Urea-N recycling and its utilization by ruminal microbial populations in lactating dairy cattle

by Erin Beth Recktenwald

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UREA-N RECYCLING AND ITS UTILIZATION BY RUMINAL MICROBIAL POPULATIONS IN LACTATING DAIRY CATTLE

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UREA-N RECYCLING AND ITS UTILIZATION BY RUMINAL MICROBIAL POPULATIONS IN LACTATING DAIRY CATTLE

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Two experiments were conducted to quantify urea-N kinetics and ruminal microbial populations and their nitrogen (N) transactions in lactating dairy cows under a variety of dietary nitrogen and carbohydrate conditions. In the first experiment, twelve ruminally fistulated Holstein cows were fed one of three diets to provide adequate MP supply and ruminal N balance (Control, 16.3% CP), adequate ruminal N but deficient MP supply (LoMP, 14.1% CP), or adequate MP supply but deficient ruminal N balance (LoRumN, 14.1% CP). A continuous jugular infusion of $^{15}N^{15}N$-urea was conducted for a minimum of 72 hours to label the respective pools. Plasma, milk, urine, feces, and ruminal contents, including the liquid associated bacteria, particle associated bacteria, and protozoa, were collected before and after urea infusion for N enrichment analysis.

Urea-N synthesis was greatest for cows fed the Control diets and lowest for the LoMP diets, with the LoRumN diets demonstrating intermediate urea-N synthesis regardless of the low N intake of cows on this diet. A greater proportion of urea-N entered the GIT instead being excreted in the urine of cows fed the LoRumN diet (75% vs. 63% for both the Control and LoMP diets). Approximately 8-14% of the microbial N pool was present as protozoal N, and the protozoa predated 4-17% of the total bacterial N yield, with the highest values observed for cows fed the Control diet.

A second study was conducted that fed differing amounts of dietary CP (14.3 vs. 15.8%), starch (23 vs. 29%), with or without the ionophore Rumensin. Urea-N kinetics were determined by dietary CP concentration, with little impact of dietary starch or Rumensin. Microbial pool sizes and N transactions were dependent on dietary interactions, suggesting the effect of Rumensin on these populations to be mediated by rumen conditions. Overall, these studies demonstrate control over urea-N kinetics by N intake, and particularly by urea-N synthesis, over a variety of dietary conditions. Low ruminal N balance, in conjunction with starch fermentation and ionophore effects, was able to stimulate urea-N entry to the GIT, improving efficiencies of N use. The amount of recycled N contributing to microbial N supply, in addition to protozoal predation of bacteria, varied by dietary conditions.

BIOGRAPHICAL SKETCH

Erin Beth Recktenwald is originally from Frederic, WI. Growing up in the countryside of northwest Wisconsin, she learned the fine arts of hiking, hunting, fishing, and horseback riding. She was always interested in animals, horses and cows in particular, and began attending UW-River Falls in the fall of 1999 for a B.S. in animal science. Cattle became her species of interest for career work, and combining her love of nutrition and chemistry, she pursued a graduate degree at in ruminant nutrition at Cornell. After completing her M.S. degree in 2007, she continued work on nitrogen metabolism in cows which is the topic of this thesis.

During her tenure at Cornell, Erin has also enjoyed living in the city of Ithaca with all it has to offer. She thoroughly enjoys rollerblading at the park, hiking near any of the falls, strolling through the commons and its festivities, and meeting people in the area. She attends Christ Chapel church, where she's learned a lot about coming together as a group of people who care for one another and having a good time. Erin also has been a member and leader of the Cornell Graduate Christian Fellowship, which has been a great experience in discussing the hard topics of life and faith and has been a place to form connections with other grad students who enjoy these topics in addition to socializing. Erin married Geoff Recktenwald in 2006 and gave birth to Vincent Recktenwald as this thesis was finishing.

To all of those in Ithaca who have enhanced my life and work.

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LIST OF ABBREVIATIONS

AA	Amino acid
ADF	Acid detergent fiber
ADIN	Acid detergent indigestible fiber
AOAC	Association of Official Analytical Chemists
APE	Atom percent excess
BCS	Body condition score
BCVFA	Branched chain volatile fatty acids
bST	Bovine somatotropin
BW	Body weight
CNCPS	Cornell Net Carbohydrate and Protein System
CP	Crude protein
CPM	Cornell Penn Miner
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EMPS	Efficiency of microbial protein synthesis
FCM	Fat corrected milk
GER	Gastrointestinal tract urea-N entry rate
GIT	Gastrointestinal tract
HPLC	High performance liquid chromatography
ME	Metabolizable energy
MFD	Milk fat depression
MP	Metabolizable protein
MUN	Milk urea nitrogen

N	Nitrogen
NAN	Non-ammonia nitrogen
NDF	Neutral detergent fiber
NDIN	Neutral detergent indigestible nitrogen
NH_3	Ammonia
NPN	Non-protein nitrogen
NRC	National Research Council
NSC	Non-structural carbohydrate
OM	Organic matter
PUFA	Polyunsaturated fatty acid
PUN	Plasma urea nitrogen
RDP	Rumen degradable protein
ROC	Urea-N returning t the ornithine cycle
RUP	Rumen undegradable protein
TDN	Total digestible nutrient
TMR	Total mixed ration
UER	Urea-N entry rate
UFE	Urea-N excreted in the feces
UUA	Urea-N utilized for anabolic purposes
UUE	Urinary urea elimination rate
VFA	Volatile fatty acid

CHAPTER ONE: LITERATURE REVIEW

The Purpose of Reducing Nitrogen Excretions from Cattle

Nitrogen (N) is an essential component of living organisms, contributing to the backbone of protein and is a component of a variety of other organic and inorganic compounds. Technologies that promote the availability of N for living and growing organisms have allowed for greater productivity. However, these advances have also shifted the amount and source of N flows throughout the environment. For example, the Haber-Bosch process utilizes the abundant supply of gaseous N_2 to produce NH_3, which accounts for 40% of crop N worldwide (FAO, 2006). It is estimated that 40% of people currently owe their lives to fertilizer made by this process (Galloway et al., 2003). Unfortunately, it also converts nonreactive N_2 into various reactive N forms. The Haber-Bosch process has increased anthropogenic reactive N emissions by more than 100 teragrams (Tg) N/yr, with 85% of this for fertilizer production (Galloway et al., 2003). This is a significant amount, considering approximately 165 Tg of reactive N/yr are produced in total. Of the 170 Tg N/yr added to croplands, only 16 Tg N/yr are consumed by humans and 33 Tg N/yr by animals, of which 5 Tg N/yr are eventually consumed by humans (Galloway et al., 2003). Improving efficient use of N requires cooperation among a variety of scientists and producers involved in stages of N formation and utilization.

Reactive N contributes to a variety of environmental and human health problems. Nitrogen can form numerous volatile or aqueous compounds:

nitrous oxide (N_2O), nitric oxide (NO), nitrogen dioxide (NO_2), nitrite ions (NO_2), and nitrate ions (NO_3-). Nitrate and nitrite can be deposited on earth's surface and contribute to ground water accumulation and eutrophication. Nitrous oxide depletes ozone and is a potent greenhouse gas. On the animal side, urinary urea is quickly hydrolyzed to ammonia (NH_3), which can form ammonium (NH_4+) in the atmosphere, which then contributes to acid rain and particulate matter formation of diameter sizes less than 2.5 um (VandeHaar and St Pierre, 2006). Approximately 40% of excreted N is lost in the form of NH_3 volitalization and 2% in the form of N_2O volitalization (FAO, 2006). These forms can also change into other types of reactive N and can enter the land and water supply via leaching. According to the FAO, livestock contribute approximately 65% of global anthropogenic nitrous oxide and ammonia emissions (FAO, 2006).

Urea Production

The amount of urea produced by an animal each day is dependent on a variety of factors. Initially, one would suggest N intake to have a major impact on urea production, as higher intakes would lead to more N catabolism, NH_3 formation, and NH_3 absorption and therefore more urea formation. In animals at low N intakes or low production, this appears to be the case. In a review by Lapierre and Lobley, (2001), they reported cattle at low N intakes and close to zero N balance to have a correlation (R^2) of 0.78 between N intake and hepatic urea production. However, in cattle with high N intakes this correlation was only 0.45. When these correlations were based on portal drained viscera

NH_3 absorption instead of N intake, they increased to 0.84 across all cattle (Lapierre and Lobley, 2001).

It can be presumed from these studies that urea production heavily depends on the amount of N either absorbed as NH_3 or catabolized to NH_3 by the PDV and liver. For animals with low N requirements, a larger and more consistent proportion of N intake undergoes this process, whereas in those with high productive requirements the N is more commonly and more variably absorbed as AA and utilized for anabolism in the body rather than deaminated to NH_3. Therefore, N intake, the form in which N is absorbed by the PDV, and N requirements each play a role in urea production.

In a variety of studies in sheep and cattle using the urea-N kinetic model detailed by Lobley et al., (2000), hepatic urea production (UER = urea-N entry rate, g urea-N/d) demonstrated a wide range of values compared to N intake. These experiments involved either feeding different levels of N (Archibeque et al., 2001; Archibeque et al., 2002; Lapierre et al., 2004; Marini et al., 2004; Marini and Van Amburgh, 2003; Sarraseca et al., 1998; Sunny et al., 2007; Valkeners et al., 2007) or changing the diet slightly to enhance either ruminal microbial fermentation or rumen N balance (Baker et al., 2007; Gozho et al., 2008; Kiran and Mutsvangwa, 2007; Lobley et al., 2000; Ouellet et al., 2004), or the urea/NH_3 barrier by the rumen wall (Marini et al., 2003) as their main objective. Among all animals, UER:N intake ranged from 0.27 to 1.19 and there were no consistent differences among species or productive state of the animal (Figure 1.1).

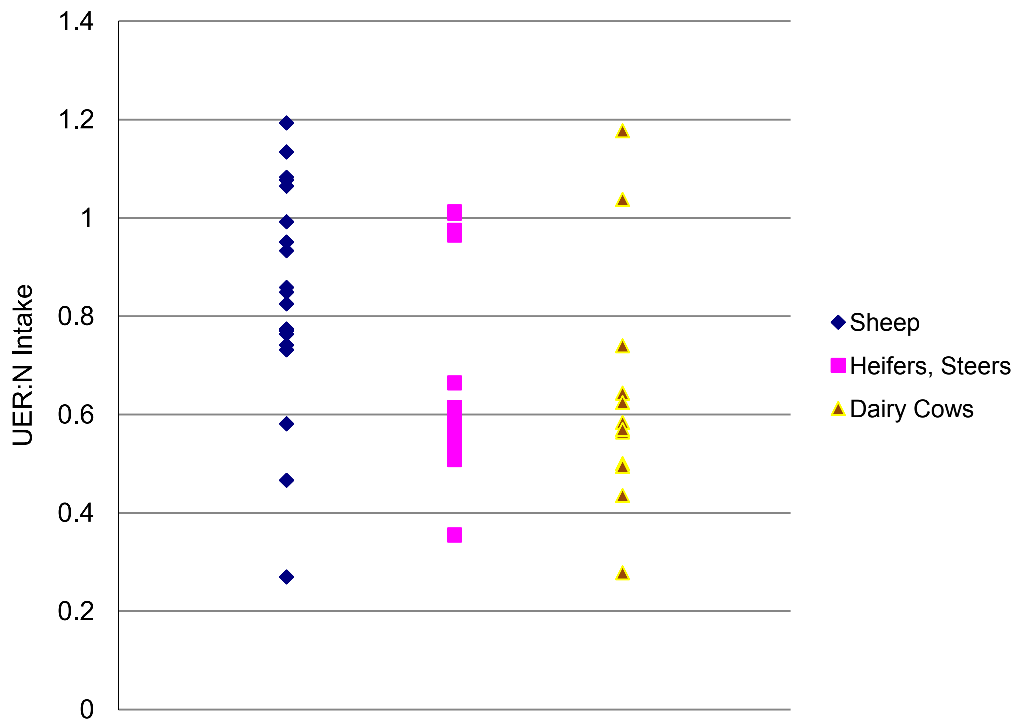

Figure 1.1. The ratio of daily urea-N entry rate (UER) to N intake across several sheep and cattle studies.

Gastrointestinal Tract Urea-N Entry

After synthesis, urea-N may either enter the gastrointestinal tract (GIT) or be excreted in urine via the kidneys. Urea-N that enters the rumen is able to be utilized by the microbial population and thereby improve whole animal N utilization. The quantity of urea-N that enters the GIT (GER) varies widely across and among species. Values of GER:N intake ranged from 0.19 to 0.80 among species and physiological status, with no apparent patterns observed among them (Figure 1.2). Because urea-N that enters the GIT must be produced, it is possible to refine estimates of GER flow in reference to the total

N intake by examining GER as a proportion of UER. When the GER:UER ratio is calculated, there again is a broad range of values (0.29 to 0.92), but there appears to be more consistency at least in the lactating animals, the dairy cows (Figure 1.3). Their ratio ranges from 0.58 to 0.75, with one data point at 0.92, but this was in animals fed at only 70% of MP requirements (Valkeners et al., 2007). With this data, it appears that at least in lactating dairy cows, the amount of urea-N entering the GIT may be relatively consistent, around 0.6 to 0.75 of the urea production. If urea-N production were able to be estimated with some accuracy through dietary and endogenous NH_3 formation, it is possible to also have a fairly accurate estimate of GER, and therefore, the ability to estimate a more accurate ruminal N balance.

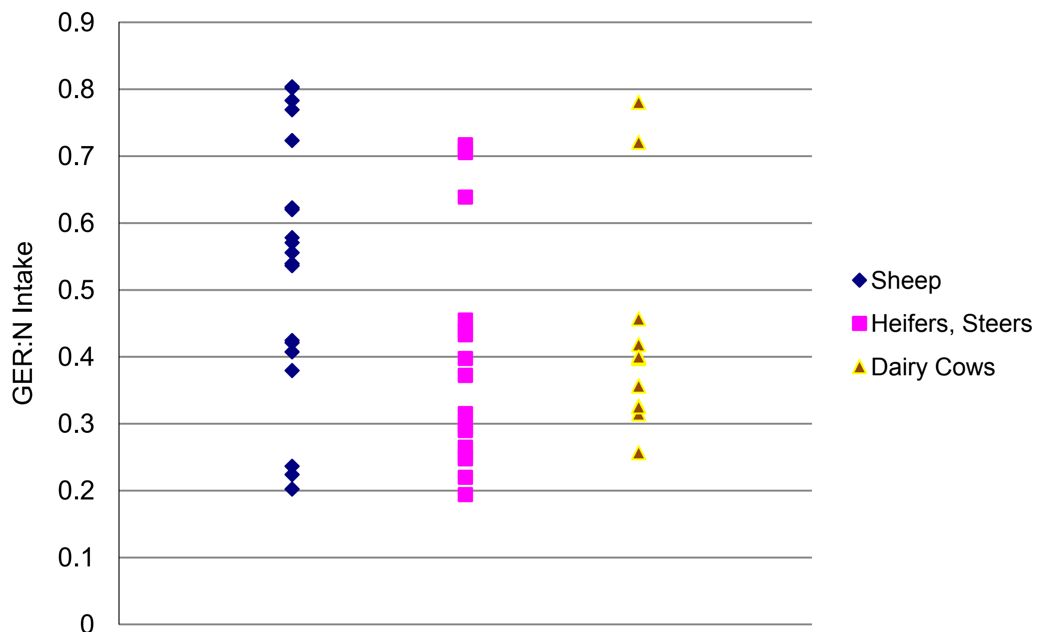

Figure 1.2. Gastrointestinal urea-N entry rate (GER) as a proportion of N intake in sheep and cattle studies.

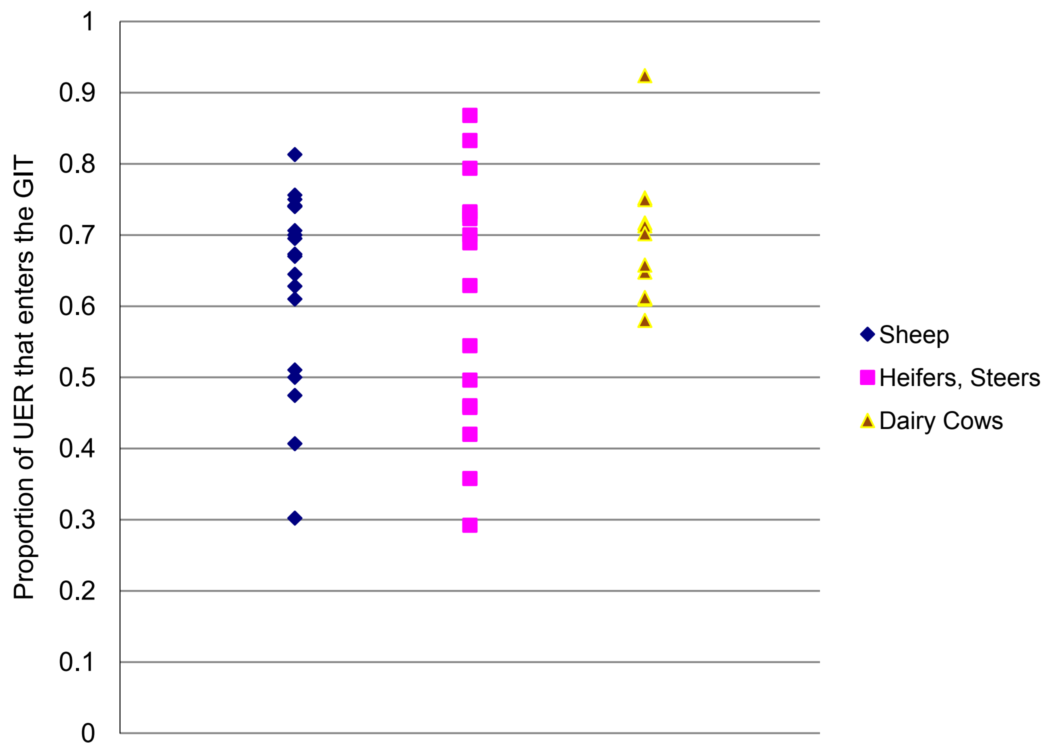

Figure 1.3. Gastrointestinal urea-N entry rate (GER) as a proportion of urea-N entry rate (UER) in sheep and cattle studies.

In addition to the rumen, however, urea-N enters the hindgut, albeit to a lower extent. In steers fed high concentrate diets, approximately 65-75% of the recycled urea-N entered the rumen, although these values were higher in animals fed forage based diets vs. concentrate based diets (90 vs 19%) (Huntington, 1989; Lapierre and Lobley, 2001; Reynolds and Huntington, 1988). In another study with steers, steam flaked instead of dry rolled corn increased urea-N entry to the PDV by 34%, but the proportion of this entry to ruminal tissues remained approximately 75% for both diets (Theurer et al., 2002). However, the amount of PDV urea-N entry that entered intestinal tissues increased from 14 to 26% with steam-flaked corn (Theurer et al., 2002). It is possible that supply of fermentable substrate in the post-ruminal

GIT shifts urea-N to this area, leading to more microbial fermentation in the hindgut and potentially more fecal N loss (Oncuer et al., 1990). Although this loss does occur, it is relatively minimal at 1-12% of the urea-N entry rate to the GIT in dairy cattle (Gozho et al., 2008; Lapierre and Lobley, 2001; Valkeners et al., 2007). However, it is also possible that stimulating fermentation in the rumen may similarly lead a larger proportion of the urea-N entry to the rumen. Factors affecting urea-N flow across the GIT will be discussed in the next section.

Factors affecting nitrogen recycling

Early research

A variety of studies were conducted in the 1960s to determine the relationship between blood urea concentration and urea transfer into the rumen. Some studies showed a linear relationship between the two, with zero transfer at zero blood urea concentration, suggesting simple diffusion only. Alternatively, some studies demonstrated fast increases in the transfer rate at low blood urea concentrations which slowed down or leveled off as concentrations became higher, suggesting a transport mechanism (Houpt, 1969). Bacterial urease may have played a role in the opposing results, as well rinsed pouches nearly devoid of urease demonstrated linear relationships, whereas unrinsed rumen pouches demonstrated near curvilinear responses for urea transport (Houpt and Houpt, 1968). Removal of the urease enzymes from the ruminal wall not only linearized the response, but it decreased the rate of urea transfer into the rumen approximately 2-3 times (Houpt and Houpt,

1968). There were two main barriers to urea or NH_3 transport across the epithelial wall that they noted. The first was the outer epithelial layer, the stratum corneum. When this layer of cells was removed by an alkaline solution (without damage to the underlying cells), urea transfer into the rumen pouches increased fifty times (Houpt and Houpt, 1968). The second was simply the diffusive coefficient of ammonia. Previous work had speculated NH_3 transfer to occur throughout the epithelial layers by either simple diffusion or a transport mechanism, but both dependent on NH_3 concentrations. They estimated that if the difference in pH was increased, then the NH_3 transfer rate into the rumen would be increased and given an advantage over urea transfer. Therefore, both these factors may modulate N flows across the rumen, and other GIT, walls.

Nitrogen gradient across the rumen wall

In a variety of early sheep and cattle studies, ruminal NH_3 concentration was found to be inversely related to the transfer of plasma urea-N to the rumen, with maximal transfer rates at ruminal NH_3 concentrations of 5-8 mg NH_3-N/dl in cattle and no further increases above 9-10 mg NH_3-N/dl (Kennedy and Milligan, 1978). In addition, an analysis of seven studies in sheep fed hay based diets demonstrated not only a negative correlation between rumen NH_3 concentration and urea transfer into the rumen, but a positive correlation between PUN concentration and urea transfer (Kennedy and Milligan, 1980).

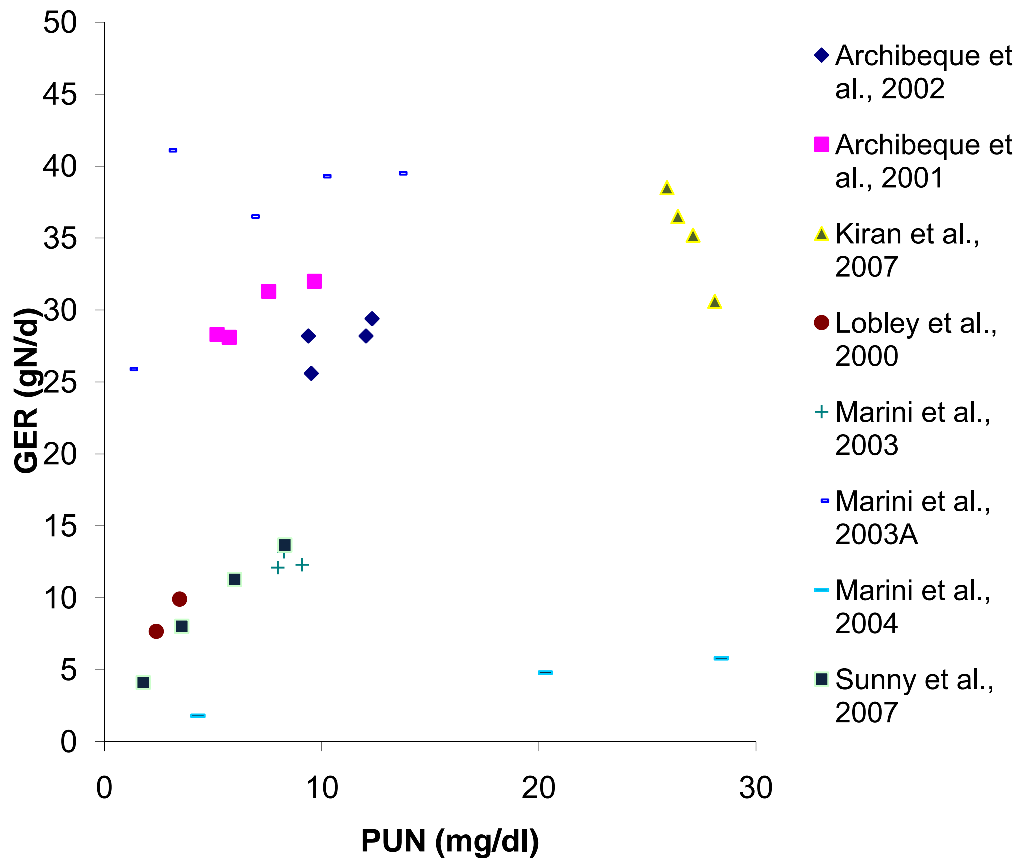

Figure 1.4. Relationship between gastrointestinal urea-N entry rate (GER) and plasma urea-N concentration (PUN) across a variety of physiological and nutritional states in cattle and sheep.

More recent work in sheep given jugular infusions of urea confirmed a positive relationship between urea-N entry and PUN concentration, although the relationship observed in this study was curvilinear, whereas the others were linear (Sunny et al., 2007). There might be slight differences in overall N handling to explain this difference, as the study demonstrating curvilinearity was for animals fed at maintenance and jugularly infused with urea, and the others were a combination of urea or NH_3 infusions and additions to the diet.

In general, among the array of studies previously examined in this review, urea-N entry to the GIT was positively associated with PUN concentration; however, the magnitude of this relationship varied widely across and among species (Figure 1.4).

Fermentation or microbial activity

Sucrose supplementation has been shown to increase urea-N recycling to the rumen. In steers fed hay diets and supplemented with 0, 150, or 300 g sucrose/d, plasma urea-N entry to the rumen was increased by 35% across all PUN concentrations with increasing sucrose supplementation (Kennedy, 1980). Improved urea-N transfer to the rumen was also observed in sheep fed brome grass pellets and supplemented with up to 300 g sucrose/d. But while the urea transfer to the rumen increased 23%, the PUN clearance to the rumen increased 179% due to 2-3 times lower PUN concentrations in sucrose supplemented animals (Kennedy et al., 1981). Because improved fermentation status will create a higher demand for N, it is likely that some of the effect of sucrose supplementation is due to changes in ruminal NH_3-N and PUN concentrations over time. However, although both supplemented and non-supplemented diets demonstrate positive or negative relationships for ruminal urea entry with PUN or ruminal NH_3-N concentrations, respectively, the effect of sucrose is independent from either N concentration (Kennedy, 1980; Kennedy et al., 1981). Therefore, something other than the N concentration gradient is in effect, or at least something is impacting the gradient.

Similarly, processing can affect urea-N transfer by modifying fermentation and/or ruminal N status. Steam flaking versus steam rolled corn increased urea-N cycling to the PDV by 140% in lactating cows producing approximately 29 kg milk/d (Delgado-Elorduy et al., 2002). In cows producing 28-33 kg milk/d, dry rolled barley compared to pelleted barley supplementation increased urea-N entry to the GIT by 35% (Gozho et al., 2008). Although improvements in N supply to the rumen might be increased by such dietary changes, it should be noted that these changes are only beneficiary if anabolic use (microbial N utilization) is improved as well. Various studies have shown improved urea-N recycling, but no or little improvement in microbial yield (Gozho et al., 2008; Marini and Van Amburgh, 2003; Sunny et al., 2007).

Carbon dioxide and short chain volatile fatty acids

Carbon dioxide bubbled into digesta from cattle in isolated rumen pouches increases urea clearance and blood flow, but the response in urea clearance is comparatively much slower (Dobson et al., 1971; Thorlacius et al., 1971). Thus the stimulation of urea clearance by CO_2 appeared to be dissociated from the stimulation of blood flow, suggesting changes in urea clearance were not directly caused by changes in blood flow but by differences in permeability within the rumen epithelia. In addition, the effects of CO_2 were localized, acting only on the area administered CO_2 (Thorlacius et al., 1971). These results were confirmed in wethers fed orchardgrass hay at maintenance level intake. Carbon dioxide again increased urea clearance (negligible changes in PUN, but urea transfer to the rumen increased up to 62%), and it increased

NH_3 absorption from the rumen up to 16% with negligible changes in ruminal NH_3 concentration (Remond et al., 1993). Further, two doses of approximately 7 ml butyric acid was injected into the rumen, and ruminal blood flow increased up to 57%. Urea transfer into the rumen decreased 29% and NH_3 absorption from the rumen increased 40%, again with negligible changes in urea and NH_3 concentrations (Remond et al., 1993). Although butyrate has been shown to stimulate urea transfer (Norton et al., 1982), it may have been possible that NH_3 absorption may have negated its stimulatory effect, as NH_3 administration (40 mL of 1.7 M ammonium sulfate) caused urea transfer to decrease 18% while NH_3-N absorption increased 101% (Remond et al., 1993).

More recent work using isolated ruminal epithelium in Ussing chambers has demonstrated urea flux into the rumen to be increased four-fold by the addition of SCVFA (Abdoun et al., 2009). This stimulation occurred within the pH range of approximately 5.4 to 7.4 and was maximal at pH 6.2 to 6.4. Microelectrodes were used to confirm that the SCFA did in fact acidify the cytosol of the apical membrane, potentially allowing for greater NH_4^+ formation, or a higher NH_4^+/NH_3 ratio (Abdoun et al., 2009). This work demonstrates the feasibility previously suggested by Houpt in the 1960s and later by others such as (Bodeker et al., 1992) and (Abdoun et al., 2006), in which compounds such as SCFA could aid in NH_4^+ formation intracellularly, maintaining a concentration gradient favorable for NH_3 uptake across the membrane and stimulating NH_4^+ export from the cell by apical sodium-hydrogen exchanger (NHE) (Figure 1.5).

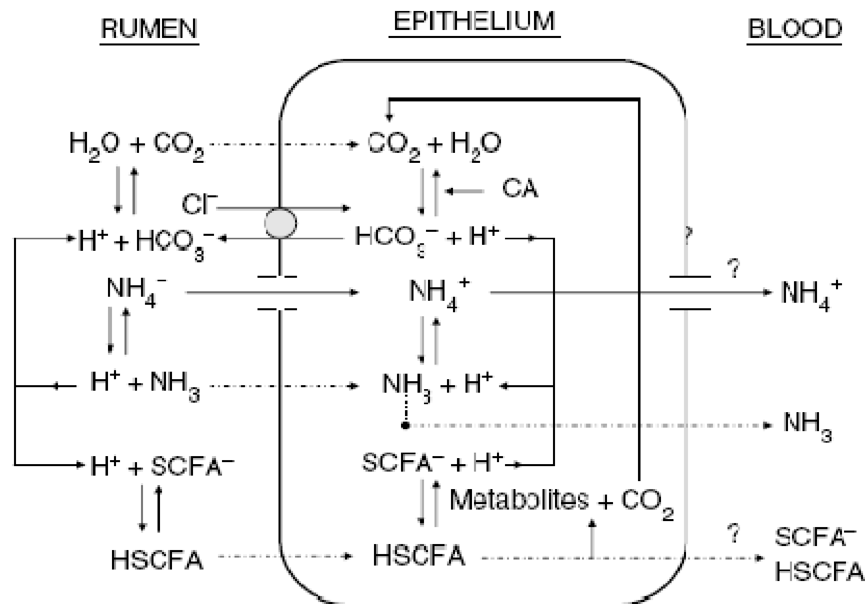

Figure 1.5. Scheme of pathways of NH_3 and NH_4^+ across the rumen epithelium and modulation by CO_2/HCO_3^- and SCFA. The luminal uptake of NH_4^+ and NH_3 is mediated by a putative K channel and by diffusion, respectively. The relative transport rates of both forms depend on the ruminal pH and the concentration of protons just above the luminal membrane. Availability of protons can be reduced by reaction with secreted HCO_3^- or by protonation of SCFA. In both cases, the NH_3 concentration increases and hence NH_3 uptake. Intracellularly, NH_3 will be protonated to NH_4^+ by protons of dissociation of HSCFA or H_2CO_3. This intracellular protonation of NH_3 maintains the NH_3 gradient and uptake across the luminal membrane. The exit of both forms of ammonia across the basolateral membrane is not clear. Modified from Abdoun et al., (2006).

Urea transporters

Urea transporters have been observed in the GIT of sheep (Marini et al., 2004; Ritzhaupt et al., 1998) and cattle and in many other tissues throughout the body (Marini and Van Amburgh, 2003; Stewart et al., 2005). Despite numerous studies, their exact role in urea transport across the ruminal wall remains unclear due to inconclusive or apparently conflicting results. Urea transporter B (UT-B) expression increased proportionally to N intake, despite urea-N production increasing over three fold while urea-N GIT entry did not significantly change over a range of N intakes (Marini and Van Amburgh, 2003). If UT-B did in fact aid in urea transfer, it would be intuitive to expect higher expression with lower N intakes (and/or higher GER:UER ratios). Surprisingly, expression of the transporters did not appear to play a major role in urea clearance, as this measure decreased seven fold as N intake increased.

More recent work has generally demonstrated a lack of response of UT expression in relation to N intake or to urea transport in ruminants. Urea transporter B abundance was not different among 3 diets containing 1.55, 2.84, or 4.13% N given to ewe lambs (Marini et al., 2004). No difference in UT-B expression was noted in lactating dairy cows fed differing amounts of RUP and RDP, but UER and GER were also unaffected by treatment (Dihn, 2007). In sheep fed wheatgrass hay and supplemented to achieve 7, 10, or 13% total dietary CP, UT-B expression did not differ by treatment. Nor were there differences in sheep fed varying amounts of RDP and RUP either daily or on alternate days (Ludden et al., 2009) and oscillating protein intake should create conditions conducive to altered transport. However, some differences

in tissue expression of 32 vs 47 kDa UT-B proteins occurred, suggesting glycosylation may play a role in UT-B activity. Defaunation in lambs fed 10.3 and 15.6% CP diets decreased UER by 33 and 19%, respectively, and GER by 28 and 6%, respectively (Kiran and Mutsvangwa, 2009). It also increased UT-B mRNA abundance 13 and 40%, respectively, although this was not significant, nor was the effect of dietary CP concentration.

Immunoblotting cattle rumen epithelial tissue revealed UT-B expression in the three inner layers (stratum basale, stratum spinosum, stratum granulosum), but not in the outermost layer (stratum corneum) (Stewart et al., 2005). The tissue was taken from slaughterhouse animals, so it is difficult to know the dietary conditions prior to excision. In a later study, UT-B was expressed in the stratum corneum as well in steers fed concentrate based diets, but not in those fed silage based diets (Simmons et al., 2009). It is possible that the stratum corneum aids in urea flux regulation via urea transporter expression, narrowing or lengthening the effective barrier between the ruminal fluid and the blood. Either dissolution of the structure or UT would be effective to allow for greater N flux across the epithelium by diffusion, which is the most likely proposed mechanism of urea transport.

This array of work on UTs demonstrates the complexity of urea transport, but due to the magnitude and variety of correspondence among UT expression and actual urea transport, it does seem that urea movement is dependent more heavily on functional effects, such as CO_2, SCFA, NH_3 and urea concentrations, than on transcriptional effects.

Anabolic utilization of recycled urea-N

After hydrolysis primarily along the rumen wall, recycled N is then converted into microbial protein, passed out of the rumen in the liquid phase, or absorbed by the animal into the mesenteric vein. Virtually all of the absorbed NH_3-N is converted into urea in the liver, with less than 5% potentially formed into AA via transamination reactions. This number is supported by very low levels of $^{15}N^{15}N$ urea formed under NH_3 mesenteric infusions, which would be expected to produce high amounts of $^{15}N^{15}N$ urea if NH_3-N was utilized to form AA, then the ^{15}N-aspartate could be used for urea cycle N donation (Lobley et al., 1995). In wether lambs infused with up to 235 umol/min of NH_4Cl into the mesenteric vein, over 97% of the urea was single-labelled, and 93.5% of the ^{15}N recovered across the liver was as urea (Lobley et al., 1995). In a similar study, approximately 80-90% of the infused ^{15}N was estimated to appear in urea (Lobley et al., 1996). It is possible that some of the ^{15}N could originate from muscle or liver glutamine release. However, glutamine formed from GLDH is restricted to the mitochondrion instead of mixing with the cytosol, inferring that much of the NH_3-N incorporated into AA via transamination is also mainly used for transfer to aspartate and entry to the ornithine cycle (Lobley et al., 1996).

