FACTORS REGULATING UREA-NITROGEN RECYCLING IN RUMINANTS

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ABSTRACT

A series of experiments were conducted to investigate how dietary and ruminal factors regulate urea-N recycling in ruminants. In Experiments 1, 2, and 3, urea-N kinetics were measured using 4-d intra-jugular infusions of [$^{15}N^{15}N$]-urea. In Experiment 1, the objective was to determine how interactions between dietary ruminally-degradable protein (RDP) level and ruminally-fermentable carbohydrate (RFC) may alter urea-N transfer to the gastrointestinal tract (GIT) and the utilization of this recycled urea-N in rapidly-growing lambs fed high N diets. The dietary factors were: 1) dry-rolled barley (DRB) vs. pelleted barley (PB) as the principal source of RFC; and 2) dietary levels of RDP of 60 vs. 70% (% of CP). Nitrogen intake, fecal and urinary N excretion increased as dietary RDP level increased; however, method of barley processing had no effect on N use. Dietary treatment had no effect on urea-N kinetics; however, endogenous production of urea-N (UER) exceeded N intake. For all diets, 0.669 to 0.742 of UER was recycled to the GIT; however, 0.636 to 0.756 of the GER was returned to the ornithine cycle. In Experiment 2, the objective was to delineate the effects of partial defaunation of the rumen on urea-N kinetics in lambs fed low or high N diets. Treatments were: 1) partial defaunation (PDFAUN) vs. faunation (FAUN); and 2) low (10%, LOW) vs. high (15%, HIGH) dietary CP. Linoleic acid-rich sunflower oil was fed as a partially-defaunating agent. Partial defaunation decreased ruminal NH_3-N concentrations. The UER and urinary urea-N excretion (UUE) were lower, and the GER tended to be lower in PDFAUN as compared to FAUN lambs; however, as a proportion of UER, GER was higher and the proportion of recycled urea-N that was utilized for anabolism (i.e., UUA) tended to be higher in PDFAUN lambs. The UER, GER and UUE were higher in lambs fed diet HIGH; however, as a proportion of UER, GER and its anabolic use were higher in lambs fed diet LOW. In Experiment 3, the objective was to delineate how, at similar N intakes, interactions between ruminal partial defaunation and altering dietary RFC may alter urea-N kinetics and N metabolism in lambs. Treatments were: 1) PDFAUN vs. FAUN; and 2) DRB vs. PB. Urinary N excretion was lower and retained N was higher in PDFAUN compared to FAUN lambs. The UER was similar across treatments; however, the GER, expressed as absolute amounts or as a proportion of UER, UUA, and microbial N supply were higher in PDFAUN compared to FAUN lambs. As a proportion of UER, GER was higher, whereas UUE was lower in lambs fed PB compared to those fed DRB. In Experiment 4, the

objective was to determine the effects of feeding oscillating dietary CP compared to static dietary CP concentration on N retention and *in vitro* urea flux across ruminal epithelia. Dietary treatments consisted of a medium CP diet (MEDIUM; 12.8% CP) or diets with oscillating CP content (OSC) fed in two different sequences i.e., 2 d of low CP (9.7% CP) followed by 2 d of high CP (16.1% CP; OSC-HIGH) or vice-versa (OSC-LOW). Ruminal epithelial tissues were collected and mounted in Ussing chambers under short-circuit conditions and the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) was measured using ^{14}C-urea. Although N intake was similar, retained N and microbial N supply were greater in lambs fed the OSC diets compared to those fed the MEDIUM diet. The total $J_{sm\text{-}urea}$ was higher in lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet. Across diets, the addition of phloretin (a known specific inhibitor of facilitative urea transporter-B; UT-B) reduced $J_{sm\text{-}urea}$; however, phloretin-insensitive $J_{sm\text{-}urea}$ was the predominant route for transepithelial urea transfer. In summary, data presented in this thesis provide new insights that the improved N retention typically observed in defaunated ruminants and in ruminants fed oscillating dietary CP concentrations is partly mediated via increased urea-N recycling to the GIT and utilization of recycled urea-N for anabolic purposes.

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Dedicated to

My Late Grandmother Smt. Channamma Hallur

For her sacrifices, support, and unconditional love to all the family members. Your demise is an irreparable loss to the whole family, but you are still with us. I pledge to keep your dreams and visions.

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LIST OF ABBREVIATIONS

[$^{15}N^{15}N$]-urea	Double-labelled urea
AA	Amino acids
ADF	Acid detergent fiber
AOAC	Association of Official Analytical Chemists
BW	Body weight
CP	Crude protein
DM	Dry matter
DRB	Dry-rolled barley
FAUN	Faunated
FAB	Fluid-associated bacteria
GER	GIT entry rate (amount of recycled urea-N entering the GIT)
GIT	Gastrointestinal tract
G_t	Transepithelial conductance
$J_{sm\text{-}urea}$	Serosal-to-mucosal urea flux
MEDIUM	Medium CP diet
MDV	Mesenteric-drained viscera
N	Nitrogen
NAN	Non-NH_3-N
NDF	Neutral detergent fiber
NH_3	Ammonia
NH_4^+	Ammonium ion
NPN	Non-protein N
OM	Organic matter
OSC	Oscillating CP content
OSC-HIGH	Feeding 2 d of low CP followed by 2 d of high CP
OSC-LOW	Feeding 2 d of high CP followed by 2 d of low CP
PAB	Particle-associated bacteria
PB	Pelleted barley
PD	Purine derivatives

PDFAUN	Partially-defaunated
PDV	Portal-drained viscera
PUN	Plasma urea-N
RDP	Ruminally-degradable protein
RFC	Ruminally-fermentable carbohydrate
ROC	Urea-N re-entering the ornithine cycle in the liver
RUDP	Ruminally-undegradable protein
SCFA	Short-chain fatty acids
SFO	Linoleic acid-rich sunflower oil
TMR	Total mixed rations
UER	Urea-N entry rate (total endogenous urea-N production)
UFE	Urea-N in feces
UT	Urea transporter(s)
UUA	Urea-N utilized for anabolism
UUE	Urinary urea-N elimination
UUN	Urinary urea-N
VFA	Volatile fatty acids

1. GENERAL INTRODUCTION

In ruminants, under a wide range of dietary conditions, the efficiency of dietary nitrogen (N) conversion into edible protein products such as milk and meat is often low. In dairy cows, only 25 to 30% of dietary N is utilized for milk protein synthesis (Tamminga, 1992), while 70 to 75% of dietary N is excreted in urine and feces. The efficiency of N utilization in beef cattle is even lower with only 10 to 15% of dietary N retained in tissues (Bierman, 1999; Galyean, 1996). Inefficient rates of dietary N utilization, accompanied by extensive losses of N in the manure leads to environmental degradation. Partitioning of N excretion in urine and feces is also very important. Nitrogen excreted in feces is composed mostly of undigested feed, microbial protein and endogenous sources, while N excreted in the urine is predominantly from ruminal N loss due to extensive degradation of protein in the rumen. About 60 to 80% of total N is excreted through the urine, and large proportion of that N is in the form of urea-N (Van Horn et al., 1996), which accounts for about 29 to 81% of total urinary N (Marini et al., 2004). Urinary urea-N (UUN) is rapidly lost (as ammonia; NH_3) into the environment via volatilization (CAST, 2002). Ammonia (from urine and feces) is also released into the soil, where it undergoes nitrification through the action of soil and fecal microbes and resultant nitrate or nitrous oxide acts as a source of N for plants (Van Horn et al., 1996). However, excess nitrate or nitrous oxide production in the soil will contaminate ground water posing health hazards in humans through drinking water. Reports from Environment Canada (2008) indicate that animal agriculture account for 65.5% of national NH_3 inventory in Canada. Hence, considerable research efforts have been directed towards improving the efficiency of N utilization in ruminants, so as to reduce feed costs and environmental pollution.

One of the major problems associated with inefficient utilization of N in ruminants is the significant loss of N from the rumen as a result of extensive ruminal degradation of dietary protein. Major end products of ruminal dietary protein degradation are peptides, amino acids (AA) and NH_3. Ammonia, along with AA and peptides, are N precursors for microbial protein synthesis. However, under most dietary conditions, ruminal NH_3-N concentrations are usually in excess of microbial requirement, because of extensive proteolytic activity of ruminal protozoa and bacteria (Broderick et al., 1991). In addition, microbial lysis and bacterial protein breakdown (intra-ruminal N recycling) also increase ruminal NH_3-N concentration. Ammonia-N in excess of

microbial requirement is absorbed across the ruminal wall into portal blood, and most of it is detoxified to urea in the liver. In most mammalian species, a large amount of endogenous urea-N is excreted via the urine. However, ruminants have evolved a mechanism that allows constant recycling of urea-N to the gastrointestinal tract (GIT), particularly to the rumen, where urea-N can be used as a source of N for microbial protein, which is the major contributor to the metabolizable protein supply to the small intestine.

Urea-N recycling to the GIT and its utilization for anabolic use is influenced by several dietary and ruminal factors. Major dietary factors which regulate the proportion of hepatic urea-N output returning to the GIT and its subsequent fate are: dietary N concentration and N intake (Bunting et al., 1987; Marini et al., 2004); total dry matter intake (Sarraseca et al., 1998); feed processing (Kennedy and Milligan, 1980; Huntington, 1989; Theurer et al., 2002); oscillating dietary N levels (Cole, 1999; Archibeque et al., 2007); and amount as well as frequency of feeding dietary N that is degraded in the rumen (Wickersham et al., 2008a; Rémond et al., 2009). In juxtaposition with dietary factors, ruminal factors such as ruminal NH_3-N concentration, ruminal bacterial urease activity, ruminally-fermentable carbohydrate (RFC), ruminal concentrations of volatile fatty acids (VFA) and CO_2, and ruminal pH also play a significant role in trans-epithelial movement of blood urea-N into the rumen (Kennedy and Milligan, 1980). However, there is limited research on how manipulating dietary factors and associated ruminal factors could impact urea-N recycling to the GIT and its subsequent utilization for anabolic purposes (primarily microbial protein synthesis in the rumen). Hence, the aim of this thesis was to delineate how various dietary and ruminal factors interact to influence urea-N recycling in ruminants.

2. REVIEW OF LITERATURE

The ruminant stomach is complex in its nature as compared to monogastrics, because of its anatomical structure and the presence of diverse microorganisms (bacterial, protozoa, fungi, archaea and bacteriophages) in the rumen (Orpin and Joblin, 1988). As the rumen hosts several groups of microbes and these microbes, in turn, provide nutrients for the host animal, the whole system can be termed as a "cooperative" or "symbiotic" system in which both the microbes and the animal benefit. Major advantages of this symbiotic relationship to the host animal are degradation of cellulolytic material by microbial cellulases, as well as synthesis of microbial protein from non-protein N (NPN). However, the proteolytic activity of microbes within the rumen poses several disadvantages in terms of dietary protein utilization. To this end, one of the major disadvantages is loss of dietary protein from the rumen in the form NH_3, due to the extensive degradation of dietary protein by ruminal microbes. Even though ruminal NH_3-N is utilized for microbial protein synthesis, most of the NH_3-N in excess of bacterial requirements is absorbed across the ruminal wall and detoxified to urea in the liver, part of which is excreted in urine. Since, feed protein ingredients are expensive, and there is ever-increasing public pressure to reduce the environmental pollution that is caused by intensive livestock operations, efforts have been directed in recent years towards optimizing N utilization in ruminants.

2.1 Nitrogen Metabolism in the Rumen

Nitrogen metabolism in the rumen is a result of mainly the metabolic activity of rumen microbes as the majority of microbes have proteolytic activity (Prins et al., 1983). Degradation activity of these proteolytic microbes depends on the chemistry and structure of dietary proteins, as well as ruminal pH and predominant species of microbes present in the rumen (Huntington and Archibeque, 2000). Dietary protein entering the rumen is degraded (RDP) and the undegraded portion (ruminally-undegraded dietary protein; RUDP) enters the small intestine, where it is further digested (**Figure 2.1**). The RDP is comprised of true protein and NPN. True protein is degraded to peptides, AA and NH_3-N, whereas NPN is comprised of N present in nucleic acids, NH_3-N, AA, small peptides, amides and amines (Bach et al., 2005). Microbial protein synthesized in the rumen, along with RUDP and endogenous N, are the major sources of AA available at the small intestine.

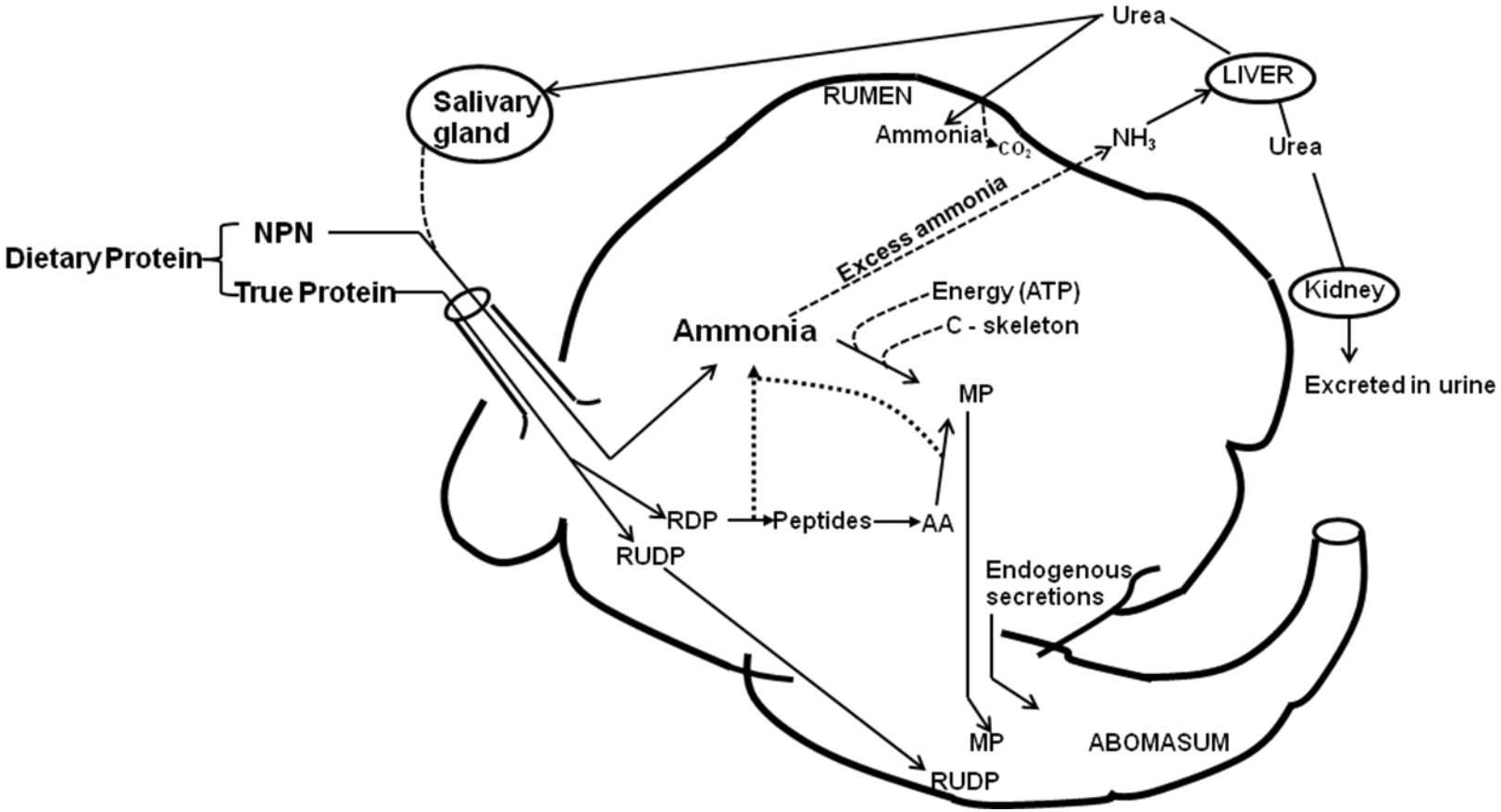

Figure 2.1 Overview of nitrogen metabolism in the rumen. NPN, non-protein nitrogen; RDP, ruminally-degradable protein; RUDP, ruminally-undegradable protein; AA, amino acids; MP, microbial protein.

Among these three fractions, microbial protein is the major contributor of AA entering the duodenum, and it accounts for about 50 to 80% of total absorbable metabolizable protein from the small intestine (Storm and Orskov, 1983).

2.1.1 Mechanisms of Dietary Protein Degradation in the Rumen

The first step in ruminal protein degradation is attachment of rumen microbes to feed particles followed by the action of extra-cellular microbial proteases (Brock et al., 1982). About 30 to 50% of ruminal bacteria that attach to undigested feed particles in the rumen have proteolytic activity (Prins et al., 1983). The major proteolytic ruminal bacteria are *Prevotella spp., Butyrivibrio sp., Ruminobacter sp.,* and *Selenomomas sp.* (Prins et al., 1983). Dietary protein is comprised of a large number of various types of bonds, hence a combination of different proteases are necessary to complete protein degradation (Wallace et al., 1997). The resultant NH_3, AA and peptides are translocated into the bacterial cell. Inside the cell, peptides are further degraded to AA by intracellular peptidases and the resulting AA, along with NH_3, are utilized for synthesis of microbial protein. The majority of the ruminal bacteria do not have a mechanism or transport system to excrete AA out of cell; hence, AA are deaminated to NH_3, VFA (including branched chain VFA), and CO_2 (Tamminga, 1979). Utilization of NH_3 and AA for microbial protein synthesis or deamination depends on ruminal available energy (Bach et al., 2005).

Apart from ruminal bacteria, protozoa also play a major role in ruminal protein degradation. Though available literature indicates that protozoa can contribute to about 20 to 70% of the total ruminal microbial biomass, their contribution to the microbial protein outflow is very low because of their longer generation time (6 to 60 h) and slower turn-over rate (Jouany, 1996). Ruminal protozoa consist largely of *Entodinium spp*., Holotrichs and cellulolytic protozoa (Ogimoto and Imai, 1981), with *Entodinium spp.,* representing up to 90% of the total ruminal protozoal population with their preferred N source being insoluble protein (Jouany, 1996). Proteases are present at high concentration inside the entodiniomorphid cells and, thus, aid in the degradation of insoluble protein to peptides, AA and NH_3. As opposed to the ruminal bacteria, ruminal protozoa cannot utilize NH_3-N as a source of N for protein synthesis; instead, they require preformed AA. Ruminal protozoa indiscriminately degrade dietary, bacteria and endogenous proteins, thus elevating ruminal NH_3-N levels; consequently, defaunation i.e., the

removal of ruminal protozoa, is consistently associated with decreased ruminal NH_3-N levels (Jouany, 1996).

Free AA concentration in the ruminal fluid is usually low (Wright and Hungate, 1967), possibly because of high microbial deaminase activity (Chalupa, 1976). Approximately 50% of the total N supplied to the rumen enters the ruminal NH_3-N pool (Huntington and Archibeque, 2000). Ruminal NH_3-N concentration varies between 0.8 to 56 mg/dL and increases as the dietary CP concentration increases (Satter and Roffler, 1974). Because of extensive fermentative activity of ruminal microbes, ruminants are relatively inefficient in converting dietary protein into usable N, as compared to non-ruminants (Broderick et al., 1991). However, Bryant (1973) demonstrated that 82% of cellulolytic and methanogeneic ruminal bacteria can utilize NH_3-N for protein synthesis. As NH_3-N is the primary source of N for several species of ruminal bacteria including, *Bacteroides amylophilus*, *Bacteroides succinogenes*, *Eubacterium ruminantium*, *Methanobacterium ruminantium, Ruminicoccus albus*, and *Ruminicoccus flavefaciens* (Hungate, 1966), sequestration of ruminal NH_3-N into bacterial protein is the primary route of its disappearance from the rumen (Leng and Nolan, 1984). Studies with ^{15}N indicate that 50 to 75 % of the bacterial N in the rumen of animals fed common diets is derived from the ruminal NH_3-N pool (Oldham, 1980). Even though, available literature indicates that NH_3-N can potentially be used as a source of N for microbial protein synthesis (Bryant and Robinson, 1963), a significant portion of dietary N that is degraded to NH_3-N in the rumen is not incorporated into microbial protein, thus elevating ruminal NH_3-N concentration and is eventually absorbed into the portal blood across the ruminal wall.

2.1.2 Ammonia Absorption Across the Ruminal Epithelium

Ammonia-N is absorbed across all the sections of the GIT and, on average 77% of NH_3-N is absorbed from the reticulo-rumen, while the lower GIT, including the small and large intestines, and cecum accounts for only 33% (Reynolds and Huntington, 1988), however, these proportions vary with the dietary characteristics (Huntington, 1989). Ammonia-N absorbed across the ruminal wall into the portal blood accounts for up to 50% of total NH_3-N flow to the liver (Parker et al., 1995). The quantity of NH_3-N absorbed across the ruminal wall is mainly determined by dietary as well as ruminal factors, with the most important factors being dietary protein that is degraded in the rumen, contributions of endogenous sources (e.g., urea) to the

ruminal NH_3-N pool, and dietary ruminally-available energy (Reynolds and Kristensen, 2008). Under a wide variety of dietary and physiological conditions in growing and lactating cattle, Firkins and Reynolds (2005) concluded that NH_3-N absorption across the GIT accounts for about 42% of dietary N intake. Sequestration of ruminal NH_3-N into the bacterial protein in the rumen is energy dependent and, hence, providing adequate ruminally-available energy is associated with lower ruminal NH_3-N concentration and, consequently, reduced NH_3-N absorption into portal blood. Using the arterio-venous difference technique, Delgado-Elorduy (2002) demonstrated that feeding steam flaked sorghum grain to increase ruminal degradable starch decreased net NH_3-N absorption across the portal-drained viscera (PDV) as compared to feeding dry-rolled sorghum grain. In addition, Reynolds (1996) demonstrated a decrease in NH_3-N absorption across the PDV when starch was infused intra-ruminally and intra-abomasally, possibly due to increased microbial protein synthesis in the rumen and hind gut, respectively.

Ruminal ammonia is present in two forms i.e., the unionized lipid-soluble form (NH_3) or the ionized less lipid-soluble form (NH_4^+). At normal ruminal pH i.e., pH 6 to 7, ammonia will be present in the form of NH_4^+, which is converted to NH_3 at the entry site in the rumen epithelium before being absorbed into portal blood (**Figure 2.2**). It is generally considered that the absorption of NH_3 (lipid soluble) occurs via simple diffusion, while putative K^+ channels are involved in transport of NH_4^+ (less lipid soluble) across the ruminal wall (Bodeker and Kemkowski, 1996). In addition, Abdoun et al. (2007) also suggested that the absorption of NH_4^+ may occur through some transport proteins and the movement of NH_4^+ across the ruminal epithelium is probably regulated by both chemical and electrical gradients. Absorption of both forms of ammonia across the ruminal wall increases with the increase in ruminal pH and total NH_3-N concentrations. At ruminal pH of 6.5 and low, which is normally observed in most feeding conditions, most of the ammonia is absorbed in the form of NH_4^+ (Abdoun et al., 2007).

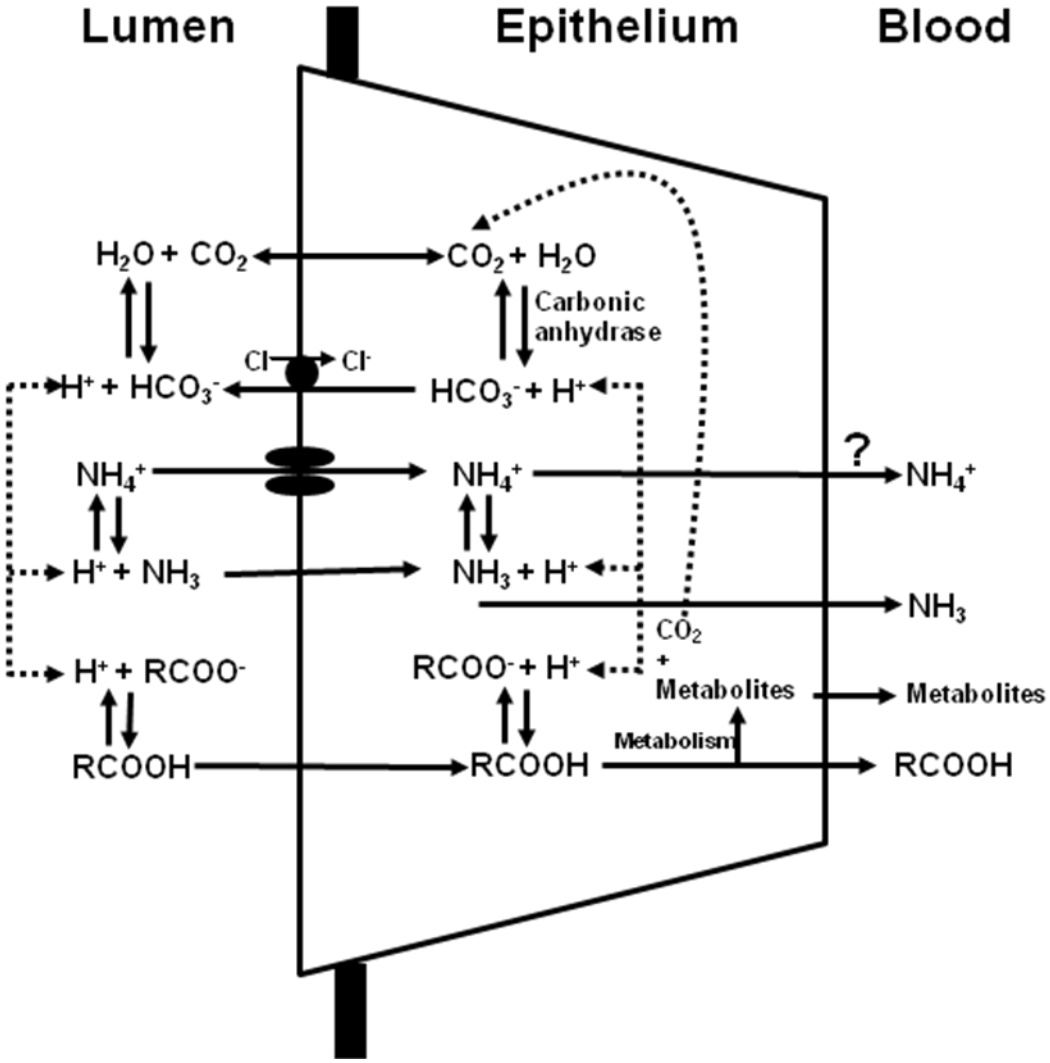

Figure 2.2 Ammonia absorption across the ruminal epithelium (modified from Rémond et al., 1996 and Abdoun et al., 2007). Ammonia diffuses down the concentration gradient into the epithelial cell by simple diffusion. However, under most dietary conditions ruminal pH is between 6 to 7 and most of the ammonia is absorbed as less lipid soluble form i.e., NH_4^+. After absorption, NH_4^+ dissociates into NH_3 and H^+. Thus, this mechanism favors absorption of NH_4^+ into the epithelial cell. Absorption of more lipid-soluble form of ammonia i.e., NH_3 is favored by forming NH_4^+ using H^+ ions produced from the dissociation of H_2CO_3 to HCO_3^- + H^+ and release of H^+ from dissociation of ionized SCFA (RCOOH) to unionized SCFA ($RCOO^-$). Less lipid soluble form of ammonia i.e., NH_4^+ diffuses into the epithelial cell facilitated by putative K channel compared to simple diffusion of more lipid soluble form of ammonia i.e., NH_3.

2.2 Ammonia Detoxification in the Liver

Ammonia that is absorbed into the portal blood is highly toxic and can lead to tetany and death of an animal, if not detoxified. Hence, NH_3 reaching the liver in portal blood is detoxified primarily to urea in the ornithine cycle, which occurs in periportal cells of the liver which is 'low affinity, high capacity' system (Haussinger, 1983; Haussinger et al., 1992). Urea synthesis in the liver occurs in five major steps, each step catalyzed by key enzymes distributed both in the cytosol and mitochondrial compartments (**Figure 2.3**). The first step in the ornithine cycle is the formation of carbamoyl phosphate by condensation of NH_3 with bicarbonate (HCO_3^-) in the mitochondria, a reaction catalyzed by carbamoyl phosphate synthetase (Meijer et al., 1990). The second step is formation of citrulline, which occurs when carbamoyl-phosphate reacts with ornithine in the mitochondria, a reaction that is catalyzed by ornithine transcarbomoylase. Citrulline is then translocated across the mitochondrial membrane into the cytosol, where it condenses with aspartate to form arginosuccinate, a reaction catalyzed by arginosuccinate synthase. Subsequently, in the fourth step, argininosuccinate lyase removes fumarate, which can enter the tricarboxylic acid cycle and serve as an intermediate for aspartate production, leaving arginine. Finally, in the fifth step, arginase completes the ornithine cycle by hydrolyzing arginine to ornithine and urea. Any NH_3 that escapes ureagenesis in periportal hepatocytes enters perivenous hepatocytes, which are a 'high affinity, low capacity' system (Haussinger et al., 1992). Perivenous hepatocytes have high glutamine synthetase activity which eliminates any excess NH_3 that was not removed by periportal hepatocytes via glutamine synthesis. Thus, synthesis of glutamine also serves as a mechanism for NH_3 detoxification. In addition, glutamine that passes through periportal cells is catalyzed by glutaminase, and thus provides amide-N for urea synthesis through the ornithine cycle. Carbamoyl phosphate provides the first N for urea synthesis, with the second N being provided by aspartate. It is most essential that the supply of N sources from mitochondrial NH_3 and cytosolic aspartate for hepatic ureagenesis is coordinated (Lobley et al., 1995). A significant portion of urea formed in the liver is excreted in the urine; however, in ruminants 40 to 80% of total endogenous urea production is recycled to digestive tract via saliva (Huntington, 1989) or by direct transfer from blood to the lumen of GIT via simple diffusion down the concentration gradient (Houpt and Houpt, 1968) and/or via carrier-mediated facilitative transport (Ritzhaupt et al., 1998; Stewart et al., 2005).

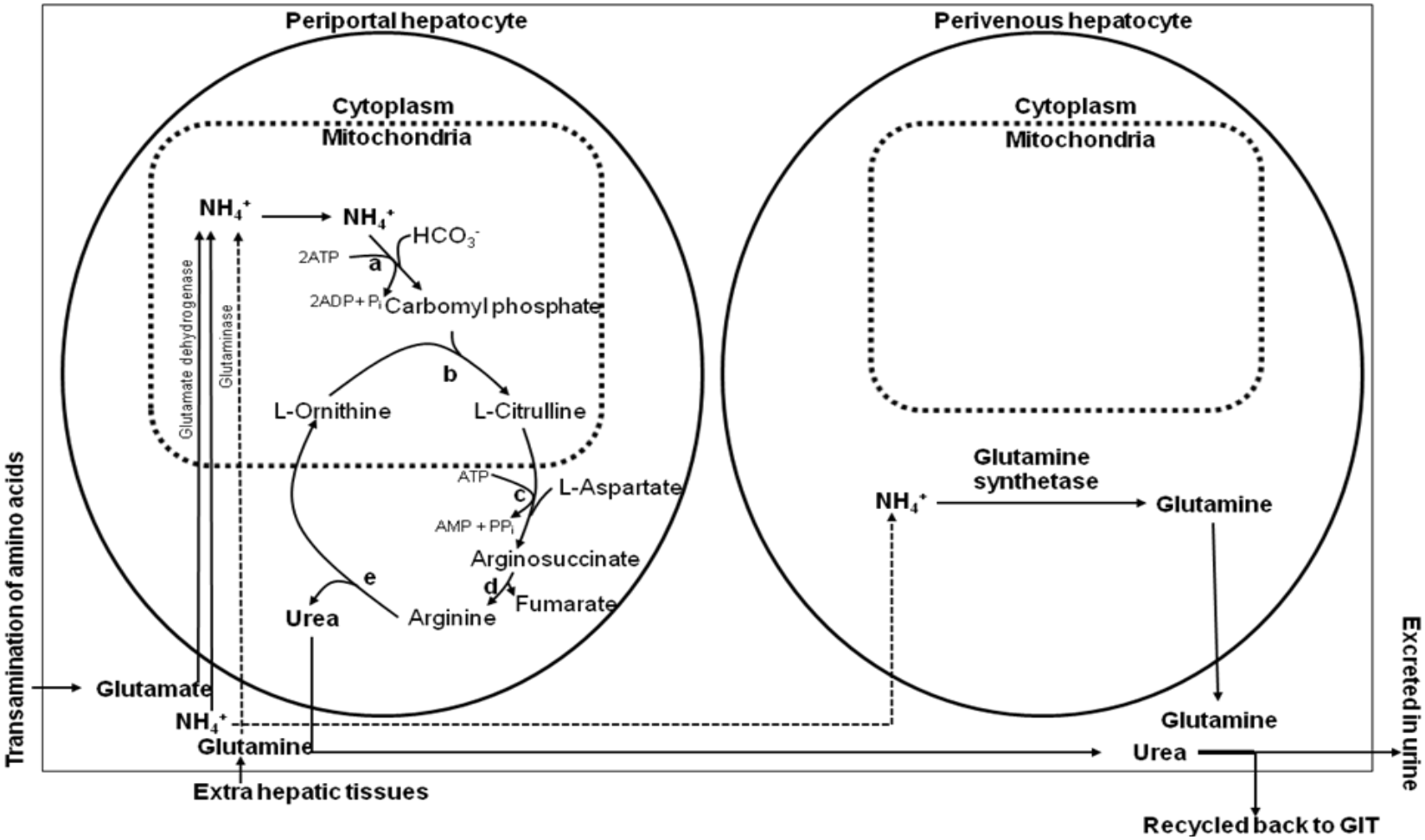

Figure 2.3 Detoxification of ammonia in the liver. Adapted from Meijer et al., 1990. Two N required for urea synthesis through ornithine cycle are provided by mitochondrial NH_4^+ and cytosolic aspartate, respectively. Urea synthesis in the liver is a five step process with each step catalyzed by specific enzymes. **a**. carbomoyl phosphate synthetase, **b**. transcarbamoylase, **c.** arginosuccinate synthase, **d.** argininosuccinate lyase, and **e**. arginase.

The urea-N recycled to the GIT can be an important N source for ruminal microbial protein synthesis, particularly under conditions of dietary N deficiency or low supply of RDP.

2.3 Urea-N Recycling

Hepatic urea-N synthesis has two fates i.e., it is either excreted in the urine or is recycled back to the GIT via salivary secretions or by the direct transfer across the epithelial tissues of the digestive tract (Reynolds and Kristensen, 2008). All mammalian species have the mechanism of urea-N recycling to the GIT (**Table 2.1**). However, in ruminants, data compiled from a variety of studies (Table 2.1) show that the amount of urea-N recycled to the GIT (as a proportion of total hepatic urea-N output) varies between 29 to 99%, which is much greater compared to non-ruminants (15 to 39%). This highlights the potential importance of the mechanism of urea-N recycling in ruminants as opposed to non-ruminants. In ruminants, N transfer across the GIT can be much greater than N intake, and urea-N recycling to the GIT is considered as an evolutionary mechanism, wherein, under conditions of N deficiency, urea-N recycled to the rumen serves as a N precursor for microbial protein synthesis and as a result ruminants can survive when N supply through diet is inadequate to meet their N requirement (Reynolds and Kristensen, 2008). However, for high producing and rapidly growing ruminants, dietary N supply is usually high enough to meet their protein requirement. Even under such conditions where in animals are fed high N diets, total hepatic urea-N production often exceeds apparent digestible N, and if some of the urea-N is not recycled to the GIT, then those animals would be in negative or zero N balance (Lapierre and Lobley, 2001). Hence, the mechanism of urea-N recycling plays an important role to maintain ruminant animals in positive N balance, and also to meet their protein requirement. In addition, the mechanism of urea-N recycling provides an opportunity to decrease excretion of total N (mainly UUN) into the environment, is part of that recycled urea-N is incorporated into microbial protein.

Table 2.1. Urea-N kinetics in different species

Item	N intake, g N/d	Digestible N, g N/d	UER, g N/d	GER, g N/d	UER: N intake, %	GER: UER, %	Citations
Dairy cow	450 - 653	301 - 463	262 - 483	171 - 483	58 - 74	65 - 99	Lapierre et al., unpublished; Gozho et al., 2008
Steers	39 - 128	14 - 88	20 - 127	20 - 106	51 - 99	84 - 99	Archibeque et al., 2001; Wickersham et al., 2008a,b;
Dairy Heifers	87 - 203	47 - 75	31 - 135	26 - 39	36 - 67	29 - 84	Marini and Van Amburgh, 2004
Sheep	9 - 29	3 - 21	3 - 22	2 - 17	33 - 76	67 - 77	Sarasecca et al., 1998; Lobley et al., 2000; Marini et al., 2004; Sunny et al., 2007
Human	10.3	-	11.3	4.4	109	39.0	McClelland and Jackson, 1996
Cat	1.7	1.5	1.1	0.2	65	15.0	Russell et al., 2000

UER = total endogenous urea-N production; GER = urea-N entry to the GIT

2.4 Sites of Urea-N Recycling to the GIT

Urea-N can enter all compartments of the GIT (i.e., the fore-gut: the rumen, mid-gut: small intestine and the hind-gut: the large intestine) through transfer across epithelial lining, and also through salivary, gastric, biliary and pancreatic secretions (Nolan and Leng, 1972; Varady et al., 1979). Even though up to 70% of urea-N may be recycled (as proportion of total portal drained viscera flux) to the post-stomach compartments (mesenteric drained viscera flux) , utilization for anabolic purposes in post-stomach compartments in terms of amino acid supply to productive functions is still questionable (Lapierre and Lobley, 2001). However, transfer of urea-N into the rumen (Houpt, 1959; Kennedy and Milligan, 1980; Egan et al., 1986) is very important in ruminants, wherein urea-N can be used as a source of N for microbial protein synthesis (Lapierre and Lobley, 2001).

2.4.1 Urea-N Entry into the Rumen

Available data from literature (Kennedy and Milligan, 1978; Siddons et al., 1985; Koenig et al., 2000) shows that between 27 to 60% (combined salivary contributions and transfer across the rumen wall) of the GIT entry is to the rumen. The quantity of urea-N transfer to different sections of the GIT is regulated by characteristics of the ruminant diet. Huntington (1989) demonstrated that in steers fed high concentrate diets, up to 95% of urea-N (as a proportion of urea-N entry to the GIT) enters the rumen, as compared to 62.5% in steers fed high forage diet. Urea-N can enter the rumen via direct transfer of blood urea-N across the ruminal wall or via salivary secretions.

2.4.1.1 Urea-N Entry into the Rumen via Transfer Across the Ruminal Wall

Urea-N transfer across the ruminal epithelium is the major route of urea-N transfer into the rumen especially when ruminants are fed high concentrate diets. The mechanism behind blood urea-N transfer across the ruminal epithelium was earlier thought to be only by simple diffusion (Houpt and Houpt, 1968) down a concentration gradient facilitated by bacterial urease activity (**Figure 2.4**). The mechanism behind simple diffusion is that bacterial urease penetrates the stratum corneum layer of ruminal epithelia and hydrolyses the urea molecule, creating a positive concentration gradient favorable for diffusion of urea-N into the rumen (Wallace et al., 1979; Rémond et al., 1996).

