**CRISTHIANE VIOL RIBEIRO DE OLIVEIRA**

# UREA SUPPLEMENTATION IN RUMEN AND POST-RUMEN FOR CATTLE FED A LOW-QUALITY TROPICAL FORAGE

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.*

Adviser: Edenio Detmann

Co-Adviser: Erick Darlisson Batista

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Assent:

Cristhiane Viol Ribeiro de Oliveira
Author

Edenio Detmann
Adviser

**DEDICATION**

I dedicated my Ph.D. to my brother Victor, who during two of these four years, fought his most difficult fight and overcame all challenges. You inspire me. Thank you for all the teaching. I love you forever, “my miracle”.

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## BIOGRAPHY

Cristhiane Viol Ribeiro de Oliveira, daughter of Danilo Pires Ribeiro de Oliveira and Laura de Fatima Viol Ribeiro de Oliveira, was born in Conselheiro Lafaiete, Minas Gerais, Brazil, in 16$^{th}$of August, 1991. She started the undergrad in Animal Science at *Universidade Federal de Lavras* in 2009, and obtained a Bachelor of Science degree in Animal Science in 2014.

She started the Master's program in August 2014, with major in ruminant nutrition and production at the same University, submitting your dissertation defense on 26$^{th}$ of February, 2016.

In February 2016, she started the Doctorate program in Animal Science with a major in ruminant nutrition and production, working on nitrogen metabolism in beef cattle, at *Universidade Federal de Viçosa*. On 18$^{th}$ of February 2020, she presented his dissertation to the evaluation committee to obtain the *Doctor Scientiae* degree in Animal Science.

## ABSTRACT

OLIVEIRA, Cristhiane Viol Ribeiro, D.Sc., Universidade Federal de Viçosa, February, 2020. **Urea supplementation in rumen and post-rumen for cattle fed a low-quality tropical forage.** Adviser: Edenio Detmann. Co-adviser: Erick Darlisson Batista.

The aim of this study was to evaluated differences between the supplementation or infusion of urea in rumen and/or abomasum on forage digestion, nitrogen (N) metabolism, urea kinetics and urea transporters in heifers fed a low-quality tropical forage. Five heifers (283±23 kg, Body Weight, BW) were fitted with rumen and abomasum fistulas and assigned to a 5 ×5 Latin square design. The treatments were: control (only forage), continuous infusion of urea in the abomasum (AC), continuous infusion of urea in the rumen (RC), a pulse dose of urea in the rumen every 12 hours (PR), and a combination of PR and AC, with half of the urea dose delivery in each supplementation site (PRAC). General, treatments did not influence ($P>0.10$) forage intake and fiber digestion. The ruminal ammoniacal nitrogen concentration was higher ($P<0.10$) when urea was totally or partially supplied in the rumen. The control exhibited the lowest ($P<0.10$) fecal and urinary N losses, which were, overall, increased by supplementation. The highest urinary N losses ($P<0.10$) was observed when urea was either totally or partially supplied as a ruminal pulse dose. The rumen N balance was negative for the control and when urea was totally supplied in the abomasum. The greatest microbial N production ($P<0.10$) was obtained when urea was partially or totally supplied in the abomasum. The highest efficiency of microbial production (EFM) was observed ($P<0.10$) when urea was partially or totally supplied in the abomasum, and the lowest EFM ($P<0.10$) occurred for the control. The amount of urea recycled to the gastrointestinal tract was increased ($P<0.10$) by providing urea. The amount of N-urea returned to the ornithine cycle was increased ($P<0.10$) by supplemental urea and did not differ ($P>0.10$) amongst supplementation forms. The greatest ($P<0.10$) amounts of N-urea used for anabolism was observed when urea was totally and continuously infused in the abomasum. The continuous abomasal infusion of urea caused the highest ($P<0.10$) assimilation of microbial N from recycling, whereas the remaining treatments did not differ ($P>0.10$) to each other. The serum urea N (SUN) was higher ($P<0.10$) and the blood 3-methyl histidine was lower ($P<0.10$) when supplemental urea was provided compared to the control. The blood activity of aspartate transaminase and alanine transaminase was higher ($P<0.10$) when urea was totally supplied as a ruminal pulse dose. The genes associated with aquaporin

3 and urea transport protein UT-B were more expressed (P<0.10) in the control compared to the supplemented treatments. The continuous releasing of urea along the day either in the rumen or abomasum, is able to improved N accretion in the animal body, despite of mechanism responsible for that be different. The supply of urea as a pulse dose in the rumen is not able to confer the same benefits, probably because a greater amount of N is lost in the urine. Current technologies for delaying urea releasing in the rumen are not totally effective in terms of achieving a steady state condition. Therefore, the protection of urea aiming to its releasing into a post-rumen compartment seems a promising technology that may improve the N utilization from a non-edible N source.

Keywords: Non-protein nitrogen. Ruminant. Supplementation.

## RESUMO

OLIVEIRA, Cristhiane Viol Ribeiro, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Suplementação de ureia no rúmen e/ou pós-rúmen em bovinos alimentados com forragem de baixa qualidade.** Orientador: Edenio Detmann. Coorientador: Erick Darlisson Batista.

Objetivou-se avaliar as diferenças entre suplementação ou infusão de ureia no rúmen e/ou abomaso sobre a digestão da forragem, metabolismo de nitrogênio, cinética de ureia e transportadores de ureia em novilhas alimentadas com forragem de baixa qualidade. Cinco novilhas (283±23 kg, Peso Vivo) fistuladas no rúmen e no abomaso foram utilizadas em um delineamento em quadrado latino 5 x 5. Os tratamentos foram: controle (somente forragem), infusão contínua de ureia no abomaso (AC), infusão contínua de ureia no rúmen (RC), uma dose pulso de ureia no rúmen a cada 12 horas (PR); e uma combinação de PR e AC, com metade da dose de ureia fornecida em cada local de suplementação (PRAC). De maneira geral, os tratamentos não influenciaram ($P>0.10$) o consumo de forragem e a digestão da fibra. A concentração de nitrogênio amoniacal ruminal (NAR) foi maior ($P<0.10$) quando a ureia foi totalmente ou parcialmente fornecida no rúmen. O controle exibiu as menores ($P<0.10$) perdas de N fecal e urinária, que foram, em geral, aumentadas pela suplementação. As maiores perdas urinárias de N ($P<0.10$) foram observadas quando a ureia era total ou parcialmente fornecida como dose pulso no rúmen. O balanço de N no rúmen foi negativo para o controle e quando a ureia foi totalmente fornecida no abomaso. As maiores produções microbianas de N ($P<0.10$) foram obtidas quando a ureia foi parcial ou totalmente fornecida no abomaso. A maior eficiência de produção microbiana (EFM) foi observado ($P<0.10$) quando a ureia foi parcial ou totalmente fornecida no abomaso, e a menor EFM ($P<0.10$) ocorreu para o controle. A quantidade de ureia reciclada para o TGI (taxa de entrada no TGI, GER) aumentou ($P<0.10$) ao fornecer a ureia. A quantidade de N-ureia retornada ao ciclo da ornitina (ROC) foi aumentada ($P<0.10$) pela suplementação de ureia e não diferiu entre as formas de suplementação. As maiores ($P<0.10$) quantidades de N-ureia usadas para anabolismo (UUA) foram observadas quando a ureia era total e continuamente infundida no abomaso. A infusão abomasal contínua de ureia causou a maior assimilação ($P<0.10$) de N microbiano da reciclagem (g/d), enquanto os demais tratamentos não diferiram ($P>0.10$). O N-ureia sérico (SUN) foi maior ($P<0.10$) e a 3-metil histidina foi menor ($P<0.10$) quando a ureia suplementar foi fornecida em comparação ao controle. A atividade sanguínea da

aspartato transaminase e alanina transaminase foi maior (P<0.10) quando a ureia foi totalmente fornecida como dose pulso no rúmen. Os genes associados a aquaporinas 3 (AQP3) e a proteína transportadora de ureia UT-B (SLC14A1) foram mais expressos (P<0.10) no tratamento controle. A liberação contínua de ureia ao longo do dia, no rúmen ou abomaso, é capaz de melhorar o acréscimo de N corporal, apesar de o mecanismo responsável ser diferente. O suprimento de ureia como dose pulso no rúmen não e capaz de conferir os mesmo benefícios, provavelmente porque uma quantidade maior de N é perdida na urina. As tecnologias atuais para retardar a liberação de ureia no rúmen não são totalmente eficazes em termos de alcançar uma condição de liberação lenta. Portanto, a proteção de ureia visando sua liberação em um compartimento pós-ruminal parece uma tecnologia promissora que pode melhorar a utilização de N a partir de uma fonte de N não comestível.