Anabolic use of recycled urea-N is the portion of “excess” N that has the most potential use to the animal, as it is synthesized into AA and able to be utilized for both protein synthetic and catabolistic reactions. In the previously mention set of studies using the technique of Lobley et al., (2000), between 0.16 and 0.62 of the GER was utilized for anabolic purposes (Table 1.6). These values were lowest for sheep and also for beef heifers (Baker et al.,

2007), but were higher for dairy heifers (0.49 - 0.62 vs 0.15 - 0.29) fed 30% brome hay and 70% pellets, even though body weights and microbial production was similar. The primary difference among these studies was that the GER observed with the beef heifers was much higher than that in the dairy heifers, so the microbial populations may have been less able to utilize as much of the total recycled urea-N in these diets. In dairy cows, proportion of GER to UUA values were relatively high (0.44-0.59) except for one study with cows on a 17% CP diet with differently processed barley and fat supplements (GER to UUA was 0.16-0.21) (Gozho et al., 2008). It was speculated in their report that the limit of N use for anabolic purposes was achieved by these high CP diets, and therefore little additional N supplied via the recycling pathway was incorporated into microbial protein. Therefore, to maximize effective utilization of recycled N, it appears that ruminal N balance should be relatively low, but not low enough to limit microbial activity.

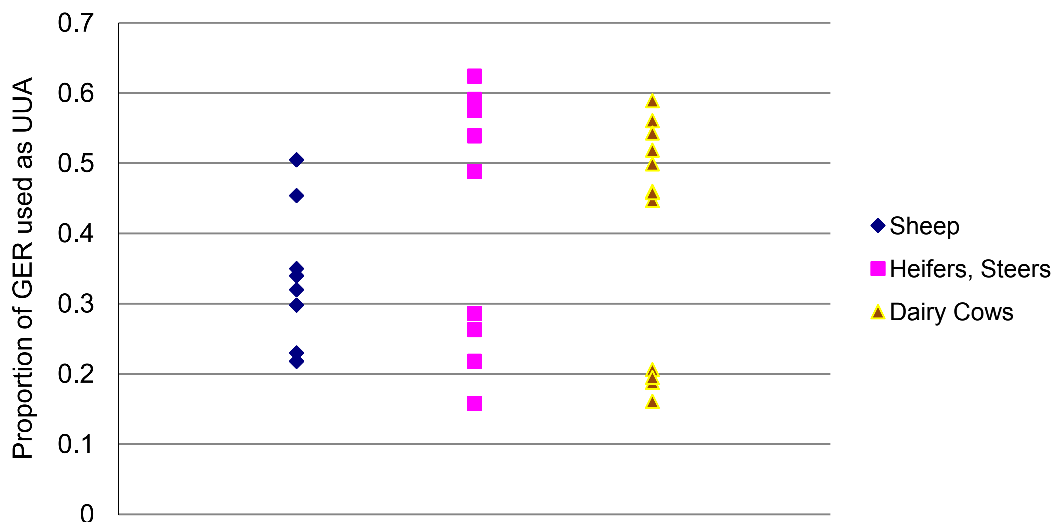

Figure 1.6. Proportion of urea-N entering the gastrointestinal tract (GER) that is used for anabolic purposes (UUA).

Microbial use of different forms of nitrogen

Microbes are able to convert inorganic N into organic N, thus providing biologically usable material not only for itself but for its host. The contribution of NH_3 versus AA and peptides to microbial N varies under different dietary conditions. In mixed ruminal microbes, ammonia contributed only 26% of the microbial N in a medium with high concentrations of AA and peptides, and 100% when NH_3 was the only available N source (Wallace et al., 1999). In a review by Bach et al., (2005), it was suggested that based on a variety of *in vitro* and *in vivo* work, on average, approximately 80% of bacterial N is derived from NH_3-N. Cellulolytic bacteria appear to be less able to use AA and peptides, but even they were able to derive up to 50% of their cell N from non-NH_3 sources (Wallace et al., 1999). This effect was also observed by higher cellulolytic bacterial counts in sheep receiving AA infusions and higher growth rates in culture with AA or peptide supplied in the medium (Cruz Soto et al., 1994). In the CNCPS, nonstructural carbohydrate fermenting bacteria can obtain up to 67% of their N from AA or peptides (Russell et al., 1992) but with emerging data, this value should be reconsidered as a constant and be considered for the structural carbohydrate bacteria.

Microbial nitrogen requirements under deficient conditions

Low ruminal N concentrations have been associated with depressed DMI and DM digestibility. In lactating cows, DMI decreased from 21.2 to 17.6 kg/d as rumen ammonia concentrations declined from 10.0 to 1.2 mg NH_3-N/dl

(Ruiz et al., 2002). Improvements in DMI and DM digestibility were observed among all three treatments, but the differences were not significant between the two highest rumen NH_3-N concentrations, 4.5 and 10.0 mg NH_3-N/dl. Concentrations above 5 mg NH_3-N/dl lead to accumulation of bacteria in incubations of rumen contents, with concentrations higher than this not increasing microbial growth (Satter and Slyter, 1974). These authors claimed that the limitation could be as low as 2 mg NH_3-N/dl, but they set a slightly higher point to account for margin of error. These results support a lower limit of 4-5 mg NH_3-N/dl approaching microbial inhibition and fit with the concept that bacteria with faster metabolism and uptake potential could outcompete slower fermenting bacteria for rumen NH_3 when concentrations are low.

Urea-N recycling can aid in overcoming potential ruminal N deficiencies by providing a constant, variable supply of N. This proves to be most important in low N diets, as observed in Holstein heifers fed incremental amounts of N. (Marini and Van Amburgh, 2003) reported that 18.7% of bacterial N originated in plasma urea N for animals on 1.45% N diets, while this number was only 4.3% for those fed 3.4% N diets. In addition, up to 43% of the GER was utilized for microbial protein synthesis. Similarly, in wether lambs, 77% of the bacterial N was derived from blood urea-N when fed a low N diet and 30% when fed a high N diet, even though comparable proportions of bacterial N originated from NH_3 (51-64%) (Bunting et al., 1987).

Protozoal predation of bacteria

Protozoa are not effectively able to utilize NH_3-N to synthesize AA and proteins (Wallace et al., 1999). Instead, they rely on bacterial engulfment, or predation, and on digestion of insoluble protein to fulfill their N requirement, and are able to occupy up to 31% of their volume as engulfed bacteria (Coleman, 1967). Approximately half of these bacteria were incorporated into protozoal N; the other half is released back into the rumen medium as AA and peptides, which are then available for reuse by bacteria (Hristov and Jouany, 2005a). This ruminal microbial turnover plays a large role in determining the amount and form of N available in the rumen for microbial utilization or rumen wall absorption, the efficiency of microbial N use, and the amount and form of N passing out of the rumen.

The majority of bacterial turnover is caused by protozoal predation, with 88% caused by predation by small entodiniomorphid ciliates *in vitro* (Hristov and Jouany, 2005b). The autolysis rate of bacteria due to starvation was less than 3%/h, while the rate of bacterial breakdown was 5.3-28.6%/h. In sheep, fractional turnover of bacterial N was 7.9%/h, and this was reduced to 5.7%/h after defaunation (Koenig et al., 2000). One protozoan can engulf 100-10,000 bacteria per hour; thus, the entire bacterial population could be turned over each hour in a rumen with high protozoal concentrations (10^5-10^6/ml) (Jouany, 1996).

Much of the actions of protozoa on N transactions have been deciphered through observing the effects of their absence in the rumen, or defaunation studies. In a quantitative meta-analysis of 90 publications with cattle, sheep, and goat, defaunation increased duodenal microbial N flow, decreased NH_3-N

concentration, and increased microbial N efficiency (Eugene et al., 2004). Defaunation in sheep fed dried grass resulted in an 87% decrease in intraruminal bacterial N recycling (Newbold et al., 2000). In a review by (Walker et al., 2005), he used data from two defaunation studies in sheep to claim that approximately 50% of microbial protein formed in the rumen was also recycled in the rumen, and that this recycling as a percent of the total N flux through the bacterial N pool decreased 25-100% with defaunation (Koenig et al., 2000). The amount of microbial N flow to the duodenum originating from NH_3-N was approximately twice as high with defaunated versus faunated sheep, although the proportion of the microbial flow from NH_3-N was slightly higher in defaunated animals (63 vs 79% for faunated and defaunated, respectively) (Koenig et al., 2000). By increasing bacterial protein synthesis and reducing OM degradation, defaunation results in improvements in microbial protein synthesis (40-125% increase in g microbial N/kg DOM) (Jouany, 1996). Defaunation also reduces urinary N and increases fecal N, effectively shifting N excretion patterns (Eugene et al., 2004; Jouany, 1996). This is partially due to reduced ruminal NH_3-N concentrations, leading to less urea synthesis and urinary excretion, and to increased hindgut fermentation because of less ruminal OM degradation, leading to more microbial growth in the lower GIT (Hristov and Jouany, 2005a).

Microbial turnover by autolysis

Besides protozoal predation, microbial turnover occurs due to autolysis. Protozoa burst after consuming sugar or starch beyond their physical capacity,

as they cannot moderate their intake (Hristov and Jouany, 2005a). In addition, protozoa are sensitive to low pH conditions and might be more prone to lysis if rumen pH drops below 6.0. Therefore, high concentrate diets or those with high amounts of rapidly fermentable material will increase protozoal turnover. Protozoa are also selectively retained in the rumen, having slower turnover rates than either liquid or solid rumen contents. It was estimated by Leng and Nolan, (1984) that two-thirds of the total rumen protozoal mass turns over in the rumen daily.

Passage rate also affects turnover, as protozoa, and potentially all microbes, are able to adapt to faster passage rates by shortening their generation intervals (Sylvester et al., 2009). In essence, more resources are directed to growth rather than maintenance, allowing for higher efficiencies. It is also possible that microbes more able to decrease their generation interval or more efficiently utilize resources are at an advantage, and are selectively retained with faster passage rates. Increases in bacterial N synthesis and EMPS have been observed with faster liquid and/or solid dilution rates, although there is less OM available for growth due to less degradation time in the rumen (Bach et al., 2005).

Monensin impacts on microbes

Monensin (Rumensin) is a carboxylic polyether that disrupts the potassium gradient across microbial membranes, transporting K^+ ions out of the cell and H^+ into the cell, then reversing the H^+ flux and transporting Na^+ ions into the cell (Russell and Strobel, 1989). The cell then expends energy in attempt to

retain its ionic equilibrium, resulting in cell death or reduced growth. It is thought that monensin affects mainly Gram-positive bacteria, due to the lack of an outer membrane (Chen and Russell, 1991). However, this may not always be the case, as determined by rRNA sequencing and RT-PCR (Callaway et al., 1999; Weimer et al., 2008). Rumensin does inhibit some hyper-NH_3 producing bacteria, which make up a small percentage of the total bacterial population but may contribute large proportion of ruminal NH_3 (Chen and Russell, 1989). In ruminal fluid from sheep, they produced 23-36% of the rumen NH_3 (Eschenlauer et al., 2002).

The main effects of monensin in vitro and in vivo with respect to N are decreasing ruminal NH_3 concentration and production (Yang and Russell, 1993a), decreasing protein degradation (Whetstone et al., 1981), increasing ruminal NAN and peptides (Chen and Russell, 1991; Van Nevel and Demeyer, 1977), and increasing peptide flow from the rumen (Lana et al., 1997; Poos et al., 1979). The decrease in NH_3 concentration and production has been linked to the activity of bacteria that utilize only peptides and AA as energetic sources. When monensin was added to the diets of nonlactating Holstein cows, these bacteria decreased in number by 10 times (Yang and Russell, 1993b). Similarly, Eschenlauer et al., (Eschenlauer et al., 2002) observed only 1.4% of the total ruminal bacterial population to grow on only Trypticase, and 93% of them were eliminated by 5 μM monensin in vitro, although they displayed variations in monensin sensitivity and NH_3 production rates. These hyper-ammonia producing bacteria are considered to have only minor involvement in peptide degradation, but exert their influence on N dynamics through deamination (Eschenlauer et al., 2002; Russell and Strobel, 1989).

The effects of reducing NH_3 production and peptide degradation leads to higher levels of peptide and AA in the rumen, and therefore, potentially more outflow of these N compounds from the rumen. Previously, this has been termed the “protein sparing effect”. In 262 kg Angus steers fed corn cob and sorghum grain based diets, dietary monensin increased essential and nonessential AA flow when animals were fed brewers dried grains, but not an equivalent N amount of urea (Poos et al., 1979). Monensin feeding also shifted N flow from bacterial to feed N exiting the rumen, and this effect has also been observed in lactating dairy cows (Martineau et al., 2007). Similar results were obtained by Lana et al., (1997), in which Holstein steers fed corn grain and corn silage with SBM or urea supplements. Monensin improved feed efficiency more for SBM supplemented steers than for urea supplemented steers (7.8 vs 1.9% improvement), suggesting there may have been more NAN flow for the animal due to effects previously mentioned in this review.

An interesting effect of monensin feeding is an increase in PUN concentration (Duffield and Bagg, 2000; Martineau et al., 2007; Poos et al., 1979). As ruminal NH_3-N concentrations typically decrease with monensin supplementation, it would be expected that PUN would correspondingly decrease. It is currently unknown why this disparity exits. One potential may be reduced activity of bacterial urease in the rumen. Starnes et al., (1984) demonstrated a 66% decrease in urease activity in rumen fluid from Hereford steers supplemented with 33 ppm monensin. This would most likely lead to reductions in urea recycling to the GIT. Indeed, monensin decreased net portal NH_3-N flux and urea-N recycling in Holstein steers fed 85% concentrate

diets, although arterial urea-N concentrations were not affected (Harmon and Avery, 1987).

Protozoa grown with monensin in culture appeared shrunken and translucent and had abnormal divisions (Sylvester et al., 2009). Concentrations greater than 0.25 M killed nearly all of the three entodiniomorphid strains used. This may be due to its ability to inhibit lysosomal fusion of the food vacuole and proteolysis within it. It also inhibited their ability to decrease generation time with faster passage rates.

Summary

The work presented in this thesis focuses on quantifying the amount of urea synthesized, recycled to the GIT, and utilized by the microbial populations in the rumen for the purpose of refining our understanding of true ruminal N balance and transactions in the rumen and for adjusting current nutritional models to account for this knowledge. Through more accurate recycled N estimates, we are able to reduce N feeding and minimize N waste while maintaining microbial growth in the rumen and therefore MP supply to the cow. Previous work has shown that N intake is a major factor in the amount of N recycled, but its relationship to urea production and GIT entry is not consistent.

Several factors may stimulate urea-N entry to the rumen, such as low ammonia concentrations, carbohydrate fermentation, VFA production, and CO_2 concentration in the ruminal fluid. Therefore, it was presumed that low CP diets or those with readily fermentable carbohydrates would stimulate urea-N entry, possibly overcoming the effects of what otherwise would be a

ruminal N deficiency without detrimental effects on the microbial population or the cow's milk production. In addition, Rumensin was utilized in these studies because of its ability to reduce ruminal protein and AA degradation, leading to less NH_3 formation and absorption. This was assumed to not only make the microbial population more N efficient directly, but to enhance their efficiency by stimulating urea-N recycling into the rumen due to the lower ruminal NH_3 concentrations. Overall, we anticipated stimulating urea-N entry to the rumen via low NH_3 concentrations, microbial growth (direct stimulation due to high amounts of fermentable material producing VFAs in the diet and indirectly due to the resulting lower NH3 concentrations caused by high microbial N demand), and Rumensin. By stimulating urea-N recycling in the presence of an active microbial population, we expected to improve N efficiency in the lactating dairy cow.

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CHAPTER TWO: NITROGEN RECYCLING IN LACTATING DAIRY COWS CONSUMING DIETS DEFICIENT IN EITHER PREDICTED RUMINAL NITROGEN OR METABOLIZABLE PROTEIN

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ABSTRACT

The objectives of this study were 1) to determine the urea-N kinetics in high producing lactating dairy cows fed diets with different formulated MP supply and ruminal N balance as estimated by CPM Dairy and 2) to estimate recycled urea-N utilization and N transactions among the ruminal microbial populations, particularly in reference to protozoal predation of bacteria. Twelve ruminally fistulated Holstein cows were fed one of three diets which would supply adequate MP supply and ruminal N balance (Control, 16.3% CP), adequate ruminal N but deficient MP supply (LoMP, 14.1% CP), or adequate MP supply but deficient ruminal N balance (LoRumN, 14.1% CP) at isocaloric ME concentrations (2.7 Mcal ME/kg DM). The cows were given a continuous jugular infusion of $^{15}N^{15}N$-urea for a minimum of 72 h at 0.0208 g urea/h to label the respective pools. Plasma, milk, urine, feces, and ruminal contents, including microbial populations, were sampled before and after urea infusion for N enrichment analysis. Milk yields and DMI were measured daily during the four days of infusion. DMI and milk yield were lowest for cows fed the LoRumN diet, although the differences were not significant. Milk, plasma, and

ruminal NH_3 concentrations followed dietary CP concentrations, with values of less than 9 mgN/dl for the low CP diets, suggesting near zero ruminal N balances, especially for the LoRumN diets. Urea-N synthesis was greatest for cows fed Control diets and intermediate for LoRumN diets, but a greater proportion of the urea entered the gastrointestinal tract (GIT) instead of the urine for animals fed LoRumN (75% vs. 63% for both the Control and LoMP diets), suggesting the difference in ruminal N balance between these diets stimulated greater GIT urea-N entry. In addition, a higher proportion (60% vs. 51%) of the recycled urea-N was utilized for anabolic purposes. Ruminal microbial pool sizes and estimated bacterial N yield were not affected by treatment. Approximately 8-14% of the microbial N pool was present as protozoal N, and the protozoa predated 4-17% of the total bacterial N yield, with highest values observed for cows fed the Control diet. Recycled N contributed 26-65% of the bacterial N yield, equivalent to 14-33% of the total intake N. This study demonstrated the potential for diets with predicted low ruminal N balance and adequate MP supply to enhance urea-N entry to the GIT and improve the utilization of this N by the microbial population with minimal detrimental effects on microbial growth or milk production. Further, this study demonstrates the potential of this experimental approach to describe N transactions among the rumen microbial populations.

INTRODUCTION

Nitrogen excretion by ruminants is a growing concern because of the negative impacts on the environment and because it represents an inefficient use of nitrogen (N). By the dairy cow, N is excreted in both urine and feces

then volatilized to ammonia (NH_3), which forms precipitates, acidifies precipitation, and decreases general air quality (National Research Council, 2002). As N intake increases, the majority of excess N is excreted in the urine, with fecal N excretion increasing proportionately less (Marini and Van Amburgh, 2005). Urinary urea and purine derivatives are major contributors to NH_3 and nitrous oxide emissions from manure, with N in feces contributing less due to its stability as undigested feed N or microbial N (Van Horn et al., 1994). The ability to understand N requirements of the cow and utilize the N partitioning scheme to enhance efficiency of use by the cow allows nutritionists to focus their efforts on reducing urinary N. An important consideration in achieving this is properly balancing the N requirements of the animal, particularly the rumen N, with high milk yield.

The rumen N balance plays an important role in microbial protein synthesis and efficiency, and therefore, metabolizable protein (MP) supply to the animal. Low balances, indicated by rumen NH_3 of <5 mg/dl, can lead to poor microbial growth, lower fiber digestibility, and reduced DMI (Bryant, 1973; Satter and Slyter, 1974), whereas high balances can lead to excess NH_3 absorption, urea formation, and urine N excretion. Understanding the ruminal N balance may help optimize microbial growth without reductions in production. Field applicable nutritional models such as the NRC (2001) and CNCPS (Tylutki et al., 2008) do not currently include accurate urea-N recycling estimates. By improving these predictions and developing better estimates of microbial use, rumen N balances will become more robust, allowing producers to improve the efficiency of N utilization.

Current research has shown that approximately 15-40% of N intake can be recycled, so it is important to describe this accurately for nutritional estimates

(Lapierre et al., 2004; Ouellet et al., 2004; Valkeners et al., 2007). Further, enhancements of models like the CNCPS (Tyluki et al. 2008) require quantitative data to improve the predictions from the rumen submodel. To refine the predictions of the models, data supporting more of the dynamics of N utilization, including the modeling of the protozoa and their use or incorporation of recycled N and microbial protein are needed to better predict energy and amino acid yields from fermented organic matter intake.

The conversion of N intake into urea ranged from 27-117% among several lactating dairy cow experiments (Baker et al., 2007; Lapierre et al., 2004; Ouellet et al., 2004; Valkeners et al., 2007). However, excluding a couple potential outliers and based on more typical MP balances, this value is more consistently in the range of 50-70%. The proportion of N intake that reenters the gastrointestinal tract (GIT) as urea-N in dairy cows has been reported to range from 25-78%, but it is most commonly 30-45%. Thus, in a typical high producing cow with 600 g N intake, this means that approximately 360 g N is formed into urea and 225 g N reenters the GIT as urea-N (Gozho et al., 2008; Valkeners et al., 2007).

A variety of factors appear to affect N reentry into the GIT. High rumen NH_3-N concentrations have been reported to be negatively correlated to urea-N transfer across the rumen wall, while PUN concentration is positively correlated (Kennedy and Milligan, 1980). It is possible that a combination of diffusion and urea transporter activity adjust accordingly to change rumen plasma clearance rates to allow for urea-N movement to a favorable environment, one where N is either low or in demand by the microbial population. The exact mechanisms behind this transfer are currently un-described, but previous work has given insight into patterns of behavior. The

kidney is playing a major role in the movement of urea back into circulation, where facilitated transporters exist all along the GIT and provide the mechanism for transfer into the GIT, but how all of this redirection is controlled is still not fully appreciated (Marini and Van Amburgh, 2003; Stewart et al., 2005).

In addition to N concentrations across the rumen wall, fermentation of readily available carbohydrates have demonstrably increased urea-N transfer into the rumen. In sheep supplemented with sucrose and/or urea, increases in urea-N recycling were observed for increasing amounts of sucrose supplementation and decreasing concentrations of ruminal NH_3-N concentration (Kennedy et al., 1981) There might also be influences on recycling via VFA production, carbon dioxide solubility, and pH, but the impact of these factors are currently less well defined in the context of the high producing dairy cow (Remond et al., 1993; Thorlacius et al., 1971).

Information on how the microbial population utilizes recycled urea N is lacking, especially quantitative data on the relationship between inter- and intra-ruminal N recycling and utilization among the microbial population. An important value is the amount of recycled urea-N that is incorporated by the microbes and formed into microbial protein. This is the fraction that is able to be utilized by the animal and represents a large proportion of the anabolic fraction of recycled urea N in the model of Lobley et al. (2000). High concentrations of ruminal NH_3-N from the diet will decrease the capture of recycled N due to the dilution effect of the dietary NH_3-N supply. For example, in lactating cows fed high concentrate diets, recycled urea-N contributed 37.5% of the duodenal bacterial N, while this was only 12.7% for those fed high forage diets with similar amounts of N (Al-Dehneh et al., 1997). And in

Holstein heifers fed isocaloric diets, increasing N intake from 1.45 to 3.4% of DM decreased the proportion of bacterial N from recycled urea-N from 18.7% on the low N diet to only 4.3% on the high N diet (Marini and Van Amburgh, 2003). If producers are to reduce N waste in cattle, the most beneficial improvements will be made by capture of this recycled N into the microbial populations, and that is really the balancing procedure the industry has been working towards since the inception of protein solubility and rumen degradable protein concepts.

The objectives of this study were to determine the amount of urea-N recycled in lactating dairy cows consuming diets predicted to be deficient in either MP or rumen N with the purpose of using these estimates for improving N recycling predictions. Diets deficient in rumen N balance were hypothesized to have a greater amount of urea-N recycling, due to the predicted low rumen N status and high potential microbial growth. Diets deficient in MP were hypothesized to recycle relatively less urea-N due to the predicted high rumen N status and to excrete a larger amount of excess rumen N via urine. A further objective was to evaluate the potential of the double labeled urea procedure of Lobley et al. (2000) to be applied to compartmental movements of N among the rumen microbial populations in order to estimate the consumption or predation of bacteria by protozoa and further the estimates of anabolism.

MATERIALS AND METHODS

Cows and Experimental Design

Twelve multiparous, lactating dairy cows (68 DIM +/- 6 days at initiation of treatment diets; or DIM 162 +/- 20 days, BW 613 kg +/- 53 kg at the time of urea infusions) fitted with ruminal fistulas were fed three diets in a study designed as a randomized complete block, blocking by average milk yield over the first 50 days of production, to investigate the effects of N intake and form on urea N recycling and efficiency of use. The Cornell University Institutional Animal Care and Use Committee approved all animal-related procedures. Animals were housed in individual stalls and fed a TMR once daily at approximately 0900 for 10% refusals. The TMR consisted of approximately 45% corn silage, 2% wheat straw, and 53% concentrate mix specific to the diet objectives (Table 2.1).

This study was a subset of a larger lactation study conducted on 88 cows (Recktenwald, 2007). The twelve animals described in this report were randomly assigned to treatments on the lactation study and were utilized in this more intensive N recycling study. Diets were formulated in CPM Dairy v3.0 (Tedeschi et al., 2008) for a 625 kg cow producing 36.3 kg milk/d at 3.70% fat and 2.95% true protein and consuming 22.2 kg DMI/d. The Control diet (16.3% CP) was formulated to represent a diet in which both rumen N and MP were positive. The LoMP treatment diet (14.1% CP) was designed to provide adequate ruminal N, but be deficient in MP relative to ME allowable and actual milk yield. Finally, the LoRumN diet (14.1% CP) was formulated to

Table 2.1. Proportional composition of ingredients for Control, LoMP, and LoRumN diets.

	% of total ration DM		
Ingredient	**Control**	**LoMP**	**LoRumN**
Corn silage, processed	46.13	45.24	45.34
Wheat straw, chopped	2.10	2.06	2.06
Soybean hulls	4.19	4.11	4.12
Corn grain, finely ground	11.12	11.31	10.92
Cottonseed, whole with lint	8.39	8.23	8.45
Citrus pulp	5.24	9.26	9.28
Soybean meal (47.5% CP)	5.77	7.81	......
Barley grain, ground	4.19	8.23	6.18
Expeller soybean meal[1]	6.29	......	5.15
Animal protein blend[2]	1.05	......	2.27
Mepron[3]	0.05	......	0.05
Sugar[4]	2.62	0.82	3.09
Nitroshure[5]	0.38	0.41	0.35
Vitamin pre-mix[6]	0.25	0.24	0.25
Salt	0.50	0.55	0.50
Calcium diphosphate	0.11	0.16	0.29
Calcium sulfate	0.21	0.24	0.25
Magnesium oxide	......	0.02	0.04
Magnesium sulfate	0.21	0.20	0.25
Limestone	1.20	0.97	0.86
Potassium chloride	......	0.12	0.31

[1]Soyplus, West Central Cooperative, Ralston, IA
[2]Provaal, Venture Milling, Fulton, NY
[3]Degussa Corp. Parsippany, NJ
[4]Blend of 50% sucrose and 50% confectioner sugar. (Round House Mills, Cortland, NY)
[5]Balchem Corp., New Hampton, NY
[6]Formulated to provide (per kg of DM) 30 g Ca, 250 g Mg, 60 g K, 88 g S, 3.7 g Cl, 8.6 g Fe, 18.6 g Zn, 6 g Cu, 16 g Mn, 100 mg Se, 330 mg Co, 570 mg I, 3022 KIU vitamin A, 1027 KIU vitamin D, 20264 IU vitamin E.

be negative in ruminal N balance, but have a positive MP balance relative to ME allowable and actual milk yield (see Table 2.2).

All diets were formulated to be isocaloric at 2.72 Mcal ME/kg and formulated ME allowable milk was similar (Table 2.2). Monensin (Elanco Animal Health, Greenfield, IN) was included in the TMR at 300 mg per cow per day and cows were given bST per label (Posilac, Monsanto Co., St. Louis, MO) and administration was timed to be uniform around all of the urea infusion measurements to prevent bias due to bST cycle; all urea infusions and collections were conducted from day 4 to day 10 of the bST cycle. Cows were fed their respective treatments diets for 68 +/- 6 days before urea infusion.

Table 2.2. The CPM Dairy V3.0 predicted performance for a 626 kg cow consuming 22.2 kg dry matter intake per day of Control, LoMP, or LoRumN diets and producing 36.3 kg milk per day at 3.70% fat and 2.95% true protein.

Predicted performance	Control	LoMP	LoRumN
ME allowable milk, kg/d	40.4	39.4	39.4
ME balance, Mcal/d	4.48	3.43	3.38
MP allowable milk, kg/d	42.1	33.1	38.3
MP balance, g/d	264	-145	91
Peptide and NH_3 balance, g/d	29	27	-39
Peptide and NH_3 balance, % req.	107	107	90
Peptide balance, g/d	18	4	-46
Peptide balance, % req.	109	102	79
MP from bacteria, g/d	1434	1478	1347
MP from RUP, g/d	1248	808	1185

Cows were milked 3x/d at 0800, 1600, and 0000 h by an inline milking system and weighed via digital scale. Milk samples were taken via subsampling the milk pail and preserved with 2-bromo-2-nitropropane-1,3-dial for component analysis (Dairy One, Foss Milkoscan 6000, Ithaca, NY). Feces, urine, bacteria, protozoa, rumen fluid and plasma samples were taken prior to the initiation of isotope infusion. Feces were collected by voluntary elimination in plastic bags, weighed, and frozen. Urine was collected by manual agitation of the lower vulva, 50% H_2SO_4 was added until pH < 2, and samples were frozen in conical vials. Blood was collected into Vacutainers containing heparin (Becton Dickinson, Rutherford, NJ) via the coccygeal vessels, placed on ice, and then centrifuged at 1500xg for 15 min to obtain plasma. Ruminal contents were taken from five different locations around the rumen, then fluid was squeezed through 4 layers of cheesecloth, acidified with 1 ml 50% (v/v) H_2SO_4 in 40 ml rumen fluid, and frozen for ruminal ammonia analysis. Formalin was added at 2.6% (v/v) to strained ruminal fluid and frozen for AA analysis or 0.3 ml 50% H_2SO_4 was added to 12 ml of fluid for VFA analysis. The remaining fluid was centrifuged at 27000xg for 30 min and frozen for collection of bacteria free fluid. Protozoa were isolated from strained rumen fluid by flocculation, addition of 1% formalin, then centrifugation at 500xg for 5 min. at 10ºC (Sylvester et al., 2004). Fluid associated bacteria were isolated from the supernatant by centrifugation at 27000xg for 30 min. at 4ºC (Martin et al., 1996). Particle associated bacteria were prepared from the solid rumen contents by a 30 min incubation in 0.1% methylcellulose followed by blending in a Waring blender then 24 h incubation in a solution of 0.1% Tween 80 and 1% methanol in saline at pH 2. Contaminant particles were eliminated via fluid

and saline washed solids were centrifuged at 1000xg for 15 min at 4ºC. Bacteria were pelleted by a final centrifugation at 27000xg for 20 min at 4ºC (Whitehouse et al., 1994). All microbial pellets were freeze dried for analysis (Virtus Freeze dryer).

Cows were prepared with indwelling catheters (Micro-renethane, Braintree Scientific, Inc., Braintree, MA) in the jugular vein for infusing urea solution. The next day, a continuous infusion of double-labeled urea ($^{15}N^{15}N$-urea, 98% purity, Cambridge Isotope Laboratories Inc., Andover, MA) in sterile saline (9 g NaCl/L) was conducted at a rate of 0.0208 g urea/h. After 72 hours, fecal, urine, bacterial, protozoal, rumen fluid, and plasma were again collected, with the addition of a milk sample for analysis of milk protein enrichment. Milk protein was precipitated from defatted milk with a 65% TCA solution (Hristov and Ropp, 2003) and freeze-dried for analysis. On the final day of urea infusion, rumen contents were emptied from each animal, weighed, and subsampled. Subsamples were freeze dried and used to determine rumen DM content.

Urine samples were collected via an external funnel system; however, significant urine leakage occurred, confounding the measurement. In lieu of total urine collection, urine spot samples were used for isotopic urea measurements and CNCPS v6.1 (updated from Tylutki et al., (2008)) predictions for daily urine N excretion were used for daily N manure output. The equations for the CNCPS predictions were recently modified and evaluated by Higgs et al., (2009), and provided a more accurate partitioning between urinary and fecal N excretions. Incorporating a more accurate fecal N prediction into the current CNCPS framework established a new excretion prediction of urinary N (MSPE = 970, R^2_{MP} = 0.86, CCC = 0.90). The changes

to fecal N and urinary N translate into an improved prediction of total manure N (MSPE = 623, R^2_{MP} = 0.96, CCC = 0.97) and have been incorporated into the latest version of the CNCPS v6.1. Variation in total manure collection studies ranges from 10-30% (Firkins and Reynolds, 2005; Reynolds and Kristensen, 2008); thus, the CNCPS estimates with actual diet chemistry, DMI inputs and animal characterization values are most likely more accurate than actual total excretion measurements on average. Therefore, these predictions, especially the urinary N excretion were considered adequate for providing inputs in the urea-N kinetics calculations.

Fecal, bacterial, protozoal, milk protein, and plasma ^{15}N enrichment were determined on the freeze dried sample with a NC2500 Carlo Erba elemental analyzer (Milan, Italy) interfaced to a ThermoFinnigan Delta Plus isotope ratio mass spectrometer (Bremen, Germany). Urinary urea was isolated using a cation exchange resin (AG 50W-X8, 100-200 mesh hydrogen form, Bio-Rad Laboratories, Hercules, CA), diluted to 6 mmol/L, and reacted with lithium hypobromite under vacuum (Marini et al, 2006). The N_2 gas was analyzed in Exetainers I (Labco Limited, Buckinghamshire, UK) using a PDZ Europa Geo 20/20 isotope ratio mass spectrometer (Cheshire, UK) attached to the ANCA-trace gas/liquid system. The model of Lobley et al., (2000) was used to calculate urea kinetics.

Feed, fecal, and urinary N were determined by a macro Kjeldahl procedure (Association of Official Analytical Chemists International, 1990). Ruminal fluid ammonia and plasma urea concentrations were measured colorimetrically using the procedure of Chaney and Marbach, (1962). Volatile fatty acids were measured by HPLC using crotonic acid as an internal standard (Seigfried et al., 1984). Purine base concentrations for guanine and adenine were also

determined in the urine samples via HPLC procedures using the method of Shingfield and Offer, (1999).

As previously indicated, the actual milk yield, DMI, fat %, protein %, BW, and BCS were used as inputs to CNCPS v6.1 to determine predicted milk yield, urine N, and fecal N. In addition, urinary N excretion was determined by the equation: Urine N (g/d) = BW*0.0259 + MUN*2.25 + 6.5 (Nennich et al., 2006) as a check on the values measured from the cows and the CNCPS predictions.

