



**BRUNO GROSSI COSTA HOMEM**

**PASTURE NITROGEN INPUT THROUGH FERTILISER OR  
LEGUME INTEGRATION: EFFECTS ON CANOPY  
STRUCTURE, FORAGE NUTRITIVE VALUE, ANIMAL  
PRODUCTION AND NITROGEN CYCLING**

**LAVRAS – MG  
2020**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para obtenção do título de Doutor.

Prof. Dr. Daniel Rume Casagrande  
Orientador

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**ENTRADA DE NITROGÊNIO EM PASTAGEM ATRAVÉS DE FERTILIZANTE OU  
INTEGRAÇÃO COM LEGUMINOSA: EFEITOS NA ESTRUTURA DO DOSSEL,  
VALOR NUTRITIVO DA FORRAGEM, PRODUÇÃO ANIMAL E CICLAGEM DE  
NITROGÊNIO**

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APROVADA em 28 de setembro de 2020.

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**LAVRAS – MG  
2020**

*Aos meus pais, Roberto e Maria, por serem os maiores exemplos da minha vida e pelo amor incondicional.*

*Dedico*

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*Algo só é impossível até que alguém duvide e resolva provar  
o contrário.*

Albert Einstein

## GENERAL ABSTRACT

Nitrogen (N) application or the integration with legumes into pasture systems coupled with grazing management strategies based on canopy structure may provide conditions for enhanced forage nutritive value, greater forage and animal productivity, and increased rates of N cycling. This study assessed canopy structure, forage nutritive value, animal performance and metabolism, and N cycling responses of three pasture treatments, namely, 1) mixed Marandu palisadegrass {*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]} and forage peanut (*Arachis pintoi* Krapov. & W.C. Greg cv. BRS Mandobi) pastures without N fertiliser (GRASS+LEGUME); 2) monoculture Marandu palisadegrass pasture with 150 kg N/ha/yr (GRASS+N); and 3) monoculture Marandu palisadegrass without N fertiliser (GRASS). Grazing management was similar across pasture treatments, using continuous stocking and a canopy height target of 20 to 25 cm. Concerning the canopy structure and forage nutritive value, herbage mass was greater in GRASS+N and GRASS+LEGUME pasture than in GRASS in summer and spring seasons ( $p = 0.014$ ). Grass crude protein (CP) and in vitro digestible dry matter (IVDDM) were greatest in the GRASS+N pasture ( $p < 0.001$ , and  $p < 0.001$ , respectively). Forage peanut had greater CP and IVDDM, and lower neutral detergent fibre (NDF) than Marandu palisadegrass. Concerning animal performance and metabolism, the average daily gain was greater in the GRASS+N and GRASS+LEGUME pastures than in the GRASS ( $p = 0.081$ ). GRASS+N pasture had the greatest stocking rate and liveweight gain per area ( $p < 0.001$  and  $p < 0.001$ , respectively), followed by GRASS+LEGUME pasture. No differences between treatments were found for the dry matter forage intake ( $p = 0.729$ ); however, GRASS+N and GRASS+LEGUME pastures had greater crude protein and digestible organic matter intakes than GRASS pasture ( $p = 0.007$  and  $p = 0.083$ , respectively). Apparent efficiency of N utilisation and microbial protein/CP intake ratio were greatest in the GRASS+LEGUME pastures ( $p = 0.009$  and  $p = 0.042$ , respectively). Concerning N cycling, existing litter and litter deposition rate were greatest in GRASS pasture ( $p = 0.005$  and  $p = 0.005$ , respectively). Litter decomposition rate was greater, and half-life time was lower in the GRASS+LEGUME and GRASS+N pastures than in the GRASS pasture ( $p = 0.079$  and  $p = 0.050$ , respectively). GRASS+N and GRASS+LEGUME pastures had the greatest deposited litter N ( $p = 0.004$ ). The greatest faecal N excretion, and urinary N excretion per seasons occurred in GRASS+N ( $p = 0.002$ , and  $p < 0.001$ , respectively). Nitrogen application or the integration of forage peanut in a grass pasture increased green herbage mass and improved forage nutritive value, increased animal performance, and improved the conservation of soil N reserves.

Keywords: *Arachis pintoi*. Beef cattle. *Brachiaria*. Fertilized pasture. Mixed pasture. Warm-season legume.

## RESUMO GERAL

A aplicação de nitrogênio (N) ou a integração com leguminosas em sistemas de pastagens em conjunto com estratégias de manejo do pastejo baseadas na estrutura do dossel podem fornecer condições para o aumento do valor nutritivo da forragem, maior produtividade de forragem e animal, e aumento da ciclagem de N. Este estudo avaliou a estrutura do dossel, o valor nutritivo da forragem, o desempenho e o metabolismo dos animais e as respostas do ciclo do N de três tratamentos de pastagem, a saber, 1) pastagem consorciada de capim-marandu (*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]) e amendoim forrageiro (*Arachis pintoi* Krapov. & W.C. Greg cv. BRS Mandobi) sem fertilizante nitrogenado (GRAMÍNEA+LEGUMINOSA); 2) monocultura de pasto de capim-marandu com 150 kg de N/ha (GRAMÍNEA+N); e 3) monocultura de pasto de capim-marandu sem fertilizante nitrogenado (GRAMÍNEA). O manejo do pastejo foi semelhante em todos os tratamentos de pastagem, usando lotação contínua e uma meta de altura do dossel de 20 a 25 cm. Com relação a estrutura do dossel e valor nutritivo da forragem, a massa de forragem foi maior na pastagem de GRAMÍNEA+N e GRAMÍNEA+LEGUMINOSA do que na GRAMÍNEA no verão e na primavera ( $p = 0,014$ ). A proteína bruta (PB) e a digestibilidade *in vitro* da matéria seca (DIVMS) do capim-marandu foram maiores na pastagem GRAMÍNEA+N ( $p < 0,001$ , and  $p < 0,001$ , respectivamente). O amendoim forrageiro apresentou maior PB e DIVMS e menor fibra em detergente neutro (FDN) do que o capim-marandu. Em relação ao desempenho e metabolismo dos animais, o ganho médio diário foi maior nas pastagens GRAMÍNEA+N e GRAMÍNEA+LEGUMINOSA do que GRAMÍNEA ( $p = 0,081$ ). A pastagem de GRAMÍNEA+N teve a maior taxa de lotação e ganho por área ( $p < 0,001$  e  $p < 0,001$ , respectivamente), seguida da pastagem de GRAMÍNEA+LEGUMINOSA. Não foram encontradas diferenças entre os tratamentos para o consumo de matéria seca de forragem ( $p = 0,729$ ); entretanto, as pastagens GRAMÍNEA+N e GRAMÍNEA+LEGUMINOSA tiveram maior ingestão de proteína bruta e matéria orgânica digestível do que as pastagens GRAMÍNEA ( $p = 0,007$  and  $p = 0,083$ , respectivamente). A eficiência aparente da utilização do N e a relação síntese de proteína microbiana/ingestão de PB foram maiores na pastagem de GRAMÍNEA+LEGUMINOSA ( $p = 0,009$  e  $p = 0,042$ , respectivamente). Em relação à ciclagem de N, a serapilheira existente e a taxa de deposição de serapilheira foram maiores na pastagem GRASS ( $p = 0,005$  e  $p = 0,005$ , respectivamente). A taxa de decomposição da serapilheira foi maior e a meia-vida foi menor nas pastagens GRAMÍNEA+LEGUMINOSA e GRAMÍNEA+N do que nas pastagens GRASS ( $p = 0,079$  and  $p = 0,050$ , respectivamente). A pastagem de GRAMÍNEA+N e GRAMÍNEA+LEGUMINOSA teve a maior quantidade de N ciclado via serapilheira depositada ( $p = 0,004$ ). As maiores excreções faecal e urinária de N por estação ocorreu em GRAMÍNEA+N ( $p = 0,002$  e  $p < 0,001$ , respectivamente). A aplicação de nitrogênio ou a integração do amendoim forrageiro em uma pastagem de gramínea aumentou a massa de forragem verde e melhorou o valor nutritivo da forragem, aumentou o desempenho animal e aumentou a conservação das reservas de N do solo.

Palavras-chaves: *Arachis pintoi*. *Brachiaria*. Pecuária de corte. Pastagem fertilizada. Pastagem consorciada. Leguminosa de clima tropical.

## INTERPRETIVE SUMMARY AND GRAPHICAL ABSTRACT

Nitrogen (N) is an essential element for the productivity of forage grasses. However, N application may cause significant negative environmental impacts, and its use is often limited by the high cost. Legumes, such as forage peanut (*Arachis pintoi*), will be an attractive alternative to provide N through biological N<sub>2</sub> fixation. Almost no information is available regarding canopy structure, forage nutritive value, animal performance and metabolism, and N cycling responses of fertilized and mixed pastures when compared with non-N fertilized systems under the same grazing management targets. Nitrogen input from fertiliser or legume integration increased herbage mass in canopies with similar height. The leaf:stem ratio in mass and forage allowance were similar among systems. Pastures managed under the same target of grazing management have little variation in the canopy structure. Thus, the heifers had similar dry matter forage intake. However, the improved forage nutritive value by N application or the presence of the legume increased the nutrient intake and digestibility, affecting positively the ruminal microbial protein synthesis and animal performance. Furthermore, N application or the integration of forage peanut in a grass pasture increased the conservation of soil N reserves by the enhancement of the total N recycled through litter and livestock excretion. The use of N application is the fastest and easiest way of intensification. However, sustainable production is a demand for grass-fed beef operations in Brazil, which ensures that the forage peanut has great potential for use in the humid tropics. Nevertheless, the economic advantages of the adoption of forage peanut by farmers will largely depend on the availability and cost of legume seeds.

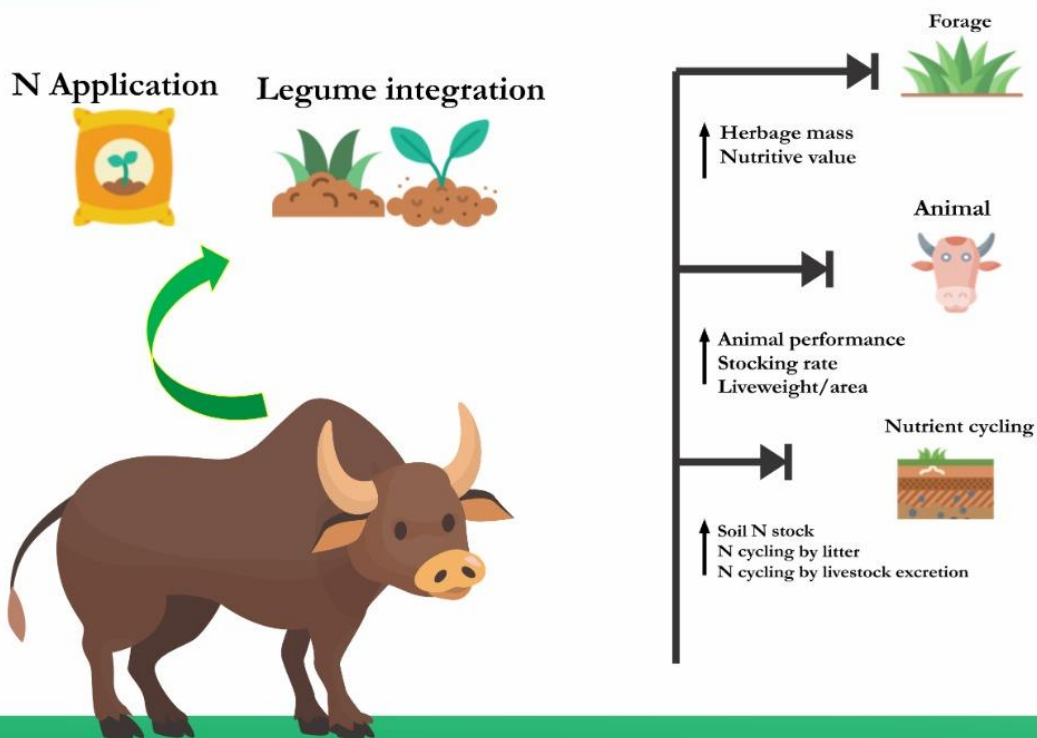
## Pasture nitrogen input through fertiliser or legume integration: effects on canopy structure, forage nutritive value, animal production and nitrogen cycling



Nitrogen (N) is an essential element for the productivity of forage grasses.

Legumes, such as forage peanut (*Arachis pintoi*), will be an attractive alternative to provide N across biological N fixation.

Nitrogen input from fertilizer or legume integration increased herbage mass in canopies with similar height.



The use of N application is the fastest and easiest way of intensification.

However, sustainable production is a demand for grass-fed beef operations in Brazil, which ensures that the forage peanut has great potential for use in the humid tropics.

Nevertheless, the economic advantages of the adoption of forage peanut by farmers will largely depend on the availability and cost of legume seeds.

## LIST OF FIGURES

### 1. CHAPTER 1

#### **Article 1 - Pasture nitrogen input through fertiliser or legume integration. 1. Effects on canopy structure and forage nutritive value**

**FIGURE 1** Mean monthly temperatures and rainfall in Lavras, Brazil, during the experimental period ..... 22

**FIGURE 2** Canopy height (cm) throughout the experimental period..... 27

**FIGURE 3** Canopy stratification of grass leaf and total leaf mass in rainy (A) and dry (B) seasons of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means ..... 32

**FIGURE 4** Canopy stratification of grass stem and total stem mass in rainy (A) and dry (B) seasons of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means ..... 33

**FIGURE 5** Canopy stratification of grass dead material and total dead material mass in rainy (A) and dry (B) seasons of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means ..... 34

**FIGURE 6** Crude protein (CP; A), neutral detergent fibre (NDF; B), in vitro digestible dry matter (IVDDM; C), and CP:IVDDM ratio (D) of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means ..... 37

### 2. CHAPTER 2

#### **Article 2 - Pasture nitrogen input through fertiliser or legume integration. 2. Effects on animal performance, forage intake and digestion, and nitrogen metabolism**

**FIGURE 1** Mean monthly temperatures and rainfall in Lavras, Brazil, during the experimental period ..... 53

**FIGURE 2** Canopy height (cm) throughout the experimental period ..... 55

**FIGURE 3** Herbage mass and botanical composition of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means ..... 61

**FIGURE 4** Stocking rate (SR) as a function of the legume mass (LM) of mixed Marandu palisadegrass with forage peanut pasture. (Stocking rate =  $1.467 + 0.0007 \cdot \text{legume mass}$ ;  $p < .001$  and  $p < .001$  for intercept and slope, respectively;  $R^2 = 0.582$ )..... 63

**FIGURE 5** Legume intake proportion of beef cattle as a function of the percentage of legume in the canopy (BC) of mixed Marandu palisadegrass with forage peanut pasture. (Legume intake =  $0.394 + 0.919 \cdot \text{legume proportion of DM}$ ;  $p = .091$  and  $p < .001$  for intercept and slope, respectively;  $R^2 = 0.689$ ) ..... 66

### 3. CHAPTER 3

#### Article 3 - NITROGEN CYCLING IN PASTURES WITH NITROGEN INPUT THROUGH FERTILISER APPLICATION OR LEGUME INTEGRATION

**FIGURE 1** Mean monthly temperatures and rainfall in Lavras, Brazil, during the experimental period (figure before shown at Homem *et al.*, 2021a) ..... 85

**FIGURE 2** Canopy height (cm) throughout the experimental period (figure previously shown in Homem *et al.*, 2021a) ..... 87

**FIGURE 3** Herbage mass (left) and grass mass (right) of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Errors bars represent  $\pm$  standard errors of the means ..... 96

**FIGURE 4** Grass N concentration of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Errors bars represent  $\pm$  standard errors of the means ..... 97

**FIGURE 5** Total N stock on herbage mass (left) and grass N stock on grass mass (right) of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Errors bars represent  $\pm$  standard errors of the means 98

**FIGURE 6** Schematic of the nitrogen annual cycle in a Marandu palisadegrass pasture fertilised with  $150 \text{ kg N ha}^{-1}$  and continuously stocked by Nellore heifers..... 108

**FIGURE 7** Schematic of the nitrogen annual cycle in a mixed Marandu palisadegrass and forage peanut pasture continuously stocked by Nellore heifers ..... 108

**FIGURE 8** Schematic of the nitrogen annual cycle in a Marandu palisadegrass pasture without N fertiliser application continuously stocked by Nellore heifers ..... 109



## LIST OF TABLES

### 1. CHAPTER 1

#### **Article 1 - Pasture nitrogen input through fertiliser application or legume integration. 1. Effects on canopy structure and forage nutritive value**

**TABLE 1** Structural characteristics of Marandu palisadegrass pastures with application or not of N fertiliser, or mixed with forage peanut during the seasons of the experimental period ... 29

**TABLE 2** Nutritive value of Marandu palisadegrass pastures with application or not of N fertiliser, or mixed with forage peanut during the seasons of the experimental period ..... 36

### 2. CHAPTER 2

#### **Article 2 - Pasture nitrogen input through fertiliser application or legume integration. 2. Effects on animal performance, forage intake and digestion, and nitrogen metabolism**

**TABLE 1** Animal performance of Nellore heifers on Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period ..... 62

**TABLE 2** Diet, intake and apparent digestibility of forage consumed by Nellore heifers on Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period..... 65

**TABLE 3** Nitrogen metabolism of Nellore heifers on Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period ..... 68

### 3. CHAPTER 3

#### **Article 3 - NITROGEN CYCLING IN PASTURES WITH NITROGEN INPUT THROUGH FERTILISER APPLICATION OR LEGUME INTEGRATION**

**TABLE 1** Legume characteristics on Marandu palisadegrass and forage peanut mixed pastures during the seasons of the experimental period ..... 95

**TABLE 2** Balance of existing litter of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period..... 101

**TABLE 3** Balance of deposited litter, decomposition, and nitrogen cycling of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period..... 102

<b>TABLE 4</b> Forage and N intake by beef heifers in Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period .....	104
<b>TABLE 5</b> Nitrogen excretion by beef heifers in Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period .....	105
<b>TABLE 6</b> Nitrogen cycling balance of beef heifers in Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period .....	107

## TABLE OF CONTENTS

<b>1. CHAPTER 1.....</b>	<b>20</b>
<b>Article 1 - Pasture nitrogen input through fertiliser or legume integration. 1. Effects on canopy structure and forage nutritive value.....</b>	<b>20</b>
<b>1. INTRODUCTION.....</b>	<b>21</b>
<b>2. MATERIAL AND METHODS .....</b>	<b>22</b>
<b>3. RESULTS .....</b>	<b>27</b>
<b>4. DISCUSSION .....</b>	<b>38</b>
<b>5. CONCLUSIONS .....</b>	<b>42</b>
<b>REFERENCES.....</b>	<b>43</b>
<b>2. CHAPTER 2.....</b>	<b>50</b>
<b>Article 2 - Pasture nitrogen input through fertiliser application or legume integration. 2. Effects on animal performance, forage intake and digestion, and nitrogen metabolism.....</b>	<b>50</b>
<b>1. INTRODUCTION.....</b>	<b>51</b>
<b>2. MATERIAL AND METHODS .....</b>	<b>52</b>
<b>3. RESULTS .....</b>	<b>60</b>
<b>4. DISCUSSION .....</b>	<b>69</b>
<b>5. CONCLUSIONS .....</b>	<b>73</b>
<b>REFERENCES.....</b>	<b>74</b>
<b>3. CHAPTER 3.....</b>	<b>82</b>
<b>Article 3 - NITROGEN CYCLING IN PASTURES WITH NITROGEN INPUT THROUGH FERTILISER APPLICATION OR LEGUME INTEGRATION.....</b>	<b>82</b>
<b>1. Introduction.....</b>	<b>83</b>
<b>2. Materials and methods .....</b>	<b>85</b>
<b>3. Results .....</b>	<b>94</b>
<b>4. Discussion.....</b>	<b>109</b>
<b>5. Conclusions.....</b>	<b>114</b>
<b>REFERENCES.....</b>	<b>115</b>

## 1. CHAPTER 1

### Article 1 - Pasture nitrogen input through fertiliser or legume integration. 1. Effects on canopy structure and forage nutritive value

Running head: **Nitrogen inputs to warm-climate pastures**

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**Preliminary version. The manuscript is following the guidelines in this Journal.**

#### **Abstract**

Nitrogen inputs to pasture systems coupled with grazing management strategies based on monitoring and control of canopy structure, may provide conditions for greater productivity and enhanced forage nutritive value. This 2-yr study assessed canopy structure and nutritive value responses of three pasture treatments, namely, 1) mixed Marandu palisadegrass {*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]} and forage peanut (*Arachis pintoi* Krapov. & W.C. Greg. cv. BRS Mandobi) pastures without N fertiliser (GRASS+LEGUME); 2) monoculture Marandu palisadegrass pastures fertilised with 150 kg N/ha (GRASS+N); and 3) monoculture Marandu palisadegrass without N fertiliser (GRASS). Grazing management was similar across pasture treatments, using continuous stocking and a target canopy height of 20 to 25 cm. Herbage mass was greater in GRASS+N and GRASS+LEGUME pastures than in GRASS in summer and spring ( $p = .014$ ). The leaf:stem ratio in the grass mass ( $p = .731$ ) was similar between treatments. Grass crude protein (CP) and *in vitro* digestible dry matter (IVDDM) were greatest in the GRASS+N pasture ( $p < .001$ , and  $p < .001$ , respectively). Grass neutral detergent fibre (NDF) concentration was greater at GRASS and GRASS+LEGUME pastures than in GRASS+N ( $p < .001$ ). Forage peanut had greater CP and IVDDM, and lesser NDF than Marandu palisadegrass. Nitrogen application or the presence of the legume increased green herbage mass and improved forage nutritive value in canopies with similar height.

#### **KEYWORDS**

*Arachis pintoi*, *Brachiaria*, fertilized pasture, mixed pasture, warm-season legume

## 1. INTRODUCTION

In Brazil, the majority of grass-fed beef operations rely on pastures of the *Brachiaria* genus. The use of fertiliser on these pastures has been rare until recently (Pereira et al., 2020). Currently, N fertiliser application is becoming a more common practice, which results in increases in productivity and protein concentrations of tropical forages (Delevatti et al., 2019). However, its use may be limited by cost and distribution logistics, since the beef industry is based on extensive operations. In the last 20 years, the average price of N fertiliser has increased by more than 500% on the Brazilian market (ANDA, 2015). An attractive alternative would be the establishment of a forage legume in the sward to provide N (Muir, Pitman, Dubeux, & Foster, 2014; Muir, Pitman, & Foster, 2011; Pereira et al., 2020), which along with other benefits would minimize dependence of farmers on synthetic N fertiliser (Alencar et al., 2018). Biological nitrogen fixation (BNF) is extremely relevant to sustainable agriculture (Oldroyd, Murray, Poole, & Downie, 2011; Udvardi & Poole, 2013) since it represents the primary N input for several terrestrial ecosystems (Carvalho, Pereira, Hungria, Camargo, & Da Silva, 2019). Among the tropical legumes, forage peanut has been the most promising for use in mixed pastures in warm-climate grassland ecosystems (Gomes et al., 2018; Hernandez, Argel, Ibrahim, and 'tMannetje, 1995; Pereira et al., 2020; Tamele, Lopes de Sá, Bernardes, Lara, & Casagrande, 2018).