Palavras-chave: Nitrogênio não-proteico. Ruminante. Suplementação.

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# INTRODUCTION

The nitrogen (N) conservation occurs in all animal species (Singer et al., 2003); however, as ruminants evolved, they developed a way to conserve N more pronouncedly as a survival mechanism (Reynolds and Kristensen, 2008; Detmann et. al, 2014). As a consequence, ruminant animals became able to produce milk and meat feeding on diets with low protein content or exclusively from non-protein N (NPN) sources (Virtanen, 1966). In cattle, as much as 40 to 80% of urea produced by the liver may re-enter the gastrointestinal tract (GIT), being the rumen the main site to receive this urea (Lapierre and Lobley, 2001). The urea entry into the rumen occurs through combinations of the saliva flow and the transfer through the rumen epithelium (Kennedy and Milligan, 1978). The transfer of urea across the rumen epithelium also can occur via process mediated by urea transporters and aquaporins (Abdoun et al., 2010; Røjen et al., 2011).

The amount of recycled urea into GIT is directly associated with dietary N intake (Lapierre and Lobley, 2001). Under low protein intake, the proportion of dietary N recycled as urea is increased to assure a continuous N supplying for microbial growth (Egan, 1965a; Van Soest, 1994). At same time, the animal may decrease the urinary N losses as an attempt to keep N status in metabolism (Batista et al., 2017a). There is a relationship between dietary N intake and urinary N excretion, whereby increasing dietary N will increase urinary N excretion and consequently the N wasted (Li et al., 2019).

Besides the amount of dietary N, the absorption site may also influence the N excretion, urea recycling, and efficiency of N utilization. When the amount of dietary rumen undegradable protein is proportionally increased, the N excretion can be decreased and the efficiency of N utilization can be improved (Batista et al, 2017b; Silva et al., 2018). Therefore, if the N source is provided in the abomasum, the forage intake can increase (Egan,

1965a; Carvalho et al., 2020), the urinary N excretion decreases, and efficiency of N utilization is enhanced (Batista et al. 2016). In others words, as an alternative to decrease N excretion and improve efficiency of N utilization, the dietary N source may be provided in the post-rumen. Thus, further studies are needed varying the site of N supply to understand N metabolism and its utilization by the animal.

Thus, considering that NPN sources, such as urea, can be absorved in the intestine, we hypothesize that moving supplementation site from rumen to abomasum can improve the efficiency of N utilization without compromise productive characteristics, such as intake and digestibility.

Therefore, our objective was to evaluated differences between the supplementation or infusion of urea in rumen and/or abomasum on forage digestion, N metabolism, urea kinetics, and urea transporters in heifers fed a low-quality tropical forage. From this study, it will be possible to improve the use of non-edible sources, which may increase the profitability and sustainability of the livestock production, reducing the waste and the emission of polluting compounds in the environment.

# MATERIAL AND METHODS

This experiment was carried out at the Department of Animal Science of the Universidade Federal de Viçosa, Viçosa, Brazil. The Institutional Animal Care and Use Committee of the Universidade Federal de Viçosa approved all surgical and animal care procedures before commencing this experiment (protocol number 015/2019).

### *Animals and Management*

Five rumminally and abomasally fistulated Nellore heifers, averaging 283±23 kg of body weight (BW), were used. The heifers were housed in individual stalls (2 × 5 m) with concrete floor, and had unrestricted access to water and complete macro/micro mineral mixture (80 g/kg of phosphorous). The animals were adapted to experimental facilities, management, and basal diet for 30 days prior the beginning of the experiment. The basal diet consisted of a low-quality Tifton hay (*Cynodon* sp., Table 1) chopped to a 15-cm particle size and fed twice daily at 0600 and 1800 h. To ensure *ad libitum* forage intake, the amounts of hay and orts were monitored daily in order to assure at least 300 g of orts per kg of forage offered.

### *Treatments and experimental design*

The experiment was performed according to a 5 × 5 Latin square design, with five treatments, five animals, and five experimental periods. Each experimental period lasted 25 days, with 14 days for supplement adaptation (Machado et al., 2016) and 11 days for sample collection. The animals were weighed at the beginning and at the end of each experimental period in order to calculate the average BW and relative voluntary intake.

The five treatments were: control (only forage), continuous infusion of urea in the abomasum (AC), continuous infusion of urea in the rumen (RC), a pulse dose of urea in the

rumen every 12 hours (PR), and a combination of PR and AC, with half of the urea dose delivery in each supplementation site (PRAC).

The amount of supplemental urea (a mixture of urea and ammonium sulfate at the ratio of 9 to 1) was calculate in order to increase the crude protein (CP) content of the diet up to 100 g/kg dry matter (Sampaio et al., 2010). From the average forage intake obtained during the 30-d adaptation of the animals to experimental facilities, we obtained a first estimate of the supplemental urea amount. Then, that amount was supplied during the first seven days of the adaptation period. As the forage intake changed due to supplemental N, the urea amount was adjusted based on average forage intake during the first seven days and offered during the last seven days of adaptation period. The average forage intake of the last seven days of adaptation period was then used to estimate the supplement amount to be offered during the collection period. As the second period began, we proceeded in the same manner and so on.

For the pulse dose of urea, the supplement was packed into paper bags and provided into the rumen twice a day concurrently with forage feeding. For the continuous infusion either in rumen or abomasum, urea was previously diluted in 1.5 L of a NaCl solution (9 g/L). The solution was infused continuously into the rumen and/or abomasum via the cannula using a peristaltic pump (BP600/4, Milan® Scientific Equipment, Inc., Colombo, Parana, Brazil) and polyvinyl chloride tubing (4.75 mm i.d.) at a rate of approximately 64 mL/h. Daily infusions were designed to last about 23.5 hours, 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. In order to assure a more homogenous experimental condition, the animals not assigned to continuous urea infusion were infused with the NaCl solution into the rumen and abomasum, as described above.

***Sample collections***

***Dry Matter Intake.*** Voluntary forage intake was quantified from d 15 to d 19 of each experimental period. The calculations took into account the amount of forage offered from d 15 to d 18 and the orts obtained from d 16 to d 19. 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. All pooled samples were ground in a knife mill to pass through a 2-mm screen sieve. After that, half of each sample was ground again to pass through a 1-mm screen sieve. Samples were then stored for subsequent chemical analyses. Urea samples were retained from each 25-kg package of product and pooled for subsequent analysis.

***Fecal Collection.*** Total fecal output was measured from d 19 to d 22. Feces were collected immediately after each spontaneous defecation and stored in 35-L buckets. At the end of each 24-h period, buckets were changed and the feces weighed and manually blended, and an aliquot (50 g/kg) was collected daily. Each daily fecal sample was oven-dried (60°C) and ground as previously described for hay and orts samples. After grinding, samples were pooled per animal and period in proportion to the daily excretion to measure digestibility and N balance. Fecal samples from d 19 were used to quantify the background enrichment of $^{15}N$, and those from d 22 were used to measure enrichment of $^{15}N$ for calculating urea kinetics, according to the sampling protocol validated by Wickersham et al. (2008).

