurer, 1986), with steam-flaking corn shown to increase energy availability primarily through the process of starch gelatinization (Theurer, 1986; Zinn et al., 2002). In regards to urea metabolism, Oncuer et al. (1990) demonstrated the significant influence that improved starch and energy availability can have on stimulating urea recycling to post-ruminal regions. The group reported that terminal ileal infusions of fermentable carbohydrates (starch in combination with cellulose vs. water) influenced the partitioning of endogenous urea-N to the lower GIT as opposed to urinary N excretion, and that this finding was due to altering the fermentative profile in the hindgut of sheep. In the current study, a similar finding was reported in lambs fed SF corn: as a proportion of UER, lambs fed SF corn had lower amounts of UUE compared to those fed WS corn. These findings could be attributed to a potential increase in fermentable substrate reaching the hindgut of lambs fed more extensively processed grains, facilitating partitioning of UER to the hindgut (away from urinary excretion). This leads to improved NH_3 sequestration in the hindgut, resulting in losses of urea-N in the feces as microbial protein.

Besides the rumen, it is well-established that urea can be transferred from the bloodstream to other compartments of the GIT, including the small intestine (Varady et al., 1979; Marini et al., 2004) and the cecum (Thornton et al., 1970; Oncuer et al., 1990; Marini et al., 2004). As already discussed elsewhere, urea-N that is secreted into the rumen can be used for anabolic purposes through sequestration as microbial protein, thus providing amino acids to the host animal after gastric digestion in the small intestine. The anabolic benefit of endogenous urea-N recycled into post-ruminal sites requires further research (Lapierre and Lobley, 2001); however, post-ruminal

recycling of urea-N has shown importance in the repartitioning of PUN from urinary excretion to quantitative recovery in feces (Ørskov et al., 1970; Thornton et al., 1970). In monogastrics, Columbus et al. (2014) found that when pigs were provided with cecal infusions of a supplemental N source (casein or urea infused at 40% of N intake compared to a saline control), use of the [$^{15}N^{15}N$]-urea isotopic infusion model indicated increases in large intestinal N disappearance (absorption), PUN, and body protein deposition (g/d and as a percent of N intake). The group proposed that free NH_3 derived from the cecal infusions was absorbed from the large intestine and utilized for hepatic ureagenesis (Columbus et al., 2014). Then, this endogenously produced urea was recycled back to the small intestine, wherein it was subject to sequestration into microbial protein, and made available for further digestion and utilization for anabolic purposes (Columbus et al., 2014). With this evidence of NH_3 absorption in the intestinal regions resulting in anabolic benefit to the animal, the whole-body urea kinetics data reported in this thesis becomes increasingly interesting. With the GER:UER ratio being influenced by dietary treatment, it leads one to question where exactly the endogenously produced urea was entering the ovine GIT. Unfortunately, one of the major limitations of implementing the isotopic method to determine whole-body urea kinetics is that the model fails to differentiate the site of urea transfer to the GIT; the entire GIT is treated, in essence, as one single compartment (Lobley et al., 2000; Lapierre and Lobley, 2001). In order to provide more insight into the regulatory mechanisms of urea recycling and the effects of dietary composition on the site of urea secretion, the current study utilized the Ussing chamber technique to obtain qualitative data on urea fluxes across the ruminal, duodenal, and cecal epithelia. Ruminal, duodenal, and cecal epithelial tissue samples that were obtained when animals were killed after exposure to experimental diets for 23 d were mounted in Ussing chambers and used to assess $J_{sm\text{-}urea}$. With this approach, it was anticipated that any adaptations that occurred in transepithelial urea secretion due to dietary treatments would be maintained ex vivo as has been reported by others (Doranalli et al., 2011; Walpole et al., 2015). In the present study, urea flux across the ruminal epithelium was unaffected by dietary treatment. In the past, studies have assessed the influence of rapidly fermentable diets (Walpole et al., 2015) and fermentative end-products such as SCFA and CO_2 (Abdoun et al., 2010) and pH (Abdoun et al., 2010; Lu et al., 2014) on ruminal urea flux. Collectively, the reported findings have led to a consensus that provision of highly digestible diets (and their subsequent fermentative end-products) have a stimulatory effect on $J_{sm\text{-}urea}$. When assessing total flux, Walpole et al. (2015)

