ERICK DARLISSON BATISTA

STUDIES ON NITROGEN UTILIZATION IN RUMINANTS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRAZIL
2015

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
B333s
2015

Batista, Erick Darlisson, 1984-
Studies on nitrogen utilization in ruminants / Erick Darlisson Batista. – Viçosa, MG, 2015.
xiii, 111f. : il. ; 29 cm.

Inclui apêndices.
Orientador: Edenio Detmann.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bovino - Alimentação e rações. 2. Metabolismo do nitrogênio. 3. Proteínas. 4. Digestão. I. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-graduação em Zootecnia. II. Título.

CDD 22. ed. 636.08521

ERICK DARLISSON BATISTA

STUDIES ON NITROGEN UTILIZATION IN RUMINANTS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: November 25, 2015.

Evan Charles Titgemeyer
(Co-adviser)

Sebastião de Campos Valadares Filho
(Co-adviser)

Rilene Ferreira Diniz Valadares

Thierry Ribeiro Tomich

Edenio Detmann
(Adviser)

The greatest enemy of knowledge is not ignorance,

it is the illusion of knowledge.

Stephen Hawking

The pursuit of knowledge is never ending.

The day you stop seeking knowledge is the day you stop growing.

Brandon T. Ciaccio

DEDICATION

For those who I owe my existence, who had not spared love, prayers, teaching and support: my parents João Batista Filho and Ilma Batista. I will seek every day give pride to you!

To my siblings Daniela Oliveira and Michel Batista whom always have shared with me every challenge, defeat or victory. It is great to know that I can always get your support!

To my beloved wife Patrícia Games who has been a constant source of support and encouragement during my Ph.D. program. I am truly thankful for having you in my life!

To my wonderful relatives that always pray and cheer for me.

In memory of my grandfathers, grandmothers, uncle Luiz Costa, and cousin Alex Cruz: you always will be in my heart and my mind!

ACKNOWLEDGEMENTS

I thank God for giving me the opportunity to work and learn with goods scientists, meet awesome people, and achieve greater goals than I had never expected.

To my wife´s relatives, especially Maria Selma Games and José Mezine, for their support and cheers.

To the Department of Animal Science of the *Universidade Federal de Viçosa* for the 10 years of knowledge and where I had the opportunity to develop a passion for science. Also, to the Department of Animal Sciences and Industry of Kansas State University by receiving me during a year for my PhD sandwich program.

To the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), for all scholarship in Brazil, and to the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for sponsored me during the visit scholar program at Kansas State University.

To the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG), *Instituto Nacional de Ciência e Tecnologia de Ciência Animal* (INCT-CA), CNPq, and Kansas Agricultural Experiment Station for the financial support.

I would like to express my sincere appreciation and gratitude to my major advisor during eight years, Prof. Edenio Detmann, for the opportunity that he gave me, as well as his example of ethics, dedication, support, and especially for his confidence in me. You always will be my example of professor and scientist!

I would also like to acknowledge the efforts of Profs. Sebastião C. Valadares Filho, Rilene F. D. Valadares, Mário F. Paulino, Luciana N. Rennó, Hilário C. Mantovani, and Cláudia B. Sampaio. Each of these individuals has always been willing to help whenever they could.

To Prof. Evan Titgemeyer for accepting to be my co-adviser in my Ph.D. I am grateful for the opportunity, teachings, patience, and constant support. Thank you very much for all!

I thank you to committee member, Dr. Thierry R. Tomich for his contributions.

To all Department of Animal Science staff that helped me, specially Joélcio, Natanael, Wellington, Fernando, Mário, Monteiro, and Plínio for their assistance in animal care and feeding, and laboratory analysis. I would like to extend my sincere thanks to my undergraduate student workers: Amanda, Annelise, Antônio, Danilo, Luis Márcio; and my dear friends Luciana Prates and Cláudia Bento for their assistance with animal care, sample collection and laboratory analysis.

I gratefully thank Cheryl Armendariz, research assistant of Department of Animal Science and Industry at Kansas State University, for friendship, teachings, and valuable help. I miss her kindness and expertise.

I thank my great friends: Aline Silva, André Mauric, Camila Cunha, Dani Oss, Lays Mariz, Laura Prados, Leandro Silva, Paloma Amaral, Pedro Benedetti, and to all countless friends from my city, São José da Lapa, and UFV.

To my workmates and friends: Gabriel Rocha, Hugo Bonfá, Luana Rufino, Malber Palma, Marcelo Machado, Márcia Franco, Marcília Medrado, Tadeu Silva, and William Reis.

To Prof. Dr. James Drouillard and family for friendship and excellent moments spent together in Manhattan, KS.

I thank you the Brazilian friends in Manhattan: Diego Piovezan, Daniel Vicari, Lucas Miranda, Lucas Rocha, Luiz Mendonça, Luiz Roberto Neto, Pablo Paiva, Renê Couto, Rodrigo Pedrozo, Gabriel Granço, Sara Hirata, Luciana Pinto, Maurícia Silva, and others.

To my foreign colleagues in Manhattan, KS: Ali Hussein, Karl Yuan, Sina Samii, Mehrnaz Ardalan, Fabian Vargas and Vivian Solano, Jorge Simroth and Daniela Flores, Jared Johnson, Cadra Van Bibber-Krueger, Justin Axman, Jake Thieszen, Gail Carpenter and others not less important. You make my transition to Manhattan easier!

To everyone who contributed to this work and cheers to complete my Ph.D. program. Thank you very much!

BIOGRAPHY

Erick Darlisson Batista, son of João Batista Filho and Ilma Maria Batista, was born in Pedro Leopoldo, Minas Gerais, Brazil, on 16th of February 1984. He started the undergrad in Animal Science at *Universidade Federal de Viçosa* in 2006, and obtained a Bachelor of Science degree in Animal Science in 2010.

He started the Master's program in August 2010, with major in ruminant nutrition and beef cattle production at the same University, submitting to the dissertation defense on 14th of February 2012.

In March 2012, he started the Doctorate program, continuing work on nitrogen metabolism in beef cattle. From January of 2014 to December of 2014 he was a visiting scholar at Kansas State University, Manhattan, KS, USA where part of his research was developed.

On 25th of November 2015, Mr. Batista defended his dissertation to the thesis committee to obtain the *Doctor Scientiae* degree in Animal Science.

TABLE OF CONTENTS

ABSTRACT

BATISTA, Erick Darlisson, D.Sc., Universidade Federal de Viçosa, November of 2015. **Studies on nitrogen utilization in ruminants.** Adviser: Edenio Detmann. Co-Advisers: Evan Charles Titgemeyer, Mário Fonseca Paulino, and Sebastião de Campos Valadares Filho.

In cattle, efficiency of nitrogen (N) utilization (g N in product/g N intake) is lower compared to others species (e.g., pig, chicken). For that reason, there is an extensive loss of N in manure, leading to environmental pollution. However, understanding the key mechanisms involved in control of N metabolism, such as efficiency of N capture in the rumen from recycled N and metabolism of amino acids (AA) in the body can improve efficiency of N utilization. To understand these factors, this dissertation was developed based on three studies. The objective of the first study was to evaluate the effects of supplemental ruminally degradable (RDP) and undegradable protein (RUP) on nutrient digestion, N metabolism, urea kinetics, and muscle protein degradation in Nellore heifers (*Bos indicus*) consuming low-quality signal grass hay [5% of crude protein (CP), 80% of neutral detergent fiber (NDF); dry matter (DM) basis). Five ruminally and abomasally cannulated Nellore heifers (248 ± 9 kg) were used in a 5 × 5 Latin square. Treatments were: control (no supplement); and RDP supplementation to meet 100% of the RDP requirement plus RUP provision to supply 0%, 50%, 100%, or 150% of the RUP requirement. Supplemental RDP (casein plus nonprotein N) was dosed ruminally twice daily, and RUP supply (casein) was continuously infused abomasally. Jugular infusion of [$^{15}N^{15}N$]-urea with measurement of enrichment in urine was used to evaluate urea kinetics. The ratio of urinary 3-methylhistidine to creatinine was used to estimate skeletal muscle protein degradation. Forage NDF intake (2.48 kg/d) was not affected ($P \geq 0.37$) by supplementation, but supplementation did increase ruminal NDF digestion ($P < 0.01$). Total N intake (by design) and N retention increased ($P < 0.001$) with supplementation and also increased linearly with RUP provision. Urea entry rate (UER) and gastrointestinal entry rate of urea (GER) were increased by supplementation ($P < 0.001$). Supplementation with RUP linearly increased ($P = 0.02$) UER and tended ($P = 0.07$) to linearly increase GER. Urea use for anabolic purposes tended ($P = 0.07$) to be increased by supplementation, and RUP provision also tended ($P = 0.08$) to linearly increase the amount of urea used for anabolism. The fraction of recycled urea-

N incorporated into microbial N (MNU) was greater ($P < 0.001$) for control (22%) than for supplemented (10%) heifers. Urinary 3-methylhistidine:creatinine of control heifers was more than double that of supplemented heifers ($P < 0.001$). Control heifers reabsorbed a greater ($P < 0.001$) fraction of urea from the renal tubule than did supplemented heifers. Overall, unsupplemented heifers had greater mobilization of AA from myofibrillar protein, which provided N for urea synthesis and subsequent recycling. Supplemental RUP, when RDP was supplied, not only increased N retention, but also supported increased urea-N recycling and increased ruminal microbial protein synthesis. In the second chapter, urea kinetics and microbial assimilation of recycled urea N in ruminants were evaluated using a meta-analytical approach. Treatment mean values were compiled from 25 studies with ruminants (beef cattle, dairy cows, and sheep) which were published from 2001 to 2016, totaling 107 treatment means. The dataset was analyzed according to meta-analysis techniques using linear or non-linear mixed models, taking into account the random variations among experiments. Urea N synthesized in the liver (UER) and urea N recycled to the gut (GER) linearly increased ($P < 0.001$) as N intake (g/$BW^{0.75}$) increased, with increases corresponding to 71.5% and 35.2% of N intake, respectively. The UER was positively associated ($P < 0.05$) with dietary CP and the ratio of CP to digestible OM (CP:DOM). Maximum curvature analyses indicate that above 17% of CP there is a prominent increase on hepatic synthesis of urea N due to an excess of dietary N and NH_3 input. The GER:UER decreased with increasing dietary CP content ($P < 0.05$). At dietary CP $\geq$ 19%, the fraction of GER became constant. The fraction of UER eliminated as urinary urea N and the contribution of urea N to total urinary N were positively associated with dietary CP ($P < 0.05$), plateaued at about 17% of CP. The fractions of GER excreted in the feces and utilized for anabolism decreased, whereas the fraction of GER returned to the ornithine cycle increased with dietary CP content ($P < 0.05$). Recycled urea N assimilated by ruminal microbes (as a fraction of GER) decreased as dietary CP and CP:DOM increased ($P < 0.05$). The efficiency of microbial assimilation of recycled urea N plateaued at 194 g CP/kg DOM. The models obtained in this study can to contribute to the knowledge on N utilization in feeding models and optimizing urea recycling, reducing N losses that contribute to air and water pollution. The objective of the third chapter was to evaluate the efficiency of lysine (Lys) utilization by growing steers. Five ruminally cannulated Holstein steers (165 kg ± 8 kg) housed in metabolism crates were used in a 6 × 6 Latin square design; data from a sixth steer was excluded due to erratic feed intake. All steers were limit fed (2.46 kg DM/d) twice daily diets low in RUP (81% soybean hulls, 8% wheat straw, 6% cane molasses, and 5% vitamins and minerals). Treatments were: 0, 3, 6, 9, 12, and 15 g/d of L-Lys abomasally infused

continuously. To prevent AA other than Lys from limiting performance, a mixture providing all essential AA to excess was continuously infused abomasally. Additional continuous infusions included 10 g urea/d, 200 g acetic acid/d, 200 g propionic acid/d, and 50 g butyric acid/d to the rumen and 300 g glucose/d to the abomasum. These infusions provided adequate ruminal ammonia and increased energy supply without increasing microbial protein supply. Each 6-d period included 2 d for adaptation and 4 d for total fecal and urinary collections for measuring N balance. Blood was collected on d 6 (10 h after feeding). Diet OM digestibility was not altered ($P \geq 0.66$) by treatment and averaged 73.7%. Urinary N excretion decreased from 32.3 to 24.3 g/d by increasing Lys supplementation to 9 g/d, with no further reduction when more than 9 g/d of Lys was supplied (linear and quadratic $P < 0.01$). Changes in total urinary N excretion were predominantly due to changes in urinary urea-N. Increasing Lys supply from 0 to 9 g/d increased N retention from 21.4 to 30.7 g/d, with no further increase beyond 9 g/d of Lys (linear and quadratic $P < 0.01$). Break-point analysis estimated maximal N retention at 9 g/d supplemental Lys. Over the linear response surface of 0 to 9 g/d Lys, the efficiency of Lys utilization for protein deposition was 40%. Plasma urea-N tended to be linearly decreased ($P = 0.06$) by Lys supplementation in agreement with the reduction in urinary urea-N excretion. Plasma concentrations of Lys increased linearly ($P < 0.001$), but leucine, serine, valine, and tyrosine ($P \leq 0.02$) were reduced linearly by Lys supplementation, likely reflecting increased uptake for protein deposition. In our model, Lys supplementation promoted significant increases in N retention and was maximized at 9 g/d supplemental Lys with efficiency of utilization of 40%.

RESUMO

BATISTA, Erick Darlisson, D.Sc., Universidade Federal de Viçosa, novembro de 2015. **Estudos sobre a utilização de nitrogênio em ruminantes.** Orientador: Edenio Detmann. Coorientadores: Evan Charles Titgemeyer, Mário Fonseca Paulino e Sebastião de Campos Valadares Filho.

Em ruminantes, a eficiência de utilização do nitrogênio (N; g de N em produto/g de N consumido) é baixa quando comparada a outras espécies (e.g., suínos, aves). Por esta razão, há uma excreção excessiva de compostos nitrogenados para o meio ambiente. No entanto, entendendo os mecanismos envolvidos no controle do metabolismo de N, tais como a eficiência de captura do N reciclado no rúmen e o metabolismo de aminoácidos (AA) pode melhorar a eficiência de utilização de N. Objetivando o entendimento destes fatores, esta tese foi desenvolvida a partir de três estudos. O objetivo do primeiro estudo foi avaliar os efeitos da suplementação com proteína degradável (PDR) e não-degradável no rúmen (PNDR) sobre a digestão de nutrientes, metabolismo de N, cinética de ureia, e degradação de proteína muscular em novilhas Nelore (*Bos indicus*) consumindo feno de capim-Braquiária [5% de proteína bruta (PB); 80% de fibra em detergente neutro (FDN); ambos em % da matéria seca (MS)]. Foram utilizadas cinco novilhas Nelore canuladas no rúmen e abomaso (248±9 kg) distribuídas em um quadrado latino 5 × 5. Os tratamentos foram: controle (sem suplemento); e suplementação com PDR para atender 100% das exigências de PDR mais suplementação com PNDR visando suprir 0%, 50%, 100% ou 150% das exigências de PNDR. O suplemento com PDR (caseína e N não-proteico) foi fornecido duas vezes ao dia, enquanto a PNDR suplementar foi continuamente infundida no abomaso. Infusão venosa de [$^{15}N^{15}N$]-ureia com a avaliação do enriquecimento urinário foi realizada para mensurar a cinética de ureia. A relação entre 3-metil-histidina e creatinina foi utilizada para estimar a degradação de proteína muscular. O consumo de FDN (2,48 kg/dia) não foi afetado pela suplementação ($P \geq 0,37$), mas elevou a digestão ruminal de FDN ($P<0,01$). O consumo total e a retenção de N aumentaram ($P<0,001$) com a suplementação e linearmente com os níveis de PNDR. A produção hepática de ureia (UER) e a reciclagem de ureia para o trato gastrointestinal (GER) foram ampliados pela suplementação ($P<0,001$). A suplementação com PNDR incrementou linearmente UER ($P=0,02$) e tendeu a aumentar linearmente GER ($P=0,07$). A ureia reciclada utilizada para fins anabólicos tendeu

(P=0,07) a ser ampliada pela suplementação e os níveis de PNDR também tenderam (P=0.08) a aumentar linearmente a quantidade de ureia reciclada para o anabolismo. A fração de N microbiano assimilado a partir da ureia reciclada (MNU) foi maior (P<0,001) para novilhas controle (22%) do que para as novilhas suplementadas (10%). A relação urinária 3-metil-histidina:creatinina foi cerca de duas vezes superior (P<0,001) em novilhas controle do que suplementadas. Novilhas não-suplementadas reabsorveram uma fração maior de ureia a partir dos túbulos renais do que as novilhas suplementadas (P<0,001). No geral, novilhas não-suplementadas apresentaram maior mobilização de AA a partir da proteína miofibrilar para fornecer N para síntese de ureia e subsequente reciclagem. Suplementação com PNDR, associada a suplementação com PDR, além de ampliar a retenção de N, também aumenta a reciclagem de N-ureia e a síntese de proteína microbiana. No segundo capítulo, foram avaliadas a cinética de ureia e assimilação microbiana de N-ureia reciclado em ruminantes utilizando meta-análise. Valores de 107 médias de tratamentos foram compiladas a partir de 25 estudos com ruminantes (bovinos de corte, vacas de leite e ovinos) publicados entre 2001 e 2016. O conjunto de dados foi analisado de acordo com técnicas de meta-análise utilizando modelos mistos lineares e não-lineares, considerando a variação aleatória entre experimentos. Houve um aumento linear (P<0,05) entre UER e GER em função do consumo de N (g/$BW^{0,75}$), correspondendo a cerca de 71,5% e 35,2% do consumo de N, respectivamente. A UER foi positivamente associada (P<0,05) com os níveis de PB na dieta e PB em relação à matéria orgânica digerida (PB:MOD). A análise da máxima curvatura indicou que dietas com níveis de PB acima de 17% promovem uma sobrecarga na síntese hepática de ureia, devido a um possível excesso de N dietético, produção de amônia e detoxificação no fígado. A relação entre GER e UER reduziu com o aumento do conteúdo de PB na dieta (P<0,05). A fração GER:UER torna-se relativamente constante quando são fornecidas dietas com níveis de PB acima de 19%. A fração de UER excretada como N-ureico e a contribuição deste para excreção total de N urinário foram positivamente associadas com o teor de PB na dieta (P<0,05), atingindo o platô em níveis de PB próximo de 17%. Em relação à cinética de ureia, a fração de GER excretada nas fezes e utilizada para o anabolismo foram reduzidas, enquanto a fração que retorna para o ciclo da ornitina ampliou com níveis de PB (P<0,05). A fração de N microbiano assimilado a partir da ureia reciclada foi reduzida (P<0,05) com níveis de PB e PB:MOD da dieta. Considerando o intervalo de confiança da assíntota do modelo de predição de MNU em função da PB:MOD, a eficiência de assimilação microbiana do N-ureia reciclado estabilizou (P>0,05) a partir de 194 g PB/kg MOD. Os modelos obtidos neste estudo podem contribuir para o atual conhecimento da utilização de N nos sistemas de predição de dietas para otimização da

reciclagem de ureia, reduzindo perdas de N que contribuem para a poluição do ar e da água. O objetivo do estudo descrito no terceiro capítulo foi avaliar a eficiência de utilização de lisina em novilhos em crescimento. Cinco novilhos holandeses fistulados no rúmen (165±8 kg) e mantidos em gaiolas metabólicas foram utilizados segundo delineamento em quadrado latino 6 × 6. Todos os novilhos receberam dieta restrita (2,46 kg de MS/dia) fornecida duas vezes ao dia, contendo baixo teor de PNDR (81% de casca de soja, 8% de palha de trigo, 6% de melaço e 5% de vitaminas e minerais). Os tratamentos foram: 0, 3, 6, 9, 12 e 15 g/dia de L-lisina infundida continuamente no abomaso. Uma mistura de todos os AA essenciais foram também infundidos em conjunto para prevenir a limitação de outros AA, exceto lisina. Adicionalmente, os novilhos receberam infusão contínua de 10 g/dia de ureia, 200 g/dia de ácido acético, 200 g/dia de ácido propiônico e 50 g/dia de ácido butírico no rúmen; e 300 g/dia de glicose no abomaso. Estas infusões forneceram concentração de amônia no rúmen e energia suplementar adequados sem promoverem alteração sobre a produção de proteína microbiana. Cada período experimental foi constituído de seis dias, sendo dois dias de adaptação e quatro dias de coleta total de fezes e urina para mensurar o balanço de N. Amostras de sangue foram coletadas no sexto dia (10 horas após alimentação). A digestibilidade de MO da dieta não foi alterada ($P \geq 0,66$) pelos tratamentos sendo, em média, 73,7%. A excreção urinária de N reduziu de 32,3 para 24,3 g/dia entre os níveis de 0 a 9 g/dia de suplementação com lisina, com nenhum aumento verificado com níveis superiores a 9 g/dia (efeito linear e quadrático, $P<0,01$). Os efeitos sobre a excreção urinária total de N foram principalmente devido a excreção de N-ureia. O aumento da suplementação com lisina de 0 para 9 g/dia ampliou a retenção de N de 21,4 para 30,7 g/dia, com nenhum aumento verificado após este último nível de suplementação (efeito linear e quadrático, $P<0,01$). Sobre a resposta linear verificada com a suplementação de lisina variando de 0 a 9 g/dia a eficiência de utilização de lisina para deposição de proteína foi de 40%. A concentração plasmática de N-ureia tendeu a reduzir linearmente ($P=0,06$) com a suplementação de lisina, conforme observado para a redução na excreção urinária de N-ureia. A concentração plasmática de lisina aumentou linearmente ($P<0,001$), mas as concentrações de leucina, serina, valina e tirosina foram reduzidas linearmente ($P<0,02$) com os níveis de lisina suplementar, provavelmente devido a maior utilização destes AA para deposição de proteína. De acordo com este modelo, a suplementação com lisina promoveu aumento significativo na retenção de N que foi maximizada com a suplementação de 9 g/dia de lisina, apresentando 40% de eficiência de utilização.

GENERAL INTRODUCTION

For many years animal nutritionists, physiologists and microbiologists have sought and demanded efforts to improve the efficiency of nitrogen (**N**) retention by ruminant animals. Such a concern is based on the fact that protein is the most expensive nutrient in animal diets and because the N excreted by animals (feces and urine) causes air and water pollution, and decreases the economic efficiency of production.

Dietary protein consumed by ruminants is divided into ruminally degradable protein (**RDP**), composed of nonprotein N and true protein, and ruminally undegradable protein (**RUP**). The breakdown of true protein in the rumen is a complex process, which encompasses many different microorganisms that provide the necessary enzymes to hydrolyze peptide bonds (Walker et al., 2005). Protein is then hydrolyzed, releasing oligopeptides, which are further catabolized to smaller peptides and amino acids (**AA**), and eventually deaminated into ammonia (**NH_3**) or directly incorporated into microbial protein. Nonprotein N consists of N present in NH_3, AA, small peptides, urea, DNA, and RNA, which can be used for microbial growth (Bach et al., 2005).

After ruminal fermentation of dietary RDP, ruminal N output is composed of some dietary protein that escapes from ruminal degradation (RUP), microbial N (true protein and nonprotein compounds), endogenous protein, and NH_3. In intensive production conditions, RDP often is consumed in excess of the amount required by ruminal microorganisms. In this situation, the surplus of NH_3 produced is absorbed, metabolized to urea in the liver, and may be lost in the urine, contributing to inefficient N retention and utilization of dietary N (Walker et al., 2002; Bach et al., 2005).

However, some of the blood urea N can be recycled to the rumen through saliva and mainly from the rumen epithelium, which could be also used for microbial growth. Therefore, a better control of ruminal N metabolism, particularly the reutilization of NH_3, is an obvious way to achieve an improvement in the efficiency of N utilization by ruminants and to limit the excessive N excretion that results in environmental pollution around animal production areas (Hristov and Jouany, 2005).

In ruminants fed under a wide range of diets, efficiency of N utilization (g N in product/g N intake) is often low. In beef cattle, efficiency of N utilization is even lower, averaging only 14% of dietary N retained in tissues of grazing young bulls (Valente et al., 2014) or 22% in bulls finished in a feedlot (Silva, 2011). In dairy cows, only 21 to 33% of dietary N is utilized for milk protein synthesis (Tamminga, 1992; Calsamiglia et al., 2010). Furthermore, once AA are absorbed, efficiency of utilization is in the range 30-50%, much lower when compared to the 60-70% observed in pigs (MacRae et al., 1996). The reasons for this low efficiency of N utilization are variable from the use of absorbed AA for hepatic and renal gluconeogenesis to AA transactions in the portal-drained viscera and peripheral tissues that support protein turnover (Bergman and Heitmann, 1978). Additionally, inefficient dietary N utilization is accompanied by extensive losses of N in the manure, leading to environmental pollution. The N excreted in feces is composed mostly of microbial and fecal metabolic N, while N excreted in the urine is predominantly from ruminal N loss due to extensive degradation of protein in the rumen. Under a wide range of crude protein (**CP**) content in the diet (9 to 21%), about 32 to 71% of total N is excreted through the urine, and large proportion of that N is in the form of urea N, which accounts for about 17 to 79% of total urinary N (Marini and Van Amburgh, 2003). Most urinary urea N is lost as NH_3 into the environment via volatilization (Lockyer and Whitehead, 1990).

Thus, improving the utilization of NH_3 in the rumen has the potential to reduce the environmental impact of ruminant production. Feeding medium- to low-CP diets can decrease the detrimental loss of ruminal NH_3, and the subsequent synthesis and excretion of urea N in urine (Marini and Van Amburgh, 2003; Reynolds and Kristensen, 2008). In addition, restricting N intake up-regulates urea N recycling to the rumen. Besides compensating for the limited supply of dietary N to support microbial protein synthesis, this increase in urea N transfer to the rumen also reduces urinary excretion of urea N (Røjen et al., 2011). Additionally, as ruminant nutritionists must feed both rumen microorganisms and their host, the requirements of each one are different and should be considered. The AA profile entering the duodenum of ruminants is different from the AA composition of the diet because most of it is composed of microbial proteins synthesized in the rumen and of dietary proteins that have been partly degraded in the rumen. Ammonia is the main product of protein catabolism in the rumen and is also the principal substrate for microbial protein synthesis (Hristov and Jouany, 2002), especially for fibrolytic bacteria (Russell et al., 1992). Most protein supplied to the small intestine of ruminants is provided by the microbial protein synthesis in the rumen, corresponding to 50 to 80% of total absorbable protein (Storm and Ørskov, 1983). Thus, optimizing ruminal N metabolism is key to maximizing the supply of AA to the small intestine and limiting N losses from the rumen as NH_3 and, ultimately, as urea N in urine.

Overall, N utilization by cattle key mechanisms involved in the control of N metabolism by cattle are the efficiency of N assimilation from N recycled into the rumen, the factors that control it, and metabolism of AA in the body (Calsamiglia et al., 2010).

Urea Recycling

Urea recycling to the rumen is an important mechanism that confers an evolutionary advantage to ruminants. Although all mammalian species have the mechanisms of urea recycling to the gastrointestinal tract (**GIT**), the amount of urea N recycled to the GIT in ruminants (as a proportion of total hepatic urea N synthesis) is more than two-fold that for nonruminants (Lapierre and Lobley, 2001). This greater salvage of synthesized urea highlights the potential importance of the mechanisms of urea N recycling in ruminants.

When dietary N supply is inadequate to meet the ruminant N requirements, such as for cattle grazing low-quality forage, urea N recycling to the GIT can be greater than N intake. In this situation, recycled urea N serves as the N precursor for microbial protein synthesis and ensures survival of the animal (Reynolds and Kristensen, 2008). Nevertheless, in intensive production systems for growing beef cattle and high-production dairy cows, dietary N supply is usually high enough to meet microbial N requirements. Even under such conditions, total hepatic synthesis of urea N often exceeds apparent digestible N, and if no mechanism exists to save some N (i.e., urea N recycling), then those animals could present a negative or zero N balance (Lapierre and Lobley, 2001). Hence, the mechanism of urea N recycling plays an important role in maintaining ruminant animals in a positive N balance, and helps them to meet protein requirement.