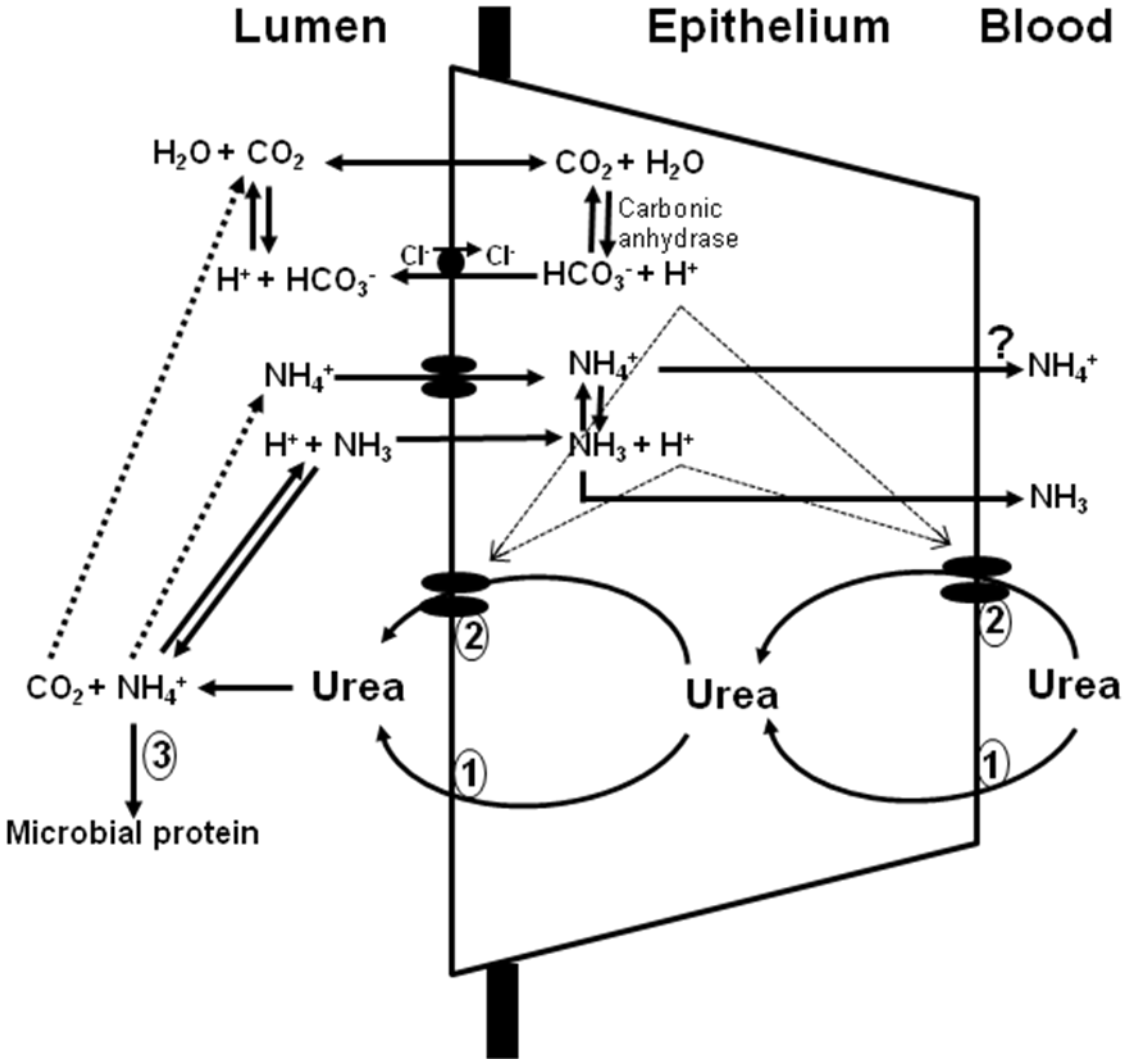

Figure 2.4 Urea transport across the ruminal epithelium (modified from Abdoun et al., 2010). Blood urea is transferred into the rumen by two mechanisms; 1) simple diffusion down the concentration gradient, and 2) carrier-mediated facilitated diffusion by urea transporter-B proteins. Urea that is transferred into the rumen is instantaneously hydrolyzed to ammonia and CO_2; ammonia-N serves as a N source for microbial protein synthesis and ammonia in excess of microbial requirement is absorbed into the portal blood and is detoxified to urea in the liver that cycle continues. Recently, Abdoun et al. (2010) contemplated that, protons (H^+) in the epithelial cell may form hydrogen bonds with urea transporter proteins and result in their conformational changes, thus activating urea transfer into the rumen by carrier-mediated process.

Ritzhaupt et al. (1997; 1998) reported the existence of bidirectional facilitative urea transporter (UT) proteins in the ovine colon and ruminal epithelia (Figure 2.4) and demonstrated that urea-N transport across the ruminal epithelium also occurs by carrier-mediated facilitative transport. Additionally, functional studies to measure whether urea transporter proteins plays a role in facilitative urea transport across the ruminal epithelium were made by mounting isolated ruminal epithelium in the Ussing chambers under short-circuit conditions (Stewart et al. 2005; Abdoun et al., 2010: Muscher et al., 2010) using phloretin as a urea transporter inhibitor. Phloretin inhibited the trans-epithelial urea flux by 50% compared to control tissues (Abdoun et al., 2010) and thus, signifies that urea transport occurs through both paracellular (simple diffusion) and transcellular (carrier-mediated diffusion) pathways. The UT are derived from two major gene variants, namely UT-A and UT-B (Stewart et al., 2005), and mRNA expression of UT-A has been characterized in ovine duodenum as well as that of UT-B in the ruminal epithelium of bovine (Marini and Van Amburgh, 2003; Stewart et al., 2005), ovine (Marini et al., 2004) and caprine (Muscher et al., 2010). Recently, Ludden et al. (2009) reported that UT-B mRNA expression was higher in response to daily supplementation of RDP as opposed to alternate day supplementation in lambs. Oba et al. (2004) demonstrated *in vitro* that ureagenesis may take place in the ruminal epithelial and duodenal mucosal cells; consequently, UT-B may serve as an excretory role rather than secretory role in the ruminal epithelium. Simmons et al. (2009) demonstrated that UT-B mRNA and protein expression in ruminal epithelium were higher in steers fed high concentrate as compared to high roughage diets. However, the mechanisms involved in the regulation of different gene variants of UT and their role in transepithelial urea-N transfer in ruminants in response to dietary characteristics needs future investigations (Reynolds and Kristensen, 2008).

2.4.1.2 Urea-N Entry into the Rumen via Salivary Secretions

As outlined in the review by Lapierre and Lobley (2001), earlier studies in sheep have shown that contributions from salivary flow to urea-N entry to the rumen can vary between 15 to 100% depending on the type of the diet. Salivary urea-N entry to the rumen calculated as difference between total splanchnic flux and urinary excretions rate as a percent of total hepatic urea-N production represented 72% in steers fed high forage diets as compared to 21% in those fed high concentrate diet (Huntington, 1989). High roughage diets stimulate rumination, thus

increasing the flow of salivary secretions to the rumen. Reports from other studies also show that salivary flow of urea-N into the rumen as a percent of total urea-N entry to the GIT was 36% in forage-fed (Taniguchi et al., 1995) and 16% in concentrate-fed (Guerino et al., 1991) ruminants. Recently, Ludden et al. (2009) showed that UT-B proteins are present in the parotid gland in sheep and may be involved in the facilitated carrier-mediated transfer of urea-N into the saliva.

2.4.2 Urea-N Entry into the Small Intestine

In ruminants, up to 70% of the total portal-drained viscera flux of urea can enter post-stomach compartments (Lapierre and Lobley, 2001) of which up to 90% of total portal-drained viscera flux of urea is to the mesenteric-drained viscera in animals fed high fiber diets (Huntington, 1989) as compared to only 19% in animals fed high concentrate diets (Reynolds and Huntington, 1988). However, most of the urea-N that enters post-stomach compartments is returned back to the ornithine cycle as NH_3 for re-synthesis of urea (Lapierre and Lobley, 2001).

2.4.3 Urea-N Entry into the Large Intestine

Small amounts of urea-N are recycled to the hind gut (cecum and colon) and, even though bacteria residing in the hind gut utilize recycled urea-N for protein synthesis, because there are no mechanisms for digestion and absorption of microbial protein formed in the hind gut, it is eventually lost in the feces. Kennedy and Milligan (1980) estimated that about 2 to 10% of urea-N entering the GIT is lost in the feces and that proportion increases to 10 to 25% by providing fermentable energy sources to the hind-gut.

2.5 Magnitude of Urea-N Recycling to the GIT

Urea-N recycling to the GIT occurs in all mammals; however, the magnitude of urea-N recycling is much greater in ruminants. Data from a variety of studies indicate that hepatic urea-N synthesis may be as high as digestible N intake (33 to 99%) and often exceeds digestible N intake (**Figure 2.5**). In high-producing and rapidly growing ruminants, urea-N recycling to the GIT is so important that it can increase the N availability to the GIT from 43 to 130% (Lapierre and Lobley, 2001).

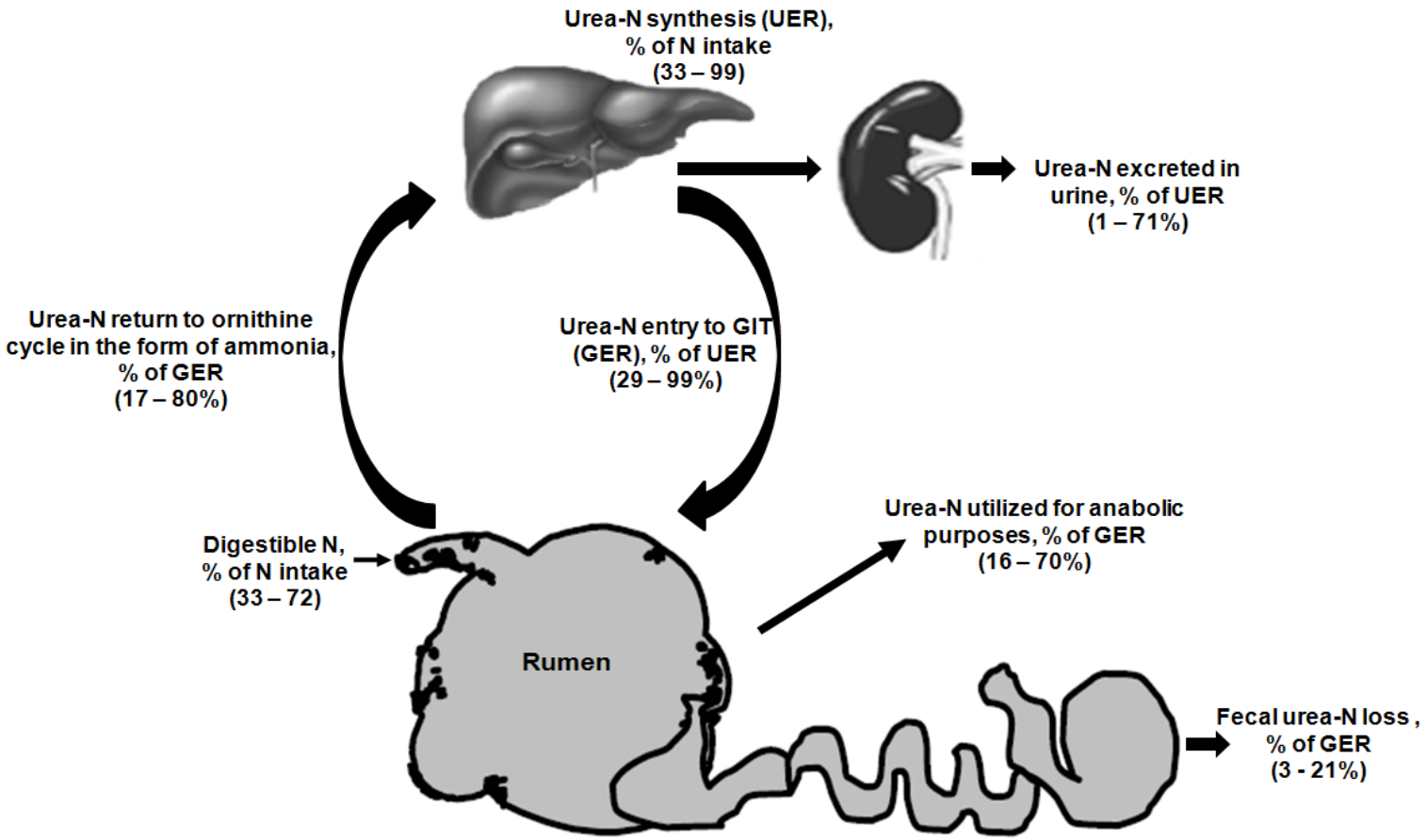

Figure 2.5 Fates of urea-N synthesized in the liver in ruminants. Compiled from: Sarasecca et al., 1998; Lobley et al., 2000; Archibeque et al., 2001; Marini and Van Amburgh, 2003; Marini et al., 2004; Sunny et al., 2007; Gozho et al., 2008; Wickersham et al., 2008a, b. Total urea synthesis in the liver can be as high as 33 to 99% of N intake. Of that total endogenous hepatic urea-N production in the liver, 1 to 71% of urea-N is excreted in the urine and about 29 to 99% enters the GIT. In the GIT 16 to 70% of urea-N (as a proportion that enters the GIT) is utilized for anabolic purposes and 3 to 21% is lost in feces. Unutilized urea-N (i.e., NH_3-N) is returned to the ornithine cycle (17 to 80% of urea-N that enters GIT) for urea synthesis. The data depicted in this figure are obtained from urea-N kinetic measurements obtained from intra-jugular infusion of $^{15}N^{15}N$-urea (Lobley et al., 2000).

On average, between 29 to 99% of the total hepatic urea-N output is recycled to the GIT, with 16 to 70% of urea-N that is recycled to the GIT could potentially being utilized for anabolic purposes, 3 to 21% lost in feces, and 17 to 80% returned to ornithine cycle for re-synthesis of urea (Figure 2.5). Recent observations from Wickersham et al. (2008a, b) showed that as much as 72% of GER can be incorporated into microbial protein. Other research has shown that 46 to 63% of urea-N that is recycled to the GIT can be utilized for anabolic purposes depending on the dietary factors such as level of feed intake, type of the diet and degradable energy intake (Sarasecca et al., 1998; Lobley et al., 2000; Archibeque et al., 2000). Data compiled from numerous studies in steers fed a wide variety of diets showed that the proportion of urea-N that was recycled to the ornithine cycle as a proportion of total endogenous urea-N production ranged from 12 to 32% (Huntington et al., 2009). In addition, there was no correlation between the proportion of urea-N that was returned to the ornithine cycle and total endogenous production of urea-N or the amount of urea-N that was recycled to the GIT. The magnitude of urea-N recycling to the GIT, and its utilization for anabolic purposes is regulated by several dietary and ruminal factors.

2.6 Factors Regulating Urea-N Recycling to the GIT

2.6.1 Dietary Factors

2.6.1.1 Dietary N Concentration and Intake

Dietary N intake and total endogenous urea-N production have a positive relationship (Kennedy and Milligan, 1980). As N intake increases, total endogenous production of urea-N also increases and as percent of N intake, total endogenous urea-N production varied from 77 to 95% (Lobley et al., 2000), clearly indicating the magnitude of the transit of N into the urea pool and the perpetual reliance of ruminants on urea-N recycling to the GIT in order to maintain a positive N balance. Several studies have demonstrated that feeding diets low in dietary N content results in lower total endogenous urea-N production, lower plasma urea-N concentration (PUN), and lower urinary excretions of urea-N, associated with decreased urea-N recycling to the GIT (in absolute amounts; Marini and Van Amburgh, 2003; Marini et al., 2004; Reynolds and Kristensen, 2008; Huntington et al., 2009). On the contrary, it is important to note that, in

ruminants fed low N diets, the quantity of urea-N recycled to the GIT, as a proportion of total endogenous urea-N production, and its utilization for anabolic purposes is greater compared to those fed high N diets; consequently, ruminants can survive for limited period of time under protein deficient situations through the mechanism of urea-N recycling. A study was conducted to examine the effect of two forages (gama grass and switch grass) at two levels of N fertilization application, and found an improved N efficiency at low N intakes as a result of higher absolute movement of N across the GIT (by 11.4% units) in steers fed forage fertilized with low N fertilization compared to those fed forages fertilized with high N (Archibeque et al., 2001).

Marini et al. (2004) demonstrated that as dietary N concentration increased, the absolute amount (g N/d) of urea-N that is recycled to GIT also increased; however, as a percent of total dietary N intake and as proportion of total endogenous urea-N production, the quantity of urea-N recycled to the GIT was greater in sheep fed a low N diet compared to those fed a high N diet. In addition, renal urea clearance rates decreased as a result of decreasing dietary N content in sheep (Marini et al., 2004) and heifers (Marini and Van Amburgh, 2003). Furthermore, urea-N recycled to the GIT as a proportion of total endogenous urea-N production varied from 29 to 42% even when dietary N concentrations were 34.0 to 25.0 g N/kg of DM (Marini and Van Amburgh, 2003). According to NRC (2001), dietary N content in dairy diets varies from 25.6 to 32.0 g N/kg of DM, the range similar to that was used in the study of Marini and Van Amburgh (2003). Hence, such a wide range in the amount of urea-N that is recycled to the GIT (as a percent of total endogenous urea-N production) paves way to manipulate urea-N recycling to the GIT in ruminants to increase utilization of recycled urea-N for microbial use such that excretions of total N in the manure decreases and efficiency of dietary N utilization increases in ruminants fed high N diets.

2.6.1.2 Frequency of Dietary Protein Supplementation

Oscillating dietary CP concentrations on a 2-d basis enhanced N retention in ruminants (Cole, 1999; Cole et al., 2003; Ludden et al., 2003; Kiran and Mutsvangwa, 2009), possibly due to an increase in urea-N recycling to the GIT (Cole, 1999). Archibeque et al. (2007), using the venous-arterial difference technique, observed a tendency for a greater net flux of urea-N across the PDV in growing wethers fed oscillating dietary CP concentrations compared to those fed a medium (12.5%) dietary CP concentration. A recent study (Kiran and Mutsvangwa, 2009)

showed an increase in N retention associated with improved microbial NAN supply to the duodenum in lambs fed oscillating dietary CP compared to those fed medium CP.

In cow-calf beef operations, supplementing protein to low quality forages is commonly practised. Such supplementation strategies are associated with increase in cost of production in terms of labour and machinery. Hence, attempts were made to increase N efficiency by increasing urea-N recycling to the GIT and its capture for microbial protein synthesis in ruminants by altering the frequency of RDP supplementation (Wickersham et al., 2008b). The urea-N entry to the GIT (g N/d) was higher and its anabolic use were lower in steers fed a RDP source (Casein) on daily basis (daily supplementation of 183 mg of N/kg of body weight; BW) as compared to those fed a similar amount of RDP once in three days (549 mg of N/kg of BW fed every third day) (Wickersham et al., 2008b). Adopting such strategies (i.e., infrequent protein supplementation) can potentially increase dietary N utilization and decrease N losses in to the environment as well as reduces cost of labour, time and machinery for producers. Though several studies have shown an improvement in N retention by adopting oscillating dietary regimen, there is no definitive evidence to prove that the increase in N utilization is due to an increase in urea-N recycling to the GIT when ruminants are fed low N diets for 2-d in a 4 d oscillating cycle.

2.6.1.3 Ruminally-Degradable Protein and Protein Solubility

The ruminal NH_3-N concentration is negatively correlated with rate of urea-N transfer across the ruminal wall (Kennedy and Milligan, 1980). Hence, the form of N in the diet, particularly RDP and/or protein solubility, are important and determine how much of the dietary protein is directed towards ruminal NH_3-N (Lapierre and Lobley, 2001). In ruminants fed high RDP level, an increase in ruminal NH_3-N concentration is associated with a decrease in ruminal urease activity, thus a decrease in urea-N transfer from blood into the rumen. Recently, Wickersham et al. (2009) showed that in steers fed low dietary N, increasing the amounts of RDP by infusing casein into the rumen linearly increased the quantity of urea-N that was recycled to the GIT (in absolute amounts) and the amount of recycled urea-N that was sequestered into microbial protein (using ^{15}N isotope) ranged from 55.9 to 64.0%. Fecal excretion of recycled urea-N linearly increased as dietary RDP level was increased (Wickersham et al., 2009). These data indicate that, in ruminants fed low N diets supplying additional RDP could benefit to conserve N (i.e., increased urea-N recycling to the GIT and supply of readily

available N source for microbial protein synthesis) for productive functions. Using arterio-venous difference Ferrell et al. (2001) demonstrated in sheep that urea-N transfer to the GIT averaged 62.3, 47.2, 47.5, and 39.2% for control, urea, soybean meal and mixture of feather/blood meal, respectively. These data indicate that with higher RUDP and lower RDP fraction in feather/blood meal diet, urea-N transfer to the GIT was lower. Recently, Wickersham et al. (2008a) also showed in steers consuming low quality forage that increasing the amount of digestible protein intake increased total endogenous urea-N production as well as urea-N transfer to the rumen. Archibeque et al. (2002) showed that urea-N entry rate was greater in steers fed gamagrass and switchgrass as compared to those fed tall fescue, due to higher slowly RDP (i.e., B2 fraction of protein) in gamagrass and switchgrass as compared to tall fescue. Processing of legume seeds especially extrusion is commonly practised to decrease RDP and increase RUDP. Recently, Rémond et al. (2009) using arterio-venous difference technique reported that the urea-N transfer across the ruminal epithelium (in absolute amounts) was not altered in sheep fed either extruded or raw pea. However, urea-N transfer across the rumen as a percent of total portal-drained viscera (PDV) flux was 72% in sheep fed extruded compared to 52% in those fed raw pea, possibly due to a lower ruminal NH_3-N concentration prevailing in sheep fed raw pea (Rémond et al., 2009). The overall conclusion from that study was that even though the net transfer of urea-N to the rumen did not differ, shifting protein digestion from the rumen to the small intestine by feeding extruded pea increased overall urea-N efficiency by higher transfer of hepatic urea-N output to GIT.

2.6.1.4 Dietary Ruminally-Fermentable Carbohydrate

Dietary energy content can be increased by supplementing grain, starch or sucrose, which would significantly provide ruminal available energy, thus increasing the utilization of NH_3-N for microbial protein synthesis. Greater sequestration of ruminal NH_3-N into microbial protein would decrease the ruminal NH_3-N concentrations, which, in turn, would increase urea-N transfer to the rumen. In addition, higher urea-N transfer to the rumen could probably be attributable to an increase in the amount and rate of organic matter degradation or decrease in the ruminal NH_3-N concentration or combination of both (Kennedy and Milligan, 1980). Providing higher amounts of dietary RFC is associated with increased urea-N transfer to the rumen (Kennedy, 1980; Kennedy and Milligan, 1980; Huntington, 1989) as opposed to post gastric

tissues (Reynolds and Huntington, 1988; Huntington, 1997), and also increases sequestration of NH_3-N into microbial protein. Recently, Huntington et al. (2009) demonstrated that supplementing carbohydrate in forage fed steers was accompanied by decreased return of urea-N to the ornithine cycle (as a proportion of recycled urea-N to the GIT). In addition, urea-N utilized for anabolic purposes (as a proportion of recycled urea-N to the GIT) was associated with improved efficiency of N utilization (Huntington et al., 2009). Kim et al. (1999) observed improved N retention and an increase in urea-N entry to the GIT and its utilization for anabolic purposes when propionate was infused into the abomasum, however underlying mechanisms needs future investigations.

Processing of feeds, especially dietary starch sources, is done to shift the site of digestion from post-ruminal compartments to the rumen particularly to synchronize the supply of starch and N to ruminal microbes, thereby increasing microbial protein synthesis and reduce losses of N from the rumen (Huntington, 1997). Grain processing is associated with increase in the ruminal available energy (i.e. increased ruminal starch fermentation) that increases utilization of NH_3-N for microbial protein synthesis. As a result, ruminal NH_3-N concentration decreases and thus urea-N recycling to rumen increases. Theurer et al. (2002) demonstrated that shifting carbohydrate digestion from the small intestine to the rumen via steam-flaking compared to dry-rolling of sorghum grain increased urea-N transfer to the rumen by 30% in beef steers, possibly because of increased sequestration of N into microbial protein, thus resulting in a greater post-ruminal flow of microbial protein in beef and dairy cattle (Theurer et al., 1999). Alio et al. (2000) also demonstrated that urea-N recycling to the PDV (as a percent of total hepatic urea-N output) was 64% in beef steers fed steam-flaked compared to 50% in those fed dry-rolled sorghum grain and in addition, increasing the degree of processing by decreasing sorghum grain flake-density resulted in greater transfer of urea-N to the PDV. Barley is the major source of carbohydrates in beef and dairy diets across Western Canada and processing of barley (grinding, pelleting and dry-rolling) is commonly practiced to increase the RFC from cereal grains (Huntington, 1997). However, there is limited research on how grain processing would impact urea-N recycling in ruminants.

2.6.2 Ruminal Factors

2.6.2.1 Ruminal NH_3-N Concentration

Ruminal NH_3-N concentration has direct effect on urea-N transfer in to the rumen by increasing the ruminal epithelium's permeability to urea-N (as ruminal NH_3-N concentration decrease). Houpt and Houpt (1968) demonstrated using ruminal pouch preparations that urea-N transfer across the ruminal epithelium decreased in response to a decrease in urease activity. Urea-N transfer across the ruminal wall is facilitated by bacterial urease activity (Rémond et al., 1996). Bacterial ureases rapidly hydrolyze urea-N entering the rumen to NH_3 and CO_2 and thus favor the diffusion of urea-N across the rumen wall by maintaining a positive concentration gradient (Rémond et al., 1996). Cheng and Wallace (1979) demonstrated that as ruminal NH_3-N concentration increases, bacterial urease activity decreases. Therefore, high ruminal NH_3-N concentrations will decrease urea-N transfer into the rumen (Kennedy and Milligan, 1980), possibly due to decrease in bacterial urease activity and the ruminal epithelium's permeability to the urea-N. Recently, Marini et al. (2004) reported a linear decrease in the ruminal bacterial urease activity as a result of increasing dietary N levels. Though ruminal NH_3-N concentrations were not measured in that study (Marini et al., 2004), it is plausible that higher N intakes would have led to increased degradation of dietary N in the rumen, thus increasing ruminal NH_3-N concentrations resulting in decreased bacterial urease activity. A possible theory behind the decrease in ruminal urease activity as ruminal NH_3-N concentration increases is that the rumen is a highly reducing environment with a pKa of NH_3 (unionized lipid-soluble form) of 9.3, which quickly gets converted to NH_4^+ (ionized less lipid-soluble form) leading to its accumulation in the rumen, and thus an increase in ruminal NH_4^+ concentration would probably inhibit ruminal urease activity; however, ruminal NH_3-N concentrations in the range of 5 to 8 mg/dL in cattle are associated with maximum urea-N transfer across the ruminal wall (Kennedy and Milligan, 1978). Contrary to other reports, Bunting et al. (1987) reported only a marginal decrease in ruminal urease activity as a result of higher ruminal NH_3-N concentrations in growing heifers fed high dietary N as compared to those fed low dietary N.

In order to maximize urea-N transfer into the rumen and its subsequent utilization for microbial protein synthesis, ruminal NH_3-N levels have to be reduced by means of altering dietary protein degraded in the rumen. One possible method to decrease RDP in feed ingredients

(especially leguminous seeds) is by extrusion. Rémond et al. (2009) demonstrated that ruminal N loss (as NH_3-N) was lower in sheep fed extruded pea (low RDP) compared to those fed raw pea (high RDP) associated with increased efficiency in urea-N recycling the GIT i.e., greater urea-N flux into the rumen compared to post-stomach compartments in sheep fed extruded pea. As ruminal protozoa are highly proteolytic, eliminating ruminal protozoa (i.e., defaunation) is another possible means to reduce dietary protein degradation in the rumen (and thus reduce ruminal NH_3-N concentrations). Microbial lysis as well as bacterial protein degradation (by ruminal protozoa) termed as 'intra-ruminal N recycling' (Jouany, 1996) contribute to ruminal NH_3-N concentrations. Available data from the literature indicates that complete or partial defaunation is consistently associated with a decrease in the ruminal NH_3-N concentrations (Jouany, 1996; Ivan et al., 2001), in addition to improving bacterial protein supply to the duodenum (Koenig et al., 2000). As ruminal NH_3-N concentration is negatively correlated with urea-N transfer to the rumen (Kennedy and Milligan, 1980), it is possible that the removal of ruminal protozoa may increase urea-N transfer to the rumen.

2.6.2.2 Ruminal CO_2

Stimulatory effects of CO_2 on urea-N transfer across the ruminal wall were first demonstrated *in vivo* using isolated rumen pouches (Thorlacius et al., 1971). Further, Rémond et al. (1993) demonstrated that urea-N transfer across the ruminal epithelium increases in response to bubbling of CO_2 and this response is seen as early as 15 min after CO_2 bubbling. A possible theory for increased urea-N transfer into the rumen could be due to increased blood flow to the ruminal wall, which in turn increases the rate of absorption of permeable substances (e.g., urea) from interstitial spaces in response to CO_2 bubbling (Mailman, 1982). Recently, Abdoun et al. (2010) demonstrated *in vitro* using isolated ruminal epithelium in Ussing chambers that the magnitude of urea-N transfer across the ruminal epithelium increased in response to CO_2 supply, and this increase was highest at a mucosal pH of 6.4 compared to marginal increase at a pH of 7.4.

2.6.2.3 Ruminal VFA and pH

Effects of ruminal VFA and pH are closely related and therefore will be discussed collectively. Engelhardt et al. (1978) reported that ruminal VFA, particularly butyrate, has a

stimulatory effect on urea-N transfer across the ruminal epithelium. In addition, Norton et al. (1982) demonstrated in sheep that prolonged exposure (2 weeks) to increased ruminal butyrate concentration increased urea-N transfer into the rumen. However, Rémond et al. (1993) found that infusing butyrate into the rumen for short duration (1 h) did not increase urea-N transfer and, in fact, butyrate infusion reduced urea-N transfer into the rumen. Simmons et al. (2009) reported a higher bUT-B2 mRNA and protein expression in steers fed concentrate-based diet compared to those fed silage-based diet. In that study, ruminal butyrate concentration was numerically higher (9.3 vs. 11.7 as % of total VFA) in steers fed concentrate-based compared to silage-based diet and, may play a role in expression of bUT-B2 thus, increasing urea-N transfer into the rumen.

Earlier work of Rémond et al. (1993) showed that shifts in the ruminal pH relative to fed and fasting-state of an animal may play a role in urea-N transfer across the ruminal epithelium. Recently, Abdoun et al. (2010) demonstrated *in vitro* using isolated ruminal epithelium in Ussing chambers that in presence of short-chain fatty acids, reducing ruminal mucosal buffer pH from 7.4 to 5.4 showed a bell-shaped curve for urea transport from serosal to mucosal direction with highest rate of urea transport between pH 6.0 to 6.4. If the ruminal pH is approximately in the range of 6.0 to 6.4, the range which is typically observed under *in vivo* physiological conditions in the rumen, changing the ruminal factors (e.g., VFA) may have a positive impact on urea-N recycling to the rumen (Abdoun et al., 2010). The ruminal pH in the study of Rémond et al. (1993) was 6.75 and because of such a high pH, ruminal concentration of NH_4^+ (less lipid soluble) rise in turn elevating total ruminal NH_3-N levels, and thus decrease urea-N transfer into the rumen. In addition, it was also demonstrated *in vitro* that, cytosolic pH of an isolated ruminal epithelial cell may play an important role in conformational changes to the UT-B proteins that are involved in the transport of urea across the ruminal epithelium (Abdoun et al., 2010).

2.6.3 Plasma Urea-N

Plasma urea-N (**PUN**) concentration is another major factor that influences urea-N recycled to the GIT. Houpt and Houpt (1968) proposed a mechanism that blood urea-N enters rumen by simple diffusion across the ruminal wall. As proposed by Huntington and Archibeque (2000), urea-N entry into the rumen depends on the amount of urea-N circulating in the blood (supply or push) and the amount of urea-N hydrolyzed by microbial ureases in the rumen after its transfer into the rumen (use, or pull). Harmeyer and Martens (1980) indicated that amount of

urea-N recycled to the GIT (through salivary secretions and transfer across the ruminal wall) is positively correlated to the PUN concentration; however, this relationship is true only if the PUN are below 6 mM (sheep) and below 4 mM (cattle) (Lapierre and Lobley, 2001). Plasma urea-N concentrations were lower and its entry across the PDV was higher in sheep fed high concentrate diets compared to high forage diets (Reynolds and Huntington, 1988). Recently, Sunny et al. (2007), using an intra-jugular urea infusion protocol to increase blood urea-N concentrations, clearly demonstrated that urea-N transfer from blood to the GIT was elevated as PUN concentration increased. In addition, there was a positive correlation between PUN and urea-N recycling to the GIT in sheep fed a basal diet low in dietary N content, and these data provide evidence that PUN plays a very important role in regulating urea-N transfer to the rumen at least in ruminant fed low N diets. However, across a wide range of PUN in cattle fed a wide variety of diets, Lapierre and Lobley (2001) did not find any relationship between arterial urea concentration, and net PDV removal of urea in ruminants fed high N diets (PDV removal, however, did not include salivary urea-N recycling). Hence, in ruminants fed high N diets, whether PUN has any positive relationship with urea-N recycling to the GIT needs future investigations.

2.7 Conclusions

Dietary N consumed by ruminants is extensively degraded in the rumen, leading to significant loss of N from the rumen in the form of ammonia. Most of that ammonia that is absorbed into the portal blood is converted to urea-N in the liver. Ruminants have developed an evolutionary mechanism of urea-N recycling, wherein part of that urea-N is recycled back to the GIT. Part of urea-N that is recycled to the rumen can potentially be incorporated into the microbial protein, thus increasing the AA supply to the duodenum. Hence, mechanism of urea-N recycling plays a very important role in keeping ruminants in positive N balance. Both dietary and ruminal factors play an important role in regulating urea-N recycling in ruminants. However, there is limited research on how interaction between different dietary as well as ruminal factors could influence urea-N recycling in high-producing and rapidly growing animals. Hence, the broad objective of this thesis was to understand the factors regulating urea-N recycling and its impact on the efficiency of N utilization in ruminants.

3. EFFECTS OF BARLEY GRAIN PROCESSING AND DIETARY RUMINALLY-DEGRADABLE PROTEIN ON UREA-NITROGEN RECYCLING AND NITROGEN METABOLISM IN GROWING LAMBS[1]

3.1 Abstract

The objective of this study was to determine how interactions between dietary ruminally-degradable protein (RDP) level and ruminally-fermentable carbohydrate (RFC) may alter urea-N transfer to the gastrointestinal tract (GIT) and the utilization of this recycled urea-N in rapidly-growing lambs fed high N diets. Four Suffolk ram lambs (34.8 ± 0.5 kg BW) were used in a 4 x 4 Latin square design with 21-d periods and a 2 x 2 factorial arrangement of dietary treatments. The dietary factors studied were: 1) dry-rolled barley (DRB) vs. pelleted barley (PB) as the principal source of RFC; and 2) dietary levels of RDP of 60 vs. 70%. All diets contained 28.8 g N/kg DM. Experimental diets were fed twice daily at 0900 and 1700 h as total mixed rations, composed of 80% concentrate mixture and 20% barley silage (DM basis). Nitrogen balance was measured from d 15 to d 20, while urea-N kinetics were measured from d 15 to d 19 using intra-jugular infusions of [$^{15}N^{15}N$]-urea. Nitrogen intake ($P = 0.001$), and fecal ($P = 0.002$) and urinary ($P = 0.034$) N excretion increased as dietary RDP level increased; however, method of barley processing had no effect on these parameters. Feeding DRB compared to PB ($P = 0.04$), and feeding 60% RDP compared to 70% RDP ($P = 0.04$) resulted in a higher N digestibility. Whole-body N retention was unaffected ($P > 0.05$) by dietary treatment. Dietary treatments had no effect on endogenous production of urea-N and its recycling to the GIT; however, across dietary treatments, endogenous production of urea-N was high (45.8 to 50.9 g/d), exceeding N intake (42.3 to 47.9 g/d). Across dietary treatments, 30.6 to 38.5 g/d of urea-N were recycled to the GIT, representing 0.669 to 0.742 of endogenous urea-N production; however, 0.636 to 0.756 of urea-N recycled to the GIT was returned to the ornithine cycle. In summary, although dietary treatment did not alter urea-N kinetics, however substantial amounts of hepatic urea-N output were recycled to the GIT under the dietary conditions employed in this study, and additional

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research is required to determine how this recycled urea-N can be efficiently captured by bacteria within the GIT.

3.2 Introduction

In ruminants, hepatic urea-N output often exceeds apparent digestible N intake; however, animals maintain a positive N balance primarily by recycling 40 to 80% of hepatic urea-N output to the GIT (Lapierre and Lobley, 2001). Urea that is recycled to the GIT is an important source of N for microbial growth. Increasing dietary amounts of RFC (Huntington, 1989; Rémond et al., 1996) or shifting carbohydrate digestion from the small intestine to the rumen via steam-flaking compared with dry-rolling of sorghum grain (Theurer et al., 2002) increased urea-N transfer to the rumen, in addition to increasing N sequestration into microbial protein.

In ruminants fed low-N diets, a greater proportion of endogenous urea-N production is recycled to the GIT compared with animals fed adequate levels of N (Siddons et al., 1985; Marini et al., 2004). However, under practical feeding conditions, high-producing ruminants (e.g., dairy cows fed for high levels of milk yield) are usually fed high levels of dietary N (25.6 to 32.0 g of N/kg of DM; NRC, 2001) to adequately meet protein requirements. Marini and Van Amburgh (2003) demonstrated in Holstein heifers that, even at high levels of N intake (25.0 to 34.0 g of N/kg of DM), there was a wide range (29 to 42%) in the proportion of hepatic urea-N output that was recycled to the GIT. Clearly, therefore, there is opportunity for manipulation of urea-N recycling to the GIT, even in ruminants fed high-N diets, to improve N efficiency of ruminants. Because ruminal NH_3-N concentration is negatively correlated with the rate of urea-N transfer into the rumen (Kennedy and Milligan, 1980), the form of the N fed, particularly the proportion of RDP, is important because it determines how much N is directed toward ruminal NH_3-N (Lapierre and Lobley, 2001). However, limited information is available on how concomitant changes in dietary content of RFC and RDP might influence urea-N kinetics in ruminants fed high N diets.

The hypothesis was that changes in the proportion of dietary N that is degraded in the rumen (by varying dietary RDP level) would alter urea-N recycling to the rumen, and that this effect would be more pronounced with more extensive barley grain processing, which would increase ruminal starch digestion and subsequently urea-N recycling to the rumen and microbial N sequestration. The objective of this study was to determine how interactions between dietary

concentrations of RDP and RFC alter urea-N transfer to the GIT and the utilization of this recycled urea-N in lambs.

3.3 Materials and Methods

3.3.1 Animals and Experimental Design

The lambs were cared for and handled in accordance with regulations of the Canadian Council on Animal Care (1993), and their use in this experiment was approved by the University of Saskatchewan Animal Care Committee (UCACS Protocol No. 20040048). Four Suffolk ram lambs (34.8 ± 0.5 kg of initial BW) were used in this study. The experiment used a 4 x 4 Latin square design with 21-d periods and a 2 x 2 factorial arrangement of dietary treatments. Each experimental period consisted of 14 d of dietary adaptation and 7 d of data collection. Throughout the experiment, lambs were housed at the Livestock Research Building (University of Saskatchewan) in a temperature-controlled environment (18 to 22°C). Lambs were housed in individual floor pens (during dietary adaptation) or in metabolism crates (during the 7-d data collection period) to facilitate total urine and feces collection. It was decided to use rapidly growing lambs as an experimental model because of their high N demands for rapid growth. Most previous research investigating regulatory mechanisms that impact urea-N recycling in ruminants has been conducted using slow-growing ruminants, ruminants fed at low intakes, non-lactating cows, or low-producing lactating cows (Lapierre and Lobley, 2001), yet the productive state of the animal is important because it dictates biological N requirements.

3.3.2 Experimental Treatments and Feeding Management

Four dietary treatments were formulated by combining 2 factors, each with 2 levels. The dietary factors studied were 1) dry-rolled barley (DRB) vs. pelleted barley (PB) as the principal source of RFC and 2) dietary levels of RDP of 60 vs. 70% (% of CP, DM basis). The ingredient and chemical composition for 4 concentrate mixtures used to formulate the experimental diets are presented in **Table 3.1**. Barley grain obtained from one source was used for both DRB and PB throughout the experiment. The DRB was prepared by passing whole barley grains through large rollers (23 x 58 cm). For pelleting, whole barley grains were ground through a 6.35-mm screen in a hammer mill and then pelleted using a California pellet mill.

Table 3.1 Ingredient and chemical composition of concentrates mixtures with low or high ruminally-degradable protein (RDP) containing dry rolled barley (DRB) or pelleted barley (PB)

	Low RDP		High RDP	
Item	DRB	PB	DRB	PB
Ingredients, % DM				
Barley, dry rolled	45.0	-	45.0	-
Barley, pelleted	-	45.0	-	45.0
Alfalfa meal	30.0	30.0	30.0	30.0
Soybean meal	1.0	1.0	1.0	1.0
Tallow	1.0	1.0	1.0	1.0
Molasses	2.0	2.0	2.0	2.0
Canola meal	2.5	2.5	8.0	8.0
Corn gluten meal	9.5	9.5	1.5	1.5
Distillers grain, dry	2.0	2.0	3.5	3.5
Ground limestone	4.0	4.0	4.0	4.0
Urea	-	-	1.0	1.0
Salt, Co-I[1]	0.7	0.7	0.7	0.7
Salt, white	1.3	1.3	1.3	1.3
Mineral vitamin supplement[2]	1.0	1.0	1.0	1.0
Chemical composition				
DM, %	92.1	92.4	92.0	92.0
OM, % of DM	89.2	88.9	88.0	88.3
CP, % of DM	20.5	20.5	21.3	21.4
RDP, % of CP	56.9	57.7	68.9	67.8
NDF, % of DM	30.2	28.4	31.5	30.2
ADF, % of DM	17.4	18.4	19.2	18.9
EE, % of DM	3.6	4.1	4.1	4.2

[1]Salt, cobalt and iodine mix

[2]Contained per kg of supplement DM : CuO, 3.3 mg; ZnO, 22 mg; MnO, 18.5 mg; vitamin A, 1,495 IU; vitamin D, 125.5 IU; vitamin E, 22 IU; sodium selenite, 0.1 mg; and rumensin, 10.2 mg.