***Total Urine Collection.*** Also from d 19 to d 22, 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). Vessels were kept all time into Styrofoam boxes with ice in order to avoid N losses. At the end of each 24-h period, urine output was weighed and mixed thoroughly, and an aliquot (10 mL/L) was filtered through four layers of cheesecloth and frozen (-20°C) for later analysis. Total collections of urine from d 19 and d

22 were used to measured $^{15}N$ background and enrichment, respectively, for later urea kinetics calculations (Wickersham et al., 2008).

***Catheter Placement and [$^{15}N^{15}N$]-Urea Infusion.*** On d 18 of each experimental period, heifers were fitted with temporary catheters (CVC; 14Ga × 20 cm; Biomedical®, São Paulo, SP) 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 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 hours, from the time the catheter was placed until 0600 h on d 20, 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 wich a 1.3 g/L stock solution of [$^{15}N^{15}N$]-urea, was dissolved in sterile saline solution (9 g NaCl/L). The [$^{15}N^{15}N$]-urea solution was prepared using a 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 its use. The infusion rate was 5 mL/h which delivered 0.200 mmol of urea N/h using a syringe infusion pump (BS-9000 Multi-Phaser; Braintree Scientific Inc., Braintee, MA) until 1600 h of d 23, when the last sample was collected. To quantify the exact volume infused, syringes were weighed before and after infusion.

***Blood Samples.*** On d 19, blood was collected via catheter at 0600, 1200, 1800, and 2400 h using syringes. Two 5-mL aliquots of blood were discarded before obtaining samples. Blood samples (10 mL) were injected immediately into vacuum tubes (BD Vacutainer,

Franklin Lakes, NJ) containing heparin (143 IU), placed in ice immediately after collection, and centrifuged (1,200 ×*g*, 15 min at 4°C). Plasma and serum were frozen (-20°C) for later analysis.

***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) were diluted in 4 L of water and infused in the rumen continually from d 14 tod22 of each period using a peristaltic pump (Milan Scientific Equipment, Inc., Colombo, Paraná, Brazil). Eight abomasal samples (1,300 mL per sample) were collected from d 19 to d 22 of each experimental 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 hours of a 24-h period in order to account for diurnal variation. Sampling was on d 19 at 0000 and 1200 h, d 20 at 0900 and 2100 h, d 21 at 0600 and 1800 h, and on d 22 at 0300 and 1500 h. After collection, abomasal samples were split in two parts: 800 mL were filtered through a nylon filter (100 µm, SefarNitex 100/44; Sefar, Thal, Switzerland) for separation of the particle phase from the fluid plus the small particle phase. Fluid plus small particle and particle phase samples were weighed, frozen at -80°C, freeze-dried, ground as previously described, pooled per animal, and analyzed separately in order to estimate abomasal digesta flow. The remaining 500 mL were used to isolate bacteria associated with the fluid and particle phases, according to the procedures described by Reynal et al. (2005). The bacterial pellets (from solid and liquid phases) were weighed, frozen (-80°C), freeze-dried, and grounded using a mortar and pestle.

On d 23 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 and at 2, 4, 6, 8, and 10 hours after feeding, frozen at -80°C, and freeze-dried. These sampling times represented 72 to 82 hours 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 sampling times on an equal weight basis.

***Ruminal Fermentation and Microbial N Synthesis.*** On d 23, at the same times as abomasal sampling for $^{15}N$ enrichment, ruminal fluid samples were obtained to evaluate pH, ruminal ammonia nitrogen (RAN), and volatile fatty acids (VFA; acetate, propionate, and butyrate), 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 four layers of cheesecloth. The pH samples were measured using a digital potentiometer. After that, an 8-mL aliquot of rumen fluid was combined with 2 mL of a 250 g/L solution of meta-phosphoric acid and frozen for subsequent VFA analysis. Another 40 mL aliquot was combined with 1 mL of a 9 *M* $H_2SO_4$solution 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.

***Rumen papillae biopsy.*** On d 25 at 0600 h, rumen papillae were biopsied from ventral sac as described by Kelly et al. (1993). Approximately 150 mg of rumen pappilae were collected during each biopsy and washed in PBS (pH = 7.4, 1X) 10 times prior to immediately frozen in liquid nitrogen and then kept at -80°C for subsequent analysis.

***Laboratory Analysis***

Samples of hay, orts, feces, and abomasal digesta, processed to pass through a 1-mm sieve, were analyzed with regards dry matter (DM; dried overnight at 105ºC; method G003/1), ash (complete combustion in a muffle furnace at 600ºC; method M-001/1), and N (Kjeldahl procedure; method N-001/1) contents according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science

(INCT-CA; Detmann et al., 2012). The neutral detergent fiber (NDF) contents were evaluated using a heat-stable α-amylase and omitting sodium sulfite according to Mertens (2002). The NDF contents were expressed exclusive of contaminant ash and protein (NDFap). Urea samples were only analyzed regarding DM and CP contents. Samples of hay, orts, and abomasal digesta, processed to pass through a 2-mm screen sieve, were evaluated with regards indigestible NDF (iNDF) content using F57 filter bags (Ankom Technology Corp., Macedon, NY, USA) and a 288-h *in situ* incubation procedure (Valente et al., 2011).Cobalt concentration in abomasal samples (both fractions) was quantified through atomic absorption spectrometry (GBC Avanta∑ atomic absorption spectrophotometer, Scientific Equipment, Braeside, Victoria, Australia).

Abomasal digesta (both fractions) and the microorganisms isolated from it were analyzed for purine bases (Ushida et al., 1985) and N (method N-001/1; Detmann et al., 2012) contents. The $N_{RNA}$:$N_{total}$ ratio in the microorganisms was used as the marker for estimating microbial production in the rumen.

Samples of rumen fluid and blood were pooled per animal and experimental period. The RAN concentration was quantified using a colorimetric technique (method N-006/1; Detmann et al., 2012). The VFA analysis, rumen fluid samples collected over time were pooled (2.0 mL per sample) and centrifuged (12,000 ×*g* for 10 min at 4°C). 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.

Urinary urea (colorimetric kinetic test, Bioclin® K056), urinary creatinine (enzymatic-colorimetric method, Bioclin® K067), and urinary ammonia (method N-006/1;

Detmann et al., 2012) concentrations were quantified with an AutoAnalyzer (BS200E, Mindray, China). Total urinary N was obtained by the Kjeldahl procedure.

Blood plasma samples were analyzed for urea (enzymatic-colorimetric method, Bioclin® K056), glucose (enzymatic glucose oxidase-peroxidase method, Bioclin® K082), creatinine (enzymatic-colorimetric method, Bioclin® K067), total protein (colorimetric kinetic test, Bioclin® K031), albumin (bromocresol green method, Bioclin® K040), aspartate transaminase (AST, U.V. kinetic – IFCC, Bioclin® K048), and alanine transaminase (ALT, U.V. kinetic – IFCC, Bioclin® K049). Blood 3-methylhistidine was analyzed in a commercial laboratory (Hermes Pardini Laboratory, Belo Horizonte, MG). The concentration of blood globulin was estimated as the difference between the total protein and albumin concentrations.

The $^{15}N$ enrichment in fecal, 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) 250µmol of urea urinary 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 IMRS ($^{15}N$ Analysis Laboratory, University of Illinois, Urbana, IL). Results were corrected for [$^{14}N^{15}N$]-$N_2$ produced by non-monomolecular reactions (Lobley et al., 2000).