Microbial Calculations

The following calculations were used to derive the various microbial pool sizes. The acronyms LAB, PAB, and PZ will be used for liquid associated bacteria, particle associated bacteria, and protozoa, respectively.

$$\text{Microbial N (LAB, PAB, or PZ) APE} = \frac{\text{enriched }\%^{15}\text{N} - \text{background }\%^{15}\text{N}}{\text{background }\%^{15}\text{N}}$$

Protozoal N APE was corrected for 8% liquid associated bacterial contamination according to previous work using similar microbial isolation methods (Sylvester et al., 2005):

$$\text{PZ N APE} = \frac{\text{PZ N APE} - 0.08 * \text{LAB N APE}}{0.92}$$

The proportion of protozoal N that originated from liquid or particle associated bacteria was calculated based on fact that the fraction of Pool B

from Pool A = Pool B APE / Pool A APE (Nolan and Leng, 1972), or in this case sum of the fraction of PZ N from LAB N (equal to PZ N APE / LAB N APE) and the fraction of PZ N from PAB N (equal to PZ N APE / PAB N APE) equals unity:

$$\frac{\text{PZ N APE}}{\text{LAB N APE}} + \frac{\text{PZ N APE}}{\text{PAB N APE}} = 1$$

which leads to:

$$\frac{\text{PZ N APE}}{\text{LAB N APE}} = \frac{\text{PAB N APE}}{\text{PAB N APE + LAB N APE}}$$

$$\frac{\text{PZ N APE}}{\text{PAB N APE}} = \frac{\text{LAB N APE}}{\text{PAB N APE + LAB N APE}}$$

The amount of bacteria present in the rumen at the time of sampling was calculated through the microbial mass isolated, the mass of liquid or particles in the rumen sample, and the total rumen liquid or particulate mass.

$$\text{LAB in rumen, g} = \frac{\text{g bacterial DM isolated in sample}}{\text{g liquid in sample}} * \text{g liquid in rumen}$$

$$\text{PAB in rumen, g} = \frac{\text{g bacterial DM isolated in sample}}{\text{g particles in sample}} * \text{g DM in rumen}$$

$$\text{PZ in rumen, g} = \frac{\text{g bacterial DM isolated in sample}}{\text{g liquid in sample}} * \text{g liquid in rumen}$$

Liquid associated bacteria and protozoa mass in the rumen was corrected for liquid associated bacterial contamination in the protozoal isolation:

$$\text{LAB in rumen, g} = \text{initial LAB in rumen} + 0.08 * \text{initial PZ in rumen}$$

$$\text{PZ in rumen, g} = \text{initial PZ in rumen} * 0.92$$

The nitrogen concentration of each microbial pool was measured by the IRMS, and the protozoal %N was corrected for liquid associated bacterial contamination as follows:

$$\text{PZ \%N} = \frac{\text{PZ \%N measured} - \text{LAB \%N} * 0.08}{0.92}$$

Grams of microbial N in each pool were calculated based on the rumen microbial pool size and the N concentration:

$$\text{grams LAB N} = \frac{\text{g LAB} * \text{LAB \%N}}{100\%}$$

$$\text{grams PAB N} = \frac{\text{g PAB} * \text{PAB \%N}}{100\%}$$

$$\text{grams PZ N} = \frac{\text{g PZ} * \text{PZ \%N}}{100\%}$$

$$\text{Fraction of microbial N} = \frac{\text{g PZ N}}{}$$

pool as PZ N $\overline{\text{g LAB N + g PAB N + g PZ N}}$

^{15}N enrichment was measured via IRMS, and the following correction was made for both the background and enriched protozoal N samples due to 8% liquid associated bacterial contamination:

$$\text{PZ } ^{15}\text{N enrichment} = \frac{\text{initial PZ } ^{15}\text{N enrichment} - \text{LAB } ^{15}\text{N enrichment} * 0.08}{0.92}$$

The ^{15}N pool size for each of the microbial populations was calculated based on the rumen microbial mass, ^{15}N enrichment prior to (bgrd) and after 72 hours of ^{15}N^{15}N-urea infusion (enr), and the N concentration of the appropriate microbe:

$$\text{Rumen LAB } ^{15}\text{N, g} = \frac{\text{g LAB * LAB } ^{15}\text{N enr * LAB \%N}}{100\% * 100\%} - \frac{\text{g LAB * LAB } ^{15}\text{N bgrd * LAB \%N}}{100\% * 100\%}$$

$$\text{Rumen PAB } ^{15}\text{N, g} = \frac{\text{g PAB * LAB } ^{15}\text{N enr * PAB \%N}}{100\% * 100\%} - \frac{\text{g PAB * PAB } ^{15}\text{N bgrd * PAB \%N}}{100\% * 100\%}$$

$$\text{Rumen PZ } ^{15}\text{N, g} = \frac{\text{g PZ * PZ } ^{15}\text{N enr * PZ \%N}}{100\% * 100\%} - \frac{\text{g PZ * PZ } ^{15}\text{N bgrd * PZ \%N}}{100\% * 100\%}$$

The mass of liquid associated bacteria consumed by protozoa (at one point in time, or present in the rumen at the time of isolation) if all of the rumen

protozoal N originated from the liquid associated bacteria or particle associated bacteria was calculated as follows:

$$\text{grams LAB consumed by PZ} = \frac{\text{g PZ }^{15}\text{N}}{\dfrac{(\text{LAB }^{15}\text{N enr} - \text{LAB }^{15}\text{N bgrd})}{100\%} * \dfrac{\text{LAB \%N}}{100\%}}$$

$$\text{grams PAB consumed by PZ} = \frac{\text{g PZ }^{15}\text{N}}{\dfrac{(\text{PAB }^{15}\text{N enr} - \text{PAB }^{15}\text{N bgrd})}{100\%} * \dfrac{\text{PAB \%N}}{100\%}}$$

As protozoa consumed both liquid and particle associated bacteria, the amount of each bacterial pool consumed by protozoa if they were consumed in the proportions suggested by APE measurements is calculated below (at one point in time, or present in the rumen at the time of isolation) from the prior two equations and those supplying the proportion of protozoal N from each pool (see above):

$$\text{LAB and PAB consumed by PZ, g} = \text{grams of LAB consumed by PZ} * \text{Fraction of PZ N from LAB N} + \text{grams of PAB consumed by PZ} * \text{Fraction of PZ N from PAB N}$$

The mass of bacterial N that was consumed by protozoa (at one point in time, or present in the rumen at the time of isolation) if they were consumed in the proportions suggested by APE measurements (see equations above) is as follows:

$$\text{LAB N and PAB N consumed by PZ, g} = \frac{\text{grams of LAB consumed by PZ (if all PZ N from LAB) * LAB \%N * Fraction of PZ from LAB}}{100\%} + \frac{\text{grams of PAB consumed by PZ (if all PZ N from PAB) * PAB \%N * Fraction of PZ from PAB}}{100\%}$$

Since we did not measure daily omasal or duodenal flows, the daily bacterial N yield was based on CNCPS estimates (Tylutki et al., 2008), and bacterial turnover rates were calculated based on the daily bacterial N yield and the rumen bacterial pool sizes.

$$\text{Bacterial N turnover (d-1)} = \frac{\text{CNCPS bacterial N yield (g N/d)}}{\text{LAB N in rumen + SAB N in rumen}}$$

Protozoal turnover rates were based on bacterial turnover rates and estimated to be equivalent (100%) to, three-fourths of (75%), or half of (50%) the bacterial turnover rates, as no protozoal yield measurement was available (Dehority, 2003; Sylvester et al., 2005).:

$$\text{Protozoal N turnover (d-1)} = \text{bacterial N turnover } (d^{-1}) \text{ * [0.50, 0.75, 1.00], depending on estimated protozoal:bacterial turnover rate}$$

The amount of bacteria consumed by the protozoa daily is calculated based on the amount of equivalent amount of each bacterial type that was consumed by the protozoa at the time of isolation multiplied by the turnover rate of the protozoal population:

$$\text{grams of bacteria consumed by protozoa per day} = \text{g LAB and PAB consumed by PZ * PZ turnover}$$

$$\text{grams of bacterial N consumed by protozoa per day} = \text{g LAB N and PAB N consumed by PZ * PZ N turnover}$$

The proportion of the total bacterial N produced each day that was consumed by the protozoa was calculated with daily bacterial N yield and protozoal consumption:

$$\text{Fraction of total bacterial N yield consumed by protozoa} = \frac{\text{bacterial N yield (g N/d)}}{\text{g bacterial N consumed by protozoa per day}}$$

Daily protozoal N yield was calculated based on the rumen protozoal N pool size and the turnover rate:

$$\text{Protozoal N yield (g N/d)} = \text{g protozoal N in rumen * protozoal turnover}$$

Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS with cow as a random factor and repeated measures by day of infusion with diet as the treatment and cow as the subject were used in the analysis of milk yield, milk components and component yield, DMI, N and feed efficiency (SAS Inst., Cary, NC). The following model was used:

$Y_{ijk} = \mu + C_i + T_j + \varepsilon_{ijk}$

where C_i is the effect of the ith cow, T_j is the effect of the jth treatment, and ε_{ijk} is random error.

A first order autoregressive structure [AR(1)] was used as the covariance structure with the Kenward-Roger adjustment. This structure was chosen due to its low Bayesian information criteria compared to the simple, unstructured, compound symmetry, or heterogeneous compound symmetry structures (Littell et al., 1996). DIM and milk yield before treatment were used as covariates in the analysis of milk yield. DIM was not significant in the analysis of DMI, so it was not used in the reported analysis. Contrasts of the treatment means were conducted with the pdiff option. The LSMEANS option was used to determine treatment means. Significance was declared at $P < 0.05$ and a trend was declared at $P < 0.10$.

RESULTS AND DISCUSSION

Dry Matter and Nitrogen Intakes

Data from the corresponding lactation study conducted simultaneously on 88 cows over a 100 d period, including the 12 presented in this paper, are shown in Table 2.3 for comparison.

As observed in the larger study, cows fed the LoRumN diet consumed numerically less than those fed either LowMP or Control (Table 2.4). However, due to the fewer numbers of cows over a shorter period of time, the differences were not significant in the infusion study as in the larger study. These DMI were similar to that observed in other studies feeding approximately 50% corn silage diets (Cabrita et al., 2007; Groff and Wu, 2005; Wattiaux and Karg, 2004a). The N intake followed formulation, and combined

with lower DMI for cows fed LoRumN, the intake of N was highest for animals on Control, with LoMP and LoRumN N intakes approximately 80 and 140 g N/d lower, respectively (Table 2.4). The diets LoMP and LoRumN were formulated to contain similar amounts of CP, but due to greater DMI of the animals consuming LoMP, the N intakes for these cows were numerically greater by approximately 60 g N/d.

Table 2.3. Production and nitrogen concentration measurements for 88 cows fed Control, LoMP, and LoRumN diets for 100 days.

Performance	Control	LoMP	LoRumN	SEM	Diet effect
n	29	29	30		
DMI, kg/d	25.66^{a}	25.45^{a}	24.21^{b}	0.40	0.02
Milk yield, kg/d	45.00^{a}	42.62^{b}	43.29^{ab}	0.75	0.06
Fat %	2.68	2.67	2.54	0.08	0.37
Protein %	2.93	2.92	2.90	0.04	0.85
Fat yield, kg/d	1.20^{a}	1.12^{ab}	1.09^{b}	0.03	0.09
Protein yield, kg/d	1.32^{a}	1.23^{b}	1.24^{b}	0.02	0.01
PUN, mg/dl	11.31^{a}	8.40^{b}	7.13^{c}	0.14	<0.001
MUN, mg/dl	11.11^{a}	8.74^{b}	8.43^{b}	0.20	<0.001

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by means separation procedure in the Mixed procedure of SAS (2001).

Table 2.4. Least square means of body weight, body condition score, and nutrient intake measurements for cows fed Control, LoMP, and LoRumN diets for the four days of $^{15}N^{15}N$ urea infusion and total collection.

	Control	LoMP	LoRumN	SEM	Diet effect
DIM, d	178[a]	158[a]	156[a]	14	0.51
BW, kg	646[a]	607[a]	586[a]	36	0.30
BCS (1-5)	2.63[a]	2.66[a]	2.63[a]	0.21	0.99
DM intake, kg/d	24.3[a]	25.2[a]	22.0[a]	2.8	0.70
N intake, gN/d	659[a]	583[a]	521[a]	66	0.35
NDF intake, kg/d	8.34[a]	8.50[a]	7.88[a]	1.01	0.90

[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by means separation procedure in the Mixed procedure of SAS (2001).

Milk Yield and Components

Milk yield was numerically lowest for cows consuming LoRumN, with marginal differences between yield for LoMP and Control treatments (Table 2.5). This differed from the results of the full study, where milk yields were highest for animals fed Control, with LoMP cows having the lowest milk yield and significantly different from Control, while LoRumN cows had intermediate production (Table 2.3). Also, the set of cows used for infusions produced approximately 10-15 kg milk/d less than their herdmates on the full study, and inclusion of milk production of these cows one week before and after infusion did not drastically change these results, as they were only 1-2 kg/d different. Thus, the lower milk yields observed on the infusion study were due to use of

lower production, and in this case, fistulated animals rather than infusion conditions and were similar to previously reported yields of cows consuming this type of diet (Cabrita et al., 2007; Weiss and Wyatt, 2006).

Table 2.5. Least square means of milk yield and milk composition measurements for cows fed Control, LoMP, and LoRumN diets for the four days of $^{15}N^{15}N$ urea infusion and total collection.

	Control	LoMP	LoRumN	SEM	Diet effect
Milk yield, kg/d	32.5^{a}	33.0^{a}	27.3^{a}	3.3	0.28
Milk fat %	3.16^{a}	3.17^{a}	2.71^{a}	0.24	0.33
Milk protein %	3.04^{a}	2.87^{a}	2.87^{a}	0.17	0.72
Milk lactose %	4.72^{a}	4.86^{a}	4.71^{a}	0.09	0.49
Milk fat yield, kg/d	0.99^{a}	1.03^{a}	0.75^{a}	0.09	0.10
Milk protein yield, kg/d	0.93^{a}	0.88^{a}	0.88^{a}	0.05	0.71
Milk lactose yield, kg/d	1.46^{a}	1.49^{a}	1.45^{a}	0.03	0.62
MUN, mg/dl	9.74^{b}	6.32^{a}	8.82^{a}	0.17	0.72
PUN, mg/dl	13.10^{a}	9.16^{b}	7.67^{b}	0.94	<0.0001
N efficiency, milk N/intake N	0.25^{a}	0.26^{a}	0.25^{a}	0.03	0.96
Feed efficiency, kg feed/kg milk	0.72^{a}	0.79^{a}	0.79^{a}	0.07	0.71

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by means separation procedure in the Mixed procedure of SAS (2001).

No significant differences were observed among treatments in milk fat and protein concentrations, and in general, the cattle on the infusion study appeared to have less severe milk fat depression than the remainder of the treatment animals (Tables 2.3 and 2.5). There was a trend for cows consuming LoRumN for lower milk fat yield (25% less than observed in other diets), due to lower fat percentage and this was due to the combination of unsaturated fat intakes, Rumensin, and potentially limiting rumen N levels (Table 2.5). Milk protein was numerically highest for cows fed Control, which translated into slightly higher milk protein yield for these cows, but not to a level of significance, as was observed in the full study. Milk lactose percent and yield were not observed to be significantly different among treatments.

Milk MUN, as anticipated, was significantly higher for animals fed the Control diet (9.7 mg/dl) and corresponds to other studies in which the majority of the forage was corn silage with similar dietary CP concentrations (Wattiaux and Karg, 2004b). However, cows fed the LoMP diet had exceptionally low MUN values (6.3 mg/dl), while those fed the LoRumN diet were intermediate (8.8 mg/dl) (Table 2.5). According to Broderick et al., 2009, PUN and MUN values of 8 mg/dl are near zero ruminal N balance, and animals fed LoMP and LoRumN appeared to be at this level. The low MUN values for cows on the LoMP diet were not expected and might be due to NIR analysis error, as calibration standards are not typically used in these ranges (Kohn et al., 2004). In addition, these MUN values were lower than the PUN measurements, which were approximately 2.8 mg/dl higher (Table 2.5), indicating some bias in the MUN readings. This has been addressed with Dairy One (Ithaca, NY) and we believe that an upgrade in technology has alleviated the bias in MUN measurements.

Feed efficiency and the use of intake N for milk production were not affected by the treatments. Although a small sample size and with instrumented animals, the lower CP diets were not more efficient than the Control diet and given the objectives of this study, this was not expected to be a primary outcome. In the more complete lactation study, the low CP diets did in fact improve N efficiency (0.36 for LoRumN and 0.33 for LoMP vs. 0.31 for Control) and were consistent with previous data (Wattiaux and Karg, 2004a).

Ruminal NH_3-N concentrations were numerically highest for animals fed LoMP (8.3 mg/dl), followed by Control (7.4 mg/dl) then LoRumN (6.6 mg/dl) (Table 2.6). Cows fed Control and LoMP would be expected to have higher ruminal NH_3-N concentrations due to the formulated higher rumen ammonia balance and dietary RDP compared to LoRumN (9.6 vs 7.8 % of DM). Rumen NH_3-N concentrations below 5 mg/dl are anticipated to limit microbial growth, and the levels measured in cows fed LoRumN suggest that the bacterial N supply might have been near a deficiency (Satter and Slyter, 1974). Diminished microbial activity due to low NH_3-N concentrations and poor fermentation might have partially explained the lower DMI observed for the cows fed LoRumN consistent with the data of Ruiz et al., (Ruiz et al., 2002). Overall, rumen NH_3 and PUN concentrations generally followed the total N intake and reflect the total pool size of intake N and this will subsequently be reflected in the total amount of hepatic urea production.

No significant differences among treatments were observed in ruminal VFA concentrations; however, total VFAs and most of the branched chain VFAs were numerically greatest for animals fed LoRumN (Table 2.6). It was anticipated that cows fed the Control and LoMP diets to potentially have higher ruminal BCVFA concentrations, as they contain higher RDP levels due to

more ruminally degradable protein sources. These effects were not observed, but there was much variation in VFA concentrations and concentration is not always positively related to VFA production (Sutton et al., 2003). Fiber digesting bacteria have a requirement for BCVFA

Table 2.6. Ruminal volatile fatty acids, ammonia, and pH concentrations in cows fed Control, LoMP, and LoRumN diets over the four days of $^{15}N^{15}N$ urea infusion and total collection.

	Control	LoMP	LoRumN	SEM	Diet effect
Total VFA, mM	117	119	138	14	0.52
Lactate, mM	0.38	0.04	0.82	0.58	0.64
Formic acid, mM	7.9	10.0	8.8	1.8	0.71
Acetic acid, mM	65.0	65.1	68.8	5.3	0.85
Propionic acid, mM	30.1	29.6	43.6	6.8	0.27
Acetate:propionate	2.41	2.44	1.97	0.30	0.46
Isobutyric acid, mM	0.86	0.73	1.14	0.26	0.53
Butyric acid, mM	10.5	10.0	13.6	2.05	0.44
Isovaleric acid, mM	1.79	2.39	1.61	0.26	0.14
Valeric acid, mM	1.00	1.43	1.59	0.44	0.62
Ruminal pH	5.95	6.02	5.94	0.23	0.97
Ruminal NH_3, mg/dl	7.35^a	8.32^a	6.57^a	2.91	0.91

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Urea-N Kinetics

Urea synthesis (urea-N entry rate, UER) was greatest for those animals fed the Control diet, which also had the highest N intakes (Table 2.7). Correlations between N intake and UER have been reported by various studies and overall, N intake appears to have the greatest influence on UER (Baker et al., 2007; Lapierre and Lobley, 2001; Valkeners et al., 2007). However, animals fed the LoRumN diet had approximately 30 gN/d more UER than those fed the LoMP diet, even though their N intakes were approximately 60 gN/d lower. Given the expected route of N metabolism, this was most likely due to partitioning the urea-N to GIT entry rather than urine excretion and then recycling more urea-N through absorption of bacterial N and re-recycling into the ammonia pool through microbial turnover in the rumen or intestinal uptake. In addition, given the differences in rumen NH_3 concentration and the form of the intake N in cows fed the LoMP diet, the dietary N was more quickly deaminated to NH_3-N for absorption as compared to the LoRumN diet, negating the direct impact of N intake on UER and GER absolute amounts. In a study referenced by Lapierre and Lobley, (2001), cows fed less degradable protein but a similar amount of total NH_3-N and AA-N had lower urea-N synthesis, demonstrating that the form of dietary N impacts urea-N synthesis. However, this is not consistent among all experiments as N intake appears to be the predominant factor affecting urea-N synthesis.

Cows fed the LoRumN diet had numerically the highest GER, which supported our hypothesis that these animals would be recycling a larger amount of N due to the lower formulated ruminal N balance (Table 2.7). Based on the lower DM and N intakes, ruminal NH_3 concentrations, and PUN

values, it appears that these animals were indeed approaching zero ruminal N balance. Previous work has found more urea-N entry to the rumen or GIT with low NH_3 concentrations, and it appears that this might have enhanced urea-N entry in our cows as well (Kennedy, 1980). However, rumen NH_3-N concentrations do not appear to regulate urea-N entry alone, but it is a combination of a variety of factors such as CO_2 and SCVFA concentrations and pH (Abdoun et al., 2009). The values from this experiment are similar to those reported by (Valkeners et al., 2007) in lactating cows fed diets of similar N intake but lower GER than cows fed higher N diets (Gozho et al., 2008).

The proportion of urea-N synthesis entering the GIT (0.63-0.75) corresponded well with other urea-N kinetic studies performed in a range of animals, from lactating dairy cows (Gozho et al., 2008; Ouellet et al., 2004; Valkeners et al., 2007), sheep (Kiran and Mutsvangwa, 2007; Lobley et al., 2000; Marini et al., 2003; Sarraseca et al., 1998; Sunny et al., 2007), and heifers (Baker et al., 2007). Given the results from this study in combination with previous work, it is apparent that dairy cows consistently recycle 60-75% of their ureagenesis, with slight variations depending on rumen N and fermentation status. It also supports work describing the high transfer capacity of urea transporters in the GIT, suggesting that these transporters have an almost requisite capacity or ability to transfer urea across the GIT wall based on the presence of plasma urea N.

Urea-N excretion rates (UUE) corresponded with N intakes, albeit the differences were non-proportional (Tables 2.4 and 2.7). Very low urinary urea-N excretion was observed for animals fed the LoRumN diet, which was most likely due to the high efficiency of urea-N transfer to the GIT, as discussed earlier. The proportion of urea-N synthesis diverted to urinary N in this study

(0.25-0.38) was similar to other work in dairy cattle consuming similar amounts of N (Gozho et al., 2008; Lapierre et al., 2004; Valkeners et al., 2007).

Table 2.7. Urea-N recycling kinetics for cows fed Control, LoMP, and LoRumN diets over the four days of $^{15}N^{15}N$ urea infusion and total collection.

	Control	**LoMP**	**LoRumN**	**SEM**	**Diet effect**
UER[a] (gN/d)	293.3	221.6	253.8	44	0.49
UUE[b] (gN/d)	108.8	84.3	48.9	19.6	0.22
GER[c] (gN/d)	184.6	137.4	204.8	45.6	0.57
ROC[d] (gN/d)	86.1	63.2	70.9	14.2	0.49
UFE[e] (gN/d)	1.19	0.80	0.42	0.32	0.35
UUA[f] (gN/d)	97.3	73.4	133.6	37.9	0.58
UER to urine[g]	0.37	0.38	0.25	0.09	0.62
UER to GIT[h]	0.63	0.63	0.75	0.09	0.62
GER to ROC[i]	0.49	0.49	0.39	0.10	0.80
GER to UFE[j]	0.007	0.006	0.003	0.002	0.53
GER to UUA[k]	0.51	0.51	0.60	0.10	0.79

[a]UER = urea-N entry rate
[b]UUE = urinary urea elimination rate
[c]GER = gastrointestinal tract (GIT) urea entry rate, or recycled urea-N
[d]ROC = urea returning to the ornithine cycle
[e]UFE = urea-N excreted in the feces
[f]UUA = urea-N utilized for anabolic purposes
[g]Proportion of the urea entry rate (UER) that enters the urine
[h]Proportion of the urea entry rate (UER) that enters the gastrointestinal tract (GIT)
[i]Proportion of the gastrointestinal tract urea entry rate (GER) that is returned to the ornithine cycle (ROC)
[j]Proportion of the gastrointestinal tract urea entry rate (GER) that enters the feces (UFE)
[k]Proportion of the gastrointestinal tract urea entry rate (GER) that is utilized for anabolic purposes (UUA)

The cows fed the LoRumN diet had a slightly lower than average proportion of urea-N entering the urine, which may have been due to a combination of factors stimulating urea-N entry and increasing urea reabsorption in the kidney. Altogether, although cows fed LoRumN did not have better overall N efficiency, they might have been more efficient in their utilization of potentially wasteful N (NH_3-N). This potential is highly valued when considering environmental impacts, as urea-N is wasted in the urine and quickly converted to NH_3, which can have detrimental effects on air quality. Both N intake and ruminal N status appeared to affect urine urea-N excretion in this study, suggesting that nutritionists combine low N diets and low but adequate rumen N balances to lower urinary N excretion.

Anabolic utilization of urea-N recycled to the GIT (UUA) was approximately 51% in cows fed the Control and LoMP diets, but increased to 60% in cows fed LoRumN (Table 2.7). These values correspond with similar work in lactating cows (Lapierre et al., 2004; Ouellet et al., 2004; Valkeners et al., 2007), but are slightly higher than those reported by (Gozho et al., 2008). A simple explanation might be that cattle in the latter study had high ruminal NH_3-N concentrations, resulting in proportionally less utilization of recycled urea-N. These results support our hypothesis that predicted low rumen N balances provide conditions for the microbial population to more efficiently utilize recycled urea-N due to lower dilution of recycled N by intake N sources. This was further confirmed by the observation of higher ^{15}N enrichments in both the rumen NH_3-N pools and the microbial populations in cattle fed the LoRumN diet (Table 2.8). Since net NH_3 sequestration by the animal is negligible, it was assumed in this study that virtually all UUA-N was utilized for microbial AA and protein synthesis (Lobley et al., 1995; Lobley et al., 1996). It

is unknown the extent to which AA and peptides are absorbed from the hindgut, but work so far has suggested that these contributions, if any, are negligible (Lapierre and Lobley, 2001). Therefore, UUA-N results in potential MP supply to the cow via microbial protein incorporation. This explains higher ^{15}N enrichments in both milk protein and fecal N, as this microbial N was utilized for milk synthesis and undigested and indigestible microbial material is excreted in the feces. Overall, the N recycling data support the formulation of the diets and proportional differences in rumen N balance indicated by CPM Dairy despite the known lack of robust prediction of urea N recycling.

Table 2.8. The ^{15}N enrichments (enr.) of microbial, fecal, rumen NH_3, and milk protein samples, and nitrogen content of microbial samples for animals fed Control, LoMP, and LoRumN diets and infused for 72 hours with $^{15}N^{15}N$ urea. Enrichments are expressed as atom percent excess (APE).

	Control	LoMP	LoRumN	SEM	Diet effect
Fecal enr.[1]	4.59^{a}	5.08^{a}	6.29^{a}	1.07	0.32
Liquid bact. enr.	6.35^{a}	6.85^{a}	9.54^{a}	1.88	0.24
Particle bact. enr.	4.83^{a}	5.79^{a}	4.73^{a}	1.12	0.59
Protozoa enr.	5.50^{a}	5.56^{a†}	7.80^{a‡}	1.21	0.15
Rumen NH_3 enr.	6.09^{b}	5.43^{b}	11.44^{a}	2.28	0.05
Milk protein enr.	5.54^{a}	5.98^{a}	6.82^{a}	0.78	0.30

^{abc}Values in rows with different superscripts differ P < 0.05 or †‡values in a row differ P < 0.1 as evaluated by contrast in the Mixed procedure of SAS (2001).
[1]Enrichments calculated as (%^{15}N of enriched sample - %^{15}N of background sample)/%^{15}N of background sample*100%

Microbial Pool Sizes and Transactions

Cows consuming the LoRumN diet appeared to have the smallest rumen bacterial mass (Table 2.9) although the values were not significantly different. However, they also had the smallest rumen pool size, which may have been due to the lower DMI by these animals. Sylvester et al. (2009) demonstrated protozoa to be capable of adjusting their growth rate and generation interval to match turnover times, thus diminishing the potentially negative effects of protozoal predation. Based on microbial pool sizes, their N content and enrichment, 8.3-13.7% of the microbial N pool was present as protozoal N (Table 2.10). This is consistent with previous work by Sylvester et al. (2005), who quantified microbial N by rt-PCR and determined this value to be 5-13%.

Table 2.9. Rumen total contents and microbial pool sizes for animals fed Control, LoMP, and LoRumN diets and infused for 72 hours with $^{15}N^{15}N$ urea.

	Control	LoMP	LoRumN	SEM	Diet effect
Wet contents, kg	73.7	82.0	69.6	8.6	0.43
Dry contents, kg	12.8	12.8	9.4	1.7	0.29
Fraction of contents as DM	0.172	0.155	0.143	0.012	0.27
Protozoal %N	11.2	10.1	10.6	0.72	0.55
Particle bact. %N	8.3	8.0	7.9	0.31	0.65
Liquid bact. %N	10.5	11.3	10.7	0.50	0.51
Liquid bact., g	91	127	82	26	0.47
Particle bact., g	1736	1335	1739	339	0.64
Protozoa, g	204	107	126	36	0.18

To estimate microbial yield and turnovers and use the current data to enhance our understanding of how the protozoa interact with the bacteria in an effort to improve our modeling prediction, the bacterial yields were estimated with the CNCPS v6.1and were increased by 20% to eliminate internal accounting for protozoal predation (Fox et al., 2004). Estimated bacterial N production was highest for animals fed LoMP (Table 2.10) and this resulted in 67% of the CP as RDP, versus 55 and 40% for the Control and LoRumN diets, respectively (Recktenwald, 2007). Recycled N in CNCPS is based on the CP percent of the diet, which would be 80-90 g N/d for the LoMP and LoRumN treatments and 70 g N/d for the Control treatment (10-15% of dietary N) (Fox et al., 2004). Based on these urea-N kinetic results, ruminal N balance was underestimated by the CNCPS, especially for Control and LoRumN diets. Bacterial turnover rates, calculated as estimated yield divided by pool size, were highest for animals consuming LoMP (Table 2.10). This is not surprising considering the smaller ruminal pool sizes observed in these animals. Microbial turnover rates in high producing lactating dairy cattle are poorly known, with most estimates made in vitro, but these can range from 5.3 to 28.6%/h (Hristov and Jouany, 2005b). It seems reasonable to estimate turnover for the purpose of this experiment through estimated yields and measured microbial pool sizes. Our real interest was to estimate the predation of bacteria by protozoa. In the current version of the CNCPS v6.1 and CPM Dairy v3.0, the maximum microbial yield from the Pirt equation (Pirt, 1965) is reduced by 20% assuming the protozoa consume that amount of bacterial growth and at a constant rate (Russell et al., 1992).

It is commonly accepted that virtually all protozoal N originates from bacterial predation (Hristov et al., 2005). Assuming this to be true, protozoal N

pool sizes and ^{15}N APE were used to calculate the amount of bacterial N that was predated for estimated protozoal yields. This is presumably an underestimate of predation, as only approximately 50% of the predated bacteria are incorporated into protozoal N, with the rest released to the medium as AA and peptides (Hristov et al., 2005). Using the combination of microbial turnover rates calculated from the yield estimates in the CNCPS and pool sizes from the rumen, we made an assumption that the range of turnover rates could be equivalent (100%) to the bacterial turnover rate down to half (50%) of this value. Given this approach, the protozoal turnover rate was 2.0-2.6 times per day (Table 2.10). According to (Dehority, 2003), protozoa most likely turn over as fast as every 7 h to avoid becoming washed out in the rumen; thus at least three turnovers per day would be expected under those conditions, which fits with our lower estimates. From our calculations, 4.1-17.1% of bacterial N was predated by protozoa, with highest values for the Control diet, due mainly to a larger proportion of microbial N as protozoa in the cows fed these diets. The CNCPS reduces the microbial yield by 20% to account for protozoa predation. In light of this study, it appears that this estimate might be appropriate, but more variable among different diets and therefore medications should be evaluated in future CNCPS revisions.

Table 2.10. Microbial yields, protozoal contributions to the microbial pool, microbial turnover rates, and protozoal predation estimates for animals fed Control, LoMP, and LoRumN diets.

	Control	LoMP	LoRumN	SEM	Diet effect
% of microbial N pool as protozoal N	13.7	7.6	8.3	3.8	0.31
Bacterial N prod.[1], g N/d	241.6	281.1	228.9	27.0	0.40
Protozoal turnover rate	2.2	2.6	2.0	0.5	0.64
Protozoal predation of bacteria, % of bact. prod.	17.1	8.2	9.6	4.0	0.28
75% of bacterial turnover	12.9	6.1	7.2	3.0	
50% of bacterial turnover	8.6	4.1	4.8	2.0	
% of bacterial N production from recycled N	43.3	26.3	64.7	22.1	0.52
% of protozoal N production from recycled N	47.7	32.3	78.7	25.8	0.51
75% of bacterial turnover	63.6	43.1	105.0	34.5	
50% of bacterial turnover	95.4	64.6	157.5	51.9	

[1]CNCPS v6.1 estimate corrected for protozoal predation.

Table 2.11. Recycled urea-N and microbial N production as proportions of intake N for cows fed Control, LoMP, and LoRumN diets and infused with $^{15}N^{15}N$ urea for 72 hours.

	Control	LoMP	LoRumN	SEM	Diet effect
% of intake N recycled to GIT	28.5	24.6	50.0	0.11	0.35
% of intake N used for bacterial N production	36.2[b]	48.1[a]	46.7[a]	2.6	0.02
% of intake N used for protozoal N production	6.4	4.0	4.2	1.6	0.53
75% of bacterial turnover	4.8	3.0	3.2	1.2	0.53
50% of bacterial turnover	3.2	2.0	2.1	0.8	0.53
% of intake N recycled and used for microbial N production	15.3	13.9	32.6	9.0	0.39

[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by contrast in the Mixed procedure of SAS (2001).

Recycled N appears to be a major contributor to microbial N production. Microbes utilized recycled N for 26-65% of their N production, with the highest values in cattle receiving the LoRumN diet and intermediate values for cattle fed the Control diet (Table 2.11). These levels of incorporation were to be expected, because cows fed the LoRumN diet were recycling more N and incorporating it into microbial populations more efficiently than those on the other diets as evidenced by high GER and UUA values. Animals consuming

the Control diet recycled relatively high amounts of N due to higher N intakes, allowing for more recycled urea-N available to microbial populations; however, capture was lower despite the amount of N recycling due to dilution of the labeled N by higher intake N. If protozoal turnover was slower than bacterial turnover, these estimates increase proportionally (65-157%) and the upper range for these data are not realistic. Proportionally larger contributions of recycled N to microbial growth with lower N intakes were also observed by (Marini and Van Amburgh, 2003), who reported 4-20% of microbial protein contributed by recycled N in heifers with varying N intakes.

As a proportion of total N intake, animals fed the LoMP and Control diets recycled approximately 25% of their intake N, while for those fed the LoRumN diet, the value was double (Table 2.11). These measurements were similar to those found in lactating dairy cows by (Gozho et al., 2008; Ouellet et al., 2004; Valkeners et al., 2007), which reported values of 25-45%. Approximately 47-48% of the intake N was utilized for bacterial N production in animals fed low dietary CP, with this value only 36% in those fed high dietary CP. Similarly, 2.0-6.4% of intake N was utilized for protozoal N production (bacterial N production included before protozoal engulfment).

CONCLUSIONS

Lactating dairy cows fed low (14.1%) or high (16.3%) CP diets recycled approximately 25-50% of their intake N. Urea-N entry to the GIT appeared to be stimulated by both high N intake and low rumen N status. Microbial and NH_3-N ^{15}N enrichments were highest in cows fed low rumen N balance diets,

reflecting the large incorporation of recycled urea-N into the microbial population. Protozoa predated 4-17% of the bacterial yield, which is similar to, but more variable than, previous estimates. This work supports our hypothesis that lactating cows can overcome previously predicted N deficiencies with relatively low CP diets without detrimental effects on production by increasing urea-N recycling to the GIT. Further work is required to more precisely estimate recycled urea-N under a variety of whole body and rumen N status conditions. In addition, more research is required to quantify microbial N turnover between and among the bacterial and protozoal populations and its effect on ruminal N efficiency in general and in relation to utilization of recycled urea-N.