Experimental diets were fed twice daily for *ad libitum* intake at 0900 and 1700 as total mixed rations (TMR), composed of 80% concentrate mixture and 20% barley silage (DM basis), which were hand mixed thoroughly just before feeding. Barley silage contained 35.5% DM and its chemical composition (DM basis) was 90.9% OM, 11.3% CP, 54.7% NDF, 35.8% ADF, and 3.42% ether extract. At the beginning of the experiment, lambs were adapted to high-grain diets by the gradual introduction of the concentrate mixtures over a 12-d period. Briefly, on d 1, lambs were fed a TMR containing 30% concentrate and 70% barley silage (DM basis). Proportions of the concentrate mixture in the TMR were increased by 10% every alternate day, such that by d 10, the TMR contained 80% concentrate. This grain adaptation protocol was used to limit the risk of digestive upsets. Experimental TMR were formulated to contain 28.8 g of N/kg of DM (18% CP). The chemical composition of experimental TMR is presented in **Table 3.2**. Lambs had free access to water and were weighed weekly.

3.3.3 Sample Collection

Experimental lambs were moved from individual floor pens into individual metabolism crates on d 12 of each experimental period to allow acclimation before the initiation of data collection on d 15. During the 7-d data collection period, individual lamb feed intake was recorded daily. Samples of experimental TMR and orts were collected daily, stored at -20°C, and composited per lamb for each experimental period before chemical analysis.

On d 14 of each experimental period, lambs were fitted with temporary vinyl catheters (0.86-mm i.d. x 1.32-mm o.d.; Scientific Commodities Inc., Lake Havasu City, AZ) in the right and left jugular veins to allow for simultaneous isotope infusion and blood sampling. Urea transfer to the GIT and whole-body N balance were determined between d 15 and 21, as described by Lobley et al. (2000). Briefly, background samples of urine, feces, and blood were collected on d 14 to measure the natural abundance of ^{15}N.

Table 3.2 Chemical composition of total mixed rations containing 80% concentrate mixture and 20% barley silage (DM basis) with low or high ruminally-degradable protein (RDP) and dry rolled (DRB) or pelleted barley (PB)

	Low RDP		High RDP	
Item	DRB	PB	DRB	PB
DM, %	91.7	92.2	92.0	92.0
OM, % of DM	89.7	89.6	88.7	88.8
CP, % of DM	18.7	18.3	19.1	18.7
RDP, % of CP	60.3	60.8	70.1	68.6
NDF, % of DM	33.1	33.7	35.4	34.8
ADF, % of DM	20.9	21.9	23.9	23.8
EE, % of DM	3.8	3.9	4.0	4.0

Beginning on d 15 of each experimental period, double-labeled urea ([$^{15}N^{15}N$]-urea), 99.8 atom % ^{15}N, Cambridge Isotope Laboratories, Andover, MA) prepared in 0.15 *M* sterile saline was infused continuously into a jugular vein at a rate of 1.2 mmol of N/d using a peristaltic pump (Model 60 rpm/7524-10, Masterflex L/S Microprocessor Pump Drive, Vernon Hills, IL) for 96 h (d 15 to 19). Total feces and urine were collected daily between d 15 to 21 before the 0900 feeding to determine daily outputs. Feces were collected using fecal bags, which were fitted 2 d before the start of collection to allow acclimation. Bags were emptied daily at 0900 during total collection periods. Total daily fecal output for each lamb was mixed thoroughly, quantitatively transferred into a preweighed plastic container and weighed. A 25% subsample was taken daily and stored at –20°C. Urine was collected into sealed plastic containers placed below metabolic crates. Plastic containers had 40 mL of 12 *M* HCl to maintain the urine pH between 2 to 3 to prevent bacterial growth and the loss of NH_3. Total urine output was recorded daily. A 50-mL subsample of urine was collected daily (d 16 to 19) and stored at –20°C until analyzed for proportions of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]-, and [$^{14}N^{14}N$]-urea in urinary urea. In addition, a 2-mL subsample of urine was diluted with 8 mL of distilled water and stored at –20°C for later determination of urea-N and purine derivatives (PD). All daily urinary output was composited by period and animal and stored at –20°C until analyzed for total N. Blood samples were collected daily from the contralateral jugular vein in vacutainers containing heparin just before the 0900 feeding. Blood samples were centrifuged at 1,500 x *g* for 15 min at 4°C, and the plasma obtained was stored at –20°C until analyzed for urea-N.

3.3.4 Sample Analyses

At the end of the trial, frozen TMR, orts, and fecal subsamples were thawed overnight at room temperature and analyzed for DM by drying in an oven at 60°C for 48 h (AOAC, 1990; method 930.15). Dried TMR, orts, and feces were then ground through a 1-mm screen using a Christy-Norris mill (Christy and Norris Ltd., Chelmsford, UK). Ground TMR, orts, and feces samples were pooled per lamb for each experimental period and analyzed for organic matter (OM) by ashing at 600°C for at least 8 h, crude protein (CP) using the macro-Kjeldahl procedure (AOAC, 1990; method 990.03), ether extract (AOAC, 1990; method 920.39), acid-detergent fiber (ADF; AOAC, 1990), and neutral-detergent fiber (NDF; Van Soest et al., 1991). Amylase and sodium sulfite were used for NDF determination. Dietary content of RDP in experimental

TMR and ruminal starch degradation were determined using the in situ method, as described by Yu et al. (2003). Briefly, air-equilibrated experimental TMR samples (approximately 7 g) were weighed into nylon bags and incubated in the rumen of a steer fed barley silage for 2, 4, 8, 12, 24, and 48 h. The rumen incubation protocol, nylon bags, and washing and drying procedures for nylon bags were as described by Yu et al. (2003). Samples of TMR and nylon bag residues were analyzed for CP using the macro-Kjeldahl procedure (AOAC, 1990; method 990.03) and for total starch (AOAC, 1990; method 996.11) using a commercial kit (Total Starch Assay Kit, Megazyme International Ireland Ltd., Wicklow, Ireland). Rumen degradation characteristics of CP and starch were analyzed using the NLIN procedure (SAS Institute Inc., Cary, NC) using iterative least squares regression (Gauss-Newton method), as described by Yu et al. (2003).

Total N in pooled urine was determined using the macro-Kjeldahl procedure (AOAC, 1990). Daily dilute urine subsamples were pooled by lamb and experimental period and analyzed for allantoin and xanthine plus hypoxanthine (Chen and Gomes, 1992), and for uric acid by a quantitative enzymatic colorimetric method using a commercial kit (Stanbio Uric Acid Liquicolor Kit, Procedure No. 1045, Stanbio Laboratories, Boerne, TX). Total PD excretion per day was calculated as: allantoin + uric acid + xanthine plus hypoxanthine. Microbial non NH_3-N (NAN) supply was calculated based on total PD excretion in urine (Chen and Gomes, 1992), using BW measurements obtained on d 14. The ratio of purine N:total N in ruminal microbes was assumed constant at 11.6:100 (Chen and Gomes, 1992). Plasma urea-N (PUN) and UUN were determined by the diacetyl monoxime method of Marsh et al. (1957) using a commercial kit (Stanbio Urea Nitrogen Kit, Procedure No. 0580, Stanbio Laboratories).

To determine the proportions of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]-, and [$^{14}N^{14}N$]-urea in daily urine samples, urinary urea was isolated by applying urine containing 1.5 mg of urea-N through prepacked cation exchange resin columns (AG-50W-x8 Resin, 100–200 mesh, H^+ form, BioRad, Richmond, CA) as described by Archibeque et al. (2001). Previous studies have determined that this concentration of urea-N was suitable to ensure sufficient amounts of gas for analysis, yet minimized the occurrence of non-monomolecular degradation of urea (Sarasecca et al., 1998; Archibeque et al., 2001; Marini and Van Amburgh, 2003). After the urine was applied to the column, 7 mL of N-free water was applied to the columns, and the eluate discarded. Urea was then eluted by applying 20 mL of N-free water to the columns, which was collected into test

tubes. The eluate was air-dried at 60°C, and urea was quantitatively transferred into 17- x 60-mm borosilicate glass tubes using three 1-mL rinses of N-free water. The urea samples were then freeze-dried and the proportions of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]-, and [$^{14}N^{14}N$]-urea in urinary urea were analyzed by isotope ratio mass spectrometry (Lobley et al., 2000) at the N-15 Analysis Laboratory, University of Illinois (Urbana-Champaign). Under the conditions of this assay, [$^{14}N^{14}N$]-, [$^{14}N^{15}N$]-, and [$^{15}N^{15}N$]-urea molecules should yield ions with mass/charge (m/z) values of 28, 29, and 30, respectively. To account for non-monomolecular reactions, standards that were prepared from [$^{15}N^{15}N$]-urea (99.8 atom % ^{15}N) and [$^{14}N^{14}N$]-urea (natural abundance urea; 0.364 atoms % ^{15}N) were also analyzed and the necessary corrections for [$^{14}N^{15}N$]-urea that is produced by non-monomolecular reactions were then made (Lobley et al., 2000). Fecal samples collected daily (d 15 to 19) were analyzed for total ^{15}N enrichment by combustion to N_2 in an elemental analyzer and continuous flow isotope ratio-mass spectrometry, as described by Lobley et al. (2000).

3.3.5 Calculation of Urea-N Kinetics

Urea-N kinetics was calculated according to the model of Lobley et al. (2000), using urinary ^{15}N enrichment of [$^{15}N^{15}N$] and [$^{14}N^{15}N$]-urea and total ^{15}N excretion in feces. The enrichments of [$^{15}N^{15}N$]-urea are expressed relative to sum of [$^{14}N^{15}N$] and [$^{14}N^{14}N$], while enrichments of [$^{14}N^{15}N$]-urea are expressed relative to sum of [$^{15}N^{15}N$] and [$^{14}N^{14}N$]. In this model, a portion of urea-N synthesized in the liver (urea-N entry rate, UER) is lost via the urine (urinary urea-N elimination, UUE), and the remainder enters the GIT (GIT entry rate, GER). The GER undergoes bacterial degradation liberating NH_3. A portion of this NH_3 is excreted in feces (urea-N in feces, UFE), some is reabsorbed into portal blood and it re-enters the ornithine cycle in the liver (ROC), and the remainder is used for anabolic purposes i.e., synthesis of microbial protein (urea-N utilized for anabolism, UUA) (Lobley et al., 2000).

3.3.6 Statistical Analysis

All data were analyzed using PROC MIXED of SAS for a 4 x 4 Latin square design according to the following model: $Y = \mu + P + L + R + G + (R \times G) + E$, where Y is the dependent variable, μ is the overall mean, P is the effect of period, L is the effect of lamb, R is the effect of dietary RDP level, G is the effect of method of barley grain processing, R x G is the

interaction between dietary RDP level and method of barley grain processing, and E is the residual error. All terms were considered fixed, except L and E, which were considered random. When there was a significant method of barley processing x level of RDP interaction, means were separated by Tukey's honestly significant differences test. Treatment differences were considered significant when $P \leq 0.05$ and tendencies are discussed when $0.05 < P \leq 0.10$.

3.4 Results and Discussion

3.4.1 Diet Characteristics

The chemical compositions of experimental TMR fed to growing lambs are presented in Table 3.2. The TMR were formulated to be isonitrogenous at 28.8 g of N/kg of DM (18.5% CP), and chemical analysis showed only marginal deviations in dietary N content across the TMR, with a CV of 1.9% (Table 3.2). Experimental TMR contained 60 and 70% RDP (as % of CP), or 11.1 and 13.1% (as % of DM; **Table 3.3**). The actual RDP levels as determined using the in situ technique indicated only marginal deviations from these intended dietary RDP contents (Table 3.2). The NRC (2001) recommendations for dietary RDP levels are 10.9 to 11.3% (as % of DM) for cows producing up to 40 kg/d of milk. However, Reynal and Broderick (2005) fed dairy cows diets with RDP levels ranging from 13.2 to 10.6% (as % of DM) and concluded that recommended levels of RDP should fall between 11.7 and 12.2%. The RDP levels that were tested in our study are comparable to that recommended RDP range. As expected, the in situ soluble CP fraction ($P < 0.001$), the degradation rate of the degradable CP fraction ($P = 0.004$), and effective CP degradability ($P < 0.001$) of the high-RDP diet were greater than that of the low-RDP diet; however, the degradable CP fraction was smaller ($P < 0.001$) for the high-RDP diet compared with the low-RDP diet (Table 3.3). In this experiment, fine grinding of barley grain before pelleting was expected to increase ruminal starch digestion compared with dry-rolling, thus increasing the ruminal energy availability for microbial protein production. The results of the in-situ study indicated that method of barley grain processing had significant effects on ruminal starch digestion (Table 3.3).

Table 3.3 In situ ruminal degradation kinetics of CP and starch in total mixed rations with low or high ruminally-degradable protein (RDP) containing dry-rolled (DRB) or pelleted barley (PB)

	Low RDP		High RDP			*P* value[1]		
Item	DRB	PB	DRB	PB	SEM	BP	RDP	BP x RDP
CP								
Soluble fraction, %	26.5	29.5	47.5	46.6	0.92	0.32	<0.001	0.11
Degradable fraction, %	64.1	62.0	41.6	42.5	1.73	0.76	<0.001	0.43
Degradation rate, %/h	6.8	6.1	6.9	7.1	0.09	0.07	0.004	0.008
Effective degradability[2], %	60.3	60.8	70.1	68.6	0.55	0.81	<0.001	0.77
Starch								
Soluble fraction, %	10.6	15.7	11.6	16.9	1.71	0.04	0.15	0.33
Degradable fraction, %	86.0	81.1	85.0	80.8	1.83	0.07	0.11	0.14
Degradation rate, %/h	13.3	15.9	12.3	16.7	1.11	0.03	0.92	0.44
Effective degradability[2], %	71.5	76.1	71.7	76.9	1.21	0.02	0.70	0.80

[1]BP = barley grain processing (DRB vs. PB); RDP = dietary level of RDP; BP x RDP = interaction.

[2]Calculated assuming a ruminal outflow rate of 6%/h.

As expected, the in situ soluble starch fraction (P = 0.04), degradation rate of the degradable starch fraction (P = 0.03), and effective starch degradability (P = 0.02) of the TMR containing PB were greater than that of the TMR containing DRB; however, the degradable starch fraction tended to be lower (P = 0.07) in the TMR containing PB compared with those containing DRB. Data comparing ruminal starch digestion in ruminants fed DRB or pelleted barley are scarce. Feeding PB increased ruminal starch digestion, decreased ruminal pH, and increased VFA concentrations compared with feeding DRB in dairy cows (Gozho et al., 2008). We did not measure ruminal starch digestion in lambs used in the current study; however, based on the in situ ruminal starch digestion measurements, we can surmise that ruminal starch digestion was altered by barley grain processing.

3.4.2 Intakes, N Balance, Urea-N Kinetics, and Microbial NAN Supply

As expected, all experimental lambs gained weight (200 ± 3.5 g/d on average) during the experiment, however there was difference in average daily gain in body weight across treatments (P > 0.05). Interactions between the level of dietary RDP and method of barley grain processing influenced N metabolism in lambs (**Table 3.4**). Lambs fed the high dietary RDP consumed 111 g/d more DM compared with those fed the low dietary RDP (P = 0.006); consequently, lambs fed the high dietary RDP consumed 4.2 g/d more N compared with those fed the low dietary RDP (P = 0.001). Compared with the high dietary RDP, excretion of fecal N (P = 0.002) and urinary N (P = 0.03) were 1.8 and 2.9 g/d lower, respectively, in lambs fed the low dietary RDP. The greater N intake (4.2 g/d) in lambs fed the high dietary RDP was similar to the extra N (4.7 g/d) that was voided in feces and urine. Nitrogen digestibility was greater (P = 0.04) in lambs fed DRB compared with those fed PB. In addition, N digestibility was greater (P = 0.04) in lambs fed the low dietary RDP compared to those fed the high dietary RDP. Retained N was unaffected (P ≥0.74) by dietary treatment, and all experimental animals were in positive N balance. Previous research with lambs fed varying dietary N levels reported that N retention reached a plateau at dietary N contents around 28 g of N/kg of DM (Marini et al., 2004), which is equivalent to the dietary N content used in our study. Plasma urea-N concentration was unaffected (P = 0.27) by barley processing, but it tended (P = 0.06) to be greater in lambs fed the low dietary RDP compared with those fed the high dietary RDP.

Table 3.4. Intake, N digestibility, N balance and plasma urea-N in growing lambs fed total mixed rations with low or high ruminally-degradable protein (RDP) containing dry rolled (DRB) or pelleted barley (PB)

	Low RDP		High RDP			*P* value[1]		
Item	DRB	PB	DRB	PB	SEM	BP	RDP	BP x RDP
DMI, g/d	1,457.8	1,377.1	1,516.0	1,541.1	39.6	0.48	0.006	0.19
N intake, g/d	43.9	42.3	47.9	46.6	1.2	0.23	0.001	0.86
Fecal N, g/d	10.2	10.1	11.5	12.4	0.5	0.51	0.002	0.35
Urine N, g/d	25.9	23.7	28.7	26.6	1.3	0.10	0.03	0.95
Urine urea-N (UUN), g/d	16.3	16.0	17.9	14.1	0.9	0.04	0.87	0.08
UUN/Urine N, %	64.0	70.0	68.0	57.0	0.04	0.59	0.35	0.04
N digestibility, %	77.1	76.1	76.2	73.3	0.9	0.04	0.04	0.33
N retention, g/d	7.79	8.49	7.72	7.66	1.34	0.81	0.74	0.78
Plasma urea-N, mg/dL	27.1	28.1	25.9	26.4	0.6	0.27	0.06	0.75

[1]BP = barley grain processing (DRB *vs* PB); RDP = level of RDP; BP x RDP = interaction.

Lambs fed high-concentrate diets with N contents comparable to those used in our study (≥27.2 g of N/kg of DM) had similar PUN concentrations (Dabiri and Thonney, 2004; Marini et al., 2004). The greater PUN concentration in lambs fed the low-RDP diet was unexpected, especially considering that these lambs had lower intake of ruminally fermentable N, which would have decreased post-absorptive NH_3-N supply for hepatic ureagenesis; the reasons for this observation are unclear.

The primary objective of the present study was to determine how interactions between dietary RDP level and RFC might potentially alter urea-N transfer to the GIT and the utilization of this recycled urea-N in growing lambs fed high N diets (i.e., 28.8 g N/kg DM) that are more representative of practical feeding conditions in North America. Previous studies (Kennedy and Milligan, 1980; Marini and Amburgh, 2003; Marini et al., 2004) have clearly demonstrated that a higher proportion of hepatic urea-N output is transferred to the GIT in ruminants fed low N diets; however, under practical feeding conditions during lactation or growth, productive ruminants are rarely fed low N diets. In Holstein heifers fed high N diets, there was a wide range (29 to 42%) in the proportion of hepatic urea-N output that was recycled to the GIT (Marini and Amburgh, 2003), thus indicating that, even at high levels of N intake there is opportunity to enhance N efficiency of ruminants by manipulating the extent of urea-N recycling to the GIT. Therefore, it is important to delineate regulatory mechanisms that dictate urea-N transfer to the GIT and the subsequent fate of recycled urea-N in ruminants fed high N diets. Across treatments, urinary [$^{15}N^{15}N$]-urea enrichment reached a plateau within 24 to 48 h of isotopic infusion, whereas plateau enrichment of [$^{14}N^{15}N$]-urea in urine was not attained until between 48 and 72 h of isotopic infusion (**Figure 3.1**). This time course in the urinary enrichments of [$^{14}N^{15}N$]- and [$^{15}N^{15}N$]-urea is consistent with previous research using sheep (Lobley et al., 2000; Sunny et al., 2007). Fecal ^{15}N enrichments increased daily over the 4-d isotopic infusion without attaining a definite plateau (**Figure 3.2**); therefore, for calculations of urea-N kinetics, urinary [$^{14}N^{15}N$]- and [$^{15}N^{15}N$]-urea and fecal ^{15}N enrichments over the final 72 to 96 h of infusion were used, similar to previous research (Lobley et al., 2000; Sunny et al., 2007). In the present study, dietary treatment did not alter ($P > 0.05$) urea kinetics as measured by the intra-jugular [$^{15}N^{15}N$]-urea infusion protocol (**Table 3.5**); however, UFE was higher ($P = 0.01$) in lambs fed DRB compared to those fed PB (Table 3.5).

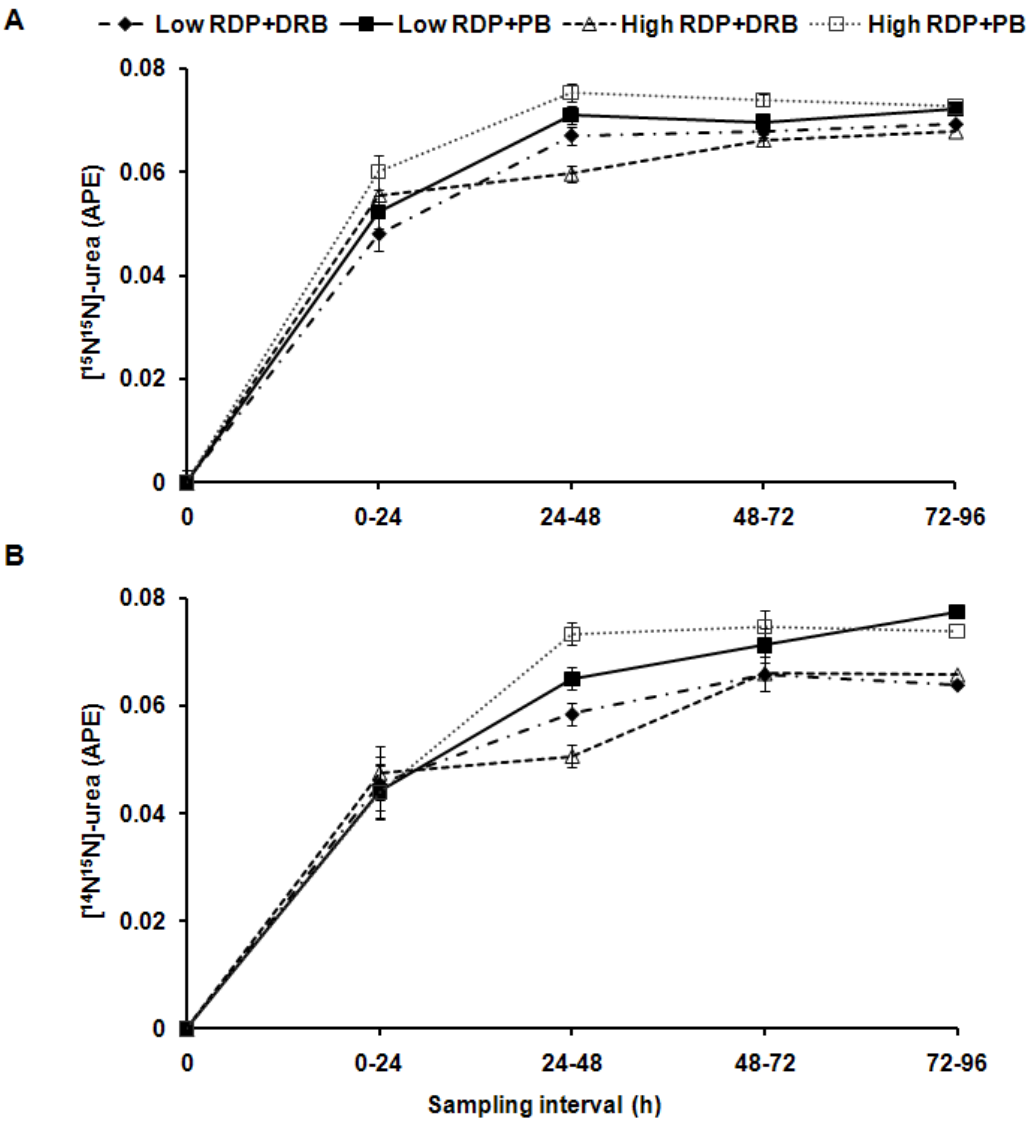

Figure 3.1 Enrichments (APE, atom percent excess) of [^{15}N^{15}N]-urea (A) and [^{14}N^{15}N]-urea (B) in urine during 4-d intra-jugular infusions of [^{15}N^{15}N]-urea (infusions were initiated at sampling interval 0 h). Treatments were: low RDP + dry-rolled barley (Low RDP-DRB); low RDP + pelleted barley (Low RDP-PB); high RDP + dry-rolled barley (High RDP-DRB); and high RDP + pelleted barley (High RDP-PB). For [^{15}N^{15}N]-urea enrichments, no differences were detected between sampling intervals 0-24, 24-48, 48-72, and 72-96 h (P > 0.10). For [^{14}N^{15}N]-urea enrichments, no differences were detected between sampling intervals 24-48, 48-72, and 72-96 h (P > 0.10). Each line represents means ± SEM for 4 lambs.

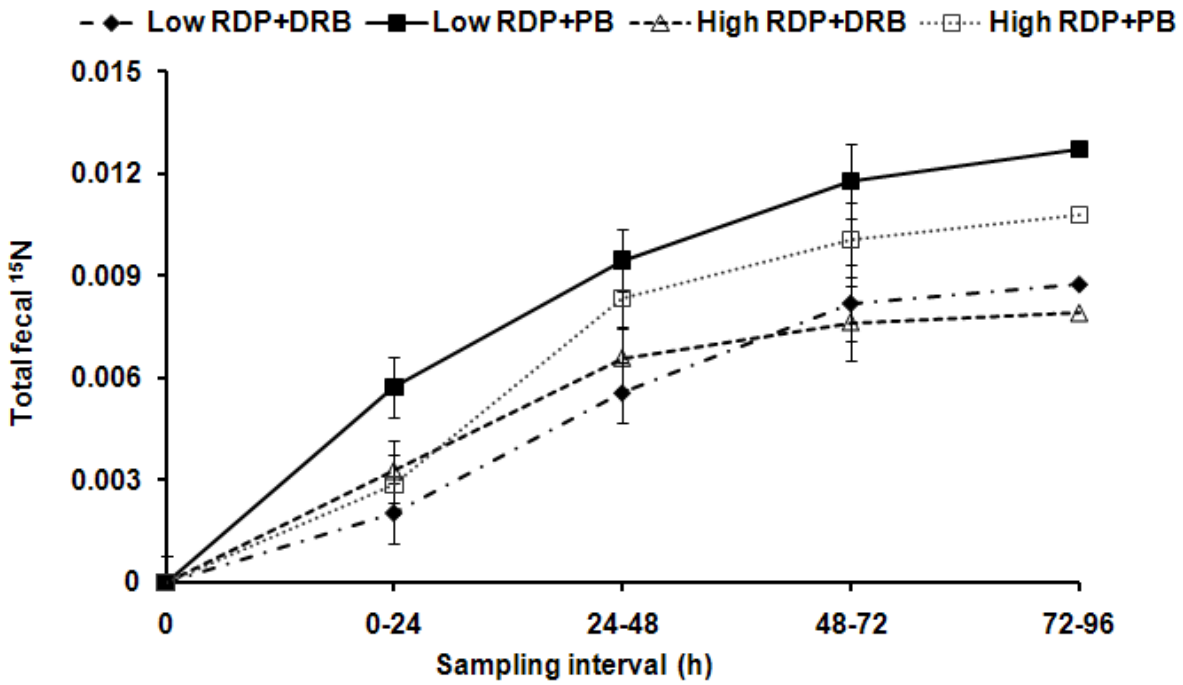

Figure 3.2 Enrichments (APE, atom percent excess) of fecal ^{15}N during 4-d intra-jugular infusions of [$^{15}N^{15}N$]-urea (infusions were initiated at sampling interval 0 h). Treatments were: low RDP + dry-rolled barley (Low RDP-DRB); low RDP + pelleted barley (Low RDP-PB); high RDP + dry-rolled barley (High RDP-DRB); and high RDP + pelleted barley (High RDP-PB). Differences were detected in fecal ^{15}N enrichments between sampling intervals 0-24, 24-48, 48-72, and 72-96 h (P = 0.001), indicating that fecal ^{15}N enrichment did not reach a definite plateau. Each line represents means ± SEM for 4 lambs.

Table 3.5. Urea-N recycling kinetics as measured using 4-d continuous jugular infusions of [$^{15}N^{15}N$]-urea in growing lambs fed total mixed rations with low or high ruminally-degradable protein (RDP) containing dry rolled (DRB) or pelleted barley (PB)

	Low RDP		High RDP			*P* value[1]		
Item	DRB	PB	DRB	PB	SEM	BP	RDP	BP x RDP
Urea-N kinetics, g/d								
Production, UER	49.76	45.78	50.98	50.18	4.99	0.22	0.35	0.58
Entry to GIT, GER	35.17	30.59	38.54	36.54	6.12	0.50	0.22	0.71
Return to ornithine cycle, ROC	22.22	23.24	23.70	24.72	3.55	0.34	0.48	0.99
Loss to feces, UFE	0.74	0.47	0.87	0.57	0.09	0.01	0.08	0.78
Loss to urine, UUE	14.59	15.19	12.44	13.63	5.19	0.68	0.54	0.92
Re-use for anabolism, UUA	12.21	6.88	13.97	11.25	5.66	0.47	0.38	0.69
Fractional urea-N transfers								
UER to urine	0.296	0.331	0.257	0.263	0.109	0.92	0.41	0.81
UER to GIT	0.704	0.669	0.742	0.737	0.109	0.92	0.41	0.81
GER to ROC	0.641	0.756	0.636	0.666	0.105	0.46	0.45	0.49
GER to feces	0.022	0.017	0.023	0.015	0.004	0.09	0.84	0.46
GER to UUA	0.338	0.227	0.340	0.319	0.106	0.51	0.45	0.47

[1]BP = barley grain processing (DRB *vs* PB); RDP = level of RDP; BP x RDP = interaction.

Previous research showed that the proportion of GER that is lost via the feces is largely influenced by the supply of fermentable energy to the hind-gut (Thornton et al., 1970; Oncuer et al., 1990). In the present study, fermentable energy supply to the hind-gut was not measured, but it can be surmised that, compared to feeding PB, feeding DRB provided more fermentable energy to the hind-gut, thus a higher transfer of GER to the hind-gut and subsequent loss via the feces. Urea-N transfer to feces tended (P = 0.08) to be higher in lambs fed the high dietary RDP compared to those fed the low dietary RDP (Table 3.5). Fractional urea-N transfers of the GER to feces ranged from 0.015 to 0.022 across diets, and this represented the smallest proportion of GER (Table 3.5). Across dietary treatments, the UER was 1.06 to 1.13 of N intake (Table 3.4). In growing ruminants, hepatic output of urea-N can exceed N intake under a wide variety of dietary conditions, and is positively correlated with N intake. The UER:N intake ratios obtained in sheep by other workers (0.77 to 0.95, Lobley et al., 2000; 0.73 to 0.99, Sunny et al., 2007) indicate that the UER:N intake ratio can vary considerably depending on dietary conditions. However, in these studies, N intakes (range of 14.3 to 22.0 g/d) were lower compared to those in the present study (42.3 to 47.9 g/d), and this difference might explain the higher UER:N intake ratios that we observed in the present study. The quantity of hepatic urea-N output transferred to the GIT (i.e., GER), expressed as a proportion of UER was high at 0.669 to 0.742 (Table 3.5). In ruminants, the proportion of hepatic urea-N output that can be recycled to the GIT is influenced by a wide variety of factors (Lapierre and Lobley, 2001), and can vary from 0.10 to 0.95 (Harmeyer and Martens, 1980). Various workers, using sheep at N intakes ranging from 8.9 to 25 g/d (i.e., lower N intakes as compared to the present study) observed that the GER:UER ratios ranged from 0.75 to 0.61 (Sarraseca et al., 1998; Lobley et al., 2001; Marini et al., 2004), and were negatively correlated with N intake. Another important factor influencing the GER:UER ratio appears to be blood concentrations of urea-N (Harmeyer and Martens, 1980). Recently, Sunny et al. (2007), using an intra-jugular urea infusion protocol to increase blood urea-N concentrations, clearly demonstrated that urea-N transfer from blood to the GIT was elevated as blood urea-N concentration increased. In the present study, PUN concentrations (25.9 to 28.1 mg/dL) across dietary treatments were higher than reported in other studies (Lobley et al., 2001; Marini et al., 2004), so it is plausible that the higher GER:UER ratios observed, even with higher N intakes, could be attributable to the higher PUN concentrations.

The GER that was used in the GIT for anabolic purposes (UUA) was unaffected by dietary treatment (Table 3.5). Across dietary treatments, UUA ranged from 0.227 to 0.340 as a proportion of the GER (Table 3.5). A significant proportion, ranging from 0.636 to 0.756, of the GER was reabsorbed into portal blood as NH_3-N and returned to the urea cycle (i.e., ROC; Table 3.5). The reasons for this observation are unclear, but it is plausible that, because of the high N intakes, a limit of N utilization for anabolic purposes in the GIT may already have been reached (Lobley et al., 2000). We had hypothesized that the provision of additional RFC via grain processing would enhance urea-N transfer to the GIT and, subsequently, its utilization for anabolic purposes. The lack of effect of grain processing suggests that energy supply did not limit utilization of the extra N provided via enhanced urea-N recycling to the GIT, likely because of the high levels of concentrate fed.

Urinary output of total urine was unaffected ($P > 0.05$) by dietary treatment (**Table 3.6**). Urinary excretion of allantoin and uric acid were unaffected ($P > 0.05$) by dietary treatment (Table 3.6); however, there was a tendency (interaction; $P = 0.08$) for urinary PD to be higher in lambs fed DRB compared to those fed PB at the low dietary RDP, but not at the high dietary RDP (Table 3.6). Microbial non-NH_3-N (NAN) flow to the small intestine, which was estimated using urinary PD excretion, tended ($P = 0.08$) to be higher in lambs fed DRB compared to those fed PB at the low dietary RDP, but not at the high dietary RDP (Table 3.6). Chamberlain et al. (1993) observed no significant difference in urinary output of PD and microbial NAN supply in sheep fed varying degradable protein and supplied with wheat starch. Similarly, Sannes et al. (2002) did not observe any difference on PD excretion in dairy cows fed protein sources of varying RDP content. Because diets were high in concentrate and N, it is likely that energy or N supply for microbial growth were not limiting, thus the lack of dietary effect on microbial NAN supply that was observed in the present study.

Table 3.6 Organic matter intake and OM digestibility, urinary purine derivative (PD) excretion and microbial non-ammonia nitrogen (NAN) supply in growing lambs fed total mixed rations with low or high ruminally-degradable protein (RDP) containing dry rolled (DRB) or pelleted barley (PB)

	Low RDP		High RDP			*P* value[1]		
Item	DRB	PB	DRB	PB	SEM	BP	RDP	BP x RDP
OM intake, g/d	1,310	1,230	1,352	1,377	11.4	0.86	0.006	0.18
OM digestibility, %	71.7	69.9	69.2	70.6	0.96	0.79	0.36	0.10
Urinary excretion								
Total urine output, g/d	2.33	2.66	2.30	2.59	0.54	0.58	0.93	0.97
Uric acid excretion, mmol/d	2.96	3.31	3.28	3.44	0.39	0.52	0.57	0.81
Allantoin excretion, mmol/d	21.6	18.9	19.9	21.6	0.72	0.06	0.45	0.12
Total PD excretion, mmol/d	24.6	22.3	23.2	23.0	0.58	0.05	0.58	0.08
Microbial NAN supply[2]								
g microbial N/d	21.3	19.3	20.1	19.9	0.49	0.05	0.58	0.08
g microbial N/kg DOMR	23.1	22.7	22.1	20.8	1.82	0.66	0.43	0.81

[1]BP = barley grain processing (DRB *vs* PB); RDP = level of RDP; BP x RDP = interaction.

[2]Microbial NAN supply was calculated according to Chen and Gomes (1992); DOMR = digestible organic matter in rumen, which was calculated as 0.65 * DOMI (Chen and Gomes, 1992).

3.5 Conclusions

Concomitant changes in dietary RDP level and method of barley grain processing did not alter hepatic urea-N output and urea-N recycled to the GIT. Although substantial amounts of hepatic urea-N output were transferred to the GIT, most of this N was returned to the ornithine cycle. Because of the high N intakes, it is plausible that a plateau in N utilization may already have been reached, thus limiting the utilization of the extra N supplied from recycled urea-N. Hence, under practical feeding systems demanding greater production in terms of protein accretion for growth, urea recycling seems to be of little importance when the dietary N concentrations are adequate or marginally above the adequate levels.

4. EFFECTS OF PARTIAL RUMINAL DEFAUNATION ON UREA-NITROGEN RECYCLING, NITROGEN METABOLISM, AND MICROBIAL NITROGEN SUPPLY IN GROWING LAMBS FED LOW OR HIGH DIETARY CRUDE PROTEIN CONCENTRATIONS[1]

4.1 Abstract

Urea-N recycling to the gastrointestinal tract (GIT), N metabolism and urea transporter-(UT)-B mRNA abundance in ruminal epithelium were evaluated in partially-defaunated (PDFAUN) and faunated (FAUN) growing lambs fed two levels (10%, LOW or 15%, HIGH) of dietary CP (DM basis). Four Suffolk ram lambs (43.9 ± 1.4 kg initial BW) were used in a 4 x 4 Latin square design with 27-d periods. Sunflower oil was fed (6%; DM basis) as an anti-protozoal agent. Nitrogen balance was measured from d 22 to d 26, with concurrent measurement of urea-N kinetics using continuous intra-jugular infusions of [$^{15}N^{15}N$]-urea. Feeding sunflower oil decreased ($P < 0.01$) total ruminal protozoa by 88%, and this was associated with a decrease ($P < 0.01$) in ruminal NH_3-N concentrations. Endogenous production of urea-N (UER; 26.1 vs. 34.6 g/d) and urinary urea-N excretion (UUE; 10.1 vs. 15.7 g/d) were lower ($P < 0.01$), and the amount of urea-N recycled to the GIT (GER; 16.0 vs. 18.9 g/d) tended to be lower ($P = 0.06$) in PDFAUN as compared to FAUN lambs. However, as a proportion of UER, GER was higher ($P < 0.01$) and the proportion of recycled urea-N that was utilized for anabolism (i.e., UUA) tended to be higher ($P = 0.09$) in PDFAUN lambs. Microbial N supply was higher ($P < 0.01$) in PDFAUN compared to FAUN lambs. The UER, GER and UUE were higher ($P < 0.01$) in lambs fed diet HIGH. However, as a proportion of UER, GER and its anabolic use were higher ($P < 0.01$) in lambs fed diet LOW. The expression of UT-B mRNA in PDFAUN lambs was numerically higher (by 20%; $P = 0.15$) as compared to FAUN lambs. In summary, results indicate that part of the mechanisms for improved N utilization in defaunated ruminants is an increase in the proportion of endogenous urea-N output that is recycled to the GIT, thus potentially providing additional N for microbial growth.

[1]A version of this chapter has been published: Kiran, D., and T. Mutsvangwa. 2010. Effects of partial ruminal defaunation on urea-nitrogen recycling, nitrogen metabolism, and microbial nitrogen supply in growing lambs fed low or high dietary crude protein concentrations. J. Anim. Sci. 88: 1034-1047.

4.2 Introduction

In ruminants, protozoa account for 20 to 70% of the ruminal microbial biomass, but their contribution to microbial protein flow at the duodenum is limited to only 20 to 40% (Jouany, 1996). Defaunation improves N utilization, mainly by decreasing intra-ruminal N recycling and the NH_3-N pool (Koenig et al., 2000), and by increasing the sequestration of NH_3-N into bacterial protein (Firkins et al., 2007). It is plausible that the positive effects of defaunation on N utilization may also result from an increase in urea-N recycling to the rumen. Protozoa degrade dietary and bacterial proteins, thus elevating ruminal NH_3-N level; consequently, defaunation consistently decreases ruminal NH_3-N level (Jouany, 1996). Ruminal NH_3-N level is negatively correlated with urea-N transfer into the rumen (Kennedy and Milligan, 1980), as a high NH_3-N level decreases the ruminal epithelium's permeability to urea-N (Egan et al., 1986). Also, urea-N transfer into the rumen is facilitated by bacterial urease activity which maintains a favorable concentration gradient (Rémond et al., 1996), and urease activity is negatively correlated with ruminal NH_3-N level (Cheng and Wallace, 1979). Therefore, it is possible that the decreased ruminal NH_3-N level with defaunation may increase urea-N recycling by increasing both urease activity and the ruminal epithelium's permeability to urea-N. Effects of defaunation on ruminal NH_3-N level decrease with dietary N level (Eugène et al., 2004), and the benefits of defaunation on N utilization are greater in protein-deficient animals (Leng and Nolan, 1984), suggesting that there might be interactions between defaunation and dietary N level on N use.

Therefore, the objective was to delineate how interactions between defaunation and dietary N level may alter urea-N kinetics in lambs. The hypothesis was that defaunation would increase urea-N recycling to the rumen and its utilization for anabolic purposes, and that these effects would be more pronounced in protein deficient lambs.

4.3 Materials and Methods

4.3.1 Animals and Experimental Design

The lambs were cared for and handled in accordance with regulations of the Canadian Council on Animal Care (1993), and their use in this experiment was approved by the University of Saskatchewan Animal Care Committee (UCACS Protocol No. 20040048). Four Suffolk ram lambs (43.9 ± 1.4 kg initial BW) fitted with ruminal cannulae were used in a 4 x 4 Latin square

design experiment with 27-d periods and a 2 x 2 factorial arrangement of dietary treatments. Each experimental period consisted of 21 d of dietary adaptation and 6 d of data collection. Throughout the experiment, lambs were housed at the Livestock Research Building (University of Saskatchewan) in a temperature-controlled environment (18 to 22°C). Lambs were housed in individual floor pens (during the 21-d dietary adaptation) or in metabolism crates (during the 6-d data collection period) to facilitate total urine and feces collection.