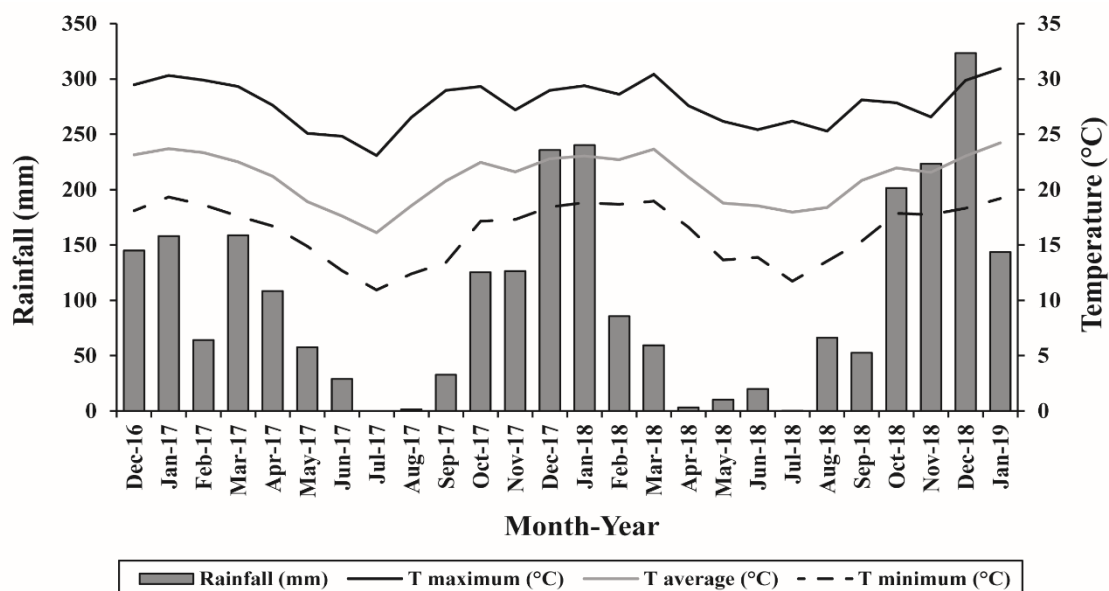
The use of grazing management strategies based on pasture targets, particularly canopy height, has become an essential requirement in pastures with N input (Rouquette Jr., 2015), which should provide conditions for better adjustments in the canopy structure for grazing (Congio et al., 2018). Canopy structure is the spatial arrangement of the aboveground organs of plants in a plant community (Norman & Champbell, 1989). The spatial arrangement of the morphological components is a balance between forage input (i.e., herbage accumulation) and forage output (i.e., senescence rate and forage intake). Nitrogen input in pasture systems may alter the morphological components of plants due to an increase in their growth rate. On the other hand, stocking rate adjustments might become the null balance, resulting in similar canopies structure with different N inputs. The tissue renovation from N input enhances the proportion of younger tissue, consequently improving forage nutritive value (Delevatti et al., 2019). However, almost no information is available regarding canopy structure and the nutritive value of fertilised and mixed tropical pastures when compared with non-N fertilised systems under the same grazing management targets.

We hypothesized that N input would not affect canopy structure if the same canopy height were used as management target for adjusting stocking rate. Forage nutritive value would improve with the N fertiliser input or the presence of a legume. Additionally, changes in grass nutritive value in mixed pastures would have less impact compared to an N-mineral input due to slower transfer of N between legume and grass. Therefore, the objective of this study was to investigate canopy structure and forage nutritive value responses of mixed pastures of Marandu palisadegrass and forage peanut compared to Marandu palisadegrass in monoculture, fertilised or not with N, grazed using continuous stocking with variable stocking rate to achieve similar canopy height across all experimental units.

## 2. MATERIAL AND METHODS

### 2.1 Experimental site and treatments

The study was carried out on the Experimental Farm of the Federal University of Lavras, Brazil (21°14'S, 44°58'W; 918 m above sea level). This area has a subtropical humid mesothermal climate with dry winters (Köppen-Geiger climate classification: Cwa; Sá Júnior, Carvalho, Silva, & Alves, 2012). Meteorological data were obtained from a weather station located 1,000 m from the experimental area (Figure 1).



**FIGURE 1** Mean monthly temperatures and rainfall in Lavras, Brazil, during the experimental period.

The soil in the area is a Ferralsol (WRB/FAO classification). Soil texture is clayey, and the clay content is similar down the profile (a characteristic of this soil type): 563 g clay/kg soil (0–10 cm) and 574 g clay/kg soil (20–40 cm). Soil analyses were conducted according to Embrapa standard techniques (Claessen, Barreto, Paula, & Duarte, 1997). The soil (0–20 cm) had the following properties: pH (H<sub>2</sub>O) = 5.9; exchangeable Al, Ca, Mg, 0.07, 2.4 and 0.7 cmol<sub>c</sub>/dm<sup>3</sup>, respectively; available P (Mehlich-I method) 7.6 mg/dm<sup>3</sup>, exchangeable K 82.8 mg/dm<sup>3</sup>, and organic matter 31.0 g/kg.

Treatments were three pastures types (**PT**), namely: 1) Marandu palisadegrass and forage peanut, mixed pasture without N fertiliser application (**GRASS+LEGUME**); 2) Marandu palisadegrass monoculture fertilised with 150 kg N/ha/year (**GRASS+N**); and 3) Marandu palisadegrass monoculture without N fertiliser application (**GRASS**).

## 2.2 Pasture and treatment establishment

In January 2014, the whole experimental area was seeded with Marandu palisadegrass at a rate of 6.0 kg/ha of pure live seeds. The 12-ha experimental area was limed (2,500 kg dolomitic lime/ha) 60 d before grass seeding. Fifty-two kg of P/ha as single super phosphate and 41.5 kg of K/ha as potassium chloride were applied during grass seeding. The whole experimental area was divided into four paddocks with three hectares each (blocks), taking into consideration the topography of the area and managed under rotational stocking until December 2015.

In December 2015, the blocks were divided into three paddocks where pasture types were randomly allocated. The size of each paddock (experimental unit) was adjusted based on previous studies in the area using these pastures types. The GRASS+N and GRASS paddocks size (0.7 and 1.3 ha, respectively) were determined by the stocking rate obtained in the study of Pereira et al. (2015) with Marandu palisadegrass monoculture pastures with 150 kg/ha of N application or no fertiliser application. In the GRASS+LEGUME treatment the size of the paddock (1.0 ha) was determined by the stocking rate obtained in the study of Gomes et al. (2018) with Marandu palisadegrass and forage peanut mixed pastures. Paddock size of each PT allowed adding an average of 3.0 animal units (AU) per paddock during the rainy season (AU: animal unit was considered a bovine weighing 500 kg; Allen et al., 2011). Furthermore, this allowed tester animals (at least two animals) to remain the maximum possible time during the dry season and that all animals were removed at the same time.

The GRASS+LEGUME paddocks were seeded with forage peanut into a previously established Marandu palisadegrass pasture in December 2015. Before the forage peanut seeding, the Marandu palisadegrass canopy was lowered to around 10 cm by mechanical cutting. The seeding rate was 10 kg/ha of pure live seeds of forage peanut planted with a no-till seeder with four rows. The planter allocated six seeds of forage peanut per linear meter with 0.5 m row spacing. After seeding, the paddocks with forage peanut were excluded from grazing during 75 d to improve the establishment.

In March 2016, grazing management targets were imposed under continuous stocking. This period up to December 2016, was considered an adaptation of the canopies to the experimental management. From December 2016 to January 2019, eight seasons were evaluated over time. The years were divided into the four seasons of each year: from December, 22 to March, 21 (Summer), from March, 22 to July, 21 (Fall), from July, 22 to September, 21 (Winter), and from September, 22 to December, 21 (Spring). Continuous stocking with variable stocking rate was used to achieve a target canopy height of 15 cm during winter and 20 to 25 cm during the remaining of the year. In each experimental year, two Nellore heifers ( $234 \pm 36$  kg of initial BW and  $12 \pm 1.3$  months of age) were used as tester animals in each paddock. At the end of the first experimental year, the animals were replaced. When necessary, put-and-take animals were used to maintain targeted canopy height (Allen et al., 2011). Water and mineral supplementation were supplied *ad libitum*. Average canopy height was measured weekly using a sward stick (Barthram, 1985) at 100 random points per paddock, and the stocking rate adjusted when necessary.

Annually, in the spring (between November and December), all paddocks received fertiliser application with single superphosphate and potassium chloride corresponding to 22 kg P/ha and 41 kg K/ha, respectively. In the GRASS+N pasture, the N fertiliser application was divided into three applications per year (50 kg N/ha each in November, January, and March), all using urea.

## **2.3 Response variables**

Herbage mass, leaf area index, canopy stratification, and forage nutritive value were evaluated every 30 d during the two years of the experimental period.

### **2.3.1 Herbage mass and leaf area index**



Herbage mass was sampled by harvesting six frames at ground level, measuring  $1 \times 0.5$  m, per paddock, at sites with average canopy height. After harvesting, the fresh material was weighed and subsampled using approximately 250 g fresh material for assessing DM concentration. A further subsample of approximately 2 kg was taken for manual separation of botanical and morphological components. Grass samples were separated into stem (stem + sheath), leaf (leaf blade), and dead material. Legume samples were separated into stem and leaf (stipule + petiole + leaflet). Forage samples were oven-dried at 55 °C for 72 h to a constant weight. Grass mass was considered as leaf + stem without dead material, and legume mass was considered to be the leaf plus stem mass. Herbage mass was considered to be the aboveground biomass of herbaceous plants (grass and legume mass, according to treatment) without dead material. The green:dead forage ratio was calculated as the division of the herbage mass by dead material mass. The leaf:stem ratio was estimated as the division of the leaf mass by stem mass on a dry weight basis. Herbage allowance was calculated as average herbage mass for a season divided by the average total weight of animals that grazed during the season (Sollenberger, Moore, Allen, & Pedreira, 2005).

The specific leaf area was measured in each paddock by harvesting the herbage within six rectangular frames of 0.5 x 0.3 m, allocated at representative points (average canopy height). These samples were separated into grass leaf blades and legume leaflets. The leaves and leaflets were scanned using a LAI 3100 leaf area integrator (LI-COR®, Lincoln, NE). Samples were dried at 55 °C for 72 hours and weighed for subsequent calculation of leaf area index (LAI). The LAI was estimated by multiplying the specific leaf area by the leaf mass of grass and legume.

### **2.3.2 Canopy stratification**

Herbage mass was evaluated in four layers of the canopy height (% of canopy height). The first layer, close to the ground, represented 0 to 25%; the second layer 25 to 50%; the third layer 50 to 75%; and the fourth layer (at the top of the canopy) 75 to 100% of the canopy height (Tamele et al., 2018). For this, the canopy structure was evaluated using an inclined point quadrat with 120 data points per paddock (Wilson, 1960). Herbage mass within each layer (% of mass per stratum) was estimated by the frequency of each botanical and morphological component multiplied by the respective mass in the four-canopy layer.

### 2.3.3 Nutritive value

Hand-plucked forage samples were collected for forage nutritive value analysis (Vries, 1995). Forage samples were oven-dried at 55 °C for 72 h to a constant weight. In the GRASS+LEGUME pasture, grass and legume were collected and separated later. The forage samples were ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. The DM of each sample was obtained by oven drying at 100 °C for 18 h (Method 934.01; AOAC, 2000). The CP concentration was calculated based on the N concentration (CP = total N × 6.25), which was determined using the Kjeldahl procedure (Method 920.87; AOAC, 2000). The ether extract (EE) was analysed according to Method 920.39 (AOAC, 2000). The ash-free NDF was determined by the autoclave method at 105 °C for 60 min (Pell & Schofield, 1993). The *in vitro* digestible DM (IVDDM) was determined by using the DAISY-II method for 48 h (Ankom Technology Corp., Fairport, NY; Holden, 1999). Rumen fluid was collected before feeding on two cannulated heifers fed a diet that consisted of Marandu palisadegrass and forage peanut mixed pasture or grass monoculture of Marandu palisadegrass according to treatment. The CP:IVDDM ratio was calculated considering what would be the CP intake (in grams) to each kg of IVDDM.

### 2.4 Statistical analysis

The data were presented as the averages of seasons. The experimental design was a randomized complete block type with three treatments (pasture type; GRASS+N, GRASS, and GRASS+LEGUME pastures), four replications, and seasons of the year considered as repeated measurements over time. Data were analysed by fitting mixed models (Littell, Pendergast, & Natarajan, 2000), using the MIXED procedure of SAS (SAS Institute, Cary NC). The effects of types of pasture and seasons were considered fixed and the effect of block and year were considered random effects. The Akaike information criterion was used to choose the best (co)variance structure (Akaike, 1974). All variance components were estimated using the restricted maximum likelihood method. The averages were estimated using the LSMEANS statement, and comparisons were made between treatments using Fisher's protected least significant difference (LSD) test at 10% probability. The statistical model for data analysis was as follows:

$$Y_{ijkz} = \mu + B_i + PT_j + \gamma_{ij} + Y_k + S_z + (PT \times S)_{jz} + \varepsilon_{ijkz}$$

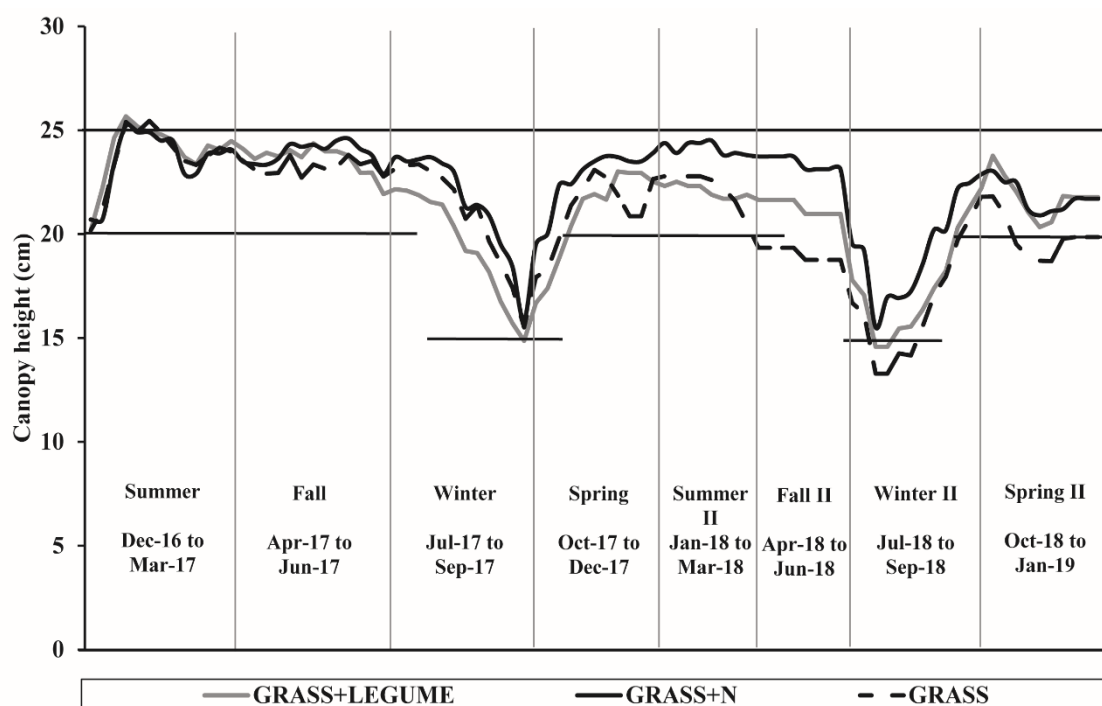
where  $Y_{ijkz}$  = value observed in the  $i$ th block of the  $j$ th PT of the  $k$ th year of the  $z$ th season;  $\mu$  = overall average;  $B_i$  = random effect associated with the  $i$ th block,  $i = 1, 2, 3, 4$ ;  $PT_j$  = fixed effect associated with  $j$ th types of pasture,  $j = 1, 2, 3$ ;  $\gamma_{ij}$  = random error associated with the  $i$ th block in the  $j$ th PT.  $Y_k$  = random effect associated with  $k$ th year,  $k = 1, 2$ ;  $S_z$  = fixed effect associated with  $z$ th season,  $z = 1, 2, \dots, 8$ ;  $(PT \times S)_{jz}$  = fixed effect of interaction  $j$ th PT with the  $z$ th season.  $\varepsilon_{ijkz}$  = random error associated with the  $i$ th block, the  $j$ th PT, the  $k$ th year, and the  $z$ th season.

The effect of canopy layers was included in the statistical model to run canopy strata data. Their interactions were also considered in the model. The canopy stratification was pooled into two seasons, being rainy season (spring, summer, and fall) and dry season (winter).

### 3. RESULTS

#### 3.1 Canopy structure

The variation in canopy height between the PT during the seasons was small; all treatments had canopy height ranging between 20 to 25 cm (Figure 2). During the dry season (winter), canopy height ranged from 15 to 20 cm (Figure 2).



**FIGURE 2** Canopy height (cm) throughout the experimental period.

Herbage mass was greater in the GRASS+N and GRASS+LEGUME pastures than in GRASS in summer and spring ( $p = .014$ ; Table 1). In fall and winter, the herbage mass was greater in GRASS+N than in GRASS and GRASS+LEGUME pastures. The grass mass was 50 to 93% greater in GRASS+N than in GRASS and GRASS+LEGUME pastures throughout the year ( $p = .028$ ; Table 1). In the GRASS+LEGUME pasture, the legume composed 25 to 33% of the total herbage mass. The greatest percentage of legume occurred in spring. The green:dead forage ratio in the grass mass was similar between GRASS and GRASS+LEGUME pastures. In both GRASS and GRASS+LEGUME, the green:dead ratio in the grass mass was less than in the GRASS+N pasture ( $p = .010$ ), except in winter when all treatments were similar (Table 1). The green:dead forage ratio in the herbage mass was greater in GRASS+LEGUME pastures than at GRASS in summer, fall and spring ( $p = .012$ ; Table 1). However, this ratio was similar for GRASS+N and GRASS+LEGUME pastures in summer and spring. In winter, all treatments were similar. The leaf:stem ratio in the grass and herbage mass were similar in all treatments throughout the year ( $p = .731$  and  $p = .116$ , respectively; Table 1). The leaf:stem ratio in the grass and herbage mass was greater in spring, followed by summer and fall, and less in winter ( $p < .001$  and  $p < .001$ , respectively). The grass LAI was greater in the GRASS+N than in the GRASS and GRASS+LEGUME pastures in summer and fall ( $p = .011$ ; Table 1). In winter, the grass LAI was similar in all treatments. In spring, the grass LAI was greater at GRASS+N than the GRASS, and both were similar to GRASS+LEGUME pastures. The total LAI was greater for the GRASS+LEGUME than for the GRASS pasture in summer and spring ( $p = .007$ ; Table 1). The herbage allowance was similar in all treatments ( $p = .293$ ), with an average value of 5.4 kg of herbage mass/kg of body weight in summer, fall, and spring (data not shown).

**TABLE 1** Structural characteristics of Marandu palisadegrass pastures with application or not of N fertiliser, or mixed with forage peanut during the seasons of the experimental period

Variables	Season (S)	Pasture type (PT)				SEM <sup>†</sup>	P-Value		
		GRASS	GRASS+N	GRASS+LEGUME			PT	S	PT*S
				Palisadegrass	Total herbage <sup>‡</sup>				
<b>Herbage mass kg DM/ha</b>	Summer	3550 B b	5343 A a	3593 B	4998 a	521	Comparing Palisadegrass only		
	Fall	3460 B b	6231 A a	3098 B	4227 b		<.001	<.001	.028
	Winter	1947 B b	3584 A a	1764 B	2349 b		Comparing Total herbage		
	Spring	3148 B b	4820 A a	2746 B	4093 a		.001	<.001	.014
<b>Green:dead mass ratio</b>	Summer	0.80 B b	1.56 A a	1.01 B	1.39 a	0.15	Comparing Palisadegrass only		
	Fall	0.60 B c	1.20 A a	0.70 B	0.95 b		<.001	<.001	.010
	Winter	0.30 A a	0.53 A a	0.34 A	0.44 a		Comparing Total herbage		
	Spring	0.58 B b	0.99 A a	0.61 B	0.89 a		<.001	<.001	.012
<b>Leaf:stem ratio</b>	Summer	0.81	0.86	0.87	0.79	0.05	Comparing Palisadegrass only		
	Fall	0.67	0.65	0.60	0.54		.731	<.001	.257
	Winter	0.53	0.45	0.46	0.40		Comparing Total herbage		
	Spring	1.00	0.91	1.01	0.92		.116	<.001	.361
<b>Leaf area index</b>	Summer	2.30 B c	3.91 A a	2.53 B	3.09 b	0.39	Comparing Palisadegrass only		
	Fall	1.71 B b	3.66 A a	1.74 B	2.14 b		<.001	<.001	.011
	Winter	0.74 A a	1.42 A a	0.72 A	0.85 a		Comparing Total herbage		
	Spring	1.91 B b	3.24 A a	2.58 AB	3.07 a		<.001	<.001	.007

<sup>a-c</sup>Comparing Total herbage; Least squares means within a row with different lowercase differ at  $p \leq .10$ ;

<sup>A-C</sup>Comparing Palisadegrass only; Least squares means within a row with different uppercase differ at  $p \leq .10$ ;

<sup>‡</sup>Grass + legume;

<sup>†</sup>Standard error of the means.

### 3.2 Canopy stratification

During the rainy season, there was an effect of PT on total leaf mass within canopy layers. In the basal canopy stratum (25%), total leaf mass was greatest for GRASS+LEGUME pasture ( $p < .001$ ; Figure 3A). The GRASS+LEGUME and GRASS+N pastures ( $p < .001$ ; Figure 3A) had greater total leaf mass in the second stratum from the bottom up (25 to 50%) than the GRASS pasture. The GRASS+N pasture had the greatest total leaf mass in the canopy stratum of 50 to 75% ( $p = .001$ ; Figure 3A), followed by the GRASS+LEGUME and GRASS pastures. In the upper canopy stratum, total leaf mass was greatest for the GRASS+N pasture ( $p = .002$ ; Figure 3A). Grass leaf mass in the rainy season was similar between PT in the canopy stratum of 25% ( $p = .293$ ; Figure 3A). The mean grass leaf mass in this stratum was 3.8 g/m<sup>2</sup>. Greater grass leaf mass in the canopy stratum of 50% was found in the GRASS+N pasture ( $p < .001$ ; Figure 3A), followed by the GRASS+LEGUME and GRASS pastures. In the canopy strata of 75% and above, grass leaf mass was greatest in the GRASS+N pasture ( $p < .001$  and  $p < .001$ , respectively; Figure 3A).

During the dry season, the greatest values of total leaf mass were found in the GRASS+LEGUME pasture in the canopy strata of 25 and 50% ( $p = .042$  and  $p = .011$ , respectively; Figure 3B). Total leaf mass was similar in all TP ( $p = .092$ ; Figure 3B) in the canopy stratum of 75%. On average, the total leaf mass was 16.7 g/m<sup>2</sup>. In the upper canopy in the dry season, total leaf mass was greatest in the GRASS+N pasture ( $p < .001$ , Figure 3B). During the dry season, there was no difference between PT for grass leaf mass in the canopy strata of 25% and 50% ( $p = .131$  and  $p = .088$ , respectively; Figure 3B). The average of grass leaf mass was 1.3 and 4.6 g/m<sup>2</sup> for the strata of 25% and 50%. The GRASS+N pasture in the dry season had the greatest values of grass leaf mass in canopy strata of 75% and the upper ( $p = .027$  and  $p < .001$ , respectively; Figure 3B).

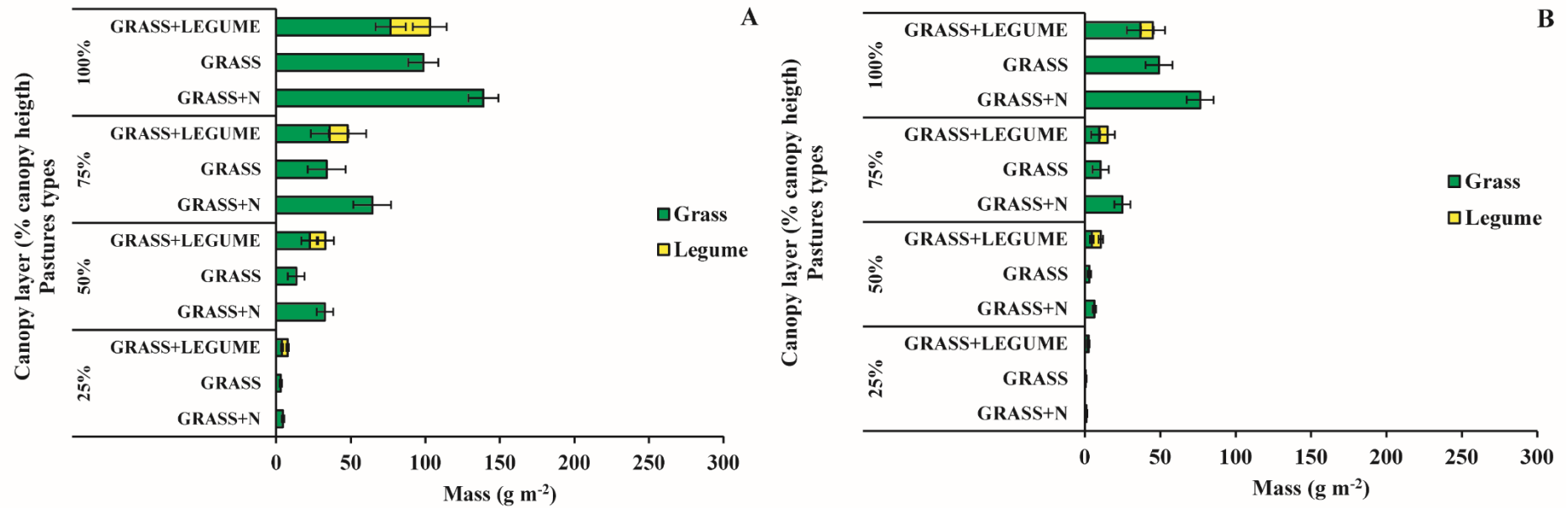
During the rainy season, greater total stem mass was found in the GRASS+N and GRASS+LEGUME pastures for the canopy strata of 25 and 50% ( $p < .001$  and  $p = .017$ , respectively; Figure 4A). However, in the 50% stratum there was no difference between the GRASS+LEGUME and GRASS pastures. The GRASS+N pasture had the greatest total stem mass in the canopy stratum of 75% ( $p < .001$ ; Figure 4A). There was no difference between PT in the upper canopy ( $p = .181$ ; Figure 4A), with an average of 47.4 g/m<sup>2</sup>. The GRASS+N pasture had the greatest grass stem mass in the canopy strata of 25, 50 and 75% in the rainy season ( $p < .001$ ,  $p = .001$ , and  $p < .001$ , respectively; Figure

4A). There was no difference between PT in the upper canopy ( $p = .168$ ; Figure 4A) in the rainy season, with an average of  $39.6 \text{ g/m}^2$ .