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CHAPTER THREE: UREA-N RECYCLING AND UPTAKE BY RUMEN MICROORGANISMS IN LACTATING DAIRY COWS FED DIETS WITH TWO DIFFERENT LEVELS OF CRUDE PROTEIN AND STARCH WITH OR WITHOUT RUMENSIN®

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ABSTRACT

This study was designed to determine urea-N kinetics in high producing lactating cows while fed differing amounts of CP and starch, with or without Rumensin. The main objectives were to evaluate the effect of Rumensin on urea-N recycling, particularly in the presence of different levels of CP and starch, and to evaluate the effects of these dietary treatments on the use of recycled urea-N by the rumen microbes and measure microbial contents under these conditions. Twelve ruminally fistulated Holstein cows were given a continuous jugular infusion of $^{15}N^{15}N$-urea over the course of 72 hours. Plasma, milk, urine, feces, and ruminal contents and microbes were sampled before and after urea infusion for N enrichment analysis. Total urine and feces were collected over the period of infusion for total N balance measurements. Prior to the 72 h infusion, a second experiment was conducted on six of the twelve cows prior to urea infusions by administration of a jugular bolus dose of $^{15}N^{15}N$-urea followed by interval sampling of ruminal fluid for analysis of $^{15}NH_3$-N enrichment. Milk yield and DMI were improved by higher dietary CP and by

Rumensin, and starch level had subtle effects. Ruminal NH_3, MUN, and PUN concentrations followed dietary CP formulation, with the lower CP diets exhibiting near ruminal N deficiency. The ruminal branched chain volatile fatty acid (BCVFA) concentrations indicated there were dietary interactions among starch and Rumensin that affected branched chain amino acid availability. Ruminal microbial contents were enhanced by high CP diets and impacted only in certain combinations of starch and Rumensin, suggesting that the ruminal environment plays a large role in the Rumensin's effect on the microbial population. High CP diets increased urinary and fecal N excretion proportionately more than milk yields, resulting in lower N efficiencies of use, whereas Rumensin also increased manure N slightly but still resulted in numerically higher N efficiency. Urea-N kinetics followed dietary CP concentrations, but were relatively unaffected by either starch or Rumensin, except for slightly better partitioning of synthesized urea-N to the GIT rather than to urine for low starch diets and those without Rumensin and better anabolic utilization of recycled N in high starch diets. Protozoal consumption of bacteria varied with dietary treatment and their interactions, being highest for the high protein, low starch, with Rumensin diet. On average, 41% of the calculated microbial N yield and 34% of protozoal N yield originated from recycled N. Microbial N from recycled N increased approximately 20% with high CP diets, and starch interacted with both CP and Rumensin mainly through its affects on urea-N utilized for anabolism.

INTRODUCTION

Improving nitrogen (N) efficiency in dairy cows is desirable for a variety of reasons. Feed costs are often reduced due to feeding less of the more costly high protein feeds such as soybean meal and rumen escape protein. It also results in less N emissions to the environment. The impact of ruminants in contributing to atmospheric particulate matter, groundwater N contamination, acid rain, and other effects of reactive N are a concern, especially in areas of dense cattle concentrations. Proper N balance in the animal allows for optimal utilization of other feed components as well, reducing not only the amount of feed and land required per animal but animal wastes while maximizing productive output.

In the case of lactating dairy cows, protein and amino acids are degraded in the rumen to form NH_3, absorbed, and then converted to urea, which then either reenters the GIT or exits the animal in urine or milk. Nitrogen efficiency is improved by increasing the amount of urea that enters the GIT instead of being excreted in the urine, because it is utilized by the microbes for growth and therefore MP supply to the animal. Several studies have indicated a relationship between dietary CP and the amount of urea formed and entering the GIT, but the partitioning between GIT and urinary N entry has been proven difficult to alter without dramatic changes in dietary CP (Marini and Van Amburgh, 2003; Ouellet et al., 2004; Valkeners et al., 2007). Prior work has suggested higher ruminal urea entry with increased starch or sugar fermentation, presumably due to higher microbial N demands (Kennedy, 1980; Kennedy et al., 1981). The hypothesis of higher GIT urea entry was supported by work in dairy cattle fed either dry rolled or pelleted barley, but the dry rolled

barley diet that supposedly improved ruminal fermentation did not appear to stimulate microbial utilization of the recycled urea-N (Gozho et al., 2008). However, microbial yield was not affected by diet, suggesting that microbial demand may not have allowed for differences in N utilization to occur (Gozho et al., 2008).

Besides improving urea-N recycling and microbial uptake, another way to increase N efficiency is to reduce ruminal N degradation. Rumensin, an ionophore routinely fed to dairy cattle, has been shown to reduce feed proteolysis, mainly through inhibiting microbial populations that are highly active in degrading amino acids (Chen and Russell, 1989), and this protein sparing effect was demonstrated by Poos et al., (1979). This results in lower NH_3 production in the rumen and, theoretically, less NH_3 absorption, urea formation and potential N waste to the urine. The effects of Rumensin on urea-N recycling have not been characterized. It is possible that lower NH_3 concentrations in the rumen might lead to higher urea-N entry and potentially better overall N efficiency.

The objectives of this study were to examine the effects of different levels of dietary CP, starch, and Rumensin on urea-N recycling kinetics in lactating dairy cows. In addition, the impacts of these diets on microbial population sizes, yields, and N utilization were examined in order to discover ways in which to stimulate higher N efficiency in both the microbial populations and the animal.

MATERIALS AND METHODS

Twelve multiparous, lactating Holstein dairy cows (107 ± 21 DIM, 647 ± 37 kg BW, 3.0 ± 0.3 BCS) fitted with rumen fistulas were fed one of eight different diets. Animals were housed at the Cornell Teaching and Research Facility, and the Cornell University Institutional Animal Care and Use Committee approved all animal-related procedures. The cows were housed in individual tie stalls and fed a TMR once daily at approximately 0900 at a 10% refusal rate. The diets were formulated to contain either 15.8 or 14.3% CP and 29 or 23% starch, resulting in four compositionally different diets. In addition, Rumensin was included in the ration for six of the cows, while the other six received no supplement, thus with the Rumensin inclusion there were eight separate diets that served as treatments for this experiment (Table 3.1). The TMR consisted of approximately 20% alfalfa hay, 40% corn silage, and 40% concentrate mix specific to dietary objectives (Table 3.2). Wheat straw (0.5 kg/d) was included in the high starch diets to enhance physically effective NDF. Diets were formulated using CNCPS v6.1 (Tylutki et al., 2008) based on a previous study that evaluated dietary CP levels and N efficiency in lactating cattle (Recktenwald, 2007).

Table 3.1. Experimental Design.

High Protein				Low Protein			
High Starch		Low Starch		High Starch		Low Starch	
w/o Rum.[1]	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.

[1]Rumensin is abbreviated as “Rum.”

Diets were formulated to be adequate for both MP and ruminal N balance (High Protein) or to be slightly deficient in ruminal N balance but adequate for MP (Low Protein) and this was done to create conditions where ureagenesis and potential urea recycling would be different. Steam flaked corn was used in all diets to provide more fermentable substrate for microbial growth and increase the ruminal requirements for N by providing greater ruminal fermentable carbohydrate (Table 3.2). This was done to create rumen conditions that should enhance urea N recycling. Finally, to determine the effect of monensin on urea recycling, Rumensin (Elanco Animal Health, Greenfield, IN) was included in the TMR of half of the high and low protein, high and low starch combinations at 400 $mg*cow^{-1}*d^{-1}$. To prevent any carryover effects of Rumensin, once a cow was assigned to the ionophore, they remained on the ionophore. Further, cows were only switched among starch levels to avoid further carry-over effects of N levels due to the length of time necessary to allow for complete adjustment to all factors. In addition, all cows were given bST per label (Posilac, Elanco Animal Health, Greenfield, IN) synchronized so that during the stable isotope infusion period, all cows were at the same stage of bST cycle (5 to 11 days). Cows were given at least 7 days adaptation to the diet before measurements were taken, with the average adaptation period being 17 days.

After the adaptation period, the cows were moved to metabolism stalls for collection of total manure. Cows were milked three times per day at 0700, 1500, and 2300 by a portable milking machine and milk was weighed via a calibrated and certified digital scale. Each milking was sub-sampled in the milk pail and preserved with 2-bromo-2-nitropropane-1,3,dial for component

analysis (Dairy One, Ithaca, NY) and a separate sample was collected without preservative and frozen at -20°C for future analyses.

Feces, urine, plasma, rumen fluid, bacterial, and protozoa samples were taken prior to either jugular $^{15}N^{15}N$-urea infusions or bolus dosing. Feces were collected by either voluntary elimination or rectal palpation into 1 gallon plastic bags then frozen. Prior to $^{15}N^{15}N$-urea infusions, urine was collected by manual agitation of the lower vulva, 50% H_2SO_4 (v/v) was added until pH < 2, then samples were frozen in conical vials. Blood was collected into Vacutainers containing heparin (Becton Dickinson, Rutherford, NJ) via the coccygeal vessels, placed on ice, and centrifuged at 1500 x g for 15 min to obtain plasma.

Table 3.2. Ingredient content (percent of dry matter) of the four treatment diets used in a study of nitrogen recycling in lactating dairy cattle.

DM % basis	High CP, High starch	High CP, Low starch	Low CP, High starch	Low CP, Low starch
Corn silage	37.71	38.10	36.77	37.99
Alfalfa hay	18.69	19.10	18.43	19.42
Wheat straw	1.90	---	1.89	---
Steam flaked corn	11.26	5.82	11.50	5.66
Cornmeal	10.92	5.78	11.37	5.49
Soybean meal	7.40	6.51	3.35	1.87
Citrus pulp	4.00	11.30	3.60	7.92
Soy hulls	---	4.02	1.72	7.45
Amino plus	3.79	4.17	5.45	6.73
Dextrose	0.83	---	0.90	---
Cargill fat	0.56	0.86	0.60	0.85
Blood meal	0.42	0.41	0.43	0.37
Sodium bicarb	0.42	0.43	0.43	0.43
Salt	0.40	0.39	0.39	0.40
Limestone	0.36	0.00	0.46	0.31
Monocalcium phosphate	0.36	0.33	0.33	0.27
Calcium sulfate	0.30	0.32	0.18	0.34
Urea	0.23	0.21	---	---
Magnesium Oxide	0.20	0.18	0.16	0.16
Wheat midds	0.08	1.92	1.76	4.17
Selenium	0.05	0.04	0.04	0.04
Alimet	0.03	0.03	0.03	0.03
1100 Dairy TM[1]	0.03	0.03	0.03	0.03
Dairy ADE-AL/MA[2]	0.03	0.02	0.02	0.03
Smartamine M	0.017	0.017	0.017	0.017
Zinc sulfate	0.004	0.004	0.004	0.004
Manganese sulfate	0.004	0.004	0.004	0.004
Agmate/KMS 050	---	---	0.12	---

[1]Contains 18.8% of DM as sulfur, 30317 mg Cu/kg, 136466 mg Mn/kg, 3393 mg Co/kg, 3039 mg iodine/kg, 153915 mg Zn/kg
[2]Contains 30464 mg Vit. A/kg, 30464 mg Vit.D/kg, 93784 mg Vit. E/kg

Table 3.3. The measured chemical composition of the diets fed to dairy cows with or without Rumensin and infused with $^{15}N^{15}N$-urea.

.% of DM	High CP, High starch	High CP, Low starch	Low CP, High starch	Low CP, Low starch
Dry matter	52.9	52.6	53.3	52.4
CP, % DM	16.4	16.1	15.0	14.4
Soluble CP, %CP	30.0	31.7	30.0	28.1
NDF, % DM	30.0	31.6	31.5	34.1
ADF, % DM	19.0	21.0	19.7	22.0
Lignin, %NDF	6.0	6.0	6.1	6.3
Starch, % DM	26.8	21.9	27.2	22.6
Sugar, % DM	6.4	7.4	6.0	6.2
Crude fat, % DM	3.7	4.1	3.8	4.1
NFC, % DM	44.7	42.6	44.5	41.1
Ca, % DM	0.74	0.75	0.72	0.80
P, % DM	0.34	0.34	0.34	0.35

Table 3.4. The measured chemical composition of the forages fed.

% of DM	Corn silage	Alfalfa hay	Wheat straw
Dry matter	31.9	88.2	88.9
CP, % DM	8.49	19.6	4.89
Soluble CP, %CP	67.7	40.1	41.8
NDF, % DM	42.9	42.4	79.8
ADF, % DM	23.5	32.3	52.7
Lignin, %NDF	6.59	14.7	12.2
Starch, % DM	31.9	1.48	1.57
Sugar, % DM	1.39	6.71	2.01
Crude fat, % DM	3.40	1.54	1.45
NFC, % DM	41.9	30.5	9.60
NDICP, %CP	10.7	18.8	31.5
ADICP, %CP	3.77	4.31	16.3
Ca, % DM	0.20	1.32	0.28
P, % DM	0.22	0.31	0.14

Ruminal contents were collected for VFA measurements by sampling at approximately 4-5 locations throughout the rumen. These were squeezed through 4 layers of cheesecloth, immediately acidified with 1 ml 50% (v/v) H_2SO_4 in 40 ml of rumen fluid, and frozen in conical vials for NH_3-N and VFA analysis. Further, rumen contents were collected from at least 5 different locations within the rumen into a 2 L cooler and the sample was intended to be representative of the total ruminal contents. Microbial isolation began approximately 30 min after collection after transport to the laboratory in Morrison Hall. Rumen contents were squeezed through four layers of

cheesecloth to separate solid and liquid portions. The solid portion was washed with an amount of Coleman buffer equivalent to the volume of rumen liquid and squeezed through four layers of cheesecloth. This liquid was added to the previously isolated fluid. Protozoa were isolated from the total fluid by flocculating in a 39°C water bath for 45 min. Formalin (37% w/w), was added at 1% of the liquid volume, then the liquid was centrifuged at 750 x g for 5 min at 10°C (Sylvester et al., 2005). Fluid associated bacteria were isolated from the supernatant by centrifugation at 27000 x g for 30 min at 4°C (Martin et al., 1996). Particle associated bacteria were prepared from approximately 20% of the sampled solid rumen content by a 30 min incubation in 0.1% methylcellulose at 37°C followed by blending in a Waring blender for 60 sec (six, 10-sec intervals), squeezed through four layers of cheesecloth and a 100μm cloth, then incubated in a solution of 0.1% Tween 80 and 1% methanol in saline at pH 2 for 24 h at 4°C. Solids were squeezed through four layers of cheesecloth and a 100μm cloth, then the solids were washed with a total of 700 ml saline over three washes (approximately 230 mL per wash). All liquids were combined and centrifuged at 1000 x g for 15 min at 4°C to eliminated contaminant feed particles. The supernatant was strained through a 100μm cloth and centrifuged at 27,000 x g for 20 min at 4°C for collection of solid associated bacteria (Whitehouse et al., 1994). All microbial pellets were transferred from centrifuge bottles with water, collected into pans, and freeze dried for composition analysis. Finally, total ruminal contents were evacuated from the rumen approximately six hours after feeding three days prior to urea infusions and approximately one hour before feeding one day prior to urea infusions. The total contents were weighed, mixed well, and a subsample taken to be freeze dried for analyses of rumen dry matter and liquid content.

For the infusion period, animals were prepared with indwelling jugular vein catheters at least one day prior the start of a continuous infusion of $^{15}N^{15}N$-urea (98% purity, Cambridge Isotope Laboratories Inc., Andover, MA) in sterile saline (9 g NaCl/L) at 0.0278 g urea/h. Feces, urine, plasma, rumen fluid, bacterial, and protozoa samples were taken at least 72 h after initiating the infusion using the same procedures described earlier.

Stable isotopic analysis proceeded as follows. Fecal, plasma, bacterial, and protozoal ^{15}N enrichment were determined on the freeze dried sample with a NC2500 Carlo Erba elemental analyzer (Milan, Italy) interfaced to a ThermoFinnigan Delta Plus isotope ratio mass spectrometer (Bremen, Germany). Urinary urea was isolated via a cation exchange resin (AG 50W-X8, 100-200 mesh hydrogen form, Bio-Rad Laboratories, Hercules, CA), diluted to 6 mmol/L, and reacted with lithium hypobromite under vacuum (Marini and Attene-Ramos, 2006). The resulting N_2 gas was analyzed with a PDZ Europa Geo 20/20 isotope ratio mass spectrometer (Cheshire, UK) attached to the ANCA-trace gas/liquid system. The model of Lobley et al. (2000) was used to calculate urea kinetics.

Total N content of the wet feces was analyzed with a Kjeltec 2300 (Foss Analytical, Hilleroed, Denmark). Ruminal ammonia and plasma urea concentrations were measured using the procedure of Chaney and Marbach (1962). Volatile fatty acids were measured by HPLC (Beckman System Gold, Brea, CA) using crotonic acid as an internal standard (Seigfried et al., 1984). Urinary purine derivatives were determined by HPLC (Beckman System Gold, Brea, CA) according to Shingfield and Offer, (1999).

Urea N Kinetic Measurements

A separate kinetics experiment was conducted on the six animals fed low CP diets, where a bolus dose of urea was given to examine differences in urea uptake and transport through the rumen contents given different dietary starch levels with or without Rumensin. Five days prior to the bolus dose, animals were fed 4x/d to create more steady-state conditions in ruminal fermentation. Animals were prepared with jugular catheters as previously described, with one catheter on each side for dosing the urea and the other side for blood sampling. Two grams of $^{15}N^{15}N$ urea dissolved in 20 ml of 0.9% saline were introduced via a jugular catheter at approximately 0900. The syringe was refilled with 20 ml saline and introduced after the urea solution to flush the syringe and catheter. Ruminal contents were taken from 3-4 locations next to the rumen wall and from 3-4 locations in the center of the rumen contents, determined by arm measurements, every 5 min for the first 15 min after dosing, then every 15 min until 2 h after dosing, then every 30 min until 6 h after dosing, then at 7, 8, 9, 10, 12, and 14 h after dosing. Rumen contents were squeezed through four layers of cheesecloth and 1 ml of 50% H_2SO_4 was added to 40 ml rumen fluid before freezing. Blood samples were also taken at these times via the non-dosing catheter and handled as described previously for urea measurement. Each animal on the low CP diet was given the bolus urea dose approximately four days prior to beginning urea infusions. All background samples were taken 1-3 days before the bolus urea dose. Ammonia was isolated from each rumen fluid sample and analyzed for ^{15}N enrichment as described previously.

Microbial Calculations

The following calculations were used to derive the various microbial pool sizes. The acronyms LAB, PAB, and PZ will be used for liquid associated bacteria, particle associated bacteria, and protozoa, respectively. The term atom percent excess (APE) is used to describe the relative increase in ^{15}N enrichment during $^{15}N^{15}N$-urea infusions or after a bolus dose. Higher APE values indicate higher ^{15}N enrichment, or therefore, greater inclusion of the infused N as a proportion of the N content. It can be used to measure the contribution of one N pool into another or the origin of a pool's N content.

$$\text{Microbial N (LAB, PAB, or PZ) APE} = \frac{\text{enriched }\%^{15}\text{N} - \text{background }\%^{15}\text{N}}{\text{background }\%^{15}\text{N}}$$

Protozoal N APE was corrected for 8% liquid associated bacterial contamination according to previous work using similar microbial isolation methods (Sylvester et al., 2005):

$$\text{PZ N APE} = \frac{\text{PZ N APE} - 0.08 * \text{LAB N APE}}{0.92}$$

The proportion of protozoal N that originated from liquid or particle associated bacteria was calculated based on the previously published observation that the fraction of Pool B from Pool A = Pool B APE / Pool A APE (Nolan and Leng, 1972), or in this case sum of the fraction of PZ N from LAB N (equal to PZ N APE / LAB N APE) and the fraction of PZ N from PAB N (equal to PZ N APE / PAB N APE) equals unity:

$$\frac{\text{PZ N APE}}{\text{LAB N APE}} + \frac{\text{PZ N APE}}{\text{PAB N APE}} = 1$$

which leads to:

$$\frac{\text{PZ N APE}}{\text{LAB N APE}} = \frac{\text{PAB N APE}}{\text{PAB N APE + LAB N APE}}$$

$$\frac{\text{PZ N APE}}{\text{PAB N APE}} = \frac{\text{LAB N APE}}{\text{PAB N APE + LAB N APE}}$$

The amount of bacteria present in the rumen at the time of sampling was calculated through the microbial mass isolated, the mass of liquid or particles in the rumen sample, and the total rumen liquid or particulate mass.

$$\text{LAB in rumen, g} = \frac{\text{g bacterial DM isolated in the liquid sample}}{\text{g liquid in sample}} * \text{g liquid in rumen}$$

$$\text{PAB in rumen, g} = \frac{\text{g bacterial DM isolated in the particle sample}}{\text{g particles in sample}} * \text{g DM in rumen}$$

$$\text{PZ in rumen, g} = \frac{\text{g protozoal DM isolated in sample}}{\text{g liquid in sample}} * \text{g liquid in rumen}$$

Liquid associated bacteria and protozoa mass in the rumen was corrected for liquid associated bacterial contamination in the protozoal isolation:

$$\text{LAB in rumen, g} = \text{initial LAB in rumen} + 0.08 * \text{initial PZ in rumen}$$

$$\text{PZ in rumen, g} = \text{initial PZ in rumen} * 0.92$$

The nitrogen concentration of each microbial pool was measured by the IRMS, and the protozoal %N was corrected for liquid associated bacterial contamination as follows:

$$\text{PZ \%N} = \frac{\text{PZ \%N measured} - \text{LAB \%N} * 0.08}{0.92}$$

Grams of microbial N in each pool were calculated based on the rumen microbial pool size and the N concentration:

$$\text{grams LAB N} = \frac{\text{g LAB} * \text{LAB \%N}}{100\%}$$

$$\text{grams PAB N} = \frac{\text{g PAB} * \text{PAB \%N}}{100\%}$$

$$\text{grams PZ N} = \frac{\text{g PZ} * \text{PZ \%N}}{100\%}$$

$$\text{Fraction of microbial N pool as PZ N} = \frac{\text{g PZ N}}{\text{g LAB N} + \text{g PAB N} + \text{g PZ N}}$$

The ^{15}N enrichment was measured via IRMS, and the following correction was made for both the background and enriched protozoal N samples due to 8% liquid associated bacterial contamination:

$$\text{PZ } ^{15}\text{N enrichment} = \frac{\text{initial PZ } ^{15}\text{N enrichment} - \text{LAB } ^{15}\text{N enrichment} * 0.08}{0.92}$$

The ^{15}N pool size for each of the microbial populations was calculated based on the rumen microbial mass, ^{15}N enrichment prior to (bgrd) and after 72 hours of $^{15}N^{15}N$-urea infusion (enr), and the N concentration of the appropriate microbe:

$$\text{Rumen LAB } ^{15}\text{N, g} = \frac{\text{g LAB} * \text{LAB } ^{15}\text{N enr} * \text{LAB \%N}}{100\% * 100\%} - \frac{\text{g LAB} * \text{LAB } ^{15}\text{N bgrd} * \text{LAB \%N}}{100\% * 100\%}$$

$$\text{Rumen PAB } ^{15}\text{N, g} = \frac{\text{g PAB} * \text{LAB } ^{15}\text{N enr} * \text{PAB \%N}}{100\% * 100\%} - \frac{\text{g PAB} * \text{PAB } ^{15}\text{N bgrd} * \text{PAB \%N}}{100\% * 100\%}$$

$$\text{Rumen PZ } ^{15}\text{N, g} = \frac{\text{g PZ} * \text{PZ } ^{15}\text{N enr} * \text{PZ \%N}}{100\% * 100\%} - \frac{\text{g PZ} * \text{PZ } ^{15}\text{N bgrd} * \text{PZ \%N}}{100\% * 100\%}$$

The mass of liquid associated bacteria consumed by protozoa (at one point in time, or present in the rumen at the time of isolation) if all of the rumen protozoal N originated from the liquid associated bacteria or particle associated bacteria was calculated as follows:

$$\text{grams LAB} = \frac{\text{g PZ } ^{15}\text{N}}{}$$

consumed by PZ

$$\frac{\text{(LAB }^{15}\text{N enr – LAB }^{15}\text{N bgrd)}}{100\%} * \frac{\text{LAB \%N}}{100\%}$$

$$\text{grams PAB consumed by PZ} = \frac{\text{g PZ }^{15}\text{N}}{\frac{\text{(PAB }^{15}\text{N enr – PAB }^{15}\text{N bgrd)}}{100\%} * \frac{\text{PAB \%N}}{100\%}}$$

Assuming protozoa consumed both liquid and particle associated bacteria, the amount of each bacterial pool consumed by protozoa if they were consumed in the proportions suggested by APE measurements is calculated below (at one point in time, or present in the rumen at the time of isolation) from the prior two equations and those supplying the proportion of protozoal N from each pool (see above):

LAB and PAB consumed by PZ, g	=	grams of LAB consumed by PZ * Fraction of PZ N from LAB N	+	grams of PAB consumed by PZ * Fraction of PZ N from PAB N

The mass of bacterial N that was consumed by protozoa (at one point in time, or present in the rumen at the time of isolation) if they were consumed in the proportions suggested by APE measurements (see equations above) is as follows:

LAB N and PAB N consumed by PZ, g	=	grams of LAB consumed by PZ (if all PZ N from LAB) * LAB %N * Fraction of PZ from LAB	+	grams of PAB consumed by PZ (if all PZ N from PAB) * PAB %N * Fraction of PZ from PAB

$$\frac{}{100\%} \qquad \frac{}{100\%}$$

The daily amount of purines absorbed by the cow was calculated as follows (based on IAEA by X. B. Chen, 2004):

$$\text{mmol purine absorbed per day} = \frac{\text{mmol purine in urine per day} - 0.385 * BW^{0.75}}{0.85}$$

The daily bacterial N yield was based on urine purine excretion (X. B. Chen et al., 2004):

$$\text{Bacterial N yield (g N/d)} = \frac{\text{mmol purine absorbed per day} * 70}{\text{purine N:total bacterial N (0.116)} * \text{nucleic acid digestibility (0.83)} * 1000}$$

Bacterial turnover rates were calculated based on the daily bacterial N yield and the rumen bacterial pool sizes. Protozoal turnover rates were based on bacterial turnover rates and estimated to be equivalent (100%) to, three-fourths of (75%), or half of (50%) the bacterial turnover rates, as no direct protozoal yield measurement was available (Dehority, 2003; Sylvester et al., 2005).:

$$\text{Bacterial N turnover } (d^{-1}) = \frac{\text{bacterial N yield (g N/d)}}{\text{g LAB N in rumen} * \text{g PAB N in rumen}}$$

Protozoal N turnover (d^{-1}) = bacterial N turnover (d^{-1}) * [0.50, 0.75, 1.00], depending on estimated protozoal:bacterial turnover rate

The amount of bacteria consumed by the protozoa daily is calculated based on the amount of equivalent amount of each bacterial type that was

consumed by the protozoa at the time of isolation multiplied by the turnover rate of the protozoal population:

$$\text{grams of bacteria consumed by protozoa per day} = \text{g LAB and PAB consumed by PZ * PZ turnover}$$

$$\text{grams of bacterial N consumed by protozoa per day} = \text{g LAB N and PAB N consumed by PZ * PZ N turnover}$$

The proportion of the total bacterial N produced each day that was consumed by the protozoa was calculated with daily bacterial N yield and protozoal consumption:

$$\text{Fraction of total bacterial N yield consumed by protozoa} = \frac{\text{bacterial N yield (g N/d)}}{\text{g bacterial N consumed by protozoa per day}}$$

Daily protozoal N yield was calculated based on the rumen protozoal N pool size and the turnover rate:

$$\text{Protozoal N yield (g N/d)} = \text{g protozoal N in rumen * protozoal turnover}$$

Statistical Analysis

Data were analyzed with the PROC MIXED procedure of SAS (SAS Inst., Cary, NC) with the model:

$$Y_{ijklm} = \mu + T_i + P_j + S_k + R_l + PS_{jk} + PR_{jl} + SR_{kl} + C_m + E_{ijklm}$$

Where Y_{ijkl} = dependent variable, μ = overall mean, T_i = effect of dietary treatment i, P_j = effect of dietary CP level j, S_k = effect of dietary starch level k, R_l = effect of feed additive l (Rumensin vs no additive), PS_{jk} = interaction of dietary CP level j and starch level k, PR_{jl} = interaction of dietary CP level j and feed additive l, SR_{kl} = interaction of dietary starch level k and feed additive l, C_m = effect of cow m, and E_{ijklm} = residual error. All terms were considered fixed except for C_m and E_{ijklm}, which were considered random.

Repeated measures was used for analysis of daily milk yield, milk components (fat %, protein %, lactose %, and MUN) and component yield, DMI, and N intake. Autoregressive, AR(1), was used as the covariance structure with repeated measures. Milk yield for an average of six days prior to initiation of treatment diets was used as a covariate for milk yield analysis. The LSMEANS option was used to determine treatment, factor, and factor interaction means, and these were tested using the PDIFF option. The Tukey-Kramer adjustment was used to separate treatment means. Type 3 tests of the fixed effects were used to determine significance, which was declared at $P < 0.05$ and a statistical trend was declared at $P < 0.10$.

RESULTS AND DISCUSSION

Body weight and condition score

The mean body weights of the cows for each treatment ranged from 584 to 688 kg and BCS ranged from 2.8 to 3.1 with no significant differences observed among treatments for either body weight or condition score (Table

Table 3.5. Body weight, body condition score, days in milk, dry matter and nitrogen intake, and dietary CP concentration for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		SEM
	w/o Rum	with Rum	w/o Rum	with Rum	w/o Rum	with Rum	w/o Rum	with Rum	
n[1]	3	3	3	3	3	3	3	3	-
BW, kg	688	637	676	646	607	670	584	665	37.3
BCS	3.0	2.8	2.8	2.8	3.0	3.0	3.1	3.0	0.29
DIM, d	117	110	113	106	117	130	126	116	20.7
DMI, kg/d	24.3	26.8	24.9	26.1	22.6	23.2	22.1	22.7	1.47
N intake, gN/d	644[b]	736[ab]	667[b]	691[b]	545[bc]	556[b]	562[b]	542[bc]	43
CP of diet, %	16.4[a]	17.1[a]	16.6[a]	16.5[a]	15.1[b]	15.0[b]	15.6[ab]	14.9[b]	0.30

[1]n = number of cows per treatment

[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.6. Main effects of dietary CP, starch, and Rumensin on animal dry matter and nitrogen intake and CP concentration of the diet of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
n[1]	6	6	-	6	6	-	6	6	-	-	-	-
DMI, kg/d	25.7	22.9	0.45	24.4	24.1	0.45	24.1	24.9	0.45	0.0001	0.72	0.058
N intake, gN/d	687.4	558.1	13.8	624.6	620.8	13.5	608.0	637.4	13.8	<0.0001	0.84	0.14
CP of diet, %	16.6	15.1	0.14	15.9	15.9	0.11	15.9	15.8	0.14	<0.0001	0.87	0.61

[1]n = number of cows per treatment

Table 3.7. The effect of two levels of dietary CP and starch, with or without Rumensin on animal dry matter and nitrogen intake and dietary CP concentration for lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	DMI, kg/d	N intake, gN/d	CP of diet, %
high	high		25.5	689.7	16.8
high	low		25.8	685.0	16.5
low	high		23.3	559.5	15.0
low	low		22.5	556.6	15.3
P value			**0.44**	**0.96**	**0.001**
high		no	24.7	657.1	16.5
high		yes	26.6	717.7	16.7
low		no	22.5	559.0	15.4
low		yes	23.2	557.1	14.9
P value			**0.39**	**0.11**	**0.086**
	high	no	23.6	598.5	15.7
	high	yes	25.2	650.8	16.0
	low	no	23.6	617.6	16.1
	low	yes	24.7	624.1	15.7
P value			**0.68**	**0.22**	**0.001**

3.5). Differences were not anticipated, as all animals were at a similar stage of lactation at treatment assignment and treatments were not long enough to cause large changes in body fat or protein reserves. The cattle were approximately 107 DIM (+/- 21 days) during the first $^{15}N^{15}N$-urea infusion and 117 DIM for the second infusion. No significant differences were observed among treatment means for DIM at initiation of infusions; thus, these animals were in a stage of lactation near their maximal milk yields and dry matter intakes.

Dry Matter Intake

Dry matter intake was significantly higher ($P < 0.05$) for cows consuming the high protein diets (25.7 vs 22.9 kg DMI/d) and tended to be higher for Rumensin fed animals (24.9 vs 24.1) (Table 3.6). These intakes are comparable to similar studies feeding high levels of corn silage in combination with alfalfa silage (Wattiaux and Karg, 2004a). Previous work has demonstrated DMI inhibition with low dietary CP concentrations, due mainly to ruminal N deficiency effects on microbial fermentation (Broderick, 2003; Ruiz et al., 2002). The lower CP diets were formulated to be close to negative rumen N balance; thus, the observation of lower DMI in these diets indicates that the formulation objectives were potentially realized. Nitrogen intake followed dietary formulation and observed DMI values (Tables 3.5-3.7).

Rumensin is known to enhance feed efficiency by reducing intake while maintaining milk yield (Duffield et al., 2008). However, in this study, inclusion of Rumensin in the diet enhanced DMI, in a nearly significant but modest amount (3.3% among treatments) (Table 3.6). This increase in DMI is not

consistent among the available data (AlZahal et al., 2008; Odongo et al., 2007; Ruiz et al., 2001) but suggests that under certain conditions, DMI can be enhanced by Rumensin inclusion. Since these diets were relatively low in CP, it may be possible that Rumensin's effects on hyper-ammonia producing bacteria allowed for better ruminal N usage by some microbial species, partially negating the low ruminal N status (Chen and Russell, 1989). There was no effect of starch on DMI, which is consistent with other research conducted on high forage fed cows over a range of 22-30% of dietary DM (Weiss et al., 2009a).