4.3.2 Experimental Treatments and Feeding Management

The treatment factors were: 1) partially-defaunated (PDFAUN) vs. faunated (FAUN) lambs; and 2) low (10%, LOW) vs. high (15%, HIGH) dietary CP (DM basis). The ingredient and chemical composition of the LOW and HIGH experimental diets are presented in **Table 4.1**. Diets were offered in the form of a meal for *ad libitum* intake twice daily in equal portions at 0900 and 1700 h ensuring that 10% of orts remained each day. Lambs had free access to water. Lambs were weighed weekly, before access to feed and water.

4.3.3 Partial Defaunation and Refaunation

Linoleic acid-rich sunflower oil (SFO; Safeway^{TM} Canada, Saskatoon, SK, Canada) was fed (6%, DM basis) as an anti-protozoal agent. The protocol for ruminal defaunation was based on previous research (Ivan et al., 2001). The SFO contained (%) 6.24, 3.87, 26.15, 67.07, and 0.49 of palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2(n-6), and linolenic (C18:3(n-3) acid, respectively. At each feeding, half the daily allotment of SFO was thoroughly hand-mixed with 250 g of feed and then the SFO-feed mixture was offered initially to experimental animals in order to ensure complete consumption of the oil. Thereafter, the rest of the feed was offered. To avoid cross-inoculation of PDFAUN lambs by FAUN lambs via direct physical contact, individual floor pens and metabolism crates housing PDFAUN lambs were spaced several meters away from those housing FAUN lambs. In addition, ruminal fluid sampling of PDFAUN lambs was conducted prior to FAUN lambs in order to minimize the risk of cross-inoculation. Ruminal contents (approximately 200 mL) were sampled at 1100 h on d 1 to d 3, d 6, d 9, d 12, d 15, d 18, and d 21 to d 26. Ruminal contents were squeezed through two layers of cheesecloth. A 10-mL aliquot of ruminal fluid was mixed with an equal amount of 10% formalin and transported to the laboratory where protozoa were enumerated immediately.

Table 4.1 Ingredient and nutrient composition of experimental diets[1]

Item	LOW	HIGH
Ingredients, % of DM		
Alfalfa meal	6.0	6.0
Barley (Dry rolled)	45.0	32.6
Canola meal	2.0	2.0
Corn gluten meal	0.5	4.0
Oat hulls	35.0	35.0
Soybean meal	1.5	10.4
Canola oil	1.0	1.0
Liquid molasses	2.0	2.0
Ground limestone	4.0	4.0
Salt, Co-I[2]	0.7	0.7
Salt white	1.3	1.3
Mineral vitamin supplement[3]	1.0	1.0
Chemical composition		
DM, %	94.6	95.1
OM, % of DM	91.7	91.6
CP, % of DM	10.3	15.6
Neutral detergent fiber, % of DM	40.2	39.1
Acid detergent fiber, % of DM	20.2	19.2
Ether extract, % of DM	3.6	3.8
Ruminally-degradable protein[4], % of CP	63.6	60.6
Metabolizable energy[4], MCal/kg DM	1.8	1.8

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Salt, cobalt and iodine mix: Co, 100 mg/kg: and I, 150 mg/kg.

[3]Provided per kg of DM: CuO, 3.3 mg; ZnO, 22 mg; MnO, 18.5 mg; vitamin A, 1,495 IU; vitamin D, 125.5 IU; vitamin E, 22 IU; and sodium selenite, 0.1 mg.

[4]Calculated according to NRC (1985) guidelines.

Ruminal protozoa were enumerated microscopically (Ogimoto and Imai, 1981) using a Neubauer improved Bright Line Hematocytometer (Hausser Scientific, Horsham, PA). Each ruminal fluid sample was counted twice and if the average of the duplicates differed by more than 10%, counts were repeated. In addition to total protozoal counts, protozoa were also differentiated into different genera as *Entodinium spp.*, Holotrichs, and cellulolytic protozoa (Ogimoto and Imai, 1981). Partially-defaunated lambs were refaunated at the start of an experimental period by a 3-d administration of approximately 300 mL of sampled ruminal contents that were obtained from 2 faunated spare lambs that were fed equal proportions of the 2 experimental diets.

4.3.4 Sample Collection

Experimental lambs were moved from individual floor pens into individual metabolism crates on d 19 of each experimental period to allow acclimation before the initiation of data collection on d 22. During the 6-d data collection period, individual lamb feed intake was recorded daily. Samples of experimental diets and orts were collected daily, stored at -20°C, and composited per lamb for each experimental period prior to chemical analysis. On d 21 of each experimental period, lambs were fitted with temporary vinyl catheters (0.86 mm I.D. x 1.32 mm O.D.; Scientific Commodities Inc., Lake Havasu City, Arizona) in the right and left jugular veins to allow for simultaneous isotope infusion and blood sampling. Urea transfer to the GIT and whole-body N balance were determined between d 22 and 26 as described by Lobley et al. (2000). Briefly, background samples of urine and feces were collected on d 21 to measure the ^{15}N natural abundance. Starting on d 22 of each experimental period, double-labeled urea ([$^{15}N^{15}N$]-urea, 99 atom % ^{15}N; Cambridge Isotope Laboratories, Andover, MA) prepared in 0.15 M sterile saline was infused continuously into a jugular vein using a peristalitic pump (Model 60 RPM/7524-10**,** Masterflex L/S Microprocessor Pump Drive, Vernon Hills, IL) for 96 h (d 22 to d 26). Daily dosage rates of [$^{15}N^{15}N$]-urea ranged from 332 to 744 mg per lamb depending on N intake, such that the predicted plateau enrichment of [$^{15}N^{15}N$]-urea in urine was 0.15 atom percent excess above the background. Total feces and urine were collected daily between d 22 and d 26 before the 0900 h feeding to determine daily outputs. Collection, processing, and sampling of feces and urine for N, fecal ^{15}N enrichment, urinary enrichment of [$^{15}N^{15}N$]-,

[$^{14}N^{15}N$]- and [$^{14}N^{14}N$]-urea, and urinary PD analysis was as previously described (Kiran and Mutsvangwa, 2007).

On d 26, ruminal contents (approximately 200 mL) were sampled at 0900, 1100, 1300, 1500 and 1700 h and squeezed through two layers of cheesecloth. Ruminal pH was then immediately determined using a Model 265A portable pH meter (Orion Research Inc., Beverly, MA). A 10-mL aliquot of ruminal fluid was preserved with 2 mL of metaphosphoric acid (25% wt/vol) and a second 10-mL aliquot of ruminal fluid was preserved with 2 mL of 1% sulfuric acid and stored at -20°C for later analyses. At the same time points corresponding to ruminal fluid sampling, blood samples were drawn from a jugular vein catheter into vacutainers containing heparin. Blood samples were centrifuged at 1,500 x *g* for 15 minutes at 4°C and the plasma obtained was stored at -20°C for later analysis. On d 27, ruminal epithelial tissue biopsies were taken at 2 h after the morning feeding. Briefly, whole ruminal contents were evacuated using suction vacuum into a large container which was placed in warm (approximately 40^{o}C) water. Approximately 6 mm of ruminal mucosal tissue with papillae was clipped carefully from the ventral sac of the rumen using a sterile clipper, immediately snap frozen in liquid N and stored at -80°C. The biopsy procedure for all the 4 lambs was completed within 1 h in order to minimize possible variations with time due to circadian effects. Ruminal contents were returned to respective lambs.

4.3.5 Sample Analyses

At the end of the trial, frozen samples of experimental diets, orts and feces were thawed overnight at room temperature and analyzed for DM by drying in an oven at 60°C for 48 h (AOAC, 1995; method 930.15). Dried diets, orts and feces were then ground through a 1-mm screen using a Christy-Norris mill (Christy and Norris Ltd., Chelmsford, England). Ground diets, orts and fecal samples were pooled per lamb and analyzed for OM by ashing at 600°C for at least 8 h (AOAC 1995; method 942.05), CP using the macro-Kjeldahl procedure (AOAC, 1995; method 2001.11), ether extract (AOAC, 1995; method 920.39), and ADF and NDF (Van Soest et al., 1991). Amylase and sodium sulfite were used for neutral detergent fiber determination.

Ruminal fluid samples that were preserved with metaphosphoric acid were thawed at room temperature, centrifuged at 18,000 x *g* for 15 min, and filtered through a 0.45-µm

membrane. A sub-sample (0.1 mL) of the supernatant was transferred into a 1.7 mL micro centrifuge tube, and 0.5 mL of 1mM trimethyl acetic acid was then added to each tube as an internal standard. The volume was made up to 1.5 mL with acetonitrile, contents were then centrifuged at 18,000 x *g* for 5 min and the supernatant was quantitatively transferred to clean, dry vials. Volatile fatty acids (VFA) were separated and quantified by GC (Agilent 6890, Mississauga, ON) as described by Erwin et al. (1961). Ruminal fluid samples that were preserved with sulphuric acid were also thawed, centrifuged for 10 min at 18,000 x *g* to obtain a clear supernatant, and analyzed for NH_3-N using a phenol-hypochlorite assay (Broderick and Kang, 1980). Total N in pooled urine was determined using the macro-Kjeldahl procedure (AOAC, 1990). Daily urine samples (2 mL) that were diluted with distilled water (8 mL) were pooled per lamb proportionally to daily urine output and analyzed for allantoin, and xanthine plus hypoxanthine (Chen and Gomes, 1992), and for uric acid by a quantitative enzymatic colorimetric method using a commercial assay kit (Stanbio Uric Acid Liquicolor Kit, Procedure No. 1045; Stanbio Laboratories, Boerne, TX). Total PD excretion per day was calculated as allantoin + uric acid + xanthine plus hypoxanthine. Microbial NAN supply was calculated based on total PD excretion in urine (Chen and Gomes, 1992), using BW measurements obtained on d 19. Plasma urea-N (PUN) and UUN were determined by the diacetyl monoxime method (Marsh et al., 1957) using a commercial kit (Stanbio Urea-N Kit, Procedure No. 0580; Stanbio Laboratories).

To determine the proportions of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]- and [$^{14}N^{14}N$]-urea in pooled urine and background urine samples, urinary urea was isolated by applying urine containing 1.5 mg of urea-N through pre-packed cation exchange resin columns (AG-50W-×8 Resin, 100-200 mesh, H^+ form; Biorad, Richmond, CA) as described by Archibeque et al. (2001). Previous studies have determined that 1.5 mg urea-N was suitable to ensure sufficient amounts of N_2 gas for analysis, yet minimizing the occurrence of non-monomolecular degradation of urea (Archibeque et al., 2001; Marini and Van Amburgh, 2003). The procedure to isolate UUN was described previously (Kiran and Mutsvangwa, 2007). The urea samples were then freeze-dried and the proportions of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]-, and [$^{14}N^{14}N$]-urea in urinary urea-N were analyzed by isotope ratio-mass spectrometry (Lobley et al., 2000) at the N-15 Analysis Laboratory, University of Illinois at Urbana-Champaign. Under the conditions of this assay, [$^{14}N^{14}N$]-, [$^{14}N^{15}N$]-, and [$^{15}N^{15}N$]-urea molecules should yield ions with mass/charge (m/z) values of 28,

29, and 30, respectively. To account for non-monomolecular reactions, standards which were prepared from [$^{15}N^{15}N$]-urea (99.8 atom % ^{15}N) and [$^{14}N^{14}N$]-urea (natural abundance urea; 0.364 atoms % ^{15}N) were also analyzed and the necessary corrections for [$^{14}N^{15}N$]-urea that is produced by non-monomolecular reactions were then made (Lobley et al., 2000). Fecal samples were analyzed for total ^{15}N enrichment by combustion to N_2 gas in an elemental analyzer and continuous flow isotope ratio-mass spectrometry (Lobley et al., 2000).

4.3.6 Urea Transporter-B Gene Expression Analysis

Ruminal epithelial tissue samples were pulverized with a mortar and pestle under liquid N. Total RNA was extracted from 20 to 30 mg tissue sample using an RNAeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada), followed by digestion with RNase-free DNase (Qiagen). Amount of RNA was quantified with PicoGreen (Molecular Probes, Eugene, OR) using a fluorometer (Fluoroscan Ascent FL, Thermo Labsystems, Waltham, MA) and 1 µg of RNA was used to generate first-strand cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA obtained was stored at -20^{o}C until analyzed. Gene transcript abundance was quantified using qPCR (iCycler iQ Real-Time PCR detection system, BioRad) using SYBR Green fluorescence detection. The primers used for UT-B and ovine glyceraldehyde 3-phosphate dehydrogenase (ovine-GAPDH; NCBI Accession no.: BC102589) were previously reported (Stewart et al., 2005; Ludden et al., 2009). Ovine-GAPDH was used as an internal reference to normalize UT-B mRNA expression. Briefly, the PCR primers were UT-B (forward, 5′ /ggacctgcctgtcttcactc/ 3′; reverse, 5′ /gatcaaggtgcttgggaaaa/ 3′) and ovine GAPDH (forward, 5′ /gattgtcagcaatgcctcct/ 3′; reverse, 5′ /ggtcataagtccctccacga/ 3′) with amplicon size of 97 and 94 bp, respectively. Amplification conditions for ovine GAPDH and UT-B included a pre-dwell for 3 min at 95^{o}C and 35 cycles of denaturing for 30 sec at 95^{o}C, and annealing for 30 sec at 58^{o}C. The qPCR reaction mixture used for each gene consisted of 12.5 µL of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Burlington, Ontario, Canada), 0.5 µL of each primer (25 µ*M*), and 1.0 µL of template cDNA, made up to 25 µL. The amplification efficiency was 100.1%. To confirm the quality of amplification, melting curve analysis was done after qPCR amplification of incubation products for 5 sec at each step with increase in temperature by 0.5^{o}C from 65^{o}C to 95^{o}C in each cycle. The results are presented as target gene transcript abundance normalized to GAPDH transcript abundance. Expression of GAPDH mRNA in equal

amounts of total RNA indicated that GAPDH in rumen epithelial tissue was stable and unaffected by the experimental conditions that were employed.

4.3.7 Calculation of Urea-N Kinetics and Statistical Analysis

Urea-N kinetics was calculated according to the model of Lobley et al. (2000), using urinary ^{15}N enrichment of [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea and total ^{15}N excretion in feces. All data were analyzed using ANOVA as a 4 x 4 Latin square using the Proc Mixed procedure of SAS (2004) with period and animal as the random effects. The model used included the following independent variables: animal, period, ruminal protozoal status (PDFAUN vs. FAUN), level of dietary CP (LOW vs. HIGH), and the ruminal protozoal status x level of dietary CP interaction. Because N intake differed due to protozoal status (**Table 4.4**), and because of the positive correlation between N intake and hepatic output of urea-N (Marini et al., 2004; Wickersham et al., 2009), data on urea-N recycling kinetics were initially adjusted by covariance analysis using N intake within sheep and collection period as a covariate (Huntington et al., 2009). For the major dependent variables quantifying urea-N recycling kinetics, statistical trends for adjusted (data not shown) and unadjusted data were similar; hence unadjusted data are presented in **Table 4.5**. Data on ruminal pH, and ruminal concentrations of VFA and NH_3-N were analyzed accounting for repeated measures as recommended by Wang and Goonewardene (2004). Data for these repeated measurements were analyzed by including in the model a REPEATED model statement, as well as terms for time (hour or day), and interactions (level of dietary CP x time, ruminal protozoal status x time, and time x level of dietary CP x ruminal protozoal status) in the model described previously. For protozoal counts, statistical analysis was performed on $\log_{10}$ transformed data. When significant treatment effects were detected, means were compared using the Tukey-Kramer multiple comparison test. Treatment differences were considered significant when $P \leq 0.05$ and tendencies are discussed when $0.05 < P \leq 0.10$.

4.4 Results and Discussion

4.4.1 Diet Characteristics

Experimental diets were formulated to have a similar chemical composition, except for the CP content (Table 4.1). Experimental diets were originally formulated to contain 10 and 15%

CP (DM basis) for the LOW and HIGH treatments, respectively, and chemical analysis showed only marginal deviations (+0.3% and +0.6% units, respectively) in dietary CP content (Table 4.1). Dietary CP was manipulated by varying the inclusion rates of corn gluten meal and soybean meal, such that both experimental diets contained the same protein ingredients (albeit, in varying amounts) in order to ensure a similar AA profile.

4.4.2 Ruminal Protozoal Numbers and Fermentation Characteristics

The experimental protocol that was used for ruminal defaunation was based on previous research (Ivan et al., 2001) which demonstrated that SFO fed at 6% of diet DM was a potent anti-protozoal agent, decreasing ruminal protozoal counts by more than 80% within 6 d when compared to a control diet. Several methods are available for eliminating protozoa from the rumen, and they are mostly based on the use of chemicals (e.g., copper sulfate and calcium peroxide) which have toxic effects on ruminal protozoa (Jouany, 1996). A major drawback of using chemicals to eliminate ruminal protozoa is that they can be toxic to experimental animals and they can also alter other aspects of the ruminal microbial ecosystem (Veira et al., 1983), hence our decision to use SFO as a defaunating agent in the present experiment. Although complete defaunation was not achieved in the present study, substantial reductions in ruminal protozoal populations were achieved, coupled with positive responses in N utilization. Also, partial defaunation can be beneficial as it has been associated with a greater flow of NAN to the duodenum (Punia and Leibholz, 1987), improved feed:gain ratio in sheep (Ivan et al., 2004) and increases in milk yield in dairy cows (Moate, 1989). Ruminal protozoa consist largely of *Entodinium spp.*, Holotrichs and cellulolytic protozoa (Ogimoto and Imai, 1981) and, in the present study the distribution of these protozoa in faunated lambs was 94% *Entodinium*, 2 to 3% Holotrichs, and 2 to 4% cellulolytic protozoa (**Table 4.2**). Feeding SFO resulted in substantial reductions in total ruminal protozoal counts by d 6, with the maximum decrease achieved by d 22 and this was maintained throughout the measurement period (**Appendix Tables I and II**). During the measurement period (d 22 to d 27), feeding sunflower oil decreased (P = 0.001) total ruminal protozoal counts by, on average, 88%, with all protozoa virtually eliminated during that period in lambs fed diet LOW (Table 4.2). In PDFAUN lambs fed diet HIGH, *Entodinium spp.* comprised virtually all of the remaining protozoal population (Table 4.2).

Table 4.2 Ruminal protozoal numbers and ruminal fermentation characteristics in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed LOW or HIGH dietary crude protein levels[1]

	LOW		HIGH			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	Protein	Protozoa x Protein
Protozoa, x 10^5/ml								
Entodinium spp.	11.2	-	16.2	3.8	0.40	0.001	0.001	0.32
Holotrichs	0.38	-	0.38	-	0.02	0.001	0.82	0.82
Cellulolytics[3]	0.29	0.09	0.74	0.13	0.04	0.001	0.02	0.02
Total	11.9	0.09	17.3	4.0	0.97	0.001	0.001	0.26
Ruminal pH	5.8	6.1	5.8	6.2	0.04	0.001	0.14	0.84
Ruminal NH_3-N, mg/dL	9.6	5.8	14.9	11.6	0.37	0.001	0.001	0.57
Ruminal VFA, mM								
Total	96.4	92.0	98.6	79.4	2.57	0.001	0.07	0.01
Acetate	60.3	52.7	59.9	45.1	1.31	0.001	0.09	0.02
Propionate	18.3	24.7	16.8	20.5	1.04	0.001	0.02	0.21
Butyrate	14.3	11.1	17.6	10.6	1.04	0.001	0.18	0.09
Isobutyrate	0.69	0.69	0.94	0.65	0.03	0.001	0.01	0.001
Valerate	1.5	1.4	1.6	1.1	0.13	0.02	0.44	0.16
Isovalerate	1.3	1.5	1.7	1.5	0.31	0.97	0.62	0.59
Acetate to propionate ratio	3.44	2.15	3.62	2.27	0.16	0.001	0.37	0.84

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Protozoa = PDFAUN vs. FAUN lambs; Protein = level of dietary CP (LOW vs. HIGH); Protozoa x Protein = interaction. PDFAUN was achieved by addition of 6% (DM basis) linoleic acid-rich sunflower oil to the diet.

[3]Includes *Polyplastron*, *Diplodinium* and *Enoplastron* sp.

This suggests that measurements (d 22 to 27) were taken when ruminal protozoal populations and ruminal function had recovered. Protozoal populations remained relatively stable after pre-defaunation levels had been restored or attained (**Appendix Table III**). Feeding diet HIGH increased (P = 0.001) ruminal protozoal counts (Table 4.2), likely reflecting a greater availability of substrates (e.g., preformed AA) for protozoal growth. As expected, ruminal defaunation was accompanied by a decrease (P = 0.001) in ruminal NH_3-N concentrations (Table 4.2). Numerous published studies have also reported decreases in ruminal NH_3-N concentrations as a result of partial (Ikwuegbu and Sutton, 1982; Ivan et al., 2001) or complete (Newbold et al., 1986; Koenig et al., 2000) defaunation. This decrease in ruminal NH_3-N concentrations is mainly attributed to decreased intra-ruminal bacterial N recycling, coupled with increased MP synthesis due to a larger bacterial population (Koenig et al., 2000), and decreased ruminal proteolysis of dietary nitrogenous compounds (Wallace et al., 1987). Ruminal pH in PDFAUN lambs was higher (P = 0.001) compared to FAUN lambs (Table 4.2), which is in agreement with other research (Ivan et al., 2001; 2004). Ruminal protozoa engulf and store starch, thereby preventing or slowing down starch fermentation by bacteria. As a result, the presence of protozoa is generally associated with a higher and more stable ruminal pH (Veira et al., 1983), which is in contrast to our observations. It is noteworthy that partial defaunation by the addition of SFO resulted in a decrease in total ruminal VFA concentrations, primarily due to a decrease in acetate and butyrate concentrations (Table 4.2). Ruminal concentration of propionate was higher (P = 0.001), whereas that of butyrate and valerate was lower (P = 0.001) in PDFAUN compared to FAUN lambs (Table 4.2), supporting previous findings (Koenig et al., 2000). Ruminal concentration of isobutyrate was higher (interaction, P = 0.001) and that of butyrate was numerically higher (interaction, P = 0.09) in FAUN lambs fed HIGH as compared to those fed LOW. Similarly, higher ruminal concentrations of butyrate and iso-butyrate have been reported in dairy cows fed high dietary CP (Ipharraguerre et al, 2005), possibly reflecting oxidative deamination and decarboxylation of AA from true protein originating from a higher dietary CP intake. This decrease in total VFA concentrations could be responsible for the higher ruminal pH that was observed in PDFAUN lambs. Ruminal fiber digestion was not measured in this study, but it is plausible that anti-microbial effects of added oil on cellulolytic bacteria (Pantoja et al., 1994) could have inhibited fiber digestion, thus decreasing VFA production. Also, some species of protozoa appear to have cellulolytic activity (Jouany et al., 1988) and their absence in

PDFAUN lambs could also have contributed to the lower VFA concentrations. However, because SFO was added only to PDFAUN, it is difficult to ascertain that alterations in ruminal function (e.g., changes in ruminal VFA concentrations) are due to the partial elimination of protozoa and not due to anti-bacterial properties of SFO.

4.4.3 Dry Matter Intake and Total Tract Digestibilities

Partially-defaunated lambs consumed 125 to 591 g/d less DM compared to FAUN lambs (P = 0.03; **Table 4.3**), supporting previous research that reported that feeding linoleic acid-rich oil sources was associated with a decrease in DM intake (Ivan et al., 2004). A preponderance of the available research indicates that defaunation has little effect on DM intake (Eugène et al., 2004). Although ruminal fiber digestion was not mesured, the decrease in DM intake that was observed in protozoa-free animals in the present study can be partly attributable to perturbations of fiber digestion in the rumen as unsaturated oils have been shown to inhibit ruminal cellulolytic bacteria (Pantoja et al., 1994). Total tract digestibilities of DM, OM, ADF, and NDF were unaffected by ruminal defaunation and dietary CP level (Table 4.3). This lack of effect of partial defaunation on total tract fiber digestion is somewhat surprising. This is particularly so considering the decrease in ruminal total VFA and acetate concentrations (and, presumably, their ruminal production) that was observed in PDFAUN lambs, which would imply reduced ruminal fiber digestion. Perusal of the literature indicates that the effects of defaunation on fiber digestion in the rumen and total tract are inconsistent. A decrease in ruminal fiber digestion has been observed in some studies (Oldick and Firkins, 2000; Koenig et al., 2000), but not others (Hristov et al., 2004). For total tract digestion, Koenig et al. (2000) observed a reduction in fiber digestion due to defaunation. More recently, Ivan et al. (2004) observed a reduction in total tract fiber digestion in defaunated lambs fed high forage diets, whereas total tract fiber digestion was increased in defaunated lambs fed high concentrate diets. It is plausible that the lack of effect of partial defaunation on total tract fiber digestion in our study could be due to a higher hindgut fermentation of fiber in PDFAUN lambs, which would compensate for any reduction in ruminal fiber digestion that might have occurred; however, this is uncertain as fiber digestion in the rumen or hindgut was not measured. Such variation in the impact of defaunation on nutrient digestion likely is attributable to dietary differences, and the associated changes in ruminal microbial populations and patterns of ruminal fermentation (Koenig et al., 2000).

Table 4.3. Dry matter (DM), energy and organic matter (OM) intakes, and total tract nutrient digestibility in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed LOW or HIGH dietary crude protein levels[1]

	LOW		HIGH			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	Protein	Protozoa x Protein
DM intake, g/d	2,244	1,653	1,807	1,682	147.2	0.03	0.19	0.14
Energy intake, MCal/kg DM[3]								
Diet	4.04	2.96	3.25	3.03	0.27	0.03	0.19	0.14
Sunflower oil	0.0	0.87	0.0	0.89	0.06	0.001	0.90	0.90
Total	4.04	3.84	3.25	3.91	0.32	0.47	0.27	0.20
OM intake, g/d	2,056	1,515	1,653	1,539	133.5	0.03	0.18	0.14
Nutrient digestibility, %								
DM	59.9	59.2	60.2	59.2	3.1	0.79	0.96	0.97
OM	63.0	62.5	62.8	62.2	2.9	0.85	0.95	0.99
N	72.3	77.5	81.7	83.2	2.2	0.15	0.005	0.41
ADF	25.3	25.8	23.7	24.8	4.1	0.84	0.76	0.95
NDF	27.6	31.5	26.9	27.2	4.1	0.62	0.55	0.67

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Protozoa = PDFAUN vs. FAUN lambs; Protein = level of dietary CP (LOW vs. HIGH); Protozoa x Protein = interaction. PDFAUN was achieved by addition of 6% (DM basis) linoleic acid-rich sunflower oil to the diet.

[3]Dietary energy intake was calculated as 1.8 * DM intake, where 1.8 is energy content (MCal/kg DM) of the diets calculated based on NRC (1985) guidelines. Energy intake from sunflower oil was calculated as (8.8 * ((DM intake in kg)* 60)) / 1000, where 8.8 is energy content (MCal/kg) of sunflower oil and 60 is amount (g) of oil fed to PDFAUN lambs per kg of DM intake.

4.4.4 Nitrogen Balance

Faunated lambs consumed 3.5 to 9.2 g/d more N compared to PDFAUN lambs (P = 0.04; **Table 4.4**), reflecting observed differences in DM intake. Fecal N excretion, expressed in absolute amounts, was unaffected (P = 0.35) by defaunation; however, when expressed as a proportion of total N excretion, fecal N excretion was greater (P = 001) in PDFAUN compared to FAUN lambs (Table 4.4). There is general agreement in the literature that defaunation increases fecal N losses (Koenig et al., 2000; Jouany, 1996). This might reflect a shift in the digestion of cell-wall carbohydrates to the hindgut, leading to greater bacterial protein synthesis (Ushida et al., 1991). Because there is no hindgut mechanism for the digestion and absorption of the resultant bacterial protein, it is subsequently excreted in the feces. Conversely, urinary N excretion, when expressed in absolute amounts (P = 0.001) or as a proportion of N intake (P = 0.001), was decreased by defaunation (Table 4.4). Ruminal absorption of NH_3-N into the portal blood is positively correlated to the ruminal NH_3-N concentration (Leng and Nolan, 1982).

As ruminal NH_3-N concentration was lower in PDFAUN compared to FAUN lambs, we can surmise that the concentration-dependent absorption of NH_3-N from the rumen was decreased in the PDFAUN lambs. Most of the NH_3-N taken up by the portal vein would be detoxified by conversion to urea in the liver, a decreased absorption of NH3-N would result in decreased total hepatic ureagenesis, and thus resulted in lower (P = 0.001) urinary urea-N excretion in PDFAUN compared to FAUN lambs. In addition, the decrease in urinary N excretion in PDFAUN lambs could also result from a greater proportion of urea-N output being recycled to the GIT and its increased sequestration for microbial protein synthesis in the rumen and/or hindgut. In agreement with our results, other workers (Punia and Leibhloz, 1987; Ikwuegbu and Sutton, 1982) have also observed decreases in urinary N excretion as a result of defaunation. Total N losses were 5.2 to 9.0 g/d lower (P = 0.004) in PDFAUN compared to FAUN lambs; as a proportion of N intake, PDFAUN lambs excreted 5.4 to 6.8% units less (P = 0.06) N compared to FAUN lambs. However, N retention (g/d) was unaffected (P = 0.63) by protozoal status, even if PDFAUN lambs consumed 8 to 25% less N compared to FAUN lambs. Daily CP requirements for lambs weighing 40 to 50 kg range from 234 to 240 g (NRC, 1985). Average CP (N x 6.25) intake in PDFAUN lambs was 216 g/d, which was deficient, yet their N retention (when expressed as a proportion of N intake) was 4.7 to 6.8 percentage units higher (P = 0.05) than in FAUN lambs (Table 4.4).

Table 4.4. Nitrogen (N) intake, N balance, and plasma-urea nitrogen in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed LOW or HIGH dietary crude protein levels[1]

	LOW		HIGH			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	Protein	Protozoa x Protein
N intake, g/d	36.6	27.4	45.3	41.8	2.8	0.04	0.001	0.33
Fecal N excretion								
g/d	8.9	8.7	9.1	10.9	0.84	0.35	0.19	0.28
% of total N excretion	31.1	44.2	25.4	36.0	2.2	0.001	0.007	0.56
Urine N excretion								
Total N, g/d	19.9	10.9	26.6	19.6	1.6	0.001	0.001	0.58
Total N, % of total N excretion	68.9	55.8	74.6	63.9	2.2	0.001	0.007	0.56
Urea-N, g/d	10.5	6.2	20.8	14.0	0.88	0.001	0.001	0.18
Total N excretion								
g/d	28.7	19.7	35.7	30.5	2.1	0.004	0.001	0.37
% of N intake	78.6	71.8	78.6	73.2	2.7	0.06	0.71	0.71
N retention, g/d								
g/d	7.8	7.7	9.6	11.3	1.6	0.63	0.12	0.58
% of N intake	21.4	28.2	21.4	26.1	2.7	0.05	0.71	0.71
Plasma urea-N, mg/dL	12.1	10.1	18.3	17.0	1.8	0.39	0.002	0.98

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Protozoa = PDFAUN vs. FAUN lambs; Protein = level of dietary CP (LOW vs. HIGH); Protozoa x Protein = interaction. PDFAUN was achieved by addition of 6% (DM basis) linoleic acid-rich sunflower oil to the diet.

Our data suggests a greater efficiency of N utilization in protozoa-free lambs, which can be partly attributed to a higher proportion of urea-N production being recycled to the GIT. Both total energy intake and the source of energy can affect the efficiency of post-absorptive N use. Assuming that the energy content of SFO is 8.8 Mcal/kg, dietary inclusion of 6% SFO contributed 0.87 to 0.89 Mcal to total energy intake of PDFAUN lambs. However, because PDFAUN lambs consumed less feed compared to FAUN lambs, total energy intake was unaffected by protozoal status. It is unlikely, therefore, that results are confounded by total energy intake. While total energy intakes did not differ between FAUN and PDFAUN lambs, it is important to note that energy sources in the diets were different. In growing steers receiving supplemental fat, the efficiency of N use (i.e., greater N retention and lower N excretion) was improved when compared to unsupplemented controls (Schroeder et al., 2006); however, in contrast to the present study, total energy intakes differed in that previous study. Supplemental energy intake as fat could provide a potential glucose precursor (glycerol) that could spare AA from being used for gluconeogenesis, thus making more AA available for protein accretion and improving N use. In the present study, N use was more efficient in lambs fed SFO; however, this cannot be definitively attributed to an effect of SFO or the absence of protozoa. Other workers (Bird et al., 1994) have reported increases in N retention due to defaunation.

The level of dietary CP also altered N balance (Table 4.4). As expected, lambs fed HIGH consumed 8.7 to 14.4 g/d more N compared to those fed LOW ($P = 0.04$), reflecting the higher dietary CP in diet HIGH as DM intake was unaltered. However, most of the additional N intake was excreted in urine as lambs fed HIGH excreted 6.7 to 8.7 g/d more urinary N compared to those fed LOW ($P = 0.001$). Consequently, N retention expressed in absolute amounts ($P = 0.12$) or as a proportion of N intake ($P = 0.71$), was unaffected by dietary CP level (Table 4.4). These observations are consistent with published literature (Kiran and Mutsvangwa, 2007; Marini et al., 2004). Surprisingly, PUN concentration was unaffected ($P = 0.39$) by protozoal status, although PUN levels were 8 to 20% numerically greater in FAUN compared to PDFAUN lambs (Table 4.4). As expected, PUN concentration was greater ($P = 0.002$) in lambs fed HIGH compared to those fed LOW, reflecting the higher N intake and higher rates of ruminal NH_3-N absorption in lambs fed HIGH.

4.4.5 Urea-N Kinetics, Expression of Urea Transporter-B, and Microbial NAN Supply

Across treatments, urinary [$^{15}N^{15}N$]-urea enrichment reached a plateau within 24 to 48 h of isotopic infusion, whereas plateau enrichment of [$^{14}N^{15}N$]-urea in urine was not attained until between 48 and 72 h of isotopic infusion (**Figure 4.1**). This time course in the urinary enrichments of [$^{14}N^{15}N$]- and [$^{15}N^{15}N$]-urea is consistent with previous research using sheep (Lobley et al., 2000; Sunny et al., 2007). Fecal ^{15}N enrichments increased daily over the 4-d isotopic infusion without attaining a definite plateau (**Figure 4.2**); therefore, for calculations of urea-N kinetics, urinary [$^{14}N^{15}N$]- and [$^{15}N^{15}N$]-urea and fecal ^{15}N enrichments over the final 72 to 96 h of infusion were used, similar to previous research (Lobley et al., 2000; Sunny et al., 2007).

The primary objective of the present study was to determine how interactions between ruminal defaunation and dietary CP level might potentially alter urea-N transfer to the GIT and the utilization of this recycled urea-N in ruminants. Endogenous production of urea-N (UER; 26.1 vs. 34.6 g/d) was lower (P = 0.001) in PDFAUN compared to FAUN lambs (**Table 4.5**), possibly reflecting differences in N intake as has been demonstrated by others (Marini et al., 2004; Wickersham et al., 2009). It is noteworthy that adjusting urea-N kinetics data using covariance analysis for differences in N intake that were observed between FAUN and PDFAUN lambs resulted in similar statistical trends (**Appendix Table IV**). This indicates that data on the effects of partial defaunation on urea-N recycling kinetics were likely not confounded by differences in N intake. Across treatments, UER ranged from 0.75 to 0.84 of N intake, which compares favorably with UER:N intake ratios obtained in sheep (0.77 to 0.95, Lobley et al., 2000; 0.73 to 0.99, Sunny et al., 2007). The quantity of hepatic urea-N output that was transferred to the GIT (i.e., GER), when expressed in absolute amounts (16.0 vs. 18.9 g/d), tended (P = 0.06) to be lower in PDFAUN compared to FAUN lambs; however, and most interestingly, when expressed as a proportion of UER (0.623 vs. 0.559), GER was greater in PDFAUN compared to FAUN lambs. Ruminal NH_3-N concentration has been reported to be negatively correlated with urea-N transfer into the rumen (Kennedy and Milligan, 1980), possibly because high ruminal NH_3-N concentration decreases the ruminal epithelium's permeability to urea (Egan et al., 1986), and ruminal urease activity (Cheng and Wallace, 1979).

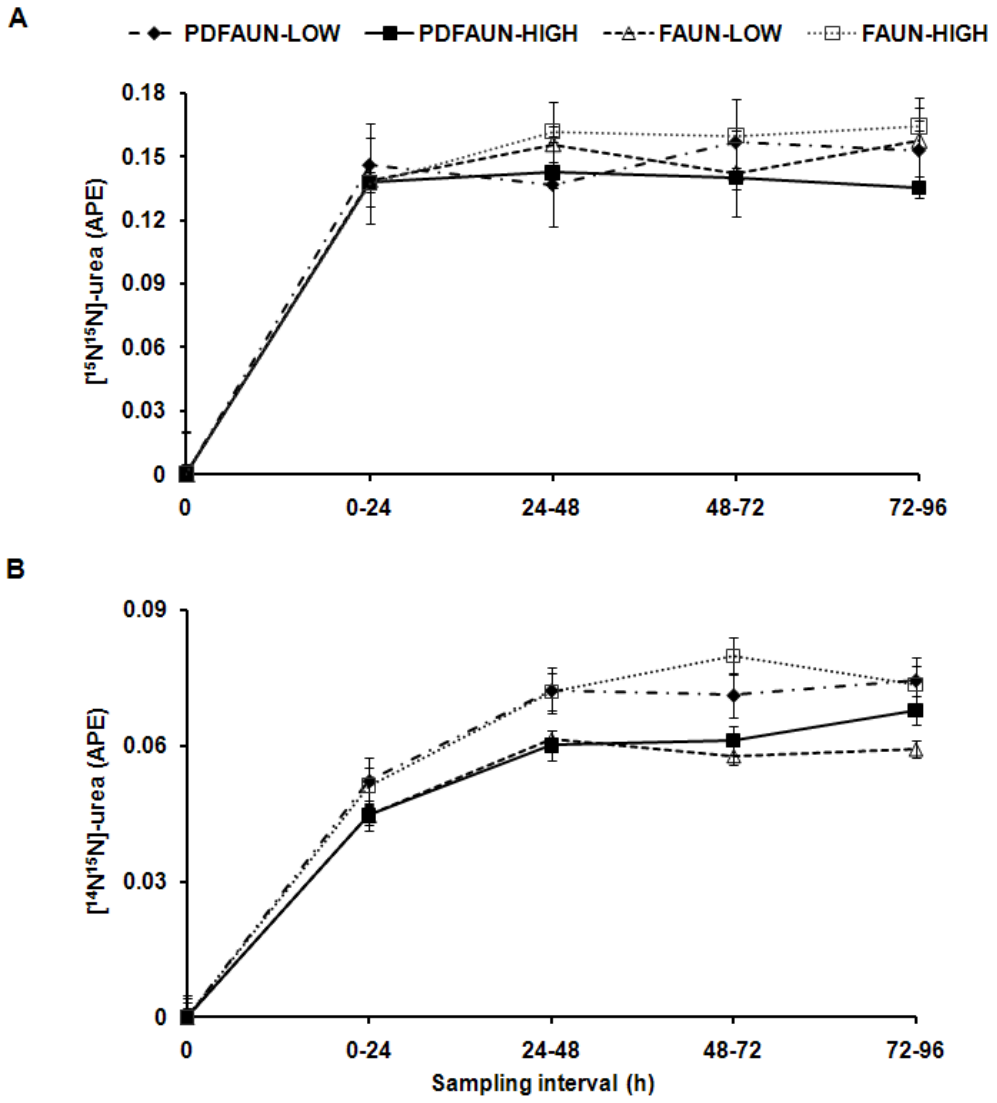

Figure 4.1 Enrichments (APE, atom percent excess) of [^{15}N^{15}N]-urea (A) and [^{14}N^{15}N]-urea (B) in urine during 4-d intra-jugular infusions of [^{15}N^{15}N]-urea (infusions were initiated at sampling interval 0 h). Treatments were: faunated + low CP (FAUN-LOW); faunated + high CP (FAUN-HIGH); partially-defaunated + low CP (PDFAUN-LOW); and partially-defaunated + high CP (PDFAUN-HIGH). PDFAUN was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet. For [^{15}N^{15}N]-urea enrichments, no differences were detected between sampling intervals 0-24, 24-48, 48-72, and 72-96 h ($P > 0.10$). For [^{14}N^{15}N]-urea enrichments, no differences were detected between sampling intervals 24-48, 48-72, and 72-96 h ($P > 0.10$). Each line represents means ± SEM for 4 lambs.