reported that when Holstein steer calves were fed a 41.5% barley grain diet, there was only a weak tendency for an increase in ruminal $J_{sm\text{-}urea}$ in steer calves fed the diet for 3, 7, 14, and 21 d (range of 112.6 to 144.2 nmol/(cm^2 × h) compared to those fed the control diet of 91.5% hay (Walpole et al., 2015). Although these findings are in agreement with the previously reported relationship between urea flux and fermentative-end products, Abdoun et al. (2010) was able to more clearly discern the effects of these end-products on ruminal urea flux by directly exposing the ruminal epithelia to the desired conditions within the closed system. Because the Ussing chamber method provides an ex vivo approach to analyzing nutrient fluxes, many physiological factors (e.g., blood supply, hormones, paracrine signalling) that may influence these fluxes in vivo are not accounted for (Clarke, 2009). This, in turn, can present difficulties in accurately interpreting in vivo dietary effects on ex vivo nutrient fluxes, as opposed to directly exposing the tissue to predicted in vivo conditions within the Ussing chamber (Abdoun et al., 2010; Doranalli et al., 2011; Lu et al., 2014). These reasons may explain why there was no dietary effect on ruminal urea flux.

It is known that endogenous urea is transferred across the small intestinal epithelia, both through passive diffusion as well as through urea transporters (UT-A and UT-B) (Marini et al., 2004). In the current study, duodenal $J_{sm\text{-}urea}$ increased by 35.7% in lambs fed HF diets compared to those fed LF diets. Though studies utilizing Ussing chambers to assess urea flux across post-ruminal sites are scarce, the V-A method is another technique that is used to measure net nutrient flux across regions of the GIT, namely the PDV and the MDV. Reynolds and Huntington (1988) reported that in steers fed high forage diets, BUN uptake by the MDV was greater compared to steers fed high concentrate diets. Similar findings were also reported in a previous study conducted by Norton et al. (1978), wherein urea metabolism of sheep fed various types of native Australian forages were assessed. All lambs used in the study exhibited a negative N balance (-0.48 to -1.62 g/d), with PUN concentrations ranging from 57 to 138 mg N/L. The study found that 70 to 72% of endogenous urea produced was recycled to post-ruminal sites, and that urea transfer to these regions had a positive linear correlation with PUN concentrations (Norton et al., 1978). In the current study, lambs fed HF diets had a 29.4% increase in PUN concentrations compared to those fed LF diets. Additionally, though not significant, a weak positive linear correlation was observed between duodenal $J_{sm\text{-}urea}$ and PUN concentrations. Moreover, there was a strong positive linear correlation between $J_{sm\text{-}mannitol}$ and $J_{sm\text{-}urea}$, suggesting that paracellular transport of urea across the duodenal epithelia is an important mechanism in the movement of urea into the intestinal region,

particularly when PUN values are elevated. In agreement with the previously discussed studies, these findings suggest that passive diffusion of endogenous urea-N may be the more prominent method of $J_{sm\text{-}urea}$ across intestinal epithelia compared to facilitative transport.

Serosal-to-mucosal urea flux across the cecal epithelia was unaffected by dietary treatment. In the current literature, there does not appear to be any studies assessing $J_{sm\text{-}urea}$ across the ruminant cecal epithelia using the Ussing chamber model; however, Stumpff et al. (2013) studied the effects of dietary fiber level on $J_{sm\text{-}urea}$ across the cecal mucosa of piglets. The objective of the study was to determine if provision of high fiber, low protein diets as well as ex vivo exposure to reduced pH and SCFA would regulate $J_{sm\text{-}urea}$ in a similar fashion as observed in ruminants (Abdoun et al., 2010). The study reported that neither dietary fiber level, nor changes in apical pH and luminal SCFA concentrations changed urea flux across the porcine cecal epithelia (Stumpff et al., 2013), perhaps suggesting that the regulatory mechanisms for $J_{sm\text{-}urea}$ differ between porcine and bovine GIT tissues, or, that $J_{sm\text{-}urea}$ across hindgut epithelia exposed to fermentative conditions responds differently than ruminal epithelia. Additionally, as observed with duodenal epithelia, a strong positive linear correlation was observed between $J_{sm\text{-}mannitol}$ and $J_{sm\text{-}urea}$. Noting that cecal mRNA expression of UT-B, and AQP-3 and -7 were largely unaffected by dietary treatment in the current study, we can surmise that perhaps diffusional transport of urea may play a more prominent role in $J_{sm\text{-}urea}$ across the cecal epithelia.