Milk yield and component yields

Milk yield was numerically higher for cattle fed high CP, high starch, and Rumensin supplemented diets, (7.7, 3.4%, and 6.3%, respectively), although the Rumensin response was a trend ($P = 0.09$) (Table 3.9). There was a significant interaction between CP and starch concentrations in the diet on milk yield ($P = 0.001$), as dietary starch concentration did not notably impact milk yield in high CP diets, but low starch concentrations resulted in nearly 3 kg/d less milk (10.4%) on the low CP diets ($P < 0.05$) (Table 3.10). Dry matter intake also followed this same pattern, but not significantly and not with enough difference in DMI for the low CP diets to fully explain the milk yield decrease in cows fed the low starch diets. Ruminal NH_3-N concentrations were not different in cows fed the low CP diets independent of starch concentration (Table 3.13) and microbial N was only approximately 6% less in the low starch-low CP diet than in the high starch-low CP diet (Table 3.21). This suggests that the low CP-low starch diets were most likely equally limiting

Table 3.8. Milk yield and composition of dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Milk yield, kg/d	30.4[a]	30.8[a]	32.9[a]	31.3[a]	27.8[a]	31.9[a]	27.0[b]	27.3[bc]	1.59
Milk fat									
%	3.86	3.97	3.79	4.01	3.71	3.74	3.83	3.58	0.35
kg/d	1.54	1.71	1.57	1.74	1.43	1.62	1.40	1.31	0.16
Milk protein									
%	2.73[ab]	2.97[a]	2.66[b]	2.98[a]	2.84[a]	2.73[ab]	2.80[a]	2.60[bc]	0.16
kg/d	1.07[a]	1.25[ab]	1.09[b]	1.28[b]	1.08[b]	1.16[b]	1.01[b]	0.96[bc]	0.08
Milk lactose									
%	4.94[a]	4.99[a]	4.99[ab]	5.01[a]	4.60[b]	4.81[ab]	4.57[bc]	4.86[ab]	0.10
kg/d	1.97[ab]	2.11[a]	2.06[a]	2.15[a]	1.75[b]	2.08[a]	1.68[c]	1.78[ab]	0.09
MUN, mg/dl	11.75[a]	10.84[a]	10.94[a]	9.30[a]	6.94[c]	8.43[b]	8.01[b]	8.95[ab]	0.75

[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.9. Effects of dietary CP, starch, and Rumensin on milk yield and composition of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	**Low CP**	**SEM**	**High starch**	**Low starch**	**SEM**	**No Rum**	**Rum**	**SEM**	**CP**	**starch**	**Rum**
Milk yield, kg/d	30.6	28.4	0.72	30.0	29.0	0.57	28.6	30.4	0.72	0.03	0.04	0.09
Milk fat %	3.90	3.72	0.23	3.81	3.81	0.17	3.80	3.82	0.23	0.57	0.96	0.95
Milk fat, kg/d	1.64	1.44	0.11	1.57	1.51	0.08	1.49	1.59	0.11	0.19	0.24	0.47
Milk protein %	2.83	2.74	0.11	2.81	2.76	0.08	2.76	2.82	0.11	0.55	0.0001	0.70
Milk protein, kg/d	1.17	1.05	0.05	1.14	1.09	0.04	1.06	1.16	0.05	0.13	0.03	0.20
Milk lactose %	4.98	4.71	0.07	4.84	4.85	0.05	4.77	4.92	0.07	0.004	0.06	0.12
Milk lactose, kg/d	2.07	1.82	0.06	1.98	1.92	0.02	1.87	2.03	0.02	0.003	0.14	0.046
MUN, mg/dl	10.71	8.08	0.50	9.49	9.30	0.37	9.41	9.38	0.50	0.0002	0.34	0.98

Table 3.10. The effect of two levels of dietary CP and starch, with or without Rumensin on milk yield and composition of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Milk yield, kg/d	Milk fat %	Milk fat, kg/d	Milk protein %	Milk protein, kg/d	Milk lactose %	Milk lactose, kg/d	MUN, mg/dl
high	high		30.3	3.91	1.62	2.85	1.16	4.96	2.04	11.30
high	low		30.9	3.90	1.65	2.82	1.19	5.00	2.10	10.12
low	high		29.8	3.72	1.52	2.78	1.12	4.71	1.91	7.69
low	low		27.0	3.72	1.37	2.71	0.99	4.71	1.74	8.47
P value			**0.001**	**0.91**	**0.09**	**0.096**	**0.0009**	**0.10**	**0.001**	**<0.0001**
high		no	29.8	3.83	1.55	2.70	1.08	4.96	2.01	11.34
high		yes	31.4	3.98	1.72	2.97	1.26	5.00	2.13	10.07
low		no	27.5	3.77	1.42	2.82	1.05	4.59	1.72	7.47
low		yes	29.4	3.66	1.47	2.66	1.06	4.84	1.93	8.70
P value			**0.88**	**0.69**	**0.70**	**0.16**	**0.29**	**0.26**	**0.57**	**0.077**
	high	no	28.7	3.78	1.48	2.78	1.08	4.77	1.86	9.34
	high	yes	31.4	3.84	1.66	2.84	1.20	4.90	2.09	9.65
	low	no	28.6	3.82	1.49	2.73	1.05	4.78	1.87	9.47
	low	yes	29.3	3.80	1.53	2.79	1.13	4.93	1.97	9.12
P value			**0.058**	**0.71**	**0.20**	**0.96**	**0.27**	**0.45**	**0.068**	**0.12**

in ruminal N supply for microbial growth independent of dietary starch, but might have been limiting either MP or specific amino acids post-ruminally that limited milk production.

Milk fat percent and yield were not significantly impacted by treatments, except for a trend ($P < 0.10$) for the interaction among CP and starch level on fat yield (Table 3.11). Various studies have observed milk fat depression with Rumensin supplemented diets, particularly high corn silage diets (Recktenwald, 2007), but no significant effect was observed. Indeed, milk fat percentages were reasonably high among all treatments, with the treatment means ranging from 3.67 to 4.01%, and were similar or higher than those reported with animals fed comparable diets (Wattiaux and Karg, 2004a) at similar milk yields (Table 3.8).

Milk protein percent and yield were significantly increased by dietary starch (2.76 vs 2.81% and 1.09 vs 1.14 kg/d, for the low and high starch diets, respectively), and the greatest increase was only in the low CP diets, mainly due to the large increase in total milk yield with a only small increase in protein concentration (Tables 3.9 and 3.10). Others have reported no significant effect of starch on milk protein yield (Hristov and Ropp, 2003; Ipharraguerre and Clark, 2005) . It is most likely that the higher protein yield was due to increases in total milk production, as both were increased by approximately the same amount (10 and 13% for total and milk protein yield, respectively). Rumensin increased milk protein yield proportionately more in the high CP diets rather than the low CP diets through both protein concentration and total milk yield changes.

Lactose yield increased 14 and 9% with high dietary starch and with Rumensin, respectively, which was associated with total milk yield increases

of 8 and 6% (Table 3.9). Similarly, lactose yields were significantly affected by the starch*CP interactions initially described in reference to total milk yield (Table 3.10). These increases in lactose concentration and yield reached significance only in with the main factor of CP, but did demonstrate trends similar to those reported for total milk yield (Table 3.9).

Nitrogen concentrations – MUN, PUN, rumen NH_3-N

Ruminal NH_3-N is absorbed, converted to urea-N, then partially excreted in proportion in milk; therefore, it is not surprising that N concentration in all three pools were related (Tables 3.11-3.13). Milk urea nitrogen concentration was significantly higher in cattle fed the high CP diets. Numerous studies have noted the close relationship between CP intake and MUN, and this study is consistent with previous data (Broderick, 2003; Ipharraguerre and Clark, 2005; Jonker et al., 2002). Milk urea N concentration might have also been impacted by milk yield, with dilution (resulting in lower concentrations) occurring in diets with proportionately higher milk yield, such as observed for low dietary CP diets with different levels of dietary starch (Table 3.13).

Table 3.11. Plasma urea-N, ruminal NH_3-N, and ruminal pH for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								**SEM**
	High Protein				**Low Protein**				
	High Starch		**Low Starch**		**High Starch**		**Low Starch**		
	w/o Rum.	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	
PUN, mg/dl	13.47[ab]	15.26[a]	13.28[ab]	12.49[ab]	7.67[cd]	8.17[cd]	6.83[cd]	8.75[cd]	0.97
Ruminal NH_3-N, mg/dl	13.99[a]	10.13[ab]	11.32[ab]	8.29[ab]	6.94[b]	4.89[bc]	5.75[bc]	5.79[bc]	1.38
Ruminal pH	5.91	6.08	5.83	5.96	5.93	6.04	6.03	5.94	0.11

[abcd]Values in rows with different superscripts differ P < 0.05 as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.12. Effects of dietary CP, starch, and Rumensin on plasma urea, ruminal NH_3-N, and ruminal pH of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	**Low CP**	**SEM**	**High starch**	**Low starch**	**SEM**	**No Rum**	**Rum**	**SEM**	**CP**	**starch**	**Rum**
PUN, mg/dl	13.66	7.85	0.59	11.24	10.27	0.47	10.31	11.20	0.59	<0.0001	0.02	0.29
Ruminal NH_3-N, mg/dl	10.94	5.84	0.72	9.00	7.77	0.67	9.50	7.27	0.72	<0.0001	0.16	0.03
Ruminal pH	5.94	5.99	0.066	5.99	5.94	0.052	5.93	6.01	0.065	0.63	0.29	0.40

Table 3.13. The effect of two levels of dietary CP and starch, with or without Rumensin on plasma urea, ruminal NH_3-N, and ruminal pH of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	PUN, mg/dl	Ruminal NH_3-N, mg/dl	Ruminal pH
high	high		14.52	12.07	6.00
high	low		12.80	9.81	5.89
low	high		7.97	5.94	5.99
low	low		7.74	5.74	5.99
P value			**0.08**	**0.24**	**0.24**
high		no	13.37	12.66	5.87
high		yes	13.95	9.22	6.02
low		no	7.26	6.35	5.99
low		yes	8.45	5.33	5.99
P value			**0.71**	**0.24**	**0.45**
	high	no	10.74	10.48	5.92
	high	yes	11.75	7.53	6.06
	low	no	9.89	8.53	5.93
	low	yes	10.65	7.02	5.95
P value			**0.76**	**0.41**	**0.23**

Plasma urea nitrogen and rumen NH_3-N concentrations were higher in cattle fed the high CP diets, consistent with MUN concentrations (Table 3.12). However, high dietary starch increased both PUN and rumen NH_3-N concentrations. Previous work has demonstrated both of these measurements to decrease with starch levels in the diet, presumably due to protein and carbohydrate synchrony, such that sugar and starch fermentation is coupled to protein availability and therefore, microbial protein growth, which in turn lowers rumen N pools (Reynolds and Kristensen, 2008). However, in this experiment, both rumen NH_3-N and PUN increased with starch level. Given the use of steam flaked corn it is likely the starch was rapidly fermentable and therefore energy spilling might have occurred without subsequent microbial protein growth, resulting in less N utilization and therefore higher N concentrations in the rumen (Van Kessel and Russell, 1996).

In addition to the higher PUN and ruminal NH_3-N concentrations, N was absorbed and converted to urea at a similar rate for animals fed either starch level (Table 3.39), but excreted proportionately more into the urine for cattle on the higher starch diets (Table 3.30-3.31). The effect of starch on ruminal to urine N exchanges occurred mainly with high CP diets, with much higher rumen and plasma N pool concentrations and subsequent urinary N excretion with high dietary starch only in the high CP diets (Tables 3.13 and 3.31). This result was not anticipated, as higher starch levels were assumed to stimulate urea-N entry to the GIT (Kennedy, 1980), but this effect may have been overpowered by the stronger effects of NH_3-N concentration at the rumen epithelial boundary or PUN concentrations (Abdoun et al., 2006; Abdoun et al., 2009; Kennedy et al., 1981).

Also, by examining the relationship between PUN and rumen NH_3-N, PUN was increased more per unit of ruminal NH_3-N concentration with high starch diets compared to low starch diets (Figure 3.1). This could be a reflection of microbial energy spilling, or poor ability to incorporate ruminal N into microbial cells with the available energy; however, since recycling did not change significantly by treatment, the differences could also be due to a large amount of available N from both feed and recycled urea N.

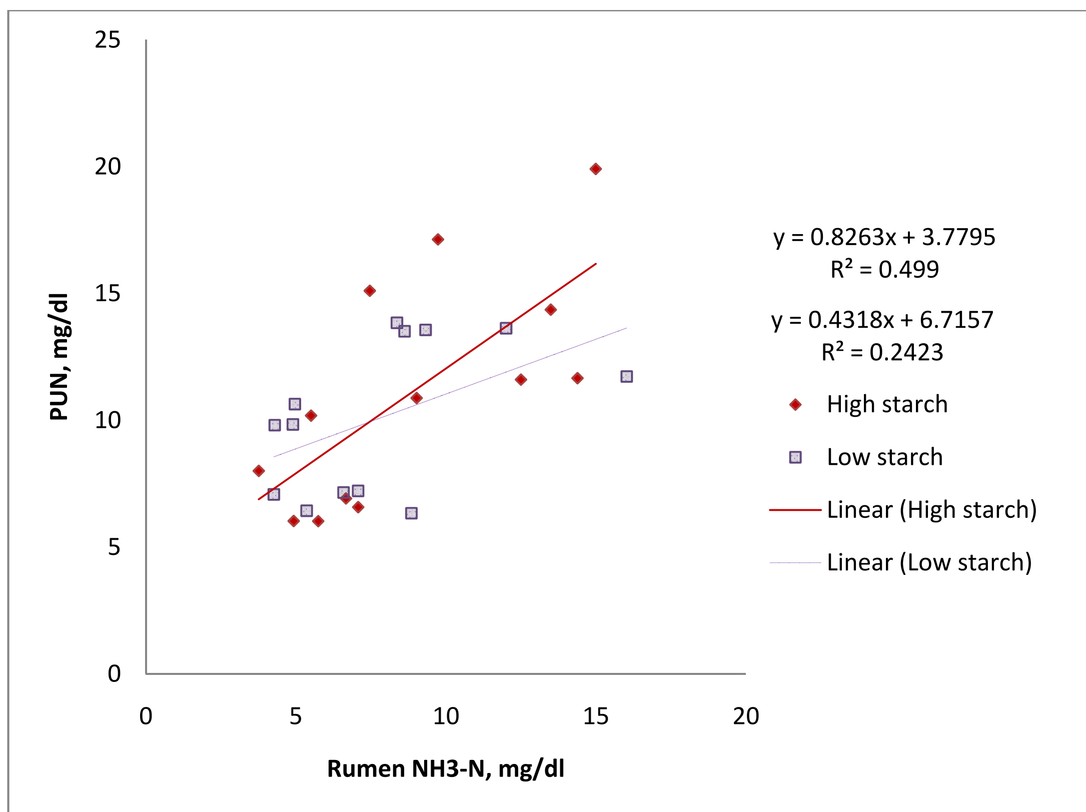

Figure 3.1. Relationship between plasma urea-N and rumen NH_3-N in lactating dairy cattle consuming diets containing different dietary starch concentrations, 22 and 27%.

An unexpected effect of Rumensin was that it numerically increased PUN and significantly decreased rumen NH_3-N among treatments (Table 3.12). This paradoxical effect has been observed in previous studies, but the cause is unclear (Duffield et al., 2003; Martineau et al., 2007). It could be indicative of a protein sparing effect of Rumensin, decreasing ruminal amino acid degradation and proportionally shifting more N degradation post-ruminally, which stimulates urea formation without concurrent ruminal NH_3-N concentration increases (Poos et al., 1979). Examining the relationship between PUN and rumen NH_3-N, Rumensin led to higher PUN concentrations among a range of rumen NH_3-N concentrations compared to cows fed diets without Rumensin, suggesting that this pattern was consistent regardless of dietary CP concentration or intake (Figure 3.2).

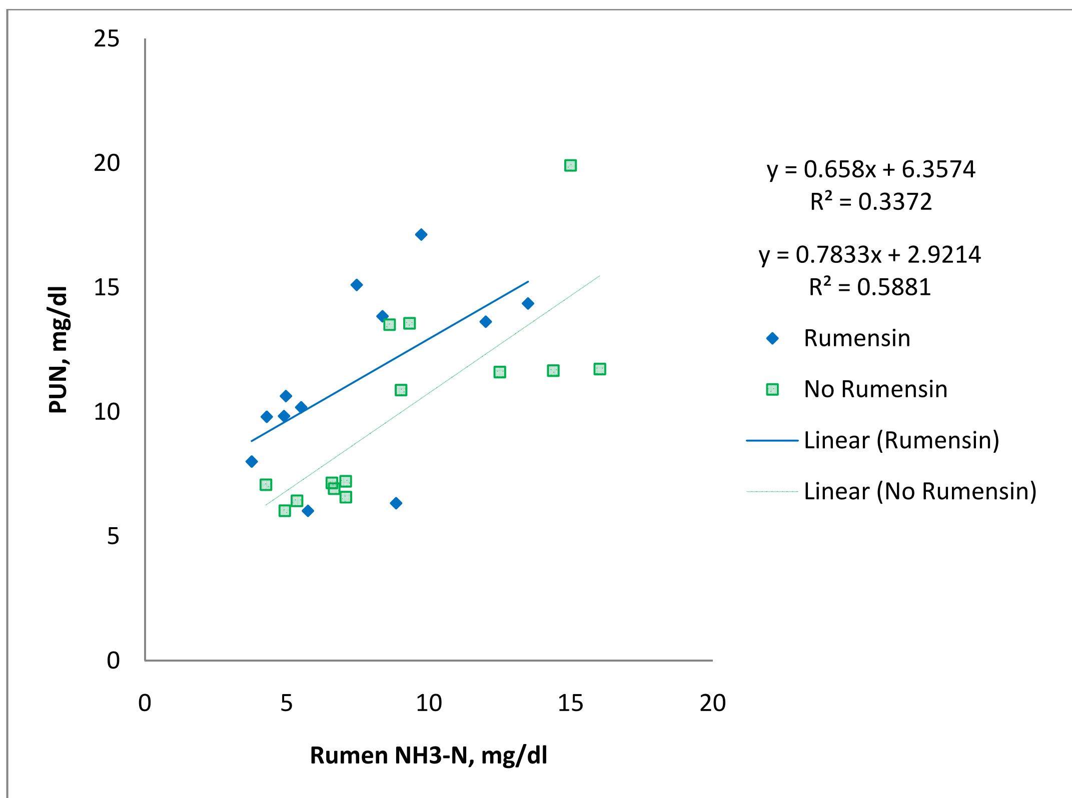

Figure 3.2. Relationship between plasma urea-N and rumen NH_3-N in lactating dairy cattle consuming diets with or without Rumensin at 400 mg/d.

It is likely that a partial explanation of the increase in PUN with Rumensin feeding was due to changes in rumen microbial pool size and N content with the main effects and interactions of dietary protein, starch and Rumensin (Table 3.17 and 3.26). The differences in N pools among level of dietary CP and starch in diets can serve as an example of this effect. Although not significantly different, there were large numerical and most likely biological differences in microbial content of the rumen in the cows fed the high protein, high starch diets with Rumensin, although the opposite was true for the lower starch diets. This shift in microbial content indicates that the previous changes in rumen ammonia under Rumensin feeding conditions might not be entirely due to protein sparing but could be due to a shift in microbial content and a

shift of ammonia from the rumen ammonia pool into the microbial pool (Tables 3.11, 3.20, and 3.26). Also, given the increase in urinary urea excretion in Rumensin fed animals, the lower levels of rumen ammonia might be due to faster clearance, but this requires further work to fully elucidate.

Rumen pH was relatively consistent among all treatments, with dietary means ranging from 5.83 to 6.08, with no significant effect of CP, starch, or Rumensin (Table 3.11). This range is typical for high producing cows in early lactation, and animal health did not seem to suggest this pH was too low (Tables 3.11-3.13).

Rumen VFA concentrations

None of the three main dietary factors significantly impacted VFA concentrations in the rumen except for isovaleric acid (Tables 3.14-3.15). Rumensin has previously been observed to increase propionic acid production, but its ruminal concentration does not always indicate this effect (Armentano and Young, 1983; Martineau et al., 2007). In this study, Rumensin decreased both propionic and acetic acid concentrations slightly, albeit not significantly, and decreased the acetate:propionate ratio (Table 3.15). However, a significant Rumensin*CP interaction in acetic acid concentration was observed, with Rumensin increasing acetic acid concentration on a high CP diets and decreasing it on low CP diets (Table 3.16). Higher dietary starch numerically reduced the acetate:propionate ratio, mainly due to decreasing acetic acid concentrations, and there were no interactive effects of dietary starch with CP or Rumensin. This result is paradoxical to previous assumptions, as starch fermentation results mainly in

Table 3.14. The concentration of volatile fatty acids in the ruminal fluid of lactating dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								**SEM**
	High Protein				**Low Protein**				
	High Starch		**Low Starch**		**High Starch**		**Low Starch**		
	w/o Rum.	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	
Total VFA, mM	46.6	50.9	53.1	49.4	53.0	42.5	46.3	49.0	2.89
Acetic acid, mM	28.4^{ab}	31.4^{ab}	32.3^{ab}	31.2^{ab}	32.9^{a}	25.2^{c}	29.4^{ab}	29.2^{ab}	1.73
Propionic acid, mM	10.0	10.9	11.8	10.3	11.5	9.24	9.58	10.6	0.87
Ac:Pr	2.89	2.89	2.82	3.03	2.93	2.75	3.12	2.81	0.13
Isobutyric acid, mM	0.82	0.42	0.52	0.27	0.33	0.72	0.40	0.94	0.24
Butyric acid, mM	4.80	5.50	5.78	5.61	6.19	5.00	5.51	6.02	0.48
Isovaleric acid, mM	0.92^{ab}	1.19^{a}	1.00^{ab}	1.04^{ab}	0.82^{ab}	0.69^{b}	0.60^{c}	0.73^{ab}	0.10
Valeric acid, mM	0.67	0.96	0.77	0.57	0.68	0.65	0.59	0.55	0.15

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.15. Effects of dietary CP, starch, and Rumensin on the concentration of ruminal volatile fatty acids in lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
Total VFA, mM	50.02	47.49	1.52	48.4	49.11	1.43	49.74	47.77	1.52	0.24	0.71	0.36
Lactate, mM	0.59	0.71	0.16	0.70	0.60	0.16	0.64	0.66	0.16	0.57	0.68	0.94
Formic acid, mM	0.20	0.16	0.18	0.20	0.17	0.18	0.20	0.17	0.18	0.31	0.29	0.43
Acetic acid, mM	30.8	29.1	0.90	29.6	30.3	0.85	30.7	29.1	0.90	0.17	0.51	0.21
Propionate, mM	10.8	10.2	0.53	10.5	10.5	0.44	10.7	10.2	0.53	0.42	0.97	0.50
Ac:Pr	2.91	2.91	0.08	2.87	2.95	0.07	2.94	2.88	0.08	0.99	0.12	0.58
Isobutyric cid, mM	0.51	0.60	0.12	0.57	0.53	0.12	0.52	0.59	0.12	0.60	0.83	0.69
Butyric acid, mM	5.43	5.66	0.29	5.39	5.69	0.24	5.57	5.51	0.29	0.58	0.24	0.89
Isovalerate, mM	1.04	0.70	0.06	0.91	0.83	0.05	0.83	0.91	0.06	0.0004	0.11	0.40
Valeric acid, mM	0.74	0.61	0.08	0.74	0.61	0.08	0.68	0.68	0.08	0.24	0.23	0.99

Table 3.16. The effect of two levels of dietary CP and starch, with or without Rumensin on ruminal fluid VFA concentration of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Total VFA, mM	Lactate, mM	Acetic acid, mM	Propionic acid, mM	Isobutyric acid, mM	Isovaleric acid, mM	Valeric acid, mM
high	high		48.8	0.62	29.9	10.5	0.62	1.06	0.82
high	low		51.2	0.56	31.7	11.1	0.40	1.02	0.67
low	high		48.0	0.77	29.2	10.4	0.52	0.76	0.67
low	low		47.0	0.65	28.9	9.89	0.67	0.65	0.56
P value			**0.37**	**0.90**	**0.38**	**0.21**	**0.28**	**0.44**	**0.85**
high		no	49.8	0.84	30.4	10.9	0.67	0.96	0.72
high		yes	50.2	0.34	31.3	10.6	0.34	1.11	0.76
low		no	49.6	0.45	31.1	10.5	0.36	0.71	0.64
low		yes	45.3	0.98	27.0	9.8	0.83	0.70	0.59
P value			**0.28**	**0.02**	**0.047**	**0.75**	**0.023**	**0.38**	**0.67**
	high	no	49.9	0.68	30.8	10.8	0.57	0.87	0.68
	high	yes	46.9	0.71	28.4	10.1	0.57	0.95	0.81
	low	no	49.5	0.61	30.7	10.7	0.46	0.79	0.68
	low	yes	48.7	0.60	29.9	10.3	0.60	0.87	0.55
P value			**0.56**	**0.93**	**0.49**	**0.73**	**0.66**	**0.98**	**0.24**

propionate formation rather than acetate production, which primarily proceeds from fiber fermentation. These results may indicate that either the highly fermentable end products such as propionate were absorbed rapidly, or the larger amount of starch and non-structural carbohydrate in the diet did not ferment in the rumen as was assumed to occur from the ingredient chemical compositions.

The dietary effects on ruminal BCVFA concentrations are of special note. Branched chain VFA originate from the branched chain amino acids and are obligate requirements for fiber fermenting bacteria (Bryant et al, 1973). Branched chain amino acids have been indicated to be deficient in high producing cows; thus, there may be a production benefit from preserving these amino acids from ruminal degradation (Mackle et al., 1999). Isovaleric acid concentration was significantly increased by dietary CP and was numerically increased by starch, potentially impacting the fiber digesting bacteria, whereas none of the other BCVFAs appeared to be significantly affected directly by any of the three main dietary factors (Table 3.15). There was no consistent pattern of dietary CP effects on BCVFA concentrations, as both increases and decreases were observed. However, there were a few interesting dietary interactions on BCVFA concentrations. The concentration of isobutyric acid was decreased by Rumensin in the high CP diets, but increased in the low CP diets (Table 3.16). However, the concentration changes occurred in the opposite directions for butyric, isovaleric, and valeric acids, albeit to a smaller magnitude. The reasons for these interactions are unclear, but they suggest changes in the availability of branch chained amino acids related to both carbohydrate availability and Rumensin.

Ruminal contents – microbial pool sizes

Dietary CP numerically increased all rumen microbial pool sizes, but on a microbial N basis the liquid associated bacterial and protozoal N pool sizes did not appreciably change (Tables 3.18 and 3.21). Dietary CP has been observed to increase microbial yield when ruminal N is limiting, and based on the ruminal NH_3-N concentrations for the low CP diets (average of 5.8 mgN/dl), the microbial populations might have been slightly N deficient. Also, given the previous discussion, the branch chain amino acids, which are obligate requirements for fiber fermenting bacteria, might have been deficient in some of these diets. Microbial pool sizes are not always directly related to total microbial yield due to the effects of rumen available carbohydrates and turnover, but in this study the low CP diets had 40% lower total microbial N pool sizes on average and 18% lower microbial yield based on urinary purine analysis (Tables 3.21 and 3.36). Although lower CP did result in reduced microbial N pool sizes, this was mainly due to its impact on the particle associated bacterial N much more than either the liquid associated bacterial and protozoal N pool sizes, both of which showed virtually no change due to dietary protein concentration. The decrease in particle associated bacterial N pool may have resulted from BCVFA deficiency, but there does not appear to be a correlation between BCVFA concentration and this microbial pool size (Tables 3.16 and 3.22).

Table 3.17. Rumen solid, liquid, and microbial pool sizes for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Rumen DM, kg	11.6	14.1	10.4	13.3	12.3	12.5	11.3	11.6	1.46
Rumen liquid, kg	68.4	79.6	71.2	78.6	68.0	69.2	65.5	64.8	5.69
Liquid assoc. bacteria, g	246	271	245	223	210	214	227	170	38.1
Particle assoc. bacteria, g	1041	1767	1912	1731	520	1366	988	527	419
Protozoa, g	252	294	268	355	337	228	308	200	49.1
Total microbes, g	1539	2333	2425	2277	1111	1808	1522	897	475

Table 3.18. Effects of dietary CP, starch, and Rumensin on rumen solid, liquid, and microbial pool sizes of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	Star-ch	Rum
Liquid assoc. bacteria, g	246	205	20	235	216	19	232	220	20	0.15	0.45	0.66
Particle assoc. bacteria, g	1609	852	277	1175	1286	211	1117	1344	277	0.06	0.47	0.57
Protozoa, g	292	268	27	278	283	25	291	269	27	0.54	0.87	0.57
Total microbes, g	2140	1336	314	1699	1777	239	1651	1825	314	0.08	0.66	0.70

Table 3.19. The effect of two levels of dietary CP and starch, with or without Rumensin on rumen solid, liquid, and microbial pool sizes of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Liquid assoc. bacteria, g	Particle assoc. bacteria, g	Protozoa, g	Total microbes, g
high	high		259	1409	273	1941
high	low		234	1808	311	2338
low	high		212	941	282	1457
low	low		198	764	255	1215
P value			**0.81**	**0.07**	**0.29**	**0.07**
high		no	246	1477	260	1982
high		yes	247	1741	324	2297
low		no	218	757	323	1320
low		yes	192	947	215	1353
P value			**0.63**	**0.92**	**0.03**	**0.75**
	high	no	228	778	294	1322
	high	yes	243	1572	262	2076
	low	no	236	1456	289	1979
	low	yes	196	1116	277	1574
P value			**0.28**	**0.0007**	**0.74**	**0.002**

Table 3.20. Rumen microbial N pool sizes for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Liquid assoc. bact. N, g	12.1	11.6	10.9	9.8	12.2	11.4	12.3	8.9	2.57
Particle assoc. bact. N, g	60.6	94.3	118.5	105.5	27.2	74.9	57.9	27.9	30.8
Protozoa N, g	13.5	17.5	15.2	21.6	20.8	12.5	20.3	10.5	3.47
Total microbial N, g	86.1	123.4	144.7	136.9	60.2	98.8	90.5	47.2	34.06

Table 3.21. Effects of dietary CP, starch, and Rumensin on rumen microbial N pool sizes of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	**Low CP**	**SEM**	**High starch**	**Low starch**	**SEM**	**No Rum**	**Rum**	**SEM**	**CP**	**starch**	**Rum**
Liquid assoc. bact. N, g	11.1	11.2	1.5	11.8	10.5	1.2	11.9	10.4	1.5	0.96	0.34	0.49
Particle assoc. bact. N, g	94.7	47.0	18.2	64.0	77.7	14.8	66.1	75.7	18.2	0.09	0.36	0.72
Protozoa N, g	17.0	16.0	1.8	16.0	16.9	1.7	17.5	15.5	1.8	0.73	0.66	0.48
Total microbial N, g	122.8	74.2	20.4	91.8	105.1	16.4	95.4	101.6	20.4	0.12	0.41	0.83

Table 3.22. The effect of two levels of dietary CP and starch, with or without Rumensin on rumen microbial N pool sizes of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Liquid assoc. bact. N, g	Particle assoc. bact. N, g	Protozo-al N, g	Total microbial N, g
high	high		11.8	77.5	15.5	104.8
high	low		10.3	112.0	18.4	140.8
low	high		11.8	50.5	16.6	78.9
low	low		10.6	43.4	15.5	69.5
P value			**0.90**	**0.18**	**0.34**	**0.17**
high		no	11.5	90.0	14.4	115.4
high		yes	10.7	100.0	19.6	130.2
low		no	12.3	42.6	20.6	75.4
low		yes	10.1	51.4	11.5	73.0
P value			**0.75**	**0.98**	**0.02**	**0.77**
	high	no	12.1	43.4	17.1	72.6
	high	yes	11.5	84.6	15.0	111.1
	low	no	11.6	88.7	17.9	118.2
	low	yes	9.3	66.7	16.0	92.1
P value			**0.55**	**0.05**	**0.95**	**0.06**

Neither dietary starch concentration nor Rumensin had any significant effects on microbial pool sizes, but several significant interactions were noted (Tables 3.20 and 3.23). Rumensin increased protozoal N pool size by 36% with high CP diets, but decreased it by 44% with low CP diets (Table 3.22). The reasons for this are not clear at this time but are most likely associated with changes in bacterial availability and changes in predation. Various studies have demonstrated Rumensin to have negative effects on protozoal viability and growth (Sylvester et al., 2009), but none of their comparisons examined differential effects of Rumensin relative to changes in dietary concentrations. They were also conducted in *in vitro* rumen fermentors that do not mimic rumen conditions, particularly the associative relationships of all the microbial populations and available substrates. Due to the complexity of changes in rumen available substrates and in microbial pool size and content, it is apparent that a variety of interactions play a major role in the final ruminal outcome, and therefore the productive outcome, of feeding Rumensin. The observations from this experiment requires further study and repeatability.

Another significant dietary interaction demonstrated between Rumensin and high starch levels an increase in the particle associated bacterial N pool by 95% with high starch diets, but decreased it by 25% with low starch diets (Table 3.22). Again, although Rumensin has been shown to eliminate or decrease the activity of certain microbes, this study suggests further consideration be made to ruminal conditions such as fermentable organic matter type and N status and indicates that the response to Rumensin is more dynamic than previously considered.

^{15}N enrichment of microbes, feces, rumen NH_3-N

As higher CP diets lead to increased N concentration in the diet, rumen contents, and subsequent pools, it is not surprising that fecal N, rumen NH_3-N, and all microbial N pool APEs were significantly lower for these treatments (Tables 3.23-3.25). Among all treatments, low dietary starch numerically decreased all microbial N APE values and slightly increased ruminal NH_3-N APE, although these effects were far from significant ($P < 0.45$ to 0.77) (Table 3.24). Because NH_3-N contributes a significant proportion of the bacterial N supply for cellular growth, bacterial N and ruminal NH_3-N APE were anticipated to be similar or at least have similar responses to dietary factors. However, in this study the ^{15}N enrichment of the microbial contents changes in opposite directions with dietary starch, with ruminal NH_3-N concentrations increasing an average of 16% and APE decreasing by 9% with high starch diets with similar urea recycling measurements (Table 3.12, 3.24, 3.38). These results, along with the observation of little change in microbial pool sizes, suggest that microbes on the high starch diets were acquiring proportionally more of their N from recycled urea-N, which was more highly labeled.

Table 3.23. The ^{15}N enrichment in atom percent excess of feces, rumen NH_3-N, and microbial N for dairy cows supplemented with different dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Fecal N APE[1]	4.40	4.14	3.68	4.15	7.17	6.03	6.36	5.32	0.83
Rumen NH_3-N APE	4.49	3.70	3.19	5.25	6.36	4.80	7.81	4.80	1.13
Liquid assoc. bacterial N APE	5.40	4.82	4.60	6.04	10.37	7.23	9.75	6.48	1.23
Particle assoc. bacterial N APE	5.38	3.96	3.49	4.85	8.43	5.73	7.89	5.43	0.99
Protozoal N APE	5.08	4.48	5.06	5.46	8.70	5.78	6.76	5.55	1.07

[1]APE = atom percent excess, (%^{15}N of enriched sample – %^{15}N of background sample)/%^{15}N of background sample*100%

Table 3.24. Effects of dietary CP, starch, and Rumensin on ^{15}N enrichment, measured in atom percent excess, of feces, rumen NH_3-N, and microbial N of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	**Low CP**	**SEM**	**High starch**	**Low starch**	**SEM**	**No Rum**	**Rum**	**SEM**	**CP**	**starch**	**Rum**
Fecal N APE[1]	4.09	6.22	0.40	5.43	4.88	0.40	5.40	4.91	0.40	0.004	0.35	0.41
Rumen NH_3-N APE	4.16	5.94	0.58	4.80	5.30	0.56	5.46	4.64	0.58	0.054	0.53	0.34
Liquid assoc. bacterial N APE	5.22	8.46	0.69	6.94	6.74	0.59	7.53	6.14	0.69	0.008	0.77	0.19
Particle assoc. bacterial N APE	4.42	6.87	0.57	5.86	5.44	0.48	6.30	4.99	0.57	0.013	0.45	0.14
Protozoal N APE	5.02	6.70	0.54	6.01	5.7	0.51	6.40	5.31	0.54	0.054	0.65	0.19

[1]APE = atom percent excess, (%^{15}N of enriched sample – %^{15}N of background sample)/%^{15}N of background sample*100%

Table 3.25. The effect of two levels of dietary CP and starch, with or without Rumensin on ^{15}N enrichment, measured in atom percent excess, of feces, rumen NH_3-N, and microbial N of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Fecal N APE[1]	Rumen NH_3-N APE	Liquid assoc. bacterial N APE	Particle assoc. bacterial N APE	Protozoal N APE
high	high		4.27	4.10	5.11	4.67	4.78
high	low		3.92	4.22	5.32	4.17	5.26
low	high		6.59	5.51	8.77	7.04	7.25
low	low		5.84	6.38	8.15	6.70	6.14
P value			**0.73**	**0.64**	**0.55**	**0.88**	**0.26**
high		no	4.04	3.84	5.00	4.44	5.07
high		yes	4.14	4.47	5.43	4.41	4.97
low		no	6.76	7.09	10.06	8.16	7.73
low		yes	5.67	4.80	6.86	5.58	5.66
P value			**0.32**	**0.105**	**0.09**	**0.15**	**0.23**
	high	no	5.78	5.36	7.85	6.86	6.90
	high	yes	5.08	4.25	6.03	4.85	5.13
	low	no	5.02	5.57	7.21	5.73	5.90
	low	yes	4.73	5.03	6.26	5.14	5.50
P value			**0.73**	**0.72**	**0.53**	**0.21**	**0.32**

[1]APE = atom percent excess, (%^{15}N of enriched sample – %^{15}N of background sample)/%^{15}N of background sample*100%

Rumensin decreased microbial N APE, and this paralleled the decrease in rumen NH_3-N APE by approximately the same amount (Table 3.24). This does not suggest a difference in proportional uptake of ruminal NH_3-N in terms of utilizing recycled N , but it does demonstrate lower ^{15}N of the ruminal NH_3-N pool itself, as Rumensin decreased NH_3-N concentrations by 23% while paradoxically decreasing NH_3-N APE by 15% (Tables 3.12 and 3.24). Since Rumensin did not significantly impact the rate of urea-N recycling nor total microbial pool size, it is unclear where the ^{15}N labeled ruminal ammonia went. It is possible that Rumensin shifted more urea-N entry to the hindgut instead of the rumen, but fecal N APE also decreased by 9%. This suggests that Rumensin may have been exerting an influence on N partitioning in the animal, with proportionally less infused $^{15}N^{15}N$-urea entering the rumen or more being excreted in the urine since the ruminal APE values were reduced but recycling was not altered. These apparent shifts in N due to Rumensin are consistent with observations made by Elsasser (1984), indicating that ionophores could impact ion movement throughout the GIT and with the entire body component. However, all of this should be viewed with some caution since these effects were not statistically significant and had various treatment interactions.