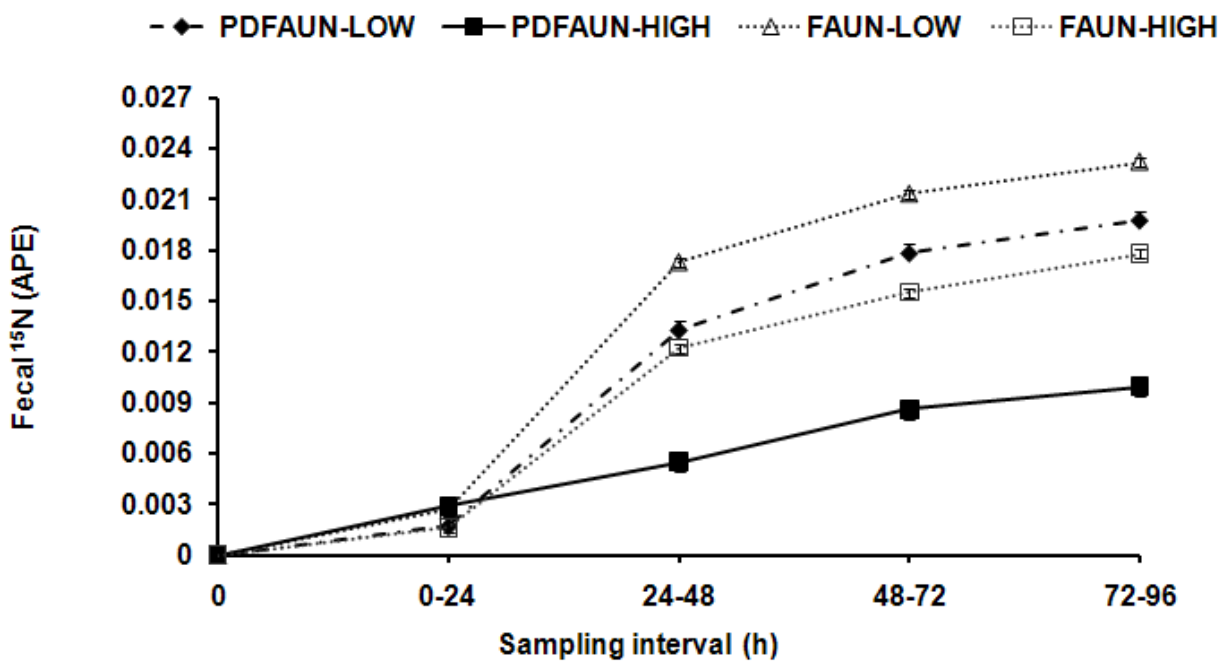

Figure 4.2 Enrichments (APE, atom percent excess) of fecal ^{15}N during 4-d intra-jugular infusions of [$^{15}N^{15}N$]-urea (infusions were initiated at sampling interval 0 h). Treatments were: faunated + low CP (FAUN-LOW); faunated + high CP (FAUN-HIGH); partially-defaunated + low CP (PDFAUN-LOW); and partially-defaunated + high CP (PDFAUN-HIGH). PDFAUN was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet. Differences were detected in fecal ^{15}N enrichments between sampling intervals 0-24, 24-48, 48-72, and 72-96 h (P = 0.001), indicating that fecal ^{15}N enrichment did not reach a definite plateau. Each line represents means ± SEM for 4 lambs.

Table 4.5 Urea-N recycling kinetics and urea transporter-B (UT-B) mRNA abundance in ruminal epithelial tissue in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed LOW or HIGH dietary crude protein levels[1]

	LOW		HIGH			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	Protein	Protozoa x Protein
Urea-N kinetics, g/d								
Production, UER	27.0	18.0	42.3	34.1	1.96	0.001	0.001	0.85
Entry to GIT, GER	16.5	11.9	21.4	20.1	1.40	0.06	0.001	0.27
Return to ornithine cycle, ROC	7.4	4.7	12.9	11.2	0.96	0.04	0.001	0.63
Loss to feces, UFE	0.95	0.73	0.77	0.72	0.18	0.48	0.61	0.64
Loss to urine, UUE	10.5	6.2	20.8	14.0	0.88	0.001	0.001	0.18
Re-use for anabolism, UUA	8.1	6.5	7.7	8.2	0.56	0.29	0.27	0.08
Fractional urea-N transfers								
UER to urine	0.388	0.342	0.494	0.413	0.016	0.002	0.001	0.31
UER to GIT	0.612	0.658	0.506	0.587	0.016	0.002	0.001	0.31
GER to ROC	0.446	0.389	0.603	0.557	0.026	0.07	0.001	0.85
GER to feces	0.057	0.056	0.035	0.036	0.009	0.97	0.04	0.91
GER to UUA	0.497	0.554	0.362	0.406	0.028	0.09	0.001	0.83
UT-B mRNA abundance, per copy of GAPDH	3.09	3.51	2.27	3.19	0.43	0.15	0.21	0.57

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Protozoa = PDFAUN vs. FAUN lambs; Protein = level of dietary CP (LOW vs. HIGH); Protozoa x Protein = interaction. PDFAUN was achieved by addition of 6% (DM basis) linoleic acid-rich sunflower oil to the diet.

In the present study, partial defaunation resulted in a lower ruminal NH_3-N concentration, which would facilitate the transfer of a greater proportion of UER to the GIT in PDFAUN compared to FAUN lambs. I am not aware of any previous research in which urea-N recycling to the GIT has been reported in defaunated compared to faunated ruminants. These data indicate that partial elimination of protozoa results in a greater proportion of endogenous urea-N output being recycled to the GIT. Endogenous urea-N can diffuse through all compartments of the GIT (Lapierre and Lobley, 2001) and, although the [$^{15}N^{15}N$]-urea infusion technique that was used in the present study cannot distinguish between urea-N transfer to the fore-stomach and post-stomach compartments of the GIT, it is generally accepted that a significant proportion of the GER is transferred to the rumen (as opposed to post-ruminal compartments). Urea-N that is recycled to the rumen would buffer the rumen from the lower NH_3-N concentrations prevailing in PDFAUN lambs, thus providing additional N that can be used for MP synthesis. Overall, this could potentially increase post-ruminal metabolizable protein supply and, consequently, improve N retention as has been consistently observed in defaunated compared to faunated ruminants.

The absolute amount of GER that was used for anabolic purposes (UUA; primarily MP synthesis) was unaffected (P = 0.56) by defaunation. However, anabolic use of recycled urea-N expressed as a proportion of GER (i.e., GER to UUA; 0.480 vs. 0.429) tended to be greater (P = 0.09) in PDFAUN compared to FAUN lambs. Although we did not measure the direct incorporation of recycled ^{15}N into microbial protein, these data suggest a greater microbial incorporation of recycled urea-N into microbial protein, as most of the anabolic use of recycled urea-N (i.e., UUA) is predominantly as sequestration of liberated NH_3-N into microbial protein (Lobley et al., 2000). As discussed elsewhere in this paper, microbial NAN supply as assessed by urinary PD excretion, was also greater (P = 0.001) in PDFAUN compared to FAUN lambs (**Table 4.6**), thus further suggesting a greater anabolic use of recycled urea-N via incorporation into microbial protein. Concomitant with the tendency for a greater utilization of recycled urea-N for anabolic purposes, urea-N that was returned to the ornithine cycle (i.e., ROC), when expressed in absolute amounts was lower (8.0 vs. 10.2 g/d; P = 0.04), or when expressed as a proportion of GER tended to be lower (0.473 vs. 0.525; P = 0.07) in PDFAUN compared to FAUN lambs, suggesting improved N efficiency.

Urinary urea-N excretion (UUE), expressed in absolute amounts (10.1 vs. 15.7 g/d; $P < 0.001$) or as a proportion of UER (i.e., fractional transfer of UER to urine; 0.378 vs. 0.441; $P < 0.001$) was lower in PDFAUN lambs compared to FAUN lambs. In ruminants, a major portion of the urea-N released from the liver originates from NH_3-N absorbed from the portal drained viscera. Because ruminal NH_3-N concentration was lower in PDFAUN compared to FAUN lambs, it is likely that ruminal absorption of NH_3-N was decreased in PDFAUN lambs as this is a concentration-dependent process. That would result in decreased UUE. Urea-N that was eliminated in feces (i.e., UFE) was unaffected ($P = 0.48$) by defaunation (Table 4.5).

Endogenous production of urea-N (38.2 vs. 22.5 g/d), GER (20.8 vs. 14.2 g/d), and UUE (17.4 vs. 8.3 g/d) were greater ($P = 0.001$) in lambs fed HIGH compared to those fed LOW diet. However, when expressed as a proportion of UER, the GER and its anabolic use (primarily microbial protein synthesis) were greater ($P = 0.001$) in lambs fed LOW compared to those fed HIGH (Table 4.5). Increases in UER, GER and UUE as dietary CP concentration increases are likely associated with the higher N intakes that were imposed. Greater levels of UER, GER and UUE with increasing N intakes have been reported in ruminants (Archibeque et al., 2001; Marini et al., 2003). Across dietary CP levels, UER was 0.70 to 0.87 of N intake, which is in agreement with previous research (Lobley et al., 2000). Clearly, these ratios indicate the magnitude of the transit of N into the urea pool and the perpetual dependence of ruminants on urea-N recycling to the GIT in order to maintain a positive N balance.

The presence of carrier-mediated, facilitative urea transporter (UT) proteins in ruminant GIT tissues was originally demonstrated by Ritzhaupt et al. (1997; 1998), and subsequently confirmed by other workers (Marini and Van Amburgh, 2003; Marini et al., 2004; Stewart et al., 2005). These UT are derived from 2 major gene variants, namely UT-A and UT-B (Stewart et al., 2005), and mRNA expression for UT-A has been characterized in ovine duodenum, and that of UT-B in the rumen of both bovines (Marini and Van Amburgh, 2003; Stewart et al., 2005) and ovines (Marini et al., 2004). In addition to measurement of urea-N kinetics, we also quantified UT-B mRNA expression using qPCR. Expression of UT-B mRNA (copies/copy of ovine-GAPDH) was unaffected by defaunation ($P = 0.15$) or dietary CP level ($P = 0.21$) (Table 4.5). However, expression of UT-B mRNA in PDFAUN lambs was numerically greater (by 20%) compared to FAUN lambs. A closer examination of these data on UT-B gene expression

indicates that there was considerable variation among experimental lambs; as such, it is conceivable that treatment differences could have approached statistical significance with a larger number of lambs per treatment. Our data on UT-B gene expression further confirm our findings using the [$^{15}N^{15}N$]-urea infusion technique where partial defaunation was associated with a greater transfer of urea-N output to the GIT. Also, UT-B mRNA abundance in lambs fed LOW was numerically greater (by 17%) compared to those fed HIGH, which is consistent with the greater GER:UER ratio that was observed for lambs fed LOW compared to those fed HIGH. The expression of UT in ruminal epithelium was altered by dietary protein level (Marini and Van Amburgh, 2003); however, a subsequent study (Marini et al., 2004) failed to detect any changes in UT expression due to dietary manipulation. The quantitative significance of these UT in trans-epithelial urea-N transfer in ruminants has yet to be determined (Reynolds and Kristensen, 2008).

In this study, the effects of ruminal defaunation on microbial NAN supply were also determined. In ruminants, the measurement of urinary PD excretion is now routinely used as an indicator of ruminal microbial protein production under a wide range of dietary conditions (Chen and Gomes, 1992). The calculation of microbial protein supply using urinary PD excretion requires knowledge of the purine:N ratio in mixed ruminal microorganisms; however, in the present study, this ratio was not measured, so assumed that it was constant at 11.6:100 and that it remained unchanged by dietary treatment (Chen and Gomes, 1992). There is evidence that factors such as diet (Ranilla and Carro, 2003) and time relative to feeding (Cecava et al., 1990) may alter the purine:N ratio of ruminal microorganisms, so the use of a constant ratio is still a matter of considerable debate. Despite these limitations, the PD technique is a non-invasive, qualitative tool that can predict relative changes in MP supply. Partially-defaunating lambs increased allantoin (P = 0.001) and total PD (P = 0.003) excretion (**Table 4.6**), supporting the findings of Fujihara et al. (2003). Microbial NAN (expressed as g/d; P = 0.03) and microbial efficiency (expressed as microbial N/kg of DOMR; P = 0.001) were higher in PDFAUN lambs compared to FAUN lambs (Table 4.6). Numerous workers have observed that MP flow to the duodenum increases after defaunation (Jouany, 1996; Koenig et al., 2000), and this response has been attributable primarily to decreased protozoal predation on bacteria and decreased competition from protozoa for growth substrates (Joauny, 1996).

Table 4.6 Urinary output, urinary purine derivative (PD) excretion, and microbial non-ammonia nitrogen (NAN) supply in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed LOW or HIGH dietary crude protein levels[1]

	LOW		HIGH			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	Protein	Protozoa x Protein
Urinary excretion								
Total output, kg/d	2.3	1.7	1.9	2.8	0.43	0.66	0.46	0.12
Allantoin excretion, mmol/d	6.1	9.6	12.1	14.0	0.59	0.001	0.001	0.19
Uric acid excretion, mmol/d	2.5	2.5	3.9	3.8	0.21	0.86	0.001	0.82
Xanthine plus Hypoxanthine, mmol/d	0.92	1.1	1.2	0.97	0.14	0.79	0.58	0.24
Total PD excretion, mmol/d	9.5	13.1	17.2	18.7	0.69	0.003	0.001	0.16
Microbial NAN supply[3]								
g microbial N/d	8.1	11.3	14.8	16.2	0.61	0.003	0.001	0.15
g microbial N/kg DOMR	9.7	18.8	22.2	26.8	1.61	0.001	0.001	0.18

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Protozoa = PDFAUN vs. FAUN lambs; Protein = level of dietary CP (LOW vs. HIGH); Protozoa x Protein = interaction. PDFAUN was achieved by addition of 6% (DM basis) linoleic acid-rich sunflower oil to the diet.

[3]Microbial NAN supply was calculated according to Chen and Gomes (1992); DOMR = digestible organic matter in rumen, which was calculated as 0.65 * DOMI (Chen and Gomes, 1992).

Also, our results provide some evidence that increased urea-N recycling to the GIT in defaunated animals might provide additional ruminally degradable N for bacterial growth, as the proportion of recycled urea-N that was used for anabolic purposes (i.e., GER to UUA) tended to increase with defaunation. When microbial NAN supply is expressed as g microbial N/kg of DOMR, the improvement in microbial protein synthesis due to defaunation ranges from 20 to 94%, which compares favorably with a range of 40 to 125% that has reported from summarized literature data (Jouany, 1996). However, these data on microbial efficiency need to be interpreted somewhat cautiously as I did not directly measure ruminal OM digestion in the present study; rather, ruminal OM digestion was estimated as 0.65 x digestible OM intake (Chen and Gomes, 1992). Although any impact of defaunation on total tract OM digestion was not observed, limited research indicates that ruminal OM digestion might be suppressed in protozoa-free ruminants (Williams and Coleman, 1992). If ruminal OM digestion was altered in the present study, then that would also alter microbial efficiency. Compared to those fed the LOW, lambs fed the HIGH excreted more allantoin ($P = 0.001$), uric acid ($P = 0.001$), and total PD ($P = 0.001$); however, xanthine plus hypoxanthine excretion did not differ ($P = 0.58$) (Table 4.6). Numerous workers have observed that MP flow to the duodenum increases after defaunation (Jouany, 1996; Koenig et al., 2000), and this response has been attributable primarily to decreased protozoal predation on bacteria and decreased competition from protozoa for growth substrates (Joauny, 1996). Similarly, Gabler and Heinrichs (2003) reported a linear increase in urinary output of allantoin and total PD as dietary CP concentration increased. Microbial NAN (expressed as, g microbial N/kg of DOMR or g microbial N/d) flow to the small intestine was higher ($P < 0.01$) in lambs fed HIGH compared to those fed LOW.

It was anticipated that there would be interactions between protozoal status and dietary CP level on N metabolism; however, no interactions were detected for any of the important measures of N balance ($P \geq 0.18$; Table 4.4), urea-N kinetics ($P \geq 0.18$; Table 4.5), or microbial N production ($P \geq 0.12$; Table 4.6). Diet LOW was formulated to contain 10% CP, which was markedly below CP requirements for growth (14.5% CP; NRC, 1985). On the other hand, HIGH was formulated to contain 15% CP, which met growth requirements. Hence, there was a large disparity in dietary CP levels. Because the benefits of defaunation on N utilization are greater when animals are protein-deficient (Leng and Nolan, 1984), it was anticipated significant interactions between protozoal status and dietary CP level would occur. Based on a meta-

analysis of available data on the effects of defaunation on N metabolism from 75 studies, the effects of defaunation on ruminal NH_3-N concentration decreased with dietary N level (Eugène et al., 2004), indicating that there might be important interactions between protozoal status and dietary CP level. In that meta-analysis, the decrease in ruminal NH_3-N concentration as a result of defaunation was >5.0 mg/dL, whereas in the present study, defaunation decreased ruminal NH_3-N concentration by only 3.74 and 3.31 mg/dL in lambs fed LOW and HIGH, respectively. It is noteworthy that the difference in ruminal NH_3-N concentration between PDFAUN and FAUN animals was similar across CP levels and, ostensibly, this decrease was not large enough to elicit any interactions between defaunation and dietary CP level on N utilization.

4.5 Conclusions

Linoleic acid-rich sunflower oil fed at 6% of dietary DM was efficacious in partially defaunating the rumen. Partially-defaunated lambs consumed less N, and exhibited lower ruminal NH_3-N concentrations compared to faunated lambs. Partially-defaunated animals excreted smaller amounts of total N and, as a result, retained more N when expressed as a proportion of N intake. Although urea-N recycling to the GIT (in g/d) was unaffected by protozoal status, partially-defaunated animals recycled a greater proportion of their endogenous urea-N output to the GIT (i.e., UER to GIT, +0.046 to 0.081) compared to faunated animals. In addition, the proportion of recycled urea-N that was used for anabolic purposes (primarily MP production) tended to be higher, and microbial N supply at the duodenum was higher, in partially-defaunated compared to faunated animals. When taken together, these findings support our hypothesis that improved N utilization in partially-defaunated ruminants is partly mediated via increased urea-N recycling to the GIT, with subsequent utilization of that recycled urea-N for anabolic purposes.

5. FEEDING SUNFLOWER OIL TO PARTIALLY DEFAUNATE THE RUMEN INCREASES NITROGEN RETENTION, UREA-NITROGEN RECYCLING TO THE GASTROINTESTINAL TRACT AND THE ANABOLIC USE OF RECYCLED UREA-NITROGEN IN GROWING LAMBS[1]

5.1 Abstract

The objective of this study was to delineate how interactions between ruminal partial defaunation and altering dietary ruminally-fermentable carbohydrate (RFC) may alter urea-N kinetics and N metabolism in lambs. Four Suffolk ram lambs (61.5 ± 4.0 kg) were used in a 4 x 4 Latin square design with a 2 x 2 factorial arrangement of treatments. Treatments were: partially-defaunated (PDFAUN) vs. faunated (FAUN) lambs, and dry-rolled barley (DRB) vs. pelleted barley (PB) barley as the principal sources of RFC. Linoleic acid-rich sunflower oil (SFO) was fed as a defaunating agent. Nitrogen balance was measured over 4 d, with concurrent measurement of urea-N kinetics using continuous intra-jugular infusions of [$^{15}N^{15}N$]-urea. Feeding SFO decreased (P = 0.001) ruminal protozoa and ammonia-N concentrations. Urinary N excretion was lower (P = 0.003) and retained N was higher (P = 0.002) in PDFAUN compared to FAUN lambs. Endogenous production of urea-N (i.e., UER) was similar across treatments. The urea-N recycled to the gastrointestinal tract (GER), expressed as absolute amounts (16.4 vs. 13.1 g/d) or as a proportion of UER (0.693 vs. 0.570), its anabolic use (9.0 vs. 6.0 g/d), and microbial N supply (14.6 vs. 10.9 g/d) were higher ($P \leq 0.001$) in PDFAUN compared to FAUN lambs. As a proportion of UER, GER was higher, whereas urinary urea-N excretion was lower in lambs fed PB compared to those fed DRB (P = 0.01). In summary, feeding SFO increased urea-N recycling to the GIT and microbial NAN supply, thus providing new evidence that the improved efficiency of N utilization in partially-defaunated ruminants could be partly mediated by an increase in urea-N recycling.

[1]A version of this chapter has been accepted for publication: Kiran, D., and T. Mutsvangwa. 2010. Feeding sunflower oil to partially defaunate the rumen increases N retention, urea-N recycling to the gastrointestinal tract and anabolic use of recycled urea-N in growing lambs . British Journal of Nutrition (in press).

5.2 Introduction

In ruminants, it is well-recognized that endogenous urea-N production often exceeds apparent digestible N intake, yet ruminants still maintain a positive N balance by recycling 40 to 80% of this urea-N to the GIT (Lapierre and Lobley, 2001). Urea-N that passes into the rumen can provide ruminally-available N for the synthesis of microbial protein, which is a major contributor to the metabolizable protein that is available for intestinal digestion (Lapierre and Lobley, 2001). In a previous experiment it was demonstrated that feeding SFO to partially defaunate the rumen was associated with: 1) an increase in the proportion of endogenous urea-N that was recycled to the GIT; and 2) a tendency to increase the proportion of recycled urea-N that was utilized for anabolic purposes (Kiran and Mutsvangwa, 2010). Previously, the removal of protozoa (i.e., defaunation) has been reported to improve N utilization, mainly by decreasing the ruminal NH_3-N pool, coupled with a greater sequestration of NH_3-N into bacterial protein (Firkins et al., 2007), and by decreasing intra-ruminal N recycling (Jouany, 1996). Previous research showed that the positive effects of defaunation on N utilization may also result from an increase in urea-N recycling to the rumen (Kiran and Mutsvangwa, 2010). Because defaunation decreases ruminal NH_3-N level (Jouany, 1996), this could potentially increase trans-epithelial urea-N transfer as ruminal NH_3-N level is negatively correlated with urea-N transfer into the rumen (Kennedy and Milligan, 1980). In addition, trans-epithelial movement of urea-N by passive diffusion into the rumen is facilitated by bacterial urease activity, which maintains a favorable concentration gradient (Rémond et al., 1996). Cheng and Wallace (1979) demonstrated that urease activity in ruminal contents was negatively correlated with ruminal NH_3-N level.

Although results of previous experiment showed that a higher proportion of endogenous urea-N production was recycled to the GIT in partially-defaunated ruminants, N intake differed with protozoal status in that study as a result of decreased DM intake (Kiran and Mutsvangwa, 2010). Because of the positive correlation between N intake and hepatic output of urea-N (Marini et al., 2004; Wickersham et al., 2009), data on urea-N recycling kinetics could have been confounded due to differences in N intake. In addition, the use of recycled urea-N for anabolic purposes showed only a tendency to be higher in partially-defaunated compared to faunated animals (Kiran and Mutsvangwa, 2010), suggesting that ruminal energy supply might have limited microbial use of recycled urea-N for protein synthesis. Increasing dietary amounts of RFC (Huntington, 1989) or shifting carbohydrate digestion from the small intestine to the rumen

via steam-flaking compared to dry-rolling of sorghum grain (Theurer et al., 2002) has been shown to increase urea-N transfer to the rumen, in addition to increasing N sequestration into microbial protein as energy supply from RFC is the major driver for microbial growth (Russell, 1998; Koenig et al., 2003). Also, substantial decreases in ruminal protozoal populations have been reported in ruminants fed high-grain diets or extensively-processed barley (Koenig et al., 2003; Eadie et al., 1970). Providing more RFC therefore could improve N utilization by stimulating both urea-N recycling to the GIT and microbial protein production. In Western Canada and parts of the Northern USA, barley grain is the principal cereal grain that is included in ruminant diets, and it is commonly fed either in a dry-rolled or pelleted form. Pelleting decreases particle size, thus shifting the site of carbohydrate digestion from post-ruminal sites to the rumen, and previous in situ studies in our laboratory clearly indicated a higher soluble starch fraction, a higher degradation rate of the degradable starch fraction, and a higher effective starch degradability of PB when compared to DRB (Kiran and Mutsvangwa, 2007). Based on this previous research, it can be expected that ruminal starch digestibility would be higher for PB compared to DRB, which could potentially alter urea-N recycling to the GIT and microbial protein synthesis in the rumen. The major objective of this study was to delineate how interactions between feeding SFO to partially defaunate the rumen and dietary RFC may alter urea-N kinetics and N metabolism in growing lambs. The hypothesis was that feeding SFO to partially defaunate the rumen would increase urea-N recycling to the GIT and the utilization of recycled urea-N for anabolic purposes, and that these effects would be more pronounced if ruminal energy supply was increased by feeding PB compared to DRB.

5.3 Materials and Methods

5.3.1 Animals and Experimental Design

Four Suffolk ram lambs (61.5 ± 4.0 kg initial BW) that were fitted with ruminal cannulae were used. The experiment was run as a 4 x 4 Latin square design with 27-d periods and a 2 x 2 factorial arrangement of treatments. Experimental periods consisted of 21 d of dietary adaptation and 6 d of data collection. Lambs were housed in individual floor pens (during dietary adaptation) or in metabolism crates (during data collection) and were handled in accordance with

the guidelines of the Canadian Council of Animal Care (1993). Their use was approved by the University of Saskatchewan Animal Care Committee (UCACS Protocol No. 20040048).

5.3.2 Experimental Treatments and Feeding Management

The treatment factors were: 1) PDFAUN vs. FAUN; and 2) DRB vs. PB as the principal source of RFC. The ingredient and chemical composition of the experimental diets containing DRB or PB are presented in **Table 5.1**. Experimental diets were formulated to be iso-nitrogenous at 16 g N/kg DM. The preparation of DRB and PB was as described by Kiran and Mutsvangwa (2007). Dry-rolled barley or PB was fed as the principal sources of dietary RFC in order to manipulate ruminal energy availability for microbial growth. Diets were offered in the form of a meal for *ad libitum* intake twice daily in equal portions at 0900 and 1700 h, ensuring that 10% orts remained each day. Lambs had free access to water.

5.3.3 Partial Defaunation and Refaunation

Linoleic acid-rich SFO (Safeway^{TM} Canada, Saskatoon, SK, Canada) was fed (60 g/kg DM) as an anti-protozoal agent. The protocol for ruminal defaunation was based on previous research (Ivan et al., 2001) and similar to Kiran and Mutsvangwa (2010). The measured fatty acid composition (%) of SFO was: 6.24, 3.87, 26.15, 67.07, and 0.49 of palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2n-6), and linolenic (C18:3n-3) acid, respectively. For lambs receiving SFO, daily allotments were calculated based on the previous day's DM intake. To avoid cross-inoculation of PDFAUN lambs by FAUN lambs via direct physical contact, individual floor pens and metabolism crates housing PDFAUN lambs were spaced several meters away from those housing FAUN lambs. In addition, ruminal fluid sampling of PDFAUN lambs was conducted prior to FAUN lambs in order to minimize the risk of cross-inoculation. To enumerate protozoa, 200 mL of ruminal contents were sampled at 1100 h on d 1 to d 3, d 6, d 9, d 12, d 15, d 18, and d 21 to d 26. The processing of ruminal contents for protozoal counting was conducted as described by Kiran and Mutsvangwa (2010), and protozoal enumeration and differentiation into different genera (i.e., *Entodinium spp.*, Holotrichs, and cellulolytic protozoa) was conducted as described by Ogimoto and Imai (1981). Partially-defaunated lambs were refaunated at the start of an experimental period as previously described (Kiran and Mutsvangwa, 2010).

Table 5.1. Ingredient and chemical composition of experimental diets

Item	Dry-rolled barley	Pelleted barley
Ingredients, % DM		
Alfalfa meal	6.0	6.0
Barley (Dry rolled)	45.0	-
Barley (Pelleted)	-	45.0
Canola meal	2.0	2.0
Corn gluten meal	0.5	0.5
Oat hulls	35.0	35.0
Soybean meal	1.5	1.5
Canola oil	1.0	1.0
Liquid molasses	2.0	2.0
Ground limestone	4.0	4.0
Salt, Co-I[1]	0.7	0.7
Salt white	1.3	1.3
Mineral vitamin supplement[2]	1.0	1.0
Chemical composition		
DM, %	93.8	93.1
OM, % of DM	91.2	90.9
CP, % of DM	9.92	9.99
NDF, % of DM	40.4	39.7
ADF, % of DM	20.7	20.9
EE, % of DM	3.6	3.7
RDP[3], % of CP	63.6	63.6
ME[3], MCal/kg	1.7	1.7

[1]Salt, cobalt and iodine mix: Co, 100 mg/kg: and I, 150 mg/kg.

[2]Provided per kg of concentrate DM: CuO, 3.3 mg; ZnO, 22 mg; MnO, 18.5 mg; vitamin A, 1,495 IU; vitamin D, 125.5 IU; vitamin E, 22 IU; and sodium selenite, 0.1 mg.

[3]Calculated according to NRC (1985) guidelines.

5.3.4 Sample Collection

Experimental lambs were moved from individual floor pens into individual metabolism crates on d 19 of each experimental period to allow acclimation before the initiation of data collection on d 22. During the 6-d data collection period, individual lamb feed intake was recorded daily. Samples of experimental diets and orts were collected daily, stored at -20°C, and composited per lamb for each experimental period prior to chemical analysis. On d 21 of each experimental period, lambs were fitted with temporary vinyl catheters (0.86 mm I.D. x 1.32 mm O.D.; Scientific Commodities Inc., Lake Havasu City, Arizona, USA) in the right and left jugular veins to allow for simultaneous isotope infusion and blood sampling. Urea-N transfer to the GIT and whole-body N balance were determined between d 22 and 26 as described by Lobley et al. (2000). Briefly, background samples of urine and feces were collected on d 21 to measure ^{15}N natural abundance. Starting on d 22 of each experimental period, [$^{15}N^{15}N$]-urea (99 atom % ^{15}N; Cambridge Isotope Laboratories, Andover, MA) prepared in 0.15 M sterile saline was infused continuously into a jugular vein using a peristaltic pump for 96 h. Daily dosage rates of [$^{15}N^{15}N$]-urea ranged from 324 to 582 mg per lamb depending on N intake, such that the predicted plateau enrichment of [$^{15}N^{15}N$]-urea in urine was 0.15 mole percent excess. Total feces and urine were collected daily between d 22 and d 26 before the 0900 h feeding to determine daily outputs. Collection, processing, and sampling of feces and urine for N, fecal ^{15}N enrichment, urinary enrichment of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]- and [$^{14}N^{14}N$]-urea, and urinary purine derivatives (PD) analysis was as previously described (Kiran and Mutsvangwa, 2010).

On d 26, 200 mL of ruminal contents were sampled at 0900, 1100, 1300, 1500 and 1700 h and squeezed through two layers of cheesecloth. Ruminal pH was immediately determined and 10-mL aliquots of ruminal fluid were preserved as previously described (Kiran and Mutsvangwa, 2010) for NH_3-N and VFA analysis. The remaining ruminal fluid was kept frozen at -20°C for the isolation of ruminal microbes. At the same time points corresponding to ruminal fluid sampling, blood samples were drawn from a jugular vein catheter into vacutainers containing heparin and plasma was obtained as previously described (Kiran and Mutsvangwa, 2010) for PUN analysis. On d 27, ruminal epithelial tissue biopsies were taken by clipping at 2 h after the morning feeding as previously described (Kiran and Mutsvangwa, 2010).

Ruminal starch degradation in diets containing DRB and PB were determined using the in situ method as described by Yu et al. (2003). Briefly, approximately 7 g of each diet were weighed into nylon bags and incubated in the rumen of a dry cow fed barley silage for 2, 4, 6, 8, 12, 24 and 48 h. The rumen incubation protocol, nylon bags, washing and drying procedures for nylon bags were as described by Yu et al. (2003). Dried samples of diet and nylon bag residues were analyzed later for starch.

5.3.5 Sample Analyses

At the end of the trial, frozen samples of experimental diets, orts and feces were thawed overnight at room temperature and analyzed for DM by drying in an oven at 60°C for 48 h (method 930.15) (AOAC, 1995). Dried diets, orts and feces were then ground through a 1-mm screen using a Christy-Norris mill (Christy and Norris Ltd., Chelmsford, England). Ground diets, orts and fecal samples were pooled per lamb and analyzed for OM (method 942.05), N using the macro-Kjeldahl procedure (method 2001.11), total starch (method 996.11) using a commercial kit (Total Starch Assay Kit, Megazyme International Ireland Ltd., Wicklow, Ireland), ether extract (method 2003.05) (AOAC, 1995), and ADF and NDF (Van Soest et al., 1991). Amylase and sodium sulfite were used for NDF determination. Total N in pooled urine was determined using the macro-Kjeldahl procedure (AOAC, 1995). Dried nylon bag residues were analyzed for total starch (method 996.11) using a commercial kit. The fatty acid composition of SFO was determined using GC (Agilent 6890, Mississauga, ON, Canada) as described by Soita et al. (2005).

Preserved ruminal fluid samples were analyzed for VFA by GC as described by Erwin et al. (1961), and for NH_3-N using a phenol-hypochlorite assay (Broderick and Kang, 1980). Frozen ruminal fluid samples for microbial isolation were thawed at room temperature and centrifuged at 20,000 x *g* at 4°C for 20 min, and the supernatant fraction was discarded. The microbial pellet was washed with double-distilled water and centrifuged at 20,000 x *g* at 4°C for 20 min and this process was repeated two more times (Makkar and Becker, 1999). The microbial pellet was then freeze-dried, ground with a mortar and pestle, and analyzed for total N and individual purines as described by Makkar and Becker (1999). Daily urine samples (2 mL) that were diluted with distilled water (8 mL) were pooled per lamb proportionally to daily urine output and analyzed for allantoin, and xanthine plus hypoxanthine (Chen and Gomes, 1992), and

for uric acid by a quantitative enzymatic colorimetric method using a commercial assay kit (Stanbio Uric Acid Liquicolor Kit, Procedure No. 1045; Stanbio Laboratories, Boerne, TX, USA). Total PD excretion per day was calculated as allantoin + uric acid + xanthine plus hypoxanthine. Microbial non-NH_3-N (NAN) supply was calculated based on total PD excretion in urine (Chen and Gomes, 1992), using the determined purine N:microbial N ratios and BW measurements obtained on d 19. Plasma urea-N (PUN) and urinary urea-N (UUN) were determined by the diacetyl monoxime method (Marsh et al., 1957) using a commercial kit (Stanbio Urea-N Kit, Procedure No. 0580; Stanbio Laboratories).

To determine the proportions of [^{15}N^{15}N]-, [^{14}N^{15}N]- and [^{14}N^{14}N]-urea in daily urine and background urine samples, urinary urea was isolated by applying urine containing 1.5 mg of urea-N through pre-packed cation exchange resin columns (AG-50W-×8 Resin, 100-200 mesh, H^+ form; Biorad, Richmond, CA) as described by Archibeque et al. (2001). The proportions of [^{15}N^{15}N]-, [^{14}N^{15}N]-, and [^{14}N^{14}N]-urea in isolated UUN were analyzed by isotope ratio-mass spectrometry (Lobley et al., 2000) at the N-15 Analysis Laboratory, University of Illinois at Urbana-Champaign. Under the conditions of this assay, [^{14}N^{14}N]-, [^{14}N^{15}N]-, and [^{15}N^{15}N]-urea molecules should yield ions with mass/charge (m/z) values of 28, 29, and 30, respectively. To account for non-monomolecular reactions, standards which were prepared from [^{15}N^{15}N]-urea (99.8 atom % ^{15}N) and [^{14}N^{14}N]-urea (natural abundance urea; 0.364 atom % ^{15}N) were also analyzed and the necessary corrections for [^{14}N^{15}N]-urea that is produced by non-monomolecular reactions were then made (Lobley et al., 2000). Fecal samples were analyzed for total ^{15}N enrichment by combustion to N_2 gas in an elemental analyzer and continuous flow isotope ratio-mass spectrometry (Lobley et al., 2000). For UT-B gene expression analysis, processing of ruminal epithelial tissue, RNA extraction, cDNA generation, and quantification of gene transcript abundance using qPCR were conducted as previously described (Kiran and Mutsvangwa, 2010). Ovine-GAPDH was used as an internal reference to UT-B mRNA expression. The results are presented as target gene transcript abundance normalized to GAPDH transcript abundance. Expression of GAPDH mRNA in equal amounts of total RNA indicated that GAPDH in rumen epithelial tissue was stable and unaffected by the experimental conditions that were employed.

5.3.6 Calculation of Urea-N Kinetics and Statistical Analysis

Rumen degradation characteristics of starch were analyzed using the NLIN procedure (SAS, 2004) using iterative least squares regression (Gauss-Newton method) as described by Yu et al. (2003). Urea-N kinetics was calculated according to the model of Lobley et al. (2000), using urinary ^{15}N enrichment of [$^{15}N^{15}N$]- and [$^{14}N^{15}N$]-urea and total ^{15}N excretion in feces. All data were analyzed using ANOVA as a 4 x 4 Latin square using the Proc Mixed procedure of SAS (2004) with animal and period as the random effects. The model used included the following independent variables: animal, period, SFO (-SFO vs. +SFO), barley grain processing (DRB vs. PB), and the SFO x barley grain processing interaction. When significant SFO x barley grain processing interactions were detected, treatments means were compared using the Tukey-Kramer multiple comparison test. Data on PUN, ruminal pH, and ruminal concentrations of VFA and NH_3-N were analyzed accounting for repeated measures (Wang and Goonewardene, 2004). For protozoal counts, statistical analysis was performed on $\log_{10}$-transformed data. Treatment differences were considered significant when $P \leq 0.05$ and tendencies are discussed when $0.05 < P \leq 0.10$. Data are presented as means ± SEM.

5.4 Results

5.4.1 In Situ Ruminal Starch Degradation Kinetics

The in situ ruminal starch degradation kinetics are presented in **Table 5.2**. The in situ soluble and degradable starch fractions, the degradation rate of the degradable starch fraction, and the effective starch degradability were greater ($P \leq 0.02$) in the diet that contained PB when compared to that containing DRB.

5.4.2 Dry Matter Intake and Total Tract Nutrient Digestibilities

Dry matter and organic matter (OM) intakes were unaffected ($P \geq 0.14$) by treatment (**Table 5.3**). Total tract digestibilities of DM, OM, N, ADF, and NDF were unaffected ($P \geq 0.24$) by feeding SFO and method of barley grain processing. Total tract digestibility of starch was greater ($P = 0.001$) in lambs fed PB compared to those fed DRB, but was unaffected by protozoal status.

Table 5.2 In situ ruminal degradation kinetics of starch in experimental diets containing dry-rolled barley or pelleted barley

Item	Dry-rolled barley	Pelleted barley	SEM	*P* value
Soluble fraction, %	9.5	15.1	0.72	0.002
Degradable fraction, %	79.6	81.0	0.35	0.02
Degradation rate, %/h	13.7	16.4	0.56	0.009
Effective degradability[1], %	65.2	74.5	0.92	0.001

[1]Calculated assuming a ruminal passage rate of 6%/h.

Table 5.3 Sunflower oil (SFO), basal dry matter (DM), and total organic matter (OM) intakes, and total tract nutrient digestibility in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed dry-rolled or pelleted barley[1]

	Dry-rolled barley		Pelleted barley			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	BP	Protozoa x BP
Intakes, g/d								
SFO	-	118.2	-	108.5	-	-	-	-
Basal DM[3]	2,074	1,970	1,856	1,809	119.6	0.54	0.14	0.82
Total OM[4]	1,901	2,039	1,701	1,872	115.9	0.21	0.14	0.88
Nutrient digestibility, %								
DM	62.4	57.9	60.9	62.4	2.4	0.55	0.54	0.24
OM	65.2	63.9	63.7	67.8	2.1	0.51	0.59	0.22
Starch	88.6	87.8	96.6	97.3	1.8	0.94	0.001	0.38
N	75.7	72.0	73.3	75.3	2.8	0.77	0.86	0.33
ADF	30.6	26.0	30.0	28.6	5.6	0.60	0.86	0.79
NDF	32.2	28.9	28.5	28.1	5.6	0.74	0.70	0.79

[1] $n = 4$ for each treatment.

[2] Protozoa = PDFAUN vs. FAUN; BP = barley grain processing (DRB vs. PB); Protozoa x BP = interaction. Partial defaunation was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet.

[3] Basal DM intake does not include SFO intake.

[4] Total OM intake includes SFO intake.

5.4.3 Ruminal Protozoa Counts and Fermentation Characteristics

Ruminal counts of total protozoa, *Entodinium spp.*, Holotrichs, and cellulolytic protozoa were greater in FAUN lambs fed DRB compared those fed PB (**Table 5.4**; $P < 0.01$); consequently, there was a greater difference in ruminal counts of total protozoa, *Entodinium spp.*, Holotrichs, and cellulolytic protozoa between FAUN and PDFAUN lambs fed DRB compared to those fed PB (interaction; $P < 0.01$). Experimental lambs were successfully refaunated by the administration of ruminal fluid from naturally-faunated sheep over 3 d, and populations (mean ± SE; $n = 4$) for total protozoa ($10.6 \pm 0.3 \times 10^5$ /ml), *Entodinium spp.* ($10.2 \pm 0.3 \times 10^5$/ml), Holotrichs ($0.24 \pm 0.02 \times 10^5$ /ml), and cellulolytic protozoa ($0.20 \pm 0.02 \times 10^5$/ml) were restored to pre-defaunation levels in previously PDFAUN lambs by d 15 of the experimental period (**Appendix Tables V, VI and VII**).