Although the amount of urea N recycled into the GIT is important, the proportion of that recycled urea N that is incorporated into microbial protein in the rumen is essential. Urea N recycled to the GIT can be hydrolyzed to NH_3 by the action of microbial urease and then utilized as a source of N for microbial protein synthesis, which is a major source of AA for maintenance and productive functions in the host (Lapierre and Lobley, 2001; Reynolds and Kristensen, 2008). Therefore, enhancing urea N recycling to the GIT is an opportunity to

decrease N excretion (mainly urinary urea N) and is a potential opportunity to improve the efficiency of N utilization.

Urea N recycling to the GIT and its utilization for anabolic purposes is influenced by several dietary and ruminal factors. Major dietary factors which regulate urea N recycling to the GIT and its subsequent fate are: N intake and dietary N concentration (Marini and Van Amburgh, 2003; Wickersham et al., 2008a), protein degradability (Atkinson et al., 2007); total dry matter intake (Sarraseca et al., 1998), feed processing and OM fermented in the rumen (Kennedy and Milligan, 1980), and frequency of RDP supplementation (Wickersham et al., 2008b). In addition, ruminal factors such as ruminal NH_3 concentration, ruminal bacterial urease activity, ruminally fermentable energy, ruminal concentrations of volatile fatty acids and CO_2 and ruminal pH also play a significant role in trans-epithelial movement of blood urea into the rumen (Kennedy and Milligan, 1980).

For cattle consuming poor-quality forages (CP less than 7%), N is the nutrient that is typically most limiting for the microbes (Köster et al., 1996; Detmann et al., 2009). In this feeding condition, protein supplementation increases forage intake and digestion (Köster et al., 1996; Lazzarini et al., 2009; Sampaio et al., 2010), improves animal performance (Mathis et al., 1999), and the total amount of urea N recycled to the rumen increases (NRC, 1985; Wickersham et al., 2008). In unsupplemented cattle fed prairie hay (temperate grass with approximately 5% CP) about 98% of the hepatic synthesis of urea is recycled to the gut (Wickersham et al., 2008ab). Thus, cattle fed low-quality forages are quite efficient in recycling urea N to the GIT. On the other hand, under this condition, breakdown of myofibrillar protein can be increased to produce energy from AA catabolism and the released N can be used for urea production (NRC, 1985). This strategy may help the animal to meet microbial N requirements through recycling, but excessive mobilization of body tissues could hinder performance.

However, there is limited research on how protein supplementation sources (RDP and RUP) could impact urea N recycling to the GIT, microbial use of recycled urea N, and its subsequent utilization for anabolic purposes in *Bos indicus* cattle fed low-quality tropical forage. Hence, the objective of the first chapter of this dissertation was to evaluate effects of increasing levels of RUP when adequate RDP is supplied on urea kinetics and efficiency of N utilization in Nellore heifers fed low-quality tropical grass hay. Also on urea kinetics, the second chapter aimed to evaluate the effect of crude protein intake on urea kinetics and microbial incorporation of recycled urea by using a meta-analytical approach.

Amino Acid Utilization by Ruminants

The RDP and fermentable energy support microbial AA production, which in combination with RUP escaping ruminal fermentation constitute the main sources of AA to the small intestine. This process makes study of the ruminant animals requirements for AA difficult because AA absorbed from the small intestine are either quantitatively or qualitatively (i.e., relative to the AA profile) different from the diet AA (Fox and Tedeschi, 2003). The absorbed AA from the small intestine (or metabolizable protein; MP) are primarily used for protein deposition in tissues of growing animals; however, not all of those AA are completely converted to product proteins. The oxidative losses are the main cause of the inefficient use of absorbed AA for protein deposition. For some AA, metabolic processes that do not contribute to protein deposition (e.g., synthesis of urea from arginine, carnitine from lysine, and polyamines from methionine) and irreversible post-translational modifications of AA (e.g., methylation of histidine residues) may also contribute to inefficiencies in AA usage. Some essential AA may also be used to synthesize non-essential AA, such as the methionine conversion to cysteine (Titgemeyer, 2003).

The animal requirements for AA are estimated by NRC (1996) separately for maintenance and growth. Maintenance protein requirements are based on estimates of requirements for scurf, endogenous urinary and endogenous fecal losses. Net maintenance needs for AA are generated from protein requirements using AA profiles of tissues (i.e., keratin for scurf and whole body for urinary and fecal losses). On the other hand, protein requirements for growth are based on net protein deposition and an efficiency of utilization; protein needs are then converted to AA requirements using the AA profile of whole body tissue (Titgemeyer, 2003). Nevertheless, the efficiency of AA utilization for protein deposition in growing cattle has remained uncertain and research to evaluate the efficiency has been rather limited.

The Cornell Net Carbohydrate and Protein System (**CNCPS**; O'Connor et al., 1993) used whole-body tissue composition and weight gain to determine net AA requirements. The efficiencies of AA for maintenance were assumed to be 85% for all essential AA, except branched-chain AA for which efficiencies of 66% were assumed. Accordingly, the efficiencies of AA utilization for maintenance were greater than those for growth (except branched-chain AA). The CNCPS estimated the efficiencies of AA utilization for growth by using a protein efficiency equation [efficiency of absorbed protein utilization for growth = 0.834 – 0.00114 × equivalent shrunk weight (kg)] developed by Ainslie et al. (1993). According to this equation, the same efficiency is applied to each essential AA, as well as to MP. If efficiency of use was the same for whole protein and all AA, one could suggest that the AA profile of MP would always be ideal. However, recent research conducted with cattle demonstrated that such assumptions are not entirely correct, because differences exist among AA with regards efficiency of utilization (e.g., methionine: Campbell et al., 1996, 1997; Löest et al., 2002; leucine: Awawdeh et al., 2005; 2006; Titgemeyer et al., 2012; and histidine: McCuistion et al., 2004). The efficiencies of AA utilization are dependent on various factors such as differences

in oxidation rates of individual AA (Heger and Frydrych, 1989) and differences in the roles of specific AA in processes other than protein synthesis (Owens and Pettigrew, 1989).

Regarding the essentiality, based on studies with growing cattle (Richardson and Hatfield, 1978) and sheep (Storm and Ørskov, 1984) receiving microbial protein as the sole source of MP, lysine has been identified as the second-limiting AA in microbial protein, with methionine identified as the first limiting. However, when growing cattle fed corn-based diets (high-methionine supply) were postruminally infused with AA, lysine was identified as the most limiting AA (Burris et al., 1976). For this reason, lysine is considered an important AA in cattle nutrition. Estimates of the lysine requirements of growing steers have been developed using plasma data (Fenderson and Bergen, 1975), modeling approaches (O`Connor et al., 1993), or growth studies (Klemesrud et al., 2000a,b). Nonetheless, the efficiencies obtained from these studies showed a broad range, from 13 to 99% (Burris et al., 1976; Klemesrud et al., 2000a).

Considering the importance of the evaluation of AA utilization by growing cattle, the third chapter of this dissertation aimed to evaluate the response surface of supplemental lysine on N retention to estimate the efficiency of lysine utilization in growing steers.

LITERATURE CITED

Ainslie, S. J., D. G. Fox, T. C. Perry, D. J. Ketchen, and M. C. Barry. 1993. Predicting amino acid adequacy of diets fed to Holstein steers. J. Anim. Sci. 71:1312-1319.

Atkinson, R. L., C. D. Toone, D. L. Harmon, and P. A. Ludden. 2007. Effects of supplemental ruminally degradable protein versus increasing amounts of supplemental ruminally undegradable protein on nitrogen retention, apparent digestibility and nutrient flux across visceral tissues in lambs fed low-quality forage. J. Anim. Sci. 85:3331–3339.

Awawdeh, M. S., E. C. Titgemeyer, G. F. Schroeder, and D. P. Gnad. 2006. Excess amino acid supply improves methionine and leucine utilization by growing steers. J. Anim. Sci. 84:1801-1810.

Awawdeh, M. S., E. C. Titgemeyer, K. C. McCuistion, and D. P. Gnad. 2005. Ruminal ammonia load affects leucine utilization by growing steers. J. Anim. Sci. 83:2448-2454.

Bach, A., Calsamiglia, S. and M. D. Stern. 2005 Nitrogen metabolism in the rumen. J. Dairy Sci. 88 (Suppl. 1):E9-E21.

Bergman, E. N., and R. N. Heitmann. 1978. Metabolism of amino acids by the gut, liver, kidneys, and peripheral tissues. Fed. Proc. 37:1228-1232.

Burris, W.R., J.A. Boling, N.W. Bradley, and A.W. Young. 1976. Abomasal lysine infusion in steers fed a urea supplemented diet. J. Anim. Sci. 42:699-705.

Calsamiglia S., A. Ferret, C. K. Reynolds, N. B. Kristensen, A. M. van Vuuren. 2010. Strategies for optimizing nitrogen use by ruminants. Animal. 4:1184–1196.

Campbell, C. G., E. C. Titgemeyer, and G. St-Jean. 1996. Efficiency of D- vs L-methionine utilization by growing steers. J. Anim. Sci. 74:2482-2487.

Campbell, C. G., E. C. Titgemeyer, and G. St-Jean. 1997. Sulfur amino acid utilization by growing steers. J. Anim. Sci. 75:230-238.

Detmann, E., M. F. Paulino, H. C. Mantovani, S. C. Valadares Filho, C. B. Sampaio, M. A. Souza, I. Lazzarini, and K. S. C. Detmann. 2009. Parameterization of ruminal fibre degradation in low-quality tropical forage using Michaelis-Menten kinetics. Livest. Sci. 126:136 –146.

Fenderson, C. L., and W. G. Bergen. 1975. An assessment of essential amino acid requirements of growing steers. J. Anim. Sci. 41:1759-1766.

Fox, D.G. and Tedeschi, L. O. 2003. Predicting Dietary Amino Acid Adequacy for Ruminants. In: J. P. F. D'Mello, editor, Amino acids in animal nutrition, 2nd ed. CAB International, Wallingford, UK. p. 389-410.

Heger, J., and Z. Frydrych. 1989. Efficiency of utilization of amino acids. In: M. Friedman (Ed.) Absorption and utilization of amino acids. pp 31-56. CRC Press, Boca Raton, FL.

Hristov, A. N. and Jouany J. P. 2005. Factors affecting the efficiency of nitrogen utilization in the rumen. Pages 117-166 in Nitrogen and phosphorus nutrition of cattle and the environment. E. Pfeffer and A. N. Hristov, ed. CAB Int., Wallingford, UK.

Kennedy, P. M., and L. P. Milligan. 1980. The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants: A review. Can. J. Anim. Sci. 60:205-221.

Kennedy, P. M., and L. P. Milligan. 1980. The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants: A review. Can. J. Anim. Sci. 60:205-221.

Klemesrud, M. J., T. J. Klopfenstein, and A. J. Lewis. 2000a. Metabolizable methionine and lysine requirements of growing cattle. J. Anim. Sci. 78:199-206.

Köster, H. H., R. C. Cochran, E. C. Titgemeyer, E. S. Vanzant, I. Abdelgadir, and G. StJean. 1996. Effect of increasing degradable intake protein on intake and digestion of low-quality, tallgrass-prairie forage by beef cows. J. Anim. Sci. 74:2473-2481.

Lapierre, H., and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84(Suppl. E):E223–E236.

Lazzarini, I., E. Detmann, C. B. Sampaio, M. F. Paulino, S. C. Valadares Filho, M. A. Souza, and F. A. Oliveira. 2009. Intake and digestibility in cattle fed low-quality tropical forage and supplemented with nitrogenous compounds. Rev. Bras. Zootec. 38:2021–2030.

Lockyer, D. R. and D. C. Whitehead. 1990. Volatilization of ammonia from cattle urine applied to grassland. Soil Biol. Biochem. 22:1137–1142.

Löest, C. A., E. C. Titgemeyer, G. St-Jean, D. C. Van Metre, and J. S. Smith. 2002. Methionine as a methyl group donor in growing cattle. J. Anim. Sci. 80:2197-2206.

MacRae, J. C., L. A. Bruce, D. S. Brown, D. A. H. Farningham, and M. F. Franklin. 1997. Absorption of amino acids from the intestine and their net flux across the mesenteric and portal drained viscera of lambs. J. Anim. Sci. 75:3307-3314.

Marini, J. C., and M. E. Van Amburgh. 2003. Nitrogen metabolism and recycling in Holstein heifers. J. Anim. Sci. 81:545–552.

Mathis, C. P., R. C. Cochran, J. S. Heldt, B. C. Woods, I. E. O. Abdelgadir, K. C. Olson, E. C. Cochran, and E. S. Vanzant. 2000. Effects of supplemental degradable intake protein on utilization of medium- to low-quality forages. J. Anim. Sci. 78:224-232.

McCuistion, K. C., E. C. Titgemeyer, M. S. Awawdeh, and D. P. Gnad. 2004. Histidine utilization by growing steers is not negatively affected by increased supply of either ammonia or amino acids. J. Anim. Sci. 82:759-769.

NRC. 1985. Ruminant nitrogen usage. Natl. Acad. Press, Washington, DC.

O'Connor, J. D., C. J. Sniffen, D. G. Fox, and W. Chalupa. 1993. A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. J. Anim. Sci. 71:1298-1311.

Owens, F.N., and J.E. Pettigrew. 1989. Subdividing amino acid requirements into portions for maintenance and growth. In: M. Friedman (Ed.). Absorption and Utilization of Amino Acids. pp 15-30. CRC Press, Inc., Boca Raton, FL.

Reynolds, C. K., and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis. J. Anim. Sci. 86: E293–E305.

Richardson, C.R., and E.E. Hatfield. 1978. The limiting amino acids in growing cattle. J. Anim. Sci. 46:740-745.

Røjen, B. A., P. K. Theil, and N. B. Kristensen. 2011. Effects of nitrogen supply on inter-organ fluxes of urea N and renal urea N in lactating Holstein cows. J. Dairy Sci. 94:2532-2544.

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

Sampaio, C. B., E. Detmann, M. F. Paulino, S. C. Valadares Filho, M. A. Souza, I. Lazzarini, P. V. Paulino, and A. C. Queiroz. 2010. Intake and digestibility in cattle fed low-quality tropical forage and supplemented with nitrogenous compounds. Trop. Anim. Health Prod. 42:1471–1479.

Sarraseca A, E. Milne, M. J. Metcalf and G. E. Lobley. 1998. Urea recycling in sheep: effects of intake. Brit. J. Nutr. 79:79–88.

Silva, L.F.C. 2011. Nutritional requirements, validation of equations to estimate the empty body and creatinine use to estimate the muscle content in Nellore bulls. MS Thesis, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Storm, E., and E.R. Ørskov. 1984. The nutritive value of rumen micro-organisms in ruminants. 4. The limiting amino acids of microbial protein in growing sheep determined by a new approach. Br. J. Nutr. 52:613-620.

Tamminga, S. 1992. Nutrition management of dairy cows as a contribution to pollution control. J. Dairy Sci. 75:345-357.

Titgemeyer, E. C. 2003. Amino acid utilization by growing and finishing ruminants. In: J. P. F. D'Mello, editor, Amino acids in animal nutrition, 2nd ed. CAB International, Wallingford, UK. p. 329-346.

Titgemeyer, E. C., K. S. Spivey, S. L. Parr, D. W. Brake, and M. L. Jones. 2012. Relationship of whole body nitrogen utilization to urea kinetics in growing steers. J. Anim. Sci. 90:3515-3526.

Valente, E. E. L., M. F. Paulino, M. I. Marcondes and I. F. T. Dias. 2014. Body composition and deposition efficiency of protein and energy in grazing young bulls. Acta Scientiarum. 36:215-224.

Walker, N. D., C. J. Newbold and R. J. Wallace. 2005. Nitrogen metabolism in the rumen. Pages 71–115 in Nitrogen and phosphorus nutrition of cattle and the environment. E. Pfeffer and A. N. Hristov, ed. CAB Int., Wallingford, UK.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, E. E. Wickersham, and D. P. Gnad. 2008a. Effect of rumen-degradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. J. Anim. Sci. 86:3079–3088.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, E. E. Wickersham and E. S. Moore. 2008b. Effect of frequency and amount of rumen-degradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. J. Anim. Sci. 86:3089-3099.

CHAPTER 1

Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage

E. D. Batista,*† E. Detmann,* E. C. Titgemeyer,† S. C. Valadares Filho,*

R. F. D. Valadares,‡ L. L. Prates,‡ L. N. Rennó,* M. F. Paulino*

*Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil;

†Department of Animal Sciences and Industry, Kansas State University, Manhattan; and

‡Department of Veterinary Medicine, Universidade Federal de Viçosa, Brazil

Published at Journal of Animal Science

doi:10.2527/jas2015-9493

ABSTRACT

Effects of supplemental RDP and RUP on nutrient digestion, N metabolism, urea kinetics, and muscle protein degradation were evaluated in Nellore heifers (*Bos indicus*) consuming low-quality signal grass hay (5% CP, 80% NDF; DM basis). Five ruminally and abomasally cannulated Nellore heifers (248 ± 9 kg) were used in a 5 × 5 Latin square. Treatments were: control (no supplement); and RDP supplementation to meet 100% of the RDP requirement plus RUP provision to supply 0%, 50%, 100%, or 150% of the RUP requirement. Supplemental RDP (casein plus NPN) was dosed ruminally twice daily, and RUP supply (casein) was continuously infused abomasally. Jugular infusion of [^{15}N^{15}N]-urea with measurement of enrichment in urine was used to evaluate urea kinetics. The ratio of urinary 3-methylhistidine to creatinine was used to estimate skeletal muscle protein degradation. Forage NDF intake (2.48 kg/d) was not affected ($P \geq 0.37$) by supplementation, but supplementation did increase ruminal NDF digestion ($P < 0.01$). Total N intake (by design) and N retention increased ($P < 0.001$) with supplementation and also increased linearly with RUP provision. Urea entry rate and gastrointestinal entry rate of urea were increased by supplementation ($P < 0.001$). Supplementation with RUP linearly increased ($P = 0.02$) urea entry rate and tended ($P = 0.07$) to linearly increase gastrointestinal entry rate of urea. Urea use for anabolic purposes tended ($P = 0.07$) to be increased by supplementation, and RUP provision also tended ($P = 0.08$) to linearly increase the amount of urea used for anabolism. The fraction of recycled urea N incorporated into microbial N was greater ($P < 0.001$) for control (22%) than for supplemented (10%) heifers. Urinary 3-methylhistidine:creatinine of control heifers was more than double that of supplemented heifers ($P < 0.001$). Control heifers reabsorbed a greater ($P < 0.001$) fraction of urea from the renal tubule than did supplemented heifers. Overall, unsupplemented heifers had greater mobilization of AA from myofibrillar protein, which provided N for urea synthesis and subsequent recycling. Supplemental RUP, when RDP was supplied, not only

increased N retention, but also supported increased urea N recycling and increased ruminal microbial protein synthesis.

Key words: *Bos indicus*, cattle, muscle degradation, protein supplementation, tropical forage, urea recycling

INTRODUCTION

During the dry season, the protein content of low-quality tropical grasses is generally less than 7%, which limits degradation of forage fiber by ruminal microorganisms (Russell et al., 1992; Detmann et al., 2009). Thus, for cattle fed low-quality grasses, RDP is a priority to optimize digestion of forage.

When protein is supplemented to cattle fed low-quality forage, urea production and urea recycling to the gastrointestinal tract **(GIT)** typically increase, but the percentage of urea production that is recycled decreases as N intake increases (Wickersham et al., 2008). By providing ammonia to ruminal microbes, urea recycling affects the amount of ruminally available N that must be provided directly from the diet. Thus, nutritional systems should to consider the recycled N during the estimation of dietary protein requirements; however, some systems, such as the Brazilian Nutrient Requirements for Zebu Beef Cattle (Valadares Filho et al., 2010), do not take recycling into account. Supplementation with RUP directly increases MP supply to ruminants (Poppi and McLennan, 1995), but RUP also may increase urea synthesis and recycling (Wickersham et al., 2009b) subsequent to deamination of AA, a process that may be sustained over time (Atkinson et al., 2007). Under low energy or protein intake, breakdown of myofibrillar protein can be increased to produce energy from AA catabolism and the released N can be used for urea production (NRC, 1985). This strategy may help the animal meet microbial N requirements through recycling, but excessive mobilization of body tissues will hinder performance.

Therefore, we hypothesized that RUP provision would increase urea recycling, effectively contribute to microbial growth, and positively impact N retention in cattle fed low-quality forage. Our objective was to evaluate effects of increasing levels of RUP when adequate RDP is supplied on nutrient digestion and urea kinetics in Nellore heifers fed low-quality tropical grass hay.

MATERIALS AND METHODS

All practices involving the use of animals were approved by the Institutional Animal Care and Use Committee of the Universidade Federal de Viçosa (protocol no. 016/2012; Appendix, pg. 110). This experiment was conducted at the Department of Animal Science, Universidade Federal de Viçosa in Viçosa, Minas Gerais, Brazil.

Animals and Management

Five ruminally and abomasally fistulated Nellore heifers (averaging 261 ± 8 kg of initial BW, 239 ± 9 kg of final BW, and 248 ± 9 kg of average BW) were used in a 5 × 5 Latin square design. The heifers were housed in individual stalls (2 by 5 m) with concrete floors and equipped with individual feeders and water dispensers. Heifers had ad libitum access to a mineral mixture (composition: ≥ 180 g/kg Ca, 80 g/kg P, 15 g/kg S, 10 g/kg Mg, 115 g/kg Na, 4.5 g/kg Zn, 1.35 g/kg Cu, 1.2 g/kg Mn, 0.09 g/kg I, 0.075 g/kg Co, 0.022 g/kg Se; Tecnophós cria, Vaccinar, Belo Horizonte, Minas Gerais, Brazil).

The basal diet consisted of a low-quality signal grass hay (*Brachiaria decumbens* Stapf.; Table 1.1) chopped to a 15-cm particle size and fed in equal amounts twice daily at 0600 and 1800 h. Hay was offered for ad libitum intake. Every day, orts from each animal were removed and weighed before the 0600 h feeding. To ensure ad libitum access to the hay, voluntary forage intake was determined daily, and hay was fed at 140% of the average intake

for the previous 5 d. The hay was produced from a dry season cutting of the forage available in a pasture located in the mid-west region of Brazil.

Treatments

Treatments were: control (no supplementation); and RDP supplementation to meet 100% of the RDP requirements plus 1 of 4 amounts of RUP supplied to meet 0, 50, 100, and 150% of the RUP requirements (**RUP0**, **RUP50**, **RUP100**, and **RUP150**). The RDP and RUP requirements were calculated according to the Brazilian Nutrient Requirements for Zebu Beef Cattle (Valadares Filho et al., 2010), based on a Nellore heifer with 250 kg BW and ADG of 300 g/d. On average, the RDP supplement provided 1.5 g of CP/kg BW, whereas RUP0, RUP50, RUP100, and RUP150 provided 0, 0.3, 0.6, and 0.9 g CP/kg BW.

The RDP supplement was administered intraruminally in 2 equal portions concurrent with the morning and evening feedings (0600 and 1800 h). A mixture of pure casein (LabSynth, Diadema, São Paulo, Brazil), urea, and ammonium sulfate (53:9:1, respectively) was used as the source of RDP (Table 1.1). This ratio was based on a previous study conducted in tropical conditions (Costa et al., 2011), where the ratio of one-third of the supplemental N from NPN (urea:ammonium sulfate, 9:1) and two-thirds of the supplemental N from true protein promoted greatest N retention in grazing cattle.

Pure casein (Table 1.1) was selected as the RUP supplement because of its high-protein content, its essentially complete digestibility in the small intestine (Wickersham et al., 2004; 2009b), and the absence of other components (e.g., carbohydrates). Abomasal supplements were prepared daily by dissolving casein with 0.53% Na_2CO_3 (wt/wt of casein; Richards et al., 2002) using a blender, and they were mixed with water to a total weight of 4.5 kg. The solution was infused continuously into the abomasum via the cannula using a peristaltic pump (Milan Scientific Equipment, Inc., Colombo, Paraná, Brazil) and polyvinyl chloride tubing (4.75 mm i.d.) at a rate of approximately 191.5 g/h. Tubing entered the abomasal cannula through a

silicone tube that extended a minimum of 20 cm into the abomasum to prevent localized accumulation of infused casein. Daily infusions were designed to last about 23.5 h, but if the infusions were not completed in that time, the remaining 0.5 h was used to ensure that the entire infusate was provided within a 24-h period. Heifers without supplementation were infused with water (4.5 kg/d) into the abomasum.

Experimental Procedures and Sample Collections

Each experimental period lasted 16 d, with 9 d for supplement adaptation and 7 d for sample collection. Heifers were weighed at the beginning and at the end of each experimental period.

DMI. Feed intake was quantified from d 9 to 12 of each period. Representative samples of hay and orts were collected daily, stored in plastic bags, and blended manually at the end of each period to obtain pooled samples per animal. Samples of hay and orts were oven-dried (60ºC) and ground in a Wiley mill (model 3, Arthur H. Thomas, Philadelphia, PA) to pass through a 2-mm screen. After that, half of each ground sample was ground again to pass through a 1-mm screen. Casein samples were retained from each 25-kg package of product and pooled for subsequent analysis.

Catheter Placement and [^{15}N^{15}N]-Urea Infusion. On d 9 of each experimental period, at 1600 h, heifers were fitted with temporary catheters (1.02 mm i.d., 1.78 mm o.d.; Tygon, S-54-HL, Buch & Holm A/S, Herlev, Denmark) in the jugular vein by percutaneous venipuncture (Holder et al., 2015) for blood collection and infusion of double-labeled urea ([^{15}N^{15}N]-urea, 99.8 atom % of ^{15}N; Cambridge Isotope Laboratories, Andover, MA). The catheter was flushed with sterile saline solution and filled with 5 mL of sterile saline solution containing heparin (100 IU/mL). Patency of the catheters was maintained by flushing with 5 mL of heparinized saline (10 IU/mL) at least every 6 h, from the time the catheter was placed until 0600 h on d 11, when infusion of [^{15}N^{15}N]-urea solution started. The concentration of [^{15}N^{15}N]-urea in the

solution was adjusted on an assumption that urea production was similar to N intake, and urea concentration of the solution was adjusted to yield a predicted enrichment of [$^{15}N^{15}N$]-urea of 0.1 atom percent excess at plateau (Marini and Van Amburgh, 2003). In each period, 500 mL of solution was produced for each treatment, from a 5 g/L stock solution of [$^{15}N^{15}N$]-urea, by dissolving in sterile saline solution (9 g NaCl/L). The [$^{15}N^{15}N$]-urea solution was prepared using sterile technique in a laminar flow hood and filtered through a 0.22-μm filter (Sterivex, Millipore Corporation, Billerica, MA) into a sterilized glass container stored at 4°C until use. The infusion rate was 5 mL/h, which delivered 0.100 to 0.490 mmol of urea N/h using a syringe infusion pump (BS-9000 Multi-Phaser, Braintree Scientific Inc, Braintee, MA) until 1600 h of d 14, when the last sample was collected. To quantify the exact volume infused, syringes were weighed before and after infusion.

Blood Samples. On d 10, blood was sampled via catheter at 0600, 1200, 1800, and 2400 h using syringes after two 5-mL aliquots of blood were discarded before obtaining samples. Blood samples (10 mL) were injected immediately into vacuum tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing heparin (143 IU), placed in ice water immediately after collection, and centrifuged (1,200 × *g*, 15 min at 4°C). Plasma was frozen (–20°C) for later analysis.

Fecal Collection. Total fecal output was collected immediately after each spontaneous defecation from d 10 through 13 of each period and stored in 20-L buckets. At the end of each 24-h collection period, buckets were changed and the feces weighed and manually blended, and an aliquot (5%) was collected daily. Each daily fecal sample was oven-dried (60°C) and ground as described for hay and orts. After grinding, samples were pooled per animal and period in proportion to the daily excretion to measure digestibility and N balance. Total collections of feces from d 10 were used to determine background enrichments of ^{15}N, and

those from d 13 were used to measure enrichments of ^{15}N for calculating urea kinetics, according to the sampling protocol validated by Wickersham et al. (2008).