In the dry season, the total stem mass and grass stem mass were similar between PT in the canopy stratum of 25% ( $p = .235$  and  $p = .139$ , respectively; Figure 4B) with an average of  $24.2$  and  $20.4 \text{ g/m}^2$ , respectively. In the 50, 75%, and upper strata of the canopy, the GRASS+N pasture had the greatest total stem mass ( $p = .031$ ,  $p < .001$ , and  $p = .013$ , respectively; Figure 4B), and grass stem mass ( $p = .002$ ,  $p < .001$ , and  $p = .008$ , respectively; Figure 4B) in the dry season.

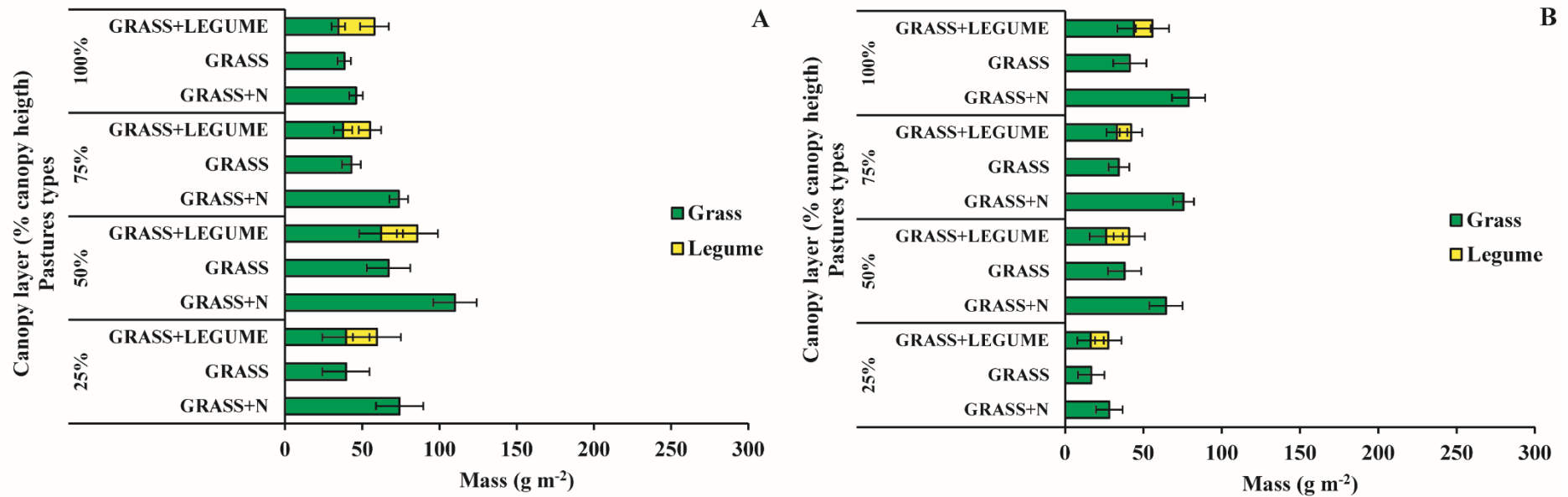
During the rainy season, there was no difference between PT in the canopy stratum for 25% for dead material mass ( $p = .193$ ; Figure 5A), with an average of  $134 \text{ g/m}^2$ . GRASS+N and GRASS pastures had the greatest dead material mass in the canopy stratum of 50% ( $p = .009$ ; Figure 5A). In the canopy stratum of 75%, the greater values of dead material mass were found in the GRASS pasture, followed by the GRASS+N and GRASS+LEGUME pastures ( $p < .001$ ; Figure 5A). The GRASS and GRASS+LEGUME pastures had the greatest mass of dead material in the upper canopy ( $p = .003$ ; Figure 5A) in the rainy season.

In the dry season, in the canopy strata of 25 and 75%, the greatest mass of dead material was found in the GRASS+N pasture ( $p = .033$  and  $p = .005$ , respectively; Figure 5B). In the canopy stratum of 50%, there was no difference between PT ( $p = .152$ ; Figure 5B), with an average of  $149 \text{ g/m}^2$ . The GRASS+N and GRASS pastures had the greatest mass of dead material in the upper canopy strata ( $p = .0212$ ; Figure 5B) in the dry season.

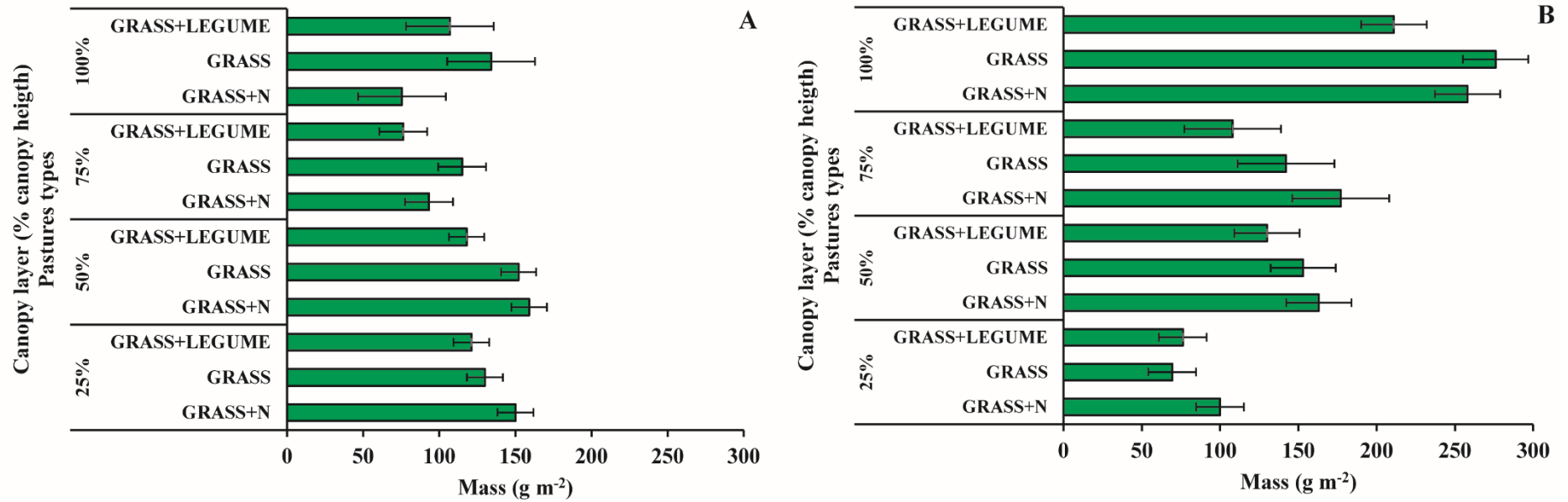


**FIGURE 3** Canopy stratification of grass leaf and total leaf mass in rainy (A) and dry (B) seasons of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means





**FIGURE 4** Canopy stratification of grass stem and total stem mass in rainy (A) and dry (B) seasons of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means.



**FIGURE 5** Canopy stratification of grass dead material and total dead material mass in rainy (A) and dry (B) seasons of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means.

### 3.3 Forage nutritive value

Grass CP and IVDDM were greater in the GRASS+N than in the GRASS and GRASS+LEGUME pastures throughout the two years ( $p < .001$  and  $p < .001$ , respectively; Table 2). Grass NDF concentration was greater in the GRASS and GRASS+LEGUME pastures than in the GRASS+N pasture ( $p < .001$ ; Table 2). The grass EE was greater in the GRASS+N pasture than in the other treatments ( $p = .001$ ; Table 2). The grass CP:IVDDM ratio was greatest in the GRASS+N pasture ( $p < .001$ ; Table 2). Greatest CP and CP:IVDDM ratios were found in spring ( $p < .001$  and  $p < .001$ , respectively; Table 2). The greatest IVDDM and EE values were observed in spring and summer ( $p < .001$  and  $p = .008$ , respectively; Table 2) compared to the fall and winter. During spring, grass had lower NDF concentration ( $p < .001$ ; Table 2) relative to other seasons. Grass nutritive value of the GRASS and GRASS+LEGUME pastures were similar throughout the experimental period (Figure 6). The CP:IVDDM ratio of the hand-plucked grass of the GRASS+LEGUME and GRASS pastures were, on average, 138 g/kg (Figure 6). The GRASS+N pasture had greater CP, IVDDM, and CP:IVDDM ratio, and lower NDF than the GRASS and GRASS+LEGUME (grass) pastures throughout the experimental period (Figure 6).

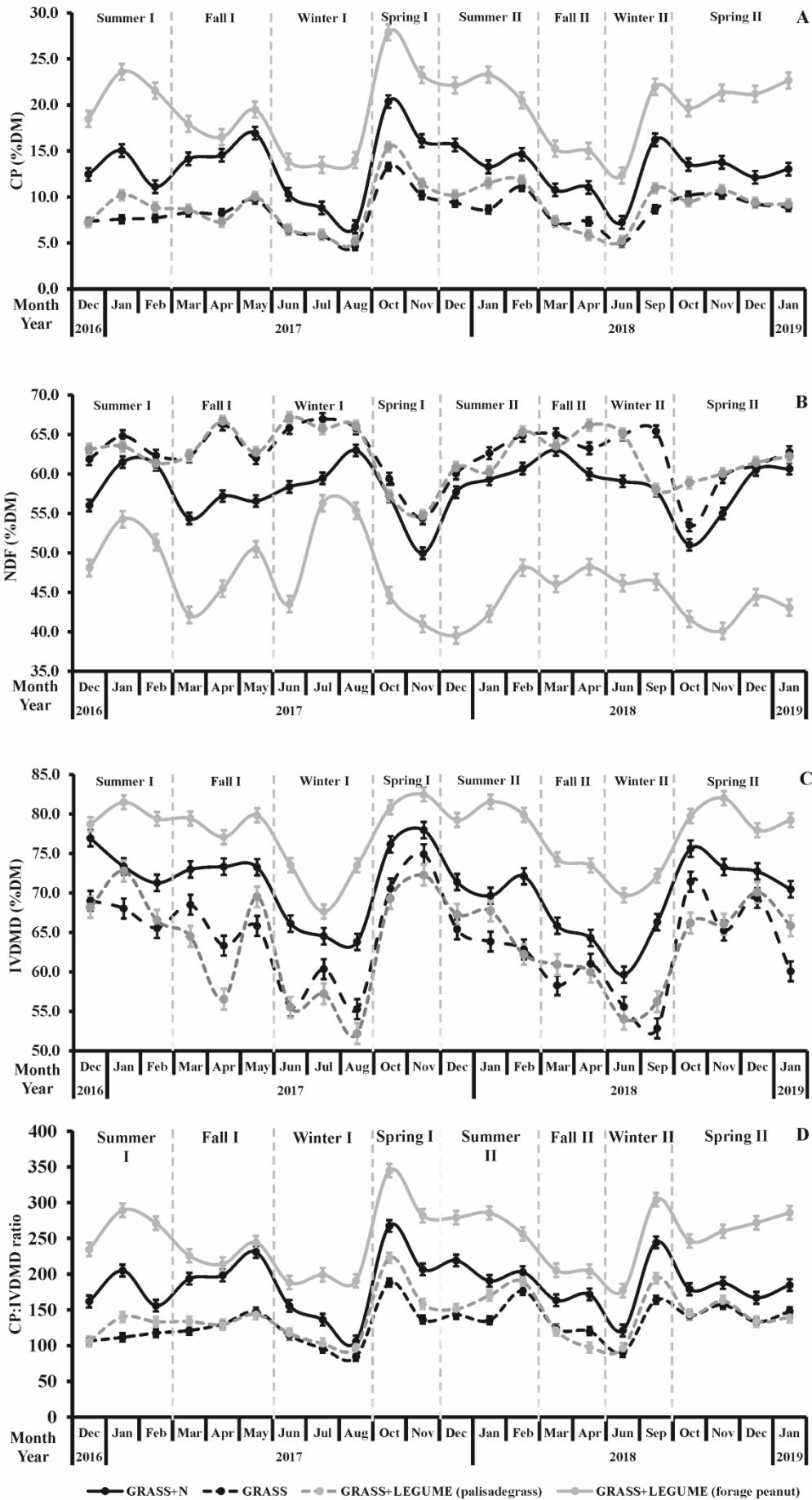
**TABLE 2** Nutritive value of Marandu palisadegrass pastures with application or not of N fertiliser, or mixed with forage peanut during the seasons of the experimental period

Variables	Pasture type (PT)			SEM	Season (S)				SEM <sup>†</sup>	<i>P-Value</i>		
	GRASS	GRASS+N	GRASS+LEGUME (grass)		Summer	Fall	Winter	Spring		PT	S	PT*S
<b>CP<sup>‡</sup>, %DM</b>	8.59b	13.4a	9.25b	0.40	10.8b	9.15c	9.09c	12.6a	0.48	<.001	<.001	.964
<b>NDF, %DM</b>	62.1a	57.8b	61.9a	0.53	61.5a	62.3a	60.8a	57.9b	0.71	<.001	<.001	.758
<b>IVDDM, %DM</b>	64.1b	70.8a	63.9b	1.31	68.6a	63.7b	61.9b	70.8a	1.46	<.001	<.001	.698
<b>EE<sup>a</sup>, %DM</b>	1.65b	1.88a	1.52b	0.06	1.68ab	1.72a	1.42b	1.92a	0.09	.001	.008	.812
<b>CP:IVDDM ratio, g/kg DM</b>	133.0b	187.0a	143.0b	10.0	156.0b	141.0b	144.0b	176.0a	10.0	<.001	<.001	.920

<sup>a-d</sup>Least squares means within a row with different lowercase differ at  $p \leq .10$ ;

<sup>‡</sup>CP: Crude protein; NDF: Neutral detergent fibre; IVDDM: *in vitro* dry matter digestibility; EE: Ether extract.

<sup>†</sup>Standard error of the means.



**FIGURE 6** Crude protein (CP; A), neutral detergent fibre (NDF; B), in vitro digestible dry matter (IVDDM; C), and CP:IVDDM ratio (D) of Marandu palisadegrass pastures with N application or not, and mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means.

#### 4. DISCUSSION

The similarity of leaf:stem ratio and herbage allowance between pastures (Table 1) indicates that canopy height is an adequate criterion for controlling and monitoring grazing management. Pastures under the same criterion of grazing management will have little variation in canopy structure (Congio et al., 2018; Rouquette Jr., 2015). In the current experiment, the canopy height ranging from 20 to 25 cm was utilized as a target for grazing management (Figure 2). During the rainy season, the canopy height of all treatments was within the management target. Canopy structure is a critical factor that determines the degree of grazing exercised by the animals (Fonseca et al., 2012; Mezzalira et al., 2014). In this way, pastures with similar canopy structures, even with different N availability, lead to similar forage intake (Amaral et al., 2013). Most of the variation on canopy structure was found between the rainy and dry seasons not between pasture treatments. The lowering of the canopy from 25 to 15 cm in the winter was the main responsible for the variation on canopy structure between seasons.

Herbage mass is the net result of growth, senescence, and grazing processes (Bircham & Hodgson, 1983; Sbrissia et al., 2018). In the current experiment, even with similar canopy height targets, herbage mass of the GRASS+N pastures increased, ranging from 51 to 84% compared to GRASS pasture (Table 1). Application of N has an impact on tiller population density due to faster generation of new leaves from several axillary buds, which may result in the appearance of new tillers (Lafarge, 2006; Paiva, Pereira, Silva, & Dias, 2015; Paiva et al., 2012). Thus, there was probably an increase in tiller population density for the GRASS+N pasture, which had a positive impact on herbage mass (Scheneiter & Améndola, 2012). Application of N increases leaf appearance and elongation rate, which is linked to greater demand and deposition of this nutrient in the cellular division zones (Borges et al., 2017; Lemaire, Da Silva, Agnusdei, Wade, & Hodgson, 2009; Silva et al., 2016). Increasing plant N enhances leaf chlorophyll concentration, which may increase the rate of photosynthesis and increase the supply of photo-assimilate to meristematic zones of cellular differentiation and expansion (Skinner, 2013). The increase in pigmentation (chlorophyll concentration) is corroborated by the

enhanced ether extract concentration of the GRASS+N pasture (Zhao et al., 2008). Since leaf:stem ratio was similar between PT (Table 1), the greater herbage mass of the GRASS+N pasture increased the leaf mass and, consequently, the leaf area index compared to the GRASS pastures (Table 1). This increase in the leaf area index of the GRASS+N pasture is also linked to the greater leaf percentage in the upper canopy strata (Figure 3). The greater leaf mass of the GRASS+N pastures changed the canopy architecture by moving its leaf area to upper canopy layers.

The GRASS+N pastures had greater green:dead mass ratio in the rainy season (summer, fall, and spring) than the GRASS pasture (Table 1). Application of N increases plant size, which leads to a greater defoliation frequency of tillers when managing the canopy under a similar target height, resulting in greater harvested forage and reduced senescence rate (Sbrissia et al., 2018). Thus, the application of N may enhance the harvest efficiency due to the increase in growth rate and the consequent adjustment in the stocking rate (Lemaire et al., 2009). On the other hand, a decrease in rainfall and temperature in the dry season (winter) affected the green:dead mass ratio even in the GRASS+N pasture (Table 1). In the dry season, there was probably an increase in senescence rates, which explains the lower green:dead ratio. Greater N availability in the GRASS+N pasture increased green:dead ratio in the herbage mass in spring due to greater rate of tiller appearance relative to tiller death (Table 1).

Application of N fertiliser resulted in greater grass nutritive value (Table 2), probably as a result of faster tissue turnover (Delevatti et al., 2019; Paiva et al., 2015; Paiva et al., 2012). The young tillers may have leaf blades with a greater proportion of tissues of greater digestibility (Batistoti et al., 2012). Greater proportions of mesophyll cells are essential for the qualitative properties of forage grass being, along with the phloem, the tissue that presents the greatest digestibility (Batistoti et al., 2012). Thus, pastures with greater predominance of older tillers (e.g. GRASS pastures), have lesser forage digestibility which may be associated with the increase in the cell-wall constituents (Batistoti et al., 2012; Duru & Delaby, 2003). Forage CP increased by 56% for the GRASS+N pasture compared with the GRASS pasture, being the bromatological variable that was most influenced by N supply (Table 2).

Conversely, a reduction of only 6.9% in the NDF was observed between the GRASS+N and the GRASS pastures, which shows that N application has less influence on fibre concentration (Table 2), mainly when pastures are managed under variable stocking rate. The impact of the N application on the increase of CP is linked to the

increases in non-protein N (nitrates, amides, amino acids, A fraction) and small molecules such as peptides, which are all rapidly degraded in the rumen (Johnson, Reiling, Mislevy, & Hall, 2001). Furthermore, the reduction of the forage NDF in the GRASS+N pasture was associated with an increase in CP. Thus, the concentration of NDF diminished because of an increase in the CP and other soluble constituents, which accumulated in the cell causing a dilution effect, which resulted in reduced cell wall concentration (Sales et al., 2019).

The use of protein:energy ratio seems to be more plausible for understanding the metabolic effects of protein on intake because it is a more reliable indicator of the metabolic adequacy of the animal (Detmann, Valente, Batista, & Huhtanen, 2014). Considering this, the CP:IVDDM ratio was used as an indicator of protein to energy status. The CP:IVDDM ratio of the GRASS+N pasture ranged from 106 to 267 g/kg (Figure 6). In a diet with low ether extract (as in the current experiment), the IVDDM was similar to the digestibility of the organic matter (DOM). Poppi and McLennan (1995) stated that losses of protein would occur when CP concentration of the diet exceeds approximately 210 g/kg DOM. During the rainy season, the GRASS+N pasture showed values of CP:IVDDM around of 210 g/kg. On the other hand, the GRASS pasture showed values of CP:IVDDM ratio ranging from 84 to 188 g/kg, being for most of the experiment below 150 g/kg (Figure 6). The animal performance will be limited with a diet with less than 150 g/kg DOM due to lack of N for ruminal microorganisms (Detmann et al., 2014; Poppi & McLennan, 1995).

Most of the applied N mineral fertilisers, such as urea, have a fast nutrient release rate (Vaio et al., 2008; Zaman, Nguyen, Blennerhassett, & Quin, 2008), which increases soil mineral N concentration immediately after N application (Vaio et al., 2008; Zaman et al., 2008). In contrast, the N input in the GRASS+LEGUME pasture from BNF is more gradual. The grass mass, canopy structure, and nutritive value of the GRASS+LEGUME pasture were similar to those of the GRASS pasture (Tables 1 and 2). In this way, the variation in canopy structure characteristics of the GRASS+LEGUME pastures were principally due to the enhanced legume mass. Even when the legume has fixed atmospheric N, the transfer of this N to grass is likely to be slower, being dependent on the biological process of mineralization (i.e., litter decomposition) or transfer from livestock excreta.

Forage peanut has been reported to be generally slower to establish in comparison to grasses (Alencar et al., 2018; Tamele et al., 2018). Frequently, it has taken 12 months



for adequate establishment in Brazilian pastoral systems (Tamele et al., 2018). Furthermore, the legume allocates a significant portion of photoassimilates for the establishment in the canopy (Ryle, Powell, & Gordon, 1985). The equilibrium of this relationship is necessary for the continuous supply of assimilates produced by the aerial biomass to the nodules, which provide N to the plants (Carvalho et al., 2019). Biological N fixation represents a greater cost to plants than nitrate uptake and reduction (Houlton, Wang, Vitousek, & Field, 2008), since photoassimilate allocation to maintain nodule respiration may correspond to up to 23% of the gross photosynthesis (Ryle et al., 1985). Legumes seem to give priority to N uptake from the soil as a means of optimizing the use of the available energy supply (Carvalho et al., 2019), although recent studies on soybean (*Glycine max*) indicate that the demand for extra photosynthates can stimulate an increase in photosynthesis (Kaschuk, Kuyper, Leffelaar, Hungria, & Giller, 2009; Kaschuk, Hungria, Leffelaar, Giller, & Kuyper, 2010). The energetic balance mechanics is known as facultative BNF, according to which N<sub>2</sub> fixation is regulated positively or negatively depending on external factors such as the natural availability of N in the soil, severity of defoliation, or the establishment process (Menge & Hedin, 2009).

The presence of the legume, despite having little impact on grass mass, resulted in an increase in herbage mass (Table 1). The spaces in the canopy occupied by legume in the GRASS+LEGUME pasture did not negatively affect the grass. This fact explains why the GRASS+LEGUME pasture in spring and summer had herbage mass (Table 1) and canopy architecture (Figures 3, 4, and 5) similar to those of the GRASS+N pasture. In the dry season, herbage mass and canopy architecture in the GRASS+LEGUME pasture were similar to the GRASS pasture. The decrease in temperature and rainfall (Figure 1) in the dry season had a negative effect on forage peanut, which was predominantly due to leaf abscission. Thus, on average, in spring and summer, there were 2.4 times as much green legume mass as in the dry season. The increase in legume in the rainy season may enhance the productive potential of forage. Thus, in conditions where farmers do not apply N fertiliser on pastures, the adoption of a legume becomes a promising alternative. However, the adoption of forage peanut by farmers is not as simple as the adoption of new grass cultivars, and the economic advantages will largely depend on the availability and cost of legume seeds.