Mannitol is a large, hydrophilic solute that is commonly used as an indicator of the permeability of paracellular pathways (and thus, barrier function) in a given tissue (Clarke, 2009). In Ussing chamber experiments, combined measurements of paracellular tracers, such as mannitol, as well as G_t values, can act as indicators of tissue integrity ex vivo (Clarke, 2009; Abdoun et al., 2010; Pederzolli et al., 2018). Diet plays a major role in GIT epithelial health, and it is known that provision of highly fermentable diets can lead to ruminal acidosis (Penner et al., 2007) which can cause negative effects on epithelial integrity (Pederzolli et al., 2018). Damage to the epithelial tissue is in response to the high level of fermentation and SCFA concentrations associated with low forage diets, particularly when SCFA production exceeds removal, resulting in reduced pH that will alter both morphological and histological properties of ruminal papillae (Penner et al., 2010). In the current study, ruminal $J_{sm\text{-}mannitol}$ tended to be greater in lambs fed LF diets compared to those fed HF diets. Similar results were reported by Walpole et al. (2015), who observed a linear

increase in $J_{sm\text{-}mannitol}$ across ruminal epithelia obtained from Holstein steer calves fed a 41.5% barley grain diet for prolonged periods of time compared to the control group (91.5% hay). Moreover, Schurmann et al. (2014) also found a linear increase in G_t associated with ruminal epithelia obtained from the same animals. Taken together, it can be surmised from these results that the provision of highly fermentable diets increases tissue permeability (as indicated by elevated G_t values) by potentially compromising barrier function of the ruminal epithelium (Penner et al., 2010), leading to increases in paracellular passage of compounds, such as mannitol. In support of this explanation, the present study also found that ruminal G_t values were 60.8% greater in lambs fed the LF diet compared to those fed the HF diet. Interestingly, however, there was no linear correlation between ruminal $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$. When comparing paracellular transport pathways of urea and mannitol, Abdoun et al. (2010) also observed no correlation between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, leading the group to conclude that perhaps $J_{sm\text{-}urea}$ across the ruminal epithelia was transported, to a greater extent, by transcellular pathways or that the paracellular pathways involved had a specificity to urea compared to mannitol.

Duodenal $J_{sm\text{-}mannitol}$ was 33.5% greater in lambs fed HF diets compared to those fed LF diets. Duodenal epithelia permeability has not often been studied in ruminants; however, Pederzolli et al. (2018) assessed duodenal mannitol flux in Holstein steers fed a diet with a 50:50 F:C ratio, subject to one of three treatments: (1) control (4 d ad libitum DMI); (2) ruminal acidosis (2 d ad libitum DMI, 2 d feed restriction); and (3) low feed intake (4 d feed restriction). The study did not find any difference in paracellular permeability of duodenal epithelia obtained from animals challenged with ruminal acidosis or low feed intake and those fed a control diet, though it should be noted that the study recorded mucosal-to-serosal as opposed to serosal-to-mucosal flux (Pederzolli et al., 2018). As mentioned previously, there was a strong positive linear correlation observed between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, thus suggesting that paracellular movement of urea may be the preferential mode of transport across intestinal epithelia. Cecal $J_{sm\text{-}mannitol}$ was also not different between dietary treatments. Much like the duodenum, we are not aware of any studies that have assessed paracellular transport across cecal epithelia in Ussing chamber experiments.

