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Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage[1]

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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 and 80% NDF, DM basis). Five ruminally and abomasally cannulated Nellore heifers (248 ± 9 kg) were used in a 5 × 5 Latin square. Treatments were the 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 ruminally dosed 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 linearly increased 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 (9%) 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

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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). Therefore, 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. Therefore, nutritional systems should 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 number 016/2012). 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 initial BW, 239 ± 9 kg final BW, and 248 ± 9 kg 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, and 0.022 g/kg Se; Tecnophós cria; Vaccinar, Belo Horizonte, Minas Gerais, Brazil).

Table 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]NDFap = NDF corrected for ash and protein.

The basal diet consisted of a low-quality signal grass hay (*Brachiaria decumbens* Stapf.; Table 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 midwest region of Brazil.

Treatments

Treatments were the 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**, respectively). The RDP and RUP requirements were calculated according to the Brazilian Nutrient Requirements for Zebu Beef Cattle system (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, respectively.

The RDP supplement was intraruminally administered 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). 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) 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 mixing them with water to a total weight of 4.5 kg. The solution was continuously infused 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 17 d, with 9 d for supplement adaptation and 8 d for sample collection. Heifers were weighed at the beginning and at the end of each experimental period.

Dry Matter Intake. 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 (60°C) 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. and 1.78 mm o.d.; Tygon, S-54-HL; Buch and 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 percent 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 and Company, Franklin Lakes, NJ) containing heparin (143 IU), placed in ice water immediately after collection, and centrifuged (1,200 × *g* for 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 were 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 (number 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 thoroughly mixed, 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 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 –20°C 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 the small-particle phase. In all procedures (the fluid plus the small particle phase, and the particle phase), samples were weighed, frozen at –80°C, freeze-dried, and ground as previously described. Samples of abomasal digesta related to each phase (the fluid plus the small particle phase, and the 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 represented 72 to 82 h of label infusion, during which the isotopic enrichment of ^{15}N reached a 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. The ruminal pool of fiber was measured at 1000 h (4 h after morning feeding) on d 15 and at 0600 h (just before morning feeding) on d 17 of each period (Allen and Linton, 2007). Whole ruminal contents were manually evacuated through the ruminal cannula, placed into a plastic container, weighed, hand mixed, and subsampled 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 number G-003/1), ash (complete combustion in a muffle furnace at 600°C for 4 h; method INCT-CA number M-001/1), N (Kjeldahl procedure; method INCT-CA number N-001/1), ether extract (Randall procedure; method INCT-CA number G-005/1), NDF corrected for ash and protein (using a heat-stable α-amylase, omitting sodium sulfite and correcting for residual ash and protein; method INCT-CA number F-002/1), and ADL (method INCT-CA number F-005/1). Casein samples were analyzed for only 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, VIC,

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 colorimetrically quantified with an AutoAnalyzer (Technicon Analyzer II; Technicon Industrial Systems, Buffalo Grove, IL). Total urinary N was obtained by the Kjeldahl procedure (method INCT-CA number N-001/1). Urinary 3-methylhistidine concentrations were measured by cation-exchange HPLC with postcolumn *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 colorimetrically quantified 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 NH3 (from wet samples) concentrations were quantified using the colorimetric technique described by Detmann et al. (2012; method INCT-CA number N-006/1). For VFA analysis, rumen fluid samples collected over time were pooled (2.0 mL) and centrifuged (12,000 × *g* for 10 min at 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). Microbial 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) variance or covariance matrix, which was chosen based on a 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

Table 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]	CON vs. SUP	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 = 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, respectively.

[2]For $n = 4$.

[3]CON vs. SUP = 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.

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 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 linearly increased ($P < 0.001$) 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 ($P < 0.001$) postruminal CP digestibility and tended ($P = 0.09$) to increase

Table 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]	CON vs. SUP	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 = 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, respectively.

[2]For $n = 4$.