For ruminal pH, and NH_3-N and VFA concentrations, sampling time x barley grain processing x ruminal protozoal status interactions were minor, so treatment means are presented (Table 5.4). Ruminal pH was higher ($P = 0.001$) in PDFAUN compared to FAUN lambs. Ruminal pH was lower ($P = 0.02$) in lambs fed PB compared to those fed DRB. Ruminal NH_3-N concentration was lower ($P = 0.001$) in PDFAUN compared to FAUN lambs, and the difference in ruminal NH_3-N concentration between PDFAUN and FAUN lambs was greater with DRB than with PB (interaction; $P < 0.01$). Ruminal total VFA, acetate and butyrate were lower ($P = 0.001$), whereas that of propionate was higher ($P = 0.001$) in PDFAUN compared to FAUN lambs; consequently, the acetate to propionate ratio was lower ($P = 0.001$) in PDFAUN compared to FAUN lambs. Ruminal concentrations of total and individual VFA were similar in lambs fed either DRB or PB, except for a higher ($P = 0.001$) ruminal concentration of valerate and a lower ($P = 0.009$) acetate to propionate ratio in lambs fed PB compared to those fed DRB.

Table 5.4 Ruminal fermentation characteristics and protozoa numbers in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed dry-rolled or pelleted barley [1]

	Dry-rolled barley		Pelleted barley			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	BP	Protozoa x BP
Protozoa, x 10^5/ml[3]								
Entodinium spp.	10.2[a]	1.3[c]	6.2[b]	ND[4]	0.25	0.001	0.001	0.001
Holotrichs	0.28[a]	0.01[c]	0.16[b]	ND	0.02	0.001	0.007	0.008
Cellulolytics[5]	0.20[a]	ND	0.09[b]	ND	0.02	0.001	0.003	0.003
Total	10.7[a]	1.3[c]	6.4[b]	ND	0.27	0.001	0.001	0.001
pH	5.8	6.2	5.7	6.0	0.05	0.001	0.02	0.86
NH_3-N, mg/dL	9.7[a]	6.0[c]	7.7[b]	5.6[c]	0.22	0.001	0.001	0.002
VFA concentration, mmol/L								
Total	100.3	92.8	98.2	94.1	1.13	0.001	0.72	0.15
Acetate	62.1	55.4	59.2	55.3	1.08	0.001	0.19	0.22
Propionate	18.5	21.5	19.3	22.3	0.48	0.001	0.13	0.99
Butyrate	16.3	11.5	15.9	12.6	0.55	0.001	0.55	0.24
Isobutyrate	0.79	0.71	0.83	0.72	0.07	0.18	0.73	0.84
Valerate	1.3	1.4	1.6	1.6	0.06	0.26	0.01	0.32
Isovalerate	1.2	1.4	1.4	1.7	0.29	0.09	0.36	0.32
Acetate to propionate ratio	3.4	2.6	3.1	2.5	0.06	0.001	0.009	0.17

[1] $n = 4$ for each treatment.

[2] Protozoa = PDFAUN vs. FAUN; BP = barley grain processing (DRB vs. PB); Protozoa x BP = interaction. Partial defaunation was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet.

[3] Treatment means for ruminal counts of protozoa during the measurement period (d 22 to d 27).

[4] Not detectable

[5] Includes *Polyplastron*, *Diplodinium* and *Enoplastron* sp.

[a-c] Means within a row with different superscripts differ ($P < 0.05$).

5.4.4 Nitrogen Balance and Plasma Urea-N Concentration

Intakes of N were similar across treatments (**Table 5.5**). Urinary excretion of total N was 2.7 to 4.9 g/d less (P = 0.003) in PDFAUN compared to FAUN lambs; consequently, total N excretion, when expressed as absolute amounts (P = 0.02) or as a proportion of N intake (P = 0.001), was lower, and N retention, when expressed as absolute amounts (P = 0.002) or as a proportion of N intake (P = 0.001), was higher in PDFAUN compared to FAUN lambs. Lambs fed PB excreted 2.5 to 4.7 g/d less (P = 0.004) urinary N compared to those fed DRB. Total N loss, when expressed in absolute amounts (P = 0.01) or as a proportion of N intake (P = 0.001), was lower in lambs fed PB compared to those fed DRB. Consequently, N retention when expressed in absolute amounts tended to be greater (P = 0.09), or when expressed as a proportion of N intake was greater (P = 0.001) in lambs fed PB compared to those fed DRB. The PUN concentration was greater (P = 0.001) in FAUN compared to PDFAUN lambs, but method of barley grain processing had no effect on PUN concentration.

5.4.5 Urea-N Kinetics, mRNA Abundance of Urea Transporter-B and Microbial NAN Supply

Across treatments, urinary [$^{15}N^{15}N$]-urea enrichment reached a plateau within 24 to 48 h of isotopic infusion, whereas plateau enrichment of [$^{14}N^{15}N$]-urea in urine was not attained until between 48 and 72 h of isotopic infusion (**Figure 5.1**). Fecal ^{15}N enrichments increased daily over the 4-d isotopic infusion without attaining a definite plateau (**Figure 5.2**); therefore, for calculations of urea-N kinetics, urinary [$^{14}N^{15}N$]- and [$^{15}N^{15}N$]-urea and fecal ^{15}N enrichments over the final 72 to 96 h of infusion were used, similar to previous research (Lobley et al., 2000; Sunny et al., 2007).

Table 5.5. Nitrogen (N) intake, N balance, and plasma-urea nitrogen in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed dry-rolled or pelleted barley [1]

	Dry-rolled barley		Pelleted barley			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	BP	Protozoa x BP
N intake, g/d	33.7	31.9	29.9	29.1	1.9	0.52	0.12	0.80
Faecal N excretion								
g/d	8.1	9.0	8.2	7.2	1.2	0.97	0.49	0.46
% of total N excretion	31.6	41.0	37.5	40.9	3.8	0.10	0.45	0.44
Urine N excretion								
Total N, g/d	17.7	12.8	13.0	10.3	1.1	0.003	0.004	0.29
Total N, % of total N excretion	68.4	58.9	62.5	59.1	3.8	0.10	0.45	0.44
Urea-N, g/d	11.2[a]	7.8[b]	8.4[b]	6.7[c]	0.33	0.001	0.001	0.03
Total N excretion								
g/d	25.8	21.7	21.2	17.5	1.5	0.02	0.01	0.88
% of N intake	76.8	68.2	71.0	60.2	1.1	0.001	0.001	0.33
N retention								
g/d	7.8	10.1	8.7	11.6	0.65	0.002	0.09	0.66
% of N intake	23.2	31.8	29.0	39.8	1.1	0.001	0.001	0.33
Plasma urea-N, mg/dL	9.5	6.7	8.7	6.7	0.53	0.001	0.51	0.47

[1] $n = 4$ for each treatment.

[2] Protozoa = PDFAUN vs. FAUN; BP = barley grain processing (DRB vs. PB); Protozoa x BP = interaction. Partial defaunation was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet.

[a-c]Means within a row with different superscripts differ ($P < 0.05$).

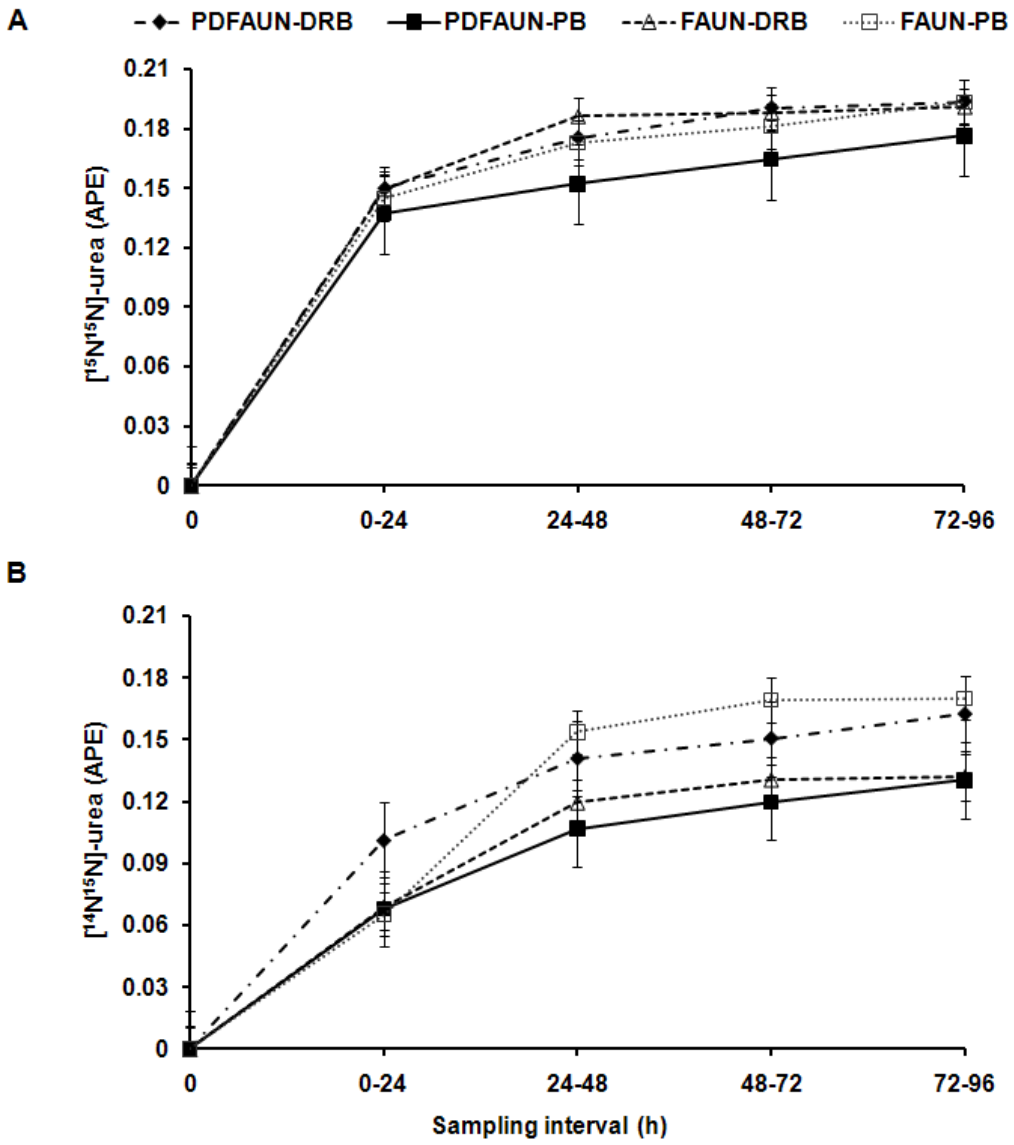

Figure 5.1 Enrichments (APE, atom percent excess) of [^{15}N^{15}N]-urea (A) and [^{14}N^{15}N]-urea (B) in urine during 4-d intra-jugular infusions of [^{15}N^{15}N]-urea (infusions were initiated at sampling interval 0 h). Treatments were: PDFAUN lambs fed dry-rolled barley (PDFAUN+DRB); PDFAUN lambs fed pelleted barley (PDFAUN+PB); FAUN lambs fed dry-rolled barley (FAUN+DRB); and FAUN lambs fed pelleted barley (FAUN+PB). For [^{15}N^{15}N]-urea enrichments, no differences were detected between sampling intervals 0-24, 24-48, 48-72, and 72-96 h (P > 0.10). For [^{14}N^{15}N]-urea enrichments, no differences were detected between sampling intervals 24-48, 48-72, and 72-96 h (P > 0.10). Each line represents means ± SEM for 4 lambs.

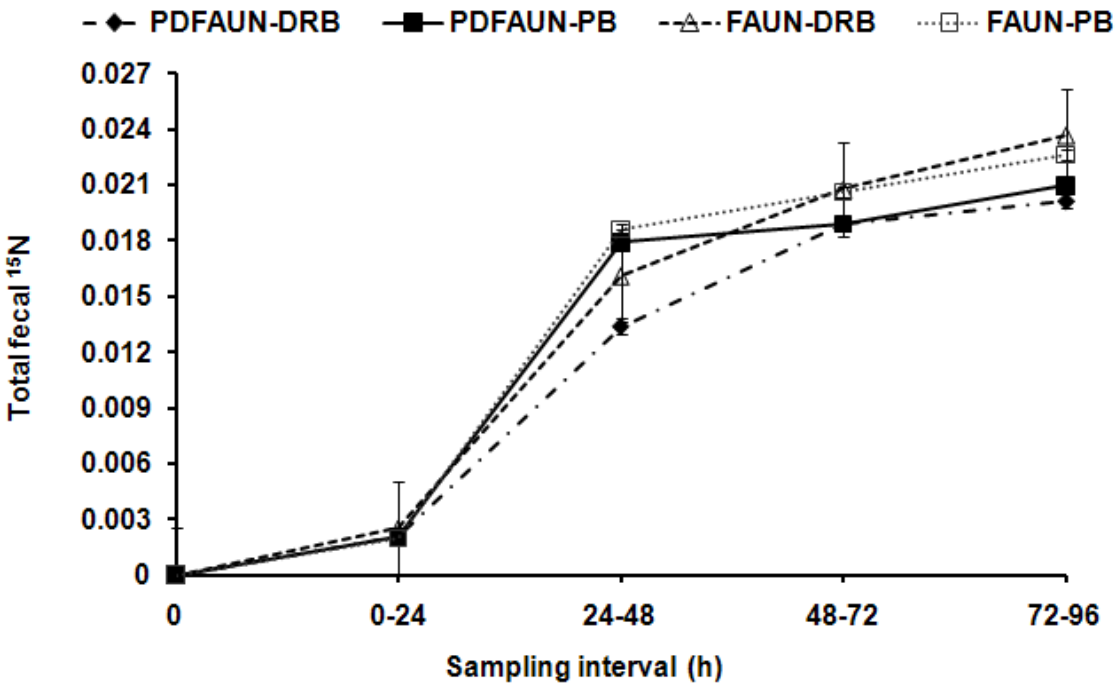

Figure 5.2 Enrichments (APE, atom percent excess) of fecal ^{15}N during 4-d intra-jugular infusions of [$^{15}N^{15}N$]-urea (infusions were initiated at sampling interval 0 h). Treatments were: PDFAUN lambs fed dry-rolled barley (PDFAUN+DRB); PDFAUN lambs fed pelleted barley (PDFAUN+PB); FAUN lambs fed dry-rolled barley (FAUN+DRB); and FAUN lambs fed pelleted barley (FAUN+PB). Differences were detected in fecal ^{15}N enrichments between sampling intervals 0-24, 24-48, 48-72, and 72-96 h (P = 0.001), indicating that fecal ^{15}N enrichment did not reach a definite plateau. Each line represents means ± SEM for 4 lambs.

Only minor interactions on urea-N kinetics were detected between feeding SFO and method of barley grain processing (**Table 5.6**). Endogenous urea-N production (i.e., UER) was similar in FAUN and PDFAUN lambs (P = 0.48). The GER, when expressed in absolute amounts (P = 0.008) or as a proportion of UER (i.e., UER to GIT; P = 0.001), was greater in PDFAUN compared to FAUN lambs. The UUA, when expressed in absolute amounts (P = 0.008) or as a proportion of GER (i.e., GER to UUA; P = 0.04), was higher in PDFAUN compared to FAUN lambs. Conversely, the amount of UER that was lost in urine (i.e., UUE), when expressed in absolute amounts (P = 0.001) or as a proportion of UER (i.e., UER to urine; P = 0.001), was lower in PDFAUN compared to FAUN lambs. The urea-N that re-entered the ornithine cycle (i.e., ROC) was unaffected by protozoal status; however, when expressed as a proportion of GER (i.e., GER to ROC), there was a tendency (P = 0.08) for it to be lower in PDFAUN compared to FAUN lambs. The UFE was unaffected by protozoal status. The UER, GER, UUA, UFE, and ROC were similar in lambs fed DRB or PB. The UUE, when expressed in absolute amounts (P = 0.001) or as a proportion of UER (P = 0.01), was lower, whereas GER when expressed as a proportion of UER was higher (P = 0.01), in lambs fed PB compared to those fed DRB. The expression of UT-B mRNA was unaffected by treatment.

Urinary excretion of allantoin and total PD was higher (P = 0.001) and, correspondingly, microbial NAN supply estimated using urinary PD excretion and measured purine N:microbial N ratios was higher (P = 0.001) in +SFO compared to –SFO lambs (**Table 5.7**). Compared to those fed DRB, lambs fed PB excreted more allantoin (P = 0.02), and tended to excrete more xanthine plus hypoxanthine (P = 0.06) and total PD (P = 0.09); however, microbial NAN supply was unaffected (P = 0.22) by barley grain processing.

Table 5.6. Urea-N recycling kinetics, as measured using 4-d continuous intra-jugular infusions of [$^{15}N^{15}N$]-urea, and urea transporter-B (UT-B) mRNA abundance in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed dry-rolled or pelleted barley[1]

	Dry-rolled barley		Pelleted barley			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	BP	Protozoa x BP
Urea-N kinetics, g/d								
Production, UER	23.2	24.6	22.5	22.6	1.04	0.48	0.24	0.53
Entry to GIT, GER	12.0	16.8	14.1	16.0	1.04	0.008	0.55	0.18
Return to ornithine cycle, ROC	5.8	6.8	6.8	6.3	0.60	0.67	0.69	0.22
Loss to feces, UFE	0.81	0.77	0.75	0.92	0.10	0.54	0.64	0.35
Loss to urine, UUE	11.2[a]	7.8[b]	8.4[b]	6.7[c]	0.33	0.001	0.001	0.03
Re-use for anabolism, UUA	5.5	9.2	6.6	8.8	0.92	0.008	0.71	0.41
Fractional urea-N transfers								
UER to urine	0.483	0.319	0.378	0.295	0.022	0.001	0.01	0.09
UER to GIT	0.517	0.680	0.622	0.705	0.022	0.001	0.01	0.09
GER to ROC	0.478	0.409	0.489	0.395	0.042	0.08	0.96	0.77
GER to feces	0.068	0.047	0.053	0.057	0.007	0.22	0.74	0.08
GER to UUA	0.453	0.544	0.458	0.548	0.040	0.04	0.91	0.99
UT-B mRNA abundance, per copy of GAPDH	10.5	11.3	10.3	11.0	1.41	0.63	0.84	0.96

[1] $n = 4$ for each treatment.

[2] Protozoa = PDFAUN vs. FAUN; BP = barley grain processing (DRB vs. PB); Protozoa x BP = interaction. Partial defaunation was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet.

[a-c] Means within a row with different superscripts differ ($P < 0.05$).

Table 5.7. Urinary output, urinary purine derivative (PD) excretion, and microbial non-ammonia nitrogen (NAN) supply in in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed dry-rolled or pelleted barley [1]

	Dry-rolled barley		Pelleted barley			*P* value[2]		
Item	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	BP	Protozoa x BP
Urinary excretion								
Total output, kg/d	2.0	1.9	1.7	1.6	0.20	0.66	0.16	0.82
Allantoin excretion, mmol/d	5.9	8.6	7.1	9.9	0.49	0.001	0.02	0.89
Uric acid excretion, mmol/d	1.6	2.2	2.2	2.0	0.30	0.53	0.57	0.22
Xanthine plus Hypoxanthine, mmol/d	0.98[ab]	1.20[a]	0.99[ab]	0.83[b]	0.09	0.76	0.06	0.04
Total PD excretion, mmol/d	8.5	12.0	10.3	12.7	0.70	0.001	0.09	0.48
Purine bases[3], g/kg dry matter	62.7	67.9	68.3	70.09	1.03	0.005	0.002	0.12
N in microbial pellet, g/kg dry matter	54.0	58.4	57.2	60.2	1.73	0.05	0.18	0.70
Puirne N/Microbial N	0.085	0.085	0.087	0.085	0.003	0.71	0.63	0.65
Microbial NAN supply[4], g/d	10.0	14.1	11.8	15.0	1.05	0.005	0.22	0.64

[1] $n = 4$ for each treatment.

[2] Protozoa = PDFAUN vs. FAUN; BP = barley grain processing (DRB vs. PB); Protozoa x BP = interaction. Partial defaunation was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet.

[3] Purine bases are expressed as g yeast RNA

[4] Microbial NAN supply was calculated according to Chen and Gomes [(24)], using determined purine N:microbial N ratios.

[a,b] Means within a row with different superscripts differ ($P < 0.05$).

5.5 Discussion

Partial defaunation had no impact on DM intake, which is in contrast to our previous study (Kiran and Mutsvangwa, 2010) in which we observed a decrease in DM intake in PDFAUN lambs. Lambs that were used in the current study were heavier (+17 kg) and older than those that were used in our initial study, and this could partly explain why responses in DM intake were different. As expected based on previous studies in which SFO was used as a defaunating agent (Kiran and Mutsvangwa, 2010; Ivan et al., 2001), ruminal protozoal counts decreased substantially within 6 d after the initiation of feeding SFO. Feeding PB also resulted in a decrease in ruminal protozoal counts when compared with DRB, supporting previous studies that have reported decreases in ruminal protozoal populations in ruminants fed extensively-processed barley (Koenig et al., 2003; Eadie et al., 1970). The major changes that were observed in ruminal fermentation characteristics i.e., a decrease in ruminal NH_3-N, total VFA, acetate and butyrate concentrations, and an increase in ruminal propionate concentration, are typical of changes that are associated with partial or complete defaunation (Ivan et al., 2001; Koenig et al., 2000; Newbold et al., 1986). Ruminal pH was higher in PDFAUN lambs compared to FAUN lambs, which also is in agreement with other research (Kiran and Mutsvangwa, 2010; Ivan et al., 2001; Ivan et al., 2004).

Total tract digestibilities of DM, OM, starch, N, ADF, and NDF were unaffected by protozoal status, supporting previous findings (Kiran and Mutsvangwa, 2010). It appears that, because of the inhibition of protozoal activities in the rumen, most of the available data when high oil diets are fed to achieve partial or complete defaunation indicate a decrease in ruminal OM or fiber digestion (Oldick and Firkins, 2000; Faichney et al., 2000). If ruminal fiber digestion was depressed by feeding SFO in the present study, this was compensated for post-ruminally as total tract digestibilities were not affected. Both in situ ruminal and total tract starch digestibility were greater with PB compared to DRB. In common with the present study, Yang et al. (2000) reported increases in ruminal and total tract starch digestion as the extent of barley grain processing was increased in dairy cows fed barley-based diets. Our results suggest that the lower ruminal digestion of DRB (as reflected by the lower in situ degradation kinetics relative to PB) was not compensated for by greater intestinal digestion, which supports previous findings (Yang et al., 2000).

In ruminants, protozoa impose inefficiencies in N utilization at a number of different levels, and the eradication of ruminal protozoa typically improves N utilization (Jouany, 1996). In the present study, FAUN and PDFAUN lambs had similar N intakes and fecal N losses; however, total N excretion decreased by 3.7 to 4.1 g/d in PDFAUN lambs, with a large fraction of this decrease in total N excretion being accounted for by a decrease in urinary urea-N excretion. Consequently, PDFAUN lambs retained 2.3 to 2.9 g/d more N when compared to FAUN lambs, thus suggesting a greater efficiency of N utilization with defaunation as has been reported by others (Kiran and Mutsvangwa, 2010; Jouany, 1996). This improved N utilization in defaunated ruminants is accounted for by various mechanisms, including a decrease in the ruminal NH_3-N pool, coupled with a greater sequestration of NH_3-N into bacterial protein (Firkins et al., 2007), a decrease in intra-ruminal N recycling (Jouany, 1996), and an increase in microbial N flow to the duodenum by 20 to 60% (Jouany, 1996; Koenig et al., 2000). Besides the change in protozoal status, associated changes in energy supply and ruminal VFA patterns could also be partly responsible for improving the efficiency of post-absorptive N use in PDFAUN lambs. Assuming that the energy content of SFO is 8.8 Mcal/kg, dietary supplementation with 6% SFO increased energy intake of PDFAUN lambs by 0.95 to 1.04 Mcal of GE/d, and this additional energy intake could have influenced post-absorptive N use. In growing steers, increasing energy intake by abomasally infusing 150 g/d of a mixture of fatty acids (equivalent to an additional 1.3 Mcal of GE/d) decreased urinary N excretion and increased N retention (Schroeder et al., 2006), similar to our current findings. Also, the shift in ruminal VFA proportions that was observed with feeding SFO, with more propionate and less acetate, provided additional glucogenic substrate that could have altered N use. In addition to improving propionate supply, supplemental SFO also could have provided another potential glucose precursor (glycerol) that could have improved N use. In ruminants wholly nourished by intra-gastric infusion, supplementation with glucogenic precursors improved N retention (Chowdhury et al., 1997; Ørskov et al., 1999). This suggests that providing glucogenic precursors can potentially spare amino acids from being used for gluconeogenesis, thus making more amino acids available for protein accretion.

A major objective of the present study was to determine the effects of defaunation on urea-N transfer to the GIT and the anabolic utilization of the recycled urea-N in lambs. Our current data on urea-N recycling kinetics offers interesting insights into additional potential

mechanisms by which ruminal defaunation could improve N utilization. The UER was similar in FAUN and PDFAUN lambs, reflecting the similar N intakes, as N intake and UER are positively correlated (Marini et al., 2004; Wickersham et al., 2009). However, the GER, when expressed in absolute amounts or as a proportion of UER, was higher in PDFAUN compared to FAUN lambs. Concomitant with these changes in GER, UUE when expressed in absolute amounts or as a proportion of UER was lower in PDFAUN when compared to FAUN lambs. Also, although there were no differences between FAUN and PDFAUN lambs in the absolute amounts of ROC, the GER/ROC ratio tended to be lower in PDFAUN compared to FAUN lambs. When taken together, these data clearly indicate that increased urea-N recycling to the GIT is also a major contributor to the improved N utilization that has been consistently observed in defaunated ruminants. In our previous research, partial defaunation was associated with a higher proportion of endogenous urea-N production being recycled to the GIT; however, N intake was lower in partially-defaunated compared to faunated sheep in that study (Kiran and Mutsvangwa, 2010). Because of the positive correlation between N intake and hepatic output of urea-N (Marini et al., 2004; Wickersham et al., 2009), data on urea-N recycling kinetics were confounded due to these differences in N intake. In the present study, N intake did not differ due protozoal status, thus allowing more definitive conclusions to be made on the effects on urea-N kinetics of defaunation.

Endogenous urea-N can diffuse through all compartments of the GIT (Lapierre and Lobley, 2001) and, although the [$^{15}N^{15}N$]-urea infusion technique that was used in the present study cannot distinguish between urea-N transfer to the fore-stomach and post-stomach compartments, measurements of urea-N recycling to the GIT obtained using arterio-venous preparations indicated that in concentrate-fed ruminants (as in the present study), 65 to 76% of urea-N transfer to the GIT entered the fore-stomachs (Huntington, 1989; Reynolds and Huntington, 1988). It is urea-N that is transferred to the fore-stomachs that can provide additional degradable N than be incorporated into microbial protein, thus contributing amino acids to the host animal after intestinal digestion. In the present study, however, direct incorporation of recycled ^{15}N into microbial protein was not measured; rather, anabolic utilization (UUA) as part of the isotopic technique was measured, in addition to estimating microbial NAN supply based on urinary excretion of PD (Chen and Gomes, 1992). The anabolic utilization of recycled urea-N within the GIT, particularly the rumen, is assumed to be

predominantly as sequestration of liberated NH_3-N into microbial protein as most of the recycled urea-N first passes through the microbial N pool (Lobley et al., 2000), but other potential anabolic uses within the body could include amination and transamination reactions (Lobley et al., 2000). The ability to partition how much of the GER is directed towards anabolic and catabolic fates is a major advantage of this isotopic technique; however, a limitation is that UUA is calculated as the difference between GER and (ROC + UFE) and therefore contains all cumulative errors. Because fecal ^{15}N enrichments failed to attain a definite plateau, UFE was likely underestimated; as such UUA was likely overestimated, so our data on UUA should be interpreted somewhat cautiously. However, when we used a curve fitting approach using the daily fecal ^{15}N enrichments over the 4-d isotopic infusions (data not shown) to estimate plateau (maximum) enrichments, the measured "plateau" enrichments were only 3.1 to 6.3% lower than the mathematically-derived "plateau" enrichments. This suggests that UUA was only slightly overestimated. Others (Lobley et al., 2000; Sunny et al., 2007) have also reported a failure of fecal ^{15}N enrichments to attain a definite plateau with 4-d isotopic infusions, so future studies should consider isotopic infusions longer than 4 d. For PDFAUN lambs, 0.546 of GER was directed towards anabolic fates, compared to only 0.456 for FAUN lambs. Furthermore, the ROC/GER ratio was 0.082 units lower for PDFAUN compared to FAUN lambs, suggesting that more of the GER was used for productive purposes in PDFAUN compared to FAUN lambs. In support of our findings of the improved productive use of recycled urea-N by partial defaunation, microbial NAN supply was 27 to 41% greater in PDFAUN compared to FAUN lambs. It is plausible that urea-N that was recycled to the rumen could have buffered the rumen from the lower NH_3-N concentrations prevailing in PDFAUN lambs, thus providing additional N that could be used for microbial protein synthesis. Additionally, because fat provides little energy for microbial growth (Wu and Palmquist, 1991), the positive response in intestinal microbial NAN flow when ruminal protozoal populations are decreased has also been attributed to decreased protozoal predation on bacteria and decreased competition from protozoa for growth substrates (Jouany, 1996). In calculating microbial NAN supply, we used purine N:N ratios that were determined in bacteria; however, microbial NAN supply in faunated and, to a much smaller extent, partially-defaunated lambs was constituted of both bacterial and protozoal N. Because the purine N:N ratio has been reported to be higher for bacteria than protozoa (Firkins et al., 1987) and only the purine N:bacterial N ratio was used to calculate microbial NAN supply, it is

possible that microbial NAN supply was underestimated particularly in faunated lambs (Koenig et al., 2000). Nonetheless, despite these limitations our data support previous findings of a greater microbial NAN flow to the intestine when ruminal protozoal populations are decreased (Jouany, 1996; Koenig et al., 2000).

The major mechanism that could be responsible for the greater urea-N transfer to the GIT that was observed in partially protozoa-free lambs is plausibly the lower ruminal NH_3-N concentration that was observed in these lambs compared to faunated lambs. Elevating ruminal concentration of NH_3-N has been demonstrated to reduce urea-N transfer from blood into the rumen (Rémond et al., 1996). Also, other evidence indicates that the trans-epithelial movement of urea-N by passive diffusion into the rumen is facilitated by bacterial urease activity, which maintains a favorable concentration gradient (Rémond et al., 1996). Cheng and Wallace (1979) demonstrated that urease activity in ruminal contents was negatively correlated with ruminal NH_3-N concentration. Because partial defaunation of the rumen resulted in a lower ruminal NH_3-N concentration, this could have facilitated the transfer of a greater proportion of UER to the GIT in PDFAUN compared to FAUN lambs. In addition, the change in propionate supply could also have altered urea-N transfer to the GIT. Feeding SFO to partially defaunate the rumen increased ruminal concentrations of propionate and, presumably, the absorption of propionate from the rumen as this is a concentration-dependent process (Van Soest, 1994). Propionate supplementation via intra-ruminal (Savary-Auzeloux et al., 2003) or intra-abomasal (Kim et al., 1999) infusion in sheep was associated with greater urea-N recycling to the GIT and N retention. The mechanism by which propionate increases urea-N recycling to the GIT is unclear, but it may be related to an alteration in ruminal bacterial activities and metabolism (Savary-Auzeloux et al., 2003), or changes in ruminal epithelial surface area and/or permeability (Abdoun et al., 2007) as propionate has been shown to stimulate epithelial cell proliferation (Sakata and Tamate, 1978).

Increasing ruminal energy availability by feeding PB compared to DRB decreased UUN and increased N retention, supporting previous studies (Theurer et al., 2002; Taniguchi et al., 1995). These positive effects on N utilization of increasing ruminal energy availability have been partly mediated via increased urea-N recycling to the rumen (Kennedy and Milligan, 1980; Huntington, 1989; Theurer et al., 2002) in addition to a greater sequestration of N into microbial protein that is then available for intestinal digestion (Theurer et al., 1999). Surprisingly, in the

present study, all the major dependent variables quantifying urea-N recycling kinetics (i.e., UER, GER, UUA, and ROC) were unaffected by barley grain processing, contradicting previous studies (Huntington, 1989; Theurer et al., 2002). The magnitude of urea-N transfer across the ruminal wall appears to be regulated by many intra-ruminal factors, including ruminal pH (Rémond et al., 1996). In the present study, lambs that were fed PB exhibited a lower ruminal pH compared to those fed DRB. A more acidic ruminal environment in lambs fed PB could have decreased bacterial urease activity in the rumen (Gozho et al., 2008) which, in turn, might have impaired urea-N transfer due to an unfavorable concentration gradient. Also, new evidence obtained using an *in vitro* isolated ruminal epithelium model indicates that urea-N transport is inhibited at luminal pH < 6.0 (Abdoun et al., 2010), and mean pH in lambs fed PB was 5.8 compared to 6.0 for those fed DRB. Another surprising observation from the present study was that feeding PB did not stimulate microbial NAN supply when compared to feeding DRB, contradicting previous studies (Reynolds and Huntington, 1988; Sunny et al., 2007). It is well-established that the amount of microbial protein that is synthesized in the rumen is largely driven by the availability of energy from RFC. In the present study, in situ measurements of ruminal starch digestion clearly indicate that starch digestion was greater for PB compared to DRB. Also, total tract starch digestion was greater in lambs fed PB compared to those fed DRB. Together, these observations would suggest that, quantitatively, ruminal energy supply was greater with PB compared to DRB, so it is not clear why microbial NAN supply was not stimulated with PB. Because the diets that were fed in this study contained only 16 g N/kg DM, it is likely that the rumen was limited in both NH_3-N and amino acids to support microbial growth. Feeding PB to increase ruminal ATP availability when NH_3-N and amino acids were potentially in short supply could have limited the use of the additional ATP for microbial protein synthesis (Russell, 2007). In that situation, the additional ATP could have been directed towards non-growth functions, a phenomenon referred to as energy spilling (Russell, 2007). In addition, Russell and Wilson (1996) observed inhibitory effects on microbial protein synthesis when ruminal pH was <6.2 and this was partly attributed to increased energy spilling (Russell, 2007). In the present study, it is plausible that the more acidic ruminal environment in lambs fed PB could have compromised microbial growth by increasing energy spilling.

The presence of carrier-mediated, facilitative UT-B proteins has been demonstrated in the rumen of bovine (Marini and Van Amburgh, 2003; Stewart et al., 2005) and ovine (Marini et al.,

2004), and these UT-B proteins have been suggested to facilitate urea-N flux into the GIT lumen (Lapierre and Lobley, 2001). Our data indicate that changes in urea-N transfer to the GIT as a result of defaunation occurred without concomitant changes in UT-B expression, suggesting that the effects of ruminal NH_3-N and propionate on urea-N transfer are probably not mediated through changes in UT-B expression. Recently, Simmons et al. (2009) reported differences in UT-B expression between forage- and concentrate-fed steers, but ruminal NH_3-N concentration was unaltered and changes in ruminal VFA profiles were minimal. The limited research that is available on dietary regulation of UT expression in ruminal epithelium is equivocal (Marini et al., 2004; Marini and Van Amburgh, 2003; Stewart et al., 2005), and additional research is needed to delineate the effects of diet and ruminal metabolites on UT expression.

5.5 Conclusions

Collectively, these results provide the first major evidence that, at similar N intakes, the improved N utilization that is typically observed in defaunated ruminants is partly mediated by an increase in urea-N transfer to the GIT and its anabolic use. This increase in urea-N recycling to the GIT in partially-defaunated ruminants could be partly mediated by the decrease in ruminal NH_3-N level, which has been demonstrated to stimulate trans-epithelial urea-N transfer. However, feeding SFO increased energy intake, in addition to altering the profile of energy metabolites entering portal blood from the rumen, and these changes in energy intake and energy metabolites could also be partly responsible for the observed changes in urea-N recycling and N utilization. Although partial defaunation increased urea-N recycling to the GIT, the response was not augmented by increasing the amount of energy that is derived from carbohydrate fermentation in the rumen.

6. FEEDING OSCILLATING DIETRAY PROTEIN CONCENTRATIONS INCREASES NITROGEN UTILIZATION IN GROWING LAMBS AND THIS RESPONSE IS PARTLY ATTRIBUTABLE TO INCREASED UREA TRANSFER TO THE RUMEN[1]

6.1 Abstract

The objective of this study was to determine the effects of feeding oscillating dietary crude protein (CP) compared to static dietary CP concentration on N retention and *in vitro* urea flux across ruminal epithelia. Twenty-seven Suffolk wether lambs (25.6 ± 4.8 kg initial body weight (BW)) were blocked by BW into 9 groups of 3 and lambs within each group were then randomly assigned to one of three dietary treatments (n = 9). Dietary treatments consisted of a medium CP diet (MEDIUM; 12.8% CP) or diets with oscillating CP content (OSC) fed in two different sequences i.e., 2 d of low CP (9.7% CP) followed by 2 d of high CP (16.1% CP; OSC-HIGH) or vice-versa (OSC-LOW). Diet adaptation was for 24 d, followed by 8 d of total urine and feces collection. On d 33, lambs were slaughtered 4 h after the morning feeding, such that those on the OSC-LOW and OSC-HIGH treatments were slaughtered on the third day of receiving the low or high CP diets, respectively. Ruminal epithelial tissues were collected and mounted in Ussing chambers under short-circuit conditions and the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) was measured using ^{14}C-urea. The serosal buffer (pH of 7.4) included 1 mmol/L urea, whereas the mucosal buffer (pH of 6.2) had no urea added. Ruminal NH_3-N concentration was lower (P = 0.001), whereas ruminal pH was higher (P = 0.03) in lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet. Although N intake was similar (P = 0.52), retained N (P = 0.001) and microbial N supply (P = 0.001) were greater in lambs fed the OSC diets compared to those fed the MEDIUM diet. The total $J_{sm\text{-}urea}$ was higher (P = 0.001) in lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet. Across diets, the addition of phloretin (a known specific inhibitor of facilitative urea transporter-B; UT-B) reduced $J_{sm\text{-}urea}$; however, phloretin-insensitive $J_{sm\text{-}urea}$ was the predominant route for transepithelial urea transfer. Taken together, these data indicate that feeding oscillating dietary CP concentrations improves N

[1]A version of this chapter has been accepted for publication: Kiran, D., G. B. Penner, and T. Mutsvangwa. 2010. Feeding oscillating dietary protein increases nitrogen utilization in growing lambs and this response is partly attributable to increased urea transfer to the rumen. Journal of Nutrition (in press).

retention partly by increasing urea recycling to the rumen when animals are fed low CP diets, but the greater rates of urea transfer cannot be attributable to upregulation of UT-B.

6.2 Introduction

In ruminants, the conversion of dietary N into edible protein products such as meat and milk is very low (20 to 30%), with the majority of dietary N (70 to 80%) being excreted in feces and urine (Tamminga, 1992; Wessels and Titgemeyer, 1997). In an effort to improve environmental stewardship associated with intensive livestock operations, there is interest to adopt feeding practices that enhance N efficiency in ruminants. Several studies have demonstrated that feeding diets with oscillating CP concentrations on a 2-d basis (e.g., feeding a low CP diet for 2 d followed by feeding a high CP diet for 2 d) can enhance N retention in growing sheep (Cole, 1999; Kiran and Mutsvangwa, 2009) and finishing cattle (Cole et al., 2003; Ludden et al., 2003). Cole (1999) proposed that the increase in N retention in ruminants fed oscillating dietary CP could, *inter alia*, be attributed to increased urea recycling to the rumen. To test that hypothesis, Archibeque et al. (2007) conducted a study using growing wethers fitted with chronic indwelling catheters and fed high concentrate diets with oscillating or static CP concentrations. They reported that, at similar N intakes, urea recycling to the PDV only tended to be greater with the oscillating treatment. Thus, it was not possible to definitively attribute the improved N retention with oscillating CP diets to greater rates of urea transfer to the gut.

Urea recycling provides a source of ruminally available N for microbial protein synthesis under most dietary conditions; however, it becomes more important when the dietary N supply is deficient (Lapierre and Lobley, 2001). Although passage of urea from blood into the rumen can occur via simple diffusion, the presence of carrier-mediated, facilitative UT-B proteins in ruminants has been demonstrated (Ritzhaupt et al., 1997; 1998), and various groups (Lapierre and Lobley, 2001; Marini and Van Amburgh, 2003; Marini et al., 2004) have proposed that UT-B may have a functional role in urea transfer into the rumen. Recently, Muscher et al. (2010) quantified urea flux across isolated ruminal epithelia in Ussing chambers and observed that when the dietary CP concentration ranged from 7 to 14%, phloretin (a known specific inhibitor of UT-B; Stewart et al., 2005) decreased the serosal-to-mucosal urea flux, but inhibitory effects of phloretin were absent at 19% CP. These observations suggest that changes in UT-B activity might modulate urea transfer depending on N status. However, in that study goats were fed static

dietary CP concentrations for 7 wk. While Muscher et al. (2010) demonstrated that the dietary CP concentration has profound impacts on urea flux mediated by UT-B, the long-term feeding strategy imposed by those authors does not explain whether short-term adaptations in urea transport occur when diets with oscillating CP content are fed.

Therefore, the objective of this study was to determine the effects of feeding oscillating dietary CP compared to static dietary CP concentrations on whole-body N retention, *in vitro* phloretin-sensitive and phloretin-insensitive urea flux across the ruminal epithelia, and microbial protein production in growing lambs. The hypothesis was that the improvement in N retention for ruminants fed oscillating dietary CP concentrations can partly be attributed to enhanced urea flux across ruminal epithelia when the low CP diet is fed during the oscillating cycle.