Marandu palisadegrass in the GRASS+LEGUME and GRASS pastures had similar nutritive value (Table 2 and Figure 4). However, the presence of legumes in the diet of grazing animals may increase the nutritive value of ingested forage. Forage peanut

(cv. BRS Mandobi) had greater crude protein concentration and dry matter digestibility, and lower fibre concentration than Marandu palisadegrass (Figure 6). Therefore, grazing animals had access to a diet of better-quality forage in the GRASS+LEGUME pasture (i.e., intake and nutritional value). Thus, if the animals feeding on the GRASS+LEGUME pasture ingested only grass, their weight gain might be limited by lower protein ingestion. In the GRASS+LEGUME pasture, the CP:IVDDM ratio for Marandu palisadegrass ranged from 96 to 223 g/kg, and in the forage peanut ranged from 177 to 345 g/kg (Figure 6). The average CP:IVDDM ratio for the GRASS+N pasture was 184 g/kg. The animals in the GRASS+LEGUME pastures would need to ingest a diet with around 45% legume DM to reach similar levels of CP:IVDDM ratio as the GRASS+N pasture.

## **5. CONCLUSIONS**

The use of N fertiliser application immediately increases herbage mass and improves grass nutritive value. Pastures managed under the same target of grazing management have little variation in canopy structure due to similar proportion of morphological components. Responses to the adoption of forage peanut in grass pasture systems require greater time due to the slower establishment of the legume. Even if there is no alteration in the Marandu palisadegrass, forage peanut in the short-term increased herbage mass and forage nutritive value. Thus, forage peanut has a great potential for use in the humid tropics.

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## **CONFLICT OF INTEREST STATEMENT**

The authors declare that there is no conflict of interest.

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## 2. CHAPTER 2

**Article 2 - Pasture nitrogen input through fertiliser application or legume integration. 2. Effects on animal performance, forage intake and digestion, and nitrogen metabolism.**

Running head: **Nitrogen inputs to warm-climate pastures**

**The manuscript will be submitted to the Journal “*Grass and Forage Science*”.**

**Preliminary version. The manuscript is following the guidelines in this Journal.**

### **Abstract**

The lack of nitrogen (N) input on pastures is the main limiting factor to increase animal performance in tropical regions. This 2.5-yr study assessed animal performance, forage intake and digestion and N metabolism responses of three pasture treatments: 1) mixed Marandu palisadegrass (*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]) and forage peanut (*Arachis pintoi* Krapov. & W.C. Greg. cv. BRS Mandobi) pastures (GRASS+LEGUME), 2) monoculture Marandu palisadegrass pastures fertilised with 150 kg N/ha (GRASS+N), and 3) monoculture Marandu palisadegrass without N fertiliser (GRASS). Continuous stocking was used with a target canopy height of 20 to 25 cm. The average daily gain was greater for the GRASS+N and GRASS+LEGUME pastures than for the GRASS ( $p = .081$ ). GRASS+N pasture had greatest stocking rate and liveweight gain per area ( $p < .001$  and  $p < .001$ , respectively), followed by the GRASS+LEGUME pasture. No differences between treatments were found for the dry matter (DM) forage intake ( $p = .729$ ); however, GRASS+N and GRASS+LEGUME pastures had greater crude protein and digestible organic matter intakes than GRASS pasture ( $p = .007$  and  $p = .083$ , respectively). Greater microbial protein synthesis and efficiency of microbial synthesis were found for the GRASS+N and GRASS+LEGUME pastures ( $p = .016$  and  $p = .067$ , respectively). Apparent efficiency of N utilisation and microbial protein/CP intake ratio was greatest in the GRASS+LEGUME pastures ( $p = .009$  and  $p = .042$ , respectively). Nitrogen application or the integration of forage peanut in grass pasture increases animal performance.

## KEYWORDS

*Arachis pintoi*, *Brachiaria*, fertilized pasture, beef cattle, mixed pasture

## 1. INTRODUCTION

Beef is rich in essential amino acids and has the greatest quality rating among food sources (Pereira & Vicente, 2013). It is considered one of the main sources of protein for the human population (Pereira & Vicente, 2013). Brazilian beef production is of great global importance as Brazil has the largest commercial cattle herd of the world (USDA, 2019). However, the majority of grass-fed beef operations in Brazil are characterized by low productivity and economic indices, mostly due to the absence of or inadequate pasture management, improper animal management, and low investment (Martha, Alves, & Contini, 2012), resulting in low productivity of the activity. The performance of grazing animals is directly related to forage intake, which in turn is affected by canopy structure and forage nutritive value (Poppi, Hugues, & L'Huillier, 1987). Forage intake is maximized under proper management of canopy structure, and stocking rate is a powerful tool to control canopy state (Davies, Fothergill, & Jones, 1989). Thus, regardless of grazing method, controlling the stocking rate by the adoption of pasture targets (i.e., canopy height and forage mass) is the best way to maximize animal performance (Congio et al., 2018; Rouquette Jr., 2015).

Low soil nutrient availability, especially N, is the main limiting factor for pasture productivity in tropical regions (Boddey et al., 2004). When pastures are under similar management, N application may improve animal productivity in two ways: influencing the average daily gain by the improvement of forage nutritive value (Delevatti et al., 2019; Sales et al., 2020) and/or increasing stocking rate because of greater forage production (Delevatti et al., 2019; Pereira et al., 2015). Nitrogen application should increase crude protein (CP) intake (Delevatti et al., 2019; Detmann, Valente, Batista, & Huhtanen, 2014). If this occurs and the digestible organic matter intake (DOMI) does not increase at the same rate, there will be lower apparent efficiency of N utilisation by the animal. The net result is the increase of N excretion to the environment, mainly via livestock urine (Detmann et al., 2014). Thus, the DOMI may be the most limiting factor to increase animal performance.

An attractive alternative would be the establishment of a forage legume in the pasture. Some studies have highlighted the effects of mixed pastures on canopy structure, nutrient cycling and animal productivity (Gomes et al., 2020; Gomes et al., 2018; Muir,

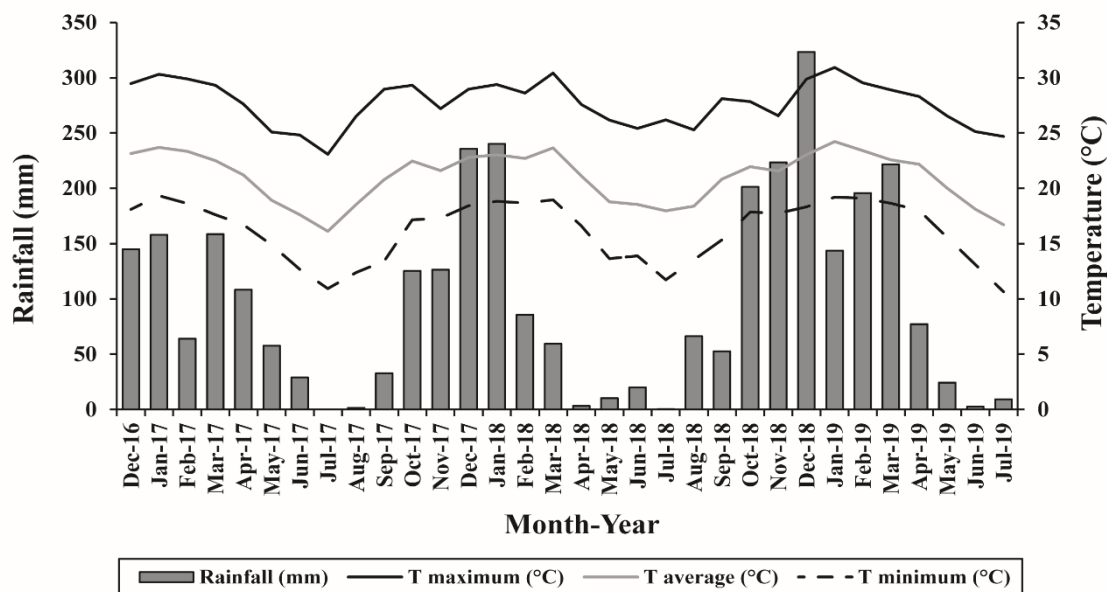
Pitman, Dubeux, & Foster, 2014; Muir, Pitman, & Foster, 2011; Pereira et al., 2020). Legumes can establish symbioses with rhizobia, which induce the formation of root nodules with the capacity of biological fixation of atmospheric nitrogen, which may increase forage production. Furthermore, tropical legumes have greater crude protein and lesser neutral detergent fibre concentrations than tropical grasses, often leading to greater digestibility (Gomes et al., 2018). Therefore, livestock grazing on grass-legume pasture may improve the balance between crude protein intake and DOMI, increasing the apparent efficiency of N utilisation and, consequently, decreasing N excretion to the environment (Detmann et al., 2014).

Thus, we hypothesized that N application or the introduction of a legume affect stocking rate more than average daily gain. Additionally, grazing animals on mixed pastures of Marandu palisadegrass and forage peanut have better balance between crude protein intake and DOMI relative to grass alone, increasing the apparent efficiency of N utilisation by the livestock. Therefore, the objective of this study was to investigate animal performance, forage intake and digestion, and N metabolism of mixed pastures of Marandu palisadegrass and forage peanut compared to Marandu palisadegrass in monoculture, fertilized or not with nitrogen, under continuous stocking.

## **2. MATERIAL AND METHODS**

### **2.1 Experimental site and treatments**

The study was carried out at the Experimental Farm of the Federal University of Lavras, Brazil (21°14'S, 44°58'W; 918 m above sea level). This area has a subtropical humid mesothermal climate with dry winters [Köppen-Geiger climate classification: Cwa; (Sá Júnior, Carvalho, Silva, & Carvalho Alves, 2012)]. Meteorological data were obtained from a weather station located 1,000 m from the experimental area (Figure 1).



**FIGURE 1** Mean monthly temperatures and rainfall in Lavras, Brazil, during the experimental period.

The soil in the area is a Ferralsol (WRB/FAO classification). Soil texture is clayey, and the clay content is similar down the profile (a characteristic of this soil type): 563 g clay/kg soil (0–10 cm) and 574 g clay/kg soil (20–40 cm). Soil analyses were conducted according to Embrapa standard techniques (Claessen, Barreto, Paula, & Duarte, 1997). The soil (0-20 cm) had the following properties: pH (H<sub>2</sub>O) = 5.9; exchangeable Al, Ca, Mg, 0.07, 2.4, and 0.7 cmol/dm<sup>3</sup>, respectively; available P (Mehlich-I method) 7.6 mg/dm<sup>3</sup>, exchangeable K 82.8 mg/dm<sup>3</sup>, and organic matter 31.0 g/kg.

Treatments were three pastures types (**PT**), namely: 1) Marandu palisadegrass (*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]) and forage peanut (*Arachis pintoi* Krapov. & W.C. Greg. cv. BRS Mandobi) mixed pasture, without N fertiliser application (**GRASS+LEGUME**); 2) Marandu palisadegrass monoculture fertilised with 150 kg N/ha/yr (**GRASS+N**); and 3) Marandu palisadegrass monoculture without N fertiliser application (**GRASS**).

## 2.2 Pasture and treatment establishment

In January 2014, the whole experimental area was seeded with Marandu palisadegrass at a rate of 6.0 kg/ha of pure live seeds. The 12-ha experimental area was limed (2,500 kg dolomitic lime/ha) 60 d before grass seeding. Fifty-two kg of P/ha as single super phosphate and 41.5 kg of K/ha as potassium chloride were applied at grass seeding. The

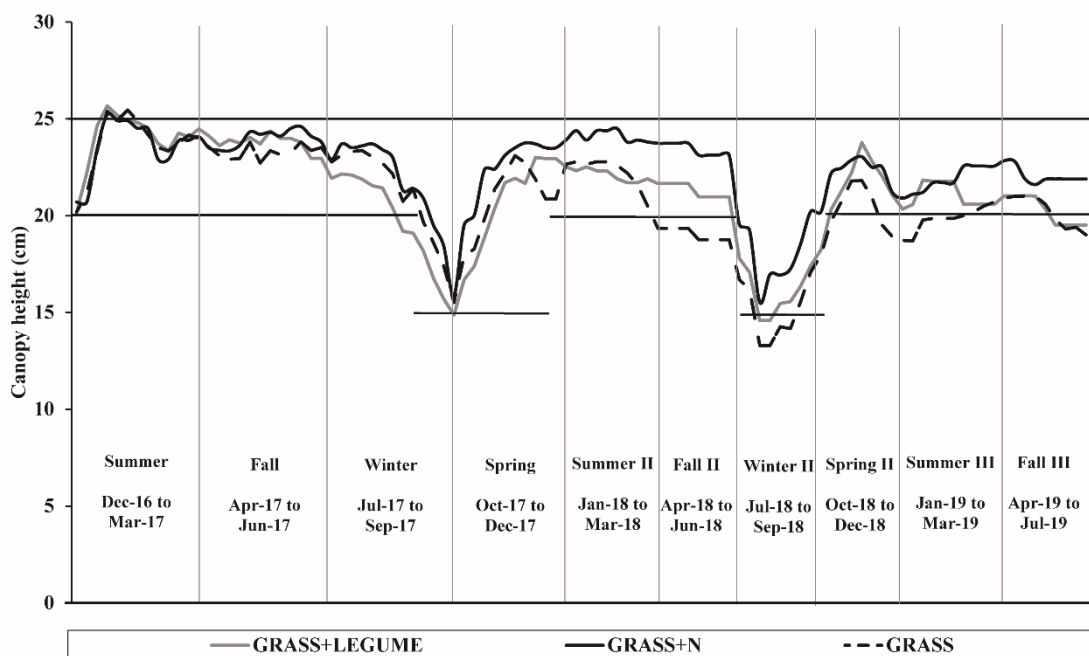
experimental area was divided into four paddocks with three hectares each (blocks) taking into account the topography of the area and managed under rotational stocking until December 2015.

In December 2015, the blocks were divided into three paddocks where pasture types were randomly allocated. The size of each paddock (experimental unit) was adjusted based on previous studies in the area using these pastures types (Homem *et al.*, 2021). The GRASS+N, GRASS+LEGUME, and GRASS paddocks size were 0.7, 1.0 and 1.3 ha, respectively. Paddock size of each PT allowed adding an average of 3.0 animal units per paddock during the rainy season. Furthermore, this allowed tester animals (at least two animals) to remain the maximum possible time during the dry season and then all animals were removed at the same time.

The GRASS+LEGUME paddocks were seeded with forage peanut into a previous established Marandu palisadegrass pasture in December 2015. Before the forage peanut seeding, the Marandu palisadegrass canopy was lowered to around 10 cm by mechanical cutting. The seeding rate was 10 kg/ha of pure live seeds for forage peanut planted with a no-till seeder with four rows. The planter allocated six seeds of forage peanut per linear meter with 0.5 m row spacing. After seeding, the paddocks with forage peanut were excluded from grazing during 75 d to improve the establishment.

In March 2016, grazing management targets were imposed under continuous stocking. This period up to December 2016 was allocated as time for the adaptation of the canopies to the experimental management. From December 2016 to July 2019, eight seasons were evaluated over time. The years were divided into the three-seasons of each year: from December 22 to March 21 (Summer), from March 22 to July 21 (Autumn), and from September 22 to December 21 (Spring). In the winter (from July 22 to September 21), the paddocks were managed until canopy height reached a minimum limit of 15 cm when the animals were removed from the experimental area. For this reason, the winter data were not recorded. Continuous stocking with a variable stocking rate was used to maintain canopy height between 20 and 25 cm (Figure 2). In each year, two Nellore heifers ( $234 \pm 36$  kg of BW and  $12 \pm 1.3$  months of age) were used as tester animals in each paddock. At the end of the first experimental year, the animals were replaced. When it was necessary, put-and-take animals were used to maintain targeted canopy height (Allen *et al.*, 2011). Water and commercial mineral supplementation [assurance levels per kilogram of product: 110 g Ca (max), 90 g Ca (min), 100 mg Co (min), 1500 mg Cu (min), 25 g S (min), 600 mg F (max), 60 g P (min), 301 mg I (min),

20 mg Se (min), 211 g Na (min), and 2500 mg Zn (min)] were supplied *ad libitum*. Average canopy height was measured weekly using a sward stick (Barthram, 1985) at 100 random points per paddock, and the results used to carrying out adjustments in stocking rate when necessary (Figure 2).



**FIGURE 2** Canopy height (cm) throughout the experimental period.

Annually, in spring (between November and December), all paddocks were fertilized with single superphosphate and potassium chloride corresponding to 22 kg/ha of P and 41 kg/ha of K, respectively. In the GRASS+N pasture, the N fertiliser application was divided into three applications per year (50 kg N/ha each in November, January, and March), all using urea.

## 2.3 Experimental evaluations

### 2.3.1 Herbage mass

Herbage mass was sampled by harvesting six frames at ground level, measuring  $1 \times 0.5$  m, per paddock, at points with average canopy height, once every 30 d. After harvesting, the fresh material was weighed. Subsamples of approximately 250 g were taken for the evaluation of dry matter (DM) concentration. Another subsample of approximately 2 kg was taken for manual separation of botanical and morphological components. Forage samples were oven-dried at 55 °C for 72 h to a constant weight. Grass mass (kg/ha) was

considered as leaf + stem without dead material and the legume mass (kg/ha) included leaf plus stem mass. Herbage mass was considered as the aboveground biomass of herbaceous plants (grass and legume mass, according to treatment). Botanical composition was measured in the samples of the GRASS+LEGUME pastures as the ratio between legume mass and total herbage mass. The results were presented as average values per season of the year.

### **2.3.2 Animal performance**

Cattle were weighed in the morning, every 28 d, without feed or water restriction. The values obtained in each weighing were submitted to individual analyses of regression per season, the result being a linear equation ( $y = ax + b$ ). In this equation, the individual initial weight in each season was the intercept, and the average daily gain (ADG) was the slope. The stocking rate [AU/ha; (AU: animal unit was considered a bovine weighing 500 kg; Allen et al., 2011)] was measured for each season as the sum of weights of all animals present in each paddock divided by the area of the paddock for each weighing period. The liveweight gain per ha was calculated by multiplying the ADG by stocking rate. All variables were averaged per paddock before analysis for each season of the year.

### **2.3.3 Diet, forage intake, nutrient intake, and total nutrient digestibility**

Hand-plucked forage samples were collected during the intake evaluation for nutritive value analysis (Vries, 1995). In the GRASS+LEGUME pasture, grass and legume were separated manually and analysed separately. Forage samples were oven-dried at 55 °C for 72 h to a constant weight. Then, a composite sample of each species was made for each paddock. The composite samples were ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. The DM of each sample was obtained by oven drying at 105 °C for 18 h (method 934.01; AOAC, 2000). A 4-h incineration process determined the ash concentration in a 600 °C muffle furnace (method 942.05; AOAC, 2000). The crude protein (CP) concentration was obtained based on the N concentration ( $CP = \text{total N} \times 6.25$ ), which was determined using the Kjeldahl procedure (method 920.87; AOAC, 2000). The ether extract (EE) was analysed according to the method 920.39; AOAC, 2000). The neutral detergent fibre corrected for ash and protein (apNDF) was determined by the autoclave method at 105 °C for 60 min (Pell & Schofield, 1993). Condensed tannin (CT) was extracted using methanol, acetone, and ascorbic acid solution; the Fe reagent and n-butanol-HCl were added to the tannin extract, which was then heated at 95 °C



(Porter, Hrstich, & Chan, 1985). The absorbance of the tannin extract solution was measured at 550 nm.

Forage intake was estimated from faecal excretion and indigestible neutral detergent fibre (iNDF) once per season. Spot faecal samples were collected once a day consistently at the same time (midday), and a composite sample was produced for each animal during the five-day period of collection. During the sampling days, the heifers were brought over from the paddocks to a barn to collect faeces directly from the rectum. Faecal production was estimated using titanium dioxide as an external marker (Titgemeyer, Armendariz, Bindel, Greenwood, & Löest, 2001) during eleven consecutive days, six for adaptation, and five for collection. The titanium dioxide was administered in the amount of 10 g/animal per day. Faecal samples were oven-dried at 55 °C for 72 h to determine DM concentration, air equilibrated, weighed, and ground in a Cyclotec mill (Tecator) to pass a 1-mm screen. The faecal samples were analysed for titanium dioxide concentration, according to Myers, Ludden, Nayigihugu, and Hess (2004).

Faecal and hand-plucked forage samples were incubated in the rumen for 288 hours to determine iNDF (Huhtanen, Kaustell, & Jaakkola, 1994). Two cannulated heifers fed with a diet that consisted of Maradu palisadegrass and forage peanut mixed forage or Marandu palisadegrass monoculture were used in the iNDF estimate, according to treatment. The faecal excretion was used to find the total amount of iNDF in faeces; thus, the estimate of iNDF intake per day was obtained. After that, iNDF from the hand-plucked samples was acquired to estimate the forage intake.

In the GRASS+LEGUME pasture treatment, the proportion of grass and legume in the forage intake was estimated using  $\delta^{13}\text{C}$  isotopes by the equation:

$$\% \text{legume} = 100 \times (\delta^{13}\text{C}_G - \delta^{13}\text{C}_S) \div (\delta^{13}\text{C}_G - \delta^{13}\text{C}_L)$$

where %legume is the proportion of carbon from a legume in the iNDF residual faecal samples (Lopes de Sá, 2017), and  $\delta^{13}\text{C}_G$ ,  $\delta^{13}\text{C}_L$ , and  $\delta^{13}\text{C}_S$  are the values of  $\delta^{13}\text{C}$  abundance of the iNDF residual in hand-plucking samples of the grass (-11.9‰), legume (27.4‰), and the iNDF residual in faecal samples, respectively. For the analysis of  $\delta^{13}\text{C}$ , samples were ground to a fine powder in a roller mill similar to that described by Arnold and Schepers (2004). Sub-samples containing between 300 to 500  $\mu\text{g}$  C were analysed for total C and  $^{13}\text{C}$  abundance using an automated continuous-flow isotope-ratio mass spectrometer consisting of a Finnigan DeltaV mass spectrometer coupled to the output of a Costech [model ECS4010] total C and N analyser (Finnigan MAT, Bremen, Germany)

in the "John Day Stable Isotope Laboratory" of Embrapa Agrobiologia. The intake of organic matter (OM), CP, EE, and apNDF were calculated.

Nutrients concentration of the diet was calculated as nutrient intake divided by forage dry matter intake. The coefficients of apparent digestibility of the DM, OM, CP, and apNDF in the total digestive tract was determined through faecal excretion of the external titanium dioxide indicator (Myers & Robbins, 1991). Furthermore, DM, OM, CP, and apNDF concentrations in faecal samples were determined in the same way as described for the hand-plucked samples. Total digestibility (g/kg) was calculated as [(% DM and nutrients in the diet - % DM and nutrients in faeces)/(% DM and nutrients in diet)]. The apparent digestibility coefficients were calculated for DM, OM, CP, and NDF.

### 2.3.4 Microbial protein synthesis

Microbial N synthesis (g N/d) was estimated by using the technique of purine derivatives in the urine (Chen & Gomes, 1992). The spot sampling was used to assess the excretion of urinary nitrogenous compounds (Valadares, Broderick, Filho, & Clayton, 1999). Spot urine samples were obtained by vulval stimulation at the same time as faecal sample collection. A 12-mL aliquot was taken, and 48 mL of 0.02 N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) were added (Chen & Gomes, 1992). A 5-d composite sample was produced and stored in a plastic flask at -20 °C. Urine volume was estimated using creatinine concentration as a marker and assuming a daily creatinine output according to the following equation (Silva et al., 2012):

$$UV = (0.0345 \times SBW^{0.9491}) \div UC_c$$

where UV (L/d) is daily total urinary production, SBW (kg) is shrunk body weight, and UC<sub>c</sub> (g/L) is urine creatinine concentration. Urine creatinine concentration was determined using a commercial kit (Creatinine K, Labtest, Lagoa Santa, Brazil).