***Rumen Papillae Analysis***

The RNA was extracted from 50 mg of rumen papillae samples using Trizol® (Invitrogen TM, Thermo Fisher Scientific®, Oregon, USA) according to the manufacturer's

recommendations. The RNA concentration was estimated by NanoVue™ Plus spectrophotometer (GE Healthcare Life Science Inc., Freiburg, Germany), and RNA integrity was evaluated through 1% agarose gel electrophoresis. The RNA samples were then reverse transcribed into cDNA using the GoScriptTM Reverse Transcription System kit (Promega Corporation, Madison, WI, USA). The primers used in this study were designed as described by Benedeti et al. (2018). The genes assessed were: aquaporins 3, 7, 8, and 10 (*AQP3, AQP7, AQP8, and AQP10*); and solute carrier family 14 members 1 (*SLC14A1*) and 2 (*SLC14A2*). The 18S ribosomal RNA (18S; NR_036642.1) was used as the endogenous control gene. Real-time quantitive PCR reactions were performed in thermal cycler ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the detection method hydrolysis probe (Kit TaqMan, Thermo Fisher Scientific®, Oregon USA) according to the following cycle parameters: 95°C for 2 min, 40 cycles at 95°C for 15 s, and 60°C for 60 s. Gene expression was calculated as described by Livak and Schmittgen (2001) using the $2^{-\Delta\Delta ct}$ method.

***Calculations***

The abomasal flow was estimated using iNDF as internal marker of the particle phase and Co-EDTA as external marker of the liquid phase. The iNDF was assumed as an ideal marker of the solid phase, while Co-EDTA was assumed as non-ideal marker of liquid phase. The reconstitution factor of the abomasal digesta was calculated as stated by France and Siddons (1986).

Urea kinetics was 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). The microbial N flow (MN) from recycled urea was calculated by multiplying MN by 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).

The N balance was calculated by subtraction fecal and urinary N from N intake. Rumen N balance was calculated by subtracting N abomasal flow from N intake.

***Statistical Analyses***

Statistical analyses were performed using the MCMC glmm package (Hadfield, 2010) of R software (R Core Team, 2018) according to a 5 × 5 Latin square design including the effects of treatment, animals, and experimental period. All data from one animal were lost because of problems during the experiment, which cause a prominent decrease in statistical power. Facing this constraint, we opted for using Bayesian inference instead a classical frequentist approach, since prior distributions are widely usually to supply information on missing experimental units (Blasco, 2017). In this context, the Latin square-based model was fitted under a MCMC (Markov chain Monte Carlo) Bayesian framework using normal prior distribution for each treatment effect, whereas inverse chi-squared distributions were used as prior for variance components associated to animal and period random effects. A total of 45,000 MCMC iterations were used in all analyses, assuming the "burn-in" and "thin" periods equal to 10,000 and 2 iterations, respectively. The MCMC convergence was evaluated via Geweke test (Geweke, 1992) implemented in "boa" package of R software (Smith, 2007). The MCMC chains for differences between treatment means were obtained, and the statistical significance was accessed through 90% credible intervals. Thus, if the interval contains the value zero, the difference between two treatments was declared non-significant. In order to simplify the notation related to significant differences, the term $P<0.10$ was used here, where P means posterior probability.

# RESULTS

## *Intake and digestibility*

General, treatments did not influence (P>0.10) voluntary intake, excepting the CP intake, which was greater (P<0.10) when urea was supplied (Table 2).

The amount of organic matter (OM) and fiber digested in the rumen or in the entire GIT was not affected (P>0.10) by the treatments (Table 3). Despite of the supplementation form, the amount of CP digested in GIT was greater (P<0.10) when urea was provided. The amount of CP digested in the rumen was increased (P<0.10) by ruminal supplementation either continuously or as a pulse dose when compared to control or abomasal infusion. An intermediary pattern was observed when the urea dose was split between rumen and abomasum. The treatments did not alter (P>0.10) the amount of fiber digested in the intestine. The amount of CP digested in the intestine increased (P<0.10) as the amount of intestinal urea supplying increased. The OM digested in the intestine was greater (P<0.10) for control and abomasal infusion and lower (P<0.10) when urea was supplied by ruminal dose pulse. The remaining treatments exhibited an intermediate pattern. Overall, the dietary ratio of CP to digested OM (DOM) was improved (P<0.10) when urea was supplemented. On average, CP:DOM was 164 g/kg for supplemented treatments compared to 72 g/kg observed in control.

## *Ruminal fermentation*

There was a treatment effect on rumen pH. When urea was totally supplied in the abomasum, the rumen pH was smaller (P<0.10) compared to the other treatments (Table 4). The RAN concentration was higher (P<0.10) when urea was totally or partially supplied in the rumen. No treatment effect was detected (P>0.10) on VFA concentration and molar proportion.

***Nitrogen metabolism***

The amount of N consumed and digested in GIT behaved as previously presented for CP intake and digestion (Table 5). The control exhibited the lowest ($P<0.10$) fecal and urinary N losses, which were, overall, increased by supplementation. The fecal N was greatest ($P<0.10$) when supplemental urea was split between rumen and abomasum. On the other hand, the highest urinary N losses ($P<0.10$) was observed when urea was either totally or partially supplied as a ruminal pulse dose. The proportion of urea-N in total urinary N increased ($P<0.10$), whereas the proportion of ammonia-N in urine decreased ($P<0.10$) with supplementation, without differences ($P>0.10$) amongst supplementation forms. The N accretion was improved ($P<0.10$) by supplementation when compared to the control, being highest ($P<0.10$) when urea was continuously provided either in rumen or abomasum.

The rumen N balance was negative for the control and when urea was totally supplied in the abomasum. An improvement on this variable was obtained ($P<0.10$) when half of the supplemental urea was delivered in the rumen, which become even higher ($P<0.10$) when urea was totally supplied in the rumen either continuously or as a pulse dose. The greatest microbial N production ($P<0.10$) was obtained when urea was partially or totally supplied in the abomasum. For this variable, the lowest values ($P<0.10$) were observed for the control and when urea was totally supplied as a ruminal pulse dose. The continuously delivering of urea in the rumen behaved intermediately. Moreover, the highest efficiency of microbial production (EFM) was observed ($P<0.10$) when urea was partially or totally supplied in the abomasum, and the lowest EFM ($P<0.10$) occurred for the control. The other treatments exhibited an intermediate pattern.

***Urea kinetics and nitrogen recycling***

In terms of absolute values (g/d), the urea synthesized in liver (urea N entry rate, UER) was increased ($P<0.10$) by supplemental urea regarding the supplementation form

(Table 6). The amount of urea recycled to the GIT (GIT entry rate, GER) was increased ($P<0.10$) by providing urea, but this was not homogenous among treatments. The greatest GER ($P<0.10$) was obtained by continuous urea infusion in the abomasum, whereas the pulse dose supplementation caused an intermediary GER between control and abomasal infusion. The other treatments placed between abomasal infusion and pulse dose. On the other hand, the amount of N-urea returned to the ornithine cycle (ROC) was increased ($P<0.10$) by supplemental urea and did not differ ($P>0.10$) amongst supplementation forms. The greatest ($P<0.10$) amounts of N-urea used for anabolism (UUA) were observed when urea was totally and continuously infused, despite of supplementation site. The lowest UUA ($P<0.10$) was verified when urea was totally supplied as a pulse dose in the rumen. Overall, urea supplementation increased ($P<0.10$) fecal excretion of N-urea from urea synthetized in the liver.

Considering the urea kinetics in terms of fractional rates (g/g), we observed that the total or partial urea supplementation as a ruminal pulse dose increased ($P<0.10$) the proportion of urea synthetized in liver that was lost in urine (UUE:UER) compared to the control (Table 6). The continuous infusion in the abomasum propitiated urinary losses similar to those observed for the control ($P>0.10$), whereas ruminal continuous infusion caused intermediary values. General, the greatest ($P<0.10$) proportion of hepatic urea recycled to the GIT (GER:UER) was obtained for the control and with total abomasal infusion. The partial or total urea supplementation as a ruminal pulse dose decreased ($P<0.10$) the proportion of urea recycled to the GIT. The continuous infusion of urea in the rumen propitiated intermediary responses. The greatest proportion ($P<0.10$) of GER used for anabolism (UUA:GER) (e.g., recycled N available for microbial production) was observed for the control and when urea was total and continuously infused in the rumen or abomasum. In this case, providing urea totally of partially as a ruminal pulse dose decreased ($P<0.10$) its availability for anabolic

purposes. In spite of some small oscillations among supplementations forms, providing urea increased (P<0.10) the proportion of GER that was lost in feces (UFE:GER). As a reflex of UFE and UUA pattern, the greatest (P<0.10) proportions of GER that returned to the ornithine cycle (ROC:GER) were obtained when urea was totally or partially provided through ruminal pulse dose. The lowest proportions (P<0.10) were obtained for the control and total abomasal infusion. Intermediary values were obtained for ruminal continuous infusion.