6.3 Materials and Methods

Twenty-seven Suffolk wether lambs were used in this study, and were cared for and handled in accordance with the guidelines of the Canadian Council of Animal Care (1993). All animal use was pre-approved by the University of Saskatchewan Animal Care Committee (UCACS Protocol No. 20040048).

6.3.1 Animals, Experimental Treatments and Feeding Management

Lambs were housed in a temperature-controlled environment (18 to 22°C) at the University of Saskatchewan. Lambs were weighed at the beginning of the experiment (26.2 ± 4.0 kg initial BW) and blocked by BW into 9 blocks of 3. Within each block, lambs were randomly assigned to 1 of 3 dietary treatments. Because only three lambs could be slaughtered per week to obtain tissue samples for *in vitro* studies at the end of the collection period, stratifying the animals into blocks was necessary so that complete blocks could be started on treatment staggered over time. The dietary treatments consisted of a static dietary CP level (MEDIUM; 127 g CP/kg DM) or 1 of 2 regimens where dietary CP was oscillated between a low CP (103 g CP/kg DM) and a high CP (161 g CP/kg of DM) diet on a 48-h basis (OSC-HIGH i.e., lambs on the OSC-HIGH received the high CP diet for 2 consecutive days followed by the low CP diet for 2 consecutive days over 8 d, such that these lambs were receiving the high CP diet when they were killed on d 33) or vice-versa (OSC-LOW). The separation of the OSC treatments into OSC-LOW and OSC-HIGH allowed for equal representation of each OSC sequence across

all days within the 8-d collection period (i.e. to cover 2 oscillating cycles of 4 d/cycle) for N balance measurements, and allowed for the measurement of urea flux across isolated ruminal epithelia obtained from lambs consuming the low CP (i.e. OSC-LOW) and the high CP (i.e. OSC-HIGH) diets within the OSC cycle. The static (MEDIUM) CP concentration met the CP requirement of 25-kg lambs for BW gains of 300 g/d (NRC, 1985), whereas the low and high CP concentrations were below and above CP requirements (NRC, 1985), respectively. However, the total CP intake in lambs fed the OSC dietary CP regimens over a 4-d oscillating cycle was similar to that of lambs fed the MEDIUM diet. All experimental diets were offered in the form of a pellet for *ad libitum* intake. Lambs were fed twice daily at 0900 and 1700 h, and had free access to water. The ingredient and chemical compositions of the diets are presented in **Table 6.1**. The *in vivo* portion of the study consisted of 24 d for dietary adaptation and 8 d for data and sample collection. Lambs were weighed at the start of the dietary adaptation and at the end of the sample collection periods. During dietary adaptation, lambs were housed individually in pens (1.5 m^2) and were placed in metabolism crates for measurement of N balance.

6.3.2 Nitrogen Balance Measurements

Lambs were provided with 3 d of acclimation to the metabolism crates prior to conducting measurements. During the 8-d data and sample collection period (d 25 to d 32), individual lamb feed intake was recorded daily. Samples of the diets and orts were collected daily, stored at -20°C, and composited by lamb prior to chemical analysis. To measure fecal N excretion, lambs were fitted with a harness attached to a nylon bag positioned for total fecal collection. Bags were emptied daily immediately prior to the 0900 feeding and the total daily fecal output for each lamb was recorded. Subsequently, individual fecal samples were mixed thoroughly and a sub-sample accounting for 50% of the total daily fecal weight (wet basis) was collected daily and stored at -20°C. Urine was collected into sealed plastic containers placed below metabolism crates and was acidified with 50 mL of 12 M HCl to maintain the urine pH between 2 to 3. Acidification was used to prevent bacterial growth and the loss of NH_3-N. Total urine output was recorded daily.

Table 6.1. Ingredient and chemical composition of experimental diets

Item	LOW	MED	HIGH
Ingredients, % DM			
Barley, dry rolled	58.9	52.2	43.5
Alfalfa meal	3.5	3.5	3.5
Canola meal	0.3	0.5	1.0
Oat hulls	24.5	24.5	24.5
Soybean meal	0.3	8.0	15.0
Corn gluten meal	0.3	0.5	1.0
Dry distiller's corn grain	1.7	1.0	1.0
Canola oil	1.0	1.0	1.0
Liquid molasses	2.0	2.0	2.0
Ground limestone	4.0	4.0	4.0
Salt, Co-I[1]	0.7	0.7	0.7
Salt white	1.3	1.3	1.3
Mineral-vitamin supplement[2]	1.0	1.0	1.0
Chemical composition			
DM, %	92.4	92.7	91.5
OM, % of DM	89.8	90.0	89.8
CP, % of DM	10.3	12.7	16.1
RDP[3], % of CP	64.8	64.9	64.5
NDF, % of DM	34.6	31.7	34.2
ADF, % of DM	15.3	14.5	15.4

[1]Salt, cobalt and iodine mix: Co, 100 mg/kg; and I, 150 mg/kg.

[2]Provided (per kg of concentrate DM): CuO, 3.3 mg; ZnO, 22 mg; MnO, 18.5 mg; vitamin A, 1,495 IU; vitamin D, 125.5 IU; vitamin E, 22 IU; and sodium selenite, 0.1 mg.

[3]Calculated according to NRC (1985) guidelines.

Each day, a sample accounting for 20% of the total urine output was collected and composited by lamb. Composited samples were stored at -20°C for determination of total N. In addition, a 2-mL sub-sample of urine was diluted with 8 mL of distilled water and stored at -20°C for determination of urea-N and PD.

6.3.3 Ussing Chamber Measurements

On d 33, lambs were killed by captive bolt stunning followed by exsanguination at 4 h after the morning feeding. This time was chosen to coincide with the post-prandial peak in PUN concentration (Kiran and Mutsvangwa, unpublished data from Chapter 4; **Appendix Figure I**). Following an incision into the abdomen, the entire gastrointestinal tract was removed from the abdominal cavity within 3 to 4 min after killing and approximately 150 cm^2 of ruminal wall tissue was collected from the ventral sac. The ruminal tissue was washed until clean using a buffer solution containing (mmol/L) 1.0 $CaCl_2 \cdot 2H_2O$, 1.0 $MgCl_2 \cdot 6H_2O$, 2.0 $Na_2HPO_4 \cdot 2H_2O$, 1.0 $NaH_2PO_4 \cdot H_2O$, 1.0 phenyl-phosphorodiamidate (urease inhibitor; ABCR GmbH and Co., Karlsruhe, Germany), 5.0 butyric acid, 60.0 NaCl, 5.0 KCl, 10.0 glucose, 25.0 Na-acetate$\cdot 3H_2O$, 15.0 Na-gluconate, 10.0 Na-propionate, and 25.0 $NaHCO_3$. The buffer solution was maintained at 37ºC, pH 7.4, and saturated with carbogen (95% O_2 and 5% CO_2). The pH of the buffer solution was adjusted using 1 mol/L NaOH. The mucosa was gently separated from the underlying muscular layers before being transported (within 20 min of slaughter) to the laboratory in the buffer solution described above.

Simultaneous to the processing of ruminal epithelial tissue for Ussing chamber experiments, total ruminal contents were evacuated into a clean plastic container. Ruminal contents were mixed and a homogeneous sample was collected and strained through two layers of cheesecloth. The pH of the strained ruminal fluid was immediately determined using a portable pH meter (Model 265A, Orion Research Inc., Beverley, MA). Subsequently, a 10-mL aliquot of ruminal fluid was preserved with 2 mL of metaphosphoric acid (25% wt/vol) and a second 10-mL aliquot was preserved with 2 mL of 1% sulfuric acid. Ruminal fluid samples were stored at -20°C for subsequent analysis. A 200-ml sub-sample of ruminal fluid was stored at -20°C for later isolation of ruminal bacteria.

In the laboratory, pieces of stripped ruminal epithelia were mounted between two halves of an Ussing chamber with an exposed surface area of 1.43 cm^2. Silicon rubber rings were positioned on both sides of the epithelia to prevent edge damage. Epithelia were then bathed in isolated buffer solutions on the mucosal and serosal sides (10 mL/side). The composition of the mucosal and serosal buffer solutions were the same as the transport buffer, except that they contained antibiotics (60 mg/L of penicillin G sodium salt, 100 mg/L of kanamycin sulphate, and 50 mg/L of flurocytosine) that, when combined with phenyl-phosphorodiamidate, were designed to prevent any potential microbial urease activity. The pH of the serosal and mucosal buffer solutions were adjusted to 7.4 and 6.2, respectively, using 3 mol/L gluconic acid or 1 mol/L NaOH to mimic physiological conditions *in vivo*. This approach has been implemented in previous studies (Penner et al., 2009, 2010). Moreover, Abdoun et al. (2010) showed that maximal transepithelial flux of urea across the isolated epithelia occurred at a pH near 6.2 (in the presence of SCFA). Epithelia were incubated under short-circuit conditions using a computer-controlled voltage-clamp device (VCC MC6; Physiologic Instruments, San Diego, CA). Throughout the incubations, measurements of transepithelial conductance ($\mathbf{G_t}$) were collected every 20 seconds and mean G_t values were then calculated for every 5-min period. Bathing solutions were maintained at 38ºC using water-jacket reservoirs and were circulated using gas lift (95% O_2/5%CO_2).

For the measurement of the serosal-to-mucosal urea flux, a 20-min equilibration period was provided for stabilization of electrophysiology, after which a urea solution spiked with ^{14}C-urea (37 kBq/10 mL; Perkin-Elmer Corp., Waltham, MA) was added to the serosal side to achieve a final urea concentration of 1 mmol/L. Thus, a urea concentration gradient from the serosal to mucosal side was imposed to mimic physiological conditions *in vivo*. This was based on the lack of urea in ruminal fluid (Muscher et al., 2010) and because the serosal-to-mucosal transepithelial movement of urea is facilitated by a concentration gradient between the blood and the rumen (Vercoe, 1969; Sunny et al., 2007). After the addition of ^{14}C-urea, an additional 45-min isotope equilibration period was implemented. For each lamb, 4 mounted tissues were ranked based on mean G_t values observed during the 20-min electrophysiology equilibration period, and then assigned to one of 2 *in vitro* treatments (SHAM vs. PHLORETIN; n = 2) such that the treatments were balanced for mean G_t values. The serosal-to-mucosal urea flux ($\mathbf{J_{sm\text{-}urea}}$) across the ruminal epithelia was measured in two 30-min consecutive flux periods, with the

initial 30-min flux period being baseline (i.e., no *in vitro* treatments were applied). At the start of the second flux period, phloretin dissolved in ethanol was added (to achieve a final concentration of 1 mmol/L) to the serosal bathing solution for 2 epithelia (PHLORETIN tissues) to measure phloretin-insensitive $J_{sm\text{-}urea}$ flux. The same volume of ethanol was added to the serosal bathing solution for the SHAM tissues. All chemicals (reagent grade), antibiotics, and phloretin were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise indicated.

6.3.4 Sample Analyses

Frozen samples of the experimental diets, orts, and feces were thawed overnight at room temperature and analyzed for DM by drying in an oven at 60°C for 48 h (AOAC, 1995; method 930.15). The dried samples were then ground to pass through a 1-mm screen using a Christy-Norris mill (Christy and Norris Ltd., Chelmsford, England). Ground samples were pooled by lamb and analyzed for OM (AOAC, 1995; method 942.05), N using the macro-Kjeldahl procedure (AOAC, 1995; method 2001.11), and ADF and NDF (Van Soest et al., 1991). Amylase and sodium sulfite were used for neutral detergent fiber determination.

Preserved ruminal fluid samples were analyzed for VFA by GC as described by Erwin et al. (1961), and for NH_3-N using a phenol-hypochlorite assay (Broderick and Kang, 1980). Frozen ruminal fluid samples for bacterial isolation were thawed at room temperature and centrifuged at 20,000 x *g* at 4°C for 20 min, and the supernatant fraction was discarded. The microbial pellet was washed with double-distilled water and again centrifuged at 20,000 x *g* at 4°C for 20 min and this process was repeated two more times (Makkar and Becker, 1999). The microbial pellet was then freeze-dried, ground with a mortar and pestle, and analyzed for total N and individual purines as described by Makkar and Becker (1999). Daily urine samples (2 mL) that were diluted with distilled water (8 mL) were pooled by lamb proportionally to daily urine output and analyzed for allantoin, xanthine and hypoxanthine (Chen and Gomes, 1992), and for uric acid by a quantitative enzymatic colorimetric method using a commercial assay kit (Stanbio Uric Acid Liquicolor Kit, Procedure No. 1045; Stanbio Laboratories, Boerne, TX). Total PD excretion per day was calculated as allantoin + uric acid + xanthine plus hypoxanthine. The microbial NAN supply was calculated based on total PD excretion in urine (Chen and Gomes, 1992), using the determined purine N:microbial N ratios and BW measurements obtained on d

33. Urinary urea-N (UUN) was determined by the diacetyl monoxime method (Marsh et al., 1957) using a commercial kit (Stanbio Urea-N Kit, Procedure No. 0580; Stanbio Laboratories).

6.3.6 Calculations and Statistical Analysis

Data on DM, OM, and N intakes, N balance, ruminal fermentation characteristics, and microbial NAN supply were analyzed using the Proc Mixed procedure of SAS (2004) for a randomized complete block design with block and dietary treatment as fixed effects and lambs within treatment as a random effect. Single degree-of-freedom orthogonal contrasts were used to determine dietary treatment effects as follows: OSC-HIGH vs. OSC-LOW and MEDIUM vs. OSC (OSC-HIGH and OSC-LOW).

The phloretin-sensitive portion of $J_{sm\text{-}urea}$ flux was calculated as the difference between $J_{sm\text{-}urea}$ flux during flux periods 1 (i.e. total $J_{sm\text{-}urea}$ flux) and 2 (phloretin-insensitive $J_{sm\text{-}urea}$ flux) for tissues assigned to the PHLORETIN treatment *in vitro*. This method of calculation was used because there were: 1) no statistical differences ($P > 0.05$) in $J_{sm\text{-}urea}$ flux between the SHAM and PHLORETIN treatments during flux period 1 (**Appendix Figure IIA**); and 2) the $J_{sm\text{-}urea}$ flux did not differ between flux periods 1 and 2 for the SHAM treatment (**Appendix Figure IIB**). Thus, differences in the $J_{sm\text{-}urea}$ flux between flux periods 1 and 2 for tissues assigned to the PHLORETIN treatment were due to the phloretin-induced inhibition of urea transport. Thus, based on these observations, within-tissue total $J_{sm\text{-}urea}$ fluxes that are reported in Table 4 are measurements from flux period 1. Phloretin-insensitive $J_{sm\text{-}urea}$ fluxes are measurements from flux period 2. The $J_{sm\text{-}urea}$ flux data were analyzed as a split-plot design using Proc Mixed procedures of SAS (2004). The model included *in vivo* dietary treatment as the whole-plot factor and *in vitro* treatment (SHAM vs. PHLORETIN) as a sub-plot factor. Single degree-of-freedom contrasts were used to determine dietary treatment effects as follows: OSC-HIGH vs. OSC-LOW and MEDIUM vs. OSC (OSC-HIGH and OSC-LOW). Within *in vivo* treatment groups, the effect of the addition of phloretin on $J_{sm\text{-}urea}$ fluxes (i.e., total vs. phloretin-insensitive $J_{sm\text{-}urea}$ flux) was analyzed using the paired Student's *t*-test in SAS (2004). The relationships between G_t and $J_{sm\text{-}urea}$ flux, and between ruminal NH_3-N concentration and $J_{sm\text{-}urea}$ flux were tested using linear regression using the Proc Reg option in SAS (2004). Significance was declared when $P < 0.05$, and tendencies were considered when $0.05 \leq P < 0.10$.

6.4 Results

6.4.1 Dry Matter and Organic Matter Intake, Organic Matter Digestibility and N Balance

Dry matter intake tended to be higher (P = 0.06) in lambs fed the MEDIUM compared to those fed the OSC diets, but it was not different between lambs fed the OSC-LOW and OSC-HIGH diets (**Table 6.2**). Organic matter intake was greater (P = 0.05) for lambs fed the MEDIUM compared to those fed the OSC diet, reflecting the observed differences in DM intake. Despite increased OM intake for lambs fed the MEDIUM diet, total-tract OM digestibility was not affected by treatment. Nitrogen intake was similar between lambs fed the MEDIUM or OSC diets. However, N excreted in feces (P = 0.009) and urine (P = 0.02) was 16% and 13% lower in lambs fed the OSC compared to those fed the MEDIUM diet. Consequently, total N excretion, when expressed as absolute amounts (P = 0.006) or as a proportion of N intake (P = 0.004), was lower, and N retention, when expressed as absolute amounts (P = 0.02) or as a proportion of N intake (P = 0.004), was higher in lambs fed the OSC compared to those fed the MEDIUM diet. Lambs fed the OSC diet gained 64.2 g/d more (P = 0.02) BW compared to those fed the MEDIUM diet. As expected, BW gain and N balance were not different between lambs fed the OSC-LOW and OSC-HIGH diets.

6.4.2 Ruminal Characteristics

Ruminal pH did not differ (P = 0.26) between lambs fed the MEDIUM or OSC diets; however, ruminal pH was higher (P = 0.03) in lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet (**Table 6.3**). As expected, ruminal NH_3-N concentration was higher (P = 0.001) in lambs fed the OSC-HIGH compared to those fed the OSC-LOW diet. There was no difference in ruminal NH_3-N concentration between lambs fed the MEDIUM or OSC diets. Ruminal concentration of total VFA tended to be higher (P = 0.09), and that of propionate, butyrate, isobutyrate, and valerate were higher (P = 0.001), in lambs fed the OSC-HIGH compared to those fed the OSC-LOW diet. In contrast, the molar proportion of acetate was lower (P = 0.001) in lambs fed the OSC-HIGH compared to those fed the OSC-LOW diet. Ruminal concentrations of total VFA, acetate, and propionate were higher (P = 0.001), whereas that of valerate was lower (P = 0.02), in lambs fed the OSC compared to those fed the MEDIUM diet.

Table 6.2 Dry matter (DM), organic matter (OM) and nitrogen (N) intakes, total-tract OM digestibility, N balance, and mean daily body weight gain (ADG) in growing lambs fed medium or oscillating (OSC) dietary crude protein content[1]

Item	Treatments[2]			SEM	Contrast, P value	
	MEDIUM	OSC-HIGH	OSC-LOW		OSC-HIGH vs. OSC-LOW	MEDIUM vs. OSC
DM intake, g/d	1,512	1,434	1,366	68.5	0.29	0.06
OM intake, g/d	1,361	1,287	1,226	61.5	0.30	0.05
OM digestibility, %	67.1	69.4	68.4	1.1	0.45	0.12
N intake, g/d	30.7	30.5	28.9	1.4	0.26	0.39
Fecal N excretion						
g/d	7.2	6.1	6.0	0.32	0.91	0.009
% of total N excretion	30.8	29.4	31.6	1.4	0.15	0.79
Urine N excretion						
Total N, g/d	16.3	15.1	13.3	1.2	0.08	0.02
Total N, % of total N excretion	66.2	67.6	65.5	2.3	0.26	0.84
Urea-N, g/d	11.9	10.5	9.7	0.95	0.19	0.004
Total N excretion						
g/d	23.5	21.6	19.4	1.3	0.15	0.006
% of N intake	76.4	69.3	66.4	2.5	0.37	0.004
N retention						
g/d	7.2	9.3	9.6	0.82	0.79	0.02
% of N intake	23.6	30.7	33.4	2.5	0.37	0.004

Initial body weight, kg/d	24.8	26.2	25.7	1.7	0.69	0.34
Final body weight, kg/d	33.6	36.6	37.1	1.8	0.72	0.03
Total body weight gain, kg	8.8	10.3	11.4	0.8	0.22	0.02
ADG, g/d	276.1	322.9	357.7	24.4	0.22	0.02

[1] $n = 9$ for each treatment.

[2] Dietary treatments included medium CP (MEDIUM; 127 g CP /kg dry matter) or diets with OSC CP content fed in two different sequences i.e., 2 d of low CP (103 g CP /kg dry matter) followed by 2 d of high CP (161 g CP /kg dry matter; OSC-HIGH) or vice-versa (OSC-LOW).

Table 6.3 Ruminal characteristics at the time of slaughter in growing lambs fed medium or oscillating (OSC) dietary crude protein content[1]

Item	Treatments[2]			SEM	Contrast, *P* value	
	MEDIUM	OSC-HIGH	OSC-LOW		OSC-HIGH vs. OSC-LOW	MEDIUM vs. OSC
pH	5.16	5.12	5.59	0.14	0.03	0.26
NH_3-N, mg/dL	7.73	10.11	6.23	0.66	0.001	0.59
VFA concentration, mmol/L						
Total	94.73	98.04	96.66	0.69	0.09	0.001
Acetate	56.21	57.68	59.60	0.35	0.001	0.001
Propionate	17.77	18.92	18.06	0.18	0.001	0.001
Butyrate	16.91	17.47	15.92	0.32	0.001	0.49
Isobutyrate	0.84	0.96	0.69	0.04	0.001	0.65
Valerate	1.44	1.54	0.94	0.09	0.001	0.02
Isovalerate	1.49	1.41	1.39	0.09	0.86	0.29
Acetate to propionate ratio	3.16	3.05	3.30	0.05	0.001	0.80

[1] $n = 9$ for each treatment.

[2] Dietary treatments included medium CP (MEDIUM; 127 g CP /kg dry matter) or diets with OSC CP content fed in two different sequences i.e., 2 d of low CP (103 g CP /kg dry matter) followed by 2 d of high CP (161 g CP /kg dry matter; OSC-HIGH) or vice-versa (OSC-LOW).

6.4.3 Microbial NAN Supply

Urinary excretions of allantoin (P = 0.002), uric acid (P = 0.006), and total PD (P = 0.001) were higher in lambs fed the OSC compared to those fed the MEDIUM diet (**Table 6.4**). Consequently, microbial NAN supply (expressed as g N/d) estimated using urinary PD excretion and measured purine N:microbial N ratios, and microbial efficiency (expressed as g/kg of digestible organic matter in the rumen) were higher (P = 0.001) in lambs fed the OSC compared to those fed the MEDIUM diet.

6.4.4 Urea Flux Across the Ruminal Epithelium

After the 20-min electrophysiology equilibration period, G_t values were stable (**Appendix Figure III**), indicating that tissues were viable throughout the incubation as G_t is an indicator of tissue integrity (Clarke, 2009). The G_t was largely unaffected ($P \geq 0.13$) by diet; however, G_t tended to be higher (P = 0.09) in ruminal epithelia that was obtained from lambs fed the MEDIUM compared to those fed the OSC diet (**Table 6.5**). The total $J_{sm\text{-}urea}$ flux across ruminal epithelia was 72% greater ($P \leq 0.001$) in lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet. Across dietary treatments, the addition of phloretin on the serosal side resulted in a 19.5 to 22.3% decrease in $J_{sm\text{-}urea}$ fluxes, and this level of inhibition was significant (P = 0.001). Both the phloretin-insensitive (P = 0.001) and phloretin-sensitive (P = 0.04) $J_{sm\text{-}urea}$ fluxes were greater in ruminal epithelia that was obtained from lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet. There were no differences in total, phloretin-sensitive and phloretin-insensitive $J_{sm\text{-}urea}$ fluxes between ruminal epithelia that were obtained from lambs fed the MEDIUM or OSC diets. There was no significant correlation (r^2 = 0.001; P = 0.99) between G_t and $J_{sm\text{-}urea}$ flux (**Figure 6.1**), but there was a significant correlation (r^2 = 0.29; P = 0.04) between ruminal NH_3-N concentration and $J_{sm\text{-}urea}$ flux (**Figure 6.2**).

Table 6.4 Urinary purine derivative (PD) excretion and microbial non-ammonia nitrogen (NAN) supply in growing lambs fed medium or oscillating (OSC) dietary crude protein content[1]

	Treatments[2]				Contrast, *P* value	
Item	MEDIUM	OSC-HIGH	OSC-LOW	SEM	OSC-HIGH vs. OSC-LOW	MEDIUM vs. OSC
Urinary excretion						
Total output, kg/d	2.13	1.96	1.61	0.19	0.19	0.15
Allantoin excretion, mmol/d	7.15	7.79	8.10	0.22	0.22	0.002
Uric acid excretion, mmol/d	0.58	0.88	0.79	0.07	0.35	0.006
Xanthine plus Hypoxanthine, mmol/d	1.23	1.01	1.11	0.09	0.45	0.12
Total PD excretion, mmol/d	8.96	9.59	10.09	0.19	0.02	0.001
Purine bases[3], g/kg dry matter	77.7	77.2	81.3	2.1	0.26	0.42
N in microbial pellet, g/kg dry matter	65.1	65.9	66.6	1.2	0.63	0.50
Puirne N/Microbial N	0.089	0.088	0.092	0.006	0.19	0.11
Microbial NAN supply[4]						
Microbial N, g/d	9.91	10.82	10.94	0.22	0.56	0.001
Microbial N, g/kg DOMR	16.80	19.09	20.61	1.01	0.12	0.001

[1] $n = 9$ for each treatment.

[2] Dietary treatments included medium CP (MEDIUM; 127 g CP /kg dry matter) or diets with OSC CP content fed in two different sequences i.e., 2 d of low CP (103 g CP /kg dry matter) followed by 2 d of high CP (161 g CP /kg dry matter; OSC-HIGH) or vice-versa (OSC-LOW).

[3]Purine bases are expressed as g yeast RNA

[4]Microbial NAN supply was calculated according to Chen and Gomes (1992) using measured purine N:microbial N ratios; DOMR = digestible organic matter in rumen, which was calculated as 0.65 x DOMI (digestible OM intake) (Chen and Gomes, 1992). Digestible OM intake was calculated as OM intake x total-tract OM digestibility (data presented in Table 6.2).

Table 6.5 Serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) rates in ruminal epithelial tissues obtained from lambs slaughtered after feeding fed a medium CP diet (MEDIUM) or oscillating (OSC) dietary CP levels[1]

	Treatments[2]				Contrast, P value	
Item	MEDIUM	OSC-HIGH	OSC-LOW	SEM	OSC-HIGH vs. OSC-LOW	MEDIUM vs. OSC
Total $J_{sm\text{-}urea}$[3], nmol/cm^2/h	148.9	120.9	208.1	14.1	0.001	0.35
Phloretin-insensitive[3]						
$J_{sm\text{-}urea}$, nmol/cm^2/h	120.8	95.2	160.0	12.2	0.001	0.57
% of total	80.5	78.5	77.7	3.6	0.87	0.61
Pholoretin-sensitive[3]						
$J_{sm\text{-}urea}$, nmol/cm^2/h	28.1	25.6	48.1	7.4	0.04	0.35
% of total	19.5	21.5	22.3	3.6	0.87	0.61

[1]n = 9 for each treatment.

[2] Dietary treatments included medium CP (MEDIUM; 127 g CP /kg dry matter) or diets with OSC CP content fed in two different sequences i.e., 2 d of low CP (103 g CP /kg dry matter) followed by 2 d of high CP (161 g CP /kg dry matter; OSC-HIGH) or vice-versa (OSC-LOW).

[3]Within each *in vivo* dietary treatment, the addition of phloretin on the serosal side resulted in a significant (P = 0.001) inhibition of $J_{sm\text{-}urea}$ flux across the ruminal epithelia.

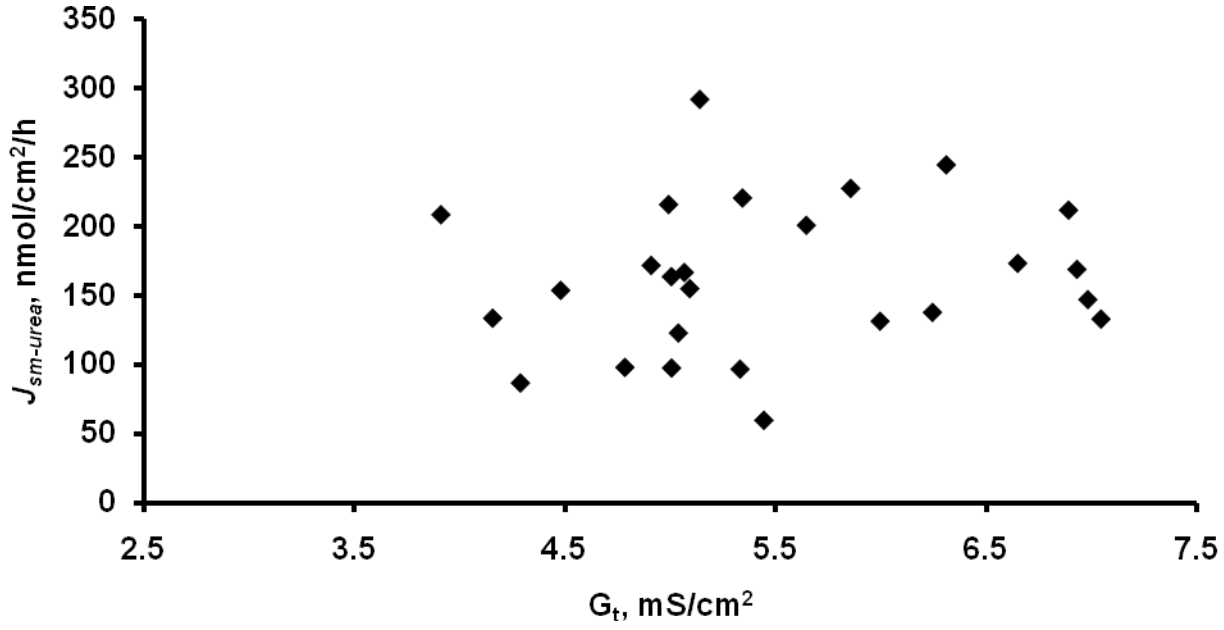

Figure 6.1 The relationship between serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) across the ruminal epithelia obtained from lambs (n = 27) with transepithelial conductance (G_t). There was no significant correlation (r^2 = 0.001, slope P = 0.99).

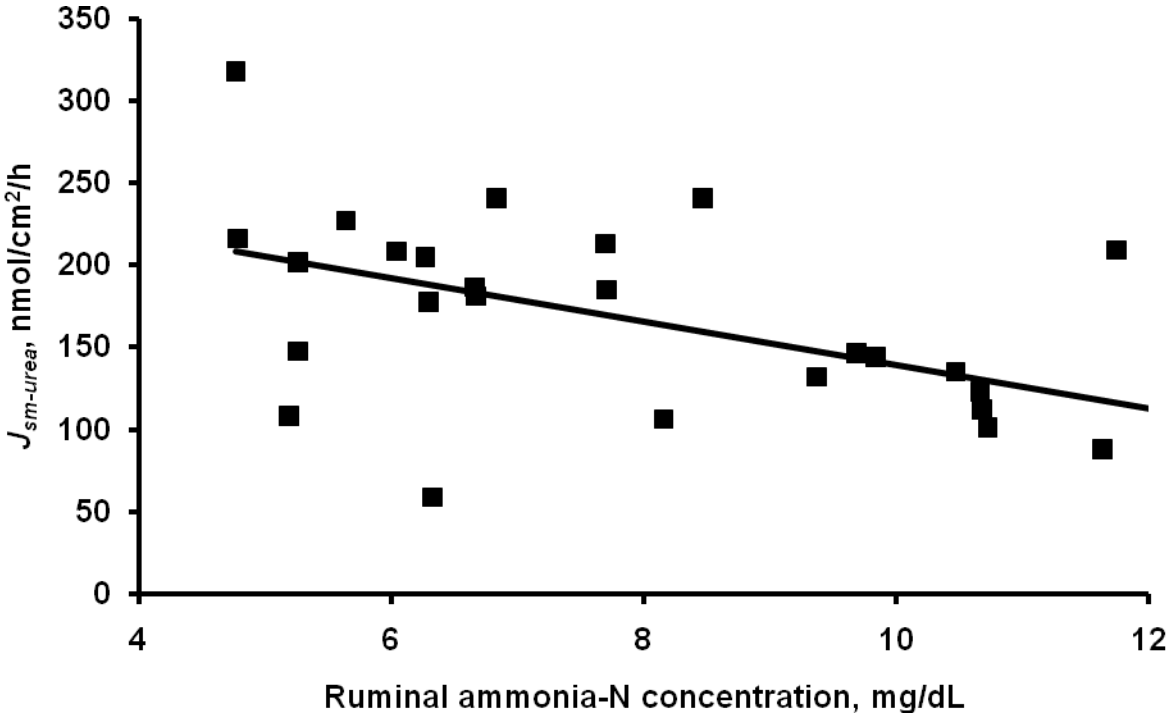

Figure 6.2 Relationship between serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) across the ruminal epithelia and ruminal ammonia-N concentration (r^2 = 0.29, slope $P < 0.001$) in lambs (n = 27) fed OSC-LOW, OSC-HIGH and MEDIUM dietary treatments.

6.5 Discussion

With growing concerns regarding the potential environmental impact arising from excessive N excretion from ruminants, there is a renewed interest to improve the efficiency of N utilization in ruminants. Perusal of the literature indicates that the major factor determining total N excretion in ruminants is total dietary N intake (Castillo et al., 2000; Yan et al., 2000). Performance studies have demonstrated that limiting dietary N intake of productive ruminants below requirements as a strategy to minimize N excretion can compromise BW gain and milk yield (Reynolds and Kristensen, 2008), so this has not been a viable strategy in commercial ruminant production systems in which edible product output needs to be optimized for profitability. As an alternative strategy, previous studies have investigated the impact of oscillating dietary CP concentrations (i.e., alternating between a low and high CP diet on a 2-d basis) on N utilization. In ruminants fed high concentrate diets, feeding oscillating dietary CP concentrations on a 2-d basis as compared to feeding static dietary CP at similar daily N intakes increased N retention in feedlot steers (Cole et al., 2003; Ludden et al., 2003) and sheep (Cole, 1999; Kiran and Mutsvangwa, 2009).

In the present study, even though N intakes were similar, N losses in feces and urine were lower and, consequently, N retention was higher when dietary CP concentration was oscillated between 103 and 161 g CP/kg of DM on a 2-d basis, compared to feeding a static (127 g CP/kg of DM) dietary CP regimen. In addition, when expressed as a percentage of N intake, lambs fed the OSC diet had a higher N retention compared to those fed the MEDIUM diet, indicating a more efficient utilization of dietary N. However, it should be noted that N retention was calculated as N intake-(fecal N + urine N), rather than being determined from slaughter studies. In such N balance studies, unaccounted N losses during sample collection and processing (e.g., volatilization of fecal or urinary N) often result in the overestimation of N retention (Spanghero and Kowalski, 1997). Assuming that N retention in sheep is 29 g/kg of BW gain (Kohn et al., 2005), the N retention values of 7.2, 9.3, and 9.6 g/d that were observed in the present study for the MEDIUM, OSC-HIGH and OSC-LOW lambs, respectively, would correspond to BW gains of 248, 320, and 331 g/d. These predicted BW gains compare well with the observed BW gains (276.1, 322.9, and 357.7 g/d), suggesting that any errors in N balance determinations were minor. Consistent with observed differences in N retention, BW gain was higher in lambs fed the

OSC compared to those fed the MEDIUM diet, supporting previous research (Cole et al., 2003). In addition to reduced N excretion, the form of N excreted was also affected. Specifically, UUN excretion was higher in lambs fed the MEDIUM compared to those fed the OSC diet. From an environmental standpoint, urinary urea is the most damaging as 50 to 90% of the excreted N is rapidly volatilized into the atmosphere (Cole, 1999). The reduction in urinary urea excretion could improve on-farm nutrient management and environmental stewardship by reducing NH_3 emissions into the environment (Erickson and Klopfenstein, 2001; Cole et al., 2005). In agreement with previous work (Cole et al., 2003), ADG was higher in lambs fed the OSC compared to those fed the MEDIUM diet, indicating that feeding oscillating dietary CP concentrations also improves animal growth in addition to the environmental benefits.

Although positive influences of feeding oscillating dietary CP concentrations on animal performance and N utilization have been reported, the underlying mechanisms have received little attention. Cole et al. (1999) was the first one to propose that, *inter alia*, the improved N retention observed in ruminants when dietary CP is oscillated could be due to increased urea recycling to the rumen. A greater transepithelial transfer of urea from blood into the rumen during periods of the oscillating cycle when animals are receiving the low CP concentration that is deficient in N relative to ruminal microbial requirements would provide additional N that can be used for microbial growth. Although we did not measure the incorporation of recycled urea into microbial protein, microbial NAN supply was greater in lambs fed the OSC compared to those fed the MEDIUM diet. Archibeque et al. (2007) reported a numerically greater absorption of α-amino acids into portal blood in sheep fed oscillating dietary CP concentrations compared to those fed static dietary CP, thereby suggesting a higher α-amino acid flow to the small intestine with oscillating dietary CP. To calculate bacterial NAN supply, we used purine N:bacterial N ratios that were determined in fluid-associated bacteria (FAB); however, the purine N:bacterial N ratio in FAB has been reported to be higher than that in particle-associated bacteria (PAB), and the fractional contributions of FAB and PAB to total purine bases arriving at the duodenum can also differ (Broderick and Merchen, 1992). Because we did not account for these differences, it is possible that bacterial NAN supply was underestimated. However, it should be noted that highly-fermentable fiber was fed in this study, so the contribution of PAB to total bacterial biomass was likely small. Besides the availability of ruminally-fermentable energy, it is well-established that ruminal NH_3 concentration is also a key driver of microbial protein synthesis. In

vitro studies conducted by Satter and Slyter (1974) indicated that the optimal ruminal NH_3 concentration for maximum microbial protein synthesis was 5.0 mg/dL. In the present study, ruminal NH_3 concentrations were >5.0 mg/dL for all diets. However, it should be noted that ruminal NH_3 concentrations were measured at 4 h after feeding when post-prandial ruminal NH_3-N concentrations were likely to be at their peak (Kiran and Mutsvangwa, unpublished data from Chapter 4; **Appendix Figure IV**) and it is possible that ruminal NH_3 concentrations were below optimal at other times of the day. It should also be noted that conflicting results have been reported regarding the optimal ruminal NH_3-N concentration for maximum microbial protein synthesis (Firkins et al., 2006). More recent in vivo studies have provided evidence that the optimal ruminal NH_3 concentrations might be in the range of 11.0 to 11.8 mg/dL (Balcells et al., 1993; Reynal and Broderick, 2005), suggesting that ruminal NH_3 concentrations that were observed in the present study might have limited microbial growth, particularly in lambs fed the OSC-LOW diet. Also, Cole (1999) suggested that any potential benefits on N retention of feeding oscillating dietary CP concentrations can only be realized if there is synchrony between changes in dietary CP and retention time of digesta in the gut. In sheep fed chopped grass hay-crushed barley, the mean retention time of digesta in the gut was 47 h (Lindberg, 1985), suggesting that changes in dietary CP concentrations with the 48-h oscillating CP regimen in the present study may have been synchronized with retention time of digesta in the gut.

To date, we are aware of only one study (Archibeque et al., 2007) that has investigated the quantitative transfer of urea into the rumen in ruminants fed high concentrate diets with oscillating dietary CP concentrations. In that study using growing wethers, measurements of PDV fluxes of nitrogenous compounds using the venous-arterial technique indicated that, at similar N intakes, PDV uptake of urea only tended to be greater with the oscillating treatment (9.9 and 14.2 g CP/kg of DM on a 2-d basis) when compared to a static (12.5 g CP/kg of DM) CP concentration. Thus, it was not possible to definitively attribute the improved N retention with oscillating CP diets to greater rates of urea transfer to the gut. In the present study, the unidirectional (i.e., serosal-to-mucosal; $J_{sm\text{-}urea}$) urea flux across isolated ruminal epithelia mounted in Ussing chambers that were obtained from lambs fed static or oscillating dietary CP concentrations was quantified. To mimic physiological conditions it was decided to measure $J_{sm\text{-}urea}$ as urea is usually not detectable in ruminal fluid (Muscher et al., 2010). These results show that $J_{sm\text{-}urea}$ was markedly greater in lambs that were killed after 2 d of receiving the OSC-LOW

diet in a 4-d oscillating cycle compared to those lambs that were killed after receiving the OSC-HIGH or MEDIUM diets. These findings provide definitive evidence that, when oscillating CP concentrations are fed, a greater N utilization can partly be attributed to enhanced urea recycling to the rumen when the low CP diet is fed within the OSC cycle. Besides the increase in urea recycling, changes in whole-body protein metabolism when oscillating CP concentrations are fed could also be partly responsible for improving the efficiency of N use. In sheep maintained by intragastric nutrient infusions, marked decreases (-30 to -21%) in urinary N excretion, total body protein flux, and both protein synthesis and degradation were observed within 1 to 2 d after animals were switched from a maintenance to a low protein intake (Liu et al., 1995). With such acute responses in body protein turnover due to changes in protein intake, it is feasible that feeding oscillating dietary CP concentrations on a 2-d basis could have altered whole-body protein metabolism.