[3]CON vs. SUP = control vs. average of all supplements. Linear and quadratic represent effects of RUP.

postruminal OM digestion. Increasing RUP supplementation linearly increased postruminal digestion of CP ($P < 0.001$) and OM ($P = 0.02$). Supplementation did not affect ($P \geq 0.63$) postruminal 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 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 4). Protein supplementation decreased ($P \leq 0.001$) the 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, respectively), 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 5). The RAN concentrations were different among sampling times ($P < 0.01$) for all treatments, except for the 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 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 linearly increased ($P = 0.04$) but urinary N excretion was not affected ($P \geq 0.14$). Urinary urea N excretion, 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 by RUP supplementation ($P > 0.20$), 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 ($P <$

Table 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]	CON vs. SUP	Linear	Quadratic
n	5	4	5	4	5				
VFA, mM	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 = 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, respectively.

[2]For $n = 4$.

[3]CON vs. SUP = control vs. average of all supplements. Linear and quadratic represent effects of RUP.

0.001) by supplementation and linearly increased ($P < 0.001$) as RUP levels increased. Protein supplementation improved N retention ($P < 0.001$), which was also linearly increased ($P < 0.001$) 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 linearly improve ($P = 0.08$) N retention as a fraction of N intake.

The ruminal N balance was increased ($P < 0.001$) by supplementation, but it linearly decreased ($P < 0.001$) 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 linearly increased ($P = 0.03$) 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 7). Moreover, UER was linearly increased ($P = 0.02$) and GER tended to linearly increase ($P = 0.07$) 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 urinary 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 ($P = 0.006$) 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 7). The fraction of MN from MNU was lower ($P < 0.001$) for the supplemented heifers (10%) compared with the 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 8). The ratio of urinary 3-methylhistine:creatinine, an indicator of skeletal muscle protein breakdown, was greater ($P <$

Table 5. Effects of RDP supplementation and provision of RUP on ruminal ammonia 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 = 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, respectively.

[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.

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 8). Renal clearance of creatinine did not differ ($P \geq 0.76$) 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 different 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., 2014b). The maximum forage intake is observed with dietary CP:DOM at 210 to 280 g/kg (Poppi and McLennan, 1995; Detmann et al., 2014b). The dietary CP:DOM for control and supplemented heifers were, on average, 136 and 404 g/kg, respectively. Therefore, they both showed an 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 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). Therefore, 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

Table 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]	CON vs. SUP	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 = 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, respectively.

[2]For $n = 4$.

[3]CON vs. SUP = 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.

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. (2014b) 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

Table 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]	CON vs. SUP	Linear	Quadratic
n	5	4	5	4	5				
Urea N kinetics, g N/d									
Urea N 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
Urea N returned to the ornithine cycle (ROC)	11.4	31.6	30.7	31.0	35.3	3.0	<0.001	0.34	0.33
Urea N utilized for anabolism (UUA)	12.9	15.9	18.6	16.3	24.7	3.3	0.07	0.08	0.34
Urea N excreted in feces (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 = 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, respectively.

[2]For $n = 4$.

[3]CON vs. SUP = control vs. average of all supplements. Linear and quadratic represent effects of RUP.

[4]GIT = gastrointestinal tract.

[5]UUE = urinary urea N excretion. Data for UUE are presented in Table 6.

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 negative only for the unsupplemented heifers, which is in agreement with Detmann et al. (2014b), 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, therefore, N recycling is important to sustain ruminal microbial growth (Detmann et al., 2014b).

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., 2014a). 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 heifers. 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

Table 8. Effectsof 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]	CON vs. SUP	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 = 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, respectively.

[2]For $n = 4$.

[3]CON vs. SUP = control vs. average of all supplements. Linear and quadratic represent effects of RUP.

[4]UER = urea N entry rate.

the rumen. Therefore, 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., 2014b). 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. (2014b).