Total Urine Collection. From d 10 through 13, urine was completely collected using a 2-way Foley probe (No. 24, Rush Amber, Kamuting, Malaysia) with a 30-mL balloon. At the free end of the probe, a polyethylene tube was attached through which the urine was conducted to a clean urine collection vessel (20-L) containing 900 mL of 10% (wt/wt) H_2SO_4. At the end of each 24-h collection period, urine output was weighed and mixed thoroughly, and an aliquot (1%) was filtered through 4 layers of cheesecloth and frozen at −20°C for later analysis. Total collections of urine from d 10 and d 13 were used to measure ^{15}N background and enrichment, respectively, for urea kinetics calculations (Wickersham et al., 2008).

Abomasal Digesta Sampling. Digesta flow into the abomasum was estimated with the double marker method, using indigestible NDF (**iNDF**) and Co-EDTA (Rotta et al., 2014). As a fluid marker, 5 g/d of Co-EDTA (420 mg of Co/d) was divided into 4 doses and infused into the rumen cannula in equidistant times (0600, 1200, 1800 and 2400 h) from d 7 through 12 of each period. Eight abomasal samples (550 mL per sample) were collected from d 10 through 12 of each period. Sample collection began after discarding digesta accumulated in the cannula neck. The schedule used sampling at 9-h intervals (Allen and Linton, 2007) to represent every 3 h of a 24-h period to account for diurnal variation. Sampling was on d 10 at 0600, 1500, and 2400 h; d 11 at 0900 and 1800 h; and d 12 at 0300, 1200, and 2100 h. At every sampling, the digesta was divided into 2 subsamples as follows: 300-mL subsamples from every time point were pooled and frozen at −20°C as they were collected to yield a 2.4-L abomasal composite samples; and 250-mL subsamples from collections were pooled and kept in a refrigerator to yield a 2.0-L digesta sample for the isolation of bacteria, according to Reynal et al. (2005). Also, an aliquot of 10 mL of the liquid fraction of digesta was added to a container and frozen at −20ºC for later analysis of NH_3. At the end of the sampling collection, 2.4 L of the composite

sample was homogenized and filtered through a nylon filter with 100-µm mesh opening (Sefar Nitex 100/44; Sefar, Heiden, Switzerland) for separation of the particle phase from the fluid plus small-particle phase. In all procedures (fluid plus small-particle phase, and particle phase), samples were weighed, frozen at −80°C, freeze-dried, and ground as previously described. Samples of abomasal digesta related to each phase (fluid plus small-particle phase, and particle phase) were pooled (10 g of predried sample of each time) for each animal and period.

On d 14 of each period, for quantifying incorporation of urea recycled into microbial protein, samples of abomasal digesta (200 mL) were collected from the abomasal cannula just before morning feeding (0 h) and at 2, 4, 6, 8, and 10 h after feeding, frozen at −80°C, and freeze-dried for 72 h. These sampling times representedg 72 to 82 h of label infusion, during which the isotopic enrichment of ^{15}N reached plateau in the collection protocol validated by Wickersham et al. (2009a). The freeze-dried samples were ground as previously described and subsequently pooled across times of collection on an equal weight basis.

Ruminal Fermentation and Microbial N Synthesis. On d 14, at the same times as abomasal sampling for ^{15}N enrichment, ruminal fluid samples were obtained to evaluate pH, ruminal ammonia nitrogen (**RAN**), and VFA and to measure the ^{15}N enrichment in bacteria. Ruminal contents (500 mL) were collected manually from the cranial, ventral, and caudal areas of the rumen and filtered through 4 layers of cheesecloth. The fluid was subjected to pH measurement (potentiometer TEC-3P-MP, Tecnal, Piracicaba, SP, Brazil). Then, an 8-mL aliquot of ruminal fluid was combined with 2 mL of 25% (wt/vol) metaphosphoric acid and frozen for subsequent analysis of VFA. Another 40-mL aliquot was combined with 1 mL of 9 *M* H_2SO_4 and frozen for later analysis of RAN. The remaining fluid and solids were used to isolate bacteria by differential centrifugation, according to Cecava et al. (1990). Bacterial pellets were freeze-dried and ground using a mortar and pestle.

Ruminal Evacuation. Ruminal pool of fiber was measured at 1000 h (4 h after morning feeding) on d 14, and at 0600 h (just before morning feeding) on d 16 of each period (Allen and Linton, 2007). Whole ruminal contents were evacuated manually through the ruminal cannula, placed into a plastic container, weighed, hand-mixed, and sub-sampled in triplicate (1 kg total) for further analysis. After sampling, the remainder was returned to the rumen of each heifer.

Laboratory Analysis

Pooled samples of each material ground through 1-mm sieves (hay, feces, abomasal digesta, and ruminal contents) and casein were analyzed according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; Detmann et al., 2012) for DM (dried overnight at 105°C; method INCT-CA no. G-003/1), ash (complete combustion in a muffle furnace at 600°C for 4 h; method INCT-CA no. M-001/1), N (Kjeldahl procedure; method INCT-CA no. N-001/1), ether extract (Randall procedure; method INCT-CA no. G-005/1), NDF corrected for ash and protein (**NDFap**; using a heat-stable α-amylase, omitting sodium sulfite and correcting for residual ash and protein; method INCT-CA no. F-002/1), and ADL (method INCT-CA no. F-005/1). Casein samples were analyzed only for DM, OM, and CP. From samples of hay, orts, feces, and abomasal digesta processed through a 2-mm sieve, iNDF content was determined as the residual NDF remaining after 288 h of ruminal in situ incubation using F57 filter bags (Ankom Technology Corp., Macedon, NY), according to Valente et al. (2011). Cobalt content in the abomasal samples was analyzed using an atomic absorption spectrophotometer (Avanta Σ, GBC Scientific Equipment, Braeside, Victoria, Australia). Potentially degradable NDF (**pdNDF**) was calculated as the difference between NFDap and iNDF.

Urinary urea (Marsh et al., 1965), urinary creatinine (Chasson et al., 1961), and urinary NH_3 (Broderick and Kang, 1980) concentrations were quantified colorimetrically with an

AutoAnalyzer (Technicon Analyzer II, Technicon Industrial Systems, Buffalo Grove, IL). Total urinary N were obtained by Kjeldahl procedure (method INCT-CA no. N-001/1). Urinary 3-methylhistidine concentrations were measured by cation-exchange HPLC with post-column *o*-phthalaldehyde derivitization and fluorimetric quantification. Blood plasma samples were analyzed for urea, creatinine, glucose, triglycerides, and β-hydroxybutyrate with an automated chemistry analyzer (BS-200E, Mindray North America, Mahwah, NJ). Concentrations of plasma urea N **(PUN)** were measured by an enzymatic kinetic test (sensitivity of 2.97 mg/dL; K056, Bioclin, Quibasa Química Básica Ltda, Belo Horizonte, Minas Gerais, Brazil), creatinine with a colorimetric kinetic test (sensitivity of 0.18 mg/dL; K067, Bioclin, Quibasa Química Básica Ltda), glucose by colorimetric kinetic test (sensitivity of 1.3 mg/dL; K082, Bioclin, Quibasa Química Básica Ltda), triglycerides by the enzymatic colorimetric test (sensitivity of 2.6 mg/dL; K117, Bioclin, Quibasa Química Básica Ltda), and β-hydroxybutyrate by kinetic test (sensitivity of 0.07 mmol/L; RANBUT – RB1007, Randox Laboratories Ltd., Crumlin, UK).

The ^{15}N enrichments of dried fecal samples (d 10 and d 13), pooled ruminal bacteria, and abomasal samples were analyzed using an isotope ratio mass spectrometer (**IRMS**; ThermoFinnigan Delta Plus, Thermo Electron Corporation, Waltham, MA). Urinary urea and ammonia concentrations were quantified colorimetrically as described before. Measurements of ^{15}N enrichment of urinary urea was conducted on N_2 samples produced from Hoffman degradation of urinary urea by using techniques similar to those described by Wickersham et al. (2009b), except 1) 250 µmol of urea was pipetted into a column; and 2) the procedures of column washing were conducted according to Archibeque et al. (2001). Samples were analyzed for the proportions of [$^{15}N^{15}N$]-, [$^{14}N^{15}N$]-, and [$^{14}N^{14}N$]-urea in urinary urea by IRMS (^{15}N Analysis Laboratory, University of Illinois, Urbana, IL). Results were corrected for [$^{14}N^{15}N$]-N_2 produced by nonmonomolecular reactions (Lobley et al., 2000).

The RAN and abomasal NH_3 (from wet samples) concentrations were quantified using the colorimetric technique described by Detmann et al. (2012; method INCT-CA no. N-006/1). For VFA analysis, rumen fluid samples collected over time were pooled (2.0 mL), centrifuged (12,000 × *g*, 10 min, 4°C), and supernatants were treated as described by Siegfried et al. (1984). Ruminal VFA were analyzed by HPLC (Shimadzu HPLC class VP series, model SPD 10A, Shimadzu Corporation, Kyoto, Japan) using a reverse phase column (mobile phase 0.15 *M* ortho-phosphoric acid) and UV detector at a wavelength of 210 nm.

Calculations

Digesta flow entering the abomasum (DM and liquid) was estimated using the double marker method described by France and Siddons (1986), using iNDF and Co-EDTA as markers. Ruminal fermented OM was calculated by correcting abomasal OM flow for the contribution of bacteria to OM. Urea kinetics were calculated according to the methods described by Lobley et al. (2000). Bacterial and abomasal ^{15}N enrichments were calculated as ^{15}N/total N and were corrected for values in the background fecal samples (Wickersham et al., 2008). Microbia N flow (**MN**) was calculated by multiplying abomasal N flow by the ratio of abomasal ^{15}N enrichment to bacterial ^{15}N enrichment. The MN derived from recycled urea N was calculated by multiplying MN by the ratio of bacterial ^{15}N enrichment to ^{15}N enrichment of urinary urea (calculated as one-half the $^{14}N^{15}N$-urea enrichment plus the $^{15}N^{15}N$-urea enrichment). Total amounts of ruminal VFA were calculated multiplying VFA concentration by the ruminal fluid volume. Renal clearances of urea and creatinine were calculated as the rates of urea or creatinine excretion in urine divided by the concentration of the corresponding metabolite in plasma. Urea pool size and turnover time were calculated on the basis of the urea space and PUN concentration, according to Harmeyer and Martens (1980); urea space was assumed to be 55% of BW (Preston and Kock, 1973). Ruminal NDF kinetics were calculated

as the ratio of fiber intake and passage (abomasal outflow) to ruminal pool size. Degradation rate was obtained as the difference between intake rate and passage rate of pdNDF.

Statistical Analyses

Statistical analyses were performed using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) according to a 5 × 5 Latin square design including the fixed effect of treatment and the random effects of heifer and experimental period. Analyses of rumen fermentation profiles (pH and RAN) were conducted using repeated measures over time (fixed effect) using a compound symmetry (RAN) or an unstructured (pH) (co)variance matrix, which was chosen based on corrected Akaike's information criterion. The degrees of freedom were estimated by the Kenward–Roger method. All data from 1 heifer from 2 periods (treatment RUP0 and RUP100) were lost because of problems unrelated to treatment. Comparisons among treatments were conducted with orthogonal contrasts which included: an overall comparison between supplemented vs. unsupplemented treatments, and the linear, quadratic, and cubic effects associated with RUP levels (0, 50, 100 and 150% of the requirements). No cubic effects were observed ($P > 0.10$), and accordingly they were not presented in the tables and also were omitted from the discussion. Statistical significance was considered at $P \leq 0.05$, and tendencies were considered at $0.05 < P \leq 0.10$.

RESULTS

Forage Intake and Digestibility

In general, voluntary intake of forage was not affected ($P \geq 0.37$) by treatments (Table 1.2). Forage OM and NDF intake averaged 2.96 and 2.48 kg/d, respectively, across all treatments. By design, intake of CP was increased by supplementation ($P < 0.001$) and also was increased linearly ($P < 0.001$) by RUP supplementation. Accordingly, TDN and digestible

OM (**DOM**) intakes were improved in response to protein supplementation ($P < 0.001$) and linearly increased ($P = 0.03$) as RUP levels increased.

Protein supplementation improved total tract digestibilities of OM, NDF, CP, and TDN ($P \leq 0.03$), but no effect of RUP level was detected ($P \geq 0.54$) for any of these criteria. Similarly, ruminal digestibilities of OM, NDF, and CP were positively affected by supplementation ($P \leq 0.008$). In addition, ruminal CP digestibility linearly decreased ($P < 0.01$) and OM tended ($P = 0.09$) to linearly decrease as RUP increased. Supplementation increased ($P < 0.001$) post-ruminal CP digestibility and tended ($P = 0.09$) to increase post-ruminal OM digestion. Increasing RUP supplementation linearly increased post-ruminal digestion of CP ($P < 0.001$) and OM ($P = 0.02$). Supplementation did not affect ($P \geq 0.63$) post-ruminal NDF digestibility, which averaged 1.9% of the abomasal flow.

Ruminal Dynamics of NDF and Fermentation

Ruminal contents of pdNDF and iNDF were not different ($P \geq 0.24$) among treatments (Table 1.3). There was no overall effect of the supplementation ($P = 0.22$) on the rate of intake (**ki**) of pdNDF. However, providing supplements tended to increase ($P = 0.09$) the degradation rate (**kd**) and decrease ($P = 0.07$) the passage rate (**kp**) of pdNDF. Increasing RUP supplementation tended to linearly increase kd ($P = 0.06$) and ki ($P = 0.05$) of pdNDF, but did not affect ($P \geq 0.48$) the kp of either iNDF or pdNDF.

Treatments did not affect ($P \geq 0.49$) ruminal concentration of total VFA (Table 1.4). Protein supplementation decreased ($P \leq 0.001$) molar proportion of acetate and the acetate:propionate ratio, but it increased molar proportions of isobutyrate, valerate, and isovalerate ($P \leq 0.01$). No effects of RUP supplementation levels were observed for VFA concentrations ($P \geq 0.35$). Ruminal pH was not affected ($P \geq 0.29$) by treatments. However, there was a sampling time effect ($P = 0.02$). The greatest values ($P < 0.05$) were observed at 2 and 4 h after ruminal supplementation (6.82 and 6.80), and the pH did not vary across the other

times, averaging 6.69 (data not shown). The treatment × time interaction was significant ($P < 0.01$) for RAN concentration (Table 1.5). The RAN concentrations were different among sampling times ($P < 0.01$) for all treatments, except for control ($P \geq 0.96$). The greatest RAN concentrations were observed at 2 or 4 h after feeding. The RAN concentrations were greater ($P < 0.01$) for supplemented treatments than for the control. In general, supplemented heifers presented similar RAN concentrations for the early times after ruminal supplementation. However, RAN concentrations at feeding (0 h) and at 10 h after feeding exhibited a tendency to increase as RUP provision increased.

Nitrogen Metabolism

Treatment effects were not detected for N intake from forage ($P \geq 0.51$), but total N intake increased ($P < 0.001$) with supplementation and with provision of supplemental RDP (Table 1.6). Corresponding with increased N intake, urinary N excretion was greater ($P < 0.001$) with supplementation, but supplementation did not affect ($P = 0.70$) fecal N excretion. Nevertheless, in response to RUP, fecal N excretion increased linearly ($P = 0.04$), but urinary N excretion was not affected ($P \geq 0.14$). Urinary urea N excretion (**UUE**), both as an amount per day and as a fraction of urinary N excretion, increased with protein supplementation ($P < 0.001$), without effects of RUP level ($P \geq 0.27$). Total urinary excretion of ammonia-N was increased by supplementation ($P < 0.001$) but not affected ($P \geq 0.20$) by RUP supplementation, whereas ammonia excretion as a percentage of urinary N was unaffected by treatments ($P \geq 0.73$). The amount of apparently digested N was improved ($P < 0.001$) by supplementation and increased linearly ($P < 0.001$) as RUP levels increased. Protein supplementation improved N retention ($P < 0.001$), which was also increased linearly ($P < 0.001$) as RUP supplementation increased. Overall, N retention efficiency (% of N intake or % of N digested) was improved ($P < 0.001$) in supplemented heifers. Moreover, increasing supplemental RUP tended to improve linearly ($P = 0.08$) N retention as a fraction of N intake.

The ruminal N balance was increased ($P < 0.001$) by supplementation, but it decreased linearly ($P < 0.001$) as RUP supplementation increased. Both abomasal N flow and MN increased with supplementation ($P \leq 0.02$) and linearly increased with RUP supplementation ($P \leq 0.01$). The relative production of MN in the rumen in proportion to the total N intake decreased ($P < 0.001$) with supplementation, averaging 99% for the control and 46% for the supplemented treatments. Total ammonia-N entering the abomasum was increased ($P < 0.001$) by supplementation and increased linearly ($P = 0.03$) with RUP provision. There were differences ($P \leq 0.01$) between control and supplemented heifers for the microbial efficiency, with larger values observed for the treatments receiving supplementation than for the control. Moreover, there was a positive linear tendency ($P = 0.07$) for the microbial efficiency based on dietary TDN with increasing supplemental RUP.

Urea Kinetics

Supplemental protein increased ($P < 0.001$) urea N entry rate (**UER**, urea production) and GIT entry rate (**GER**; Table 1.7). Moreover, UER was increased linearly ($P = 0.02$) and GER tended to increase linearly ($P = 0.07$) as RUP supplementation increased. The amount of urea N (g/d) returned to the ornithine cycle (**ROC**) and the amount of urea N excreted in feces (**UFE**) were greater ($P < 0.001$) for supplemented treatments than for the control. The amount of urea N utilized for anabolism (**UUA**) tended ($P = 0.07$) to be increased by supplementation and to linearly increase ($P = 0.08$) with RUP provision. The urimary urea N excretion:UER was lower ($P < 0.001$), and GER:UER and ROC:UER were greater ($P \leq 0.02$) for unsupplemented than for supplemented heifers. Supplementation increased ROC:GER ($P < 0.01$) and UFE:GER ($P = 0.001$), whereas UUA:GER decreased with supplementation ($P = 0.002$).

Microbial Use of Recycled Urea Nitrogen

The microbial use of recycled urea N (**MNU**) was not different among treatments ($P \geq 0.22$), averaging 4.8 g/d (Table 1.7). The fraction of MN from MNU was lower ($P < 0.001$) for the supplemented heifers (10%) compared to control (22%). The same pattern was also observed for the percentages of UER and of GER that were captured by the ruminal microbes.

Plasma Metabolites and Urinary 3-Methylhistidine

Supplementation significantly increased ($P < 0.001$) PUN concentration, but PUN did not differ among RUP levels ($P \geq 0.51$). Treatments effects were not detected ($P \geq 0.17$) for plasma glucose and triglycerides, but β-hydroxybutyrate tended ($P = 0.10$) to be lower for supplemented heifers (Table 1.8). The ratio of urinary 3-methylhistine:creatinine, an indicator of skeletal muscle protein breakdown, was greater ($P < 0.001$) in unsupplemented heifers than in those receiving protein supplementation, but it was unaffected by RUP supplementation level ($P \geq 0.49$).

Renal Clearance, Urea Space, and Turnover

Glomerular filtration rate of PUN was greater ($P < 0.001$) in supplemented heifers than in control heifers (Table 1.8). Renal clearance of creatinine did not differ ($P \geq 0.64$) among treatments. However, renal clearance of urea N and the proportion of urea that was filtered by the kidneys was greater ($P < 0.001$) in supplemented heifers. Supplementation increased urea pool size ($P < 0.001$), corresponding to PUN concentration, but turnover time of UER was not different ($P \geq 0.13$) among treatments.

DISCUSSION

Forage intake was not significantly affected by protein supplementation or increasing delivery of supplemental RUP. Much of the positive effects of supplementation on digestion (ruminal and total tract) seem to have resulted from digestion of the protein supplements per

se, which would be highly digestible. This contention is supported by increases in DOM intake in response to supplementation without any simultaneous increase in digestible NDF intake, although both ruminal and total tract NDF digestibilities were increased by supplementation.

Increases in low-quality forage intake as a response to protein supplementation have been described by different authors. Such a pattern has been associated with improvements in fiber degradation and microbial growth (Lazzarini et al., 2009; Sampaio et al., 2010). However, the forage intake is determined by integration of diferent mechanisms. Among these the adequacy of dietary protein-to-energy ratio has been pointed out as one of the main indicators of the intake pattern of cattle fed tropical forages (Detmann et al., 2014a). The maximum forage intake is observed with dietary CP:DOM at 210 to 280 g/kg (Poppi and McLennan, 1995; Detmann et al., 2014a). The dietary CP:DOM for control and supplemented heifers were, on average, 136 and 404 g/kg, respectively. Therefore, they both showed unbalanced dietary protein-to-energy ratio when adequacy of intake is considered, which seems to support the unaltered forage intake among treatments. A similar pattern was reported in the tropics (Rufino, 2011; Lazzarini et al., 2013).

Protein supplementation increased RAN concentration and branched-chain VFA (Table 1.4), as expected. The low RAN concentrations for control heifers (4.4 mg/dL) are similar to published values for crossbred *Bos indicus* cattle consuming only low-quality tropical forage (Lazzarini et al., 2009; Sampaio et al., 2010; Rufino, 2015), and they fall bellow reported optimal levels for NDF degradation in ruminants fed tropical forage-based diets (8 mg/dL; Detmann et al., 2009). All treatments with supplementation increased RAN concentrations, which improved the N provision relative to microbial requirements for fiber degradation. Similar to previous observations (Wickersham et al., 2004; 2008), branched-chain VFA were produced from ruminal degradation of branched-chain AA in the supplemental casein, which would provide essential growth factors that stimulate growth of ruminal cellulolytic bacteria

(Russell et al., 1992). Thus, the combined effect of increases in RAN and branched-chain VFA concentrations likely promoted positive effects on NDF digestibility, kd of pdNDF, and MN production.

On the other hand, the decreased kp of pdNDF with supplementation seems to be an indirect response of the improved ruminal digestion of NDF. There was no difference among treatments with regard to the ruminal pdNDF pool size. From this, with a greater ruminal digestion of NDF, a lower escape of undigested pdNDF would be observed, causing a lower abomasal outflow of pdNDF.

Considering that supplementation had only modest effects on nutritional characteristics (i.e., forage intake and digestion), it can be inferred that the most prominent effects were on the whole body N metabolism, N retention, and efficiency of N utilization, as observed by other researchers working with cattle in tropical conditions (Costa et al., 2011; Rufino, 2011; Batista, 2012).

Detmann et al. (2014a) used a meta-analysis to evaluate the efficiency of N utilization (% of N intake) in cattle fed tropical forage and receiving N supplementation. These authors reported that there were positive relationships for N retention with the amount of supplement and the percentage of CP in the supplement; additionally, the efficiency of N retention was more associated with the N supply rather than the energy content of the diet. However, it has been hypothesized that increases in energy supply can also contribute to an improved N retention. According to Wickersham et al. (2009b), the provision of RUP can also increase the energy supply by directly providing a source of digestible AA that could be catabolized and used as a source of energy.

The absolute values of N retention should be evaluated with caution because they likely overestimate protein accretion. Gerrits et al. (1996) compared N retention obtained in digestion trials with protein accretion obtained by serial slaughter, and they reported that N retention

overestimated protein accretion of growing cattle. This seems to occur mainly due the underestimation of urine N (e.g., volatile N losses from containers), fecal N (e.g., incomplete collection, volatile losses during either collection or drying), or both (Spanghero and Kowalski, 1997). However, when experimental procedures are standardized among treatments and experimental periods within an experiment, as performed in this study, in spite of bias in the absolute values, the relative comparisons between treatments should be valid.

Fecal N excretion increased with levels of supplemental RUP; this can be explained mainly by increases in MN, which increased by 16 g N/d (from RUP0 to RUP150). If 20% of MN is indigestible (NRC, 1985), an additional 3.2 g of N/d would be excreted in the feces, which accounts for about of 60% of the increase in fecal N excretion. Furthermore, the true intestinal digestibility of casein was evaluated by using a Lucas test approach (data not showed). It was estimated that 6.3% of casein is not digested in the intestines. Therefore, the increased indigestible MN along with undigested casein seems to be the cause of the increasing fecal N as RUP provision increased.

The ruminal N balance was only negative for the unsupplemented heifers, which is in agreement with Detmann et al. (2014a) who reported that the minimal value to obtain a null estimate of ruminal N balance under tropical conditions is approximately 9.2 mg/dL of RAN or 12.4% CP in the diet. Our control treatment presented only 5.0% dietary CP and 4.4 mg/dL of RAN. The negative value for the unsupplemented heifers demonstrates that the N intake was lower than the abomasal N flow and thus N recycling is important to sustain ruminal microbial growth (Detmann et al., 2014a).

Nitrogen recycling is one of the greater priority metabolic functions in the animal because a continuous N supply for microbial growth in the rumen is a strategy for animal survival (Egan, 1965; Van Soest, 1994). When there is a dietary N deficiency, as observed for the control treatment, the animal is able to decrease urinary N excretion and increase the

fraction of dietary N that is recycled to the rumen (Harmeyer and Martens, 1980). When availability of N, energy, or both is severely deficient, the animal can increase tissue protein mobilization (Ballard et al., 1976; NRC, 1985; Detmann et al., 2014b). The N obtained from mobilized myofibrillar protein could improve the N pool that can be used to sustain N recycling. Likewise, we can confirm this by the greater urinary 3-methylhistidine:creatinine for the unsupplemented heifers than for the supplemented. The pattern observed for ruminal N balance supported the assumptions about the metabolic priorities of N. In this case, there is a more significant dependency on recycling events to provide an adequate N supply to the rumen. Thus, animals fed low-quality forage can increase muscle protein breakdown due to a low protein or energy intake or both, and this may lead to an increased uptake of AA by the liver with enhanced catabolism to produce energy with a consequent conversion of amino groups to urea N.

The MN as a percentage of N intake can be associated with ruminal N balance because both give information about the N status of the rumen (Detmann et al., 2014a). In this sense, estimates of MN greater than or close to N intake indicate a severe dietary deficiency of N and a dependency on N recycling to sustain microbial growth in the rumen. Under a deficiency of N, there would be a net gain of N in the rumen due to significant assimilation of recycled N into MN. This is similarly one of the main causes of negative ruminal N balance, as highlighted by Detmann et al. (2014a).

Across levels of RUP supplementation, forage N intake (23.9 ± 2.8 g) and supplemental N from RDP (62.0 ± 1.2 g) were similar. The linear increase in abomasal N flow and linear decline in ruminal N balance in response to increasing RUP can be attributed mainly to the increases in MN and with only a minor effect of increased abomasal ammonia flow. The increment in MN in response to RUP supplementation might be related to the increase in urea

N recycling stimulating microbial growth, although MNU was not statistically increased by RUP supplementation.

Firkins and Reynolds (2005) reported from multicatheterization studies in cattle that net urea N release by the liver accounts for 65% of increases in N intake. In the current research, synthesis of urea N corresponded to 98% of N intake, averaging 112% for the unsupplemented heifers and 92% for the supplemented heifers ($P < 0.02$; data not shown). According to Marini and Van Amburgh (2003), the ratio of urea synthesis to N intake can vary considerably depending on the N and energy content of the diet and physiological state of the animal, with greater values observed in animals fed near maintenance, as for the heifers in this work.

Alternatively, UER can be expressed as a percentage of the N apparently digested, which was 580% for the control and 115% for supplemented treatments ($P < 0.001$; data not shown). Lapierre and Lobley (2001) reported that this percentage ranged from 43 to 123%. These authors stated that, for UER to be near or in excess of apparently digested N, part of absorbed ammonia or AA or both must arise from endogenous N inputs, which primarily would be recycled urea N. From these suggestions, we hypothesize that the large ratio of UER to digestible N for control heifers may be due to the contribution of N from skeletal muscle protein mobilization, as reflected by urinary 3-methylhistidine:creatinine ratio. Thus, the net N available to the animal and intestinal AA absorption can exceed the apparent digestible N (Lapierre and Lobley, 2001), in part due to N from AA that are released from tissue protein on a net basis during submaintenance intake (Ferrell et al., 1999). It should also be noted that apparent digestible N does not reflect MP, because apparent digestible N underestimates N absorption from the gut (Lapierre and Lobley, 2001).

For the unsupplemented heifers in our trial, only 28% of urinary N excretion was urea N, whereas for the supplemented heifers 67% or urinary N was from urea. This observation is in agreement with others (Archibeque et al., 2001; Marini and Van Amburgh, 2003;

Wickersham et al., 2008) reporting greater proportions of urinary N from urea N as dietary protein increased. In our study, the urinary ammonia-N remained a constant portion of total urinary N among treatments (average 5.7%).