Allantoin was analysed as described by Chen and Gomes (1992). Uric acid was determined using a commercial kit (Uric acid monoreagent, Bioclin, Belo Horizonte, Brazil). Excretion of allantoin and uric acid were estimated multiplying their concentrations in urine by the daily urinary volume. Excretion of the purine derivatives in the urine was calculated by the sum of allantoin and uric acid excretions (mmol/d). The daily purine absorption (P<sub>a</sub>) and the production of ruminal microbial N (g/d) were calculated using the following equations, respectively (Chen & Gomes, 1992):

$$P_a = (PD_e - 0.385 \times SBW^{0.75}) \div 0.85$$

$$NMIC = 0.727 \times P_a$$

where  $P_a$  (mmol/d) is purine absorbed,  $PD_e$  (mmol/d) is purine derivatives excreted (uric acid and allantoin),  $SBW$  (kg) is shrunk body weight, and  $NMIC$  (g of N/d) is ruminal production of microbial nitrogen. The efficiency of microbial synthesis in the rumen (g microbial N/kg of digestible OM) was calculated by dividing the production of ruminal microbial N by the digestible OM intake (kg/d). The ratio of CP/digestible OM was calculated based on the intake of digestible OM and CP (g/kg). The ratio microbial protein/CP intake was calculated.

### 2.3.5 Nitrogen balance

The nitrogen balance was obtained by subtracting the total excreted N in faeces and urine from total N intake, representing the total N that was effectively retained by the animal. For this purpose, diet components, faecal, and urine samples were analysed for N concentration according to method 920.87; AOAC (2000). In order to determine the faecal N excretion, the faecal production was multiplied by total N concentration in the faeces. Urinary N excretion was calculated using urinary volume and total N concentration in the samples. Nitrogen intake was obtained based on the value of forage intake and total N concentration found in the hand-plucking samples. The apparent efficiency of N utilisation (ENU) by the animal was calculated as the ration between nitrogen balance and nitrogen intake.

### 2.4 Statistics analysis

The experimental design was a randomized complete block with three treatments (pasture type (PT); GRASS+N, GRASS, and GRASS+LEGUME), four replications, and repeated measurements over time (seasons of the year). Data were analysed by fitting mixed models (Littell, Pendergast, & Natarajan, 2000), using the MIXED procedure of SAS (SAS Institute, Cary, NC). The effects of types of pasture and seasons were considered fixed and the effects of block and year as a random effect. The Akaike information criterion was used to choose the best (co)variance structure (Akaike, 1974). All variance components were estimated using the restricted maximum likelihood method. The averages were estimated using the LSMEANS statement, and comparisons were made

between treatments using Fisher's protected least significant difference (LSD) test at 10% probability. The statistical model for data analysis was as follows:

$$Y_{ijkz} = \mu + B_i + PT_j + \gamma_{ij} + Y_k + S_z + (PT \times S)_{jz} + \varepsilon_{ijkz}$$

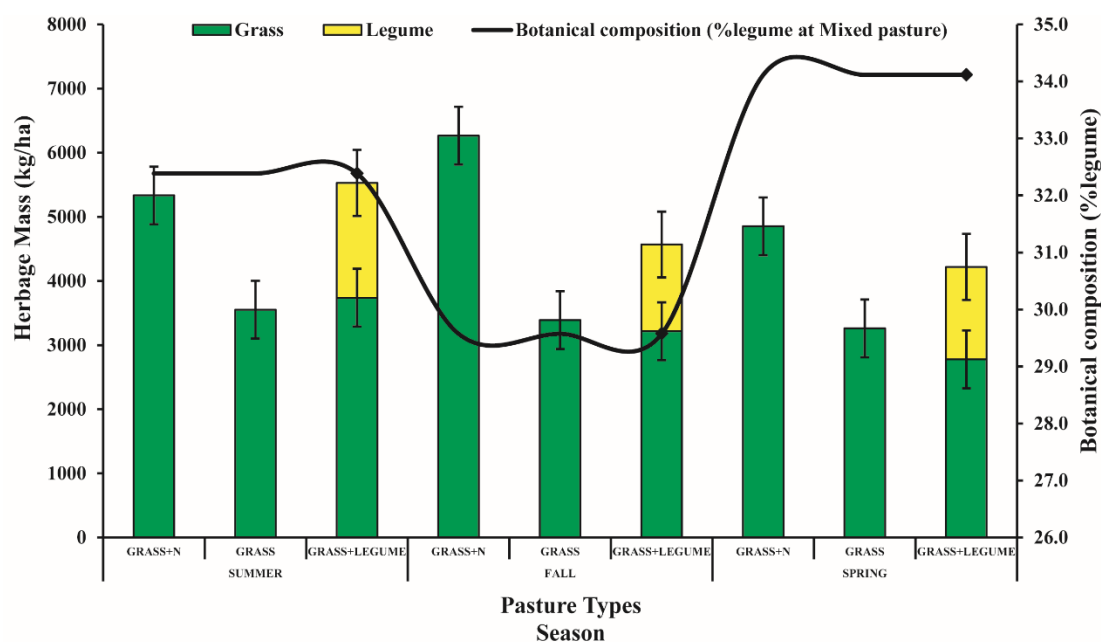
where  $Y_{ijkz}$  = value observed in the  $i$ th block of the  $j$ th PT of the  $k$ th year of the  $z$ th season;  $\mu$  = overall average;  $B_i$  = random effect associated with the  $i$ th block,  $i = 1, 2, 3, 4$ ;  $PT_j$  = fixed effect associated with  $j$ th types of pasture,  $j = 1, 2, 3$ ;  $\gamma_{ij}$  = random error associated with the  $i$ th block in the  $j$ th PT.  $Y_k$  = random effect associated with  $k$ th year,  $k = 1, 2$ ;  $S_z$  = fixed effect associated with  $z$ th season,  $z = 1, 2, \dots, 8$ ;  $(PT \times S)_{jz}$  = fixed effect of interaction  $j$ th PT with the  $z$ th season.  $\varepsilon_{ijkz}$  = random error associated with the  $i$ th block, the  $j$ th PT, the  $k$ th year, and the  $z$ th season.

The effect of legume mass (kg/ha) on stocking rate (AU/ha) and of legume proportion in the herbage mass (botanical composition) on legume intake (% of forage peanut in the forage intake) for the GRASS+LEGUME pasture were analysed using regression analysis at 10% probability by the REG procedure of SAS (SAS Institute).

### 3. RESULTS

#### 3.1 Herbage mass

Herbage mass was greater for the GRASS+N and GRASS+LEGUME pastures than the GRASS pasture in summer and spring ( $p < .001$ ; Figure 3). In the fall, the greatest herbage mass was observed for the GRASS+N pasture, followed by the GRASS+LEGUME pasture. There was no difference in herbage mass between seasons for the GRASS pasture (average value of 3402 kg DM/ha). The greatest herbage mass in the GRASS+N and GRASS+LEGUME pastures was observed in the fall and summer, respectively. Grass mass was greatest in the GRASS+N pasture in all seasons ( $p = .001$ ; Figure 3). A similar response of herbage mass between seasons of the year was found for the grass mass. The botanical composition (% of legume in the herbage mass) ranged from 29.6 to 34.1%.



**FIGURE 3** Herbage mass and botanical composition of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Error bars represent  $\pm$  standard errors of the means.

### 3.2 Animal performance

Average daily gain was greater for the GRASS+N and GRASS+LEGUME pastures ( $p = .081$ ; Table 1) than for the GRASS pasture (0.636 and 0.611 kg/d vs. 0.544 kg/d, respectively). The greatest ADG occurred in spring, followed by summer and lowest in fall ( $p < .001$ ). Stocking rate was greater in the GRASS+N pasture ( $p < .001$ ; Table 1), followed by the GRASS+LEGUME pasture, and lowest in the GRASS pasture. The greatest stocking rate was observed in summer ( $p < .001$ ; an increase of 15.8 and 31.6% compared to fall and spring, respectively). Liveweight gain per area was greatest for the GRASS+N ( $p < .001$ ; Table 1), intermediate for the GRASS+LEGUME pasture and lowest for the GRASS pasture. The greatest liveweight gain per area between seasons was found in summer and spring ( $p = .004$ ; Table 1). The stocking rate varied according to the legume mass of the GRASS+LEGUME pasture ( $p < .001$ ) and showed a positive linear relationship with legume mass (Figure 4).

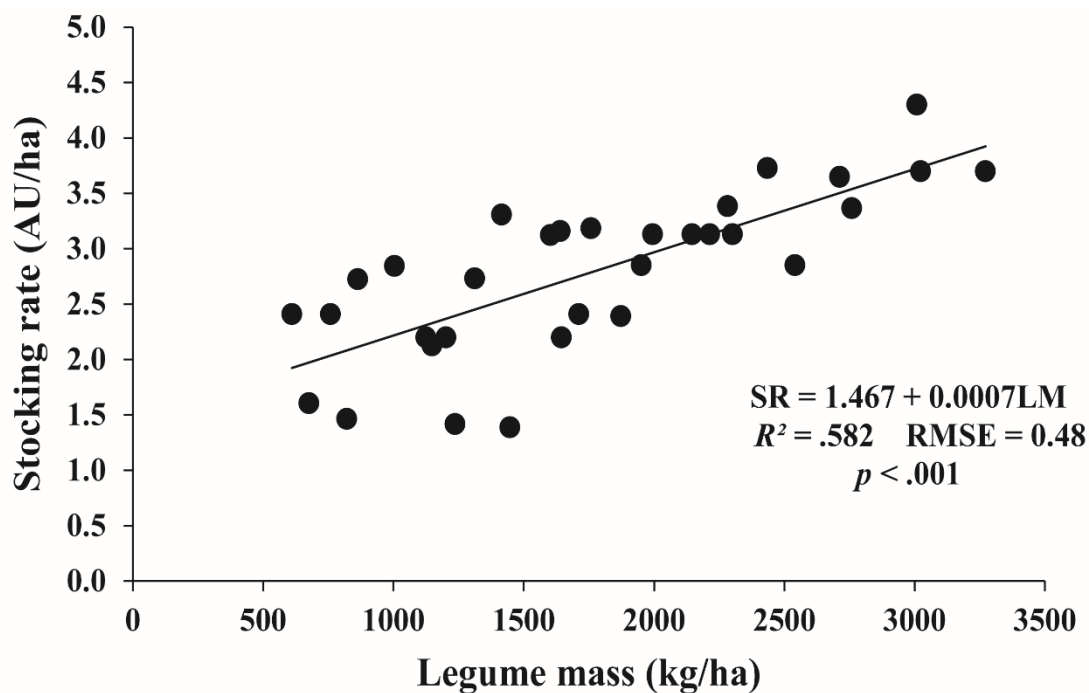
**Table 1** Animal performance of Nellore heifers on Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Variables	Pasture type (PT)			Seasons (S)			SEM <sup>†</sup>	<i>P-value</i>		
	GRASS+N	GRASS	GRASS+LEGUME	Summer	Fall	Spring		PT	S	PT*S
ADG <sup>‡</sup> , kg/d	0.636a	0.544b	0.611a	0.577b	0.453c	0.761a	0.05	.081	<.001	.820
Stocking rate, animal unit/ha	3.8a	2.3c	2.8b	3.4a	2.9b	2.6c	0.3	<.001	<.001	.948
Liveweight gain per area, kg/ha/season	219a	106c	143b	173a	125b	169a	22	<.001	.004	.961

<sup>a-c</sup>Least squares means within a row with different lowercase differ at  $p \leq .10$ ;

<sup>‡</sup>ADG: Average daily gain;

<sup>†</sup>Standard error of the means.



**FIGURE 4** Stocking rate (SR) as a function of the legume mass (LM) of mixed Marandu palisadegrass with forage peanut pasture. (Stocking rate =  $1.467 + 0.0007 \times \text{legume mass}$ ;  $p < .001$  and  $p < .001$  for intercept and slope, respectively;  $R^2 = 0.582$ ).

### 3.3 Diet, intake and apparent digestibility

No differences between PT and seasons ( $p = .632$  and  $p = .154$ , respectively; Table 2) were found for the OM concentration in the diet (average of 92.5%). The GRASS+N pasture had the greatest CP and EE concentrations in the diet ( $p < .001$  and  $p < .001$ , respectively; Table 2), followed by the GRASS+LEGUME pasture for CP concentration and by the GRASS pasture for EE concentration. Greater CP concentration was observed in the diet during summer and spring ( $p < .001$ ) than in fall. There was no difference between seasons for EE concentration in the diet ( $p = .118$ ; Table 2). The GRASS+N and GRASS+LEGUME pastures had lower apNDF concentration in the diet ( $p < .001$ ; Table 2) than the GRASS pasture. A lower apNDF concentration was observed in spring relative to the remaining seasons of the year ( $p < .001$ ). The greatest CT concentration was found in the GRASS+LEGUME pasture ( $p < .001$ ; Table 2). Throughout the year, greater CT concentration in the diet was recorded during summer and spring relative to fall ( $p = .038$ ).

No differences between PT were observed for forage DM and OM intakes ( $p = .729$  and  $p = .780$ , respectively; Table 2), with average values of 2.29 and 2.11% BW/d,

respectively. In the GRASS+LEGUME pasture, the legume DM intake was 0.79, 0.49, and 0.71% BW/d during summer, fall, and spring, respectively, and the legume OM intake was 0.63, 0.38, and 0.56% BW/d during summer, fall, and spring, respectively. Both, forage DM and OM intake were greater during summer and spring ( $p < .001$  and  $p < .001$ , respectively) relative to fall season. The GRASS+N and GRASS+LEGUME pastures had greater forage CP intake ( $p < .007$ ; Table 2) than the GRASS pasture. In the GRASS+LEGUME pasture, legume CP intake was 0.13, 0.06, and 0.13% BW/d during summer, fall and spring, respectively. Greater forage CP intake was observed in summer and spring ( $p < .001$ ) compared to the fall season. There was no difference between treatments for forage apNDF intake ( $p = .970$ ; average of 1.35 %BW/d; Table 2). In the GRASS+LEGUME pasture, legume apNDF intake was 0.32, 0.18, and 0.26 %BW/d during summer, fall, and spring, respectively. Lowest forage apNDF intake was observed during fall season ( $p < .001$ ). Greatest forage EE intake and forage CT intake occurred in the GRASS+N and GRASS+LEGUME pastures ( $p = .058$  and  $p < .001$ , respectively; Table 2). In the GRASS+LEGUME pasture, legume CT intake was 0.017, 0.006, and 0.014 during summer, fall, and spring, respectively. Greatest EE forage intake was obtained in spring ( $p < .001$ ); however, CT forage intake was greater in summer and spring relative to fall season ( $p = .001$ ).

The GRASS+N and GRASS+LEGUME pastures had greater DM and OM apparent digestibility ( $p = .013$  and  $p = .016$ , respectively; Table 2) than the GRASS pasture. Greatest DM and OM apparent digestibility was observed during the spring ( $p < .001$  and  $p < .001$ , respectively). Greatest CP and apNDF apparent digestibility occurred in the GRASS+N pasture ( $p < .001$  and  $p = .005$ , respectively; Table 2), followed by the GRASS+LEGUME pasture, and lowest in the GRASS pasture. Forages during spring had the greatest CP and apNDF apparent digestibility ( $p < .001$  and  $p < .001$ , respectively).

The legume intake varied according to the botanical composition of the GRASS+LEGUME pasture ( $p < .001$ ) and showed a positive linear relationship with legume percentage in the herbage mass (Figure 5).



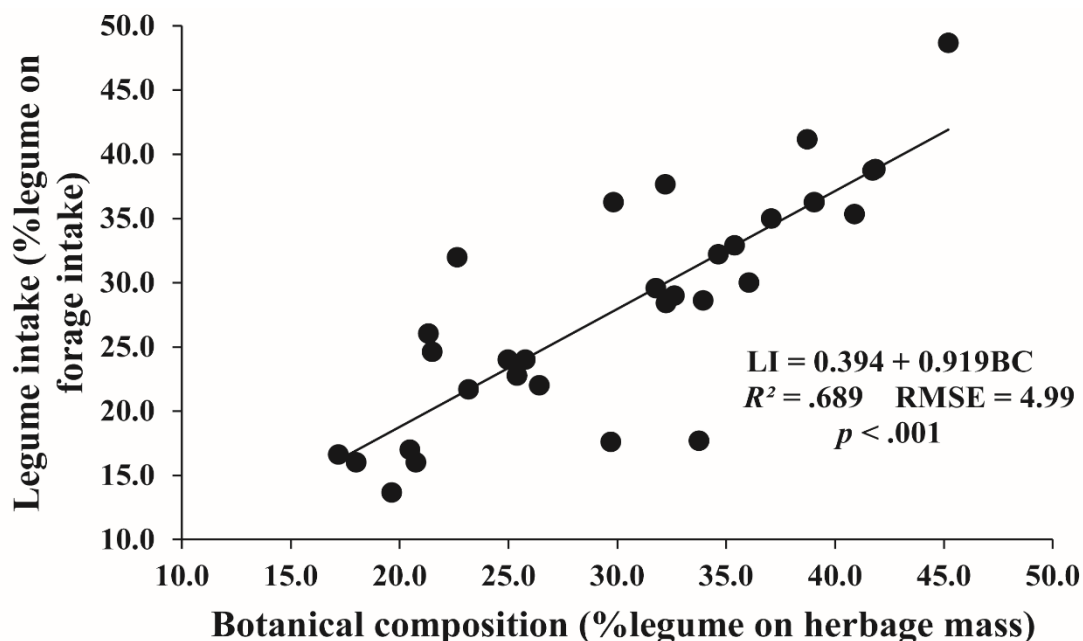
**Table 2** Diet, intake and apparent digestibility of forage consumed by Nellore heifers on Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Variables	Pasture type (PT)			Seasons (S)			SEM <sup>†</sup>	P-value		
	GRASS+N	GRASS	GRASS+LEGUME	Summer	Fall	Spring		PT	S	PT*S
<b>Diet, %</b>										
OM <sup>‡</sup>	92.5	93.0	91.9	91.7	93.3	92.4	0.7	.632	.154	.206
CP	13.8a	9.1c	11.5b	12.2a	9.7b	12.3a	0.4	<.001	<.001	.196
apNDF	58.0b	61.9a	57.7b	60.1a	60.9a	56.6b	0.6	<.001	<.001	.155
EE	1.99a	1.71b	1.54c	1.61	1.71	1.91	0.07	<.001	.118	.828
CT	0.19b	0.16b	0.61a	0.36a	0.26b	0.34a	0.06	<.001	.038	.106
<b>Intake, %BW/d</b>										
DM Forage	2.36	2.19	2.31	2.46a	1.88b	2.52a	0.17	.729	<.001	.720
OM forage	2.17	2.04	2.12	2.26a	1.75b	2.33a	0.16	.780	<.001	.703
CP forage	0.33a	0.21b	0.27a	0.30a	0.18b	0.32a	0.02	.007	<.001	.742
apNDF forage	1.36	1.35	1.33	1.48a	1.14b	1.42a	0.09	.970	<.001	.668
EE forage	0.046a	0.038b	0.036b	0.040b	0.032c	0.048a	0.003	.058	<.001	.571
CT forage	0.004b	0.003b	0.015a	0.009a	0.005b	0.009a	0.002	<.001	.001	.108
<b>Apparent Digestibility, %</b>										
DM	58.1a	51.6b	55.0a	53.5b	45.6c	65.5a	0.02	.013	<.001	.200
OM	61.8a	57.1b	60.0a	58.3b	51.5c	69.0a	0.01	.016	<.001	.142
CP	60.9a	43.0c	53.3b	53.0b	41.6c	62.7a	0.02	<.001	<.001	.563
NDF	60.2a	55.6b	56.1b	56.3b	48.4c	67.1a	0.02	.005	<.001	.516

<sup>a-c</sup>Least squares means within a row with different lowercase differ at  $p \leq .10$ ;

<sup>‡</sup>OM: Organic matter; CP: Crude protein; apNDF: Neutral detergent fiber corrected to ash and protein; EE: Ether extract; CT: Condensed tannin; DM: Dry matter;

<sup>†</sup>Standard error of the means.



**FIGURE 5** Legume intake proportion of beef cattle as a function of the percentage of legume in the canopy (BC) of mixed Marandu palisadegrass with forage peanut pasture. (Legume intake =  $0.394 + 0.919 \times \text{legume proportion of DM}$ ;  $p = .091$  and  $p < .001$  for intercept and slope, respectively;  $R^2 = 0.689$ ).

### 3.4 Microbial protein synthesis and nitrogen balance

The GRASS+N and GRASS+LEGUME pastures had greater DOMI ( $p = .083$ ; Table 3) than the GRASS pasture. The greatest DOMI was observed in spring ( $p < .001$ ). The GRASS+N pasture had the greatest N intake and CP/DOM ratio ( $p = .007$  and  $p < .001$ , respectively; Table 3), followed by the GRASS+LEGUME pasture and lesser in the GRASS pasture. Greatest N intake and CP/DOM ratio were observed during spring and summer ( $p < .001$  and  $p = .004$ , respectively). Greater microbial protein synthesis and efficiency of microbial synthesis occurred in the GRASS+N and GRASS+LEGUME pastures ( $p = .016$  and  $p = .067$ , respectively; Table 3) compared with the GRASS pasture (265 and 242 g of protein/d vs. 161 g of protein/d, and 56.4 and 57.6 g of microbial protein/kg DOM vs. 42.4 g of microbial protein/kg DOM, respectively). Greatest microbial protein synthesis was obtained in spring ( $p < .001$ ). On the other hand, greater efficiency of microbial synthesis was found in spring and fall ( $p = .031$ ) relative to summer season.

The GRASS+N pasture had the greatest urinary N excretion ( $p < .001$ ; Table 3), followed by the GRASS+LEGUME pasture, and lowest in the GRASS pasture. No

differences between PT were observed for faecal N excretion ( $p = .280$ ; average of 38.5 g of N/d; Table 3). Urinary N excretion was greatest in spring ( $p < .001$ ). However, for faecal N excretion, greater values were recorded during spring and summer ( $p = .021$ ) relative to fall season. Animal nitrogen balance was greater in the GRASS+N and GRASS+LEGUME pastures ( $p = .026$ ; Table 3). Spring had the greatest N balance ( $p < .001$ ), followed by summer and fall seasons. Microbial protein/CP intake ratio and ENU were greatest in the GRASS+LEGUME pasture ( $p = .042$  and  $p = .009$ , respectively; Table 3). In relation to season of the year, greater values of microbial protein/CP intake ratio were recorded during fall and spring relative to summer ( $p = .002$ ). On the other hand, greater values of ENU were recorded during spring and summer relative to fall season ( $p < .001$ ).

**Table 3** Nitrogen metabolism of Nellore heifers on Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Variables	Pasture type (PT)			Seasons (S)			SEM <sup>†</sup>	P-value		
	GRASS+N	GRASS	GRASS+LEGUME	Summer	Fall	Spring		PT	S	PT*S
DOMI <sup>‡</sup> , kg/d	4.7a	3.8b	4.2a	3.8b	2.8c	6.0a	0.4	.083	<.001	.491
N intake, g/d	180a	108c	143b	141b	102c	189a	16.1	.007	<.001	.593
CP/DOM, g/kg	247a	174c	206b	232a	203b	192b	10.6	<.001	.004	.323
MP, g of protein/d	265a	161b	242a	185b	152b	330a	30.9	.016	<.001	.398
EMS, g of microbial protein/kg DOM	56.4a	42.4b	57.6a	48.7b	54.3a	55.0a	8.7	.067	.031	.827
UNE, g of N/d	98.9a	55.3c	68.6b	63.8b	67.2b	91.8a	4.9	<.001	<.001	.269
FNE, g of N/d	43.4	32.9	39.2	41.2a	31.4b	43.0a	4.8	.280	.021	.996
N balance, g of N/d	34.7a	16.8b	32.2a	36.0b	3.4c	54.2a	5.1	.026	<.001	.353
MP/CP intake, g of MP/kg CP intake	271b	287b	312a	193b	360a	317a	18.2	.042	.002	.888
ENU, g/g	0.192b	0.155c	0.225a	0.255a	0.033b	0.287a	0.015	.009	<.001	.658

<sup>a-c</sup>Least squares means within a row with different lowercase differ at  $p \leq .10$ ;

<sup>‡</sup>DOMI: Digestible organic matter intake; CP/DOM: Crude protein/digestible organic matter ratio; MP: microbial synthesis; EMS: Efficiency of microbial protein synthesis; UNE: Urinary nitrogen excretion; FNE: Faecal nitrogen excretion; ENU: Apparent efficiency of nitrogen utilisation in the animals' body.