Table 3.26. Ruminal microbial N concentration for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Liquid assoc. bacteria, %N	4.93	4.22	4.10	4.37	5.20	4.85	4.91	4.70	0.64
Particle assoc. bacteria, %N	5.69	5.64	6.01	5.99	5.52	5.55	5.77	5.27	0.20
Protozoa, %N	5.74	6.37	6.14	6.69	6.64	5.74	7.17	5.59	0.48

Table 3.27. Effects of dietary CP, starch, and Rumensin on ruminal microbial N concentration of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
Liquid assoc. bacteria, %N	4.41	4.92	0.36	4.79	4.53	0.31	4.79	4.54	0.36	0.32	0.46	0.63
Particle assoc. bacteria, %N	5.83	5.53	0.12	5.60	5.76	0.10	5.75	5.61	0.12	0.09	0.09	0.41
Protozoa, %N	6.23	6.29	0.28	6.12	6.40	0.23	6.42	6.09	0.28	0.89	0.25	0.42

Table 3.28. The effect of two levels of dietary CP and starch, with or without Rumensin on ruminal microbial N concentration of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Liquid assoc. bacteria, %N	Particle assoc. bacteria, %N	Protozoa, %N
high	high		4.58	5.67	6.05
high	low		4.24	5.99	6.40
low	high		5.01	5.53	6.18
low	low		4.82	5.53	6.39
P value			**0.83**	**0.09**	**0.76**
high		no	4.52	5.85	5.94
high		yes	4.30	5.81	6.52
low		no	5.06	5.65	6.91
low		yes	4.78	5.41	5.67
P value			**0.96**	**0.57**	**0.029**
	high	no	5.05	5.60	6.18
	high	yes	4.53	5.59	6.06
	low	no	4.52	5.90	6.66
	low	yes	4.54	5.62	6.13
P value			**0.46**	**0.16**	**0.39**

Nitrogen excretion measurements

Feeding diets with greater CP content resulted in approximately 25 and 53 gN/d more fecal and urinary N excretion, respectively (Table 3.30). These results fit relatively well with current literature (Kohn et al., 2002; Nennich et al., 2006; Wattiaux and Karg, 2004a), although comparatively few studies have fed diets in the range of our low CP treatments to high producing lactating cows. This data also demonstrates larger changes in urinary N compared to fecal N excretion, which has been often observed in a variety of dietary conditions (Weiss et al., 2009a). Nitrogen intake was 129 gN/d higher on average for the high CP diets, of which 59% was excreted in the manure (19% in feces and 40% in urine) and 11% in milk protein. Because these are the treatment mean averages for high vs. low CP, they are relative in scale rather than descriptive of total N balance. However, these means are comparable to a series of trials feeding cows 8-12% MP diets as a % of DM, in which 83% of the increased MP was excreted in manure (61% via urine and 22% via feces) and 17% via milk (Weiss et al., 2009b). Our study fits well with these results in demonstrating the partitioning of excess N beyond requirements and its impact on subsequent manure N excretion. Producers must balance additional feed costs that may be incurred by CP content, but these should be weighed with information about the marginal milk outputs and subsequent revenue (Recktenwald, 2007).

Table 3.29. Fecal and urinary N concentration and daily excretion for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Fecal NH_3-N, % of wet sample	0.051^{b}	0.046^{b}	0.052^{b}	0.048^{b}	0.044^{b}	0.047^{bc}	0.047^{b}	0.054^{ab}	0.003
Fecal N, % of wet sample	0.408	0.396	0.407	0.391	0.385	0.391	0.384	0.404	0.012
Fecal N, g/d	200.5	217.2	188.0	214.9	181.0	175.9	183.1	178.9	10.7
Urinary N, g/d	206.9^{ab}	223.0^{a}	187.0^{ab}	183.3^{ab}	130.5^{b}	168.9^{ab}	147.4^{b}	146.4^{b}	16.0
Urine urea N, g/d	110.5^{b}	122.9^{ab}	103.9^{b}	83.0^{b}	65.7^{c}	88.2^{b}	75.2^{b}	82.0^{b}	11.9
N balance, g/d[1]	65.6	102.1	128.8	100.4	72.0	34.9	73.2	76.2	34.0

[1]N balance = intake N – milk N – urine N – fecal N

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.30. Effects of dietary CP, starch, and Rumensin on fecal and urinary N daily excretion of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
Fecal N, g/d	205.1	179.7	6.2	193.4	191.3	5.1	188.1	196.6	6.2	0.0042	0.69	0.33
Urinary N, g/d	200.0	148.4	10.4	182.0	166.4	8.2	167.9	180.5	10.4	0.0008	0.037	0.40
Urine urea N, g/d	105.2	77.7	7.2	97.0	85.9	6.1	88.6	94.0	7.2	0.009	0.10	0.61
N balance[1]	99.2	64.1	16.5	69.9	93.4	16.5	84.9	78.4	16.5	0.16	0.34	0.78

[1]N balance = intake N – milk N – urine N – fecal N

Table 3.31. The effect of two levels of dietary CP and starch, with or without Rumensin on fecal and urinary N daily excretion of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Fecal N, g/d	Urinary N, g/d	Urine urea N, g/d	N balance
high	high		208.7	214.6	116.9	84.9
high	low		201.5	185.5	93.5	113.6
low	high		178.2	149.5	77.1	54.9
low	low		181.1	147.3	78.3	73.3
P value			**0.35**	**0.08**	**0.07**	**0.83**
high		no	194.2	196.9	107.2	97.2
high		yes	216.0	203.1	103.1	101.2
low		no	182.0	138.9	70.5	72.6
low		yes	177.3	157.8	85.0	55.5
P value			**0.13**	**0.67**	**0.37**	**0.66**
	high	no	190.4	168.1	88.6	70.3
	high	yes	196.5	196.0	105.4	69.5
	low	no	185.8	167.7	89.1	99.6
	low	yes	196.8	165.0	82.7	87.3
P value			**0.65**	**0.04**	**0.08**	**0.81**

Urinary N was significantly higher (16 g N/d) in cows fed the high starch diets, but fecal N excretion was not different (Table 3.30). The magnitude of the increase in mean urinary N and urea N excretion for high starch diets was almost solely observed in the high CP diets, however, as there were no significant differences in urinary N or urinary urea N excretion on the low CP diets with different dietary starch concentrations (Table 3.31). Since N intakes were virtually identical for high and low dietary starch concentrations on either high or low dietary CP (Table 3.7), this increase in urinary N with high starch on high CP diets is not explained by differences in intake. Rather, it appeared to be mainly due to higher urea synthesis (Table 3.40) with no change in partitioning of that synthesized urea into the urine (Table 3.43).

Rumensin did not affect fecal and urinary N excretion; however, the combination of Rumensin and starch did have a significant effect on urinary N excretion, increasing total urinary N and urinary urea N excretion with high starch diets with relatively no change for low starch diets (Table 3.31). Increases in urinary N excretion with Rumensin feeding have been observed in previous studies (Martineau et al., 2007; Poos et al., 1979), and these observations were associated with higher PUN concentrations, which are likely to be responsible for the increased urine N output. In this study, PUN concentration increased by 9.4% with Rumensin in the high starch diets, while urinary N excretion increased by 16.6% (Tables 3.13 and 3.31). However, these changes can only be viewed as relative indicators, as PUN is a body pool concentration whereas urinary N excretion is a final pool of outputted material.

As mentioned previously, Rumensin might be sparing protein in the rumen and shifting it to the hindgut, where it is not significantly utilized by the

microbial population and absorbed as amino acids. Another potential but not mutually exclusive hypothesis for these results is that Rumensin is facilitating a shift of ammonia out of the rumen and into the plasma, directly or indirectly, which explains the higher observed PUN and increased urinary urea excretion. Therefore, in this study Rumensin appeared to cause a shift in N to occur among these pools, albeit without significance except in high CP diets. Altogether, although Rumensin increased N excretion approximately 21 gN/d on average, it also increased milk yield by 1.8 kg/d, resulting in no significant change in either milkN:urineN or milkN:manureN ratios (Tables 3.32-3.34).

Although N excretion was approximately 75 gN/d higher with high CP diets, milk yield increased 2.2 kg/d, resulting in no significant change in either milkN:urineN or milkN:manureN ratios (Tables 3.32-3.34). There were, however, significant interactions between dietary CP and starch on N efficiency ratios. These ratios were both decreased by dietary starch on the high CP diets, but increased on the low CP diets (Table 3.34). As neither fecal nor urinary N excretion were affected by dietary starch on the low CP diets (Table 3.31), it appears that the higher N efficiency with high starch diets was due to improved milk protein yield (Table 3.10), while the poorer N efficiency with high dietary starch on the high CP diets was mainly explained by increased urinary N excretion with little change in milk protein yield. These results again suggest that there were numerous interactions occurring in the animals from ruminal fermentation to milk synthesis that impacted N utilization and its ultimate destination.

Table 3.32. Ratios of daily milk N to daily urinary N excretion and to daily manure N excretion (fecal N and urinary N) for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
MilkN:UrineN	0.85	0.89	1.00	1.11	1.45	1.08	1.16	1.09	0.20
MilkN:ManureN	0.41	0.45	0.47	0.51	0.56	0.53	0.49	0.47	0.05

Table 3.33. Effects of dietary CP, starch, and Rumensin on ratios of daily milk N secretion to daily urinary N excretion and to daily manure N excretion (fecal N and urinary N) of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
MilkN:UrineN	0.96	1.19	0.13	1.07	1.08	0.10	1.11	1.04	0.13	0.24	0.85	0.70
MilkN:ManureN	0.46	0.51	0.03	0.49	0.48	0.03	0.48	0.49	0.04	0.31	0.73	0.90

Table 3.34. The effect of two levels of dietary CP and starch, with or without Rumensin on ratios of daily milk N secretion to daily urinary N excretion and to daily manure N excretion (fecal N and urinary N) of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	MilkN:UrineN	MilkN:ManureN
high	high		0.87	0.43
high	low		1.05	0.49
low	high		1.27	0.55
low	low		1.12	0.48
P value			**0.05**	**0.004**
high		no	0.92	0.44
high		yes	1.00	0.48
low		no	1.30	0.52
low		yes	1.08	0.50
P value			**0.44**	**0.56**
	high	no	1.15	0.49
	high	yes	0.99	0.49
	low	no	1.07	0.48
	low	yes	1.09	0.49
P value			**0.22**	**0.63**

Urinary purines and microbial yield estimation

Dietary crude protein, starch and Rumensin each affected allantoin, uric acid, xanthine, and hypoxanthine excretion, although the differences were significant only in cows fed the higher CP level for allantion and uric acid (Table 3.35-3.37). This translated to increases in total urine purine derivative concentrations for high CP, high starch, and Rumensin feeding among all diets, with P values of 0.026, 0.16, and 0.36, respectively (Table 3.36). Similarly, estimated microbial N yield was higher for high CP, high starch, and Rumensin included diets (by 22, 5, and 8%, respectively), but again this was only significant for dietary CP. As previously discussed in reference to DMI and ruminal NH_3-N and BCVFA concentrations, it is possible that ruminal microbial populations might have been at or nearing N deficiency in cows fed the low CP diets. No significant interactions among factors were observed for urinary purine derivatives nor microbial N yield (Table 3.37). It should be noted that there were interactions of CP with Rumensin and dietary starch on ruminal NH_3-N concentrations, but this occurred only with the high CP diets and did not reach below 7.5 mg/dl, which makes them less likely to demonstrate microbial growth inhibition (Satter and Slyter, 1974) (Table 3.13). In summary, estimated microbial yield was greatest in the cows fed the high protein diets and although not significant, the increased microbial yield on the high protein, high starch with Rumensin diet is consistent with increased microbial N yield observed with each of the dietary treatment factors.

Table 3.35. Urinary purine excretion and estimated microbial N for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Allantoin, mmol/d	495	505	448	441	352	474	361	360	52.6
Uric acid, mmol/d	58.7	56.8	49.7	53.5	37.4	51.7	37.1	38.4	7.3
Xanthine, mmol/d	6.06	6.36	3.98	5.12	4.04	5.89	4.18	4.90	1.02
Hypoxanthine, mmol/d	21.4	27.4	28.8	25.4	23.3	31.6	22.2	26.8	5.3
Creatinine, mmol/d	155.3	145.0	139.1	123.0	128.1	175.3	125.5	141.0	18.3
Total purine derivatives, mmol/d	580.9	595.4	530.2	525.4	417.8	563.1	424.5	429.6	62.9
Fraction as allantoin, %	0.844	0.849	0.845	0.842	0.825	0.843	0.848	0.844	0.016
Microbial N, gN/d	383.5	467.2	410.0	407.4	342.7	360.8	322.8	349.4	38.4

Table 3.36. Effects of dietary CP, starch, and Rumensin on urinary purine excretion and estimated microbial N of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
Allantoin, mmol/d	472.3	386.9	25.0	455.0	404.2	24.9	414.0	445.2	25.1	0.019	0.15	0.38
Uric acid, mmol/d	54.7	41.3	3.5	50.9	45.0	3.5	45.8	50.2	3.6	0.0095	0.22	0.38
Xanthine, mmol/d	5.38	4.76	0.55	5.56	4.58	0.49	4.57	5.58	0.55	0.43	0.10	0.20
Hypoxanthine, mmol/d	25.8	26.0	2.9	26.0	25.7	2.5	23.9	27.8	2.9	0.96	0.93	0.35
Creatinine, mmol/d	140.6	142.6	9.4	150.6	132.6	8.7	137.0	146.2	9.5	0.88	0.11	0.50
Total purine derivatives, mmol/d	558.0	459.4	30.6	537.8	479.5	29.8	488.5	528.8	30.6	0.026	0.16	0.36
Fraction as allantoin, %	0.845	0.840	0.008	0.840	0.845	0.007	0.841	0.844	0.008	0.64	0.65	0.72
Microbial N, gN/d	417.5	343.3	18.4	390.4	370.4	18.1	365.0	395.8	18.3	0.0058	0.43	0.24

Table 3.37. The effect of two levels of dietary CP and starch, with or without Rumensin on urinary purine excretion and estimated microbial N of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Allantoin, mmol/d	Uric acid, mmol/d	Total purine derivatives, mmol/d	Fraction as allantoin, %	Microbial N, gN/d
high	high		499.0	57.6	587.1	0.847	427.1
high	low		445.6	51.7	528.8	0.843	407.9
low	high		411.0	44.2	488.4	0.833	353.7
low	low		362.9	38.3	430.3	0.847	332.9
P value			**0.94**	**0.99**	**0.99**	**0.44**	**0.98**
high		no	471.4	54.2	555.5	0.845	397.8
high		yes	473.1	55.2	560.4	0.845	437.3
low		no	356.7	37.3	421.5	0.837	332.2
low		yes	417.2	45.2	497.2	0.843	354.4
P value			**0.41**	**0.50**	**0.42**	**0.78**	**0.74**
	high	no	420.1	47.5	495.4	0.834	366.7
	high	yes	489.9	54.4	580.1	0.846	414.1
	low	no	408.0	44.1	481.6	0.847	363.3
	low	yes	400.4	46.0	477.5	0.843	377.6
P value			**0.27**	**0.60**	**0.28**	**0.46**	**0.51**

Urea-N recycling kinetics

Urea-N recycling kinetics were heavily impacted by dietary CP, significantly increasing urea production (UER), entry to the GIT (GER), excretion to the urine (UUE), excretion via feces (UFE), and urea-N used for anabolic purposes (UUA) (Tables 3.38-3.40). However, the difference in N intake did not change partitioning of urea within the animal, as the portion of synthesized urea that entered the GIT versus the urine was not significantly altered by dietary CP, nor was what occurred to it after being recycled to the GIT (Tables 3.41-3.43). This suggests that the mechanisms responsible for controlling the partitioning of urea-N were not changed by the amount of N consumed; therefore, the amount of urea produced and its subsequent kinetics were mainly determined by N intake rather than by changes in urea-N partitioning among the pools.

Table 3.38. Urea-N recycling kinetics for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
UER[a], gN/d	437.6	432.6	388.6	393.1	275.3	290.0	342.2	328.9	55.2
UUE[b], gN/d	111.8	124.0	114.1	97.1	71.9	87.1	72.6	83.0	12.8
GER[c], gN/d	325.8	305.5	274.6	299.2	208.2	199.7	264.9	249.0	52.3
ROC[d], gN/d	111.1	121.8	118.6	124.2	84.7	85.4	116.8	93.8	21.2
UFE[e], gN/d	0.947[b]	1.308[ab]	1.007[b]	0.818[b]	0.578[bc]	0.685[b]	0.704[b]	0.655[b]	0.125
UUA[f], gN/d	213.8	182.4	155.0	174.1	122.9	113.6	115.4	154.6	30.3

[a]UER = urea-N entry rate
[b]UUE = urinary urea elimination rate
[c]GER = gastrointestinal tract (GIT) urea entry rate, or recycled urea-N
[d]ROC = urea returning to the ornithine cycle
[e]UFE = urea-N excreted in the feces
[f]UUA = urea-N utilized for anabolic purposes
[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.39. Effects of dietary CP, starch, and Rumensin on urea-N recycling kinetics of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin, infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
UER[a], gN/d	413.0	309.1	23.4	358.0	364.0	25.5	360.9	361.2	25.7	0.019	0.87	0.99
UUE[b], gN/d	111.7	78.6	6.5	99.2	91.1	6.2	92.6	97.8	6.5	0.007	0.36	0.59
GER[c], gN/d	301.3	230.4	24.4	258.6	273.1	24.2	268.4	263.4	24.4	0.07	0.68	0.89
ROC[d], gN/d	118.9	95.2	9.9	100.3	113.8	9.8	107.8	106.3	9.9	0.12	0.36	0.92
UFE[e], gN/d	1.020	0.658	0.061	0.888	0.790	0.061	0.812	0.866	0.062	0.0032	0.29	0.55
UUA[f], gN/d	181.3	126.4	15.7	158.2	149.6	14.3	151.6	156.2	15.8	0.039	0.65	0.84

[a]UER = urea-N entry rate
[b]UUE = urinary urea elimination rate
[c]GER = gastrointestinal tract (GIT) urea entry rate, or recycled urea-N
[d]ROC = urea returning to the ornithine cycle
[e]UFE = urea-N excreted in the feces
[f]UUA = urea-N utilized for anabolic purposes

Table 3.40. The effect of two levels of dietary CP and starch, with or without Rumensin on urea-N recycling kinetics of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	UER[a], gN/d	UUE[b], gN/d	GER[c], gN/d	ROC[d], gN/d	UFE[e], gN/d	UUA[f], gN/d
high	high		434.0	118.6	314.1	115.9	1.139	198.1
high	low		392.0	104.8	288.4	122.0	0.901	164.6
low	high		282.1	79.8	203.2	84.8	0.637	118.2
low	low		336.1	77.5	257.7	105.6	0.679	134.6
P value			**0.22**	**0.52**	**0.27**	**0.61**	**0.14**	**0.20**
high		no	413.1	112.9	300.2	114.8	0.977	184.4
high		yes	412.9	110.5	302.3	123.0	1.063	178.3
low		no	308.8	72.2	236.5	100.8	0.647	118.8
low		yes	309.4	85.1	224.4	89.6	0.670	134.1
P value			**0.99**	**0.43**	**0.84**	**0.51**	**0.73**	**0.64**
	high	no	354.8	92.8	264.7	97.1	0.779	168.3
	high	yes	361.3	105.7	252.6	103.6	0.996	148.0
	low	no	367.1	92.4	272.0	118.5	0.844	134.8
	low	yes	361.0	89.9	274.1	109.0	0.736	164.4
P value			**0.86**	**0.41**	**0.84**	**0.57**	**0.10**	**0.20**

[a]UER = urea-N entry rate
[b]UUE = urinary urea elimination rate
[c]GER = gastrointestinal tract (GIT) urea entry rate, or recycled urea-N
[d]ROC = urea returning to the ornithine cycle
[e]UFE = urea-N excreted in the feces
[f]UUA = urea-N utilized for anabolic purposes

Table 3.41. Partitioning of urea-N recycling kinetics for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								**SEM**
	High Protein				**Low Protein**				
	High Starch		**Low Starch**		**High Starch**		**Low Starch**		
	w/o Rum.	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	
UER to urine[a]	0.254	0.293	0.291	0.252	0.247	0.335	0.242	0.243	0.049
UER to GIT[b]	0.746	0.707	0.709	0.748	0.753	0.665	0.758	0.757	0.049
GER to ROC[c]	0.338	0.400	0.436	0.414	0.414	0.446	0.439	0.384	0.034
GER to UFE[d]	0.0029	0.0043	0.0037	0.0029	0.0029	0.0039	0.0034	0.0026	0.0006
GER to UUA[e]	0.659	0.597	0.561	0.584	0.584	0.550	0.555	0.613	0.035

[a]Proportion of the urea entry rate (UER) that enters the urine
[b]Proportion of the urea entry rate (UER) that enters the gastrointestinal tract (GIT)
[c]Proportion of the gastrointestinal tract urea entry rate (GER) that is returned to the ornithine cycle (ROC)
[d]Proportion of the gastrointestinal tract urea entry rate (GER) that enters the feces (UFE)
[e]Proportion of the gastrointestinal tract urea entry rate (GER) that is utilized for anabolic purposes (UUA)

Table 3.42. Effects of dietary CP, starch, and Rumensin on partitioning of urea-N recycling kinetics of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
UER to urine[a]	0.273	0.267	0.025	0.282	0.257	0.023	0.259	0.281	0.025	0.88	0.40	0.55
UER to GIT[b]	0.727	0.733	0.025	0.718	0.743	0.023	0.741	0.719	0.025	0.88	0.40	0.55
GER to ROC[c]	0.397	0.421	0.021	0.399	0.418	0.016	0.407	0.411	0.021	0.44	0.17	0.90
GER to UFE[d]	0.0035	0.0032	0.0003	0.0035	0.0032	0.0003	0.0032	0.0034	0.0003	0.59	0.29	0.70
GER to UUA[e]	0.600	0.576	0.021	0.598	0.578	0.016	0.590	0.586	0.021	0.45	0.20	0.91

[a]Proportion of the urea entry rate (UER) that enters the urine
[b]Proportion of the urea entry rate (UER) that enters the gastrointestinal tract (GIT)
[c]Proportion of the gastrointestinal tract urea entry rate (GER) that is returned to the ornithine cycle (ROC)
[d]Proportion of the gastrointestinal tract urea entry rate (GER) that enters the feces (UFE)
[e]Proportion of the gastrointestinal tract urea entry rate (GER) that is utilized for anabolic purposes (UUA)

Table 3.43. The effect of two levels of dietary CP, starch, and Rumensin on urea-N recycling kinetics of lactating dairy cows consuming diets differing in CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	UER to urine[a]	UER to GIT[b]	GER to ROC[c]	GER to UFE[d]	GER to UUA[e]
high	high		0.273	0.727	0.368	0.0036	0.628
high	low		0.272	0.728	0.425	0.0033	0.572
low	high		0.291	0.709	0.430	0.0034	0.567
low	low		0.243	0.757	0.411	0.0030	0.584
P value			**0.43**	**0.43**	**0.02**	**0.93**	**0.03**
high		no	0.273	0.728	0.387	0.0033	0.610
high		yes	0.273	0.727	0.406	0.0036	0.590
low		no	0.245	0.755	0.426	0.0031	0.569
low		yes	0.289	0.711	0.415	0.0032	0.582
P value			**0.55**	**0.55**	**0.63**	**0.86**	**0.62**
	high	no	0.250	0.750	0.376	0.0029	0.622
	high	yes	0.314	0.686	0.422	0.0041	0.574
	low	no	0.267	0.733	0.437	0.0036	0.558
	low	yes	0.248	0.752	0.399	0.0027	0.599
P value			**0.17**	**0.17**	**0.009**	**0.01**	**0.013**

[a]Proportion of the urea entry rate (UER) that enters the urine
[b]Proportion of the urea entry rate (UER) that enters the gastrointestinal tract
[c]Proportion of the gastrointestinal tract urea entry rate (GER) that is returned to the ornithine cycle (ROC)
[d]Proportion of the gastrointestinal tract urea entry rate (GER) that enters the feces (UFE)
[e]Proportion of the gastrointestinal tract urea entry rate (GER) that is utilized for anabolic purposes (UUA)

Starch had little effect on overall urea kinetics, but cows fed the higher dietary starch concentrations did appear to have a small but non-significant decrease in recycling to the GIT and more excretion via urine (Tables 3.39 and 3.30), which is contrary to our expectations about higher starch consumption. Our hypothesis was that increased fermentable substrate availability would stimulate microbial N production, leading to a greater N gradient across the ruminal wall (by depleting ruminal N) and therefore higher gastrointestinal urea N entry. In actuality, high dietary starch did not cause lower ruminal NH_3-N concentrations overall (9.0 vs 7.8 mg/dl), but only caused NH_3-N concentration increases in the high CP diets (Table 3.13). When examining urea kinetics in these diets, GER was numerically increased in the high dietary starch, high CP diets as compared to the low dietary starch, high CP diets, which does not coincide with our hypothesis about starch fermentation nor about NH_3-N gradient differences stimulating urea-N entry into the rumen. We anticipated that the NH_3-N concentrations would be lower and this would have potentially increased urea-N recycling. However, PUN concentrations were also higher by approximately the same amount as the ruminal NH_3-N concentration increases (Table 3.13). Therefore, this might have negated the effect of lower ruminal NH_3-N concentrations. It is very likely that it was difficult to observe our initial hypothesis of starch fermentation stimulating urea entry into the GIT due to a variety of factors that included changes in N gradients, microbial growth and N demand, and even fermentation byproducts. High dietary starch did cause proportionately more of the recycled urea-N to be used for anabolic purposes (i.e. microbial N synthesis), although this increase was only with the high CP diets (Table 3.40). This indicates that starch could in fact stimulate

use of recycled urea-N for microbial growth, but it might be better utilized if there is already enough ruminal N to maintain healthy microbial growth.

Rumensin was not observed to have any direct effects on urea-N kinetics, regardless of previously discussed effects on N excretion and ruminal NH_3-N and PUN concentrations (Table 3.30 and 3.12). There were highly significant interactions of Rumensin with dietary starch on the fate of recycled urea-N, increasing the proportion of recycled urea-N the exits the animal in feces on high starch diets, but decreasing it on low starch diets (Table 3.43). One possible reason why Rumensin could increase this pathway is through its effects on PUN concentration. By increasing PUN, potentially more urea can enter the hindgut, kidney, and rumen. However, PUN concentrations did not align with the GER to UFE ratio for starch*Rumensin interactions (Tables 3.13 and 3.43), suggesting that PUN concentration was not a factor. Another possibility is that Rumensin might have shifted N degradation to the hindgut, increasing NH_3-N concentrations or stimulating microbial growth in this region and therefore potentially impacting urea-N entry to the hindgut.

Rumensin also interacted with dietary starch to improve the proportion of recycled urea-N used for anabolism by 7% in low starch diets but decrease it by 8% in high starch diets (Table 3.43). This increase in anabolic use of recycled urea-N by Rumensin in low CP diets does fit with our original hypothesis about Rumensin. However, it was not recycling to the GIT that was affected (Table 3.40), but only improved utilization of that urea. Again, these results were all due to a combination of Rumensin's effects on ruminal NH_3-N and PUN concentrations, microbial growth, and general urea kinetics. It is possible that Rumensin improved microbial N and energy efficiencies, resulting in better utilization of less available NH_3-N and also more microbial

incorporation of non-NH_3-N sources rather than stimulating urea-N recycling such as previously hypothesized.

To further determine relationships among the urea-N recycling measurements, the various kinetic measurements were plotted by cow within treatment. Urea-N entry rate (UER) and gastrointestinal urea-N entry rate (GER) appeared to have a very strong relationship (R^2 of approximately 0.93 on average), regardless of dietary starch concentration or Rumensin addition (Figures 3.3 and 3.4).

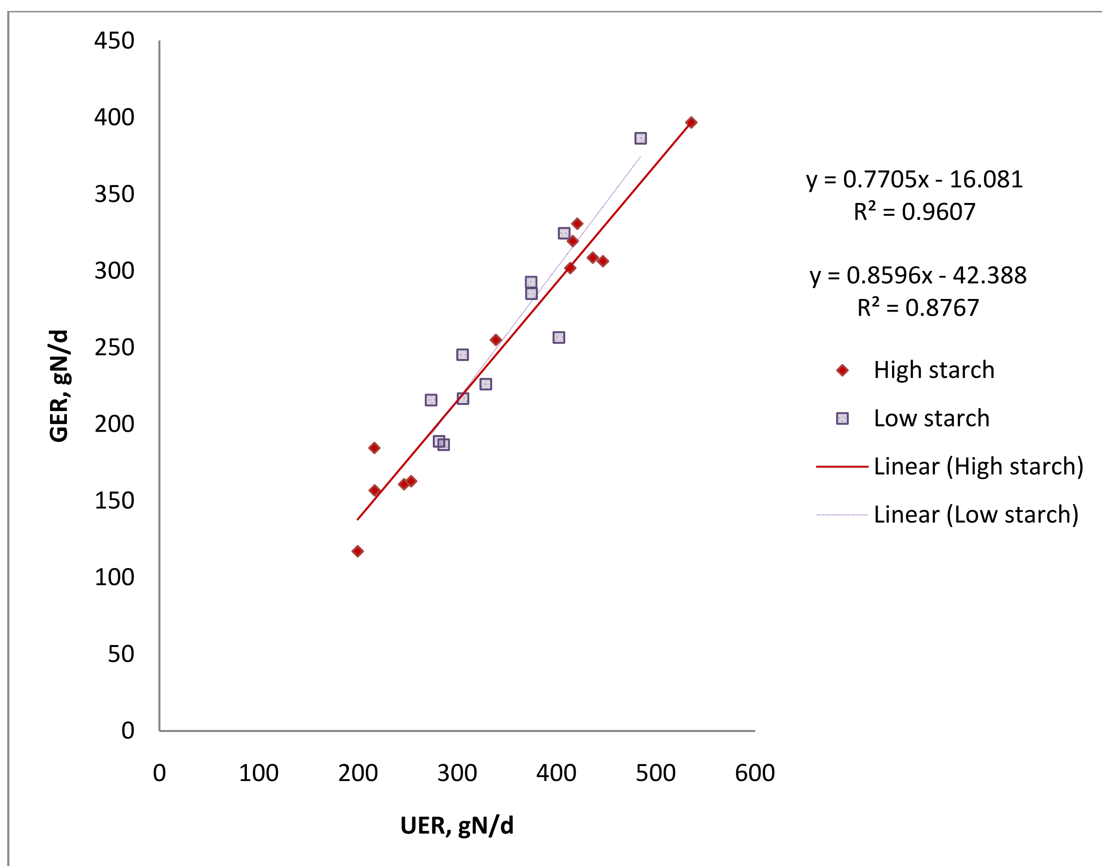

Figure 3.3. The relationship between urea-N synthesis rate (UER) and gastrointestinal urea-N entry rate (GER) in lactating dairy cattle consuming diets with differing starch contents, 22 and 27%, and infused with $^{15}N^{15}N$-urea.

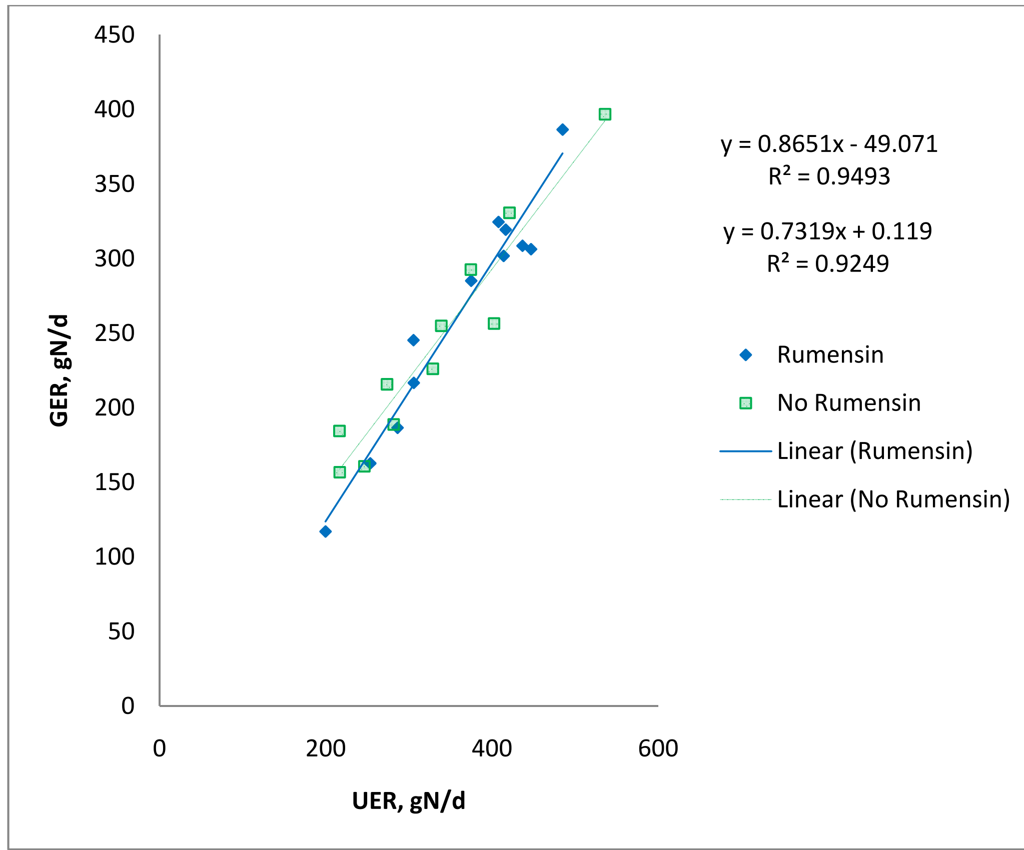

Figure 3.4. The relationship between urea-N synthesis rate (UER) and gastrointestinal urea-N entry rate (GER) in lactating dairy cows consuming diets with or without Rumensin at 400 mg/d and infused with $^{15}N^{15}N$-urea.