In response to supplemental RUP, UER increased linearly, but urinary excretion of urea did not statistically increase, resulting in the tendency for increased GER. The urea N synthesized in the liver can be excreted in the urine or be recycled to the GIT. Urea that is produced and subsequently enters the GIT can serve a productive function if it is incorporated into MNU. Kennedy and Milligan (1980) postulated that the UER, PUN concentration, RAN concentration, and ruminal OM digestion typically are positively related to each other, whereas GER is negatively related to RAN concentration, and positively related to PUN concentration. If recycled urea N generated from tissue mobilization comprises a large proportion of the total supply of ruminally available N, the long-term protein requirements of the animal may be underestimated, resulting in decreased production if the predicted, but suboptimal, concentrations of protein are fed (NRC, 1985). Thus, Atkinson et al. (2007) hypothesized that to counterbalance this effect, provision of RDP along with additional RUP supplementation will not only provide additional MP for tissue deposition, but a portion of that RUP will serve as a source of N for endogenous recycling. The prolonged time required for absorption and deamination of AA contained in supplemental RUP may support a more stable ruminal environment by providing the animal with a sustained source of recyclable N (Bohnert et al., 2002).

The hypothesis of Atkinson et al. (2007) along with the data from the current study would support that RUP supplementation provided a greater RAN concentration at times distant from supplementation. Such a pattern can be supported by the increased RAN caused by RUP provision just before and 10 h after RDP supplementation. In turn, the greater GER may have contributed to the stimulated ruminal microbial growth. This result suggests that

supplemental RUP stimulated urea N recycling and sustained RAN. Thus, in agreement with Atkinson et al. (2007), we can suggest that prediction equations for endogenous urea N recycling should include a protein degradability component to better predict overall ruminal N status as well as RDP requirements.

In agreement with other studies that have evaluated urea recycling (Marini and Van Amburgh, 2003; Wickersham et al., 2008; 2009b), UUA:GER was greater for treatments with low protein than with greater protein intake. These results demonstrated the importance of urea N salvage to support anabolic purposes in ruminants fed low-quality diets, because GER:UER was greater for control heifers (85%) than for those that were supplemented (58%). Reynolds and Kristensen (2008) stated that UUA:UER was typically greater in cattle fed low-protein diets (<12%) than in those fed high-protein diets, but other dietary factors also may influence the fate of urea N in the GIT. According to Lapierre and Lobley (2001), part of the reason for the efficient reuse of urea N for anabolism in cattle fed low-protein diets is because the urea N atoms can return to the GIT more than once, which increases the overall probability of sequestration towards an anabolic fate. This multiple-recycling process can result in improvements of 22% in UUA:GER in cattle fed grass (Lapierre and Lobley, 2001).

The greater ROC:GER observed for supplemented treatments than for control (52 vs. 34%, respectively) supports that protein supplementation stimulates NH_3 absortion from the rumen, which, at first glance, seems a futile and costly cycle of urea synthesis. However, such a mechanism to provide RAN for microbial synthesis may be an adaptative tool to retain N within the system (Marini and Van Amburgh, 2003). If RAN is maintained at a greater concentration, then more ammonia would be returned to the ornithine cycle for re-synthesis of urea, resulting in greater ROC (Holder et al., 2015). Moreover, urea may be hydrolyzed by ruminal microbes at the epithelial border, and the resulting RAN may never enter the rumen pool, yet it would be accounted as ROC (Kristensen et al., 2010).

The UFE observed in our study (2 to 7% of GER) was similar to others trials evaluating cattle receiving protein supplementation (Wickersham et al., 2009a; Bailey et al., 2012ab), where UFE:GER ranged from 1 to 12%. This loss of urea N in feces is influenced by the supply of fermentable energy to the lower GIT, and the evidence so far would suggest that hindgut usage of urea N involves only catabolic fates, at least in terms of AA supply to the animal (Lapierre and Lobley, 2001). Much of UFE would represent undigestible N present in ruminally synthesized microbial cell mass.

The enhancement of ruminal digestion with supplementation suggested an improved environment for the rumen microbes, which tended to increase the amount (g/d) of GER and UUA. The UUA is calculated by subtracting ROC and UFE from GER (Lobley et al., 2000). The UUA would predominantly represent recycled urea N that was incorporated into microbial AA, intestinally absorbed, and used for net deposition of body protein (Wickersham et al., 2008). Although UUA tended to increase with supplementation, UUA:GER decreased with supplementation as a result of the increase in the proportion of urea N directed to ROC, likely as ammonia absorbed across the ruminal wall.

In our study, UUA (17.7 g N/d) was greater than MNU (4.8 g N/d). Similar observations were made by Marini and Van Amburgh (2003). Theoretically, UUA should be largely dependent on microbial synthesis of AA that could subsequently be used for protein deposition (Wickersham et al., 2008). According to Wickersham et al. (2009b), an observation of MNU less than UUA should suggest that UUA is overestimated, either due to underestimation of UFE or urinary excretion of label in unmeasured forms (e.g., other than urea N), which would be calculated as part of UUA; the methodology might be improved by measuring total ^{15}N in urinary excretion, which would prevent non-urea losses from being accounted for as anabolic use (Wickersham et al., 2008; 2009b). The increase in UUA with RUP provision (from RUP0 to RUP150) was from 15.9 to 24.7 g/d, which represented 19 and 25% of the N intake,

respectively. In contrast, MNU was not affected by RUP supplementation and represented 5% of N intake. Thus, with increasing delivery of supplemental RUP, a greater proportion of N intake was used for urea N synthesis and subsequently destined for anabolic purposes, which could have contributed to a part of the increase in N retention when RUP was supplied.

Urea N recycling to the rumen is an important mechanism for ruminants to survive. Benefits from urea recycling occur when PUN is converted to RAN and used by microbes. To conserve N in times of a shortfall, ruminal microrganisms use the recycled urea N. The microbial use of recycled urea N observed in our research agrees with observations of Marini and Van Amburgh (2003) and Bailey et al. (2012b), who found that protein supplementation decreased MNU as a proportion of GER. According to Bailey et al. (2012b), this probably occurred because microbes had access to a greater supply of N provided directly from the supplemental N, which competes with ammonia generated from recycled urea for use by the microbes. The effect of supplemental N on MNU is related to the effects of supplements on urea recycling to the rumen and on availability of N directly from the RDP source. However, contrary to our hypothesis, we did not observe significant effects of RUP supplementation on MNU. It would be expected that RUP could improve MNU because recycling is positively correlated with RUP provision. However, the forage utilized here presented a very low energy content. The increase in dietary TDN with RUP supplementation did not contribute to ruminal fermentation. Therefore, any positive association between RUP provision and MNU may be limited by the low availability of energy for rumen fermentation and consequentely for microbial assimilation of recycled N.

If all GER entered the rumen, it would be expected that the relationship between GER and GER plus dietary RDP would be similar to the percentage of MN that was synthesized from recycled urea (Brake et al., 2010). In our study, GER as a percentage of GER plus measured RDP (data not presented tabularly) was greater ($P < 0.001$) for the control (65%)

than for the supplemented treatments (41%). For the control, the observed MNU:MN (22%) were approximately one-third of the values for GER:(GER + RDP), suggesting that about one-third of GER was recycled to the rumen and completely mixed with the RAN pool. For the supplemented treatments, MNU:MN (10%) was approximately one-fourth of the GER:(GER + RDP). These differences between control and supplemented treatments for the relationships between GER:(GER + RDP) and MNU:MN may be due to urea N that was recycled to the rumen but did not mix with the RAN pool before absorption (accounted for as ROC by the methodology and expected to be greater for the supplemented treatments than for control) as well as to urea recycled to the postruminal GIT (Brake et al., 2010).

The renal excretion of urea N increases when N intake is sufficient, but it is reduced when the availability of dietary N is limited (Harmeyer and Martens, 1980). In this sense, the movement of urea into the GIT is in competition with its movement into urine, and changes in renal clearance thus impact recycling to the GIT (Bailey et al., 2012a). In the present study, protein supplementation raised the glomerular filtration rate of PUN, renal clearance of urea N, and the proportion of urea filtered by the kidneys. These responses were expected because PUN increased with supplementation. The amount of urea excreted by the kidneys may be influenced by three factors: changes of PUN and corresponding changes of filtered urea loads; changes of the glomerular filtration rates; and changes of tubular resorption of urea (Harmeyer and Martens, 1980). Similar to our study, Marini and Van Amburgh (2003) reported that, in heifers fed a low-protein diet (9.1% of CP), 47% of urea filtered by the kidney was reabsorbed and that reabsorption decreased to 8% as dietary CP content increased to 21.4%. In our study, the reabsorption of urea filtered by the kidney in supplemented heifers averaged 69%, whereas 88% was reabsorbed by heifers receiving only forage, representing a significant increase.

The urea turnover time for the control treatment was similar ($P \geq 0.13$) to that for supplemented treatments (averaged 530 min). Marini and Van Amburgh (2003) observed that heifers fed low-N diets had a smaller urea pool turning over faster than when the heifers were fed high-N diets. In our study, the increased UER in response to protein supplementation were matched in magnitude by increases in the urea pool size.

As in other studies with cattle fed low-quality grass, protein supplementation did not affect blood glucose (Bailey et al., 2012ab; Rufino, 2015) or trygliceride (Rufino, 2015). The tendency for greater plasma β-hydroxybutyrate concentration for control than for supplementation may reflect greater body fat mobilization, which would match the nutritional status.

CONCLUSIONS

Protein supplementation improved nitrogen utilization in cattle fed low-quality tropical forage. Unsupplemented heifers had greater muscle protein degradation, which released AA for hepatic urea production in support of urea N recycling. The RUP supplementation not only provided additional MP for tissue deposition, but a portion of the RUP served as a source of N for endogenous recycling and promoted increases in ruminal microbial protein synthesis.

LITERATURE CITED

Allen, M. S., and J. A. V. Linton. 2007. In vivo methods to measure digestibility and digestion kinetics of feed fractions in the rumen. In: Proc. Simpósio Int. Avanços Téc. Pesq. Nutrição Rumin. Univ. de São Paulo, São Paulo, Brazil. p. 72–89.

Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2001. Urea flux in beef steers: Effects of forage species and nitrogen fertilization. J. Anim. Sci. 79:1937–1943.

Atkinson, R. L., C. D. Toone, D. L. Harmon, and P. A. Ludden. 2007. Effects of supplemental ruminally degradable protein versus increasing amounts of supplemental ruminally undegradable protein on nitrogen retention, apparent digestibility and nutrient flux across visceral tissues in lambs fed low-quality forage. J. Anim. Sci. 85:3331–3339.

Ballard, F. J., O. H. Filsell, and I. G. Jarrett. 1976. Amino acid uptake and output by the sheep hind limb. Metabolis. 25:415-418.

Bailey, E. A., E. C. Titgemeyer, K. C. Olson, D. W. Brake, M. L. Jones, and D. E. Anderson. 2012a. Effects of supplemental energy and protein on forage digestion and urea kinetics in growing beef cattle. J. Anim. Sci. 90:3492–3504.

Bailey, E. A., E. C. Titgemeyer, K. C. Olson, D. W. Brake, M. L. Jones, and D. E. Anderson. 2012b. Effects of ruminal casein and glucose on forage digestion and urea kinetics in beef cattle. J. Anim. Sci. 90: 3505-3514.

Batista, E. D. 2012. Ruminal and/or abomasal nitrogen supplementation in cattle fed high-quality tropical forage. M.S. Thesis. Univ. Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Bohnert, D. W., C. S. Schauer, S. J. Falck, and T. DelCurto. 2002. Inflence of rumen protein degradability and supplementation frequency on steers consuming low-quality forage: II. Ruminal fermentation characteristics. J. Anim. Sci. 80:2978–2988.

Brake, D. W., E. C. Titgemeyer, M. L. Jones, and D. E. Anderson. 2010. Effect of nitrogen supplementation on urea kinetics and microbial use of recycled urea in steers consuming corn-based diets. J. Anim. Sci. 88:2729–2740.

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

Cecava, M. J., N. R. Merchen, L. C. Gay, and L. L. Berger. 1990. Composition of ruminal bacteria harvest from steers as inflenced by dietary energy level, feeding frequency, and isolation techniques. J. Dairy Sci. 73:2480–2488.

Chasson, A. L., H. J. Grady, and M. A. Stanley. 1961. Determination of creatinine by means of automatic chemical analysis. Am. J. Clin. Pathol. 35:83–88.

Costa, V. A. C., E. Detmann, M. F. Paulino, S. C. Valadares Filho, L. T. Henriques, and I. P. C. Carvalho. 2011. Total and partial digestibility and nitrogen balance in grazing cattle supplemented with non-protein and, or true protein nitrogen during the rainy season. Rev. Bras. Zootec. 40:2815–2826.

Detmann, E., M. F. Paulino, H. C. Mantovani, S. C. Valadares Filho, C. B. Sampaio, M. A. Souza, I. Lazzarini, and K. S. C. Detmann. 2009. Parameterization of ruminal fibre degradation in low-quality tropical forage using Michaelis-Menten kinetics. Livest. Sci. 126:136 –146.

Detmann, E., M. A. Souza, S. C. Valadares Filho, A. C. Queiroz, T. T. Berchielli, E. O. S. Saliba, L. S. Cabral, D. S. Pina, M. M. Ladeira, and J. A. G. Azevedo. 2012. Métodos para análise de alimentos. (In Portuguese) Suprema, Visconde do Rio Branco, Minas Gerais, Brazil.

Detmann, E., E. E. L. Valente, E. D. Batista, and P. Huhtanen. 2014a. An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. Livest. Sci. 162:141–153.

Detmann, E., S. C. Valadares Filho, M. F. Paulino, and P. Huhtanen. 2014b. Nutritional aspects applied to grazing cattle in tropics: a review based on Brazilian results. Semin-Cienc. Agrar. 35:2829-2854.

Egan, A. R. 1965. The fate and effects of duodenally infused casein and urea nitrogen in sheep fed on a low-protein roughage. Aust. J. Agric. Res. 16:169–177.

Ferrell, C. L., K. K. Kreikemeier, and H. C. Freetly. 1999. The effect of supplemental energy, nitrogen, and protein on feed intake, digestibility, and nitrogen flux across the gut and liver in sheep fed low-quality forage. J. Anim. Sci. 77:3353-3364.

Firkins, J. L., and C. K. Reynolds. 2005. Whole animal nitrogen balance in cattle. In: Nitrogen and Phosphorus Nutrition of Cattle and the Environment. CAB Int., Wallingford, UK. Pages 167–186.

France, J., and R. C. Siddons. 1986. Determination of digesta flow by continuous marker infusion. J. Theor. Biol. 121:105–120.

Gerrits, W. J. J., G. H. Tolman, J. W. Schrama, S. Tamminga, M. W. Bosch, and M. W. A. Verstegen. 1996. Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. J. Anim. Sci. 74:2129–2139.

Harmeyer, J., and H. Martens. 1980. Aspects of urea metabolism in ruminants with reference to the goat. J. Dairy Sci. 63:1707–1728.

Holder, V. B., J. M. Tricarico, D. H. Kim, N. B. Kristensen, and D. L. Harmon. 2015. The effects of degradable nitrogen level and slow release urea on nitrogen balance and urea kinetics in Holstein steers. Anim. Feed Sci. Technol. 200:57–65.

Kennedy, P. M., and L. P. Milligan. 1980. The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants: A review. Can. J. Anim. Sci. 60:205-221.

Kristensen, N. B., A. C. Storm, and M. Larsen. 2010. Effect of dietary nitrogen content and intravenous urea infusion on ruminal and portal-drained visceral extraction of arterial urea in lactating Holstein cows. J. Dairy Sci. 93:2670–2683.

Lapierre, H., and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84(Suppl. E):E223–E236.

Lazzarini, I., E. Detmann, C. B. Sampaio, M. F. Paulino, S. C. Valadares Filho, M. A. Souza, and F. A. Oliveira. 2009. Intake and digestibility in cattle fed low-quality tropical forage and supplemented with nitrogenous compounds. Rev. Bras. Zootec. 38:2021–2030.

Lazzarini, I., E. Detmann, M. F. Paulino, S. C. Valadares Filho, F. A. Oliveira, P. T. Silva, and W. L. S. Reis. 2013. Nutritional performance of cattle grazing on low-quality tropical forage supplemented with nitrogenous compounds and/or starch. Rev. Bras. Zootec. 42:664-674.

Lobley, G. E., D. M. Bremner, and G. Zuur. 2000. Effects of diet quality on urea fates in sheep assessed by a refined, non-invasive [$^{15}N^{15}N$]-urea kinetics. Br. J. Nutr. 84:459–468.

Marini, J. C., and M. E. Van Amburgh. 2003. Nitrogen metabolism and recycling in Holstein heifers. J. Anim. Sci. 81:545–552.

Marsh, W. H., B. Fingerhut, and H. Miller. 1965. Automated and manual direct methods for the determination of blood urea. Clin. Chem. 11:624–627.

NRC. 1985. Ruminant Nitrogen Usage. Natl. Acad. Press, Washington, DC.

Poppi, D. P., and S. R. McLennan. 1995. Protein and energy utilization by ruminants at pasture. J. Anim. Sci. 73:278–290.

Preston, R. L., and S. W. Kock. 1973. In vivo prediction of body composition in cattle from urea space measurements. Proc. Soc. Exp. Biol. Med. 143:1057–1061.

Reynal, S. M., G. A. Broderick, and C. Bearzi. 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. J. Dairy Sci. 88:4065–4082.

Reynolds, C. K., and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis. J. Anim. Sci. 86: E293–E305.

Richards, C. J., A. F. Branco, D. W. Bohnert, G. B. Huntington, M. Macari, and D. L. Harmon. 2002. Intestinal starch disappearance increased in steers abomasally infused with starch and protein. J. Anim. Sci. 80:3361–3368.

Rotta, P. P., S. C. Valadares Filho, E. Detmann, L. F. Costa e Silva, M. F. Paulino, M. I. Marcondes, A. A. G. Lobo, and F. A. C. Villadiego. 2014. Digesta sampling sites and marker methods for estimation of ruminal outflow in bulls fed different proportions of corn silage or sugarcane. J. Anim. Sci. 92:2996-3006.

Rufino, L. M. A. 2011. Ruminal and/or abomasal nitrogenous supplementation in cattle fed tropical forage. M.S. Thesis. Univ. Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Rufino, L. M. A. 2015. Nutritional performance and metabolic characteristics in cattle fed low-quality tropical forage in response to infrequent supplementation with nitrogenous compounds. PhD Diss. Univ. Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

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

Sampaio, C. B., E. Detmann, M. F. Paulino, S. C. Valadares Filho, M. A. Souza, I. Lazzarini, P. V. Paulino, and A. C. Queiroz. 2010. Intake and digestibility in cattle fed low-quality tropical forage and supplemented with nitrogenous compounds. Trop. Anim. Health Prod. 42:1471–1479.

Siegfried, R., H. Ruckemann, and G. Stumpf. 1984. Method for the determination of organic acids in silage by high performance liquid chromatography. Landwirt Forsch. 37:298–304.

Spanghero, M. and Z. M. Kowalski. 1997. Critical analysis of N balance experiments with lactating cows. Livest. Prod. Sci. 52: 113–122.

Valadares Filho, S. C., M. I. Marcondes, M. L.Chizzotti, and P. V. R. Paulino. 2010. Nutrients requirements of Zebu beef cattle BR-CORTE. 2nd ed. Viçosa: DZO-UFV. Viçosa, Minas Gerais, Brazil.

Valente, T. N. P., E. Detmann, A. C. Queiroz, S. C. Valdares Filho, D. I. Gomes, and J. F. Figueiras. 2011. Evaluation of ruminal degradation profiles of forages using bags made from different textiles. Rev. Bras. Zootec. 40:2565–2573.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. 2nd ed. Cornell Univ. Press, Ithaca, NY.

Wickersham, T. A., R. C. Cochran, E. C. Titgemeyer, C. G. Farmer, E. A. Klevesahl, J. I. Arroquy, D. E. Johnson, and D. P. Gnad. 2004. Effect of postruminal protein supply on the response to ruminal protein supplementation in beef steers fed a low-quality grass hay. Anim. Feed Sci. Technol. 115:19–36.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, E. E. Wickersham, and D. P. Gnad. 2008. Effect of rumen-degradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. J. Anim. Sci. 86:3079–3088.

Wickersham, T. A., E. C. Titgemeyer, and R. C. Cochran. 2009a. Methodology for concurrent determination of urea kinetics and the capture of recycled urea nitrogen by ruminal microbes in cattle. Animal 3:372–379.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, and E. E. Wickersham. 2009b. Effect of undegradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. Br. J. Nutr. 101:225–232.

Table 1.1 Composition of forage, ruminal protein supplement, and casein

Item	Hay[1]	Ruminal supplement[2]	Casein
DM, % (as fed)	87.6 ± 0.4	90.4	89.1
	——————————— % of DM ———————————		
OM	96.2 ± 0.1	97.9	97.6
CP	5.0 ± 0.1	119.8	90.0
NDFap[3]	80.1 ± 0.3	–	–
Indigestible NDF	44.4 ± 0.4	–	–
ADL	8.1 ± 0.2	–	–

[1]Signal grass (*Brachiaria decumbens* Stapf.).

[2]Composed of casein, urea, and ammonium sulfate in a ratio of 53:9:1.

[3]NDF corrected for ash and protein.

Table 1.2 Effects of RDP supplementation and provision of RUP on voluntary intake and digestibility in beef heifers consuming low-quality signal grass hay

	Treatment[1]						Contrast *P*-value[3]		
Item	Control	RUP0	RUP50	RUP100	RUP150	SEM[2]	C vs. S	Linear	Quadratic
n	5	4	5	4	5				
Intake, kg/d									
Total DM	3.13	3.25	3.37	3.52	3.86	0.35	0.30	0.18	0.70
Forage DM	3.13	2.93	2.94	3.05	3.31	0.35	0.82	0.38	0.70
Total OM	3.01	3.13	3.21	3.40	3.72	0.34	0.29	0.18	0.70
Forage OM	3.01	2.82	2.83	2.93	3.19	0.33	0.82	0.39	0.69
Digestible OM	1.10	1.46	1.48	1.57	1.78	0.11	<0.001	0.03	0.34
NDFap[4]	2.53	2.34	2.39	2.45	2.67	0.28	0.80	0.37	0.75
Digestible NDFap	1.08	1.12	1.11	1.11	1.24	0.12	0.56	0.44	0.61
Indigestible NDF	1.33	1.18	1.25	1.27	1.36	0.16	0.69	0.39	0.93
CP	0.15	0.53	0.60	0.67	0.75	0.02	<0.001	<0.001	0.76
TDN	1.10	1.57	1.58	1.68	1.89	0.11	<0.001	0.03	0.35
Total tract digestibility[5], %									
OM	36.8	47.5	46.3	47.5	48.2	2.1	<0.001	0.60	0.54
NDFap	43.3	47.2	46.7	46.6	46.7	1.6	0.03	0.78	0.81
CP	24.3	80.6	79.3	81.1	81.4	2.8	<0.001	0.65	0.72
TDN	35.7	49.2	47.8	49.3	49.3	2.3	<0.001	0.81	0.65

Apparent ruminal digestibility,[6] % of intake									
OM	24.7	37.5	33.0	33.8	31.2	3.1	<0.001	0.09	0.67
NDFap	41.2	46.4	44.9	46.2	46.1	1.9	0.008	0.95	0.62
CP	-68.1	48.1	35.0	24.9	7.5	10.1	<0.001	<0.01	0.82
Postruminal digestibility,[7] % of abomasal flow									
OM	15.7	15.7	20.2	22.9	26.0	3.7	0.09	0.02	0.80
NDFap	3.1	1.4	2.7	1.2	0.9	3.4	0.63	0.81	0.79
CP	51.8	61.2	69.7	76.6	79.3	3.3	<0.001	<0.001	0.28

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] For $n = 4$.

[3] C vs. S = Control vs. average of all supplements. Linear and quadratic represent effects of RUP.

[4] NDFap = NDF corrected for ash and protein.

[5] Calculated using intakes that included OM and CP from the diet and from ruminal and abomasal infusions.

[6] Calculated using intakes that did not include OM and CP from abomasal infusions and using abomasal flows from which OM and CP from abomasal infusions had been subtracted.

[7] Calculated using abomasal flows that included OM and CP from abomasal infusions.

Table 1.3 Effects of RDP supplementation and provision of RUP on ruminal contents and intake, passage, and digestion rates of potentially digestible NDF (pdNDF) and indigestible NDF in beef heifers consuming low-quality signal grass hay

	Treatment[1]						Contrast *P*-value[3]		
Item	Control	RUP0	RUP50	RUP100	RUP150	SEM[2]	C vs. S	Linear	Quadratic
n	5	4	5	4	5				
Ruminal contents, g/kg BW									
pdNDF	6.9	6.4	6.2	5.4	5.5	0.9	0.24	0.34	0.78
Indigestible NDF	28.8	24.6	26.8	28.1	27.8	3.2	0.49	0.37	0.64
Rates, %/h									
pdNDF									
Intake	3.06	3.08	3.21	4.01	4.03	0.44	0.22	0.05	0.89
Passage	0.40	0.20	0.21	0.25	0.26	0.01	0.07	0.48	0.92
Digestion	2.66	2.94	3.00	3.76	3.77	0.41	0.09	0.06	0.95
Indigestible NDF									
Passage	0.80	0.82	0.78	0.77	0.83	0.10	0.80	0.96	0.53

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] For $n = 4$.

[3] C vs. S = Control vs. average of all supplements. Linear and quadratic represent effects of RUP.

Table 1.4 Effects of RDP supplementation and provision of RUP on ruminal fermentation characteristics in beef heifers consuming low-quality signal grass hay

	Treatment[1]						Contrast *P*-value[3]		
Item	Control	RUP0	RUP50	RUP100	RUP150	SEM[2]	C vs. S	Linear	Quadratic
n	5	4	5	4	5				
VFA, m*M*	63.9	65.4	68.8	76.0	66.7	9.49	0.59	0.79	0.49
VFA, mol/100 mol									
Acetate	73.5	68.2	67.7	68.3	67.5	1.29	<0.001	0.76	0.92
Propionate	16.2	16.5	16.4	16.5	16.9	0.34	0.30	0.35	0.48
Butyrate	6.0	6.3	6.6	6.2	6.3	0.30	0.30	0.67	0.81
Isobutyrate	1.5	2.8	2.8	2.7	2.7	0.42	0.01	0.93	0.99
Valerate	1.4	2.5	2.6	2.4	2.6	0.40	0.01	0.95	0.87
Isovalerate	1.4	3.7	3.9	3.8	4.0	0.58	<0.001	0.74	0.94
Acetate:propionate	4.5	4.1	4.1	4.1	4.0	0.13	0.001	0.34	0.51
pH	6.72	6.85	6.66	6.68	6.73	0.13	0.90	0.51	0.29

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] For n = 4.

[3] C vs. S = Control vs. average of all supplements. Linear and quadratic represent effects of RUP.

Table 1.5 Effects of RDP supplementation and provision of RUP on ruminal ammnonia-N (mg/dL) concentration in beef heifers consuming low-quality signal grass hay

Hour after feeding and ruminal supplementation	Treatment[1,2]					*P*-value[3]
	Control	RUP0	RUP50	RUP100	RUP150	
0	3.8 (1.6) [c]	10.3 (1.9) [b]	13.0 (1.6) [ab]	13.4 (2.2) [ab]	15.2 (1.6) [a]	<0.001
2	5.0 (4.8) [b]	35.8 (5.3) [a]	28.0 (4.8) [a]	20.4 (6.1) [a]	30.2 (4.8) [a]	<0.001
4	5.8 (4.0) [b]	30.8 (4.5) [a]	36.0 (4.0) [a]	35.6 (5.2) [a]	34.1 (4.0) [a]	<0.001
6	5.4 (3.2) [c]	21.5 (3.6) [b]	22.0 (3.2) [b]	32.4 (3.6) [a]	28.6 (3.2) [ab]	<0.001
8	3.4 (3.0) [b]	15.6 (5.4) [a]	17.4 (3.0) [a]	17.5 (4.0) [a]	23.5 (3.0) [a]	<0.01
10	3.0 (2.4) [c]	9.5 (2.6) [b]	13.7 (2.3) [ab]	14.2 (3.0) [ab]	19.0 (2.3) [a]	<0.01
P-value[4]	0.96	<0.001	<0.001	<0.001	<0.001	

[a–c] Means in the same row with different superscripts differ ($P < 0.05$).