The continuous abomasal infusion of urea caused the highest (P<0.10) assimilation of microbial N from recycling (g/d), whereas the remaining treatments did not differ (P>0.10) to each other (Table 6). The highest proportion of microbial N coming from recycling (P<0.10) was also observed when urea was continuously infused in the abomasum, being followed by control and split infusion, and ruminal supplementation (continuous and pulse dose), which produced the lowest proportions (P<0.10). The evaluation of microbial N assimilation as a fraction of total recycled N (GER), indicated the highest dependency on recycling for the control (P<0.10), while the supplemented treatments did not differ to each other (P>0.10).

***Blood characteristics and liver function***

The different treatments did not influence (*P*>0.10) blood glucose, total protein, albumin, and globulin (Table 7). The serum urea N (SUN) was higher (P<0.10) and the blood 3MH was lower (P<0.10) when supplemental urea was provided compared to the control, regarding supplementation form.

The blood AST was higher (P<0.10) when urea was totally supplied as a ruminal pulse dose compared to the control (Table 7). The remaining treatment behaved intermediary. The blood ALT was greatest (P<0.10) when urea was totally supplied as a ruminal pulse dose.

***Gene Expression***

We did not detect any gene expression for aquaporin 8, aquaporin 10, and urea transport protein UT-A (SLC14A2) in the rumen epithelium. Moreover, there was no treatment effect ($P>0.10$) on the gene expression for aquaporin 7. The genes associated with aquaporin 3 (AQP3) and urea transport protein UT-B (SLC14A1) were more expressed ($P<0.10$) in the control compared to the supplemented treatments, which did not differ ($P>0.10$) to each other (Table 8).

# DISCUSSION

Overall, the supplements did not alter the voluntary intake of forage regarding the supplementation form. Normally, it is assumed that N deficiency in rumen is the main constraint to intake and fiber degradation of low-quality forages (Egan and Doyle, 1985; Detmann et al., 2009). However, several authors have found no supplemental N effects on voluntary intake in cattle fed low-quality tropical forages (Batista et al., 2016; Rufino et al., 2016; Franco et al., 2017). The control of voluntary intake in ruminants cannot be ascribed to a single factor as it has a multifactorial influence (Forbes, 2003). The protein-to-energy ratio in the total diet seems a more valuable indicator of the supplementation influence on forage intake, as it integrates the supplemental effects on both rumen and metabolism (Poppi and McLennan, 1995; Detmann et al., 2014). The voluntary intake of tropical forages would be maximized when the CP:DOM of the diet is raised close to 210 g/kg through supplementation (Reis et al., 2016). In this experiment, the CP:DOM in the basal forage was 72 g/kg and was increased, on average, up to 164 g/kg by supplementation, being very lower than the ratio expected to increase forage intake.

The total protein digestion was improved by N supplementation. This pattern should be expected as the apparent digestibility of a non-fibrous compound is positively associated with its intake, which causes a dilution of the metabolic fecal fraction (Van Soest, 1994). Such a behavior can be corroborated by the digestibility pattern of CP in both rumen and abomasum, which was also increased when urea was supplied inside those compartments.

Amid all digestibility measurements, there was an oscillating pattern for the intestinal OM digestibility, which cannot be solely explained by the post-rumen N infusion. At first glance, the intestinal fiber digestibility could not help for any explanation here, because the amount of fiber digested in the intestine did not vary among treatments. However, the ratio of

fiber digested in the rumen to the fiber digested in the intestine was quite different between control (2.70) and supplemented animals (3.75 on average, Table 3). This pattern brings into evidence two facts. First, the supplementation increased the proportion of fiber digested in the rumen despite the absence of difference amongst treatments concerning the amount of ruminal digested NDF. Such effects seemed almost homogenous for all supplementation forms. Therefore, a rumen N deficiency did occur in non-supplemented animals and constrained ruminal fiber digestion. Second, any constraint to rumen degradation may imply an increased intestinal fiber digestion (Dixon and Stockdale, 1999). The main explanation for that relies on a greater escape of potentially digestible NDF from rumen to the hindgut. Thus, the control exhibited greater intestine fiber digestion due to constraints on rumen digestion. A differential escape of pdNDF among treatments may be responsible, at least in part, for the oscillating intestinal digestion of OM of supplemented animals.

Despite of the unclear effects of the supplemental N on intake and digestibility, we can state the rumen of non-supplemented animals did work under N deficiency. Such a statement is supported by the negative N balance in the rumen, the limited microbial growth, and the increased expression of auxiliary mechanisms of urea carriage with in rumen epithelium in the control. Even though urea can be passively carried through rumen wall, an improvement in auxiliary transportation mechanisms, sub as UT-B, would provide an increased N supply for rumen bacterial growth (Simmons et al., 2009) by optimizing ruminal urea inflow. Thus, considering the low blood urea availability in the control, the increased expression of UT-B and AQP3 constitutes an attempt by the animals to assure a continuous microbial growth on the N-deficient basal substrate.

In agreement with several reports from the tropics (Batista et al., 2017b; Franco et al., 2017; Rufino et al., 2016; 2020), the impacts of N supplementation were more prominent on metabolic characteristics than on intake and digestibility. The metabolic action of

supplemental N encompasses two different effects, which may or not occur simultaneously: an improvement in anabolism and a decrease in catabolism (Detmann et al., 2017). The anti-catabolic effect of supplemental N occurred similarly for all supplementation forms, as demonstrated by the decrease in the blood 3MH-to-creatinine ratio. The 3MH is an amino acid formed from methylation of histidine after its inclusion in the muscle proteins. When muscle proteins are degraded, the 3MH cannot be reused for protein synthesis and is excreted in the urine (Waterlow, 2006). The greatest blood 3MH in the control indicates a greater muscle catabolism, as an attempt to mobilize N for an additional rumen supply (Batista et al., 2016; Rufino et al., 2016), which, in turn, may compromise the overall efficiency of N utilization in the animal body (Detmann et al., 2017).

On the other hand, the anabolic effects of the supplemental N were not homogenous among supplementation forms. Such a pattern is supported mainly by the differences in N accretion and efficiency of N utilization. These specific measurements allow giving a holistic view over animal efficiency, as the N accretion is dependent on both energy and protein metabolism (Detmann et al., 2017) and is directly associated with the animal performance. As indicated by catabolic aspects, the control had a negative N balance, an effect of the dietary N deficiency. However, when supplements were provided, the N accretion was greater for continuous infusion compared to total or partial urea supplying through a pulse dose, despite the similar effects in decreasing muscle catabolism.

We would like to emphasize that the present study has a theoretical purpose, where the effects of NPN supplementation were studied avoiding influences of other compounds such as carbohydrates or true protein. It has been demonstrated that interactions between supplemental N and carbohydrates do exist on N accretion and microbial assimilation (Souza et al., 2010; Lazzarini et al., 2016; Franco et al., 2017). However, we can qualify our

hypothesis here as exploratory rather than any applied statement, as we intended to understand the dynamics of NNP supplied at different sites of GIT.

Considering this, we start stating that a more positive and consistent effect on N accretion was obtained when urea was totally supplied by continuous infusion in either rumen or abomasum. Compared to the dose-pulse treatments, the continuous infusion promoted a lower urinary loss of N, which was, at least partially, responsible for its grater effect on N accretion. A greater N loss would decrease the availability of metabolizable N for anabolic purposes, as it was somehow indicated by UUA pattern.