The role of UT-A and UT-B in the facilitative transport of urea have been studied extensively throughout the literature, as reviewed by Sands et al. (1997). Various studies have isolated UT-A1 and -A2 isoforms in the kidney, and UT-B1 and -B2 in the kidney, bone marrow,

and brain of numerous species (Sands et al., 1997). In the kidney, urea transporters are responsible for maintaining the urea concentration gradient within the inner medulla of the kidney, facilitating adequate concentration of urea in the urine via reabsorption of urea from the vasa recta to the descending loop of Henle (Knepper and Françoise, 1987; Sands et al., 1997). Collectively, UT-A exists in six different isoforms (Stewart et al., 2005), whereas UT-B exists in two slightly different transcript variants (UT-B1 and -B2) (Sands, 2003). Characterization of UT-B expression in the ruminal mucosa was first reported by Marini and Van Amburgh (2003) on Holstein heifers using western blot analysis. The following year, Marini et al. (2004) validated the presence of UT-B in the rumen mucosa, as well as the presence of UT-A in the kidney, liver, and duodenum in sheep using western blot analysis. Stewart et al. (2005) conducted the first study that reported the presence of UT-B in the bovine rumen epithelium using real-time qPCR, and validated its functional role with the use of phloretin, which is an inhibitor of UT-B. Since then, more recent studies have assessed the influence of dietary factors such as dietary N content (Ludden et al., 2009; Røjen et al., 2011) and level of dietary concentrate (Simmons et al., 2009; Walpole et al., 2015) on the expression of these transporters in the rumen. In addition to UT-A and -B, AQP have also been reported to play a role in facilitative urea transport, in particular, the variants AQP-3, -7, -9, -10 (Rojek et al., 2008). As reviewed by Rojek et al. (2008), the different isoforms of AQP are present in a wide variety of tissues in the body, including the kidney, GIT, epidermis, adipose, and eyes. To our knowledge, Røjen et al. (2011) and Walpole et al. (2015) are the only studies that have reported AQP-3, -7, and -10 mRNA abundance in the ruminal epithelia of ruminants, with Walpole et al. (2015) validating their functional role in urea transport with the use of $NiCl_2$, which is an inhibitor for AQP.

One of the objectives of this thesis research was to improve our understanding of the mechanisms involved in in the transepithelial movement of urea in the rumen, duodenum, and cecum. As already discussed elsewhere, there is ample evidence that urea is secreted from the bloodstream into these GIT compartments. To achieve this objective, the influence of dietary F:C ratio and grain processing on the mRNA expression of UT-B, and AQP-3 and -7 in the ovine rumen, duodenum, and cecum was investigated. In the current study, there were no dietary effects observed on the mRNA expression of UT-B or AQP-7 in the ruminal epithelium; however, mRNA for both transporters were expressed. Expression of AQP-3, however, was greater in lambs fed LF diets compared to those fed HF diets. The lack of response of UT-B expression to our dietary

treatments is inconsistent with the available literature. Simmons et al. (2009) fed beef steers either a silage-based diet or a concentrate-based diet and reported that steers fed the concentrate-based diet had greater UT-B2 mRNA abundance as well as increased UT-B2 protein expression compared to those steers fed the silage-based diet. More recently, Walpole et al. (2015) reported a linear increase in ruminal UT-B expression in Holstein steer calves fed a 50:50 F:C diet compared to steers fed a high forage control diet (91.5% hay). As discussed by Lu et al. (2015), mRNA abundance of UT-B in the rumen has been reported to be upregulated by SCFA production and reduced pH (typical of a high concentrate diet) and downregulated by the presence of NH_3. Though further research is needed to validate the mechanism, Lu et al. (2015) noted a positive correlation between GPR4 and UT-B expression in cultured ruminal epithelial cells from goats fed high RFC diets, and proposed that GPR4 receptors in the rumen (which are sensitive to changes in ruminal pH), might elicit intracellular signaling cascades (e.g. mitogen-activated protein kinases, cyclic adenosine monophosphate pathways) that result in upregulation of UT-B mRNA expression (Lu et al., 2015). With evidence supporting the theory that high concentrate diets upregulate UT expression, it is not clear why the same relationship was not observed in the present study. It should be noted, however, that $J_{sm\text{-}urea}$ was also not influenced by dietary treatment in the present study so one could surmise that the physiological stimulus was not adequate to upregulate the expression of ruminal UT-B.