<sup>†</sup>Standard error of the means.

#### 4. DISCUSSION

Animal productivity of pasture systems is dependent on animal performance and stocking rate (Jones & Sandland, 1974; Pereira et al., 2020). In the current experiment, the ADG was greater in the GRASS+N and GRASS+LEGUME pastures than in the GRASS pasture (Table 1). The maximization of animal performance is linked to the capacity of nutrient intake by the grazing animals and forage digestibility (Chapman et al., 2007). As the forage DM and OM intakes were similar for all PT (average of 2.29 and 2.11 %BW/d, respectively; Table 2), the difference in ADG was due differences in forage nutritive value and nutrient digestibility. Nitrogen application and the presence of the legume increased forage CP concentration and decreased apNDF concentration in the diet (Table 2). The enhancement of CP concentration in the GRASS+N pasture is linked to increases in non-protein N such as nitrates, amides, amino acids, and small molecules such as peptides, all of which rapidly degraded in the rumen (Johnson, Reiling, Mislevy, & Hall, 2001). The reduction in apNDF concentration of the GRASS+N pasture is linked to the increase in CP concentration, causing a dilution effect in the cell wall concentration, as well as tissue renovation and enhancement of young tillers in this pasture (Paiva, Pereira, Silva, & Dias, 2015; Paiva et al., 2012; Sales et al., 2020). In the GRASS+LEGUME pasture, the presence of the legume improved forage nutritive value since grass nutritive value was similar between the GRASS+LEGUME and the GRASS pastures (Homem et al., 2021). Forage peanut often has a greater CP concentration and lesser apNDF concentration than Marandu palisadegrass (Gomes et al., 2018). Therefore, as heifers in the GRASS+LEGUME pasture ingested around 29% of their daily intake from forage peanut (0.66% BW/d), grazing animals had access to forage of greater nutritive value (Table 2). In the current experiment, the legume proportion in the diet was proportional to their contribution to the botanical composition (Figure 5; Curll, Wilkins, Snaydon, & Shanmugalingam, 1985; Gomes et al., 2018). The legume percentage was on average 32.0%. As forage intake was similar between PT, the increased CP concentration in the diet for the GRASS+N and GRASS+LEGUME enhanced CP intake from those pastures (an increase of 57.1% and 28.5% than in the GRASS pasture, respectively).

In forage diets, the NDF concentration is one of the determinants of forage intake (Baumont, Cohen-Salmon, Prache, & Sauvant, 2004). All pasture types had apNDF intake close to 1.3% of BW/d (Table 2), even with a reduction in apNDF concentration in the GRASS+N and GRASS+LEGUME pastures (Table 2). This probably indicates that non-nutritional factors did not affect forage intake. Canopy structural characteristics of

tropical forages are relatively more important than nutritional factors in terms of regulation of herbage intake (Poppi et al., 1987; Da Silva et al., 2013; Kunrath et al., 2020). Non-nutritional characteristics affect mainly the ingestive behaviour of grazing animals (i.e., bite rate, bite mass and bite volume), which will impact positively or negatively on forage intake (Carvalho et al., 2015; Da Silva et al., 2013). Thus, pastures with similar management targets, as in the current experiment, would have the same forage intake due to similar canopy structure (Forbes, 1988; Homem et al., 2021). In the GRASS+N pasture, there was an increase in leaf density at the upper canopy stratum, which could enhance forage intake due to easier formation and realization of the bite (Laca, Ungar, Seligman, Ramey, & Demment, 1992; Mezzalira et al., 2014). However, canopies managed with similar targets and lower leaf density in the upper stratum (as in the GRASS pasture) could result in grazing animals changing their ingestive behaviour (i.e., greater bite rate and grazing time) to maintain a steady forage intake (Da Silva et al., 2013). Therefore, even with N input in the GRASS+N and GRASS+LEGUME pastures, canopies managed under similar canopy height will have the same forage intake, probably due to changes in ingestive behaviour.

Nitrogen input in the GRASS+N and GRASS+LEGUME pastures increased DM and apparent OM digestibility, which would explain the greater ADG on those pastures (Table 2). Application of N results in faster tissue turnover and faster renovation in tiller population, generating younger tiller which have leaf blades with greater proportion of digestible tissues (Batistoti et al., 2012; Duru & Delaby, 2003). Young leaf blades have greater proportions of mesophyll cells that are tissues with greater digestibility than cell-wall constituents (Akin, 1989; Wilson, 1994). In the GRASS+LEGUME pasture, greater DM and apparent OM digestibility is likely linked to the legume intake. Forage peanut had greater digestibility than Marandu palisadegrass (Gomes et al., 2018), most likely because of greater proportion of mesophyll cells, which represents around 60-70% of the leaf blade constituents (Minson & Wilson, 1980; Valente et al., 2016).

Another critical point is the mesophyll cell arrangement. The C<sub>4</sub> mesophyll cells are more densely arranged and form a radial structure around the vascular bundles, which is referred to as Kranz anatomy and is absent in C<sub>3</sub> plants (Valente et al., 2016). Legume mesophyll has cells in irregular formats, delimiting intercellular spaces with variable amplitude (called lacunae parenchyma; Valente et al., 2016). Therefore, ruminal microorganisms have greater access to the different tissue through the intercellular

spaces, increasing the legume leaf blade digestibility (Minson & Wilson, 1980; Valente et al., 2016).

Even with similar CP intake in the GRASS+LEGUME pasture, the GRASS+N pasture had the greatest apparent CP digestibility (Table 2). As discussed previously, the N application increases the proportion of A and B1 fractions of the protein. These fractions are rapidly degraded in the rumen, which explains the greater apparent CP digestibility (Tylutki et al., 2008). In the GRASS+LEGUME pasture, the lower apparent CP digestibility than in the GRASS+N pasture may be explained by the protein:tannin association, in view of the fact that the CT intake in the GRASS+LEGUME pasture was 3.28 times more than in GRASS+N and GRASS pastures (Table 2). Condensed tannins reduce the digestion of protein in the rumen, enhancing the proportion of ruminally undegradable protein. However, part of the protein complex may not be released in the abomasum, being excreted in the faeces (Mueller-Harvey et al., 2019).

The greatest apparent NDF digestibility in the GRASS+N pasture is probably consequence of greater tissue renovation and younger tillers as a result of greater stocking rate and consequent defoliation frequency relative to the remaining treatments (Table 2). The NDF of young tillers is composed of greater proportions of hemicellulose and cellulose and lesser lignin concentrations than old tillers (Delevatti et al., 2019; Gomes et al., 2018). Conversely, the presence of the legume did not increase apparent NDF digestibility in the GRASS+LEGUME pasture (Table 2). Although forage peanut had a lower apNDF than Marandu palisadegrass (Gomes et al., 2018), its fibre is of inferior quality. On average, 50% of the forage peanut NDF is composed of acid detergent fibre (ADF), and 20% of the forage peanut ADF is composed of lignin (Gomes et al., 2018), whereas Marandu palisadegrass, 45% of the NDF is composed of ADF, and 9% of the ADF is composed of lignin (Gomes et al., 2018).

The CP/DOM ratio is used as an indicator of protein to energy status (Detmann et al., 2014; Poppi & McLennan, 1995). The response of the OM intake was similar for all PT; however, differences in OM apparent digestibility caused greater digestible OM intake for the GRASS+N and GRASS+LEGUME pastures (Table 3). Greater CP/DOM ratio in the GRASS+N and GRASS+LEGUME pastures was due to a greater increase of the CP intake than OM digestibility. This greater CP/DOM ratio in these pastures had a positive effect on microbial synthesis and efficiency of microbial synthesis (Table 3). Using a protein to energy ratio seems to be more plausible to understand the metabolic effects of protein on intake, because it is a more reliable indicator of the metabolic

adequacy of the animal (Detmann et al., 2014). Poppi and McLennan (1995) stated that animal performance would be limited with a diet with less than 150 g CP/kg DOM due to lack of N to ruminal microorganisms. The value of the CP/DOM ratio in the GRASS pasture was closer to the minimum level considered. Thus, the heifer ADG in the GRASS pasture was likely limited because of the smaller N intake, as in this pasture there was a decrease of 40% and 24.5% on N intake, and 51.6% and 47.8% in the N balance compared to the GRASS+N and GRASS+LEGUME pastures, respectively (Table 3).

In the GRASS+N pasture, the CP/DOM ratio was 16.5% greater than in the GRASS+LEGUME pasture (Table 3). Poppi and McLennan (1995) stated that losses of protein would occur when the CP concentration of the diet exceeded approximately 210 g CP/kg DOM. Thus, in the GRASS+N, there was an increase of 30.6% in urinary N excretion and a decrease of 13.1% and 14.7% in the microbial synthesis/CP intake ratio and ENU compared with GRASS+LEGUME pasture (Table 3). The surplus of N with respect to the energy availability for animal metabolism has several adverse effects on voluntary intake (Detmann et al., 2014). These include ATP deficiency in liver metabolism due to excessive utilisation of the urea cycle (Visek, 1984), increased body heat production (Poppi & McLennan, 1995), and animal indisposition due to excess ammonia in the blood (Detmann et al., 2014). Thus, the heifer ADG in the GRASS+N pasture was likely limited due to the lack of energy to meet the CP intake and to increase the rumen protein:energy synergism. The GRASS+LEGUME pasture had greater rumen protein:energy synergism, which leads to greater microbial synthesis/CP intake ratio, resulting in better ENU. Furthermore, the GRASS+LEGUME pasture had similar ADG to the GRASS+N pasture with a smaller excretion of urinary N.

Spring in regions with Cwa climate type (Köppen-Geiger climate classification) are a transition season between dry and rainy seasons. During winter, it is normal for forages to lose their nutritive value due to the unfavourable climatic conditions. However, at the beginning of the rainy season (spring) there are greater plant growth rates (leaf appearance and elongation) and tissue renovation. Thus, forages in spring had greater CP and lesser apNDF concentration than other seasons (Table 2), resulting in the increase of nutrient intake and digestibility at that time of the year (Table 2), enhancing the ADG by 31.9% and 68.0%, compared to summer and fall, respectively.

Nitrogen input increased the stocking rate (Table 1). Nitrogen application or the presence of the legume led to an increase in stocking rate of 72.4% and 25.3%, respectively, compared with the GRASS pasture (Table 1). Thus, N input affects the



stocking rate more than the ADG, as N application or the presence of the legume only increased the ADG by 16.9% and 12.3% compared with the GRASS pasture (Table 1). Nitrogen application to pastures increases the morphogenetic rhythm, population density, and tiller appearance rate, which culminates in greater forage accumulation rate (Lafarge, 2006; Paiva et al., 2015; Paiva et al., 2012). Thus, for canopies managed with similar targets, greater forage accumulation results in increased herbage mass and ultimately, stocking rate (Figure 4 and Table 1). In the GRASS+LEGUME pasture, the presence of the legume had little impact on the grass mass but resulted in an increase in herbage mass (Homem et al., 2021; Figure 3). The stocking rate was increased in the GRASS+LEGUME pasture due to the addition of the legume mass, resulting in a greater forage accumulation rate (Alves, Menezes, Lara, Casagrande, & Bernardes, 2016; Pereira et al., 2020).

In Brazil, the increase in productivity of livestock production on pasture in a sustainable manner is a necessity. In this scenario, the N application is the fastest and easiest way to produce more meat in smaller areas (Delevatti et al., 2019; Pereira et al., 2020). In the present study, the GRASS+N pasture had the greatest liveweight gain per area (Table 1), with an increase of 106% compared to the GRASS pastures. Liveweight gain per area in the GRASS+N pasture was 658 kg/ha/yr, six to seven times greater than the Brazilian average (Martha et al., 2012). In the GRASS+LEGUME pasture, there was an increase of 34.5% in the liveweight per area compared with GRASS pasture. Therefore, liveweight gain per area in the GRASS+LEGUME pasture was 429 kg/ha/yr, four times that of the Brazilian average (Martha et al., 2012). Mixed pastures have direct benefits to farmers, with a reduction of the cost of maintenance N application and an increase in gross income. Moreover, legumes provide significant environmental benefits, since the need for fossil fuel for N fertiliser synthesis is eliminated (Pereira et al., 2020). However, the adoption of forage peanut by farmers is not as simple as the adoption of new grass cultivars, and the economic advantages will largely depend on the availability and cost of legume seeds.

## 5. CONCLUSIONS

Nitrogen application or the integration of forage peanut in a grass pasture increased the animal performance; however, the nitrogen input by either way affected the stocking rate more than the average daily gain. Application of N fertiliser is the fastest and easiest way to increase the liveweight gain per area; however, in N-fertilized pastures there is a greater

nitrogen excretion by the grazing animal due to imbalance between protein and digestible organic matter in the diet. The presence of forage peanut increased the liveweight gain per area and improved the protein:energy synergism in the rumen. Thus, forage peanut has great potential for use in the humid tropics.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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### 3. CHAPTER 3

#### Article 3 - NITROGEN CYCLING IN PASTURES WITH NITROGEN INPUT THROUGH FERTILISER APPLICATION OR LEGUME INTEGRATION

**The manuscript will be submitted to the Journal “*Agriculture, Ecosystems & Environment.*” Preliminary version. The manuscript is following the guidelines in this Journal.**

#### ABSTRACT

Nitrogen (N) is the most limiting plant nutrient for the growth of grasses and the low amount of this nutrient in the soil is one of the main causes of pasture degradation. The understanding of the effects linked to N application or to introduction of a legume on N cycling is critical for achieving productive and sustainable grassland systems. This 2-yr study assessed the dynamics of existing and deposited litter, N intake, and N cycling via litter and excreta of three pasture treatments, namely, 1) mixed Marandu palisadegrass {*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]} and forage peanut (*Arachis pintoii* Krapov. & W.C. Greg. cv. BRS Mandobi) pastures without N fertiliser (GRASS+LEGUME); 2) monoculture Marandu palisadegrass pasture fertilised with 150 kg N ha<sup>-1</sup> yr<sup>-1</sup> (GRASS+N); and 3) monoculture Marandu palisadegrass without N fertiliser (GRASS). Grazing management was similar across pasture treatments, using continuous stocking and a target canopy height of 20 to 25 cm. Existing litter and litter deposition rate were greatest in the GRASS pasture ( $p = 0.005$  and  $p = 0.005$ , respectively). Litter decomposition rate was greater, and half-life time was lower in the GRASS+LEGUME and GRASS+N pastures than in the GRASS pasture ( $p = 0.079$  and  $p = 0.050$ , respectively). The GRASS+N and GRASS+LEGUME pastures had the greatest deposited litter N ( $p = 0.004$ ). The greatest faecal N excretion, and urinary N excretion per season occurred in the GRASS+N ( $p = 0.002$ , and  $p < 0.001$ , respectively). In the GRASS+N and GRASS+LEGUME pastures, there was a positive overall change of N in the system of 19 and 18 kg N ha<sup>-1</sup> year<sup>-1</sup>, respectively. However, in the GRASS pasture, there was an overall negative change of N in the system of -41 kg N ha<sup>-1</sup> year<sup>-1</sup>. Nitrogen application or the integration of forage peanut in a grass pasture increased the conservation of soil N reserves.

Keywords: *Arachis pintoi*, *Brachiaria*, fertilized pasture, litter deposition and decomposition, livestock excretion, warm-season legume

## 1. Introduction

Brazilian beef production is of great global importance as Brazil has the largest commercial herd of cattle in the World (USDA, 2019). For a long time, Brazilian pastures were mostly established and maintained with little or no chemical fertiliser and their productivity was dependent on the nutrients in the ash resulting from the burning of the forest (Boddey *et al.*, 2004; Pereira *et al.*, 2020). For the first few years after establishment, liveweight gain per area on these pastures were between 200 and 400 kg ha<sup>-1</sup> y<sup>-1</sup> (Martha *et al.*, 2012). However, subsequently pasture and animal productivity decline, and the areas become invaded by weed species, bare soil starts to appear, characterizing a process called pasture degradation (Boddey *et al.*, 2004; Pereira *et al.*, 2020). Thus, typical low productivity and economic indices on beef pasture operations are common in degraded pastures (Martha *et al.*, 2012), as well as reduction of biological, chemical and physical traits related to soil quality (Sollenberger *et al.*, 2019). The delivery of ecosystem services by grasslands agroecosystems may be severely compromised in degraded pasture (Kohmann *et al.*, 2018; Sollenberger *et al.*, 2019).

Nitrogen (N) is the most limiting plant nutrient for the growth of C<sub>4</sub> grasses. Additionally, the low amounts of N in the soil is one of the main factors causing pasture degradation (Boddey *et al.*, 2004; Dubeux Jr. *et al.*, 2007). Nitrogen fertiliser application has become a more common practice to increase N input in Brazilian pastures (Martha *et al.*, 2012; Pereira *et al.*, 2020). However, its use may be limited by its cost and distribution logistics, since the beef industry is based on extensive operations. Incorporating N<sub>2</sub>-fixing legumes with grasses has been suggested to be one of the best alternatives to achieve sustainable grazing systems (Muir *et al.*, 2011; Muir *et al.*, 2014). Among the legumes, forage peanut has been the most productive and persistent over a relatively wide range of grazing management practices in warm-climate grasslands ecosystems (Hernandez *et al.*, 1995; Gomes *et al.*, 2018; Tamele *et al.*, 2018; Gomes *et al.*, 2020; Pereira *et al.*, 2020). Nevertheless, forage legume adoption has been limited even though there are successful cases reported in the literature (Boddey *et al.*, 2020). Nitrogen application or legume integration increases the overall forage production and forage nutritive value, allowing an increase in stocking rate and liveweight gain per area (Homem *et al.*, 2021a; Homem *et al.*, 2021b). Also, N input enhances nutrient cycling, acting as a catalyst for the vital

recycling processes, particularly in low-soil-fertility environments (Dubeux Jr. *et al.*, 2007; Liu *et al.*, 2017).

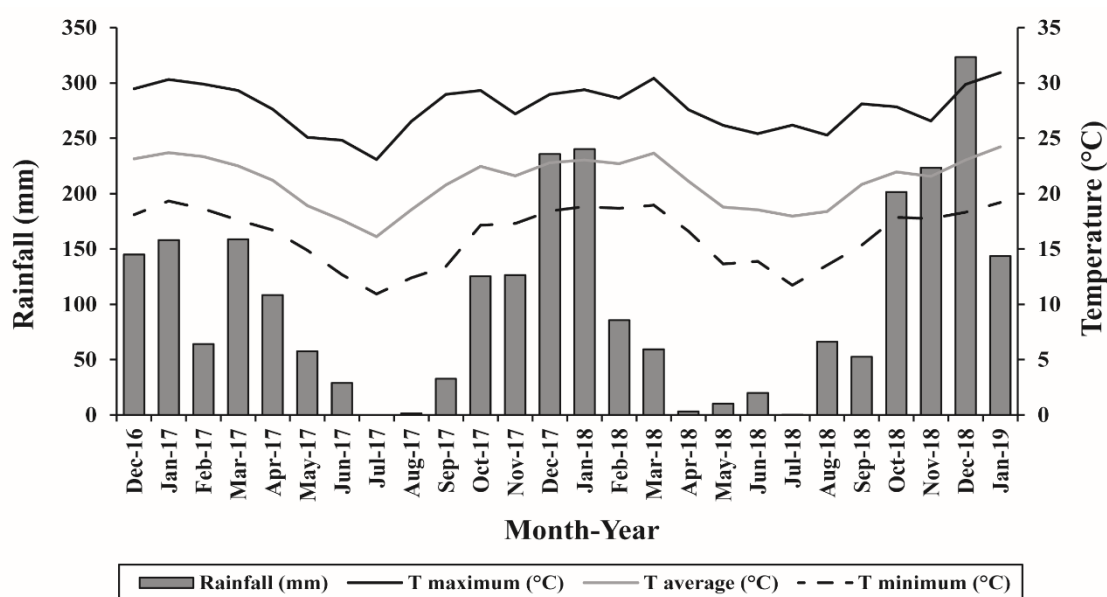
Interest in nutrient dynamics in managed grasslands has been intensified in recent years (Dubeux Jr. *et al.*, 2007). Even in the pasture with N application, the return of N via residues may reduce the dependency of intensified livestock systems on chemical fertiliser use, decreasing the production costs (Wang *et al.*, 2018). On mixed pastures, N cycling is a determinant of grass production, since it is necessary to transfer the fixed N from the legume to the companion grass (Kohmann *et al.*, 2018). Thus, the most important pathways of N recycled in pastures are via litter or livestock excretion (Dubeux Jr. *et al.*, 2007). Plant litter has an essential role in nutrient return and soil organic matter (OM) formation in warm-climate grassland ecosystems (Cantarutti *et al.*, 2002; Dubeux Jr. *et al.*, 2006a; Dubeux Jr. *et al.*, 2006b). Litter decomposition is one of the main processes controlling the availability of carbon and nitrogen in terrestrial ecosystems (Wardle *et al.*, 2004; Wang *et al.*, 2020). Climate and quality of deposited litter have been traditionally considered as the main drivers of litter decomposition (Cornwell *et al.*, 2008; García-Palacios *et al.*, 2013). Thus, N input may enhance the litter decomposition by improving plant litter quality. Concerning livestock excretion, when pastures are under similar management, N input increases stocking rate and N excretion per area (Boddey *et al.*, 2004). Nevertheless, recovery of N from excreta by plants is poor due to heterogenous excreta distribution (White *et al.*, 2001) and the potential nutrient losses due to leaching and gaseous N losses (Dubeux Jr. *et al.*, 2007).

Understanding the effects of N application or the introduction of a legume on the cycling of N is critical for achieving productive and sustainable grassland systems. However, knowledge is still limited regarding the effects of N application or legume integration on the distribution and cycling of N among significant nutrient pools. Thus, we hypothesized that N input on pasture systems would positively impact the overall change of N in the system by increasing the total N recycled through primary aerial productivity. Additionally, even under target of grazing management, the efficiency of N utilization by heifers (NUE;  $\text{kg N ingested kg N in plant uptake}^{-1}$ ) will be greater with the input of N on pastures. Therefore, the objective of this study was to investigate the dynamics of existing and deposited litter, N intake, and N cycling via litter and excreta of mixed pastures of Marandu palisadegrass and forage peanut compared to Marandu palisadegrass in monoculture, fertilized or not with N, subjected to continuous stocking with variable stocking rate to maintain the same canopy height of 20 to 25 cm.

## 2. Materials and methods

### 2.1 Experimental site and treatments

The study was carried out at the Experimental Farm of the Federal University of Lavras, Brazil (21°14'S, 44°58'W; 918 m above sea level). This area has a subtropical humid mesothermal climate with dry winters (Köppen-Geiger climate classification: Cwa; Sá Júnior *et al.*, 2012). Meteorological data were obtained from a weather station located 1,000 m from the experimental area (Fig. 1).



**Fig. 1.** Mean monthly temperatures and rainfall in Lavras, Brazil, during the experimental period (figure before shown at Homem *et al.*, 2021a).

The soil in the area is a Ferralsol (WRB/FAO classification). Soil texture is clayey, and the clay content is similar down the profile (a characteristic of this soil type): 563 g clay kg soil<sup>-1</sup> (0–10 cm) and 574 g clay kg soil<sup>-1</sup> (20–40 cm). Soil analyses were conducted according to Embrapa standard techniques (Claessen *et al.*, 1997). The soil (0–20 cm) had the following properties: pH (H<sub>2</sub>O) = 5.9; exchangeable Al, Ca, Mg, 0.07, 2.4, and 0.7 cmol<sub>c</sub> dm<sup>-3</sup>, respectively; available P (Mehlich-I method) 7.6 mg dm<sup>-3</sup>, exchangeable K 82.8 mg dm<sup>-3</sup>, and soil organic matter 31.0 g kg<sup>-1</sup>.

Treatments were three pastures types (PT), namely: 1) a mixed pasture of Marandu palisadegrass {*Brachiaria brizantha* (Hochst. ex A. Rich.) R.D. Webster [syn. *Urochloa brizantha* Stapf cv. Marandu]} and forage peanut (*Arachis pintoi* Krapov. &

W.C. Greg. cv. BRS Mandobi), without N fertiliser application (**GRASS+LEGUME**);  
2) Marandu palisadegrass monoculture fertilised with 150 kg N ha<sup>-1</sup> year<sup>-1</sup> (**GRASS+N**);  
and 3) Marandu palisadegrass monoculture without N fertiliser application (**GRASS**).