When urea was totally or partially supplied as ruminal dose pulse, the pattern of RAN throughout time after feeding was less stable than patterns obtained with continuous infusion (Figure 1), promoting high-concentration peaks between 2 and 4 hours after supplementation. A similar pattern was also detected in the study by Carvalho et al. (2020). The transfer of ammonia excess from portal drained viscera to the blood is proportional to the ammonia concentration in the rumen fluid (Kennedy and Milligan, 1978; Abdoun et al., 2007). Moreover, when cattle are fed low-quality forages, the RAN accumulation becomes more prominent when concentrations exceed 10-12 mg/dL (Detmann et al., 2009; 2014), which seems associated with a limitation in the energy supply for microbial N uptake. Therefore, the excessive ammonia represented by the peaks after feeding was transferred to the blood, however its return to the rumen as urea was compromised, which is supported by the increased urinary N loss and the higher UUE-to-UER ratio.

When we planned to supply all urea as a ruminal pulse dose, our intention was to simulate a situation more similar to a N delivery with unprotected feed grade urea through a low-intake supplement (Rezende et al., 2008). However, this type of urea releasing proved to be inefficient in terms of anabolism and even detrimental to the animals, as an increased AST and ALT blood activities were detected. The ALT and AST are important for transferring of

amino groups to α-ketoglutarate and producing glutamate, which, in turn, can serve as amino-group donor for hepatic urea synthesis (Nelson and Cox, 1999). Normally, the blood activity of both enzymes is extremely lower compared to their liver activity (Panteghini and Bais, 2008). Therefore, their increased blood activity indicates that liver injury (Giannini et al., 2005) took place when this treatment was applied. As a consequence, the probable overload of ammonia in the liver leaded to hepatocyte damages and released more AST and ALT into bloodstream. A similar pattern was detected by Palma (2018), who observed an increased blood ALT when cattle fed tropical forage received a high-urea supplement.

Overall, the continuous delivery of supplemental N either in rumen or abomasum has the greatest effect on improving N accretion and utilization efficiency. However, continuous delivery of N in the rumen seems to constitute only a theoretical procedure and must be seen here as a "positive control" compared to the other supplemented treatments. The current technologies applied for delaying urea hydrolysis in the rumen either propitiate similar ammonia concentrations (Mahmoudi-Abyane et al., 2020) or are only able to create a lag for ammonia releasing (Joysowal et al., 2019) compared to a feed-grade urea. Therefore, those technologies, as used nowadays, cannot confer a ruminal releasing close to a steady state condition. On the other hand, any technology capable to propitiate a complete covering or protection of the urea particles regarding ruminal degradation, but being soluble or hydrolysable either in the abomasum acid or in the intestinal alkali, would be able to promote a steady state releasing of urea in the intestine. Such a behavior would be likely because the liquid rate of passage from rumen to the intestine tends to be approximately constant throughout the day.

Despite of conferring the same N accretion and efficiency of N utilization, the continuous infusion in rumen and abomasum presented some remarkable differences in terms of N metabolism. Even though being statistically similar to each other, the microbial N

production and efficiency were slightly superior when urea was continuously provided in the abomasum. Moreover, the efficiency of microbial N uptake from recycling was remarkably higher with the abomasal supplementation. That improved microbial production can imply a greater metabolizable protein supply from bacterial protein, which in turn, may support the better N accretion compared to the control. In this sense, the enhanced microbial production in the rumen obtained by abomasal infusion is a direct reflex of recycling.

Several reports have brought into evidence that a N supplying in either abomasum or duodenum increase the amount of N recycled to the rumen (Egan et al., 1965a; 1965b; Batista et al., 2016). Indirect evidences of a greater recycling with abomasal supplementation can be obtained by evaluating the rumen N balance and RAN concentration in comparison to the control. The ruminal N balance became more negative, showing that rumen N output was much compared to N intake and recycling is the potential source for that, such as reported by Batista et al. (2017b) and Rufino et al. (2016). Such a pattern is also directly evidentiated by the higher GER obtained through abomasal infusion. Despite of conferring the same RAN concentration compared to the control, the grater microbial N production indicates that ruminal ammonia availability did increase with abomasum supplementation. However, the delivering of N through recycling presents a delay compared to the rumen supplementation (Egan, 1965b) as the hepatic pathways intermediate its return to the rumen. It caused the N delivering slower and approximately constant along the day (Figure 1). Considering that substrate has a limited energy supply, this slow ammonia supply seemed allowing microorganisms work on a more coupled way. In other words, the slow energy extraction coupled with a slow N delivery conferred a greater and more efficient microbial synthesis.

It important to note that total continuous abomasal infusion cased a decrease in rumen pH compared to the other treatments. It seems to be a reflex of the greater microbial growth obtained with this supplementation form. Despite of presenting a similar microbial N

production, the split supplementation was not able to decrease rumen pH, a direct effect of the buffering capacity caused by ruminal urea delivery. The lower pH obtained with complete infusion of urea in the abomasum can be positive to keep a more adequate N supply for microbial growth in the rumen. A lower pH decreases the permeability of the rumen epithelium to ammonia (Abdoun et al., 2007), then avoiding an excessive ammonia transfer to the bloodstream. Moreover, a decrease in rumen pH down to 6.2 optimizes urea transportation from blood to rumen (Lu et al., 2014), which seems a very interesting effect when a low-quality quality forage is fed to ruminant animals. Therefore, such a pattern brings into evidence other positive mechanism associate with post-ruminal urea supplementation.

On the other hand, the continuous supplementation in the rumen caused a slightly lower microbial production compared to abomasum infusion. It must be noted that RAN was increased by rumen N infusion, however the pattern throughout time after feeding highlights an interesting difference compared to the other treatments. When urea was completely delivered in a continuous way in the rumen, there was a peak of RAN, but this one took place latter compared to pulse-dose treatments (Figure 1). At first glance, a RAN peak would not be expected by using a continuous infusion system. However, the releasing of ammonia from urea can behave faster for dietary urea compared to recycled urea. Therefore, even though being both continuous delivering ways, the ruminal infusion is able to increase ruminal ammonia pool faster compared to abomasal infusion, as previously discussed. Considering the low availability of energy in the basal substrate, there would be a break point in time where energy and N availability will be no longer coupled, causing the accumulation of the compounds with greater availability, the N in this case. This pattern is supported by the latter ammonia peak observed with ruminal continuous infusion of urea.

From this argument, the ruminal continuous infusion should have conferred a lower supply of metabolizable protein from microorganism compared to abomasal infusion.

However, both supplementations forms gave the same N accretion and efficiency of N utilization. There are two likely explanations for that behavior. First, the continuous ruminal infusion allowed keeping the urinary N losses lower compared to pulse-dose treatments, being similar to the continuous abomasal infusion. Despite of presenting a later RAN peak, the RAN profile along time was more stable compared to pulse dose treatments (Figure 1), then supporting the lower urinary N loss obtained with continuous ruminal infusion.

Second, compared to abomasal infusion, the ruminal continuous infusion propitiated a slightly lower amount of N recycled to GIT. However, the improved N availability for metabolism (e.g., blood urea) coupled with the lower urinary losses may have alter the N status in the animal body. In theoretical terms, the expression "N status" defines the quantitative and qualitative availability of N compounds for different physiological functions in animal metabolism, including functions associated with the metabolism of other compounds, such as energy (Detmann et al., 2014). When the N status is improved, the metabolism can achieved a better adjustment. In other words, molecules of NPN can be direct towards less exigent metabolic pathways, such as urea cycle. Thus, amino acids utilization for those pathways will be decrease, which, in turn, improve the availability of metabolic precursors for protein synthesis (Detmann et al., 2014; 2017; Reis et al., 2020).