The effect of RFC on mRNA abundance of AQP-3, -7, and -10 in the rumen have been studied previously by Walpole et al. (2015). The study reported that the mRNA expression of AQP-3, -7, and -10 was increased in Holstein steer calves fed a 41.5% concentrate diet for 21 d compared to those fed a 91.5% forage-based control diet. As with UT, the mechanism for regulation of AQP is not well understood; however, recent literature suggests that because AQP play a role in the movement of water across tissues, hypertonicity and hyperosmolality in the environment surrounding these transporters will have an up-regulatory effect on mRNA abundance in various tissues, as observed in the kidney (Matsuzaki et al., 2001). Additionally, high levels of plasma osmolality is typically indicative of a dehydrative state, resulting in increased secretion of vasopressin, which is known to upregulate expression of AQP so as to assist water reabsorption from the kidney and into the blood (Robertson and Athar, 1976; Rojek et al., 2008). Provision of fermentable carbohydrates will increase ruminal osmolality via increased production of SCFA (Huber, 1976; Owens et al., 1998). Therefore, if the proposed regulatory relationship between AQP

and high osmolality is to be considered, it could be suggested that the increase in AQP-3 expression reported in the ruminal epithelium of lambs fed LF diets could be attributed to increased fermentative activity in the rumen compared to lambs fed HF diets. It should be noted, however, that ruminal osmolality was not measured in the present study, so any potential dietary effects on osmolality are unknown.

Few studies have assessed mRNA abundance of UT and AQP in post-ruminal tissues like the duodenum and cecum. In the current study, there were no dietary effects on duodenal UT-B expression. In ruminants, the presence and expression of UT (both UT-A and UT-B) in the duodenum of lambs has been reported by Marini et al. (2004) using Western blot analysis. Although the study attempted to assess the influence of N intake on duodenal expression of UT-A and UT-B, no differences were observed (Marini et al., 2004). Knowing that the duodenum is a region where $J_{sm\text{-}urea}$ occurs (Lapierre and Lobley, 2001), the current study reported a strong, positive correlation between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, indicating that paracellular transport of urea was a major mechanism for urea secretion from the bloodstream into the duodenum. Moreover, the lack of dietary effects on duodenal expression of UT-B further suggests that facilitative transport may not be the primary mechanism responsible for urea-N salvaging within the small intestine.

With the intestinal regions of the GIT responsible for transporting large volumes of fluid (i.e., bile, pancreatic secretions) and absorbing water from the lumen (Ma and Verkman, 1999), AQP expression and regulation in the body has become a subject of increasing interest. As reviewed by Ma and Verkman (1999), the past two decades of research on AQP have reported the expression of various isoforms of AQP in the stomach, small intestine (jejunum, ileum), and colon of both humans and rats. In ruminants, the expression of AQP-3, -7, and -10 have been reported in the rumen (Røjen et al., 2011; Walpole et al., 2015); however, studies reporting the expression of AQP in the small intestine of ruminants are lacking in the literature. In the present study, dietary treatments only tended to influence duodenal mRNA expression of AQP-3 and -7. In lambs fed LF diets, there was a tendency for a 28.3% increase in mRNA expression of AQP-3 compared to those fed HF diets. Moreover, lambs provided with WS corn also tended to exhibit greater levels of AQP-7 compared to their SF fed counterparts. The mechanism behind these tendencies are unknown, as $J_{sm\text{-}urea}$ across the duodenal epithelia was not affected by dietary treatment in the

current study, and validation of the functional role of AQP in the duodenum was not tested in the Ussing chamber experiment.