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] Means (SEM); SEM were obtained using a heterogeneous compound symmetry matrix.

[3] Differences between treatments within each sampling time.

[4] Differences between sampling times within treatment.

Table 1.6 Effects of RDP supplementation and provision of RUP on N intake, excretion, digestion, retention, ruminal balance, and abomasal flows in beef heifers consuming low-quality signal grass hay

	Treatment[1]						Contrast *P*-value[3]		
Item	Control	RUP0	RUP50	RUP100	RUP150	SEM[2]	C vs. S	Linear	Quadratic
n	5	4	5	4	5				
N intake, g/d									
Forage	25.8	23.2	23.3	23.1	25.9	2.8	0.91	0.51	0.60
Ruminal supplement	0	62.3	61.5	62.4	61.7	1.0	–	–	–
Abomasal supplement	0	0	10.8	22.0	32.2	1.1	–	–	–
Total	25.8	85.3	95.6	107.6	119.9	3.9	<0.001	<0.001	0.76
N excretion									
Fecal N, g/d	19.2	16.8	19.9	20.5	22.3	2.2	0.70	0.04	0.71
Urine N, g/d	14.9	61.1	57.5	57.4	65.6	4.2	<0.001	0.45	0.14
Urea N, g/d	4.2	38.5	39.3	39.1	44.7	3.8	<0.001	0.27	0.52
% of urine N	28.2	63.3	68.4	69.0	68.2	5.4	<0.001	0.52	0.58
Ammonia-N, g/d	0.9	3.6	3.3	2.8	3.7	0.4	<0.001	0.96	0.20
% of urine N	5.9	5.8	6.0	5.2	5.7	0.9	0.79	0.73	0.80
N digested, g/d	6.6	67.9	75.7	86.9	97.5	2.7	<0.001	<0.001	0.54
N retention									
g/d	-8.3	6.9	18.2	29.4	31.9	4.7	<0.001	<0.001	0.33
% of N intake	-38	8	19	27	27	9.0	<0.001	0.08	0.45
% of digested N	-235	10	24	34	32	60	<0.001	0.77	0.89
Ruminal N balance, g/d	-15.1	40.9	29.7	20.3	5.9	6.8	<0.001	<0.001	0.80

Abomasal N flow									
Total N[4], g/d	40.8	44.6	55.2	65.1	81.7	8.3	0.02	0.002	0.69
Microbial N, g/d	25.3	38.2	43.1	51.5	53.9	5.4	<0.001	0.01	0.77
% of total N intake	98.7	45.5	45.1	47.2	44.4	4.6	<0.001	0.93	0.76
Ammonia-N, g N/d	0.9	5.0	5.2	6.4	7.4	0.9	<0.001	0.03	0.68
Microbial efficiency									
g CP/kg TDN	144	167	176	194	185	16	0.001	0.07	0.22
g CP/kg RFOM[5]	150	189	207	224	218	30	0.01	0.25	0.54

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] For $n = 4$.

[3] C vs. S = Control vs. average of all supplements. Linear and quadratic represent effects of RUP.

[4] Total N flow was calculated by deleting N provided by abomasal infusion from the measured N flow.

[5] RFOM = rumen fermented OM.

Table 1.7 Effects of RDP supplementation and provision of RUP on urea kinetics and ruminal microbial capture of urea N recycled in beef heifers consuming low-quality signal grass hay

	Treatment[1]						Contrast *P*-value[3]		
Item	Control	RUP0	RUP50	RUP100	RUP150	SEM[2]	C vs. S	Linear	Quadratic
n	5	4	5	4	5				
Urea N kinetics, g N/d									
Entry rate (UER)	28.8	86.9	89.7	94.4	104.6	6.0	<0.001	0.02	0.45
GIT[4] entry rate (GER)	24.7	49.8	51.8	49.8	63.0	4.7	<0.001	0.07	0.21
Returned to ornithine cycle (ROC)	11.4	31.6	30.7	31.0	35.3	3.0	<0.001	0.34	0.33
Utilized for anabolism (UUA)	12.9	15.9	18.6	16.3	24.7	3.3	0.07	0.08	0.34
Fecal excretion (UFE)	0.4	3.2	2.6	2.8	3.0	0.5	<0.001	0.91	0.38
Fractional urea kinetics									
UUE[5]:UER	0.15	0.42	0.42	0.46	0.40	0.03	<0.001	0.81	0.31
GER:UER	0.85	0.58	0.58	0.54	0.60	0.03	<0.001	0.81	0.31
ROC:UER	0.39	0.36	0.34	0.33	0.34	0.02	0.02	0.37	0.49
ROC:GER	0.46	0.62	0.59	0.63	0.56	0.04	0.006	0.43	0.64
UUA:GER	0.52	0.32	0.36	0.31	0.39	0.05	0.002	0.36	0.71
UFE:GER	0.02	0.07	0.05	0.06	0.05	0.01	0.001	0.32	0.73
Ruminal microbial capture of recycled urea N									
g N/d	5.5	4.0	4.7	4.3	5.6	1.0	0.36	0.22	0.73
% of total microbial N	21.6	10.3	10.7	8.5	10.0	1.4	<0.001	0.58	0.67
% of urea production	18.9	4.8	5.2	4.8	5.3	1.4	<0.001	0.84	0.96
% of GER	22.1	8.1	9.0	8.9	8.8	1.8	<0.001	0.77	0.78

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] For $n = 4$.

[3] C vs. S = Control vs. average of all supplements. Linear and quadratic represent effects of RUP.

[4] GIT = gastrointestinal tract.

[5] UUE = Urinary urea excretion. Data for UUE are presented in Table 1.6.

Table 1.8 Effects of RDP supplementation and provision of RUP on plasma metabolites, the ratio of urinary 3-methylhistidine (3MH) to creatinine, plasma urea N filtration, renal clearance, and urea pool and turnover in beef heifers consuming low-quality signal-grass hay

	Treatment[1]						Contrast *P*-value[3]		
Item	Control	RUP0	RUP50	RUP100	RUP150	SEM[2]	C vs. S	Linear	Quadratic
n	5	4	5	4	5				
Plasma, mg/dL									
Urea N	6.5	25.5	25.6	25.7	27.0	1.7	<0.001	0.51	0.72
Glucose	56.8	61.6	59.6	61.4	57.1	2.5	0.17	0.22	0.58
Triglycerides	14.6	14.2	15.2	16.9	16.2	2.6	0.57	0.32	0.61
β-hydroxybutyrate	2.8	2.6	2.2	2.6	2.5	0.2	0.10	0.82	0.25
Urinary 3MH:creatinine, mg/g	45.9	16.5	20.6	18.0	22.3	5.1	<0.001	0.49	0.99
Plasma urea N filtration									
g/d	36	139	136	128	132	12	<0.001	0.36	0.94
% reabsorption	88	70	70	69	66	4	<0.001	0.40	0.66
Renal creatinine clearance, L/d	440	429	453	424	424	47	0.84	0.79	0.76
Renal urea clearance, L/d	64	156	155	152	169	17	<0.001	0.36	0.94
% of filtered	15	37	36	36	42	6	<0.001	0.52	0.48
Urea N pool size, g N	9.0	34.6	35.0	35.5	36.9	2.1	<0.001	0.42	0.78
Urea turnover time UER[4], min	460	587	570	543	530	64	0.13	0.45	0.97

[1] Control = no supplementation; RUP0, RUP50, RUP100, and RUP150 = supplemental RDP to meet 100% of the RDP requirements plus supplemental RUP to meet 0, 50, 100, or 150% of the RUP requirement.

[2] For $n = 4$.

[3] C vs. S = Control vs. average of all supplements. Linear and quadratic represents effects of RUP.

[4] UER = urea N entry rate.

CHAPTER 2

The effect of crude protein content in the diet on urea kinetics and microbial usage of recycled urea in ruminants: A meta-analysis

E. D. Batista,*† E. Detmann,* S. C. Valadares Filho,* E. C. Titgemeyer,†

R. F. D. Valadares‡

*Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil;

†Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS; and

‡Department of Veterinary Medicine, Universidade Federal de Viçosa, Brazil

ABSTRACT

In ruminants, urea recycling is considered an evolutionary advantage. The amount of urea recycled mainly depends of the CP content in the diet and OM digested in the rumen. As recycled nitrogen (**N**) contributes to meeting microbial N requirements, accurate estimates of urea recycling can improve the understanding of efficiency of N utilization and N losses to environment. The objective of this study was to evaluate urea kinetics and microbial usage of recycled urea N in ruminants using a meta-analytical approach. Treatment mean values were compiled from 25 studies with ruminants (beef cattle, dairy cows, and sheep) which were published from 2001 to 2016, totaling 107 treatment means. The dataset was analyzed according to meta-analysis techniques using linear or non-linear mixed models, taking into account the random variations among experiments. Urea N synthesized in the liver (**UER**) and urea N recycled to the gut (**GER**) linearly increased ($P < 0.001$) as N intake (g/$BW^{0.75}$) increased, with increases corresponding to 71.5% and 35.2% of N intake, respectively. The UER was positively associated ($P < 0.05$) with dietary CP and the ratio of CP to digestible OM (**CP:DOM**). Maximum curvature analyses indicate that above 17% of CP there is a prominent increase on hepatic synthesis of urea N due to an excess of dietary N and NH_3 input. The GER:UER decreased with increasing dietary CP content ($P < 0.05$). At dietary CP ≥ 19%, the fraction of GER became constant. The fraction of UER eliminated as urinary urea N and the contribution of urea N to total urinary N were positively associated with dietary CP ($P < 0.05$), plateaued at about 17% of CP. The fractions of GER excreted in the feces and utilized for anabolism decreased, whereas the fraction of GER returned to the ornithine cycle increased with dietary CP content ($P < 0.05$). Recycled urea N assimilated by ruminal microbes (as a fraction of GER) decreased as dietary CP and CP:DOM increased ($P < 0.05$). The efficiency of microbial assimilation of recycled urea N plateaued at 194 g CP/kg DOM. The models

obtained in this study can to contribute to the knowledge on N utilization in feeding models and optimizing urea recycling, reducing N losses that contribute to air and water pollution.

Key words: mixed models, protein, recycling, ruminant, ruminal bacteria, urea

INTRODUCTION

Urea recycling to the rumen is an evolutionary advantage for ruminants because it provides a source of N for microbial protein synthesis and enhances survival (Reynolds and Kristensen, 2008). This process is called a protein regeneration cycle (Houpt, 1959), because the animal can use the AA from microbial protein synthesized from NPN sources to use for anabolic purposes. In addition, even in ruminants with high CP intake, urea recycling is important to maintain a positive N balance (Lapierre and Lobley, 2001).

Urea kinetics have been measured successfully in a range of studies on ruminants by using the relatively non-invasive technique developed in sheep (Sarraseca et al., 1998; Lobley et al., 2000). This procedure involves infusion or injection of [$^{15}N^{15}N$]-urea in the jugular vein followed by the analysis of the chemical forms of urea formed within the body and excreted in the urine (i.e., [$^{14}N^{14}N$]-, [$^{15}N^{14}N$]-, and [$^{15}N^{15}N$]-urea), and fecal ^{15}N excretion. However, this original method was unable to accurately estimate the ruminal microbial incorporation of recycled urea N. Supplemental methodologies have been described to measure the capture of recycled urea N by using bacterial and plasma urea (Marini and Van Amburgh, 2003) or bacterial and duodenal ^{15}N enrichments (Wickersham et al., 2009a).

Urea N recycling and assimilation by ruminal microbes are influenced by dietary and ruminal factors, and intakes of CP and digestible OM are the major factors (Harmeyer and Martens, 1980; Kennedy and Milligan, 1980). For that reason, a better understanding of the relationships between dietary factors and urea kinetics and microbial usage of recycled urea

would allow for a more accurate estimation of N supply and N requirements by current feeding models.

Therefore, we evaluated urea kinetics and microbial assimilation of recycled urea N by ruminants according to the protein content the diet using a meta-analytical approach.

MATERIAL AND METHODS

Data acquisition

The dataset used was collected from 25 experiments published from 2001 to 2016, including a total of 107 treatment means (Appendix). The prerequisites for a study to be included in the dataset were an evaluation of the nutritional characteristics [i.e., DMI, CP intake or N intake, OM intake and digestibility or digestible OM (**DOM**)] and an evaluation of urea kinetics using the model developed by Lobley and collaborators (2000) [i.e., urea N entry rate (**UER**, g/d), urinary urea N elimination (**UUE**, g/g UER), gastrointestinal entry rate of urea N (**GER**, g/g UER), re-entry to ornithine cycle (**ROC**, g/g GER), urea N to fecal excretion (**UFE**, g/g GER), and urea N utilized for anabolism (**UUA**, g/g GER)], and microbial incorporation of recycled urea according to methods validated by Wickersham et al. (2009a) [i.e., microbial N from recycled urea N (**MNU**, g/g GER)]. The dataset encompassed information from different animal species and categories (growing beef cattle, dairy cattle, and sheep). Information concerning N intake, UER, and GER (g/d) was scaled to $BW^{0.75}$ to allow comparations from different species and categories, considering that metabolic processes may be described as a function of metabolic BW (Brody, 1945).

It must be emphasized that we tried to collect additional information from the dataset that might be useful in describing the responses, such as ruminally degradable protein (**RDP**), ruminally undegradable protein (**RUP**), and starch concentration in the diets. Nevertheless,

most of these data were either absents or incomplete, which would compromise the inferences. Therefore, such information was not included in the dataset.

Statistical methods

The data were analyzed by meta-analysis techniques (St-Pierre, 2001; Van Houwelingen et al., 2002), using the mixed procedures of SAS (version 9.4; SAS Inst. Inc., Cary, NC) for non-linear (NLMIXED) and linear (MIXED) models (Littell et al., 2006). The random effect of the different subjects (experiments) was considered in the regression parameters for both linear and non-linear models. For the linear models, the adequacy of the models and the best covariance structures were evaluated using the corrected Akaike's information criterion (AICC). All variance components in the linear models were estimated using the restricted maximum likelihood method.

For the non-linear models, the random variance representative of the variation among experiments (subjects) was associated to each parameter of the model. When this random variance associated to the subjects was found non-significant ($P > 0.05$) for a specific parameter of the model, a new model was then adjusted, excluding that specific random effect. The dual quasi-Newton method was used as optimization technique. Additionally, the integration was performed using the adaptive Gaussian quadrature (Littell et al., 2006).

For selecting a linear or non-linear model for a specific relationship, a graphic analysis of the ordinary residuals was performed. It must be emphasized that structure of models was also determined based on biological meaning of the parameters and curve shape. The coefficients of determination (r^2/R^2) of adjusted models were calculated as the square of the correlation between predicted and observed values.

When non-linear models were adjusted, two specific evaluations were performed to improve the biological interpretation of data. For non-linear exponential models without an asymptotic value, the point of maximum curvature was evaluated. This point indicates the

value of the independent variable where there is a modification of the response pattern with a more prominent increase or decrease in the first derivative of the adjusted model. This specific point is defined as the point with the maximum distance between the adjusted model and a straight line linking the predicted response for minimum and maximum values of the independent variable. For non-linear models with an asymptotical response, it was identified the point where the response become stable as the value of the independent variable where the adjusted model enters the asymptotic confidence interval for the asymptote (Valente et al., 2011).

All statistical evaluations were considered significant at $P \leq 0.05$ as the critical (asymptotical) level for the occurrence of type I error.

RESULTS AND DISCUSSION

Description of Database

A description of the animal factors, dietary characteristics, urea kinetics, and microbial incorporation of recycled urea N collected and used in the current meta-analysis are shown in Table 2.1. There was a broad range of variation to dietary characteristics: CP in the diet ranged between 4.7 and 27.7% of DM, and ratio of CP to DOM (**CP:DOM**), an indicator of dietary protein-to-energy ratio, ranged from 84 to 428 g CP/kg DOM, respectively. These broad ranges indicate that the data set consists of low- to high-protein diets.

Initially, information regarding N intake, UER, and GER as a function of $BW^{0.75}$ was compared across species/categories to evaluate if they could be analyzed as a homogenous dataset. However, there was a significant difference between species for all these variables (data not shown). The N intake, UER, and GER in a $BW^{0.75}$ basis and UER and GER per unit of N intake were greater for sheep compared to growing beef cattle and dairy cattle ($P < 0.001$), although all these relationships were found similar for cattle categories ($P \geq 0.52$). Besides

these differences, the number of treatment means for sheep was quite low (n = 16). Therefore, accurate models could not be obtained for sheep and only the datasets from beef and dairy cattle were used in the meta-analysis.

Urea Kinetics

Urea N entry rate. Urea N synthesized in the liver was positively correlated with N intake (**NI**, g/BW$^{0.75}$; Fig. 2.1). From the linear regression between NI and UER, net urea N release by the liver accounted for 71.5% of N intake in cattle (Table 2.2). The slope estimate was close to that reported by Reynolds and Kristensen (2008), which was 72% for growing cattle and 60.2% for dairy cows. However, it must be considered that the intercept of the linear regression observed by these authors was significant, whereas in this current meta-analytical approach it was not significant (P > 0.05) and the intercept was set to zero.

According to Harmeyer and Martens (1980), daily N intake is the major factor influencing UER, because the relationship between these variables presented a correlation of 0.88, which was similar to this current study (r = 0.91, Table 2.2). These authors found that the feeding schedule (twice daily or feeding hourly), site of N supplied to the animal (oral or abomasal), and type of N fed (NPN vs. true protein) were not able to affect significantly the amount of urea synthesized in the liver. Therefore, as the source of N (ammonia or AA) would not affect UER, the form of absorbed N would not be relevant, and the same proportion of the absorbed N is converted to urea N, maintaining whole body N balance close to zero (Lapierre and Lobley, 2001). Based in studies on trans-hepatic veno-arterial differences across a range of BW and diet types (high-forage or high-concentrate), correlations between N intake and liver production of urea N varied between 0.57 (for sheep) to 0.98 (for beef and dairy cattle; Huntington and Archibeque, 1999; Lapierre and Lobley, 2001).

The UER, on a g/$BW^{0.75}$ basis, increased exponentially ($P < 0.05$) according to dietary CP content (Table 2.2 and Fig. 2.2). Considering the exponential relationship between CP and UER, the maximum curvature analyses indicate that above 17% of CP the hepatic synthesis of urea N became more prominent than below 17% of CP.

Under normal feeding situations, Parker et al. (1995) reported that ammonia (**NH_3**) absorbed into the portal vein is efficiently extracted by the liver and detoxified by conversion to urea or glutamine. These authors stated that, over a wide range of portal NH_3 concentrations, the liver is able to extract about 82% of portal NH_3 resulting in a hepatic removal that is, on average, very slightly higher (4%) than portal absorption. In this situation, arterial NH_3 concentrations are maintained constant even when portal NH_3 absorption varies threefold (Parker et al., 1995). This demonstrates the ability of NH_3 detoxification in ruminants under feeding conditions.

However, some authors have used broken-line models to describe ruminal NH_3 concentration according to dietary CP (Satter and Slyter, 1974; Detmann et al., 2009). In those approaches, there is a threshold point from which NH_3 accumulation becomes more intense, which has been attributed to the saturation of microbial NH_3 uptake. Considering the exponential relationship between UER and dietary CP, it seems to be reasonable that the maximum curvature point (17% CP) is associated with a decreased NH_3 uptake in the rumen and, consequently, a more prominent flow of blood NH_3 to the liver.

Dietary factors that influence the relative amounts of NH_3 absorbed from the gastrointestinal tract (**GIT**) include degradability of dietary N, as well as DOM (i.e., dietary carbohydrate; Parker et al., 1995). Because microbial protein synthesis in the rumen uses NH_3 and is energy-dependent, DOM can influence NH_3 absorption and, consequently, urea synthesis in the liver. Thus, we considered that dietary CP:DOM as an indicator of dietary

protein:energy might be a useful predictor of UER. However, it was not possible to adequately fit a model to describe the relationship between UER and CP:DOM ratio.

The UER expressed as a fraction of N intake (**UER:NI**, g UER/g N intake) was inversely associated with the dietary CP content and the relationship was described by a hyperbolic model (Table 2.3). In some feeding conditions, urea N synthesis in the liver can exceed N intake in ruminants. According to the adjusted model it should be necessary fed a diet with at least 7.84% of CP to obtain a UER:NI lower than 1. Nevertheless, not all the NH_3 and/or AA absorbed originates directly from dietary N because there is a contribution to digested N of other (non-measured) N components such as nucleic acids, amino sugars, peptides, etc (Lapierre and Lobley, 2001). Lapierre and Lobley (2001) showed the relationships between ureagenesis and digestible N, which ranged from 88 to 161% for ruminants species (lactating dairy cows, growing cattle, and growing and young mature sheep). Thus, it becomes obvious that part of absorbed NH_3 and/or AA were generated from endogenous N inputs which contributed to achieve apparent 'recoveries' close to, or in excess of, 100%. These inputs are primarily as urea N that may be metabolized either to NH_3 or AA (Lapierre and Lobley, 2001).

Urinary Urea N Elimination. The fraction of UER eliminated as urinary urea N (**UUE:UER**, g UUE/g UER) was positively associated with CP content ($P < 0.05$) and was described by a Gompertz function (Table 2.3; Fig. 2.3A). The relationship demonstrated that increasing CP content increases the fraction of UER that appeared in the urine. However, by evaluating the asymptotic confidence interval ($1 - \alpha = 0.95$) for the asymptote of the model, it was obtained that UUE:UER stabilized ($P > 0.05$) from 17.6% CP. In other words, this indicated that the fraction of UER excreted in urine did not statistically change when CP in the diet is greater than 17.6%. From the linear relationship between UUE and NI ($g/BW^{0.75}$), UUE accounted for 32.6% of N intake in cattle (Table 2.2). Regarding the amount of urinary

urea N excretion, there was an exponential increase ($P < 0.05$) with dietary CP levels (Table 2.3). Considering the exponential relationship, the maximum curvature analyses indicate that above 14.8% of CP the hepatic synthesis of urea N became more prominent than below 14.8% of CP.

Urinary urea N elimination is one of the fates of the urea synthesized in the liver and released into blood and factors such as fermentability of dietary carbohydrates and N intake relative to nutritional requirements affect UUE:UER (Huntington and Archibeque, 1999). According to Harmeyer and Martens (1980), the kidney has specific mechanisms that modify urinary excretion (or reabsorption) of urea N. The amount of urea excreted by the kidneys may be influenced by three main factors: changes of plasma urea N (**PUN**) and corresponding changes in filtered urea loads, changes of the glomerular filtration rates, and changes of tubular reabsorption of urea (Harmeyer and Martens, 1980). The contribution of urea N to total urinary N may indicate the priority for excretion of excess N in relation to muscle mass, muscle growth, or perhaps acid-base regulation (Huntington and Archibeque, 1999).

Considering a broad range of dietary CP, we can expects a lesser contribution of urea N to total urinary N (**UUE:TUN**, g UUE/g TUN) in ruminants fed low protein diets and, on the other hand, greater proportions when fed high protein diets (Lapierre and Loble, 2001; Marini and Van Amburgh, 2004; Reynolds and Kristensen, 2008; Wickersham et al., 2008). In this sense, as for UUE:UER, UUE:TUN was positively associated with dietary CP ($P < 0.05$) and the fitted model was a Gompertz function (Table 2.3; Fig. 2.4). From this equation considering the asymptotic confidence interval ($1 - \alpha = 0.95$), it would be expected a stabilization on UUE:TUN when dietary CP is greater than 15.9%, which this fraction is 0.65.

The evaluation of UUE:UER and UUE:TUN must be connected to the evaluation of UER as a function of dietary CP content. As previously discussed, considering that levels of CP in the diet from 17% shows an excess of dietary N, NH_3 input in the liver and its hepatic

detoxification, close to this value we observed a stabilization on UUE:UER and UUE:TUN (all from asymptotic confidence interval), which was 17.6 and 15.9 %. According to dietary change, the kidney has specific mechanisms to handle the tubular permeability and modify excretion or retention of urea (Harmeyer and Martens, 1980). Then, it is possible that cattle fed diets greater than 17% of CP presents a limit to reabsorb urea from renal tubules, which may be confirmed by the constant UUE:UER and UUE:TUN. However, although both fractions remained statistically constant, there is an increase on the total amount of urea N reabsorbed and excreted as corroborated by Harmeyer and Martens (1980). This seems to indicate that when dietary CP is greater than 17%, the excess of N intake decrease the efficiency of urea N utilization by the animal metabolism, decreasing renal reabsorption, but increasing the amount of urinary urea N excretion. These results emphasize the inherent ability of ruminants to salvage urea N for recycling and anabolic purposes when dietary protein is limited (Reynolds and Kristensen, 2008).

Gastrointestinal Entry Rate of Urea N. The amount of urea N transferred to the GIT (g/d) is estimated as the difference between UER and UUE (Lobley et al., 2001). On the other hand, fraction of UER that is recycled to GIT is calculated as:

$$GER{:}UER = 1 - UUE{:}UER, \quad [1]$$

in which: GER:UER is the fraction of UER recycled to the GIT and UUE:UER is the fraction of UER eliminated in the urine.

From Eq. [1], the relationship between dietary CP and CP:DOM, and GER:UER (g GER/g UER) can be calculated by using the prediction models to UUE (Table 2.3), respectively, as follows:

$$GER{:}UER = 1 - [0.558 \times e^{(-8.641 \times e^{(-0.213 \times CP)})}] \quad [2]$$

From the relationship between UUE:UER and CP or GER:UER and CP, both varied in a reciprocal manner (Fig. 2.3A and 2.3B), as highlighted by Reynolds and Kristensen

(2008). According to the models, GER:UER was negatively associated with dietary CP content. As for UUE:UER, at 17.6% of dietary CP content and above the fraction of recycled urea N (GER:UER) was relatively constant.

Urea N recycling provides a continuous source of NH_3 to support microbial fermentation in the rumen as well as other regions of the GIT (Huntington and Archibeque, 1999). Ruminal NH_3 concentration, DOM, and PUN concentration are the principal factors affecting urea recycling from blood to the lumen of the GIT (Kennedy and Milligan, 1980). Ruminal concentrations of NH_3 and OM digestion tend to be inversely related to each other, whereas GER is negatively related to ruminal NH_3 concentration, and positively related to PUN concentration.

As highlighted by Detmann et al. (2014), ruminants utilize nitrogenous compounds in different metabolic functions following the order of priority: survival, maintenance, and production (e.g., growth, reproduction, etc.). In this sense, urea N recycling can be considered as one of the possible high-priority metabolic functions, because a continuous N supply for microbial growth in the rumen is a strategy for animal survival (Egan, 1965; Van Soest, 1994). When there is a N deficiency, the animal is able to decrease urinary urea N excretion (Fig. 2.3A) and increase the fraction of urea N recycled to the rumen (Fig. 2.3B). The low UUE:UER at low dietary CP concentrations demonstrates the remarkable ability of ruminants to conserve N through urea recycling mechanisms in the face of a severe N deficiency (Wickersham et al., 2009a). Additionally, when N and/or energy availability is severely deficient, tissue protein mobilization can increase the N pool available for urea N synthesis and subsequent recycling (Ballard et al., 1976; NRC, 1985; Batista et al., 2016).

In a normal feeding situation (> 12% of CP), the amount of N recycled (g/d) through the rumen wall may be relatively constant (Marini and Van Amburgh, 2003; Maltby et al., 2005; Reynolds and Kristensen, 2008). However, in the current analysis, increases of N intake

were linearly associated ($P < 0.001$) with increases in the amount of urea N recycled, with the recycling accounting for about 35% of increases in N intake (Table 2.2; Fig. 2.5). Additionally, the amount of urea N recycled increased exponentially ($P < 0.05$) as the CP of the diet increased (Table 2.3).