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. Therefore, 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% of 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 linearly increased 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 makes up 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). Therefore, 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. Therefore, 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 toward 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 the control (52 vs. 34%, respectively) supports that protein supplementation stimulates NH_3 absorption 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 resynthesis 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; Table 7) was similar to others trials evaluating cattle receiving protein supplementation (Wickersham et al., 2009a; Bailey et al., 2012a,b), 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. Therefore, 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 MNU observed in our research agrees with observations of Marini and Van Amburgh (2003) and Bailey et al. (2012a), who found that protein supplementation decreased MNU as a proportion of GER. According to Bailey et al. (2012a), 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 used 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, consequently, 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%) was 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., 2012b). 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 3 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 ($P > 0.13$) 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 was 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., 2012a,b; Rufino, 2015) or tryglicerides (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. doi:10.2527/jas.2006-418.

Bailey, E. A., E. C. Titgemeyer, K. C. Olson, D. W. Brake, M. L. Jones, and D. E. Anderson. 2012a. Effects of ruminal casein and glucose on forage digestion and urea kinetics in beef cattle. J. Anim. Sci. 90:3505–3514. doi:10.2527/jas.2011-4459.

Bailey, E. A., E. C. Titgemeyer, K. C. Olson, D. W. Brake, M. L. Jones, and D. E. Anderson. 2012b. Effects of supplemental energy and protein on forage digestion and urea kinetics in growing beef cattle. J. Anim. Sci. 90:3492–3504. doi:10.2527/jas.2011-4458.

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

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

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. doi:10.2527/jas.2009-2641.

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

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. doi:10.3168/jds.S0022-0302(80)82888-8.

Cecava, M. J., N. R. Merchen, L. C. Gay, and L. L. Berger. 1990. Composition of ruminal bacteria harvest from steers as influenced 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. R. Bras. Zootec. 40:2815–2826. doi:10.1590/S1516-35982011001200028.

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. doi:10.1016/j.livsci.2009.06.013.

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., S. C. Valadares Filho, M. F. Paulino, and P. Huhtanen. 2014a. Nutritional aspects applied to grazing cattle in the tropics: A review based on Brazilian results. Semina: Cienc. Agrar. 35:2829–2854.

Detmann, E., E. E. L. Valente, E. D. Batista, and P. Huhtanen. 2014b. An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. Livest. Sci. 162:141–153. doi:10.1016/j.livsci.2014.01.029.

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. doi:10.1071/AR9650169.

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. p. 167–186.

France, J., and R. C. Siddons. 1986. Determination of digesta flow by continuous marker infusion. J. Theor. Biol. 121:105–120. doi:10.1016/S0022-5193(86)80031-5.

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. doi:10.3168/jds.S0022-0302(80)83132-8.

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. doi:10.1016/j.anifeedsci.2014.12.009.

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. doi:10.4141/cjas80-030.

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. doi:10.3168/jds.2010-3067.

Lapierre, H., and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84(Suppl. E):E223–E236. doi:10.3168/jds.S0022-0302(01)70222-6.

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. R. Bras. Zootec. 42:664–674. doi:10.1590/S1516-35982013000900009.

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. R. Bras. Zootec. 38:2021–2030. doi:10.1590/S1516-35982009001000024.

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. doi:10.3181/00379727-143-37470.

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. doi:10.3168/jds.S0022-0302(05)73091-5.

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. doi:10.2527/jas.2007-0475.

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. doi:10.2527/jas.2013-7364.

Rufino, L. M. A. 2011. Ruminal and/or abomasal nitrogenous supplementation in cattle fed tropical forage. MS 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. doi:10.1007/s11250-010-9581-7.

Siegfried, R., H. Ruckemann, and G. Stumpf. 1984. Method for the determination of organic acids in silage by high performance liquid chromatography. Landwirtsch. 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. doi:10.1016/S0301-6226(97)00138-3.

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. Departamento de Zootecnia – Universidade Federal de Vicosa, 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. R. Bras. Zootec. 40:2565–2573. doi:10.1590/S1516-35982011001100039.

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. doi:10.1016/j.anifeedsci.2004.03.005.

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. doi:10.1017/S1751731108003704.

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. doi:10.1017/S0007114508995672.

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. doi:10.2527/jas.2007-0325.