Expression of cecal UT-B mRNA in lambs fed HF diets had a weak tendency to be greater compared to those fed LF diets in the current study. Both Marini et al. (2004) and Ludden et al. (2009) have successfully reported the presence of UT-B in the hindgut of lambs, with Marini et al. (2004) observing abundance of the 98-kD UT-B bands in the cecum, and Ludden et al. (2009) observing both 32- and 47-kD UT-B bands in the cecum and spiral colon. In the Ussing chamber experiment, cecal $J_{sm\text{-}urea}$ was not affected by dietary treatment; however, cecal $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ had a strong, positive linear correlation, suggesting paracellular movement of urea across the cecal epithelia may be a mechanism of importance. Stumpff et al. (2013) measured $J_{sm\text{-}urea}$ across the cecal epithelia of piglets, and assessed the effects of fermentative end-products (i.e., SCFA, reduced pH) on urea transport across the tissue. Though the study did not attempt to sequence UT-B expression in this region, exposure to a fermentative environment did not enhance $J_{sm\text{-}urea}$ in the same way Abdoun et al. (2010) observed when assessing ruminal epithelia. Previous tracer studies in piglets have reported that the proportion of endogenous urea secreted to the intestine is greater than that of the cecum (Mosenthin et al., 1992a, 1992b), meaning that rather than urea being recycled directly to the GIT across the cecal epithelia, $J_{sm\text{-}urea}$ occurs more extensively across the intestinal epithelia (based on our findings, presumably through paracellular transport), providing N sources for hindgut microbiota through the digesta (Stumpff et al., 2013). Perhaps the same explanation could be used to explain the numerically lower cecal $J_{sm\text{-}urea}$ values obtained in the current study (relative to the ruminal and duodenal $J_{sm\text{-}urea}$ across dietary treatments), as well as the weak response of UT-B expression to dietary treatment. Expression of AQP isoforms were unaffected by dietary treatment in the current study. In humans, it has been reported that AQP-3 is present in the colon (Ishibashi et al., 1995), though to my knowledge, no studies have assessed the expression of AQP mRNA in the hindgut of ruminants. The lack of dietary influence on UT and AQP in post-ruminal sites of the ruminant GIT could be attributed to a few possible explanations: (1) it could be possible that expression of these facilitative transporters are not regulated by the same mechanisms in the hindgut as they are within the rumen; (2) The studied transporters do not play as major of a role in the transport of urea across hindgut epithelia compared to diffusional transport; and (3) That the hindgut is not a major site for urea secretion compared to the proximal intestinal segments.

Although dietary treatments largely had a negligible influence on the mRNA expression of UT and AQP in ruminal, duodenal, and cecal epithelia, a major contribution of this research is the characterization of these urea transport mechanisms in all 3 GIT compartments in the same study. To my knowledge, no other studies have characterized the expression of these urea transport mechanisms across the various GIT compartments. More research is needed to investigate if the expression of UT and AQP in various GIT compartments, particularly the rumen where there is the greatest opportunity for the anabolic use of recycled urea, mediate the known effects of dietary composition (e.g., dietary content of N and RFC) and end-products of fermentation (e.g., SCFA and NH_3 concentrations, and pH) on urea secretion from the bloodstream into the GIT.