Urea N returned to Ornithine Cycle. Urea N recycled returned to ornithine cycle as a fraction of GER (**ROC:GER**, g ROC/g GER) exponentially increased ($P < 0.05$) with dietary CP concentration (Table 2.3 and Fig. 2.6). In addition, ROC linearly increased with NI, accounting for about 23% of N intake (Table 2.2). The increases of ROC can be explained by the greater rumen NH_3 concentration with increasing CP supply. According to Holder et al. (2015), when rumen NH_3 concentration is maintained at high concentrations, then more NH_3 would be returned to the ornithine cycle for re-synthesis to urea, resulting in greater ROC:GER. Additionally, urea may be hydrolyzed by ruminal microbes at the epithelial border, and the resulting NH_3 may never enter the rumen pool resulting in increased ROC (Kristensen et al., 2010). It should be emphasized that it was not possible to fit a model to describe the relationship between ROC ($g/BW^{0.75}$) and dietary CP.

Urea N to Fecal Excretion. Prediction models for relationships between fecal urea N excretion as a fraction of GER (**UFE:GER**, g/g GER) and dietary CP showed exponential decreases ($P < 0.05$) with greater provision of CP (Table 2.3). According to Lapierre and Lobley (2001), the loss of urea N in feces is influenced by the supply of fermentable energy to the lower GIT, suggesting that hindgut usage of urea N involves only catabolic fates, at least in terms of AA supply to the animal. Much of UFE would represent undigestible N present in ruminally synthesized microbial cell mass (Van Soest, 1994). Thus, the decrease in UFE:GER with increasing protein in the diet (Fig. 2.6) may be a result of lower proportion of recycled urea N being captured as bacterial N. However, there is an increase on the total amount of UFE with NI ($g/kg^{0.75}$), corresponding for about 2.9% of increases in N intake (Table 2.2).

Neverthless, these model did not explain most of the variation in the amount of urea N excreted in feces with R^2 lower than 0.14 (Table 2.2). A part of this may be associated with low precision of fecal ^{15}N measurements, because ^{15}N enrichments are lower than those of urine and blood.

Urea N Utilized for Anabolism. Lobley et al. (2000) defined anabolic utilization of recycled urea N as the portion of urea N recycled to the GIT that is used to support anabolism. It is calculated as GIT entry rate of urea N minus urea N returned to the ornithine cycle and fecal excretion of recycled urea N:

$$UUA{:}\,GER = 1 - ROC{:}\,GER - UFE{:}\,GER \quad [4]$$

From equation [4] and models presented in Table 2.3 for ROC:GER and UFE:GER, the follow model can be described for UUA:GER:

$$UUA{:}\,GER = 1 - \left[0.625 \times \left(1 - e^{(-0.107 \times CP)}\right)\right] - \left[0.098 \times e^{(-0.037 \times CP)}\right] \quad [5]$$

Urea N utilized for anabolism as a fraction of GER (**UUA:GER**, g/g GER) presented an exponential relationship with the dietary CP content, decreasing as CP increased ($P < 0.05$). Regarding the relationship between UUA and NI (g/kg$^{0.75}$), the increases of N intake was linearly associated ($P < 0.001$) with increases in the amount of urea N utilized for anabolism, showing an increase of 14.8% in N intake (Table 2.2). However, there was no relationship between UUA (g/BW$^{0.75}$) and dietary CP to fit a model.

Because almost all urea N synthesized in the liver is recycled to the GIT in ruminants fed low-protein diets (Fig. 2.3B), UUA:GER is typically greater for low-protein diets than for high-protein diets (Reynolds and Kristensen, 2008). At least partially, the efficient reuse of N in ruminants fed low-CP diets occurs because urea N atoms can return to the GIT more than once, which increases the overall probability of sequestration towards an anabolic fate. This multiple-recycling process can result in improvements in UUA:GER of 22 to 49% in cattle and sheep (Lapierre and Lobley, 2001).

In theory, UUA:GER would predominantly represent recycled urea N that was incorporated into microbial AA, intestinally absorbed, and used for net deposition of body protein. For this reason, UUA:GER and MNU as a fraction of GER (**MNU:GER**; g/g GER) presents a similar pattern with the dietary CP content (Fig. 2.6 and 2.7). Nevertheless, according to Wickersham et al. (2008), UUA may include urea N that, subsequent to GIT entry, is excreted in the urine in forms other than urea, because the urinary excretion of label in forms other than urea is not measured by the methodology described by Lobley et al. (2001). Additionally, it is possible for urea N to be retained in the body without first being used for microbial production of AA. In spite of this, several studies reported that MNU:GER is frequently lesser than UUA:GER (Wickersham et al., 2008; 2009b; Davies et al., 2013; Batista et al., 2015) suggesting that UUA:GER is overestimated. This can be either due to underestimation of UFE or urinary excretion of label in unmeasured forms (e.g., other than urea N), which would be calculated as part of UUA; the methodology might be improved by measuring total ^{15}N in urinary excretion, which would prevent non-urea losses from being accounted for as anabolic use (Wickersham et al., 2008; 2009b).

Microbial Use of Recycled Urea N. Although urea N recycling can occur across the entire GIT, only urea N that is recycled into the rumen can potentially be incorporated in microbial protein and contribute AA to the host animal (Davies et al., 2013). In our analysis, MNU:GER (g/g GER) exponentially decreased ($P < 0.05$) with increasing dietary CP contents and CP:DOM (Table 2.3). For instance, when ruminants are fed with a diet with 5% CP, 53% of recycled urea N is assimilated by ruminal microbes. However, when a diet with 15% CP is provided, only 21% of recycled urea N is incorporated into microbial N in the rumen. Regarding CP:DOM ratio, evaluating the asymptotic confidence interval ($P > 0.05$) the efficiency of microbial assimilation of recycled urea N stabilized in diets with more than 194 g CP/kg DOM (MNU:GER is 0.18). For the relationship between MNU and N intake in a

$BW^{0.75}$ basis it was not found a linear ($P > 0.05$), although a non-linear model was fitted for describing the pattern between these variables. From the adjusted model, MNU ($g/BW^{0.75}$) exponentially increased ($P < 0.05$) with increasing N intake (Table 2.3), showing a prominent decrease on the total amount of MNU above 2.4 $g/BW^{0.75}$ of N intake (from evaluation of the maximum curvature).

Ruminants fed low-protein diets incorporated more recycled N into microbial products likely because ruminal NH_3 concentration was limiting and the efficiency of N utilization for microbial N synthesis is greater (Firkins et al., 2007). On the other hand, as dietary CP supply increases (i.e., RDP), therefore, ruminal bacteria become less dependent on recycled urea N as a source of NH_3 (Davies et al., 2013). According to Brake et al. (2010), efficiency of microbial capture of recycled urea N is associated with the availability of fermentable energy to the ruminal microbes, the fraction of GER recycled to the rumen, and the availability of competing N sources within the rumen.

Theoretically, increases in the proportion of GER being recycled to the rumen, rather than to postruminal segments of the GIT, would increase the opportunity of ruminal microbes to assimilate recycled urea N. In addition, when NH_3 is limiting, the ruminal microbes are more dependent on recycling mechanisms to meet their needs for N. The fraction of recycled urea N assimilated by ruminal microbes (i.e., MNU:GER) appeared to be dependent on the ruminal NH_3; when ruminal NH_3 increased, the efficiency of recycled-N assimilation by microbes decreased (Brake et al., 2010). Additionally, urea N can be recycled to the postruminal GIT. However, urea N recycled to the hindgut involves only catabolic fates considering MP provision to the ruminant (Lapierre and Lobley, 2001).

CONCLUSIONS

Based on the present meta-analysis, the amounts of urea N synthesized in the liver and recycled to the GIT were linearly related to N intake. However, increasing dietary CP intake (dietary CP concentration and CP relative to DOM) led to decreases in the fractions of urea N recycled to the GIT and of recycled urea N incorporated into microbial N. Urea recycling to the GIT and microbial assimilation of recycled urea N in ruminants can be predicted using dietary CP content. These models could be utilized in current feeding models to improve the knowledge on N utilization, optimize urea recycling, and consequently reduce N losses that contribute to air and water pollution.

LITERATURE CITED

Ballard, F. J., O. H. Filsell, and I. G. Jarrett. 1976. Amino acid uptake and output by the sheep hind limb. Metabolis. 25:415-418.

Batista, E. D., E. Detmann, E. C. Titgemeyer S. C. Valadares Filho, R. F. D. Valadares, L. L. Prates, L. N. Rennó, and M. F. Paulino. 2016. Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage. J. Anim. Sci. 94:201–216.

Brake, D. W., E. C. Titgemeyer, M. L. Jones, and D. E. Anderson. 2010. Effect of nitrogen supplementation on urea kinetics and microbial use of recycled urea in steers consuming corn-based diets. J. Anim. Sci. 88:2729–2740.

Brody, S. 1945. Bioenergetics and Growth. Reinhold, New York, NY.

Detmann, E., M.F. Paulino, H.C. Mantovani, S.C. Valadares Filho, C.B. Sampaio, M.A. Souza, I. Lazzarini, and K.S.C. Detmann. 2009. Parameterization of ruminal fibre degradation in low-quality tropical forage using Michaelis–Menten kinetics. Liv. Sci 126:136–146.

Detmann, E., E. E. L. Valente, E. D. Batista, and P. Huhtanen. 2014. An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. Livest. Sci. 162:141–153.

Egan, A. R. 1965. The fate and effects of duodenally infused casein and urea nitrogen in sheep fed on a low-protein roughage. Aust. J. Agric. Res. 16:169–177.

Firkins, J. L., and C. K. Reynolds. 2005. Whole animal nitrogen balance in cattle. In: Nitrogen and Phosphorus Nutrition of Cattle and the Environment. CAB Int., Wallingford, UK. Pages 167–186.

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: Perspectives for integration of microbiology and nutrition for dairy. J. Dairy Sci. 90(Suppl. E):E1–E16.

Harmeyer, J., and H. Martens. 1980. Aspects of urea metabolism in ruminants with reference to the goat. J. Dairy Sci. 63:1707–1728.

Holder, V. B., J. M. Tricarico, D. H. Kim, N. B. Kristensen, and D. L. Harmon. 2015. The effects of degradable nitrogen level and slow release urea on nitrogen balance and urea kinetics in Holstein steers. Anim. Feed Sci. Technol. 200:57–65.

Houpt, T. R. 1959. Utilization of blood urea in ruminants. Am. J. Physiol. 197:115.–120.

Huntington, G. B., and S. L. Archibeque. 1999. Practical aspects of urea and ammonia metabolism in ruminants. Proc. Am. Soc. Anim. Sci. 1999. 1–11.

Davies, K. L., J. J. McKinnon, and T. Mutsvangwa. 2013. Effects of dietary ruminally degradable starch and ruminally degradable protein levels on urea recycling, microbial protein production, nitrogen balance, and duodenal nutrient flow in beef heifers fed low crude protein diets. Can. J. Anim. Sci. 93:123–136.

Kennedy, P. M., and L. P. Milligan. 1980. The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants: A review. Can. J. Anim. Sci. 60:205-221.

Kristensen, N. B., A. C. Storm, and M. Larsen. 2010. Effect of dietary nitrogen content and intravenous urea infusion on ruminal and portal-drained visceral extraction of arterial urea in lactating Holstein cows. J. Dairy Sci. 93:2670–2683.

Lapierre, H., and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84(Suppl. E):E223–E236.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger, and O. Schabenberger. 2006. SASs for mixed models, 2nd ed. Cary, SAS Institute Inc..

Lobley, G. E., and G. D. Milano. 1997. Regulation of hepatic nitrogen metabolism in ruminants. Proc. Nutr. Soc. 56:547-563.

Lobley, G. E., D. M. Bremner, and G. Zuur. 2000. Effects of diet quality on urea fates in sheep assessed by a refined, non-invasive [$^{15}N^{15}N$]-urea kinetics. Br. J. Nutr. 84:459–468.

Maltby, S. A., C. K. Reynolds, M. A. Lomax, and D. E. Beever. 2005. Splanchnic metabolism of nitrogenous compounds and urinary nitrogen excretion in steers fed alfalfa under conditions of increased absorption of ammonia and L-arginine supply across the portal-drained viscera. J. Anim. Sci. 83:1075–1087.

Marini, J. C., and M. E. Van Amburgh. 2003. Nitrogen metabolism and recycling in Holstein heifers. J. Anim. Sci. 81:545–552.

NRC. 1985. Ruminant Nitrogen Usage. Natl. Acad. Press, Washington, DC.

Parker, D. S., M. A. Lomax, C. J. Seal, and J. C. Wilton. 1995. Metabolic implications of ammonia production in the ruminant. Proc. Nutr. Soc. 54:549–563.

Reynolds, C. K., and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis. J. Anim.Sci.86:E293–E305.

Sarraseca, A., E. Milne, M. J. Metcalf, and G. E. Lobley. 1998. Urea recycling in sheep: Effects of intake. Br. J. Nutr. 79:79–88.

Satter, L.D. and L.L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr. 32:199–208.

St-Pierre, N.R. 2001. Integrating quantitative findings from multiple studies using mixed model methodology. J. Dairy. Sci. 84: 741–755.

Valente, T. N. P., E. Detmann, A. C. Queiroz, S. C. Valdares Filho, D. I. Gomes, and J. F. Figueiras. 2011. Evaluation of ruminal degradation profiles of forages using bags made from different textiles. Rev. Bras. Zootec. 40:2565–2573.

Van Houwelingen, H. C., L. R. Arends, T. Stijen. 2002. Advanced methods in meta-analysis: multivariate approach and meta-regression. Stat. Med 21: 589–624.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. 2nd ed. Cornell Univ. Press, Ithaca, NY.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, E. E. Wickersham, and D. P. Gnad. 2008. Effect of rumen-degradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. J. Anim. Sci. 86:3079–3088.

Wickersham, T. A., E. C. Titgemeyer, and R. C. Cochran. 2009a. Methodology for concurrent determination of urea kinetics and the capture of recycled urea nitrogen by ruminal microbes in cattle. Animal 3:372–379.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, and E. E. Wickersham. 2009b. Effect of undegradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. Br. J. Nutr. 101:225–232.

Table 2.1 Statistical description of the dataset used for the analysis of urea kinetics and microbial use of recycled urea in ruminants

	Statistics				
Item[1]	n	Mean	Minimum	Maximum	s
BW, kg	107	309.3	20.8	723.0	209.8
DMI, kg/d	107	7.88	0.43	26.8	7.23
N intake, g/$BW^{0,75}$	107	2.05	0.41	5.80	1.30
Sheep	16	1.79	0.56	3.34	0.97
Beef cattle	72	1.53	0.41	3.09	0.59
Dairy cattle	19	4.49	2.10	5.80	0.87
CP, % DM	107	12.8	4.7	25.7	4.3
Sheep	16	14.9	7.5	25.7	4.8
Beef cattle	72	11.5	4.7	21.4	3.8
Dairy cattle	19	16.2	9.6	20.7	2.5
DOM, kg/d	53	3.07	0.86	6.91	1.53
CP:DOM, g CP/kg DOM	53	190	84	428	79
UER, g/$BW^{0.75}$	107	1.50	0.25	4.63	1.00
Sheep	16	1.62	0.25	3.56	1.18
Beef cattle	72	1.11	0.29	2.83	0.47
Dairy cattle	19	2.91	0.74	4.63	1.05
GER, g/$BW^{0.75}$	107	1.50	0.25	4.63	1.0
Sheep	16	1.62	0.25	3.56	1.18
Beef cattle	72	1.11	0.29	2.83	0.47
Dairy cattle	19	2.91	0.74	4.63	1.05
UER, g/g N intake	107	0.749	0.270	1.431	0.261
GER, g/g UER	107	0.709	0.293	0.990	0.159
UUE, g/g UER	107	0.291	0.010	0.707	0.159
UUE, g/g TUN	107	0.523	0.012	0.886	0.227
ROC, g/g GER	98	0.449	0.152	0.860	0.165
UFE, g/g GER	90	0.059	0.003	0.211	0.043
UUA, g/g GER	90	0.478	0.110	0.750	0.153
MNU, g/g GER	46	0.322	0.059	0.720	0.206

[1] BW = body weight; DMI = dry matter intake; CP = dietary crude protein (% of DM); DOM = digestible OM content in the diet; CP:DOM = ratio between CP and DOM content; UER = urea N entry rate; GER = gastrointestinal entry rate of urea N; UUE = urinary urea N elimination; TUN = total urinary N; ROC = re-entry to ornithine cycle; UFE = urea N to fecal excretion; UUA = urea N utilized for anabolism; and MNU = microbial incorporation of recycled urea N.

Table 2.2 Summary of the linear models for describing the pattern of urea N entry rate and gastrointestinal entry rate of urea N and urea kinetics in function of N intake in cattle

Y[1]	X[1]	Linear term			Intercept			RSD[2]	r^2
		Estimate	SE	*P*-value	Estimate	SE	*P*-value		
UER	NI	0.7146	0.0490	< 0.001	–	–	–	3.935	0.825
GER	NI	0.3525	0.0557	< 0.001	0.2514	0.0883	0.010	2.510	0.728
UUE	NI	0.3264	0.0505	< 0.001	- 0.2413	0.0897	0.014	2.343	0.702
ROC	NI	0.2320	0.0259	< 0.001	–	–	–	1.994	0.586
UFE	NI	0.0290	0.004	< 0.001	–	–	–	0.347	0.133
UUA	NI	0.1477	0.0371	< 0.001	0.1943	0.0557	0.003	1.373	0.566

[1] UER = urea N entry rate ($g/BW^{0.75}$); NI = N intake ($g/BW^{0.75}$); GER = gastrointestinal entry rate of urea N ($g/BW^{0.75}$); UUE = urinary urea N elimination ($g/BW^{0.75}$); ROC = re-entry to ornithine cycle ($g/BW^{0.75}$); UFE = urea N to fecal excretion ($g/BW^{0.75}$); and UUA = urea N utilized for anabolism ($g/BW^{0.75}$).

[2] RSD = residual standard deviation of the relationship.

Table 2.3 Summary of the non-linear models for describing the pattern of urea kinetics and microbial use of recycled urea in cattle

Y[1]	X[1]	Model structure	α		β		γ		ASD[3]	R^2
			Estimate	Effect[2]	Estimate	Effect[2]	Estimate	Effect[2]		
UER	CP	$\hat{Y} = \alpha \times e^{(\beta \times X)}$	0.3121	Random	0.1170	Random	–	–	0.250	0.738
UER:NI	CP	$\hat{Y} = \alpha / X$	7.8402	Random	–	–	–	–	2.225	0.493
UUE	CP	$\hat{Y} = \alpha \times e^{(\beta \times X)}$	0.0491	Random	0.1610	Fixed	–	–	0.104	0.932
UUE:UER	CP	$\hat{Y} = \alpha \times e^{(-\beta \times e^{(-\gamma \times X)})}$	0.5579	Random	8.6406	Fixed	0.2133	Fixed	0.048	0.928
UUE:TUN	CP	$\hat{Y} = \alpha \times e^{(-\beta \times e^{(-\gamma \times X)})}$	0.7020	Random	11.000	Fixed	0.3167	Fixed	0.071	0.855
GER	CP	$\hat{Y} = \alpha \times e^{(\beta \times X)}$	0.3854	Random	0.0720	Fixed	–	–	0.201	0.932
ROC:GER	CP	$\hat{Y} = \alpha \times (1 - e^{(-\beta \times X)})$	0.6254	Random	0.1066	Random	–	–	0.053	0.695
UFE:GER	CP	$\hat{Y} = \alpha \times e^{(-\beta \times X)}$	0.0981	Random	0.0373	Random	–	–	0.011	0.694
MNU	NI	$\hat{Y} = 1 - e^{(-\alpha \times X)}$	0.2034	Random	–	–	–	–	0.088	0.821
MNU:GER	CP	$\hat{Y} = \alpha \times e^{(-\beta \times X)}$	0.8340	Random	0.0920	Random	–	–	0.043	0.956
MNU:GER	CP:DOM	$\hat{Y} = \alpha \times e^{(-\beta \times X)} + \gamma$	0.8719	Fixed	0.0082	Random	0.0973	Fixed	0.053	0.977

[1] UER = urea N entry rate (g/$BW^{0.75}$); CP = dietary crude protein (% of DM); UERNI = UER to N intake ratio (g UER/g N intake); UER = urinary urea N elimination (g/$BW^{0.75}$); UUE:UER = urinary urea N elimination relative to entry rate of urea N (g UUE/g UER); UUE:TUN = urinary urea N elimination relative to total urinary N excretion (g UUE/g TUN); GER = gastrointestinal entry rate of urea N (g/$BW^{0.75}$); ROC:GER = re-entry to ornithine cycle relative to gastrointestinal rate of urea N (g ROC/g GER); UFE:GER = urea N to fecal excretion relative to gastrointestinal rate of urea N (g UFE/g GER); MNU = microbial incorporation of recycled urea N (g/$BW^{0.75}$); NI = N intake (g/$BW^{0.75}$); MNU:GER = microbial incorporation of recycled urea N relative to gastrointestinal entry rate of urea N (g MNU/g GER); and CP:DOM = ratio between CP and DOM contents (g CP/kg DOM).

[2] A random effect type means that a random variance component was associated to the parameter ($P < 0.05$). A fixed effect type indicates that the random variance (among experiments) was not significant ($P > 0.05$).

[3] ASD = asymptotic standard deviation of the relationship.

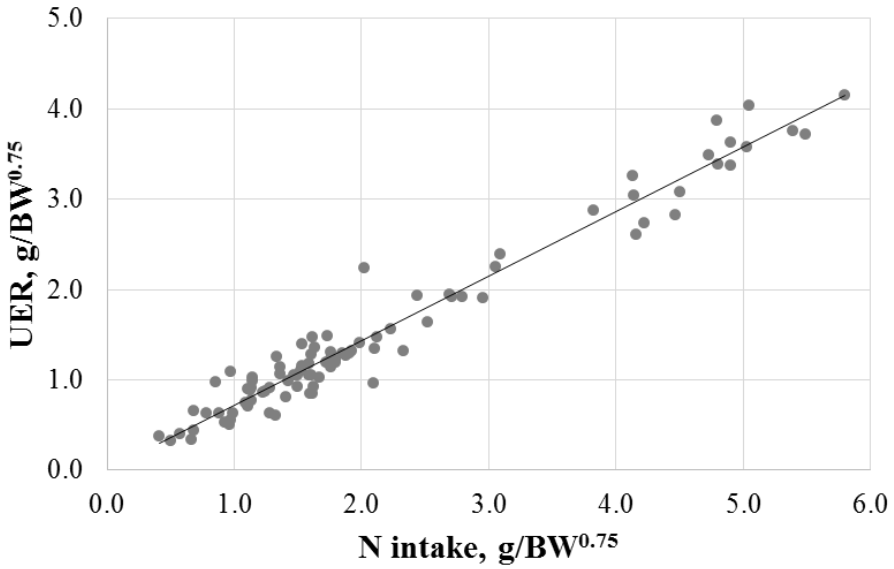


Figure 2.1 Relationship between N intake and hepatic synthesis of urea N (UER) in cattle. Dataset was adjusted for random effects of the studies. Details of the model can be seen in Table 2.2.

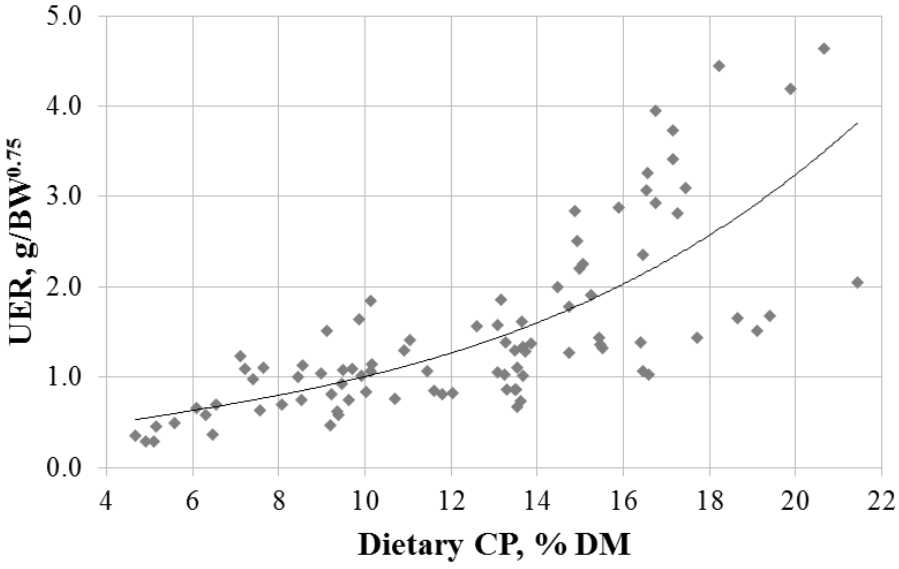
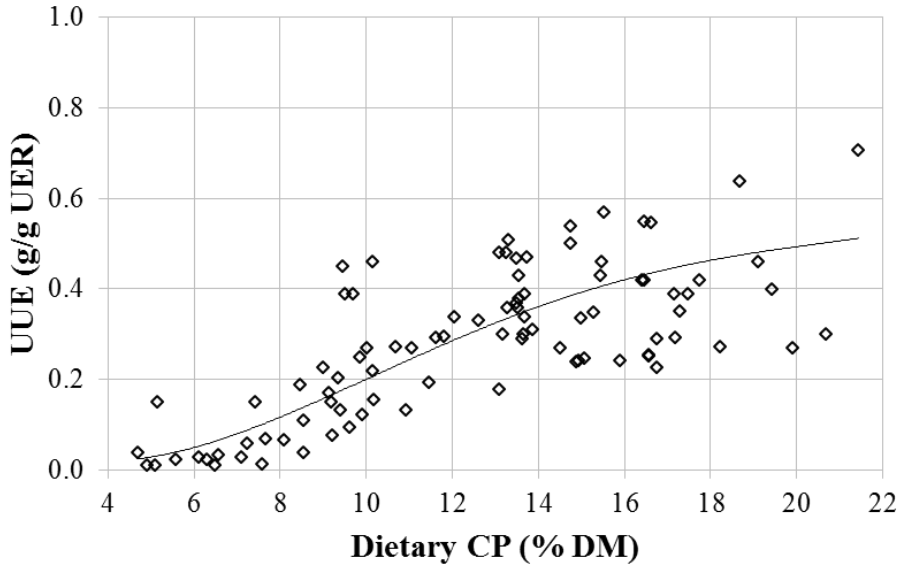


Figure 2.2 Relationship between dietary CP content and hepatic synthesis of urea N (UER) in cattle. Details of the model can be seen in Table 2.3.

A



B

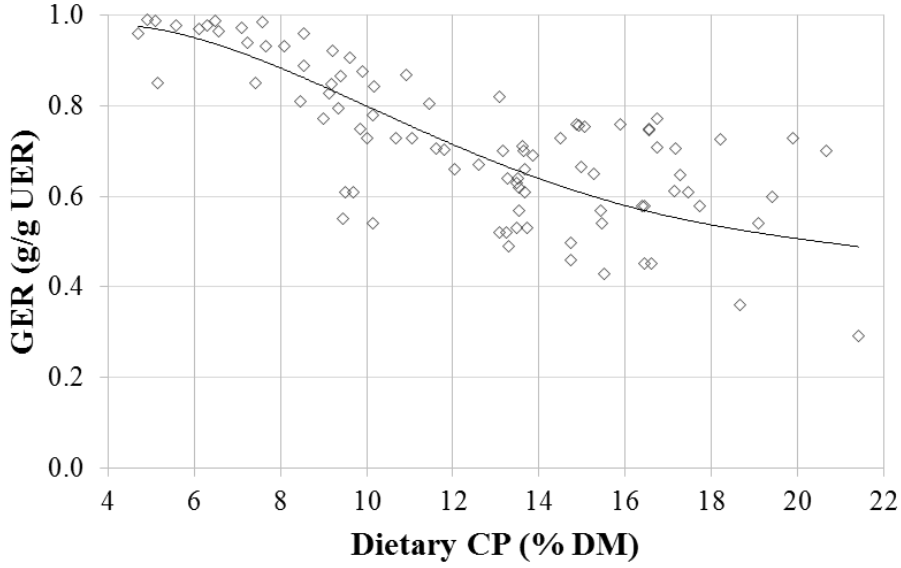


Figure 2.3 Relationship between dietary CP content and the fraction of hepatic synthesis of urea N in cattle: (A) eliminated in the urine (UUE); and (B) recycled to the gastrointestinal tract via epithelium and saliva (GER). Details of the model can be seen in Table 2.3.