## 2.2 Pasture and treatment establishment

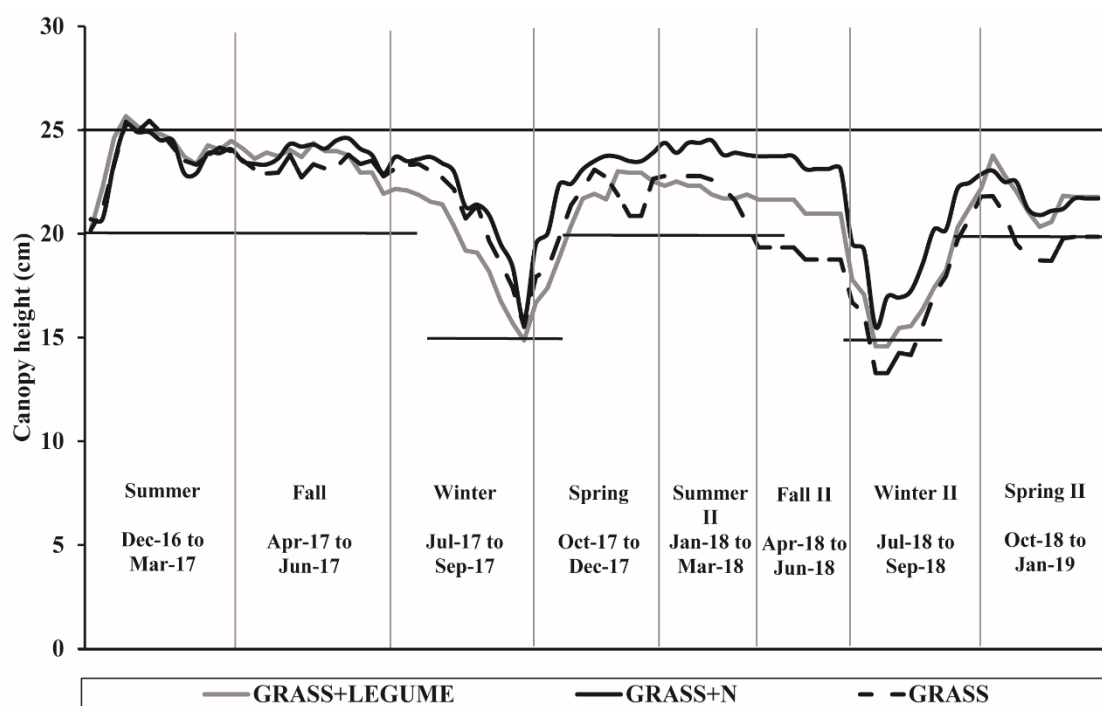
In January 2014, the whole experimental area was seeded with Marandu palisadegrass at a rate of 6.0 kg ha<sup>-1</sup> of pure live seeds. The 12-ha experimental area was limed (2,500 kg dolomitic lime ha<sup>-1</sup>) 60 d before grass seeding. Fifty-two kg of P ha<sup>-1</sup> as single super phosphate and 41.5 kg of K ha<sup>-1</sup> as potassium chloride were applied at grass seeding. The experimental area was divided into four paddocks with three hectares each (blocks) taking into account the topography of the area, and managed under rotational stocking until December 2015.

In December 2015, the blocks were divided into three paddocks where pasture types were randomly allocated. The size of each paddock (experimental unit) was adjusted based on previous studies in the area using these pastures types. The GRASS+N and GRASS paddock size (0.7 and 1.3 ha, respectively) were determined by the stocking rate obtained in the study of Pereira *et al.* (2015) with Marandu palisadegrass monoculture pastures with the application of 150 kg N ha<sup>-1</sup> or no fertiliser application. For the GRASS+LEGUME pasture, the size of the paddocks (1.0 ha) was determined by the stocking rate obtained in the study of Gomes *et al.* (2018) with Marandu palisadegrass and forage peanut mixed pastures. The paddock size of each PT allowed adding an average of 3.0 animal units (1 AU = 500 kg live weight) per paddock during the rainy season. Furthermore, this allowed tester animals (at least two animals) to remain the maximum possible time during the dry season, and then all animals were removed at the same time.

The GRASS+LEGUME paddocks were seeded with forage peanut into a previously established Marandu palisadegrass pasture in December 2015. Before the forage peanut seeding, the Marandu palisadegrass canopy was lowered to around 10 cm by mechanical cutting. The seeding rate was 10 kg ha<sup>-1</sup> of pure live seeds for forage peanut planted with a no-till seeder with four rows. The planter allocated six seeds of forage peanut per linear meter with 0.5 m row spacing. After seeding, paddocks with forage peanut were excluded from grazing during 75 d to improve the establishment.

In March 2016, grazing management targets were imposed under continuous stocking. This period up to December 2016 was considered as a time for the adaptation

of the canopies to the experimental management. From December 2016 to January 2019, eight seasons were evaluated. The years were divided into the four seasons of each year: from December, 22 to March, 21 (Summer), from March, 22 to July, 21 (Fall), from July, 22 to September, 21 (Winter), and from September, 22 to December, 21 (Spring). Continuous stocking with variable stocking rate was used to achieve and maintain the target canopy height of 15 cm during winter and 20 to 25 cm during the remaining of the year. In each experimental year, two Nellore heifers ( $234 \pm 36$  kg of initial BW and  $12 \pm 1.3$  months of age) were used as tester animals in each paddock. At the end of the first experimental year, the animals were replaced. When necessary to adjust canopy height, put-and-take animals were added or retrieved from paddocks (Allen *et al.*, 2011). Water and mineral supplementation were supplied *ad libitum*. Average canopy height was measured weekly using a sward stick (Barthram, 1985) at 100 random points per paddock, and stocking rate was adjusted when necessary (Fig. 2).



**Fig. 2** Canopy height (cm) throughout the experimental period (figure previously shown in Homem *et al.*, 2021a).

Annually, in spring (between November and December), all paddocks were fertilized with single superphosphate and potassium chloride corresponding to  $22 \text{ kg ha}^{-1}$  of P and  $41 \text{ kg ha}^{-1}$  of K, respectively. In the GRASS+N pasture, the N fertiliser

application was divided into three applications per year (50 kg N ha<sup>-1</sup> each in November, January, and March) using urea.

## 2.2 Responses variables

### 2.2.1 Herbage mass and N stock

Herbage mass was sampled by harvesting six frames at ground level, measuring 1 x 0.5 m, per paddock, on sites with average canopy height once every 30 d. After harvesting, the fresh material was weighed. Subsamples of approximately 250 g fresh material were taken for the evaluation of dry matter (DM) concentration. Another subsample of approximately 2 kg was taken for manual separation of botanical components. Forage samples were oven-dried at 55 °C for 72 h to a constant weight. Grass mass (kg ha<sup>-1</sup>) was considered as leaf + stem + dead material, and the legume mass (kg ha<sup>-1</sup>) included leaf plus stem mass. Herbage mass was considered the aboveground biomass of herbaceous plants (grass and legume mass, according to treatment).

After drying, forage samples were ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. After grinding, the DM of each sample was obtained by oven drying at 105 °C for 18 h (method 934.01; AOAC, 2000). Grass and legume N concentration was determined using the Kjeldahl procedure (method 920.87; AOAC, 2000). Grass and legume N stock was assessed by the concentration of N in the grass or legume multiplied by their respective mass. Total N stock was the sum of the grass and legume N stock, according to treatment. The results were presented as average per season.

### 2.2.2 Biological N fixation from the GRASS+LEGUME pasture

The N biological fixation (BNF) was estimated per season using the <sup>15</sup>N isotope natural abundance technique (Shearer and Kohl, 1986). The technique is based on the fact that the plant available N on the majority of the soils is lightly enriched with the <sup>15</sup>N isotope relative to the atmosphere (Okito *et al.*, 2004), indicating that an N<sub>2</sub>-fixing plant will have a lower <sup>15</sup>N abundance than a non-fixing control plant, which is entirely dependent of N from the soil. The herbage mass of the legume and non-fixing plants were analysed for <sup>15</sup>N abundance. This plant material was oven-dried at 55 °C for 72 h to a constant weight and ground to a fine powder using a roller mill similar to that described by Arnold and Schepers (2004). For the evaluation of δ<sup>15</sup>N, aliquots containing between 200 and 400 µg N were analysed using an automated continuous-flow isotope-ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to the output of a Costech



[model ECS4010] total C and N analyser – Finnigan MAT, Bremen, Germany in the "John Day Stable Isotope Laboratory" at Embrapa Agrobiologia. Secondary standards of wheat flour for  $^{15}\text{N}$  abundance were used to calibrate the mass spectrometer by including the standards at intervals of every five samples in the carousel.

According to the methodology, the N derived from the atmosphere (% Ndfa) was calculated as follows:

$$\%Ndfa = \left( \frac{\delta^{15}\text{N reference} - \delta^{15}\text{N legume}}{\delta^{15}\text{N reference} - B} \right) \times 100$$

Where:

- $\delta^{15}\text{N reference}$  =  $\delta^{15}\text{N}$  value from the soil obtained from non-fixing plants growing together with the legume;
- $\delta^{15}\text{N legume}$  =  $\delta^{15}\text{N}$  value for the  $\text{N}_2$  fixing plant;
- $B$  = the  $^{15}\text{N}$  natural abundance of the N derived from biological N fixation in the legume.

Forage peanut was the N-fixing plant. Non- $\text{N}_2$ -fixing plants growing nearby within the experimental units were used: *Sida rhombifolia* L., *Vernonia polysphaera*, *Solanum viarum*, and *Baccharis dracunculifolia*. The adjustment of the isotopic fractioning was made according to the recommendation of Shearer and Kohl (1986), and a  $B$  value of -2.27‰, proposed by Okito *et al.* (2004) for non-inoculated *Arachis hypogaea* L. was used.

The amount of N biologically fixed in the aerial biomass of forage peanut ( $\text{kg N ha}^{-1}$ ) was calculated as:

$$N_{bnf} = N_{accumulated} \times \%N_{bnf}$$

Where:

- $N_{bnf}$  = total amount of N in the aerial biomass of forage peanut derived from BNF ( $\text{kg N ha}^{-1} \text{ season}^{-1}$ );
- $N_{accumulated}$  = total amount of N of forage peanut deposited in the litter plus the amount of N of forage peanut ingested by animals ( $\text{kg N ha}^{-1} \text{ season}^{-1}$ );
- $\%N_{bnf}$  = %Ndfa in the aerial biomass of forage peanut.

### 2.2.3 Litter dynamics

Litter deposition, decomposition, and chemical composition were measured throughout the experiment. Litter accumulation and disappearance were evaluated using a procedure based on the technique described by Rezende *et al.* (1999). Every 28 d, six frames of 1 by 0.5 m were allocated in points of average canopy height and the litter harvested. Litter was considered the dead detached plant material at the soil surface (Allen *et al.*, 2011). These samples were denominated as existing litter (Rezende *et al.*, 1999). Fourteen days after the existing litter harvest, the litter from the same collection point was harvested, and these samples were denominated deposited litter. Subsequently, every 28 d, the same procedure was adopted to existing and deposited litters until the end of the experiment. The deposited litter was the sum of all the litter harvested during each season of the year (Rezende *et al.*, 1999).

After collection, litter samples were oven-dried at 55 °C for 72 h to constant weight. After drying, all litter samples were ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. After grinding, DM of each sample was obtained by oven drying at 105 °C for 18 h (method 934.01; AOAC, 2000), and OM was obtained by ashing at 500 °C for 4 h (Moore and Mott, 1974). All data were expressed on an OM basis to eliminate mineral particle effects on nutrient concentration. Nutrient ratios were obtained by dividing nutrient concentrations, all of which were expressed in an OM basis (g kg<sup>-1</sup> of OM). The decomposition constant was calculated according to the following equation described by Rezende *et al.* (1999):

$$k = [\ln (Le_{(n-1)} + Ld_n) - \ln (Le_n)] / t$$

Where:

- $Le_{(n-1)}$  = the amount of existing litter in the previous cycle;
- $Ld_n$  = the amount of deposited litter in the current cycle;
- $Le_n$  = existing litter in the current cycle;
- $t$  = the cycle time (14 d).

The half-life ( $t^{1/2}$ ) was estimated according to the following equation Rezende *et al.* (1999):

$$t^{1/2} = \ln (2) / k$$

Where:

- $k$  = decomposition constant (g g<sup>-1</sup> d<sup>-1</sup>).

Litter disappearance was estimated by the following equation Rezende *et al.* (1999):

$$\text{Litter disappearance} = \text{Le}_{(n-1)} + \text{Ld}_n - \text{Le}_n$$

Total N concentration was determined using the Kjeldahl procedure (method 920.87; AOAC, 2000). In the GRASS+LEGUME pasture, the proportion of grass and legume in the litter samples was estimated through the ratio of natural  $^{12}\text{C}$  and  $^{13}\text{C}$  isotopes by the equation:

$$\% \text{legume} = 100 (\delta^{13}\text{C}_G - \delta^{13}\text{C}_S) / (\delta^{13}\text{C}_G - \delta^{13}\text{C}_L)$$

Where:

- $\% \text{legume}$  = proportion of carbon from a legume in the litter sample;
- $\delta^{13}\text{C}_G$  = value of  $\delta^{13}\text{C}$  abundance of the dead material from the grass ( $-11.7\text{‰}$ );
- $\delta^{13}\text{C}_L$  = value of  $\delta^{13}\text{C}$  abundance of the dead material from the legume ( $-26.5\text{‰}$ );
- $\delta^{13}\text{C}_S$  = value of  $\delta^{13}\text{C}$  abundance of the litter samples from the GRASS+LEGUME pasture.

For the analysis of  $^{13}\text{C}$ , subsamples were ground to a fine powder in a roller mill similar to that described by Arnold and Schepers (2004). Sub-samples containing between 300 and 500  $\mu\text{g}$  C were analysed for total C and  $^{13}\text{C}$  abundance using the same continuous-flow isotope-ratio mass spectrometer as that used to determine  $^{15}\text{N}$  abundance.

#### 2.2.4 Forage intake

Hand-plucked forage samples were collected during the intake evaluation for nutritional value analysis (Vries, 1995). In the GRASS+LEGUME pasture, grass and legume were collected separately. Forage samples were oven-dried at  $55\text{ }^\circ\text{C}$  for 72 h to a constant weight. Then, a composite sample of each species was made for each experimental unit. The composite samples were ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. The DM of each sample was obtained by oven drying at  $105\text{ }^\circ\text{C}$  for 18 h (method 934.01; AOAC, 2000).

Forage intake was estimated from faecal excretion and indigestible neutral detergent fibre (iNDF) once per season. Spot faecal samples were collected once a day at the same hour (midday), and a composite sample was produced for each animal for the

five days of collection. During the sampling days, the heifers were brought in from the paddocks to a barn to collect faeces directly from the rectum. Faecal production was estimated using titanium dioxide as an external marker (Titgemeyer *et al.*, 2001) during eleven consecutive days, six for adaptation, and five for collection. The titanium dioxide was dosed in the amount of 10 g animal<sup>-1</sup> day<sup>-1</sup>. Faecal samples were oven-dried at 55 °C for 72 h to determine DM concentration, air equilibrated, weighed, and ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. The faecal samples were analysed for titanium dioxide concentration, according to Myers *et al.* (2004).

Faecal and hand-plucked forage samples were incubated in the rumen for 288 hours to determine iNDF (Huhtanen *et al.*, 1994). Two cannulated heifers fed with a diet that consisted of Marandu palisadegrass and forage peanut mixed pasture or Marandu palisadegrass monoculture were used in the iNDF estimate, according to treatment. The faecal excretion was used to find the total amount of iNDF in faeces; in this way, the estimate of iNDF intake per day was obtained. After that, iNDF from the hand-plucked samples was determined to estimate forage intake.

In the GRASS+LEGUME pasture treatment, the proportion of grass and legume in the forage intake was estimated through the ratio of natural <sup>12</sup>C and <sup>13</sup>C isotopes by the equation:

$$\% \text{legume} = 100 (\delta^{13}\text{C}_G - \delta^{13}\text{C}_S) / (\delta^{13}\text{C}_G - \delta^{13}\text{C}_L)$$

Where:

- %legume = proportion of carbon from a legume in the iNDF residual faecal samples (Lopes de Sá, 2017);
- $\delta^{13}\text{C}_G$  = value of  $\delta^{13}\text{C}$  abundance of the iNDF residual in hand-plucked samples of the grass (-11.9‰);
- $\delta^{13}\text{C}_L$  = value of  $\delta^{13}\text{C}$  abundance of the iNDF residual in hand-plucked samples of the legume (-27.4‰);
- $\delta^{13}\text{C}_S$  = value of  $\delta^{13}\text{C}$  abundance of the iNDF residual in faecal samples.

The <sup>13</sup>C abundance procedure was evaluated as described for the litter. Nitrogen concentration in hand-plucked forage samples was determined using the Kjeldahl procedure (method 920.87; AOAC, 2000). The N concentration in the forage samples was estimated using the Kjeldahl procedure (method 920.87, AOAC, 2000) and used to

determine the intake of total N in the forage. The N intake in  $\text{kg ha}^{-1}$  was obtained by multiplying daily N intake per animal and stocking rate for each treatment.

#### 2.2.5 Nitrogen excretion

Faecal N excretion ( $\text{g N d}^{-1}$ ) was assessed by the concentration of N in the faeces (method 920.87; AOAC, 2000) multiplied by total faecal production. The urinary excretion was estimated through spot urine samples, which were obtained by vulvar stimulation at the same time as faecal samples were collected. A 12-ml aliquot was taken, and 48 mL of 0.02 N sulfuric acid ( $\text{H}_2\text{SO}_4$ ) were added (Chen and Gomes, 1992). A 5-d composite sample was collected and stored in a plastic flask at  $-20\text{ }^\circ\text{C}$ . Urine creatinine concentration was determined using a commercial kit (Creatinine K, Labtest, Lagoa Santa, Brazil). Urine volume was estimated using creatinine concentration as a marker and assuming a daily creatinine output, according to the following equation (Silva *et al.*, 2012):

$$\text{UV} = (0.0345 \times \text{SBW}^{0.9491}) \div \text{UC}_c$$

Where:

- UV ( $\text{L d}^{-1}$ ) = daily total urinary production;
- SBW (kg) = shrunk body weight;
- $\text{UC}_c$  ( $\text{g L}^{-1}$ ) = urine creatinine concentration.

Urinary N excretion ( $\text{g of N d}^{-1}$ ) was determined by its N concentration related to urinary volume (method 920.87; AOAC, 2000). The N excreted from faeces and urine in  $\text{kg ha}^{-1}$  was obtained by multiplying daily N excretion per animal and stocking rate for each treatment. The N retained in the animal was measured by the difference between N intake and N excretion in faeces and urine.

#### 2.2.6 Nitrogen cycling balance

Nitrogen cycling balance was measured according to the values of N cycled via litter and livestock excretion. *Values in rectangular boxes* are pool sizes ( $\text{kg N ha}^{-1}$ ), and other values are annual fluxes of N in  $\text{kg N ha}^{-1} \text{ year}^{-1}$ . Plant uptake or N in annual net aerial primary productivity was considered as deposited litter N + total N intake + change in standing N biomass. Nitrogen losses were estimated based on a 5% loss of N in faeces

and 50% of N in urine (Boddey *et al.*, 2004). Nitrogen stocked in the animal carcass was calculated as live weight x 0.025 (Scholefield *et al.*, 1991).

### 2.3 Statistics analysis

The experimental design was a randomized complete block with three treatments (pasture type; GRASS+N, GRASS+LEGUME, and GRASS), four replications, and repeated measurements over time (seasons of the year). Data were analysed by fitting mixed models (Littell *et al.*, 2000) using the MIXED procedure of SAS (SAS Institute, Cary NC). The effects of pasture types and seasons were considered fixed and the effects of block and year as random effect. The Akaike information criterion was used to choose the best (co)variance structure (Akaike, 1974). All variance components were estimated using the LSMEANS statement, and comparisons were made between treatments using Fisher's protected least significant difference (LSD) test at 10% probability. The statistical model for data analysis was as follows:

$$Y_{ijkz} = \mu + B_i + PT_j + \gamma_{ij} + Y_k + S_z + (PT \times S)_{jz} + \varepsilon_{ijkz}$$

Where  $Y_{ijkz}$  = value observed in the  $i$ th block of the  $j$ th PT of the  $k$ th year of the  $z$ th season;  $\mu$  = overall average;  $B_i$  = random effect associated with the  $i$ th block,  $i = 1, 2, 3, 4$ ;  $PT_j$  = fixed effect associated with  $j$ th types of pasture,  $j = 1, 2, 3$ ;  $\gamma_{ij}$  = random error associated with the  $i$ th block in the  $j$ th PT.  $Y_k$  = random effect associated with  $k$ th year,  $k = 1, 2$ ;  $S_z$  = fixed effect associated with  $z$ th season,  $z = 1, 2, \dots, 8$ ;  $(PT \times S)_{jz}$  = fixed effect of interaction  $j$ th PT with the  $z$ th season.  $\varepsilon_{ijkz}$  = random error associated with the  $i$ th block, the  $j$ th PT, the  $k$ th year, and the  $z$ th season.

## 3. Results

### 3.1 Herbage mass, N stock, and biological N fixation

Herbage and grass mass were greatest in the GRASS+N pasture in fall and winter ( $p < 0.001$  and  $p = 0.004$ , respectively; Fig. 3). In summer and spring, there were no differences in herbage and grass mass between PTs (average values of 8,650 and 9,020 kg ha<sup>-1</sup> for herbage mass; and 8,170 and 8,560 kg ha<sup>-1</sup> for grass mass, respectively). The greatest herbage and grass mass occurred in the fall for the GRASS+N pasture. Summer, fall, and spring for the GRASS+LEGUME pasture and fall and spring for the GRASS pasture had the greatest herbage and grass mass. In all seasons, N concentration in the

grass mass was greatest in the GRASS+N pasture ( $p = 0.053$ ; Fig 4). Summer and spring seasons showed greater grass N concentration in the GRASS+N pasture than in fall and winter. The GRASS+LEGUME and GRASS pastures showed greater grass N concentration in summer, fall, and spring compared to winter. Total N stock and grass N stock were greatest in the GRASS+N pasture in all seasons ( $p = 0.008$  and  $p = 0.022$ , respectively; Fig 5). In fall, the GRASS+N pasture had the greatest total and grass N stock. In winter there was a lower total N stock in the GRASS+LEGUME pasture. There were no differences in grass N stock between seasons in the GRASS+LEGUME pasture, and in the GRASS pasture for total and grass N stock.

In the GRASS+LEGUME pasture, the legume mass ranged from 680 kg ha<sup>-1</sup> in winter to 1,470 kg ha<sup>-1</sup> in summer (average of 1,190 kg ha<sup>-1</sup>; Table 1). The legume N concentration and legume N stock were greatest in summer and spring with mean values of 24.1 g kg<sup>-1</sup> for legume N concentration, and 28.6 kg ha<sup>-1</sup> for legume N stock (Table 1). Legume N stock corresponded to 39.3, 34.9, 24.7, and 37.7% of the total N stock in the GRASS+LEGUME pasture in summer, fall, winter, and spring, respectively. The  $\delta^{15}\text{N}\text{‰}$  of the forage peanut ranged from 1.31 in summer and winter to 1.64 in spring (Table 1). Biological N<sub>2</sub> fixation in the GRASS+LEGUME pasture was greatest in summer (Table 1). Annually, forage peanut provided 94 kg N ha<sup>-1</sup> through biological N fixation.