6.0 GENERAL DISCUSSION

Urea recycling is studied extensively within the rumen, as it has the most anabolic benefit to the animal; however, it has been shown repeatedly throughout the literature that post-ruminal regions are an important site for endogenous urea-N entry into the ruminant GIT (Ørskov et al., 1970; Thornton et al., 1970; Oncuer et al., 1990; Lapierre and Lobley, 2001). When urea is recycled back to the post-ruminal regions of the GIT, N is more likely to be lost to the environment in a stable, organic form (i.e., microbial protein) in the feces as opposed to volatile NH_3 in the urine (Thornton et al., 1970), thereby reducing the environmental impact from N excretion (Dijkstra et al., 2011). In terms of dietary regulation, it is well validated in the literature that dietary protein (Wickersham et al., 2008; Doranalli et al., 2011) and RFC (Doranalli and Mutsvangwa, 2007; Davies et al., 2013) content play a major role in influencing the partitioning of endogenous urea secretion into the GIT. Though urea secretion into the GIT is known to occur through passive diffusion, more recently, studies have started to discover facilitative transport mechanisms associated with urea movement across the ruminant GIT, for example, UT-B and AQP-3, -7, and -10 (Røjen et al., 2011; Walpole et al., 2015). Interestingly, some of these transporters have also been identified in post-ruminal regions, namely UT-B in the ruminant duodenum, ileum, and cecum. Various studies have assessed the effects of dietary N intake on UT expression throughout the ruminant GIT (Marini et al., 2004; Ludden et al., 2009), with Røjen et al. (2011) also observing effects on ruminal expression of AQP. Furthering this, more recent studies have also started to assess the influence of RFC and fermentative end-products on these molecular transporters (Lu et al., 2015; Walpole et al., 2015). Knowing that the provision of RFC and its subsequent fermentative end-products have a stimulatory effect on urea recycling to the GIT (Huntington, 1989; Delgado-Elorduy et al., 2002; Abdoun et al., 2010) and ruminal expression of urea transport mechanisms (Lu et al., 2015; Walpole et al., 2015), this study sought out to determine if similar mechanisms were responsible in the post-ruminal regions of the ruminant GIT, and if altering dietary forage level and grain processing influenced our observations.

It was determined in the current study that lambs fed HF diets tended to produce greater amounts of UER compared to their LF counterparts; however, they also had greater rates of N loss through UUE (both in g/d, as well as a proportion of UER), resulting in reduced UUA (both in g/d, and as a proportion of GER). These data suggest that lambs on the HF diet were less efficient in

their ability to retain and utilize endogenously produced urea compared to lambs fed LF diets. When assessing the effects of grain processing, lambs fed SF corn had numerically greater production of UER than lambs fed WS corn and exhibited greater GER:UER. Of this urea-N returned to the GIT, lambs fed SF corn had increased UFE:GER and were able to retain numerically greater amounts of UUA:GER compared to lambs fed WS corn. Therefore, when comparing HF vs. LF fed lambs, or WS vs. SF fed lambs, the data suggest that the provision of RFC (by feeding low F:C dietary ratios or extensively processed grains) improves the ruminant's ability to retain endogenously produced urea and partition the free NH_3-N obtained to microbial usage. Such usage can result in contribution to the ruminant's anabolic requirements (Reynolds and Kristensen, 2008), or be lost in the feces as a stable, organic form of N (Thornton et al., 1970; Oncuer et al., 1990). Moreover, provision of these highly fermentable diets appeared to increase the amount of fermentative substrate present not only the rumen, but post-ruminal regions as well. This was indicated by numerical increases in total SCFA concentrations in the rumen, increases in total SCFA concentrations in the cecum, and reduced ruminal and cecal pH in lambs fed LF compared to lambs fed HF. Knowing that fermentative end-products assist in the microbial sequestration of NH_3, it can be surmised that the reduced NH_3 concentrations observed in the rumen and cecum in lambs fed LF diets compared to those fed the HF diets could be responsible for facilitating urea transfer to these regions.

Because the whole-body urea kinetics model treats the entire GIT as one compartment, it can be challenging to interpret at what region UER returns to the GIT. To obtain qualitative information regarding the magnitude of urea entry at each of the studied GIT regions, the current study utilized the Ussing chamber method to obtain $J_{sm\text{-}urea}$ measurements from the ovine rumen, duodenum, and cecum in an effort to compliment the whole-body urea kinetics data. Despite such prominent dietary effects reported from the in vivo isotopic infusion experiment, $J_{sm\text{-}urea}$ across the three regions was mostly unaffected by dietary treatment, making it difficult to interpret the influence of dietary treatment on region of endogenous urea-N entry into the GIT. For instance, $J_{sm\text{-}urea}$ across the ruminal and cecal epithelia was not affected by dietary treatment. Serosal-to-mucosal urea flux across the duodenal epithelia, however, presented a strong positive correlation with $J_{sm\text{-}mannitol}$, suggesting an increase in the paracellular transport of endogenous urea in lambs fed HF diets as opposed to LF diets. This finding was unexpected, as the duodenum is not a major site of microbial fermentation and sequestration of NH_3-N compared to the rumen and/or the