When we planned to use the treatment where urea dose was split into rumen and abomasum, our intention was to simulate the utilization of a low-intake supplement with a partial replacement of feed-grade urea by a urea molecule able to pass intact from rumen degradation. The partial urea infusion into abomasum caused a microbial growth similar to that one observed with total infusion of urea into abomasum. We believe the support for this is based on the same arguments we presented before. However, the partial pulse dose into the rumen increased the urinary N losses and, as consequence, N with anabolic potential was lost. For some key variables, this treatment behaved intermediately between total urea as a rumen

pulse dose and total infusion into abomasum (e.g., GER, UUA, rumen N balance, fraction of microbial N from recycling). However, unexpectedly, there was no improvement in N accretion compared to the control. To our knowledge, a likely explanation for that behavior could not be taken from data we have in this work. We hypothesize that some negative interactions could exist between two forms of supplementation, but that subject deserves more investigations to be adequately supported.

# CONCLUSION

The continuous releasing of urea along the day either in the rumen or abomasum is able to improved N accretion in the animal body, despite of mechanism responsible for that be different. The supply of urea as a pulse dose in the rumen is not able to confer the same benefits, probably because a greater amount of N is lost in the urine. Current technologies for delaying urea releasing in the rumen are not totally effective in terms of achieving a steady state condition. Therefore, the protection of urea aiming to its releasing into a post-rumen compartment seems a promising technology that may improve the N utilization from a non-edible N source.

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**Table 1.** Chemical composition of the Tifton hay

| Item | Content[3] |
|---|---|
| Dry matter[1] | 884±5.8 |
| Organic matter[2] | 941±2.0 |
| Crude protein[2] | 43.6±2.41 |
| NDFap[2;4] | 756±7.6 |
| iNDF[2;5] | 326±9.0 |

[1]g/kg as fed. [2]g/kg of dry matter. [3]Mean ± standard-error.[4]Neutral detergent fiber corrected for contaminant ash and protein. [5]Indigestible neutral detergent fiber.

**Table 2.**Voluntary intake in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| Item[2] | Treatments[1] | | | | | pSD[3] |
|---|---|---|---|---|---|---|
| | Control | AC | RC | PR | PRAC | |
| Intake (kg/d) | | | | | | |
| DM | 3.98 | 4.10 | 3.66 | 4.10 | 3.89 | 0.431 |
| Forage | 3.98 | 4.04 | 3.61 | 4.06 | 3.84 | 0.435 |
| Urea | - | 0.057 | 0.054 | 0.053 | 0.053 | - |
| OM | 3.74 | 3.85 | 3.46 | 3.88 | 3.69 | 0.401 |
| CP | 0.189b | 0.347a | 0.315a | 0.339a | 0.327a | 0.0421 |
| NDFap | 3.07 | 3.11 | 2.80 | 3.11 | 2.93 | 0.327 |
| iNDF | 1.255 | 1.275 | 1.131 | 1.256 | 1.246 | 0.1380 |
| Intake (g/kg BW) | | | | | | |
| DM | 13.9 | 14.4 | 13.1 | 14.5 | 13.3 | 1.41 |
| OM | 13.0 | 13.5 | 12.4 | 13.7 | 12.6 | 1.31 |
| NDFap | 10.7 | 10.9 | 10.0 | 11.0 | 10.0 | 1.10 |
| iNDF | 4.39 | 4.45 | 4.02 | 4.45 | 4.29 | 0.439 |

[1]AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2] DM, dry matter; OM, organic matter; CP, crude protein; NDFap, neutral detergent fiber corrected for contaminant ash and protein; DOM, digested organic matter, DNDF, digested neutral detergent fiber corrected for ash and protein; iNDF, indigestible neutral detergent fiber.[3] *A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals.

**Table 3.** Amount of material digested in different sites in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| | Treatments[1] | | | | | |
|---|---|---|---|---|---|---|
| Item[2] | Control | AC | RC | PR | PRAC | pSD[3] |
| Ruminal (kg/d) | | | | | | |
| OM | 1.58 | 1.72 | 1.57 | 1.85 | 1.51 | 0.327 |
| CP | -0.001c | -0.023c | 0.161a | 0.146a | 0.070b | 0.0350 |
| NDFap | 1.50 | 1.64 | 1.44 | 1.63 | 1.38 | 0.262 |
| Intestinal (kg/d) | | | | | | |
| OM | 0.704a | 0.687a | 0.469ab | 0.421b | 0.463ab | 0.1567 |
| CP | 0.084c | 0.269a | 0.058c | 0.084c | 0.153b | 0.0363 |
| NDFap | 0.555 | 0.508 | 0.393 | 0.365 | 0.378 | 0.1234 |
| Total (kg/d) | | | | | | |
| OM | 2.29 | 2.40 | 2.04 | 2.27 | 1.97 | 0.367 |
| CP | 0.084b | 0.246a | 0.219a | 0.230a | 0.223a | 0.0217 |
| NDFap | 2.05 | 2.15 | 1.84 | 2.00 | 1.76 | 0.292 |
| CP:DOM (g/kg)[4] | 72b | 156a | 163a | 157a | 181a | 21.8 |
| R:I (g/g)[5] | 2.70 | 3.23 | 3.66 | 4.47 | 3.65 | - |

[1] AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2]OM, organic matter; CP, crude protein; NDFap, neutral detergent fiber corrected for contaminant ash and protein.[3] *A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals.[4] CP:DOM, Dietary ratio of CP to digested OM.[5] R:I, ratio of fiber digested in rumen to fiber digested in the intestines.

**Table 4.** Ruminal fermentation characteristics in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| | Treatments[1] | | | | | |
|---|---|---|---|---|---|---|
| Item[2] | Control | AC | RC | PR | PRAC | pSD[3] |
| pH | 6.53a | 6.34b | 6.50a | 6.47a | 6.55a | 0.06 |
| RAN, mg/dL | 3.8b | 5.6b | 14.7a | 16.3a | 14.3a | 2.29 |
| VFA, mM | 30.2 | 41.9 | 42.4 | 39.3 | 40.0 | 10.02 |
| VFA, mol/100 mol | | | | | | |
| Acetate | 74.9 | 75.4 | 75.7 | 74.3 | 75.3 | 4.58 |
| Propionate | 16.8 | 16.4 | 15.1 | 16.4 | 16.5 | 2.98 |
| Butyrate | 8.3 | 8.2 | 9.2 | 9.3 | 8.2 | 1.43 |

[1]AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2] RAN, rumen ammonia N; VFA, volatile fatty acids. [3]*A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals.

**Table 5.** Characteristics of N utilization in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| | Treatments[1] | | | | | |
|---|---|---|---|---|---|---|
| Item | Control | AC | RC | PR | PRAC | pSD[2] |
| N intake, g/d | | | | | | |
| Forage | 29.0 | 29.5 | 25.2 | 30.5 | 28.8 | 3.33 |
| Ruminal urea | 0 | 0 | 25.3 | 23.7 | 11.8 | - |
| Abomasal urea | 0 | 25.9 | 0 | 0 | 11.8 | - |
| Total | 29.0b | 55.5a | 50.8a | 54.3a | 53.4a | 3.88 |
| N excretion | | | | | | |
| Fecal, g/d | 15.6c | 16.8ab | 15.8bc | 16.9ab | 17.0a | 1.18 |
| Urine N, g/d | 17.9c | 29.9b | 26.5b | 37.0a | 38.7a | 2.92 |
| Urea N, g/d | 8.6b | 21.8a | 18.4b | 26.2a | 25.7a | 2.90 |
| % of urine N | 49.8b | 71.3a | 68.2a | 70.1a | 66.5a | 4.86 |
| Ammonia N, g/d | 2.1 | 2.3 | 1.9 | 2.2 | 1.7 | 0.53 |
| % of urine N | 12.5a | 8.0b | 7.7b | 6.2b | 4.7b | 3.31 |
| N digested, g/d | 13.7b | 34.9a | 39.1a | 37.1a | 35.6a | 3.19 |
| N retention | | | | | | |
| g/d | -3.4c | 8.8a | 8.1a | 0.2b | -3.3c | 4.20 |
| g/g N intake | -0.17c | 0.16a | 0.16a | 0.01b | -0.06b | 0.092 |
| g/g digested N | -0.35c | 0.22a | 0.24a | -0.03b | -0.10b | 0.377 |
| Ruminal N balance , g/d | -0.09c | - 3.82c | 25.6a | 23.6a | 11.4b | 3.76 |
| Microbial N nitrogen production | | | | | | |
| g/d | 28.9b | 48.0a | 37.8ab | 32.5b | 49.8a | 9.51 |
| g/g N intake | 0.91a | 0.88a | 0.82ab | 0.62b | 0.89a | 0.152 |
| g microbial CP/g DOM | 70c | 133a | 118ab | 95bc | 145a | 21.4 |

[1]AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2]*A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals.