In light of the small effects of either Rumensin or starch on urea-N recycling but the large effect of dietary CP on all urea-N kinetic measurements, the lack of treatment variation due to either dietary starch concentration or Rumensin in these figures was expected. Stronger relationships between GER and UER were noted with high starch vs. low starch diets (R^2 = 0.96 and 0.88, respectively) (see Figure 3.3), but the difference in the R^2 value for either Rumensin or no Rumensin in the diet was small (Figure 3. 4). It is likely that high dietary starch concentrations resulted in more consistent ruminal conditions while maintaining microbial growth.

There were very slight differences in the slopes of the GER vs. UER graphs among dietary factors, with a slope of 0.77 for low starch diets (which might be interpreted as 77% of the UER entered the GIT across the range of UER measurements) and 0.88 for high starch diets (Figure 3.3). This type of analysis provides a slightly different perspective on the urea-N kinetics, and from it we can see that although high dietary starch in general appeared to decrease the proportion of UER to the GIT (Table 3.42) as compared to low dietary starch, it may have improved the efficiency of urea transfer into the GIT in relation to UER. However this effect was masked by the fact that the low dietary starch treatments also had slightly lower and more clustered UER values. Similarly, Rumensin may have improved urea transfer efficiency to the GIT because its slope was 0.87 compared to 0.73 observed in diets without Rumensin (Figure 3.4). However, this effect was not observed as a main effect (Table 3.42) potentially also due to the spread of the UER data.

The relationship between GER and both anabolic utilization of recycled urea-N (UUA) (Figures 3.5 and 3.6) and urea-N returned to the ornithine cycle (ROC) (Figures 3.7 and 3.8) were also quite strong (R^2 = 0.94 and 0.80 averaged among all treatments, respectively), demonstrating the consistency of these relationships among all dietary treatments. Due to the consistent nature of these relationships compared to those based on presumed factors influencing urea-N recycling, it seems that these measurements are better indicators for predicting urea-N recycling rather than attempting to estimate urea-N kinetics with either singular or multiple factors such as ruminal NH_3-N concentration (Figures 3.9 and 3.10) or PUN concentration (Figures 3.11 and 3.12). Although these factors can play a role in N recycling, their effect appears to be minimal when compared to that of other urea-N kinetic

interrelationships. Instead, it might be more appropriate for research to focus on determining urea synthesis rates, which can then be used to estimate further urea-N kinetics.

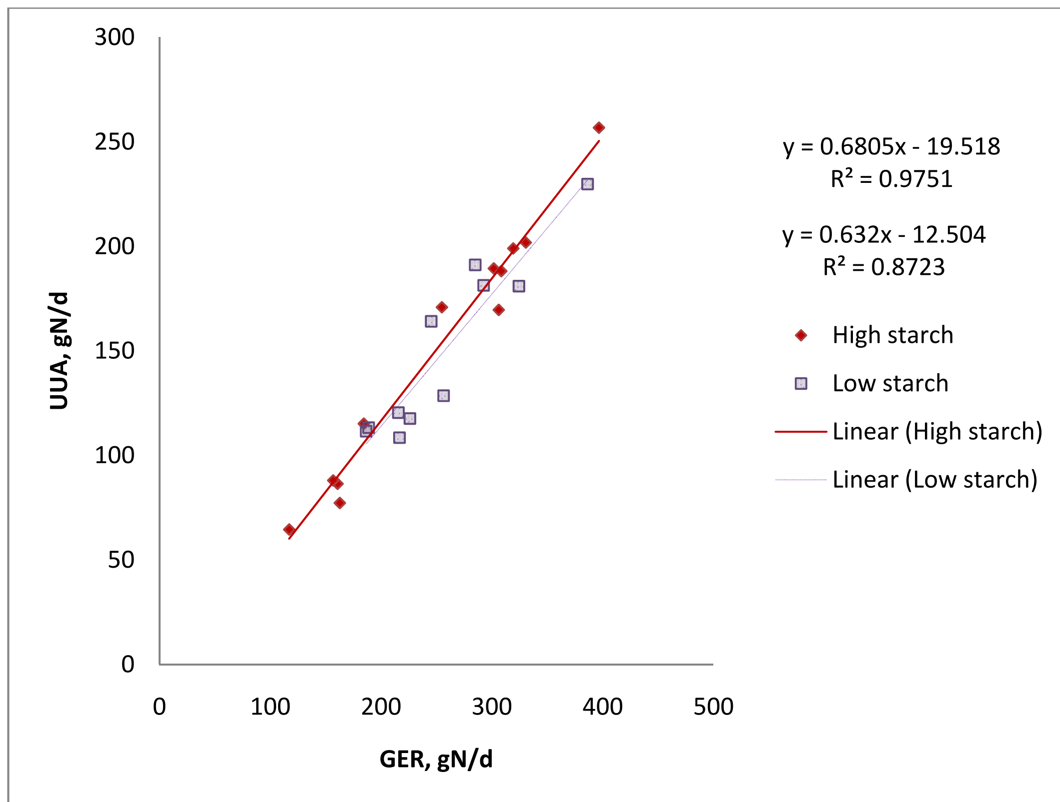

Figure 3.5. The relationship between gastrointestinal urea-N entry rate (GER) and urea-N used for anabolic purposes (UUA) in lactating dairy cattle consuming diets with differing starch contents, 22 and 27%, and infused with $^{15}N^{15}N$-urea.

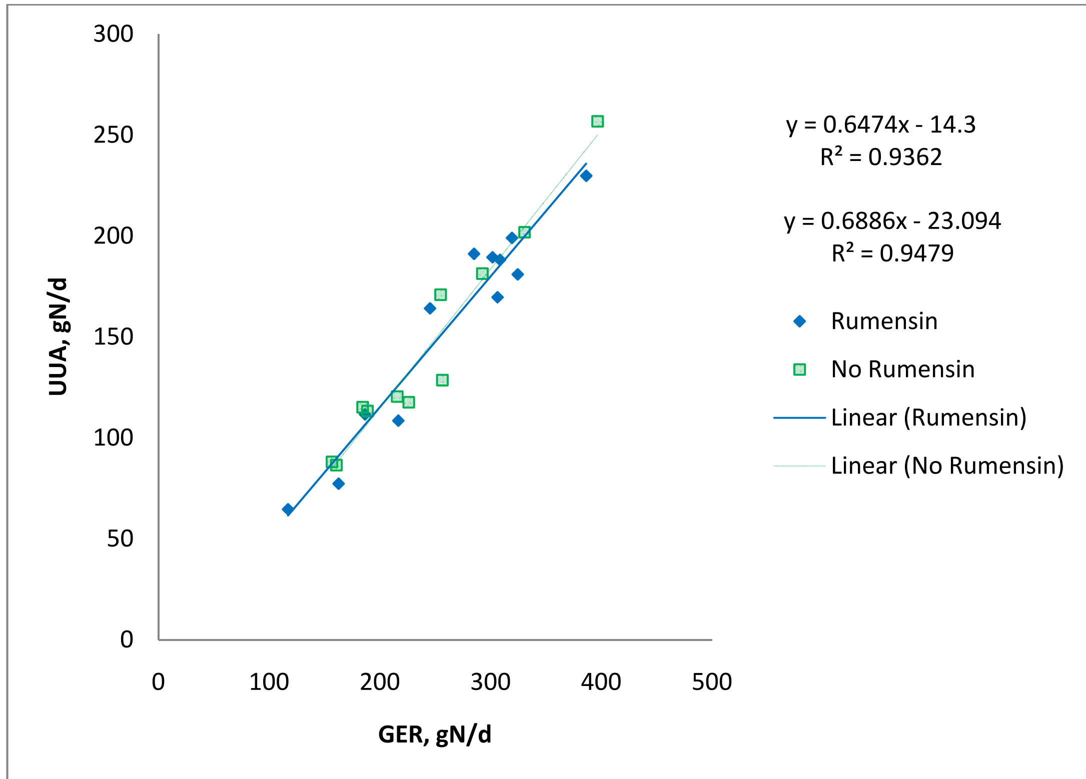

Figure 3.6. The relationship between gastrointestinal urea-N entry rate (GER) and urea-N used for anabolic purposes (UUA) in lactating dairy cattle consuming diets with or without Rumensin at 400 mg/d and infused with $^{15}N^{15}N$-urea.

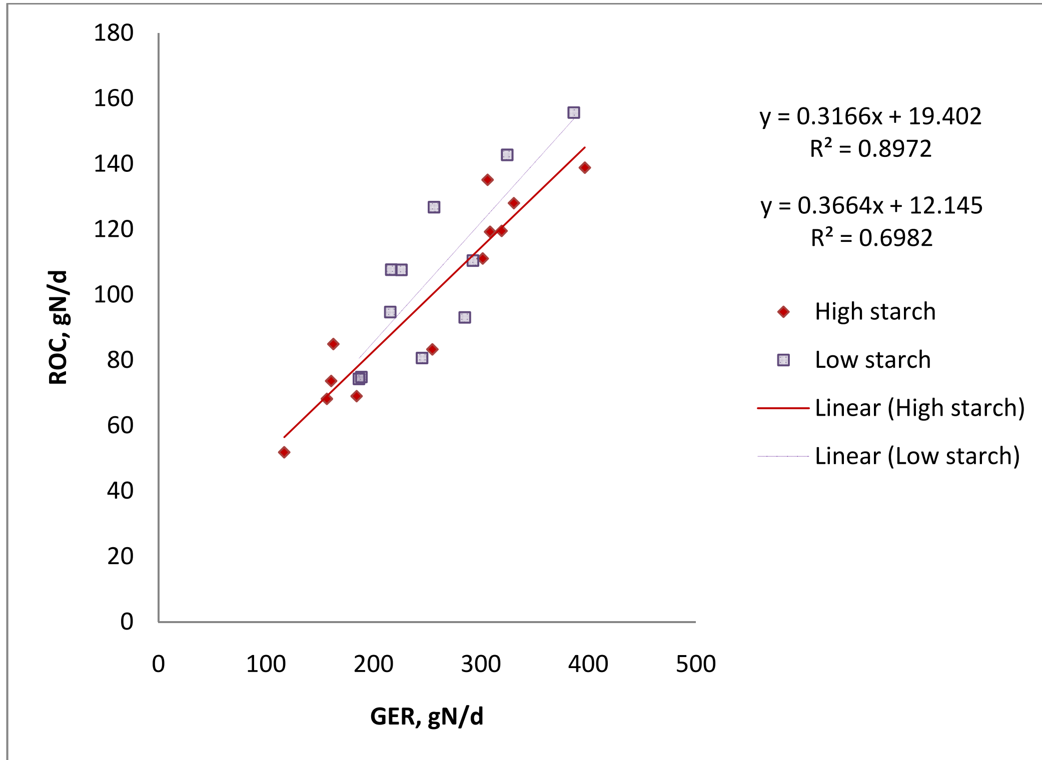

Figure 3.7. The relationship between gastrointestinal urea-N entry rate (GER) and urea-N returned to the ornithine cycle (ROC) in lactating dairy cattle consuming diets with differing starch contents, 22 and 27%, and infused with $^{15}N^{15}N$-urea.

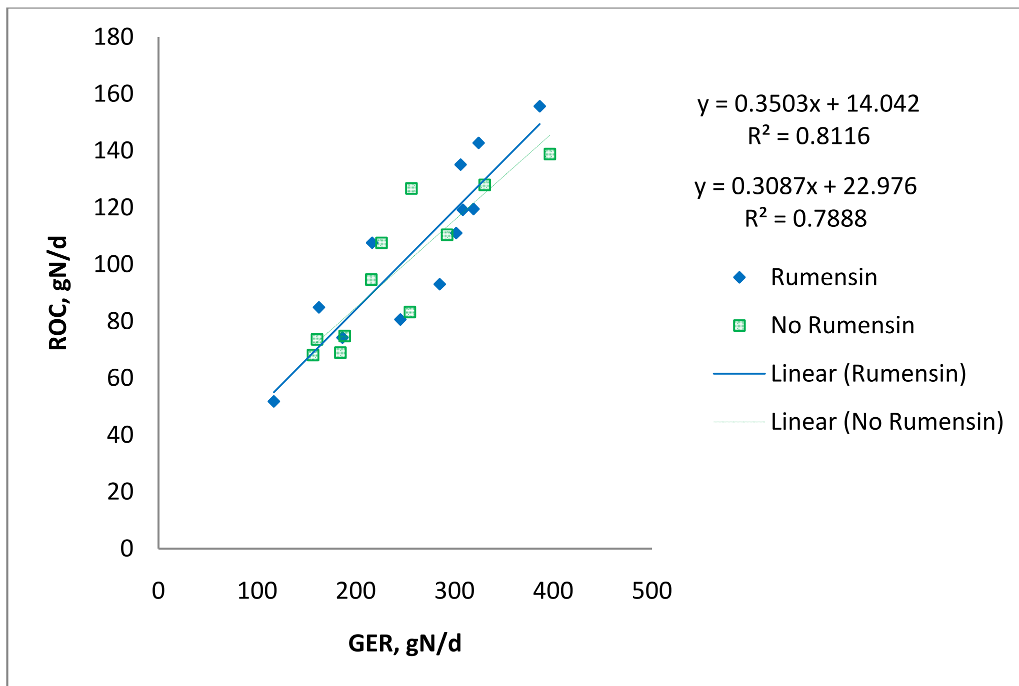

Figure 3.8. The relationship between gastrointestinal urea-N entry rate (GER) and urea-N returned to the ornithine cycle (ROC) in lactating dairy cattle consuming diets with or without Rumensin at 400 mg/d and infused with $^{15}N^{15}N$-urea.

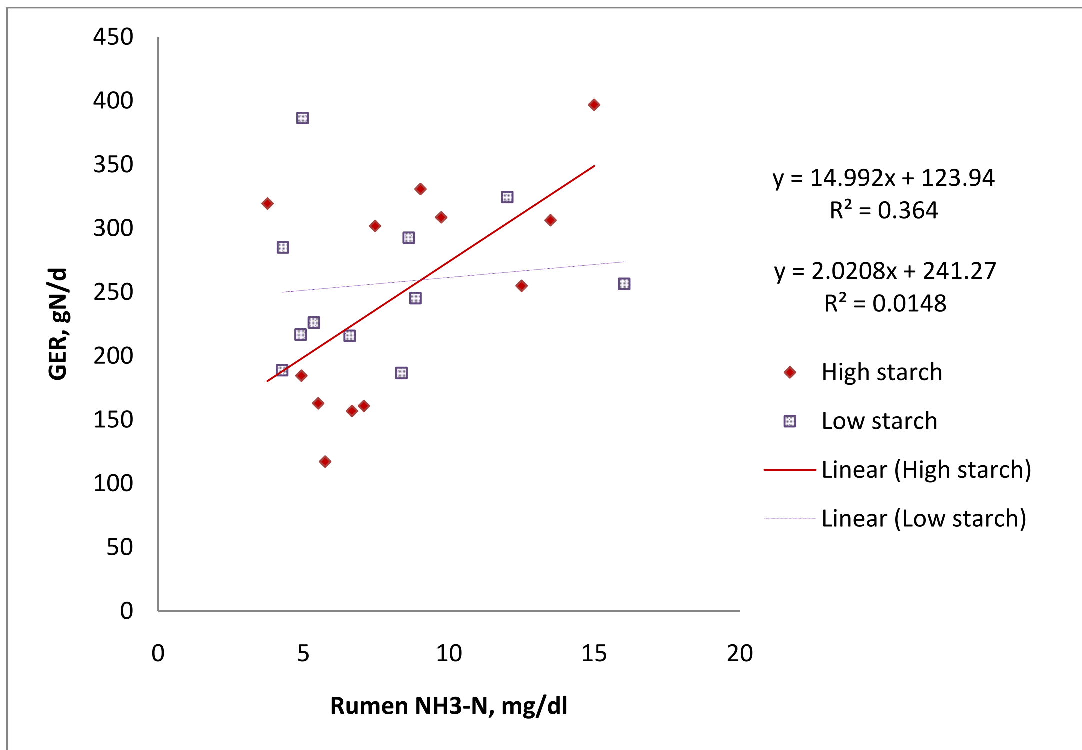

Figure 3.9. The relationship between rumen NH3-N concentration and gastrointestinal urea-N entry rate (GER) in lactating dairy cattle consuming diets with differing starch contents, 22 and 27%, and infused with $^{15}N^{15}N$-urea.

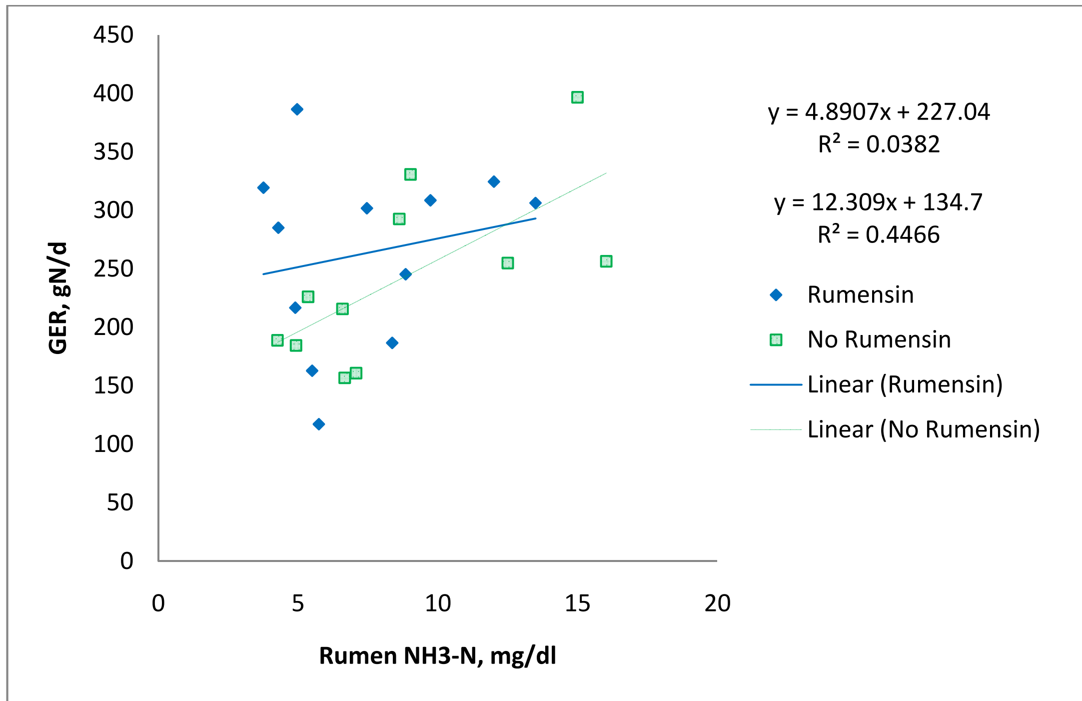

Figure 3.10. The relationship between rumen NH_3-N concentration and gastrointestinal urea-N entry rate (GER) in lactating dairy cattle consuming diets with or without Rumensin at 400 mg/d and infused with $^{15}N^{15}N$-urea.

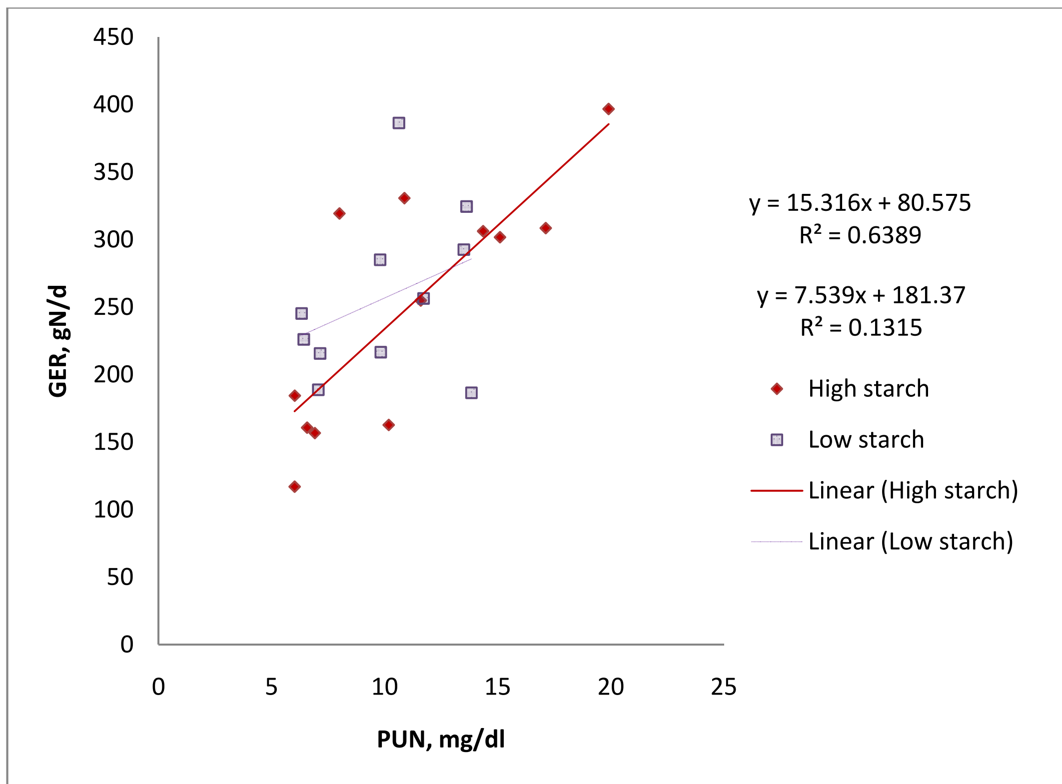

Figure 3.11. The relationship between PUN concentration and gastrointestinal urea-N entry rate (GER) in lactating dairy cattle consuming diets with differing starch contents, 22 and 27%, and infused with $^{15}N^{15}N$-urea.

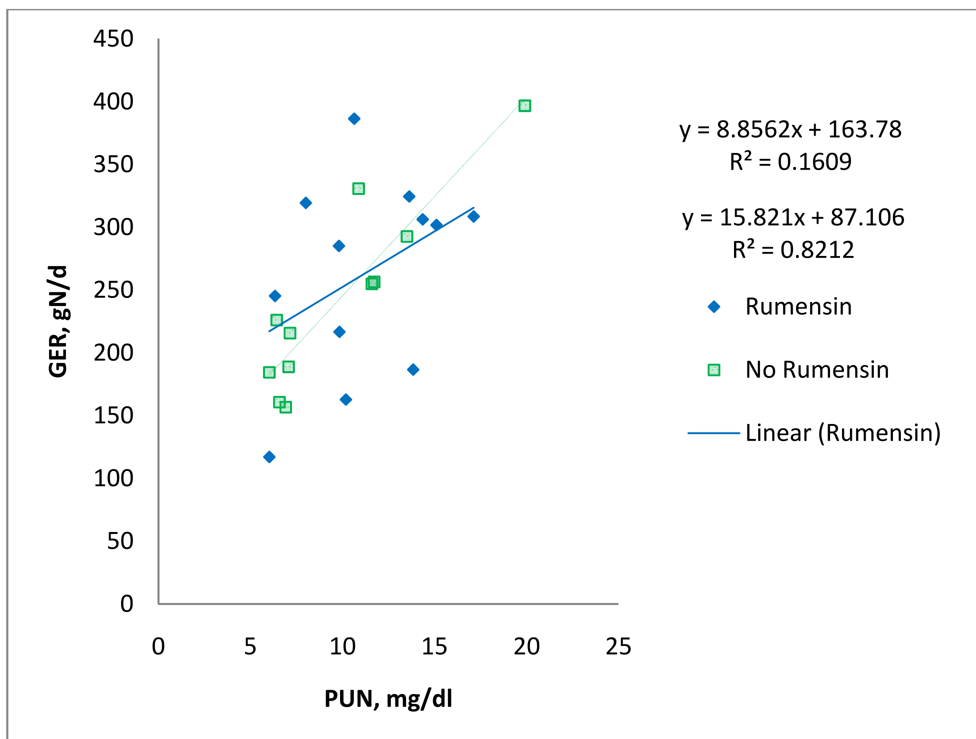

Figure 3.12. The relationship between rumen NH_3-N concentration and gastrointestinal urea-N entry rate (GER) in lactating dairy cattle consuming diets with or without Rumensin at 400 mg/d and infused with $^{15}N^{15}N$-urea.

Although PUN and ruminal NH_3-N concentrations were weakly correlated (R^2 = 0.40 among all treatments), PUN appeared to have a slightly stronger correlation to GER than rumen NH_3-N concentration, suggesting that PUN may be a better estimator of urea-N recycling than ruminal NH_3-N (Figures 3.9-3.12). However, ruminal NH_3-N concentrations vary widely, even within the same cow over the course of the day, making a single concentration less relevant than more consistent measures such as PUN. The rate of urea-N entry to the GIT increased with PUN concentrations over all starch and Rumensin levels, and the slope was quite steep (slope = 15 gN/d GER per 1 mg/dl PUN) with a relatively high correlation (R^2 = 0.64) in cattle fed the high dietary starch diets (Figure 3.11). Similar to observations for rumen NH_3-N

concentration, Rumensin seemed to have modified the relationship between PUN and GER (R^2 = 0.16), as animals not receiving Rumensin demonstrated a much tighter relationship (R^2 =0.82) (Figure 3.12). An interpretation of these results may be that PUN is a better indicator of GER than rumen NH_3-N concentration, and clearance of the plasma by the rumen epithelia may override any stimulatory or inhibitory effects of urea-N entry caused by diffusive N gradients across the wall (Abdoun et al., 2006; Abdoun et al., 2009; Kennedy et al., 1981). The apparent effect of Rumensin on altering the relationship between PUN and GER requires additional study. This data suggests altered transport of urea within the system, but the effect of Rumensin on urea transporters or kidney function is still lacking in the literature.

Although the ratio of NH_3/NH_4+ ions may play a minor role, if any, in urea-N entry, the effects of pH on GER should also be noted, as it has been implicated to affect entry rate (Abdoun et al., 2006). In this study, pH had virtually no correlation to GER among all treatments (R^2 = 0.06) (data not shown). Thus, within the conditions of this experiment, pH did not impact urea-N entry, at least in a magnitude that was large enough to be observed. However, pH values did not vary greatly in this study, so a lack of relationship might not be surprising. More recent work has suggested that rumen fluid pH might not be as significant a factor in N transport across the rumen epithelium as its relation to local pH effects across the apical epithelial wall. This pH appears to be highly impacted by SCVFAs and CO_2 concentrations (Abdoun et al., 2009).

Microbial N transaction estimates and utilization of recycled urea-N

Based on our calculations, among all diets, approximately 45% of protozoal N originated from liquid associated bacteria while 55% originated from particle associated bacteria (Tables 3.44-3.46). A small increase in protozoal N originating from the liquid associated bacterial N pool was observed in cows fed the high starch diets ($P < 0.13$) (Tables 3.45) and this was most likely due to increased growth of this microbial population compared to solid associated bacteria (Table 3.21). Overall, there was very little effect of treatment on the type of bacteria the protozoa were consuming. Approximately 21% of the microbial N was present as protozoal N, which corresponds with a previous result of 5-13% in dairy cattle fed diets similar diets with 16 and 21% forage NDF and using similar protozoal isolation methods that minimize bacterial contamination (Sylvester et al., 2005).

Table 3.44. The origin of protozoal N and estimated microbial turnover rates for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								**SEM**
	High Protein				**Low Protein**				
	High Starch		**Low Starch**		**High Starch**		**Low Starch**		
	w/o Rum.	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	**w/o Rum.**	**with Rum.**	
Proportion of protozoa from liquid assoc. bact.	0.51[ab]	0.45[b]	0.43[bc]	0.45[b]	0.45[b]	0.44[b]	0.46[b]	0.45[b]	0.019
Proportion of protozoa from particle assoc. bact.	0.49[bc]	0.55[b]	0.57[ab]	0.55[b]	0.55[b]	0.56[b]	0.55[b]	0.54[b]	0.019
Fraction of microbial N pool as protozoal N	0.16	0.16	0.13	0.28	0.34	0.13	0.24	0.22	0.062
bacterial turnover (d^{-1})[a]	6.15	5.46	4.31	12.52	9.12	4.96	5.35	8.73	3.68
protozoal turnover (d^{-1})[b]	6.15	5.46	4.31	12.52	9.12	4.96	5.35	8.73	3.68
75% bact. turn. rate	4.61	4.10	3.24	9.39	6.84	3.72	4.01	6.55	2.75
50% bact. turn. rate	3.08	2.73	2.16	6.26	4.56	2.48	2.67	4.37	1.83

[a]Bacterial turnover rate calculated from estimated bacterial N yield based on urinary purine derivative excretion divided by the sum of the liquid and particle associated bacterial N pool sizes in the rumen.
[b]Protozoal turnover rate was estimated to be equivalent to bacterial turnover rate (100%), only ¾ of the bacterial turnover rate (75%), or only half of the bacterial turnover rate (50%)
[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 3.45. Effects of dietary CP, starch, and Rumensin on the origin of protozoal N and estimated microbial turnover rates of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
Proportion of protozoa from liquid assoc. bact.	0.45	0.45	0.01	0.46	0.44	0.01	0.46	0.45	0.01	0.60	0.13	0.59
proportion of protozoa from particle assoc. bact.	0.54	0.55	0.01	0.54	0.55	0.01	0.54	0.55	0.01	0.60	0.13	0.59
Fraction of microbial N pool as protozoal N	0.18	0.23	0.03	0.20	0.22	0.03	0.22	0.20	0.03	0.25	0.64	0.65
bacterial turnover (d^{-1})[a]	7.11	7.04	1.86	6.41	7.74	1.74	6.23	7.92	1.86	0.98	0.57	0.54
protozoal turnover (d^{-1})[b]	7.11	7.04	1.86	6.41	7.74	1.74	6.23	7.92	1.86	0.98	0.57	0.54
75% bact. turn. rate	5.33	5.28	1.40	4.81	5.81	1.30	4.68	5.94	1.40	0.98	0.57	0.54
50% bact. turn. rate	3.56	3.52	0.93	3.21	3.87	0.87	3.12	3.96	0.93	0.98	0.57	0.54

[a]Bacterial turnover rate calculated from estimated bacterial N yield based on urinary purine derivative excretion divided by the sum of the liquid and particle associated bacterial N pool sizes in the rumen.
[b]Protozoal turnover rate was estimated to be equivalent to bacterial turnover rate (100%), only ¾ of the bacterial turnover rate (75%), or only half of the bacterial turnover rate (50%)

Table 3.46. The effect of two levels of dietary CP and starch, with or without Rumensin on protozoal N origins and microbial turnover rates of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Proportion of protozoa from liquid assoc. bact.	proportion of protozoa from particle assoc. bact.	Fraction of microbial N pool as protozoal N	bacterial turnover (d^{-1})	protozoal turnover (d^{-1})
high	high		0.48	0.52	0.16	5.8	5.8
high	low		0.44	0.56	0.20	8.4	8.4
low	high		0.44	0.56	0.24	7.0	7.0
low	low		0.45	0.55	0.23	7.1	7.1
P value			**0.025**	**0.025**	**0.53**	**0.58**	**0.58**
high		no	0.47	0.53	0.14	5.2	5.2
high		yes	0.45	0.55	0.22	9.0	9.0
low		no	0.45	0.55	0.29	7.2	7.2
low		yes	0.45	0.55	0.18	6.8	6.8
P value			**0.58**	**0.58**	**0.05**	**0.45**	**0.45**
	high	no	0.48	0.52	0.25	7.6	7.6
	high	yes	0.45	0.55	0.14	5.2	5.2
	low	no	0.44	0.56	0.18	4.9	4.9
	low	yes	0.45	0.55	0.25	10.6	10.6
P value			**0.05**	**0.05**	**0.06**	**0.10**	**0.10**

Bacterial N turnover, estimated from bacterial N yield based on urinary purine derivative excretion and from bacterial N pool sizes (see equations in Materials and Methods), ranged from 4.3 to 12.5 times per day and was not significantly affected by dietary factor (Tables 3.44 and 3.45). This is higher than 1.4-2.2 times per day reported by (Sylvester et al., 2005) or 1.5-3.2 times per day reported by (Karnati et al., 2007). However, these previous results were based on duodenal flows rather than urinary purine derivatives. Therefore, they do not include bacterial lysis or turnover in the rumen, which may partially explain the discrepancy between our results. Bacterial turnover rates have been estimated to be as high as 90%, but realistically this value is less than 50% (Firkins et al., 1992; Wells and Russell, 1996). However, more work needs to be done to quantitatively determine bacterial lysis more accurately.

With our estimates of protozoal turnover in comparison to bacterial turnover rate (which were set at an equivalent rate of turnover to only half the rate of bacterial turnover), the protozoa consumed between 27 and 255 g bacterial N/d, which was 7 to 45% of the estimated daily bacterial N yield (Table 3.47). None of the three main dietary factors significantly affected these measurements, but some general patterns can be observed. Higher dietary CP increased bacterial yield by 21.6% (see Table 3.36), which along with changes in microbial ^{15}N enrichment and yields, resulted in approximately 22% higher bacterial N consumption and 5% higher protozoal N yield, albeit by non-significant amounts relative to treatments (Table 3.48-3.49).

Table 3.47. Protozoal consumption of bacteria and protozoal yield for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
bacteria consumed by protozoa (g/d)[a]	1330	1924	1716	4815	3602	1014	1668	1889	1379
50% bact. turn. rate	665	962	858	2408	1801	507	834	945	689
bacterial N consumed by protozoa (gN/d)[a]	71	98	89	255	186	55	88	92	73.0
50% bact. turn. rate	35.3	49.0	44.3	127.6	93.0	27.3	44.0	45.9	36.5
Fraction of total bacterial N yield consumed[a]	0.18	0.21	0.23	0.60	0.52	0.14	0.27	0.27	0.166
75% bact. turn. rate	0.14	0.16	0.17	0.45	0.39	0.11	0.21	0.21	0.124
50% bact. turn. rate	0.09	0.11	0.11	0.30	0.26	0.07	0.14	0.14	0.08
protozoal yield (gN/d)[a]	72	88	60	251	190	57	103	96	71.0
50% bact. turn. rate	36.0	44.2	29.9	125.4	95.2	28.7	51.5	48.0	35.5

[a]Protozoal turnover rate was estimated to be equivalent to bacterial turnover rate (100%), only ¾ of the bacterial turnover rate (75%), or only half of the bacterial turnover rate (50%)

Table 3.48. Effects of dietary CP, starch, and Rumensin on protozoal consumption of bacteria and protozoal yield of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
bacteria consumed by protozoa (g/d)[a]	2446	2043	650	1970	2520	649	2079	2411	650	0.67	0.56	0.73
50% bact. turn. rate	1223	1022	325	985	1260	325	1039	1205	325	0.67	0.56	0.73
bacterial N consumed by protozoa (gN/d)[a]	128	105	34.4	102	131	34.2	108	125	34.4	0.65	0.57	0.74
50% bact. turn. rate	64.1	52.5	17.2	51.1	65.5	17.2	54.1	62.5	17.2	0.65	0.57	0.74
Fraction of total bacterial N yield consumed[a]	0.31	0.30	0.08	0.27	0.34	0.08	0.30	0.31	0.08	0.98	0.49	0.96
50% bact. turn. rate	0.15	0.15	0.04	0.13	0.17	0.04	0.15	0.15	0.04	0.98	0.49	0.96
protozoal yield (gN/d)[a]	118	112	33.5	102	128	33.5	106	123	33.5	0.90	0.59	0.73
50% bact. turn. rate	59	56	16.8	51	64	16.8	53	62	16.8	0.90	0.59	0.73

[a]Protozoal turnover rate was estimated to be equivalent to bacterial turnover rate (100%), only ¾ of the bacterial turnover rate (75%), or only half of the bacterial turnover rate (50%)

Table 3.49. The effect of two levels of dietary CP and starch, with or without Rumensin on protozoal consumption of bacteria and protozoal yield of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin.