cecum. Interestingly, PUN concentrations were also greater in lambs fed HF diets compared to those fed LF diets, and previous studies using the V-A technique have found positive correlations between BUN and urea-N transfer to the MDV (Norton et al., 1978; Reynolds and Huntington, 1988). With these data in mind, combined with the increase in duodenal $J_{sm\text{-}mannitol}$ in the HF diet compared to the LF diet, one could surmise that the functional role of diffusional transport may be more prominent in the duodenum compared to facilitated transport, and that the duodenum may play a more notable role in urea recycling than previously thought. Further research on this mechanism should be investigated.

Walpole et al. (2015) reported mRNA abundance for UT-B, AQP-3, -7, and -10 in the ruminal epithelia, and was able to elucidate dietary effects of RFC provided for prolonged periods of time on the expression of the previously mentioned transporters. Moreover, with the use of phloretin and $NiCl_2$, the authors were able to validate the functional role of these transporters by inhibiting $J_{sm\text{-}urea}$ across the ruminal epithelia. In the current study, mRNA expression of UT-B, AQP-3, and -7 were assessed in the ruminal, duodenal, and cecal epithelia. All target genes assessed were present in the three regions, providing valuable characterization of these transporters throughout the ovine GIT. In terms of dietary effects, expression of AQP-3 was greater in the rumen, and tended to be greater in the duodenum, of lambs fed LF diets compared to lambs fed HF diets. Since provision of highly fermentable diets results in increases in end-products of fermentation, the proposed mechanism of upregulation of AQP by increases in regional osmolality (Matsuzaki et al., 2001; Walpole et al., 2015) may provide an explanation for these findings. In the farther fermentative region of the ruminant GIT, the cecum, dietary treatments had negligible effects on mRNA expression of the investigated transporters.

To my knowledge, this is the first study to utilize in vivo and ex vivo approaches to study the effects of F:C ratio and extent of grain processing on urea recycling across the ovine rumen, duodenum, and cecum collectively. Moreover, it is also the first study to attempt to characterize the mRNA expression of select UT and AQP throughout the three regions assessed. To better understand the mechanisms associated with serosal-to-mucosal urea transport, ex vivo inhibitory techniques utilized by Doranalli et al. (2011) and Walpole et al. (2015) should be attempted in order to validate the functional role of these transporters in both ruminal and post-ruminal regions. Moreover, other factors associated with utilization of urea-N in the body, such as hormones (i.e.,

vasopressin, cortisol), should also be investigated, as these too could influence the partitioning of urea to the GIT. These data, in combination with in vivo whole-body urea kinetics, could uncover important information pertaining to the molecular mechanisms of urea-recycling.

7.0 CONCLUSION

Implementing dietary strategies that assist in improving N retention and utilization in ruminants is of the utmost importance for both production and environmental stewardship. In order to do this, understanding the molecular mechanisms of urea recycling is imperative. In this study, increased provision of RFC by decreasing the F:C ratio, or by feeding extensively processed corn, improved apparent N balance in lambs, increased (or numerically increased) endogenous urea-N partitioning to anabolism, and reduced urinary urea-N excretion. Provision of either LF diets or SF corn also increased the amount of endogenous urea-N excreted in the feces, which may yield an environmental benefit to intensive agricultural operations. In addition to this, characterization of the fermentative profiles of ruminal and post-ruminal sites of the ovine GIT were determined in lambs fed HF and LF diets, and WS and SF corn. Although the present study was not able to determine major dietary effects on urea flux across the studied regions (in an attempt to better interpret the whole-body urea kinetics data), the study did report the expression of UT-B and AQP mRNA throughout the ovine rumen, duodenum, and cecum. This data will prove to be valuable information as future research delves into validating their functional roles in urea recycling throughout the ruminant GIT.

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