It is well-established that urea can be transferred to both the fore-stomach (rumen) and post-stomach compartments of the digestive tract (Lapierre and Lobley, 2001), but only urea recycled into the rumen can potentially contribute amino acids to the host animal. Urea recycling to the rumen can occur via saliva or direct transfer from the blood across ruminal wall (Lapierre and Lobley, 2001). Salivary secretion increases with the physically-effective fibre content of the diet, and it is a major factor that would dictate how much urea is transferred via the salivary route. Although urea transfer via saliva was not measured in the present study, it is likely that the relative contribution of this route to total urea transfer into the rumen was small as the diets used were fed as a pellet with low physically-effective fibre. In all likelihood, most of the urea recycling was by direct transfer from blood across the ruminal epithelia, especially when dietary N intake was low (Harmeyer and Martens, 1980).

Until recently, passage of urea from blood across the ruminal wall was believed to occur largely by simple passive diffusion, with the rumen-blood urea concentration gradient facilitating urea transfer into the rumen. Ritzhaupt et al. (1997; 1998) demonstrated the presence of carrier-mediated, facilitative urea transporter-B (UT-B) proteins in ovine ruminal epithelium. That UT-B might have a role in trans-epithelial urea flux is suggested by observations in ruminal epithelia that UT-B protein abundance was upregulated when lambs were fed high N diets (Marini and Van Amburgh, 2003) and that UT-B mRNA abundance was upregulated in concentrate-

compared to forage-fed steers (Simmons et al., 2009). However, other studies have failed to detect changes in UT-B mRNA abundance when dietary CP level is manipulated (Marini et al., 2004; Muscher et al., 2010; Ludden et al., 2009), so the functional role of UT-B in trans-epithelial urea transfer remains rather obscure. In the present study, it was sought to delineate the functional role of UT-B in the movement of urea across ruminal epithelia isolated from lambs fed the OSC or MEDIUM CP diets by conducting measurements of total and phloretin-insensitive urea flux. Phloretin is a known specific inhibitor of facilitative UT-B and it has been reported to reduce the flux of urea in bovine (Stewart et al., 2005), ovine (Abdoun et al., 2010) and caprine (Muscher et al., 2010) ruminal epithelia. The current study revealed that both phloretin-insensitive and phloretin-sensitive portions of $J_{sm\text{-}urea}$ were markedly greater in ruminal epithelia isolated from lambs fed the OSC-LOW compared to those fed the OSC-HIGH diet. When expressed as a proportion of total $J_{sm\text{-}urea}$, the phloretin-sensitive portion only accounted for 18.8 to 23.1%. This suggests that although UT-B carrier-mediated urea transport was functional in epithelia from all lambs, the greater $J_{sm\text{-}urea}$ flux in lambs fed the OSC-LOW cannot be directly attributed to the upregulation of a trans-cellular pathway for urea transport involving UT-B transporters.

While numerous studies have suggested that urea transport occurs via passive diffusion in addition to UT-B-mediated transport, recent studies (Muscher et al., 2010; Abdoun et al., 2010) have reported no correlation between tissue G_t and urea flux. Past studies have examined this relationship with equimolar concentrations of urea on the mucosal and serosal sides in Ussing chambers (Abdoun et al., 2010). In the current study, a serosal-to-mucosal driven concentration gradient was imposed to mimic physiological conditions observed *in vivo*. These results support previous studies showing that changes in G_t could not account for a significant portion of the variation observed for $J_{sm\text{-}urea}$ fluxes. Thus, it appears that other potential phloretin-insensitive transporters may be involved in $J_{sm\text{-}urea}$ fluxes across the ruminal epithelium (Muscher et al., 2010). Possible candidates include aquaporins (Borgnia et al., 1999).

It is well documented that ruminal NH_3-N is negatively correlated with urea transfer to the rumen (Kennedy and Milligan, 1980). In the present study, $J_{sm\text{-}urea}$ flux was negatively correlated with ruminal NH_3-N concentration. These inhibitory effects of a high ruminal NH_3-N concentration on trans-epithelial urea flux could arise partly due to a decrease in ruminal urease

activity (Cheng and Wallace, 1979). Because bacterial urease activity facilitates the trans-epithelial movement of urea by maintaining a concentration gradient that is favorable to diffusion (Rémond et al.,. 1996), depressed bacterial urease activity might impair urea transfer. Bacterial urease activity was not measured in the present study, but Muscher et al. (2010) reported a negative correlation between ruminal urease activity and *in vitro* urea flux across ruminal epithelia. It is worth noting, though, that the incubation buffer solutions that were used in the present study contained antibiotics and an urease inhibitor that would have inhibited any bacterial urease activity. As such, it is not likely that changes in urease activity *in vitro* would have influenced trans-epithelial urea flux. It is plausible that physiological adaptations for $J_{sm\text{-}urea}$ were partially mediated via changes in ruminal NH_3-N concentration *in vivo*. In our study, the lower ruminal NH_3-N concentration that was observed in lambs fed the OSC-LOW could have stimulated a greater $J_{sm\text{-}urea}$ flux when compared to lambs fed the OSC-HIGH diet. Changes in ruminal pH could also be partly responsible for the observed differences in $J_{sm\text{-}urea}$ flux between OSC-LOW and OSC-HIGH lambs. When mucosal pH was varied between 7.4 and 5.4 in the presence of short-chain fatty acids (as in the present study), *in vitro* $J_{sm\text{-}urea}$ flux was maximal at a pH of 6.2 and there was a steep decrease in $J_{sm\text{-}urea}$ flux as pH was reduced to 5.4 (Abdoun et al., 2010). Ruminal pH in lambs fed the OSC-LOW diet was 5.59, whereas it was only 5.12 in lambs fed the OSC-HIGH diet, so the more favorable ruminal pH in lambs fed the OSC-LOW diet could have had stimulatory effects on trans-epithelial urea flux.

6.6 Conclusions

Compared to feeding a static dietary CP concentration on a daily basis, feeding oscillating dietary CP concentrations on a 48-h basis improves N retention in ruminants even when N intakes are similar. Of particular importance, our results provide new insights that the improvement in N retention with oscillating dietary CP concentrations is partly mediated by increased urea transport into the rumen during periods when animals are consuming the low CP diet. However, the phloretin-sensitive $J_{sm\text{-}urea}$ flux accounted for only a small proportion of total $J_{sm\text{-}urea}$ flux in lambs fed oscillating dietary CP concentrations, so it is not clear if UT-B plays a role in mediating the greater trans-epithelial flux of urea into the rumen when dietary CP concentration is oscillated in the short-term. Future studies should seek to discover other transporters that might be involved in urea transport across the ruminal epithelium.

7. GENERAL DISCUSSION

The physiological process of urea-N recycling to the GIT in ruminants plays a vital role in maintaining an adequate N supply for rumen functions under most dietary conditions (Lapierre and Lobley, 2001), therefore, understanding dietary and ruminal factors which regulate urea-N recycling and capture of recycled urea-N within the GIT for anabolic is important. Four experiments presented in this thesis were conducted with an overall objective of delineating how dietary factors and associated ruminal factors regulate urea-N recycling in ruminants. Of particular importance, interactions between dietary protein degraded in the rumen and ruminally-fermentable carbohydrate in high N diets (Chapter 3), defaunation of the rumen (to decrease ruminal NH_3-N levels) and dietary N levels (Chapter 4) and, dietary ruminally-fermentable carbohydrate (Chapter 5), as well as the effects of oscillating high and low dietary N levels (Chapter 6) were studied.

Numerous studies in the literature have demonstrated that urea-N recycling to the GIT is important in supplying N for sustaining microbial growth and thus keep ruminants in positive N balance when low N diets are fed. However, to meet the higher production demands, dairy and beef cattle by and large require high N in the diet. Therefore, my first question when I started this project was "can we manipulate urea-N recycled to the GIT and the utilization of this recycled urea-N for anabolic use by dietary alterations in ruminants fed high N diets"?. Barley grain processing to shift starch digestion from small intestine to the rumen or changing dietary RDP level did not alter urea-N kinetics in lambs fed high N diets (Chapter 3). However, across dietary treatments, substantial amounts (66.9 to 74.2%) of hepatic urea-N output were transferred to the GIT, but most of this N (63.6 to 75.6%) was returned to the ornithine cycle without being utilized for microbial use. Across dietary treatments, even though dietary N intakes were higher, however, small proportion of recycled urea-N was still being utilized for anabolic purposes thus illustrating the importance of urea-N recycling even when ruminants are fed high N to maintain them in positive N balance.

Previous studies (Theurer et al., 1999, 2002; Alio et al., 2000; Delgado-Elorduy et al., 2002) have shown that feeding steam flaked sorghum and corn grain to increase starch degradability in the rumen was associated with a greater transfer of urea-N across PDV and higher microbial N supply to the duodenum. Across western Canada, barley grain is the major

source of cereal grain fed to beef and dairy cattle. Barley starch is more rapidly fermented in the rumen compared to corn and sorghum. Because of higher starch digestibility, question was does barley grain processing (i.e., pelleting) further improves RFC compared to dry-rolling and can we increase urea-N recycling to the rumen and improve utilization recycled urea-N for microbial protein synthesis by feeding PB as compared to DRB.

Surprisingly, increasing the RFC by feeding PB as compared to DRB did not have any positive impact on the major parameters of urea-N recycling (Chapters 3 and 5), contradicting previous research with sorghum (Theurer et al., 2002) and corn (Delgado-Elorduy et al., 2002). The lack of effect of grain processing could be attributable to high concentrate diet, where in energy supply might not have limited the utilization of the extra N provided via enhanced urea-N recycling to the GIT. Another possible reason could be the lower ruminal pH that was observed in lambs fed PB as compared those fed DRB. A recent study (Abdoun et al., 2010) reported that ruminal pH plays an important role in regulating urea-N transfer across the ruminal wall with maximal urea flux observed at a mucosal pH of around 6.0 to 6.4 with a steep decrease in J_{sm} urea flux when pH of the mucosal buffer dropped below 6.0. Therefore, a more acidic ruminal environment in lambs fed PB compared to DRB could have impaired urea-N transfer across the ruminal epithelium possibly due to decreased urease activity (Reynolds and Kristensen, 2008). Ruminal pH in lambs fed PB and DRB were 5.8 and 6.0 (Chapter 5), respectively; thus, this lower ruminal pH could be the reason why positive effects of barley grain processing on urea-N kinetics were not observed (Chapters 3 and 5).

Two experiments presented in this thesis (Chapters 4 and 5) describe the impact of defaunation on urea-N recycling. Before conducting these experiments, the mechanism put forward as being responsible for the improved N metabolism in ruminants were: 1) decreased intra-ruminal N recycling (Koenig et al., 2000), 2) greater sequestration of NH_3-N into bacterial protein, and 3) lower ruminal NH_3-N concentrations (Firkins et al., 2007). This thesis provides new evidence that improved N utilization in ruminants can be partly attributable to increased urea-N recycling to the GIT and greater utilization of this recycled urea-N for anabolic purposes. This might be related to lower ruminal NH_3-N concentration that is consistently observed in these studies, as ruminal NH_3-N concentration is negatively correlated with urea-N transfer into the rumen (Kennedy and Milligan, 1980). Sunflower oil was fed (6%; DM basis) as an

antiprotozoal agent in both studies. Commonly used methods to eliminate ruminal protozoa involve the use of chemicals (e.g., copper sulfate and calcium peroxide), but a major disadvantage of using chemicals is that they are not only toxic to ruminal protozoa (Jouany, 1996), but also to animals and also alter other aspects of the ruminal ecosystem (Veira et al., 1983). Hence, SFO was used as a defaunating agent because it is safer and can be applied under practical feeding conditions (Ivan et al., 2001). Although complete defaunation was not achieved, substantial reductions in ruminal protozoal populations were achieved, coupled with positive responses in N utilization. However, the major issue with the design of both studies was that no additional fat source was fed as part of control diets to balance energy intakes. Therefore, differences in both energy source and total energy intake may have influenced some of the responses. Because DM intake was higher in FAUN compared to PDFAUN lambs, additional energy supplied through SFO to PDFAUN lambs was compensated by additional DM intake in FAUN lambs and, hence, total energy intake was unaffected by protozoal status (Chapter 4). However, the energy sources in the diets were different and may have influenced the post-absorptive N use. Conversely, total energy intakes differed in the subsequent study (Chapter 5) and additional energy intake in PDFAUN lambs due to feeding SFO could have influenced post-absorptive N use. Therefore, the ideal experimental design would be to feed SFO to partially-defaunate animals and concurrently feed a mixture of saturated fatty acids to control animals to equalize energy intakes. Feeding oil (i.e., extracted from seeds) in ruminants will increase the feed cost and, hence, alternative feeding strategies to eliminate ruminal protozoa have to be investigated. Ivan et al. (2003, 2004) incorporated crushed sunflower seed in sheep diets such that the SFO content of the diet was 6% of dietary DM and reported a 79% decrease in ruminal protozoa counts as compared to the control diet. Another possible means of defaunating the rumen is by separating newborn ruminants within few hours after birth, but the practical application of this approach is doubtful. By quantifying urea-N kinetics with such approaches to defaunate the rumen, definitive conclusions on the effects of defaunation on urea-N recycling can be made.

Stewart et al. (2005) demonstrated presence of UT-B in the bovine ruminal epithelium and may play role in carrier-mediated facilitated urea diffusion into the rumen. Further, Simmons et al. (2009) demonstrated that steers fed high concentrate diet expressed higher UT-B mRNA, and UT proteins in the ruminal epithelium compared to those fed high silage diets; however,

other studies have shown conflicting results on whether these transporters are regulated by dietary alterations (Marini and Van Amburgh, 2003; Marini et al., 2004; Ludden et al., 2008; Muscher et al., 2010). In addition to quantifying urea-N, mRNA expression of UT-B was quantified (Chapters 3 and 4). Surprisingly, even though, urea-N recycling increased by partial defaunation, the mRNA UT-B expression was not altered. As urea diffuses into the rumen both by simple and facilitated carrier-mediated diffusion, does UT-B play a functional role in urea transfer into the rumen? Results from my last study (Chapter 6) shows that urea entry into the rumen is largely through by simple diffusion rather than through facilitated carrier-mediated diffusion. Therefore, future research has to be directed towards delineating the effect of diet and ruminal metabolites on functional significance of UT expression. In addition, research efforts have to be directed towards investigating other possible urea-transporting molecules other than UT-B and UT-A.

This thesis also provides definitive evidence that increased urea-N recycling to the rumen is the probable mechanism for the improved N retention in ruminants fed oscillating dietary N levels (Chapter 6). Initially, Cole (1999) speculated that the increase in N retention in ruminants fed oscillating dietary CP could be attributed to increased N recycling to the GIT. Archibeque et al. (2007) observed only a tendency for a greater net flux of urea-N to the GIT in sheep fed oscillating dietary CP concentrations; however, overall results from that study did not provide definitive evidence that improved N utilization was associated with increased urea-N recycling to the rumen. Results from my last study (Chapter 6) provides definitive evidence that, compared to feeding the same amount of dietary protein on a daily basis, feeding oscillating dietary CP concentrations on a 2-d basis improves N retention in lambs, and this response is mediated at least in part by increased urea-N recycling to the rumen and improved microbial NAN supply to the duodenum.

8. OVERALL CONCLUSIONS

Results presented in this thesis provide some interesting findings on factors regulating urea-N recycling in ruminants. This research provides new evidence that the improved efficiency of N utilization in partially-defaunated ruminants could be partly mediated by an increase in urea-N recycling. In addition, improvement in the N retention with oscillating dietary crude protein concentrations is probably attributable to an increase in the urea-N transfer into the rumen. As urea-N recycling plays a critical role in salvaging N for productive purposes, additional research is required to elucidate factors regulating the mechanism of urea-N recycling in ruminants.

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10. APPENDICES

10.1 Appendix Tables

Appendix Table I. Ruminal protozoal concentration (*x* 10^5*/ml*) of *Entodinium spp*., Holotrics, Cellulolytics (*Polyplastron*, *Diplodinium* and *Enoplastron sp*.) and total in FAUN and PDFAUN lambs fed diet LOW

	FAUN				PDFAUN			
Day	Entodinium *spp.*	Holotrics	Cellulolytics	Total	Entodinium *spp.*	Holotrics	Cellulolytics	Total
1	6.163	0.150	0.238	6.550	10.29	0.18	0.20	10.67
2	5.713	0.256	0.206	6.175	9.97	0.29	0.20	10.46
3	6.225	0.250	0.206	6.681	9.02	0.22	0.12	9.36
6	7.188	0.325	0.175	7.688	5.78	0.06	0.09	5.93
9	10.031	0.431	0.313	10.775	3.11	0.00	0.08	3.19
12	9.844	0.313	0.119	10.275	1.70	0.00	0.07	1.77
15	12.963	0.506	0.181	13.650	1.18	0.00	0.08	1.25
18	12.050	0.456	0.206	12.713	0.76	0.00	0.08	0.83
21	12.350	0.413	0.294	13.056	0.19	0.00	0.07	0.26
22	11.156	0.375	0.313	11.844	0.00	0.00	0.09	0.09
23	11.375	0.400	0.306	12.081	0.00	0.00	0.09	0.09
24	11.150	0.406	0.300	11.856	0.00	0.00	0.08	0.08
25	11.063	0.375	0.313	11.750	0.00	0.00	0.09	0.09
26	11.300	0.369	0.256	11.925	0.00	0.00	0.09	0.09

Appendix Table II. Ruminal protozoal concentration (*x* 10^5*/ml*) of *Entodinium spp*., Holotrics, Cellulolytics (*Polyplastron*, *Diplodinium* and *Enoplastron sp*.) and total in FAUN and PDFAUN lambs fed diet HIGH

	FAUN				PDFAUN			
Day	Entodinium *spp.*	Holotrics	Cellulolytics	Total	Entodinium *spp.*	Holotrics	Cellulolytics	Total
1	5.038	0.125	0.094	5.256	8.544	0.269	0.355	9.168
2	5.063	0.188	0.150	5.400	8.219	0.263	0.292	8.774
3	6.231	0.131	0.163	6.525	8.331	0.281	0.223	8.835
6	7.213	0.256	0.275	7.744	5.775	0.150	0.214	6.139
9	9.569	0.225	0.231	10.025	4.175	0.038	0.149	4.361
12	12.388	0.275	0.425	13.088	3.813	0.000	0.132	3.944
15	15.700	0.431	0.669	16.800	3.694	0.000	0.134	3.828
18	16.725	0.338	0.600	17.663	3.844	0.000	0.152	3.996
21	16.669	0.388	0.563	17.619	3.756	0.000	0.071	3.827
22	16.163	0.363	0.788	17.313	3.925	0.000	0.104	4.029
23	16.206	0.400	0.756	17.363	3.819	0.000	0.126	3.944
24	16.206	0.369	0.750	17.325	3.806	0.000	0.137	3.943
25	16.256	0.394	0.700	17.350	3.725	0.000	0.078	3.803
26	15.975	0.363	0.725	17.063	3.938	0.000	0.132	4.069

Appendix Table III. Mean (n = 4) ruminal protozoal concentration (*x* 10^5*/ml*) of *Entodinium spp*., Holotrics, Cellulolytics (*Polyplastron*, *Diplodinium* and *Enoplastron sp*.) and total for re-faunated lambs starting from period 2 to period 4.

Day	Entodinium *spp.*	Holotrics	Cellulolytics	Total
1	0.298	0.000	0.013	0.310
2	1.738	0.050	0.025	1.813
3	4.381	0.156	0.050	4.588
6	6.844	0.294	0.073	7.210
9	9.513	0.350	0.163	10.025
12	11.800	0.325	0.269	12.394
15	15.313	0.438	0.419	16.169
18	15.944	0.481	0.469	16.894
21	16.288	0.463	0.475	17.225
22	14.650	0.406	0.544	15.600
23	14.506	0.413	0.544	15.463
24	14.281	0.394	0.544	15.219
25	14.731	0.388	0.531	15.650
26	14.531	0.394	0.525	15.450

Appendix Table IV. Urea-N recycling kinetics corrected using N intake as a covariate in partially-defaunated (PDFAUN) or faunated (FAUN) growing lambs fed LOW or HIGH dietary crude protein levels[1]

Item	LOW		HIGH			*P* value[2]		
	FAUN	PDFAUN	FAUN	PDFAUN	SEM	Protozoa	Protein	Protozoa x Protein
Urea-N kinetics, g/d								
Production, UER	27.63	23.88	37.98	31.85	1.53	0.008	0.001	0.38
Entry to GIT[2], GER	10.75	8.25	19.27	13.18	0.85	0.001	0.001	0.03
Return to ornithine cycle, ROC	7.61	6.79	11.40	10.40	0.99	0.38	0.02	0.92
Loss to feces, UFE	1.58	1.90	0.66	0.85	0.25	0.33	0.01	0.76
Loss to urine, UUE	10.75	8.25	19.27	13.18	0.85	0.001	0.001	0.03
Re-use for anabolism, UUA	7.69	6.90	6.65	7.72	0.59	0.98	0.74	0.15
Fractional urea-N transfers								
UER to urine	0.387	0.329	0.503	0.418	0.020	0.005	0.003	0.45
UER to GIT[2]	0.613	0.671	0.497	0.582	0.020	0.005	0.003	0.45
GER to ROC	0.447	0.401	0.594	0.553	0.032	0.20	0.005	0.95
GER to feces	0.094	0.122	0.045	0.031	0.015	0.20	0.005	0.60
GER to UUA	0.459	0.476	0.375	0.403	0.036	0.54	0.12	0.87

[1]LOW = 10% CP, HIGH = 15% CP (DM basis).

[2]Protozoa = PDFAUN vs. FAUN lambs; Protein = level of dietary CP (LOW vs. HIGH); Protozoa x Protein = interaction. PDFAUN was achieved by addition of 6% (DM basis) linoleic acid-rich sunflower oil to the diet.

Appendix Table V. Ruminal protozoal concentration (*x* 10^5*/ml*) of *Entodinium spp*., Holotrics, Cellulolytics (*Polyplastron*, *Diplodinium* and *Enoplastron sp*.) and total in FAUN and PDFAUN lambs fed pelleted barley.

	FAUN				PDFAUN			
Day	Entodinium *spp.*	Holotrics	Cellulolytics	Total	Entodinium *spp.*	Holotrics	Cellulolytics	Total
1	5.61	0.16	0.09	5.86	7.41	0.13	0.23	7.77
2	6.61	0.23	0.18	7.01	6.94	0.14	0.21	7.29
3	6.86	0.23	0.17	7.27	6.26	0.09	0.12	6.47
6	7.34	0.22	0.16	7.72	4.41	0.05	0.10	4.56
9	8.56	0.20	0.14	8.91	3.02	0.07	0.06	3.14
12	9.23	0.26	0.16	9.65	1.71	0.02	0.02	1.74
15	9.44	0.25	0.15	9.84	1.28	0.00	0.01	1.28
18	8.44	0.22	0.14	8.79	0.50	0.00	0.00	0.50
21	7.24	0.21	0.11	7.57	0.21	0.00	0.00	0.21
22	6.63	0.17	0.10	6.90	0.00	0.00	0.00	0.00
23	5.83	0.16	0.08	6.07	0.00	0.00	0.00	0.00
24	6.07	0.15	0.08	6.31	0.00	0.00	0.00	0.00
25	5.97	0.17	0.08	6.22	0.00	0.00	0.00	0.00
26	6.09	0.15	0.09	6.32	0.00	0.00	0.00	0.00

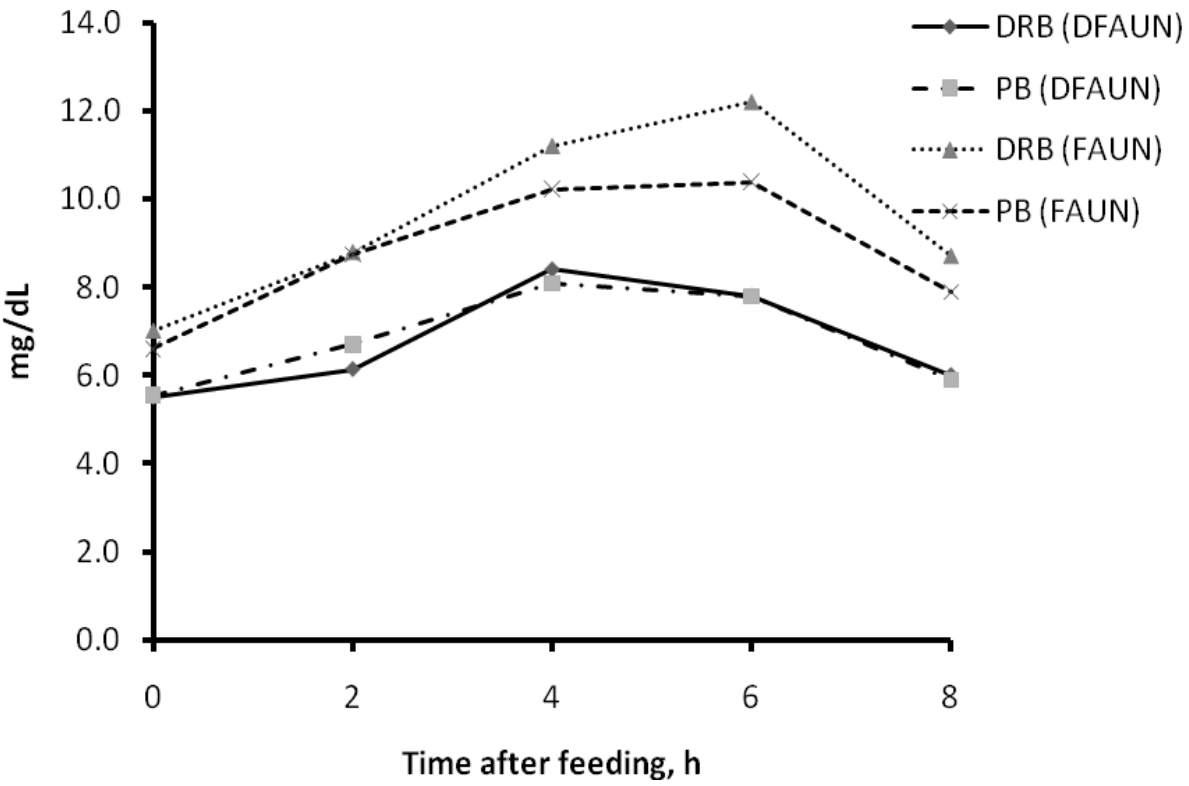
Appendix Table VI. Ruminal protozoal concentration (*x* 10^5*/ml*) of *Entodinium spp*., Holotrics, Cellulolytics (*Polyplastron*, *Diplodinium* and *Enoplastron sp*.) and total in FAUN and PDFAUN lambs fed dry-rolled barley

	FAUN				PDFAUN			
Day	Entodinium *spp.*	Holotrics	Cellulolytics	Total	Entodinium *spp.*	Holotrics	Cellulolytics	Total
1	3.31	0.11	0.05	3.47	6.82	0.20	0.19	7.20
2	5.49	0.15	0.13	5.77	6.53	0.18	0.18	6.89
3	5.94	0.18	0.18	6.30	6.01	0.12	0.14	6.27
6	7.14	0.19	0.18	7.51	5.16	0.10	0.14	5.41
9	8.84	0.22	0.19	9.25	4.32	0.05	0.09	4.46
12	10.11	0.27	0.18	10.57	3.08	0.02	0.04	3.14
15	11.36	0.27	0.21	11.84	2.44	0.02	0.04	2.50
18	11.21	0.25	0.19	11.65	2.06	0.01	0.01	2.08
21	10.19	0.27	0.22	10.68	1.31	0.01	0.00	1.32
22	10.05	0.27	0.21	10.53	1.26	0.01	0.00	1.27
23	10.29	0.28	0.19	10.76	1.34	0.00	0.00	1.34
24	9.73	0.28	0.19	10.20	1.26	0.00	0.00	1.26
25	10.27	0.28	0.20	10.75	1.33	0.00	0.00	1.33
26	10.50	0.29	0.20	10.99	1.28	0.00	0.00	1.28

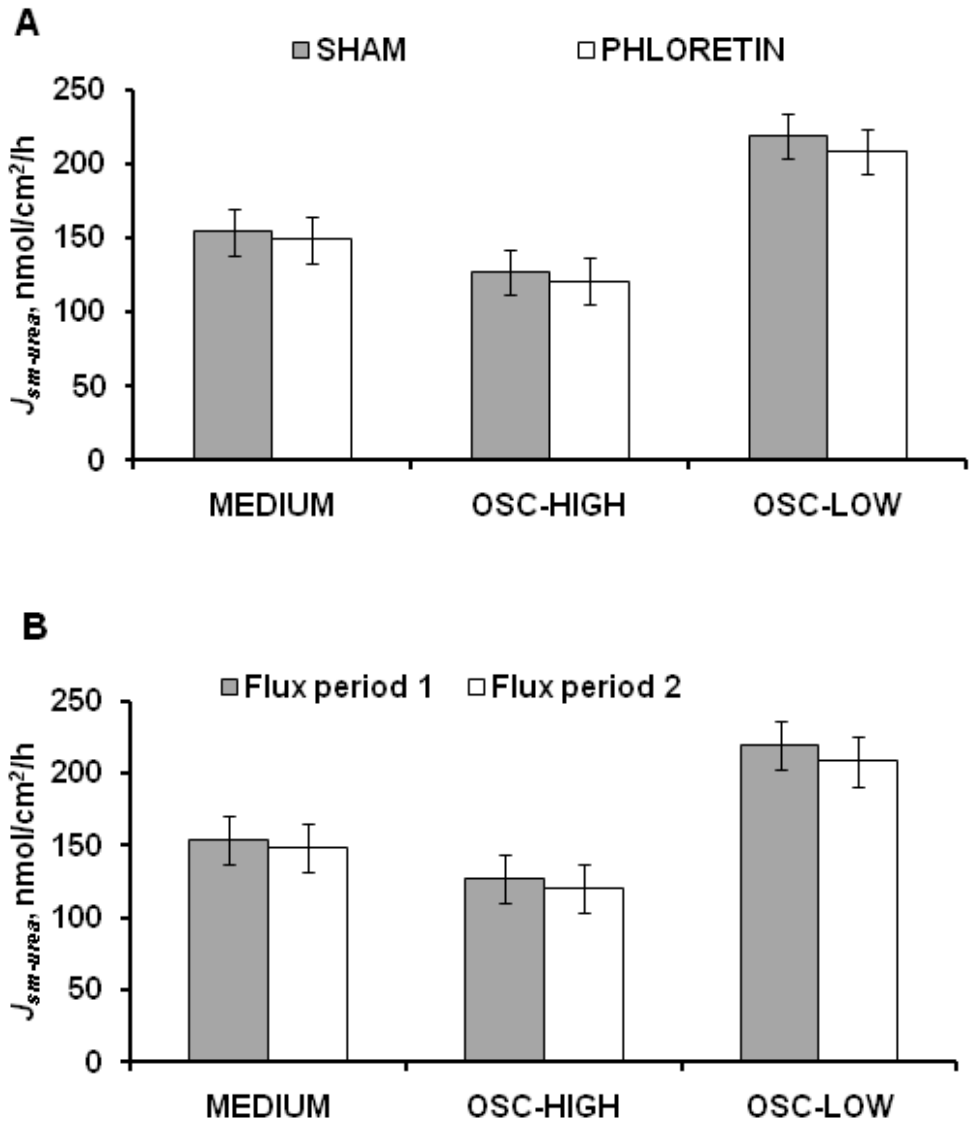
Appendix Table VII. Mean (n = 4) ruminal protozoal concentration (*x* 10^5*/ml*) of *Entodinium spp*., Holotrics, Cellulolytics (*Polyplastron*, *Diplodinium* and *Enoplastron sp*.) and total for re-faunated lambs starting from period 2 to period 4.

Day	Entodinium *spp.*	Holotrics	Cellulolytics	Total
1	2.08	0.00	0.00	2.08
2	3.95	0.05	0.08	4.08
3	4.18	0.15	0.11	4.44
6	4.93	0.12	0.12	5.17
9	6.93	0.15	0.16	7.24
12	8.68	0.28	0.17	9.12
15	10.15	0.24	0.20	10.59
18	10.35	0.22	0.19	10.75
21	9.18	0.24	0.18	9.59
22	8.58	0.18	0.14	8.90
23	7.66	0.18	0.12	7.96
24	7.87	0.17	0.12	8.17
25	8.32	0.18	0.12	8.61
26	8.02	0.17	0.12	8.32

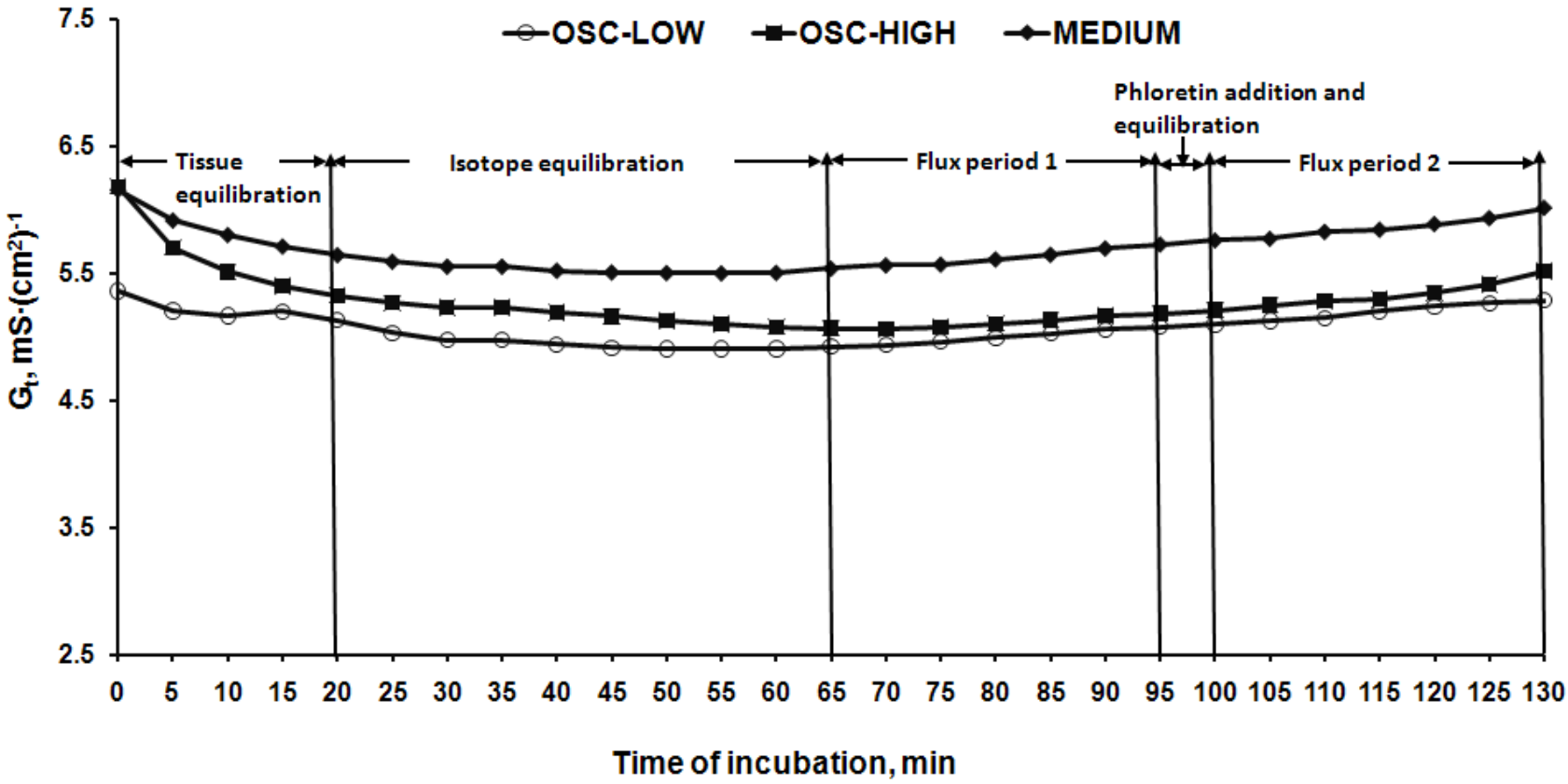
10.2 Appendix Figures.



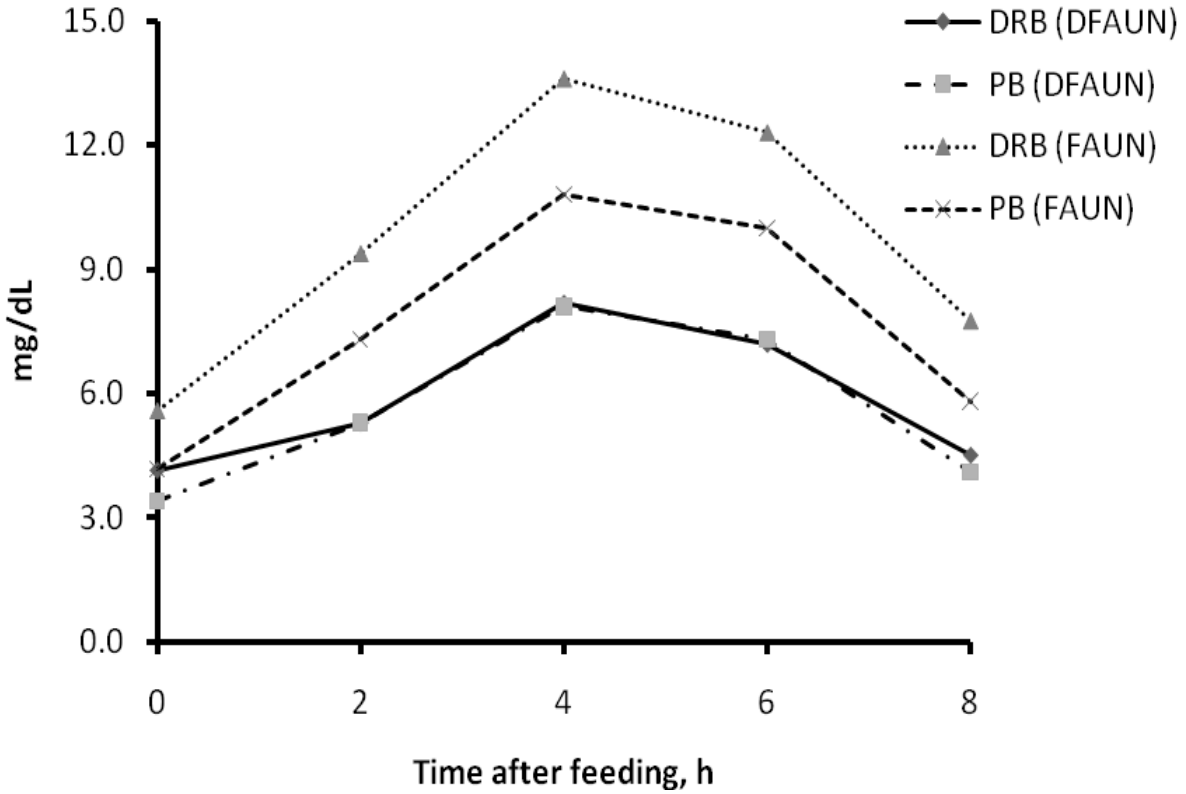
Appendix Figure I. Plasma urea-N over time post-feeding for samples collected on d 26. Treatments were: faunated + dry-rolled barley (FAUN-DRB); faunated + pelleted barley (FAUN-PB); defaunated + dry-rolled barley (DFAUN-DRB); and defaunated + pelleted barley (DFAUN-PB). DFAUN was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet. Differences were detected in PUN between time of sampling (time) 0, 2, 4, 6 and 8h (P = 0.001). There was no difference for time × barley grain processing (BP), time × protozoa and interaction (time × BP × protozoa). Each line represents means ± SEM for 4 lambs and each sample was analyzed in triplicates.



Appendix Figure II. Total serosal to mucosal urea flux ($J_{sm\text{-}urea}$, nmol/cm^2/h) across dietary treatments. **A**: compared to lambs fed MEDIUM and OSC-HIGH, $J_{sm\text{-}urea}$ was higher in lambs fed OSC-LOW treatment irrespective tissue assignment for *in vitro* treatments (P = 0.56; no difference between control and phloretin-assigned tissues) during first 30-min flux period (baseline) before addition of phloretin. **B**. Second 30-min consecutive flux period (inhibition) was done to determine the effect of addition of phloretin and $J_{sm\text{-}urea}$ did not change over time within tissues (P = 0.94) control tissues in baseline vs. inhibition period) between 30-min baseline and inhibition period. Each line represents mean ± SEM for 9 lambs.



Appendix Figure III. Time-course changes in transepithelial conductance (G_t) of ruminal epithelia of lambs fed the medium or oscillating dietary crude protein concentrations (n = 9).



Appendix Figure IV. Ruminal ammonia-N over time post-feeding for samples collected on d 26. Treatments were: faunated + dry-rolled barley (FAUN-DRB); faunated + pelleted barley (FAUN-PB); defaunated + dry-rolled barley (DFAUN-DRB); and defaunated + pelleted barley (DFAUN-PB). DFAUN was achieved by the addition of 6% (DM basis) linoleic acid-rich sunflower to the diet. Differences were detected in ruminal ammonia-N between time of sampling (time) 0, 2, 4, 6 and 8 h (P = 0.001). There was no difference for time × barley grain processing (BP), time × protozoa and interaction (time × BP × protozoa). Each line represents means ± SEM for 4 lambs and each sample was analyzed in triplicates.