CP	starch	Rum	bacteria consumed by protozoa (g/d)[a]	bacterial N consumed by protozoa (gN/d)[a]	Fraction of total bacterial N yield consumed[a]	protozoal yield (gN/d)[a]
high	high		1627	84.3	0.20	80.3
high	low		3266	172.0	0.41	155.3
low	high		2313	120.2	0.33	123.1
low	low		1773	90.0	0.27	100.3
P value			**0.26**	**0.25**	**0.24**	**0.33**
high		no	1523	79.6	0.21	65.9
high		yes	3370	176.7	0.40	169.6
low		no	2635	137.0	0.40	146.7
low		yes	1452	73.2	0.21	76.7
P value			**0.13**	**0.13**	**0.12**	**0.097**
	high	no	2471	128.2	0.35	130.4
	high	yes	1469	76.3	0.18	72.9
	low	no	1687	88.4	0.25	82.2
	low	yes	3352	173.5	0.44	173.4
P value			**0.18**	**0.19**	**0.13**	**0.15**

[a]Protozoal turnover rate was estimated to be equivalent to bacterial turnover rate (100%)

Feeding diets with higher starch concentrations decreased bacterial N turnover, thereby decreasing bacterial N consumption and protozoal N yield in conjunction with changes in microbial pool sizes and yields. Rumensin increased bacterial turnover rate, along with 16% increases in bacterial N consumption and protozoal N yield (Table 3.48). However, there was a strong interaction (P = 0.10) between Rumensin and dietary starch concentration on bacterial turnover rate that reflected its previously stated impact on microbial N yield (Table 3.37). This, along with combined effects on ^{15}N enrichment and pool sizes, then impacted bacterial N consumption and protozoal N yield, with Rumensin decreasing them by approximately 50% on high starch diets but increasing them by approximately 100% on low starch diets (Table 3.49).

In summary, approximately 45% of protozoal N was calculated to originate from liquid associated bacteria and 55% from solid associated bacteria among all treatments. Calculated bacterial turnover rates were approximately 6-8 times per day with only minor affects of dietary factor. Protozoal predation of bacterial N varied according to microbial turnover rates and pool sizes, but were on average stimulated by high dietary CP and Rumensin and by low dietary starch concentration. Although none of these factors demonstrated a significant difference, the biological differences and the implications to Rumensin interactions among dietary conditions is important for future work, especially modeling the response.

Use of Recycled Nitrogen by Microbial Populations

Recycled urea-N enters the GIT through both saliva and the rumen and post-ruminal epithelial walls. The urea kinetic method used in this experiment

does not discriminate between ruminal and post-ruminal entry; however, previous work suggests 75-90% of the urea-N entry is ruminal (Delgado-Elorduy et al., 2002; Huntington, 1989; Lapierre and Lobley, 2001). In addition, the amount of ^{15}N converted into body amino acids is minimal (<5%) (Lobley et al., 1995; Lobley et al., 1996). Therefore, for the purpose of this study we assumed all recycled urea-N to enter the rumen and to be utilized for microbial N synthesis if it was used for anabolic purposes. This will slightly overestimate microbial usage of recycled N, but is an appropriate estimate for the purposes of this study.

Using recycled urea-N kinetics and bacterial and protozoal yield estimates, approximately 41 and 34% of the bacterial and protozoal N yield, respectively, originated from recycled urea-N (Table 3.50). There were no significant main effects of dietary treatment factor on the proportion of microbial N yield from recycled N, but the value was numerically higher for high CP diets due to relatively higher UUA values than microbial N yields (Table 3.51). From the previous discussion, urea kinetics were highly dependent on dietary CP, with high CP diets resulting in more recycled N and urea utilized for anabolism but not necessarily resulting in higher microbial growth. However, averaging among studies, proportionally more microbial N originated from recycled urea-N with higher dietary CP. In a study with heifers fed incremental amounts of N, the proportion of bacterial N from recycled N decreased with increasing N intake (Marini and Van Amburgh, 2003). But GER values in that experiment did not correlate well to N intake and in fact remained relatively constant among diets, as did bacterial N yield. Therefore, it is unlike the currently reported experiment in which GER values and microbial yields did vary among

Table 3.50. Contribution of recycled urea-N to microbial yield for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet								SEM
	High Protein				Low Protein				
	High Starch		Low Starch		High Starch		Low Starch		
	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	w/o Rum.	with Rum.	
Fraction of bacterial N yield from UUA[1]	0.55	0.41	0.41	0.42	0.36	0.28	0.35	0.51	0.08
Fraction of protozoal yield from UUA[1]	0.41	0.39	0.47	0.32	0.23	0.26	0.23	0.41	0.08
75% bact. turn.	0.55	0.51	0.63	0.42	0.31	0.35	0.31	0.55	0.10
50% bact. turn.	0.82	0.77	0.94	0.63	0.47	0.53	0.47	0.82	0.15

[1]UUA = urea-N recycled to the gastrointestinal tract and utilized for anabolism

Table 3.51. Effects of dietary CP, starch, and Rumensin on contributions of recycled urea-N to microbial yield of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

	Diet									P value		
	High CP	Low CP	SEM	High starch	Low starch	SEM	No Rum	Rum	SEM	CP	starch	Rum
Fraction of bacterial N yield from UUA[1]	0.45	0.38	0.05	0.40	0.42	0.04	0.42	0.41	0.05	0.36	0.65	0.88
Fraction of protozoal yield from UUA[1]	0.40	0.29	0.04	0.33	0.35	0.04	0.34	0.34	0.04	0.10	0.60	0.95
75% bact. turn.	0.53	0.38	0.06	0.44	0.47	0.05	0.45	0.46	0.06	0.10	0.60	0.95
50% bact. turn.	0.79	0.57	0.08	0.66	0.71	0.08	0.68	0.69	0.08	0.10	0.60	0.95

[1]UUA = urea-N recycled to the gastrointestinal tract and utilized for anabolism

Table 3.52. The effect of two levels of dietary CP and starch, with or without Rumensin on contributions of recycled urea-N to microbial yield of lactating dairy cows consuming diets with different amounts of dietary CP and starch, with or without Rumensin and infused with $^{15}N^{15}N$-urea.

CP	starch	Rum	Fraction of bacterial N yield from UUA[1]	Fraction of protozoal yield from UUA[1,2]
high	high		0.48	0.41
high	low		0.41	0.39
low	high		0.33	0.25
low	low		0.43	0.32
P value			**0.05**	**0.36**
high		no	0.48	0.44
high		yes	0.42	0.35
low		no	0.36	0.24
low		yes	0.40	0.34
P value			**0.48**	**0.15**
	high	no	0.46	0.33
	high	yes	0.35	0.32
	low	no	0.38	0.35
	low	yes	0.47	0.36
P value			**0.03**	**0.79**

[1]UUA = urea-N recycled to the gastrointestinal tract and utilized for anabolism
[2]Values reported only for protozoal turnover rates equivalent to bacterial turnover rates

CP treatments, resulting in more complex relationships among N pools than simple dilution of N with higher intakes.

Ruminal N kinetics following urea bolus dose

A bolus dose of $^{15}N^{15}N$-urea was administered to six cows with subsequent sampling of the ruminal fluid in both the interior and the exterior of the rumen mass for 14 h. The ^{15}N enrichment of ammonia from these samples was analyzed to determine the rate constants for ammonia in these two pools of the rumen. Urea enters the rumen via the epithelial wall and diffuses into the rumen mass, or enters via saliva in the swallowed feed. It can be hydrolyzed by microbes to form ammonia and diffuse into the rumen mass, be reabsorbed by the epithelial wall, or utilized by microbes with the potential of returning to the rumen fluid ammonia pool via lysis or engulfment. The ^{15}N-NH_3 enrichment was plotted via time and the following equation was fit to the decay curve: $y = y_0 + A*e^{-kt}$.

The change in ^{15}N enrichment over time provides a measure of how quickly ^{15}N is exiting the pool. For example, a large rate constant (k), or decay rate in this case, for the outer rumen ammonia pool implies that the rate of ammonia entering the outer rumen fluid is much slower than the total rates of exit into the inner fluid, microbes, or back across the rumen epithelium. A large rate constant in the inner rumen ammonia pool implies that the rate of ammonia entering the inner rumen, either by diffusion from the outer pool or by saliva entry in consumed feed, is much slower than the total rates of exit back to the outer rumen ammonia pool or its utilization by microbes in this region. Small

or slow rate constants, on the other hand, imply that there is less difference between the entry and exit rates.

The constant A is a measure of the initial ^{15}N enrichment of the pool; therefore, a large constant A can be interpreted as rapid entry of the plasma urea into the rumen ammonia pool and/or a large contribution of ^{15}N-NH_3 to the entire NH_3 pool due to a large amount of ^{15}N entry or to a relatively small amount of NH_3 in the pool. The y intercept (y_0) is simply a correction factor, as natural abundance of ^{15}N is not zero, but approximately 0.40-0.43% in the rumen fluid ammonia of these particular animals. The R^2 values are also reported, providing an estimate of the adequacy of fit for the decay curves.

Graphical fit of the inner and outer rumen ^{15}N-NH_3 kinetics for each diet were relatively good, with treatment mean R^2 values ranging from 0.66 to 0.82 (Table 3.53). This suggests the $^{15}NH_3$-N decay curves were appropriate, or ^{15}N enrichment patterns over time were observed consistently in both the outer and inner rumen fluid. The Y intercepts ranged from 0.41-0.43 %^{15}N, demonstrating that these cows had consistent natural $^{15}NH_3$-N abundance in their rumen fluid (Table 3.53). The A constants varied widely (0.17-6.7 %^{15}N), suggesting large differences in either the rate of urea entry and NH_3 formation or the relative contribution of $^{15}NH_3$-N to the initial NH_3 pool. Because urea entry rates to the GIT, rumen NH_3 concentrations, and rumen pool sizes were similar among low CP treatments, large A constants may signify particularly rapid entry to the pool followed by inhibition or slower entry over time rather than a more constant rate of urea entry such as observed in treatments with

Table 3.53. Graphical constants of the $^{15}NH_3$-N decay curves for dairy cows fed low CP diets with different dietary starch concentrations with or without Rumensin after a bolus jugular $^{15}N^{15}N$-urea dose.

	Diet					P value		
	Low Starch W/o Rum	Low Starch With Rum	High Starch W/o Rum	High Starch With Rum	SEM	Starch	Rumensin	Rum*Starch
Outer Rumen								
Y intercept[1]	0.432	0.410	0.429	0.406	0.011	0.72	0.14	0.96
Constant a[2]	1.173	0.232	0.346	0.332	0.48	0.50	0.39	0.40
Constant k[3]	0.0186	0.0066	0.0314	0.0077	0.013	0.63	0.25	0.68
R squared[4]	0.79	0.66	0.67	0.82	0.09	0.80	0.95	0.14
Inner Rumen								
Y intercept	0.422	0.420	0.415	0.414	0.017	0.60	0.94	0.96
Constant a	0.168	0.273	0.959	6.715	3.26	0.33	0.42	0.43
Constant k	0.0138	0.0133	0.0166	0.0216	0.010	0.52	0.86	0.75
R squared	0.70	0.82	0.77	0.74	0.07	0.99	0.54	0.36

[1]Y intercept represents the natural abundance of ^{15}N in the ruminal fluid.
[2]Constant a represents the estimated initial ^{15}N enrichment of the ruminal fluid. Larger values signify either rapid entry of $^{15}N^{15}N$-urea or proportionally large contributions of ^{15}N to the total $^{15}NH_3$-N pool.
[3]Constant k represents the change in $^{15}NH_3$-N enrichment over time ($\Delta\%^{15}N$/minute). Larger values signify greater differences between entry and exit rates into the $^{15}NH_3$-N pool, such as slower entry rates and/or faster exit rates.
[4]R squared represents goodness of fit for the $^{15}NH_3$-N decay curves within each treatment.

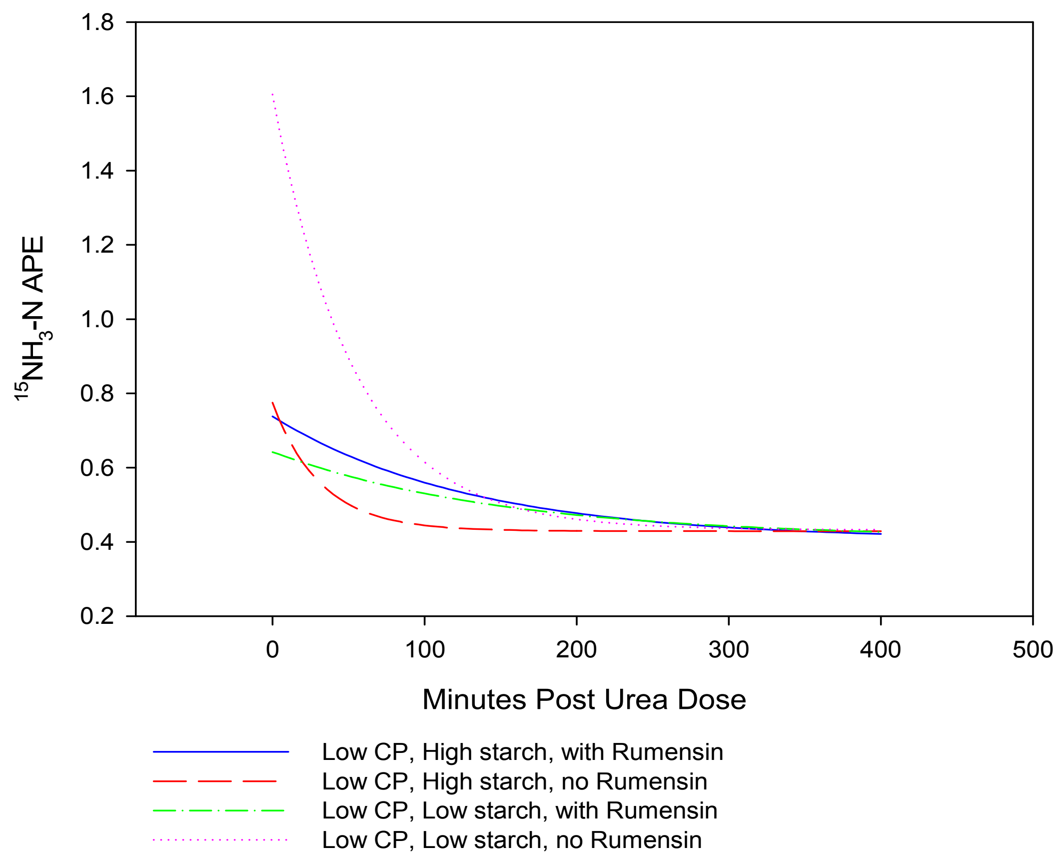

Figure 3.13. Means of Atom Percent Enrichment (APE) of the outer ruminal fluid $^{15}NH_3$-N over time after jugular $^{15}N^{15}N$-urea bolus dose by dietary treatment.

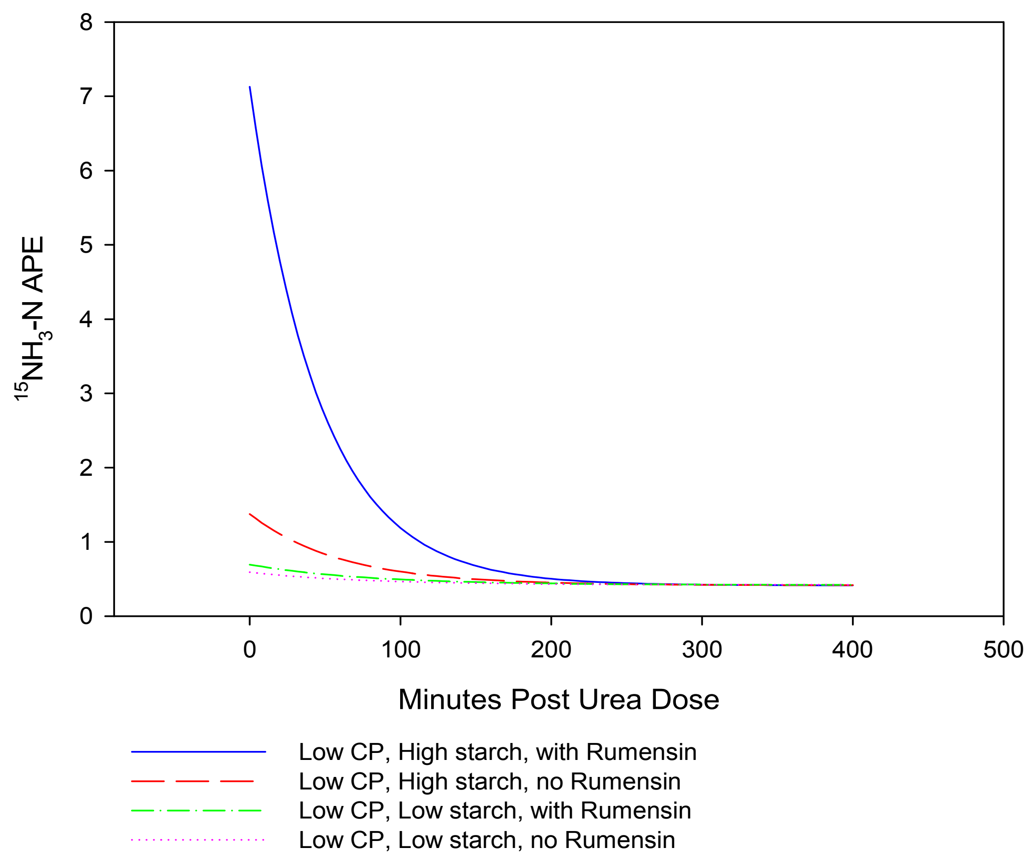

Figure 3.14. Means of Atom Percent Enrichment of the inner ruminal fluid $^{15}NH_3$-N over time after jugular $^{15}N^{15}N$-urea bolus dose by dietary treatment.

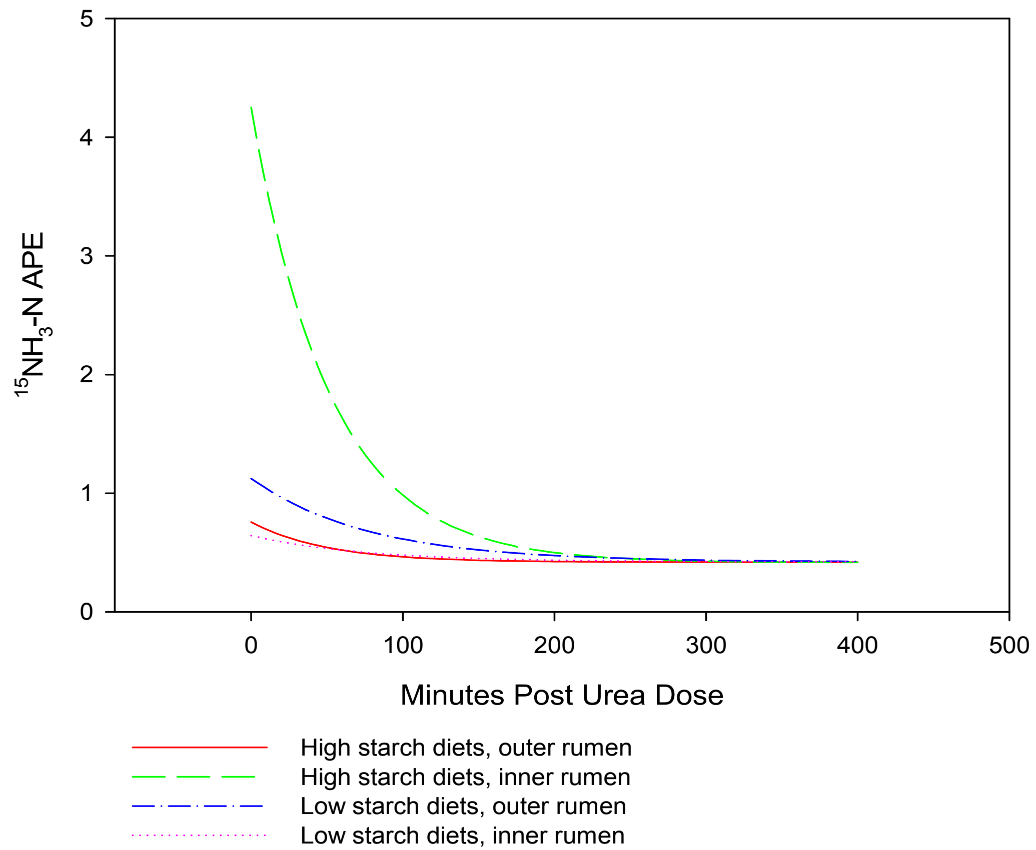

Figure 3.15. Means of Atom Percent Enrichment of the ruminal fluid $^{15}NH_3$-N over time after jugular $^{15}N^{15}N$-urea bolus dose by dietary starch levels.

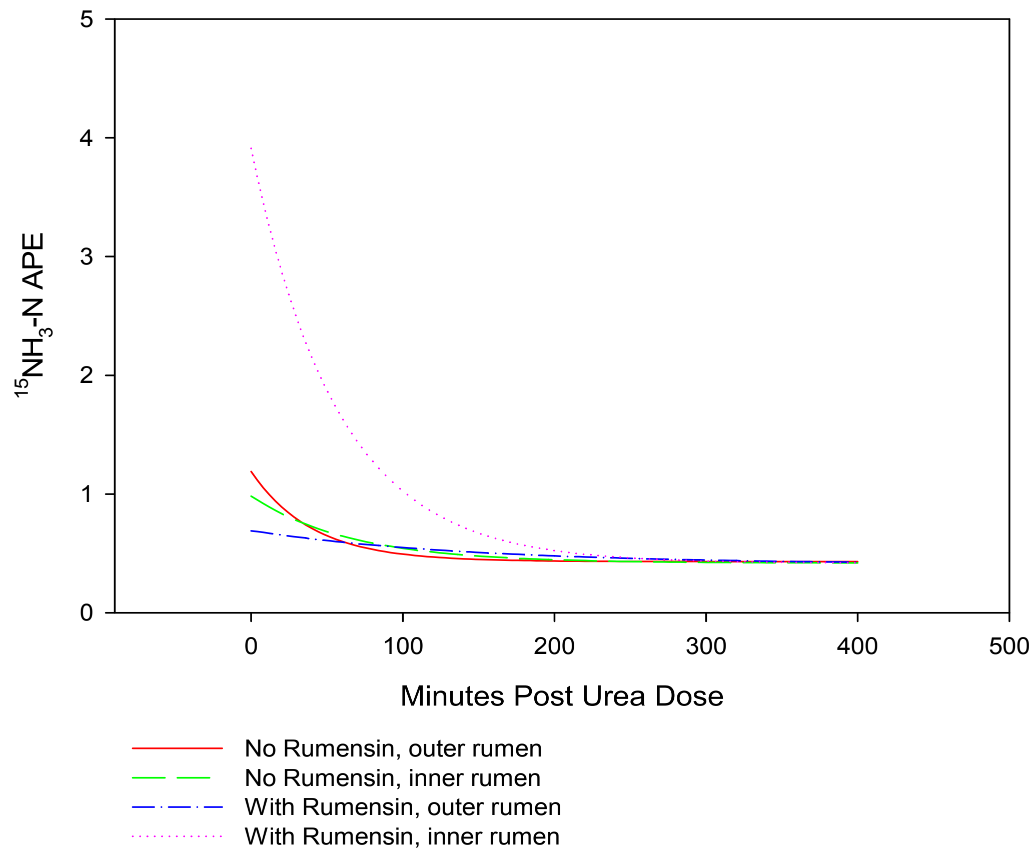

Figure 3.16. Means of Atom Percent Enrichment of the ruminal fluid $^{15}NH_3$-N over time after jugular $^{15}N^{15}N$-urea bolus dose by dietary Rumensin levels.

smaller A constants. No significant dietary treatment effects were observed due to the large standard error of the mean, suggesting that neither starch nor Rumensin affected the rate of urea entry over time. The largest a constants were observed for diets with low starch and no Rumensin and for diets with high starch with Rumensin, again implying that these dietary factors might

have not impact on urea entry rate unless they behave differently in the outer vs. the inner rumen, which is not likely.

The k constants (decay constants) in the outer rumen were numerically lower for diets including Rumensin, but this was not true in the inner rumen (Table 3.53). This suggests Rumensin might be stimulating urea entry to the outer rumen via the epithelium, which is unlikely due to no previous effects by Rumensin noted in GIT entry during continuous urea infusions. Or Rumensin could be slowing the rate of NH_3 diffusion into the inner rumen or the uptake or NH_3 by the microbes. However, there were large standard errors in these measurements. Otherwise, constant k values were similar among diets and among the dietary treatment factors of starch and Rumensin (Table 3.53 and depicted graphically in Figures 3.13-3.16). These data suggest that urea-N entry and subsequent NH_3 flows were not significantly impacted by these dietary treatments. However, Rumensin might have caused retention in the outer rumen either as $^{15}NH_3$-N or as microbial N (Figure 3.16). Also, high starch concentrations may have increased the rate of loss of $^{15}NH_3$-N in the outer rumen, either by faster sequestration by the microbial population, transit to the inner rumen, or by absorption across the ruminal epithelium.

CONCLUSIONS

The results of this study suggest that urea-N kinetics are more closely related to N intake and form than to plasma and ruminal N concentrations or pH in high producing lactating dairy cows. The amount of urea synthesized by the cow, and therefore the amount of urea recycled to the GIT and utilized by the microbial populations, was weakly related to N intake, suggesting the need for better estimations of N excess and NH3 absorption. Approximately 70-75% of the urea-N synthesized by the animal entered the GIT among all dietary treatments and 55-60% of this urea-N was utilized for anabolic purposes, with both dietary starch and Rumensin treatments stimulating the use of recycled N for anabolism. Rumensin increased PUN concentrations and slightly increased urinary N excretion, but improvements in milk yield negated changes in N efficiency. Rumen microbial pool sizes were numerically increased by addition of Rumensin to the diet, but this observation was not consistent among diets. Further work should be conducted to define the impact of Rumensin on microbial yields in a variety of fermentation and N status conditions. In addition, the extent of bacterial N predation by the protozoa and quantification of protozoal N flows in different dietary scenarios should be examined to provide more accurate estimations of MP supply and ruminal N status.

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CHAPTER FOUR: CONCLUSION

The focus of this work was to develop methods to both investigate and improve N efficiency in lactating dairy cows through a better understanding of the dietary factors regulating urea-N recycling to the rumen and to further describe the microbial uptake and interactions of recycled urea-N. This goal would decrease N feeding to dairy cattle, lowering both costs to producers and environmental waste. It was hypothesized that stimulation of urea recycling to the GIT would increase microbial N utilization and microbial protein supply to the animal, supporting milk yield while reducing urinary N excretion. Therefore, the cow would take advantage of its high, obligatory recycling capacity for productive purposes to increase N efficiency with no or minimal detriment to milk production.

Cattle converted approximately 50-70% of their intake N into urea. This amount was partially determined by the form of dietary N, with highly degradable protein converted to ammonia and then urea to a larger extent than slowly degradable protein. But dietary CP concentration played a stronger role than N form in determining the amount of urea synthesized. This was particularly evident in the second experiment, with dietary CP having much stronger effects on urea synthesis than either dietary starch concentration or Rumensin. Nitrogen intake, however, was not as consistent of an indicator of urea-N kinetics as the measurement of urea-N synthesis. Therefore, both N intake and form should be considered to estimate urea-N synthesis, from which further urea-N kinetics might be determined, such as urea-N entry to the GIT, which remained relatively consistent among all treatments at approximately 60-70% of urea-N synthesis. There appears to be

some variation in urea-N kinetics and that variation is dependent on dietary factors, such as the level of carbohydrate fermentation and microbial N demand, but these factors appeared to have a minimal effect in these studies, making them less relevant to quantify until further refinements in urea-N synthesis estimation are made.

Drastic changes in ruminal N balance appeared to affect urea entry to the GIT, but the impacts of N gradients across the ruminal epithelial wall and fermentation activity in the rumen were not as large as we had hypothesized. Because high producing lactating cows have high N requirements in the rumen and beyond, diets that significantly improve N recycling to the rumen might result in lower milk production. While the cows fed low CP diets in these studies maintained nearly equivalent milk production than those fed higher CP diets, only relatively small changes in N efficiency were observed due to slightly lower DMI and milk production. These results support the practice of feeding lower amounts of N to cows but diets should still be balanced properly for MP supply and an understanding that lower ruminal N balances might stimulate higher proportions of urea-N to enter the rumen, but less urea-N synthesis occurs in low CP diets as well.

In the second study, because urea kinetics remained relatively unchanged by Rumensin and dietary starch concentrations, it is easy to conclude that once urea synthesis is known, other urea kinetic measurements can be assumed with relative accuracy. However, this might be too broad of a conclusion as there were some impacts on urea kinetics beyond that of dietary N or urea-N synthesis. In particular, Rumensin impacted N dynamics in the animal while having minimal affects on overall urea kinetics. Rumensin feeding decreased ruminal NH_3-N concentrations while increasing PUN

concentrations in addition to having a variety of interactive effects on microbial pool sizes, resulting in complex changes in ruminal N dynamics not consistent with previous modes of action of the ionophore. In this case, because ruminal N balance, and in particular NH_3 formation, has a large impact on urea-N synthesis and kinetics, further work should be done to quantify the impact of Rumensin on NH_3 formation under a variety of ruminal fermentation and N status conditions.

The protozoa constituted approximately 8-34% of the rumen microbial N pool and predated from 4 to 60% of the total bacterial N yield. Further work should be conducted to estimate bacterial and protozoal turnover rates, which include both predation and lysis, in addition to quantifying ruminal microbial pool sizes to better estimate microbial N requirements and supply to the animal post-ruminally. The current CNCPS estimation of bacterial predation at 20% of total yield might be an accurate but imprecise estimate, as these results suggest the number to be more dynamic depending on dietary and ruminal conditions. The contribution of recycled N to microbial growth is also evident from these studies, indicating it to be equivalent to 26-65% of the bacterial N yield and 23 to over 48% of the protozoal N yield.

Improving the estimates of recycled N would allow the true ruminal N balance and microbial N yield to be determined more accurately along with protein and AA supply to the animal for milk protein synthesis. The results from this work suggest that efforts be made to more precisely estimate urea synthesis in order to estimate further utilization of excess ruminal N, such as entry to the GIT, ruminal N balance, and microbial protein supply. In addition, the impact of Rumensin on N dynamics both in the rumen and the plasma pool

warrant further investigation along with more accurate estimations of microbial turnover.

APPENDIX

Microbial amino acid values were determined from six cows described in Chapter 2. Bacterial and protozoal samples from 6 cows (two per diet) were analyzed for amino acids by three separate hydrolyses procedures (Gehrke et al., 1985). For most AA, the samples were hydrolyzed in 6 N HCl for 21 h under N gas. For sulfur AA, samples were pre-oxidized using performic acid followed by acid hydrolysis in 6 N HCl, and tryptophan was measured after hydrolysis in 1.9 M $Ba(OH)2 \cdot 8H_20$. One mL of 125 mM norleucine was added to each sample as an internal standard and samples were analyzed on a Beckman System Gold HPLC (Beckman-Coulter, Brea, CA) with a lithium cation exchange column (Pickering Laboratories Inc., Mountain View, CA).

Table 4.1. Amino acid composition of the liquid associated bacteria for animals fed Control, LoMP, and LoRumN diets.

Liquid bact. AA	Control	LoMP	LoRumN	SEM	Diet effect
Met	14.83	13.83	15.28	0.85	0.35
Met fr/HCl	8.35	5.92	8.06	1.35	0.29
Thr	23.87	23.43	24.50	1.14	0.68
Val	22.83[ab]	20.79[b]	25.22[a]	1.35	0.10
Ile	17.96[b]	16.63[b]	20.25[a]	0.69	0.03
Leu	31.40	29.76	32.96	1.92	0.37
Phe	18.80	18.15	19.80	1.14	0.45
Trp	5.66[ab]	8.96[a]	3.56[b]	1.68	0.11
Lys	15.31	13.11	15.04	0.75	0.11
His	5.79[ab]	4.81[b]	5.98[a]	0.32	0.06
Arg	21.30	18.86	21.90	1.86	0.35
Cys	5.04	5.08	5.02	0.20	0.95
Asp	50.84	52.01	48.85	1.18	0.16
Ser	20.55	18.71	20.58	0.98	0.24
Glu	53.63	49.56	55.01	2.94	0.30
Pro	14.08	12.84	14.03	0.79	0.34
Gly	22.79	21.21	23.87	0.92	0.14
Ala	32.49	30.15	33.26	1.26	0.17
Tyr	0.34	0.00	0.00	0.28	0.46
Dapa	2.02	1.74	1.65	0.13	0.12
NH3	14.05[ab]	12.43[b]	15.32[a]	0.65	0.05

[abc]Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 4.2. Amino acid composition of the particle associated bacteria for animals fed Control, LoMP, and LoRumN diets.

Particle bact. AA	Control	LoMP	LoRumN	SEM	Diet effect
Met	14.49	14.15	14.10	1.45	0.96
Met fr/HCl	7.13	4.16	5.58	0.96	0.12
Thr	21.33	19.58	18.81	1.96	0.50
Val	21.14	19.53	20.37	2.13	0.77
Ile	16.78	15.69	15.58	1.68	0.75
Leu	31.39	27.59	28.34	2.27	0.34
Phe	18.55	17.22	17.74	2.01	0.81
Trp	5.47	4.32	4.98	0.77	0.43
Lys	26.32	23.57	23.18	2.38	0.46
His	7.79	6.81	7.28	0.97	0.64
Arg	20.05	19.07	19.53	2.20	0.91
Cys	5.52	5.59	5.60	0.40	0.98
Asp	52.78^{a}	39.84^{b}	42.45^{ab}	3.90	0.09
Ser	21.76	17.75	18.22	2.02	0.24
Glu	52.71	48.67	48.14	4.37	0.58
Pro	15.04	13.51	13.76	1.08	0.42
Gly	21.77	18.99	19.08	1.60	0.29
Ala	29.46	25.14	24.48	2.29	0.21
Tyr	17.55	15.71	14.94	1.40	0.30
Dapa	1.74	1.56	1.31	0.36	0.55
NH3	12.11	10.81	11.11	0.94	0.45

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).

Table 4.3. Amino acid composition of the protozoa for animals fed Control, LoMP, and LoRumN diets.

Protozoa AA	Control	LoMP	LoRumN	SEM	Diet effect
Met	10.93	11.84	14.28	1.09	0.11
Met fr/HCl	4.43	3.87	6.52	2.13	0.51
Thr	21.16	22.87	26.00	1.77	0.15
Val	20.09^{ab}	19.03^{b}	24.88^{a}	1.51	0.06
Ile	20.44^{b}	22.86^{b}	29.19^{a}	1.59	0.02
Leu	31.63	33.61	38.90	2.43	0.12
Phe	20.03^{b}	22.40^{ab}	26.50^{a}	1.60	0.06
Trp	5.85	5.98	5.88	0.82	0.99
Lys	16.17^{b}	21.67^{ab}	24.91^{a}	2.72	0.10
His	6.45	6.03	7.07	0.56	0.31
Arg	20.89^{b}	22.12^{ab}	25.91^{a}	1.39	0.07
Cys	5.89	6.91	7.15	0.75	0.34
Asp	49.87	57.66	62.91	6.93	0.31
Ser	19.13	19.29	22.06	1.27	0.17
Glu	58.64	59.89	69.94	5.74	0.25
Pro	15.33^{b}	16.15^{ab}	17.90^{a}	0.67	0.07
Gly	18.84^{b}	18.80^{b}	22.74^{a}	0.78	0.02
Ala	22.76	21.64	25.68	1.47	0.14
NH3	16.52^{b}	17.98^{b}	22.72^{a}	0.50	0.002

^{abc}Values in rows with different superscripts differ $P < 0.05$ as evaluated by pdiff contrast in the Mixed procedure of SAS (2001).