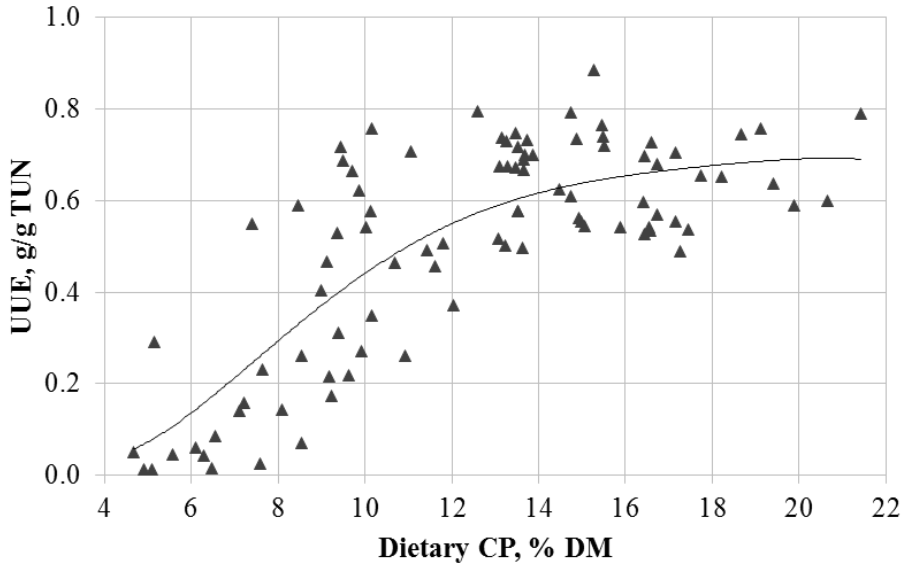


Figure 2.4 Relationship between dietary CP content and the fraction of urea N to total urinary N (UUE:TUN) in cattle. Details on the model can be seen in Table 2.3.

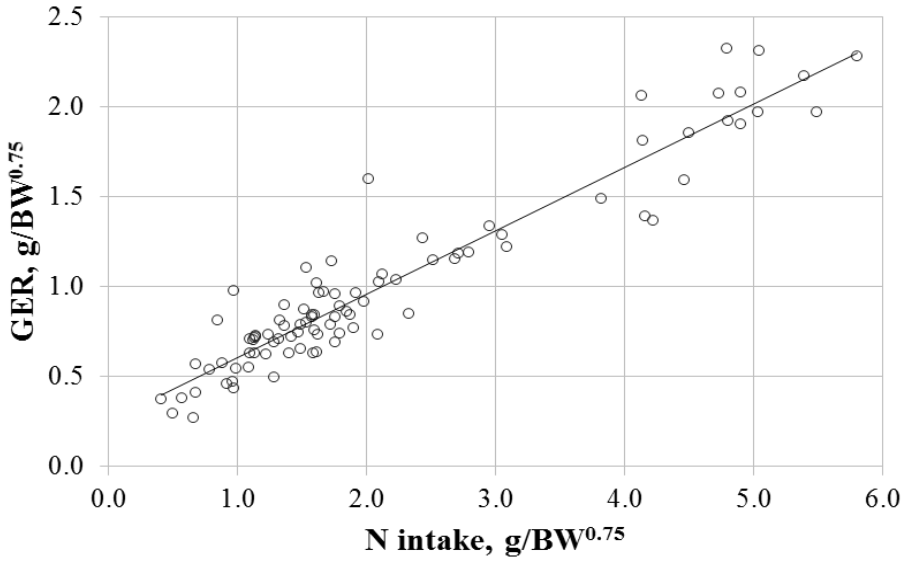


Figure 2.5 Relationship between N intake and urea N recycled to the gastrointestinal tract (GER) in cattle. Dataset was adjusted for random effects of the studies. Details on the model can be seen in Table 2.2.

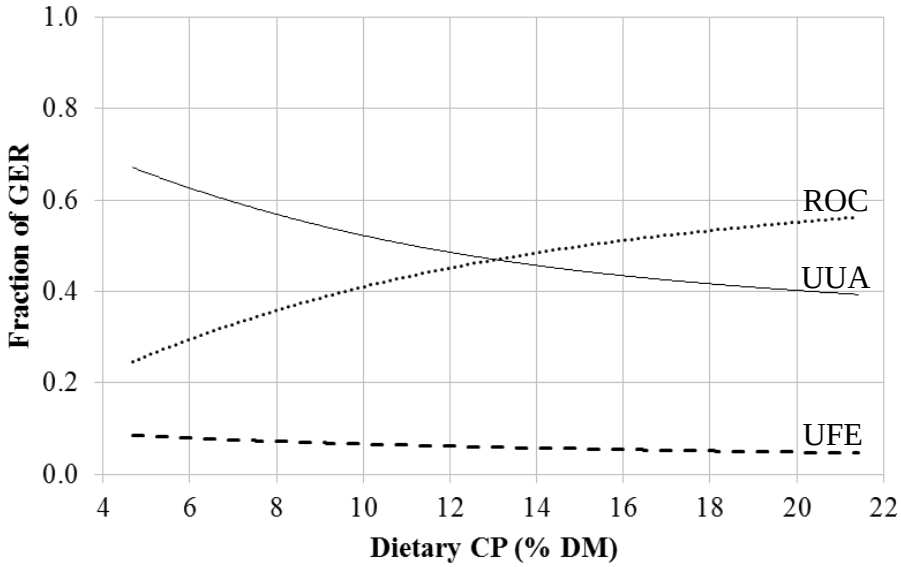


Figure 2.6 Relationship between dietary CP content and the fraction of gastrointestinal entry rate of urea N that re-entry to ornithine cycle (ROC), undergoes to anabolic use (UUA), and is excreted in the feces (UFE) in cattle. Details of the models are presented in Table 2.3, except to UUA.

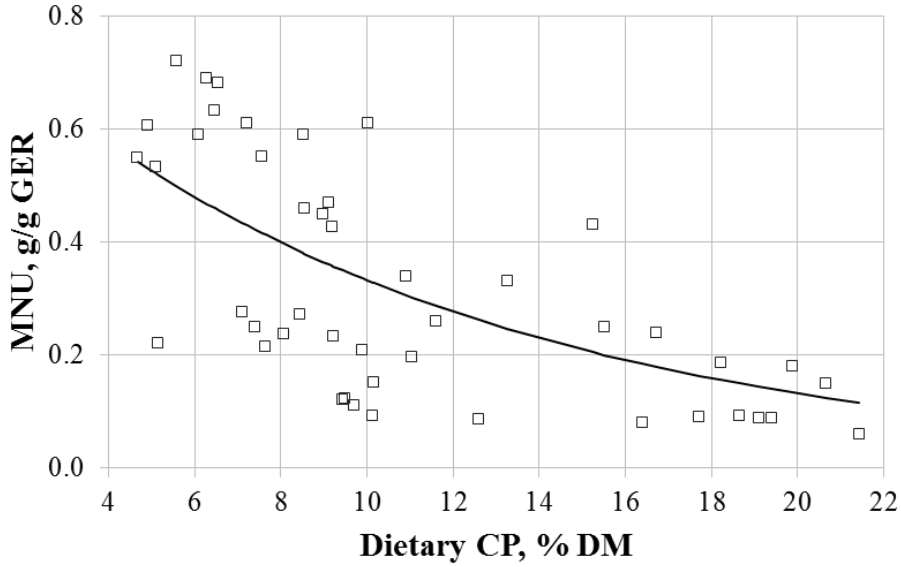


Figure 2.7 Relationship between dietary CP content and the fraction of urea N recycled to the gastrointestinal tract incorporated in microbial N in the rumen (MNU) in cattle. Details of the models are presented in Table 2.3.

APPENDIX

Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2001. Urea flux in beef steers: Effects of forage species and nitrogen fertilization. J. Anim. Sci. 79:1937–1943.

Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2002. Nitrogen metabolism of beef steers fed endophyte-free tall fescue hay: Effects of ruminally protected methionine supplementation. J. Anim. Sci. 80:1344-1351.

Bailey, E. A., E. C. Titgemeyer, K. C. Olson, D. W. Brake, M. L. Jones, and D. E. Anderson. 2012a. Effects of supplemental energy and protein on forage digestion and urea kinetics in growing beef cattle. J. Anim. Sci. 90:3492–3504.

Bailey, E. A., E. C. Titgemeyer, K. C. Olson, D. W. Brake, M. L. Jones, and D. E. Anderson. 2012b. Effects of ruminal casein and glucose on forage digestion and urea kinetics in beef cattle. J. Anim. Sci. 90: 3505-3514.

Batista, E.D., E. Detmann, E. C. Titgemeyer S. C. Valadares Filho, R. F. D. Valadares, L. L. Prates, L. N. Rennó, and M. F. Paulino. 2015. Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage. J. Anim. Sci. *in press.* doi:10.2527/jas2015-9493

Brake, D. W., E. C. Titgemeyer, M. L. Jones, and D. E. Anderson. 2010. Effect of nitrogen supplementation on urea kinetics and microbial use of recycled urea in steers consuming corn-based diets. J. Anim. Sci. 88:2729–2740.

Brake, D. W., E. C. Titgemeyer, and Jones, M.L. 2011. Effect of nitrogen supplementation and zilpaterol–HCl on urea kinetics in steers consuming corn-based diets. J. Anim. Physiol. An. N. 95:409–416.

Dinn, S.K. 2007. Urea N recycling in lactating dairy cows. (Ph.D. thesis) University of Maryland-College Park.

Davies, K. L., J. J. McKinnon, and T. Mutsvangwa. 2013. Effects of dietary ruminally degradable starch and ruminally degradable protein levels on urea recycling, microbial protein production, nitrogen balance, and duodenal nutrient flow in beef heifers fed low crude protein diets. Can. J. Anim. Sci. 93:123–136.

Gozho, G.N. M. R. Hobin, and T. Mutsvangwa. 2008. Interactions between barley grain processing and source of supplemental dietary fat on nitrogen metabolism and urea nitrogen recycling in dairy cows. J. Dairy. Sci. 91:247–259.

Kiran, D. and T. Mutsvangwa. 2007. Effects of barley grain processing and dietary ruminally degradable protein on urea Nitrogen recycling and nitrogen metabolism in growing lambs. J. Anim. Sci., 85:3391–3399.

Holder, V. B., J. M. Tricarico, D. H. Kim, N. B. Kristensen, and D. L. Harmon. 2015. The effects of degradable nitrogen level and slow release urea on nitrogen balance and urea kinetics in Holstein steers. Anim. Feed Sci. Technol. 200:57–65.

Lobley, G. E., D. M. Bremner, and G. Zuur. 2000. Effects of diet quality on urea fates in sheep assessed by a refined, non-invasive [$^{15}N^{15}N$]-urea kinetics. Br. J. Nutr. 84:459–468.

Marini, J. C., and M. E. Van Amburgh. 2003. Nitrogen metabolism and recycling in Holstein heifers. J. Anim. Sci. 81:545–552.

Marini, J. C., J. D. Klein, J. M. Sands, and M. E. Van Amburgh. 2004. Effect of nitrogen intake on nitrogen recycling and urea transporter abundance in lambs. J. Anim. Sci. 82:1157–1164.

Recktenwald, E. B., D. A. Ross, S. W. Fessenden, C. J. Wall, and M. E. Van Amburgh. 2014. Urea N recycling in lactating dairy cows fed diets with 2 different levels of dietary crude protein and starch with or without monensin. J. Dairy Sci. 97:1611–1622.

Ruiz, R., L.O. Tedeschi, J. C. Marini, D. G. Fox., A. N. Pell, G. Jarvis, and J. B. Russell. 2002. The effect of a ruminal nitrogen (N) deficiency in dairy cows: Evaluation of the Cornell Net Carbohydrate and Protein System ruminal nitrogen deficiency adjustment. J. Dairy Sci. 85:2986–2999.

Sarraseca, A., E. Milne, M. J. Metcalf, and G. E. Lobley. 1998. Urea recycling in sheep: Effects of intake. Br. J. Nutr. 79:79–88.

Sunny, N. E., S. L. Owens, R. L. Baldwin, S. W. El-Kadi, R. A. Kohn, and B. J. Bequette. 2007. Salvage of blood urea nitrogen in sheep is highly dependent on plasma urea concentration and the efficiency of capture within the digestive tract. J. Anim. Sci. 85:1006–1013.

Titgemeyer, E. C., K. S. Spivey, S. L. Parr, D. W. Brake, and M. L. Jones. 2012. Relationship of whole body nitrogen utilization to urea kinetics in growing steers. J. Anim. Sci. 90:3515-3526.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, E. E. Wickersham, and D. P. Gnad. 2008a. Effect of rumen-degradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. J. Anim. Sci. 86:3079–3088.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, E. E. Wickersham and E. S. Moore. 2008b. Effect of frequency and amount of rumen-degradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. J. Anim. Sci. 86:3089-3099.

Wickersham, T. A., E. C. Titgemeyer, and R. C. Cochran. 2009a. Methodology for concurrent determination of urea kinetics and the capture of recycled urea nitrogen by ruminal microbes in cattle. Animal 3:372–379.

Wickersham, T. A., E. C. Titgemeyer, R. C. Cochran, and E. E. Wickersham. 2009b. Effect of undegradable intake protein supplementation on urea kinetics and microbial use of recycled urea in steers consuming low-quality forage. Br. J. Nutr. 101:225–232.

CHAPTER 3

Efficiency of lysine utilization by growing steers

E. D. Batista,*† A. H. Hussein,* E. Detmann,† M. D. Miesner,‡ E. C. Titgemeyer*

*Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS;

†Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil;

‡Department of Clinical Sciences, Kansas State University, Manhattan, KS.

Published at Journal of Animal Science

doi:10.2527/jas2015-9716

ABSTRACT

This study evaluated the efficiency of lysine (Lys) utilization by growing steers. Five ruminally cannulated Holstein steers (165 kg ± 8 kg) housed in metabolism crates were used in a 6 × 6 Latin square design; data from a sixth steer was excluded due to erratic feed intake. All steers were limit fed (2.46 kg DM/d) twice daily diets low in RUP (81% soybean hulls, 8% wheat straw, 6% cane molasses, and 5% vitamins and minerals). Treatments were: 0, 3, 6, 9, 12, and 15 g/d of L-Lys abomasally infused continuously. To prevent AA other than Lys from limiting performance, a mixture providing all essential AA to excess was continuously infused abomasally. Additional continuous infusions included 10 g urea/d, 200 g acetic acid/d, 200 g propionic acid/d, and 50 g butyric acid/d to the rumen and 300 g glucose/d to the abomasum. These infusions provided adequate ruminal ammonia and increased energy supply without increasing microbial protein supply. Each 6-d period included 2 d for adaptation and 4 d for total fecal and urinary collections for measuring N balance. Blood was collected on d 6 (10 h after feeding). Diet OM digestibility was not altered ($P \geq 0.66$) by treatment and averaged 73.7%. Urinary N excretion decreased from 32.3 to 24.3 g/d by increasing Lys supplementation to 9 g/d, with no further reduction when more than 9 g/d of Lys was supplied (linear and quadratic $P < 0.01$). Changes in total urinary N excretion were predominantly due to changes in urinary urea N. Increasing Lys supply from 0 to 9 g/d increased N retention from 21.4 to 30.7 g/d, with no further increase beyond 9 g/d of Lys (linear and quadratic $P < 0.01$). Break-point analysis estimated maximal N retention at 9 g/d supplemental Lys. Over the linear response surface of 0 to 9 g/d Lys, the efficiency of Lys utilization for protein deposition was 40%. Plasma urea N tended to be linearly decreased ($P = 0.06$) by Lys supplementation in agreement with the reduction in urinary urea N excretion. Plasma concentrations of Lys increased linearly ($P < 0.001$), but Leu, Ser, Val, and Tyr ($P \leq 0.02$) were reduced linearly by Lys supplementation, likely reflecting increased uptake for protein deposition. In our model,

Lys supplementation promoted significant increases in N retention and was maximized at 9 g/d supplemental Lys with efficiency of utilization of 40%.

Key words: amino acids, cattle, efficiency, lysine, nitrogen retention

INTRODUCTION

The physiological needs of cattle for AA must be considered to optimize performance, but microbial degradation of dietary protein and AA in the rumen creates a challenge for developing accurate estimates of requirements in ruminants (Titgemeyer, 2003).

Lysine (**Lys**) was identified as the second-limiting AA when ruminal microbial protein was the main source of MP in growing cattle receiving semipurified diets (Richardson and Hatfield, 1978) as well as for sheep maintained by intragastric nutrition (Storm and Ørskov, 1984). However, for corn-based diets, which are rich in methionine supply, Lys was the first-limiting AA for growing cattle (Burris et al., 1976; Hill et al., 1980; Abe et al., 1997).

Estimates of the Lys requirements of growing steers have been developed using plasma data (Fenderson and Bergen, 1975), modeling approaches (O`Connor et al., 1993), and growth studies (Klemesrud et al., 2000a,b). The Cornell Net Carbohydrate and Protein System (CNCPS; O´Connor et al., 1993) bases efficiencies of AA utilization on relationships between absorbed and deposited protein, and equivalent shrunk BW is the sole factor used to estimate the efficiency of AA use. The same efficiency is applied to each essential AA as well as to MP. However, recent evaluations of methionine (Campbell et al., 1996, 1997; Löest et al., 2002), leucine (Awawdeh et al., 2005; 2006; Titgemeyer et al., 2012) and histidine (McCuistion et al., 2004) utilization showed that differences exist among AA for the efficiency of use. These inherent differences in the efficiency of individual AA use reflect differences in oxidation rates (Heger and Frydrych, 1989) and diverse metabolic processes for each AA (Owens and Pettigrew, 1989).

Our objective was to determine the efficiency of Lys utilization for whole-body protein deposition by growing steers.

MATERIALS AND METHODS

Animals and Treatments

The Kansas State University Institutional Animal Care and Use Committee approved procedures involving animals in this study (protocol no. 016/2012; Appendix, pg. 111).

Five ruminally cannulated Holstein steers (165 kg) were used in a 6 × 6, balanced, Latin square design. A sixth steer was included in the experiment, but erratic feed intake by this animal led to exclusion of all data collected from it. Animals were housed in individual metabolism crates in a temperature-controlled room (20°C) under 16 h of light daily. Before initiation of each experiment, steers were adapted to the basal diet (Table 3.1) for 2 wk and to ruminal and abomasal infusions for 5 d. All steers had free access to water and received the same basal diet at 2.5 kg of DM/d in equal proportions at 12-h intervals. The diet used in this study (Table 3.1) was formulated to provide small amounts of metabolizable AA to create a limitation in Lys.

Treatments included daily abomasal infusions of 0, 3, 6, 9, 12 or 15 g of L-Lys/d. Treatments were infused continuously into the abomasum via peristaltic pump (Model CP-78002-10; Cole-Parmer Instrument Company, Vernon Hills, IL) by placing Tygon tubing (2.0 mm i.d.) through the ruminal cannula and the reticulo-omasal orifice and into the abomasum. A rubber flange (10-cm diameter) was attached to the end of the abomasal infusion lines to ensure that they remained in place.

All steers received a basal infusion of a mixture providing daily 20 g of L-Leu, 15 g of L-Thr, 8 g of L-His·HCl·H_2O, 20 g of L-Phe, 5 g of L-Trp, 10 g of L-Met, 15 g of L-Ile, 15 g of L-Val, 15 g of L-Arg, 150 g of monosodium glutamate, and 40 g of Gly. The mixture was

continuously infused into the abomasum to provide all essential AA except Lys in excess of the steers' requirements and prevent limitations in protein synthesis by AA other than Lys. The profile of AA infused was based on the supplies and requirements of AA estimated for growing Holstein steers fed with a diet similar to that used in our study (Greenwood and Titgemeyer, 2000). Abomasal infusate for each steer was prepared by dissolving L-Leu, L-Ile, and L-Val in 1 kg of water containing 60 g of 6 M HCl. Once L-Leu, L-Ile, and L-Val were dissolved, the remaining AA, except Glu (monosodium glutamate), were added to the mixture. Monosodium glutamate was dissolved separately in 500 g of water. After all AA were dissolved, the 2 solutions of AA were mixed together, dextrose was added (330 g supplying 300 g glucose) to provide an additional energy source, and water was added to bring the total weight of the daily infusate to 4 kg. All steers received 10 mg of pyridoxine·HCl/d, 10 mg of folic acid/d, and 100 µg of cyanocobalamin/d in the abomasal infusate because the experimental diet was deficient in at least one of those vitamins (Lambert et al., 2004).

To ensure adequate ruminal ammonia concentrations to support microbial growth, all steers received a ruminal infusion of 10 g of urea/d; this was considered as part of dietary N intake. In addition, all steers received continuous ruminal infusions of 10 g of urea/d, 200 g of acetic acid/d, 200 g of propionic acid/d and 50 g of butyric acid/d, with the total weight of the ruminal infusate being 4 kg/d. This supplemental energy was provided because dietary energy intake was restricted and energy deficiencies would limit the ability of the steers to retain N. Additionally, these energy sources can be utilized by the steers without affecting ruminal microbial protein synthesis. Infusion lines were placed in the rumen through the ruminal cannula and a perforated vial was attached to the end of the ruminal infusion lines to avoid direct infusion of VFA onto the ruminal wall.

Sample Collection and Analyses

Each experimental period lasted 6 d, with 2 d for adaptation to treatment and 4 d for total fecal and urinary collections. The 2-d adaptation periods were adequate because cattle rapidly adapt to changes in nutrients supplied postruminally (Moloney et al., 1998; Schroeder et al., 2006). Representative samples of the basal diet for each period were collected daily and stored (−20°C) for later analysis. Orts, if any, were collected on d 2 through 5, composited, and stored (−20°C) for later analysis. Feces and urine from each steer were collected from d 3 through 6 of each period and weighed to determine total output. Urine was collected in buckets containing 900 mL of 10% wt/wt H_2SO_4 to prevent NH_3 loss. Representative samples of feces (10%) and urine (1%) were frozen daily, composited by period, and stored (−20°C) for later analysis. Before analysis, samples were thawed and homogenized. Feed and fecal samples were partly dried at 55°C for 36 h, air-equilibrated for 36 h, and ground with a Wiley mill (Thomas Scientific, Swedesboro, NJ) through a 1-mm screen. Partly dried diet and fecal samples were analyzed for DM (105°C for 24 h) and ash (450°C for 8 h). Total N content was measured for feed, orts, wet fecal samples, and urine samples using a combustion analyzer (True Mac, Leco Corporation, St. Joseph, MI). Urine samples were analyzed colorimetrically for NH_3 (Broderick and Kang, 1980) and urea concentrations (Marsh et al., 1965).

On d 6 of each period, 10 h after the morning feeding, jugular blood was collected into vacuum tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Blood samples were immediately placed on ice and then centrifuged (1,000 × *g*, 20 min, 4ºC) to obtain plasma, which was frozen (−20°C) for later analysis. Plasma urea and glucose concentrations were measured according to methods of Marsh et al. (1965) and Gochman and Schmitz (1972), respectively.

For AA analysis, plasma (750 µL) was mixed with 750 µL of 10% (wt/vol) sulfosalicylic acid containing 1 mM norleucine as an internal standard. After cooling on ice for 30 min, samples were vortexed and centrifuged (13,800 × *g*, 20 min, 4ºC). The supernatant was used to measure plasma AA by cation exchange chromatography with post-column derivitization with *o*-phthalaldehyde. A 4 × 100 mm lithium ion-exchange column (Pickering Laboratories, Mountain View, CA) and lithium eluents (Pickering Laboratories) were used. Flow rate was 0.3 mL/min and the total run time was 130 min. The initial eluant contained 0.7% lithium citrate, 0.6% lithium chloride, and 0.2% sulfolane and was pumped for 39.1 min. The second eluant contained 2.7% lithium citrate and <0.1% lithium chloride and was pumped for 18 min. The third eluant contained 3.4% lithium chloride and 1.5% lithium citrate and was pumped for 21 min. The fourth eluant contained 2.56% lithium chloride, 1.05% lithium citrate, and 0.18% lithium hydroxide and was pumped for 30 min, after which the initial eluant was used to re-equilibrate the column for 21.9 min. The column temperature was 33°C for the initial 25 min, then increased to 70°C for 85 min before being returned to 33°C for the final 20 min. The *o*-phthalaldehyde reagent (Pickering Laboratories) was mixed with column effluent at a rate of 0.3 mL/min and allowed to react for 10 s at 21°C prior to fluorescence detection with excitation at 330 nm and emission at 465 nm (HP 1046A Fluorescence Detector; Agilent Technologies, Santa Clara, CA).

Statistical Analysis

In addition to exclusion of all data from a sixth steer due to erratic feed intake, 2 observations from other steers (0 and 15 g/d of Lys) were lost due to problems not related to treatment.

Nitrogen balance and plasma metabolite data were analyzed statistically using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). The model contained the fixed effects of Lys and period, and steer was included as a random effect. Linear and quadratic effects of

Lys were were tested using single degree of freedom contrasts. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

Lysine requirements from the N retention and plasma Lys data were estimated from treatment means using a single slope, broken-line model (Robbins, 1986) and the Nonlinear procedure of SAS.

RESULTS AND DISCUSSION

Utilization of Lys for Protein Deposition

Intake, nitrogen balance and apparent diet digestibility data are presented in Table 3.2. Lysine supplementation did not affect ($P \geq 0.45$) DM or OM intake because steers were limit fed and feed refusals were absent or small. Apparent digestibilities of DM and OM were not altered ($P \geq 0.54$) by treatment and averaged 72.2 and 73.7%, respectively. These digestibilities were consistent with those reported by Löest et al. (2002) and Campbell et al. (1996, 1997) for a similar diet. Steers receiving no supplemental Lys had less fecal N output than other steers (quadratic $P < 0.01$); the difference, though significant, was neither large nor expected. Urinary N excretion was decreased from 32.3 to 24.3 g/d by increasing Lys supplementation to 9 g/d, with no further reduction when more than 9 g/d of Lys was supplied (linear and quadratic $P < 0.01$). Changes in total urinary N excretion were predominantly due to changes in urinary urea N, and urinary ammonia-N was not affected by treatment ($P \geq 0.21$). Increasing Lys supply from 0 to 9 g/d increased N retention from 21.4 to 30.7 g/d, with no further increase beyond 9 g/d of Lys (linear and quadratic $P < 0.01$). These N retention responses indicate that steers were deficient in Lys in our research model.

Break-point analysis (Figure 3.1A) estimated maximal N retention at 9.0 g/d supplemental Lys. Assuming that 1) responses to supplemental Lys up to 9 g/d are linear, 2) protein deposition is 6.25 × N retention, and 3) deposited protein contains 6.4% Lys (Ainslie

et al., 1993), the efficiency of Lys utilization between 0 to 9 g/d supplemental Lys was 40% (1.01 g retained N/g supplemental Lys × 6.25 g protein/g N × 0.064 g Lys/g protein).

This efficiency of Lys utilization is less than the efficiencies near 100% that can be calculated from the growth data of Klemesrud et al. (2000a, b). However, van Weerden and Huisman (1985) calculated an efficiency of 35% using N retention as a response variable in preruminant calves fed a milk replacer designed to be first limiting in Lys, and this represents an efficiency close to that from our study (40%). The Cornell Net Carbohydrate and Protein System as well as the NRC (1996) assume the same utilization efficiency value for all AA, and that efficiency is based only on the equivalent shrunk BW of the animal. The efficiency of AA utilization for cattle in our study (BW 165 kg) would be estimated by NRC (1996) to be 65%; our lower efficiency would question the validity of this prediction. The overestimation by the NRC (1996) for efficiency of utilization of supplemental AA has been noted previously for Met (Campbell et al., 1996). McCuistion et al. (2004) observed an efficiency of supplemental histidine utilization (65%) that was greater than observations for Leu (~41%; Awawdeh et al., 2006; Titgemeyer et al., 2012), but close to the NRC (1996) estimate for histidine utilization. The divergent estimated efficiencies among AA suggest that there are differences among AA in how efficiently they are used by cattle.