**Table 6.** Urea kinetics and microbial assimilation of recycled urea N in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| | Treatments[1] | | | | | |
|---|---|---|---|---|---|---|
| Item | Control | AC | RC | PR | PRAC | pSD[2] |
| Urea N kinetics, g N/d | | | | | | |
| Urea N entry rate (UER) | 37.5b | 63.2a | 57.1a | 60.7a | 66.8a | 7.92 |
| GIT entry rate (GER) | 28.6c | 46.3a | 38.6ab | 36.2b | 41.0ab | 8.10 |
| Urea N returned to the ornithine cycle (ROC) | 20.8b | 36.5a | 30.3a | 33.7a | 35.4a | 5.49 |
| Urea N utilized for anabolism (UUA) | 7.3ab | 8.5a | 7.5a | 1.2b | 4.4ab | 3.42 |
| Urea N excreted in feces (UFE) | 0.4b | 1.3a | 0.8ab | 1.3a | 1.2a | 0.48 |
| Fractional urea kinetics | | | | | | |
| UUE[3]:UER | 0.29c | 0.27c | 0.32bc | 0.41a | 0.39ab | 0.056 |
| GER:UER | 0.71a | 0.73a | 0.68ab | 0.59c | 0.61bc | 0.056 |
| ROC:GER | 0.79c | 0.79c | 0.80bc | 0.93a | 0.87ab | 0.050 |
| UUA:GER | 0.20a | 0.18a | 0.18a | 0.03b | 0.10b | 0.053 |
| UFE:GER | 0.014c | 0.027ab | 0.021b | 0.033a | 0.030ab | 0.0085 |
| Ruminal microbial uptake of recycled urea N | | | | | | |
| g N/d | 2.79b | 4.18a | 2.45b | 2.92b | 2.70b | 0.769 |
| % of total microbial N | 17.3b | 27.5a | 13.9c | 14.1c | 17.1b | 1.80 |
| % of GER | 10.5a | 7.9b | 5.7b | 6.1b | 5.2b | 2.20 |

[1]AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2]*A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals.[3]UUE – urinary N urea excretion (Table 4).

**Table 7**. Blood characteristics and liver function in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| Item | Treatments[1] | | | | | pSD[2] |
|---|---|---|---|---|---|---|
| | Control | AC | RC | PR | PRAC | |
| Blood characteristics | | | | | | |
| Glucose, mg/dL | 57.5 | 58.3 | 58.3 | 59.2 | 57.1 | 2.92 |
| Total protein, g/dL | 7.29 | 6.98 | 7.07 | 7.11 | 6.98 | 0.358 |
| Albumin, g/dL | 2.42 | 2.39 | 2.44 | 2.44 | 2.35 | 0.714 |
| Globulin, g/dL | 4.87 | 4.59 | 4.63 | 4.67 | 4.63 | 0.338 |
| Urea N, mg/dL | 4.82b | 13.07a | 14.07a | 12.81a | 12.01a | 1.320 |
| 3MH:creatinine, mg/g | 1.21a | 0.92b | 0.96b | 1.07b | 0.92b | 0.150 |
| Liverfunction[3] | | | | | | |
| AST, U/L | 37.0b | 45.6ab | 41.4ab | 45.9a | 40.3ab | 5.30 |
| ALT, U/L | 10.3b | 11.7b | 11.4b | 14.0a | 11.0b | 1.04 |

[1]AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2]*A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals. [3]AST, aspartate aminotransferase; ALT, alanine aminotransferase.

**Table 8.** Gene expression of aquaporins and urea transporters in rumen epithelium of heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract

| | Treatments[1] | | | | | |
|---|---|---|---|---|---|---|
| Gene | Control | AC | RC | RT | RTAC | pSD[2] |
| AQP3 | 5.749a | 4.498b | 4.998b | 3.972b | 4.58b | 0.7239 |
| AQP7 | 2.187 | 2.080 | 1.958 | 1.838 | 1.904 | 0.3398 |
| SLC14A1(UT-B) | 5.363a | 4.245b | 4.595b | 3.863b | 4.103b | 0.6334 |

[1]AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion. [2]*A posteriori* standard deviation for the difference between two treatments means. Different superscripts indicate differences based on overlapping of 90% credibility intervals.

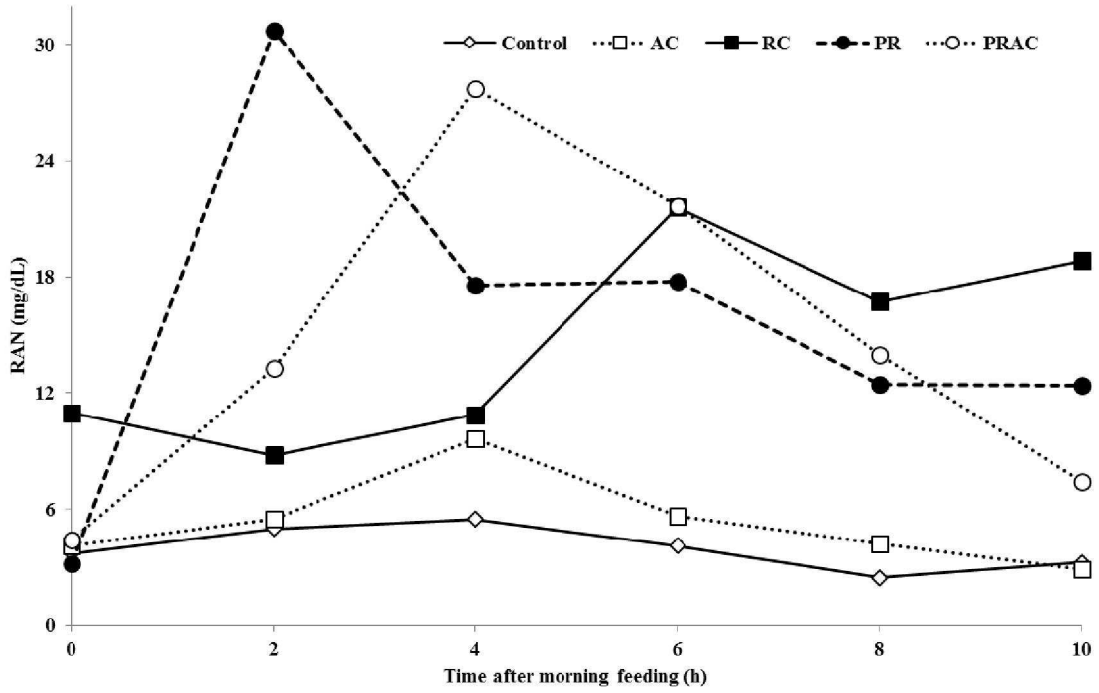


**Figure 1.** Average concentrations of rumen ammonia N according to the time after morning feeding in heifers fed a low-quality tropical forage and supplemented with urea in different sites of the gastrointestinal tract (AC, abomasal continuous infusion; RC, ruminal continuous infusion; PR, ruminal pulse dose every 12 hours; PRAC, half dose supplied as a pulse dose in the rumen every 12 hours and half dose supplied through continuous abomasal infusion).