Our basal diet provided 7.1 g of intestinally absorbable Lys/kg DMI (Campbell et al., 1997), so based on intakes of 2.46 kg DM/d (Table 3.2) the basal supplies of absorbable Lys were 17.5 g/d. Steers receiving no supplemental Lys deposited 8.6 g Lys/d, which, assuming that maintenance requirements are 0, yields an efficiency of utilization of the basal Lys supplies of 49% (Table 3.3). If the x-axis in Fig. 3.1A is converted to total Lys supply by adding the basal supply of 17.5 g Lys/d to the supplemental amounts on the current x-axis, the regression line for N retention can be extrapolated to N retention of 0 to estimate the maintenance Lys requirements of the steers (assuming efficiency remains constant). This

extrapolation yields a negative estimate for the maintenance requirement for Lys (-3.4 g/d), which is infeasible for an essential AA. The observation that maintenance requirements could be calculated to be negative may be explained by overestimation of protein deposition by N retention, efficiencies of utilization of the basal Lys that are greater than the efficiencies of utilization of the supplemental Lys, or both. Comparisons of N balance and serial slaughter techniques have demonstrated that N retention generally overestimates protein deposition for growing cattle by 10 to 17% (Gerrits et al., 1996).

The Lys maintenance requirement for our steers based on equations of O'Connor et al. (1993) was 9.9 g/d (Table 3.3). Using this estimate, 7.6 g Lys/d (basal supplies of 17.5 g/d minus 9.9 g/d for maintenance) would be available for growth when no supplemental Lys was provided, which is inconsistent with our estimate that these steers were depositing 8.6 g Lys/d (Table 3.3); estimates of efficiency of utilization above 100% are not feasible for an essential AA. Although protein deposition was calculated directly from N retention, N retention measurements would include scurf losses as part of retained N. As such, the scurf loss should be subtracted from N retention before using the relationship between N retention and Lys supply to estimate maintenance requirements. In practice, this correction will have little effect because scurf losses represent less than 4% of the estimated requirement.

Using an approach similar to our study, Löest et al. (2001) observed that the efficiency of utilization of the basal supplies of Leu was greater than 100% when estimates for maintenance requirements were based on O'Connor et al. (1993). Similar calculations based on data of Campbell et al. (1997) for Met and of McCuistion et al. (2004) for His yield similar conclusions. Taken as a whole, the calculations discussed here and presented in Table 3.3 can be interpreted to suggest for Lys, as well as for Leu, Met, and His, that the maintenance requirements of growing cattle are less than estimated by O'Connor et al. (1993). The observation that the efficiency of utilization of the basal Lys was greater than 100% may be

explained by some combination of 1) overestimation of maintenance requirements by O'Connor et al. (1993), 2) overestimation of protein deposition by N retention, and 3) efficiencies of utilization of the basal Lys that are greater than the utilization of the supplemental Lys (i.e., the efficiency of use above maintenance may not be a constant, although constant efficiencies have been observed for supplemental Lys [this study], Met [Campbell et al., 1997], and Leu [Awawdeh et al., 2005]).

Plasma Metabolites and AA

Effects of supplemental Lys on plasma metabolites and AA are shown in Table 3.4. Plasma urea N tended to be decreased linearly ($P = 0.06$) by Lys supplementation in agreement with the reduction in urinary urea N excretion, but plasma glucose was unaffected ($P \geq 0.26$) by Lys supplementation.

Plasma AA profiles are affected by different factors, which make it difficult to interpret the net result. Usually, an increase in plasma concentration of any essential AA in response to its supplementation would signify that the supply exceeds protein synthetic capacity as dictated by the first-limiting AA (Bergen, 1979). In an opposite way, a decrease in plasma concentrations of other AA, when a first-limiting AA is provided, would imply greater utilization for synthetic purposes (Gibb et al., 1992), because supplementation of the limiting AA should lift previous restrictions that the basal diet may have imposed on protein synthesis (Wessels et al., 1997).

Plasma Lys concentrations increased linearly ($P < 0.001$) with Lys supplementation. Plasma Ly remained low when 3 g/d Lys was infused and then increased with greater levels of Lys supplementation. Break-point analysis of plasma Lys concentrations (Figure 3.1B) estimated the supplemental Lys requirement of these steers to be 3.12 g/d, considering that that levels of an AA will increase in plasma once the needs for that AA have been exceeded (Bergen, 1979). The difference between requirements estimated from N retention data and

plasma Lys data suggest that plasma Lys may be a poor indicator of requirements because its plasma concentration increased at supplementation levels that were clearly less than the amount needed to maximize protein deposition. This might suggest that increases in plasma Lys are associated with the mechanisms necessary to increase protein deposition in cattle, and this linkage of the protein deposition response to elevated concentrations of Lys may be a key to explaining why cattle use AA relatively inefficiently; elevated concentrations of AA would presumably increase their oxidation at the same time that they are needed to support greater protein deposition.

Plasma α-aminoadipic acid increased linearly (P = 0.02) in response to Lys supplementation. Mammalian catabolism of lysine occurs from 2 different routes, the pipecolate pathways predominates in adult brain, whereas the saccharopine pathway is the predominant lysine degradative pathway in extracerebral tissues (Hallen et al., 2013). The main pathway yields α-aminoadipic acid as one of the intermediates (Voet et al., 2013); thus, the increase in plasma α-aminoadipic acid probably reflects an increase in Lys catabolism as more Lys was supplied. Even when Lys was deficient, 60% of the supplemental Lys was not used for protein deposition (i.e., the efficiency of use was 40%), such that production of α-aminoadipic acid would be expected to increase with all levels of Lys supplementation.

Lysine supplementation linearly decreased plasma concentrations of Leu, Ser, Tyr, and and Val ($P \leq 0.02$), and tended ($P \leq 0.07$) to linearly decrease Asn and Phe, probably reflecting the increased uptake and use of these AA for protein deposition. Previous studies also have observed that supplementation with the most limiting AA decreases plasma concentrations of some of the other AA as protein retention is increased (Campbell et al., 1997; Wessels et al., 1997; Schroeder et al., 2006). Regarding Leu, Ser, and Val, decreases in plasma concentrations of these AA in response to supplementation of a limiting AA were also observed in growing

steers limited by Met (Campbell et al., 1996; Awawdeh et al., 2004) or His (McCuistion et al., 2004).

On the other hand, plasma concentrations of Glu were linearly increased ($P = 0.04$) and Ala and Arg tended ($P \leq 0.08$) to linearly increase in response to Lys supplementation. The higher concentrations of Ala with levels of Lys possibly reflect decreases in Ala oxidation or increases in Ala synthesis via transamination reactions. Similarly, the increases in Glu might reflect less utilization of Glu for urea production because Lys supplementation decreased urea production, although increases in synthesis of Glu cannot be excluded as a possible explanation. Lysine and Arg are absorbed in the small intestine and kidney by the same transporters (Closs et al., 2004), and thus greater supplies of Lys could theoretically compete with Arg for transport. However, Abe et al. (1998) observed that antagonism between Lys and Arg did not occur in calves receiving excess Lys, demonstrated by increases in plasma Arg concentrations; in this regard, our study is in agreement with Abe et al. (1998).

CONCLUSIONS

Under our experimental conditions, Lys supplementation promoted significant increases in N retention, demonstrating that our research model was limiting in this AA. The efficiency of utilization of supplemental Lys for whole-body protein deposition was 40%. Thus, our findings suggest that the efficiency of Lys utilization by growing cattle is somewhat less than would be predicted by NRC (1996).

LITERATURE CITED

Abe, M., T. Iriki, and M. Funaba. 1997. Lysine deficiency in post-weaned calves fed corn and corn gluten meal diets. J. Anim. Sci. 75:1974–1982.

Abe, M., T. Iriki, M. Funaba, and S. Onda. 1998. Limiting amino acids for a corn and soybean meal diet in weaned calves less than three months of age. J. Anim. Sci. 76:628–636.

Ainslie, S. J., D. G. Fox, T. C. Perry, D. J. Ketchen, and M. C. Barry. 1993. Predicting amino acid adequacy of diets fed to Holstein steers. J. Anim. Sci. 71:1312-1319.

Awawdeh, M. S., E. C. Titgemeyer, K. C. McCuistion, and D. P. Gnad. 2004. Effects of ammonia load on methionine utilization by growing steers. J. Anim. Sci. 82:3537-3542.

Awawdeh, M. S., E. C. Titgemeyer, K. C. McCuistion, and D. P. Gnad. 2005. Ruminal ammonia load affects leucine utilization by growing steers. J. Anim. Sci. 83:2448-2454.

Awawdeh, M. S., E. C. Titgemeyer, G. F. Schroeder, and D. P. Gnad. 2006. Excess amino acid supply improves methionine and leucine utilization by growing steers. J. Anim. Sci. 84:1801-1810.

Bergen, W. G. 1979. Free amino acids in blood of ruminants-physiological and nutritional regulation. J. Anim. Sci. 49:1577-1589.

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

Burris, W. R., J. A. Boling, N. W. Bradley, and A. W. Young. 1976. Abomasal lysine infusion in steers fed a urea supplemented diet. J. Anim. Sci. 42:699-705.

Campbell, C. G., E. C. Titgemeyer, and G. St-Jean. 1996. Efficiency of D- vs L-methionine utilization by growing steers. J. Anim. Sci. 74:2482-2487.

Campbell, C. G., E. C. Titgemeyer, and G. St-Jean. 1997. Sulfur amino acid utilization by growing steers. J. Anim. Sci. 75:230-238.

Closs E., A. Simon, N. Vekony, and A. Rotmann. 2004. Plasma membrane transporters for arginine. J. Nutr. 134:2752S–2759S.

Fenderson, C. L., and W. G. Bergen. 1975. An assessment of essential amino acid requirements of growing steers. J. Anim. Sci. 41:1759-1766.

Gerrits, W. J. J., G. H. Tolman, J. W. Schrama, S. Tamminga, M. W. Bosch, and M. W. A. Verstegen. 1996. Effect of protein and protein-free energy intake on protein and fat deposition rates in preruminant calves of 80 to 240 kg live weight. J. Anim. Sci. 74:2129–2139.

Gibb, D. J., T. J. Klopfenstein, R. A. Britton, and A. J. Lewis. 1992. Plasma amino acid response to graded levels of escape protein. J. Anim. Sci. 70:2885−2892.

Gochman, N., and J. Schmitz. 1972. Application of new peroxidase indicator reaction to the specific automated determination of glucose with glucose oxidase. Clin. Chem. 18:943–950.

Greenwood, R. H., and E. C. Titgemeyer. 2000. Limiting amino acids for growing Holstein steers limit-fed soybean hull-based diets. J. Anim. Sci. 78:1997–2004.

Hallen, A., J. Jamie, and A. Cooper. 2013. Lysine metabolism in mammalian brain: an update on the importance of recent discoveries. Amino Acids 6:1249-1272.

Heger, J., and Z. Frydrych. 1989. Efficiency of utilization of amino acids. In: M. Friedman, editor, Absorption and Utilization of Amino Acids. CRC Press, Boca Raton, FL. p. 31-56.

Hill, G. M., J. A. Boling, and N. W. Bradley. 1980. Postruminal lysine and methionine infusion in steers fed a urea-supplemented diet adequate in sulfur. J. Dairy Sci. 63:1242-1247.

Klemesrud, M. J., T. J. Klopfenstein, and A. J. Lewis. 2000a. Metabolizable methionine and lysine requirements of growing cattle. J. Anim. Sci. 78:199-206.

Klemesrud, M. J., T. J. Klopfenstein, R. A. Stock, A. J. Lewis, and D. W. Herold. 2000b. Effect of dietary concentration of metabolizable lysine on finishing cattle performance. J. Anim. Sci. 78:1060-1066.

Lambert, B. D., E. C. Titgemeyer, C. A. Löest, and D. E. Johnson. 2004. Effect of glycine and vitamin supplementation on sulphur amino acid utilization by growing cattle. J. Anim. Physiol. Anim. Nutr. (Berl.) 88:288-300.

Löest, C. A., E. C. Titgemeyer, B. D. Lambert, and A. M. Trater. 2001. Branched-chain amino acids for growing cattle limit-fed soybean hull-based diets. J. Anim. Sci. 79:2747–2753.

Löest, C. A., E. C. Titgemeyer, G. St-Jean, D. C. Van Metre, and J. S. Smith. 2002. Methionine as a methyl group donor in growing cattle. J. Anim. Sci. 80:2197-2206.

Marsh, W. H., F. Benjamin, and H. Miller. 1965. Automated and manual direct methods for determination of blood urea. Clin. Chem. 11:624–627.

McCuistion, K. C., E. C. Titgemeyer, M. S. Awawdeh, and D. P. Gnad. 2004. Histidine utilization by growing steers is not negatively affected by increased supply of either ammonia or amino acids. J. Anim. Sci. 82:759-769.

Moloney, A. P., D. H. Beermann, D. Gerrard, T. F. Robinson, and K. D. Finnerty. 1998. Temporal change in skeletal muscle IGF-I mRNA abundance and nitrogen metabolism responses to abomasal casein infusion in steers. J. Anim. Sci. 76:1380-1388.

NRC. 1996. Nutrient requirements of beef cattle. 7th ed. Natl. Acad. Press, Washington, DC.

O'Connor, J. D., C. J. Sniffen, D. G. Fox, and W. Chalupa. 1993. A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. J. Anim. Sci. 71:1298-1311.

Owens, F. N., and J. E. Pettigrew. 1989. Subdividing amino acid requirements into portions for maintenance and growth. In: M. Friedman (Ed.). Absorption and Utilization of Amino Acids. pp 15-30. CRC Press, Inc., Boca Raton, FL.

Richardson, C. R., and E. E. Hatfield. 1978. The limiting amino acids in growing cattle. J. Anim. Sci. 46:740-745.

Robbins, K. R. 1986. A method, SAS program, and example for fitting the broken-line to growth data. Univ. of Tennessee Agric. Exp. Sta. Res. Rep. 86-09.

Schroeder, G. F., E. C. Titgemeyer, M. S. Awawdeh, J. S. Smith, and D. P. Gnad. 2006. Effects of energy source on methionine utilization by growing steers. J. Anim. Sci. 84:1505-1511.

Storm, E., and E. R. Ørskov. 1984. The nutritive value of rumen micro-organisms in ruminants. Br. J. Nutr. 52:613-620.

Titgemeyer, E. C. 2003. Amino acid utilization by growing and finishing ruminants. In: J. P. F. D'Mello, editor, Amino acids in animal nutrition, 2nd ed. CAB International, Wallingford, UK. p. 329-346.

Titgemeyer, E. C., K. S. Spivey, S. L. Parr, D. W. Brake, and M. L. Jones. 2012. Relationship of whole body nitrogen utilization to urea kinetics in growing steers. J. Anim. Sci. 90:3515-3526.

van Weerden, E. J., and J. Huisman. 1985. Amino acid requirement of the young veal calf. Z. Tierphysiol. Tierernahr. Futtermittelkd. 53:232-244.

Voet, D., J. Voet, and C. Pratt. 2013. Principles of Biochemistry. 4th ed. John Wiley & Sons Inc. Hoboken, NJ.

Wessels, R. H., E. C. Titgemeyer, and G. St-Jean. 1997. Effect of amino acid supplementation on whole-body protein turnover in Holstein steers. J. Anim. Sci. 75:3066–3073. doi:/1997.75113066x

Table 3.1 Composition of the diet fed to growing steers

Item	% of DM
Ingredient	
Pelleted soybean hulls	81.7
Wheat straw	8.1
Cane molasses	4.8
Calcium phosphate (22% P)	2.07
Sodium bicarbonate	1.31
Calcium carbonate	1.09
Magnesium oxide	0.44
Trace mineral salt[1]	0.22
Vitamin premix[2]	0.14
Sulfur	0.11
Se premix[3]	0.011
Bovatec-91[4]	0.018
Nutrient	
DM, %	89.3 $\pm$ 0.07
OM, % of DM	90.1 $\pm$ 0.17
N, % of DM	1.40 $\pm$ 0.02

[1] Composition > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.

[2] Provided 5,300 IU vitamin A/kg diet DM, 3,593 IU vitamin D/kg diet DM, and 48 IU vitamin E/kg diet DM.

[3] Provided 0.065 mg Se/kg diet DM from sodium selenite.

[4] Supplied 36 mg lasalocid/kg diet DM (Zoetis, Florham Park, NJ).

Table 3.2 Effects of Lys supplementation on diet digestion and N balance in growing steers

	Supplemental L-Lys, g/d							*P*-value	
Item	0	3	6	9	12	15	SEM[1]	Linear	Quadratic
n	4	5	5	5	5	4			
DMI,[2] kg/d	2.46	2.38	2.49	2.50	2.46	2.48	0.05	0.45	0.76
OM intake,[2] kg/d	2.22	2.14	2.25	2.25	2.21	2.23	0.05	0.51	0.77
N, g/d									
Infused[3]	36.1	36.7	37.3	37.9	38.4	39.0	-	-	
Dietary intake[4]	38.8	38.3	39.2	39.4	39.1	39.2	0.52	0.28	0.75
Total intake	74.9	75.0	76.5	77.3	77.5	78.3	0.52	<0.001	0.75
Fecal excretion	21.0	22.1	22.9	22.2	22.2	22.0	0.82	0.15	<0.01
Urinary	32.3	29.1	26.6	24.3	25.5	25.8	1.6	<0.001	<0.001
Urea	21.6	19.1	16.1	14.8	15.1	14.2	2.0	<0.001	0.05
Ammonia	0.83	0.81	0.75	0.56	0.64	0.71	0.12	0.21	0.32
Retained	21.4	23.8	27.0	30.7	29.8	30.6	1.2	<0.001	<0.01
Diet apparent digestibility,[5]									
DM	72.7	71.9	71.5	72.1	72.6	72.2	1.9	0.96	0.54
OM	74.2	73.4	73.5	73.4	74.0	73.6	1.9	0.86	0.66

[1] For n = 4.

[2] Amount provided by the diet (does not include amounts provided by infusions).

[3] Amount provided by AA.

[4] Dietary N plus N from ruminally infused urea.

[5] Based on dietary intake (infusions not considered)

Table 3.3 Estimates of the efficiency of lysine deposition above maintenance for steers receiving no supplemental lysine

Item	Lysine[1]
Basal supply[2], g/d	17.5
CP deposited[3], g/d	133.8
Lysine deposited[4], g/d	8.6
Gross efficiency[5], %	49
Maintenance requirement[6], g/d	9.9
Available for gain[7], g/d	7.6
Efficiency above maintenance[8], %	113

[1] Estimates for intestinally absorbed Lys supply to steers receiving no supplemental Lys. Supply = 2.46 kg DMI/d × 7.1 g Lys/kg DMI (Campbell et al., 1997).

[2] Based on measures of absorbable AA supplies for soybean hull-based diets (Campbell et al., 1997).

[3] Nitrogen retention (21.4 g/d) × 6.25.

[4] CP deposited × 0.064 (Lys concentration in tissue protein; Ainslie et al., 1993).

[5] (Lys deposited/Lys available) × 100%, where Lys available = basal supply.

[6] Represents the sum of scurf ($0.032 \times 0.2 \times BW^{0.6}/0.40$), endogenous urine ($0.067 \times 2.75 \times BW^{0.5}/0.40$), and metabolic fecal ($0.064 \times 0.09 \times$ g/d of indigestible DM) requirements for 165-kg steers (O'Connor et al., 1993), where 0.032 and 0.064 represent the concentration of Lys in keratin and tissue proteins, respectively. Indigestible DM was based on observed fecal DM output.

[7] Difference between basal supplies and maintenance requirements.

[8] (Lys deposited/Lys available) × 100%, where Lys available = Lys available for gain. This estimate of 113% is not feasible for an essential AA, and it likely reflects an overestimation of the maintenance requirement, an overestimation of lysine deposition based on N retention, or both.

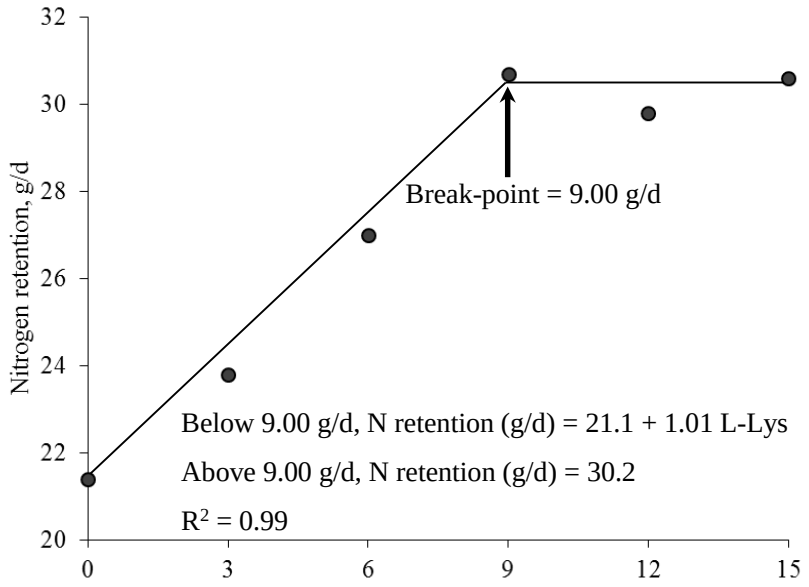
Table 3.4 Effects of Lys supplementation on plasma metabolite concentrations of growing steers

	Supplemental L-Lys, g/d							*P*-value	
Item	0	3	6	9	12	15	SEM[1]	Linear	Quadratic
n	4	5	5	5	5	4			
Plasma									
Glucose, m*M*	5.59	5.64	5.62	5.74	5.78	5.36	0.22	0.74	0.26
Urea, m*M*[1]	2.00	2.17	1.49	1.57	2.07	1.47	0.21	0.06	0.55
AA, µ*M*									
Lys	34.2	35.7	47.8	72.1	99.0	99.2	8.1	<0.001	0.44
α-aminoadipic acid	2.32	2.53	1.82	3.04	4.08	3.02	0.53	0.02	0.86
Ala	197.8	204.3	223.6	221.6	208.6	226.3	12.5	0.06	0.34
Arg	83.7	83.8	93.8	102.7	111.4	95.8	10.3	0.08	0.32
Asn	37.7	35.6	32.1	30.1	32.2	31.6	3.3	0.06	0.18
Asp	13.8	13.5	12.3	15.1	13.7	14.4	1.4	0.52	0.64
Citrulline	71.4	70.7	68.5	71.5	74.5	71.9	10.2	0.64	0.75
Glu	99.2	85.2	95.8	102.0	103.8	112.1	7.9	0.04	0.21
Gln	307.5	328.9	327.5	312.6	309.2	325.5	27.3	0.93	0.87
Gly	668.7	699.3	653.8	671.3	638.8	650.0	60.2	0.56	0.92
His	86.0	91.1	80.1	82.8	79.5	79.2	5.8	0.15	0.94
Ile	93.7	78.4	84.0	82.2	83.4	77.6	6.9	0.26	0.62
Leu	92.9	70.9	74.5	69.7	72.9	64.0	6.1	0.01	0.24
Met	47.3	42.0	45.2	44.2	40.7	42.3	5.7	0.51	0.87
Ornithine	63.3	60.1	69.8	77.9	84.2	70.1	12.4	0.17	0.41
Phe	99.4	83.9	91.3	84.3	89.7	75.9	6.5	0.07	0.99

Ser	137.4	122.4	100.3	88.1	92.3	91.9	8.7	<0.001	<0.01
Taurine	52.1	41.9	44.8	49.4	43.1	41.1	4.4	0.14	0.88
Thr	163.3	184.0	167.4	159.4	162.2	159.1	19.1	0.42	0.72
Trp	86.1	75.8	79.6	75.4	82.4	68.2	7.4	0.21	0.91
Tyr	91.1	79.1	65.7	61.2	64.5	60.2	6.9	<0.001	0.04
Val	330.6	285.3	287.2	274.5	279.5	258.3	17.5	0.02	0.38
Total AA	2,854	2,775	2,747	2,750	2,770	2,718	176	0.60	0.81

[1] For n = 4.

A



B

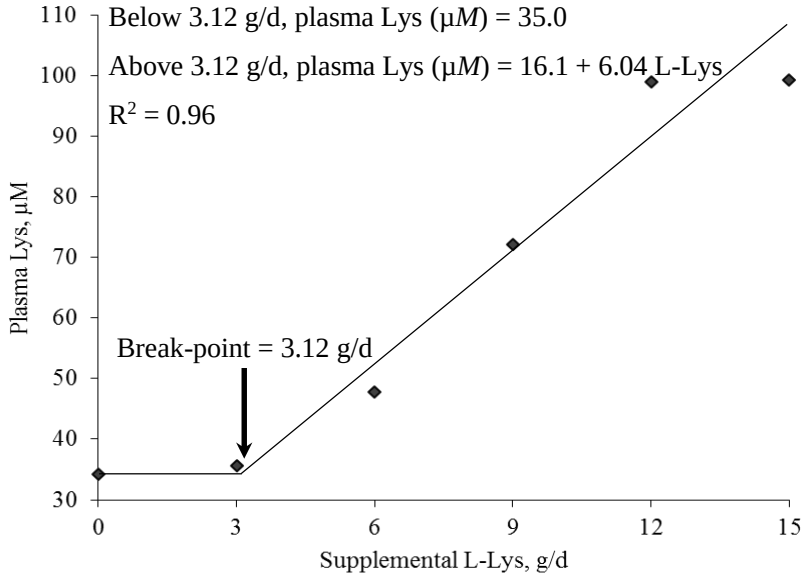


Figure 3.1 Nitrogen retention (A) and plasma Lys concentrations (B) of steers abomasally infused with L-Lys. Single-slope break-point analysis estimate of supplemental Lys requirement was 9.00 g/d based on N retention, but only 3.12 g/d based on plasma Lys.

APPENDIX

UNIVERSIDADE FEDERAL DE VIÇOSA
CENTRO DE CIÊNCIAS AGRÁRIAS
DEPARTAMENTO DE ZOOTECNIA

Campus Universitário – Viçosa, MG – 36570-000 – Telefone: (31) 3899.2262 – Fax: (31) 3899.2275 – e-mail: dzo@ufv.br

Comitê de Ética para Uso de Animais/DZO

Viçosa, 3 de abril de 2012

CERTIFICADO

O Comitê de Ética para Uso de Animais do Departamento de Zootecnia da Universidade Federal de Viçosa certifica que o **processo nº 16/2012**, intitulado "**Avaliação nutricional e metabólica em bovinos alimentados com forragem tropical de baixa qualidade recebendo suplementação protéica ruminal e diferentes níveis de suplementação abomasal**", coordenado pelo **Prof(a). Edenio Detmann**, está de acordo com os princípios éticos da experimentação animal, estabelecido pelo Colégio Brasileiro de Experimentação Animal e com a legislação vigente, tendo sido aprovado por este Comitê em **03/Abr/2012**.

CERTIFICATE

The Ethic Committee in Animal Use of Animal Science Department/Universidade Federal de Viçosa certify that the **process number 16/2012**, named "**Nutritional and metabolic assessment in cattles fed low-quality tropical forage ruminal receiving ruminal protein supplementation and abomasal supplementation different levels**", coordinated by **Prof(a). Edenio Detmann**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA) and with actual Brazilian legislation. This Institutional Committee approved this process on **Apr, 3rd, 2012**.

Marcos Inácio Marcondes
Presidente do CEUA/DZO/UFV

TO: Evan Titgemeyer
ASI
132 Call Hall

Protocol Number: 3403

FROM: Sally Olson, Chair
Institutional Animal Care and Use Committee

DATE OF APPROVAL: 03/18/2014
DATE OF EXPIRATION: 03/18/2017

RE: Approval of Animal Care and Use Protocol Entitled, "Efficiency of lysine utilization by growing cattle."

The Institutional Animal Care and Use Committee (IACUC) for Kansas State University has reviewed the protocol identified above and has approved it for three years from the date of this memo. During the period of approval, the protocol will be subject to annual monitoring, which may include the examination of records connected with the project. Announced post-approval monitoring (PAM) may be performed during the course of this approval period by a member of the University Research Compliance Office staff. Changes in the protocol affecting the care or use of animals must be reviewed by the IACUC prior to implementation. Unanticipated problems related to the humane care or use of animals must be reported to the IACUC immediately.

It is important that your animal care and use project is consistent with submissions to funding/contract entities. It is your responsibility to initiate notification procedures to any funding/contract entity of any changes in your project that affects the use of animals.