**Table 1**

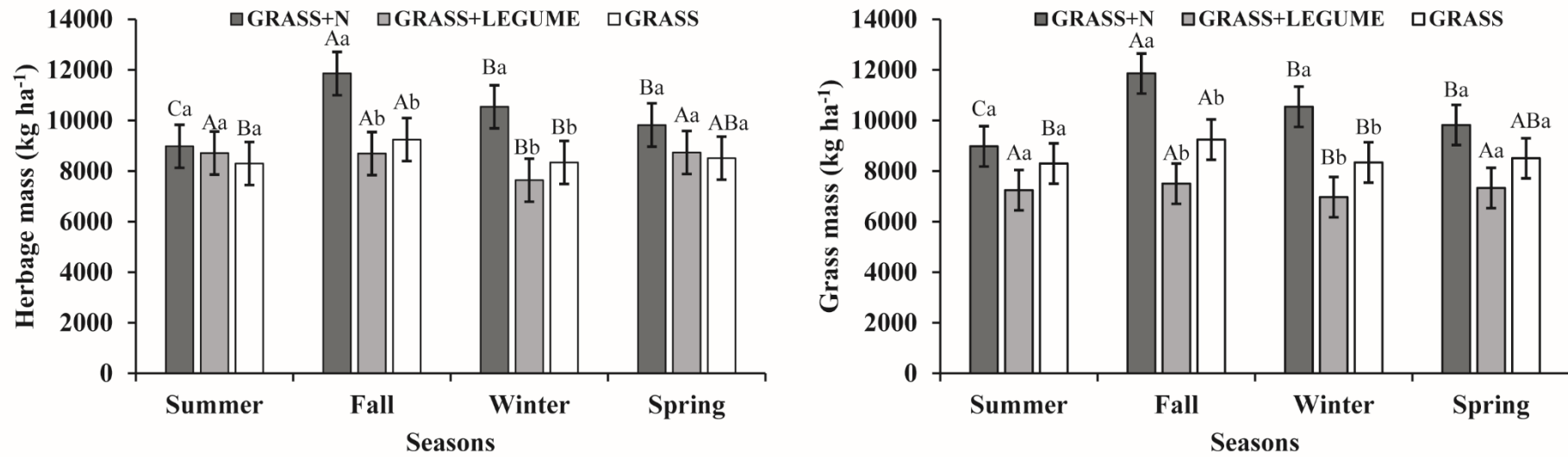
Legume characteristics on Marandu palisadegrass and forage peanut mixed pastures during the seasons of the experimental period.

Item	Seasons				SEM <sup>1</sup>
	Summer	Fall	Winter	Spring	
Legume mass, kg ha <sup>-1</sup>	1470	1190	680	1400	178
Legume N concentration, g kg <sup>-1</sup>	24.8	24.0	21.9	25.7	0.8
Legume N stock, kg ha <sup>-1</sup>	36.6	27.9	15.1	34.7	4.9
$\delta^{15}\text{N}\text{‰}$	1.31	1.22	1.31	1.64	0.05
Ndfa <sup>2</sup> , %	45.0	46.6	45.1	39.1	1.0
BNF, kg ha <sup>-1</sup>	30.3	23.5	15.7	24.7	3.0

\*The mean value of  $\delta^{15}\text{N}$  from the soil obtained from non-fixing plants was 2.34‰;

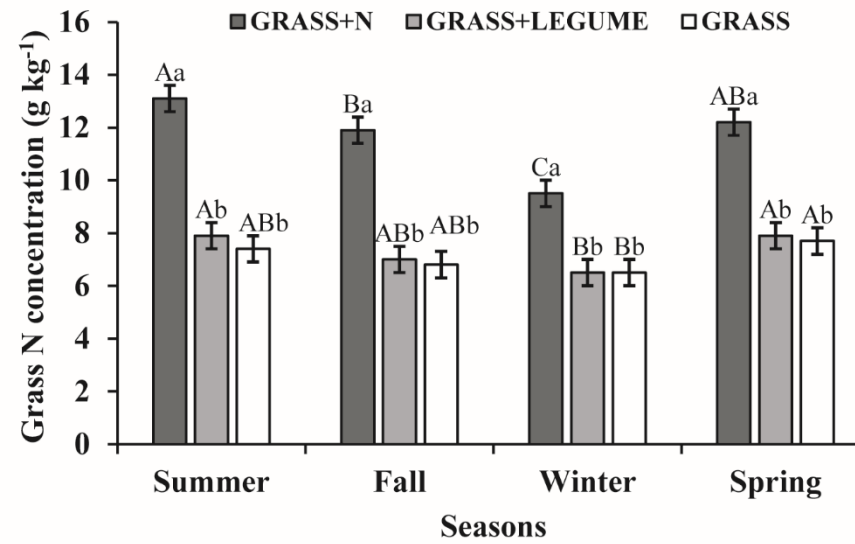
<sup>1</sup>Standard error of the means;

<sup>2</sup>Ndfa is the N derived from the atmosphere; BNF is biological N fixation.

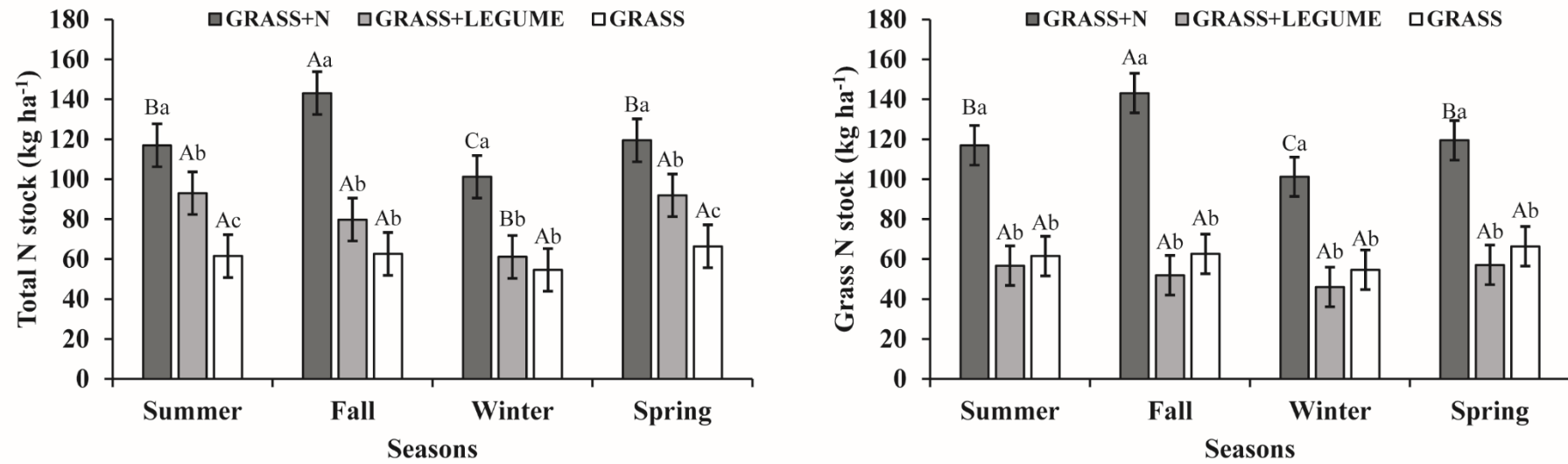


**Fig. 3.** Herbage mass (left) and grass mass (right) of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Errors bars represent  $\pm$  standard errors of the means.





**Fig. 4.** Grass N concentration of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Errors bars represent  $\pm$  standard errors of the means.



**Fig. 5.** Total N stock on herbage mass (left) and grass N stock on grass mass (right) of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period. Errors bars represent  $\pm$  standard errors of the means.

### 3.2 Litter dynamics

Total existing litter was greatest in the GRASS pasture ( $p = 0.005$ ; Table 2). Values of existing litter in the GRASS pasture were 30.2 and 28.7% greater than in the GRASS+LEGUME and GRASS+N pastures. Greatest values of existing and existing grass litters were recorded during winter and spring ( $p < 0.001$ ). In the GRASS+LEGUME pasture, the percentage of legume in the existing litter was 24.9%, and corresponded to 528 kg ha<sup>-1</sup> (Table 2). The  $\delta^{13}\text{C}$  abundance in the existing litter in GRASS+LEGUME pasture was of -14.40‰ compared to -10.40‰ for pure Marandou palisadegrass litter and -27.00‰ for forage peanut litter (Table 2). Fall and winter showed the greatest legume existing litter and the most negative  $\delta^{13}\text{C}$  in the existing litter. Greatest existing litter N concentration occurred in the GRASS+N pasture in spring ( $p < 0.001$  and  $p = 0.007$ , respectively; Table 2). The existing litter N was greatest in the GRASS+N and GRASS pastures ( $p = 0.009$ ; Table 2). The GRASS+N and GRASS pastures had 40.8 and 31.4% greater existing litter N values than the GRASS+LEGUME pasture, respectively. Winter and spring showed the greatest values of existing litter N ( $p < 0.001$ ).

The litter deposition rate was greatest in the GRASS pasture ( $p = 0.005$ ; Table 3). This treatment had deposition rates 24.7 and 21.5% greater than the GRASS+N and GRASS+LEGUME pastures. Similar response occurred for the grass deposition rate, with greatest values recorded in the GRASS pasture ( $p < 0.001$ ; Table 3). Throughout the year, greatest values of deposition and grass deposition rates were recorded during winter ( $p = 0.003$  and  $p = 0.005$ , respectively; Table 3). In the GRASS+LEGUME pasture, the mean proportion of forage peanut in the deposited litter was 20.5% amounting to 13.6 kg OM ha<sup>-1</sup> d<sup>-1</sup> (Table 3). The greatest rate of deposition of legume litter were found to spring. The  $\delta^{13}\text{C}$  abundance in the deposited litter in GRASS+LEGUME pasture was -13.89‰ and more negative in the fall (Table 3). The litter decomposition rate was greater, and half-life time was lower in the GRASS+LEGUME and GRASS+N pastures than in the GRASS pasture ( $p = 0.079$  and  $p = 0.050$ , respectively; Table 3). Faster decomposition rate and lower half-life time occurred during spring ( $p < 0.001$  and  $p = 0.008$ , respectively; Table 3). Nitrogen concentration deposited in grass litter was greatest in the GRASS+N pasture ( $p < 0.001$ ; Table 3). There were no differences between seasons for the N concentration deposited in the grass litter ( $p = 0.894$ ). In the GRASS+LEGUME pasture, N concentration deposited in legume litter was 21.4 g kg<sup>-1</sup>, and greatest values were recorded during summer (Table 3). The GRASS+N and GRASS+LEGUME pastures had the greatest amounts of N deposited in litter ( $p = 0.004$ ; Table 3). For N

deposited in grass litter, greatest values were recorded in the GRASS+N pasture ( $p < 0.001$ ; Table 3). Winter and spring had greater deposited litter N and grass deposited litter N than summer and fall ( $p = 0.058$  and  $p = 0.018$ , respectively). In the GRASS+LEGUME pasture, legume deposited litter N corresponded to 47.8% of deposited litter N (Table 3). The greatest legume deposited litter N occurred in spring.

**Table 2**

Balance of existing litter of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Item	Pasture types (PT)			Seasons (S)				SEM <sup>1</sup>	P-value		
	GRASS+N	GRASS+LEGUME	GRASS	Summer	Fall	Winter	Spring		PT	S	PT*S
Existing litter, kg OM <sup>2</sup> ha <sup>-1</sup>	2162b	2116b	3033a	2077B	2288B	2794A	2588A	336	0.005	<0.001	0.920
Grass existing litter, kg OM ha <sup>-1</sup>	2162b	1588c	3033a	1962B	2084B	2560A	2439A	350	<0.001	<0.001	0.586
Legume existing litter, kg OM ha <sup>-1</sup>	-	528	-	346	613	704	448	71	-	-	-
$\delta^{13}\text{C}$ existing litter in GRASS+LEGUME pasture, ‰	-	-14.40	-	-13.70	-15.10	-15.00	-13.90	0.28	-	-	-
Existing litter N concentration, g kg <sup>-1</sup>	14.4a	8.7b	8.8b	10.3B	10.6B	10.3B	11.4A	0.6	<0.001	0.007	0.379
Existing litter N, kg N ha <sup>-1</sup>	31.3a	18.5b	27.0a	21.1B	23.8B	28.4A	29.2A	4.5	0.009	<0.001	0.463

<sup>a-d</sup>Least squares mean within a row with different lowercase, and uppercase letters differ at  $p \leq .10$ ;

<sup>1</sup>Standard error of the means;

<sup>2</sup>Organic matter.

**Table 3**

Balance of deposited litter, decomposition, and nitrogen cycling of Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Item	Pasture types (PT)			Seasons (S)				SEM <sup>1</sup>	<i>P</i> -value		
	GRASS+N	GRASS+LEGUME	GRASS	Summer	Fall	Winter	Spring		PT	S	PT*S
Deposition rate, kg OM <sup>2</sup> ha <sup>-1</sup> d <sup>-1</sup>	63.5b	66.2b	84.3a	66.6B	67.5B	81.2A	70.1B	9.2	0.005	0.003	0.986
Grass deposition rate, kg OM ha <sup>-1</sup> d <sup>-1</sup>	63.5b	51.6c	84.3a	62.6B	62.9B	75.3A	65.1B	9.1	<0.001	0.005	0.940
Legume deposition rate, kg OM ha <sup>-1</sup> d <sup>-1</sup>	-	13.6	-	11.6	13.9	13.8	15.0	0.4	-	-	-
δ <sup>13</sup> C deposited litter, ‰	-	-13.89	-	-13.71	-14.20	-13.76	-13.80	0.15	-	-	-
<i>k</i> , g OM g <sup>-1</sup> d <sup>-1</sup>	0.023a	0.025a	0.016b	0.024B	0.023B	0.012C	0.028A	0.001	0.079	<0.001	0.202
Half-life time, d	39.6b	34.2b	55.5a	39.9A	37.8A	62.6A	27.5B	5.8	0.050	0.008	0.866
N grass deposited litter, g kg <sup>-1</sup>	10.4a	6.1b	5.9b	7.3	7.5	7.5	7.5	0.4	<0.001	0.894	0.108
N legume deposited litter, g kg <sup>-1</sup>	-	21.4	-	23.0	22.0	19.0	21.0	0.4	-	-	-
Deposited litter N, kg N ha <sup>-1</sup>	82.1a	76.2a	57.1b	66.0C	68.4BC	76.8A	75.9AB	6.8	0.004	0.058	0.398
Grass deposited litter N, kg N ha <sup>-1</sup>	82.1a	39.7c	57.1b	54.3B	55.4B	66.4A	62.4A	6.1	<0.001	0.018	0.601
Legume deposited litter, kg N ha <sup>-1</sup>	-	36.4	-	35.3	38.9	31.5	40.2	2.1	-	-	-

<sup>a-d</sup>Least squares mean within a row with different lowercase, and uppercase letters differ at  $p \leq .10$ ;

<sup>1</sup>Standard error of the means;

<sup>2</sup>Organic matter.

### 3.3 Forage and N intake

There was no difference in total DM intake between PTs, with mean of 6.7 kg heifer<sup>-1</sup> d<sup>-1</sup> ( $p = 0.179$ ; Table 4). However, the GRASS+N pasture showed the greatest grass DM intake ( $p < 0.001$ ; Table 4). The greatest total and grass DM intake occurred in spring ( $p < 0.001$  and  $p < 0.001$ , respectively; Table 4). In the GRASS+LEGUME pasture, the legume DM intake corresponded to 24.6% of the total DM intake and corresponded to 1.6 kg heifer<sup>-1</sup> d<sup>-1</sup> (Table 4). Greater values of legume DM intake were recorded during summer and spring relative to fall and winter (Table 4). The greatest grass N concentration in hand-plucked samples was obtained for the GRASS+N pasture and in the summer and spring ( $p < 0.001$  and  $p < 0.001$ , respectively; Table 4). In the GRASS+LEGUME pasture, legume N concentration in the hand-plucked samples was 28.1 g kg<sup>-1</sup> with greatest values occurred in spring (Table 4). Total and grass N intake were greatest for the GRASS+N pasture ( $p = 0.002$  and  $p < 0.001$ , respectively; Table 4). Greatest valued of total and grass N intake were recorded in spring ( $p < 0.001$  and  $p < 0.001$ , respectively; Table 4). In the GRASS+LEGUME pasture, legume N intake corresponded to 41.4% of the total N intake and corresponded to 48.9 g heifer<sup>-1</sup> d<sup>-1</sup> (Table 4). Greatest values of legume N intake were recorded in spring (Table 4).

### 3.4 Nitrogen excretion

Total N excretion and urinary N excretion were greatest in the GRASS+N pasture ( $p < 0.001$  and  $p < 0.001$ , respectively; Table 5). Throughout the year, greatest values of total and urinary N excretion were recorded in spring ( $p < 0.001$  and  $p < 0.001$ , respectively). The GRASS+N and GRASS+LEGUME pastures had the greatest faecal N excretion, with an increase of 21.3 and 16.7% relative to the GRASS pasture ( $p = 0.039$ ; Table 5). Greatest values of faecal N excretion were recorded during summer and spring ( $p < 0.001$ ; Table 5). The GRASS+N and GRASS+LEGUME pastures had greater N retained than the GRASS pasture, however there was no difference between GRASS+LEGUME and GRASS pastures ( $p = 0.055$ ; Table 5). Greatest values of N retained were recorded in spring ( $p < 0.001$ ).

**Table 4**

Forage and N intake by beef heifers in Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Item	Pasture types (PT)			Seasons (S)				SEM <sup>1</sup>	P-value		
	GRASS+N	GRASS+LEGUME	GRASS	Summer	Fall	Winter	Spring		PT	S	PT*S
Total DM <sup>2</sup> intake, kg heifer <sup>-1</sup> d <sup>-1</sup>	7.4	6.5	6.2	7.0B	5.9C	4.7D	9.3A	0.4	0.179	<0.001	0.862
Grass DM intake, kg heifer <sup>-1</sup> d <sup>-1</sup>	7.4a	4.9c	6.2b	6.4B	5.5C	4.3D	8.5A	0.4	<0.001	<0.001	0.590
Legume DM intake, kg heifer <sup>-1</sup> d <sup>-1</sup>	-	1.6	-	2.0	1.2	1.1	2.3	0.3	-	-	-
Grass N, g kg <sup>-1</sup>	20.0a	13.2b	13.1b	18.3A	14.5B	10.8C	18.3A	0.9	<0.001	<0.001	0.245
Legume N, g kg <sup>-1</sup>	-	28.1	-	30.7	25.2	20.9	35.5	0.6	-	-	-
Total N intake, g heifer <sup>-1</sup> d <sup>-1</sup>	154.0a	118.2b	88.4c	140.5B	92.1C	58.8D	189.4A	12.5	0.002	<0.001	0.668
Grass N intake, g heifer <sup>-1</sup> d <sup>-1</sup>	154.0a	69.3b	88.4b	120.5B	81.4C	50.9D	162.8A	10.5	<0.001	<0.001	0.287
Legume N intake, g heifer <sup>-1</sup> d <sup>-1</sup>	-	48.9	-	60.2	32.1	23.7	79.6	8.1	-	-	-

<sup>a-d</sup>Least squares mean within a row with different lowercase, and uppercase letters differ at  $p \leq .10$ ;

<sup>1</sup>Standard error of the means;

<sup>2</sup>Dry matter.



**Table 5**

Nitrogen excretion by beef heifers in Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

Item	Pasture types (PT)			Seasons (S)				SEM <sup>1</sup>	P-value		
	GRASS+N	GRASS+LEGUME	GRASS	Summer	Fall	Winter	Spring		PT	S	PT*S
Total N excretion, g heifer <sup>-1</sup> d <sup>-1</sup>	119.3a	96.3b	77.9c	103.1B	87.4B	71.9C	126.9A	6.3	<0.001	<0.001	0.602
Faecal N excretion, g heifer <sup>-1</sup> d <sup>-1</sup>	48.7a	46.0a	38.3b	52.0A	33.7B	36.1B	53.5A	3.3	0.039	<0.001	0.959
Urinary N excretion, g heifer <sup>-1</sup> d <sup>-1</sup>	70.6a	50.3b	39.6c	51.1B	53.7B	35.8C	73.4A	6.0	<0.001	<0.001	0.189
N retained, g N heifer <sup>-1</sup> d <sup>-1</sup>	34.7a	21.9ab	10.5b	37.5B	4.7C	-13.1D	62.4A	8.9	0.055	<0.001	0.576

<sup>a-d</sup>Least squares mean within a row with different lowercase, and uppercase letters differ at  $p \leq .10$ ;

<sup>1</sup>Standard error of the means;

### 3.5 Nitrogen cycling and balance

The greatest stocking rate was recorded in the GRASS+N pasture, with an increase of 25.0 and 35.4% relative to the GRASS+LEGUME and GRASS pastures ( $p < 0.001$ ; Table 6). Summer was the season of the year when greatest values were recorded ( $p < 0.001$ ). Greatest total N intake, grass N intake, total N excretion, faecal N excretion, and urinary N excretion occurred in the GRASS+N pasture ( $p < 0.001$ ,  $p < 0.001$ ,  $p < 0.001$ ,  $p = 0.002$ , and  $p < 0.001$ , respectively; Table 6), and during summer ( $p < 0.001$ ,  $p < 0.001$ ,  $p < 0.001$ , and  $p < 0.001$ , respectively). In the GRASS+LEGUME pasture, legume N intake corresponded to 42.4% of the total N intake, which was 18.3 kg N ha<sup>-1</sup> season<sup>-1</sup> (Table 4). Greatest legume N intake was recorded during summer and spring (Table 4). The GRASS+N and GRASS+LEGUME pastures resulted in greater N retained by the animals than for the GRASS pasture, however there was no difference between the GRASS+LEGUME and GRASS pastures ( $p = 0.059$ ; Table 6). Greatest values of N retention occurred in spring and summer ( $p < 0.001$ ).

Using the data from Tables 1, 3, and 6, it is possible to mount flow diagrams for all the important N fluxes in the soil/plant/animal system for all pasture types, GRASS+N (Fig. 6), GRASS+LEGUME (Fig. 7), and GRASS (Fig. 8). The boundaries of the system were regarded as the atmosphere and below the rooting depth of the grass. The N inputs to the systems that were not quantified were N from rainfall or in atmospheric deposition. The N outputs which were not evaluated were the losses of N via leaching, denitrification, or volatilization of ammonia. In the GRASS+N pasture (Fig. 6) and GRASS+LEGUME pasture (Fig. 7), there was a positive overall change of system N of 13 and 18 kg N ha<sup>-1</sup> year<sup>-1</sup>, respectively. However, at GRASS pasture (Fig. 8), there was an overall negative change in system N of -41 kg N ha<sup>-1</sup> year<sup>-1</sup>.

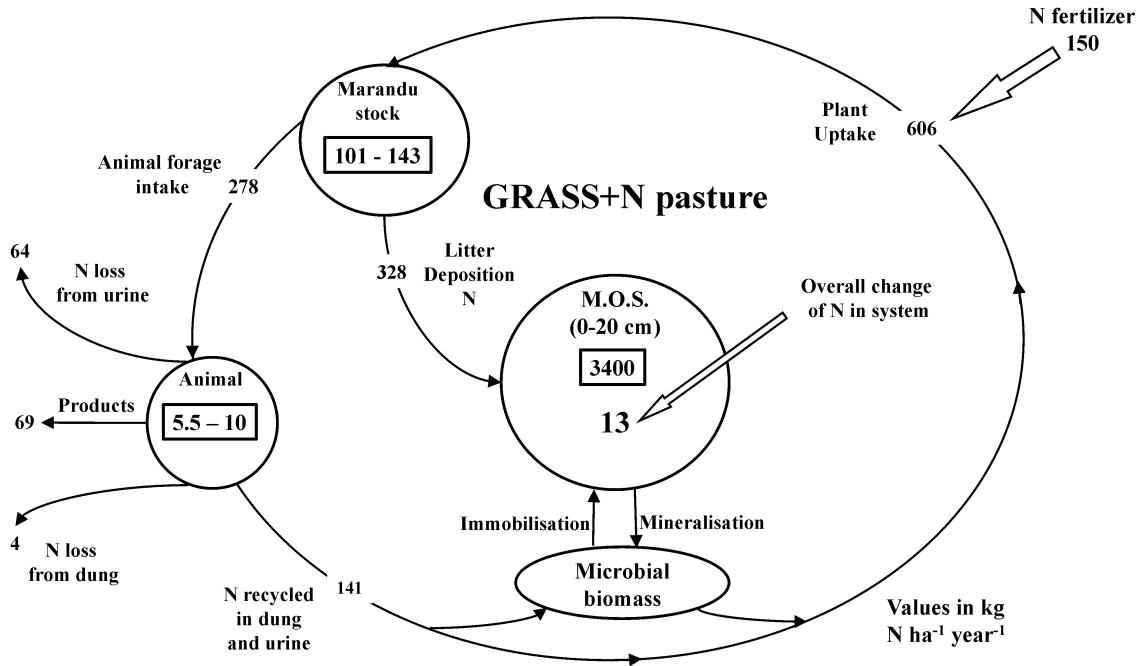
**Table 6**

Nitrogen cycling balance of beef heifers in Marandu palisadegrass pastures with N application or not, or mixed with forage peanut during the seasons of the experimental period.

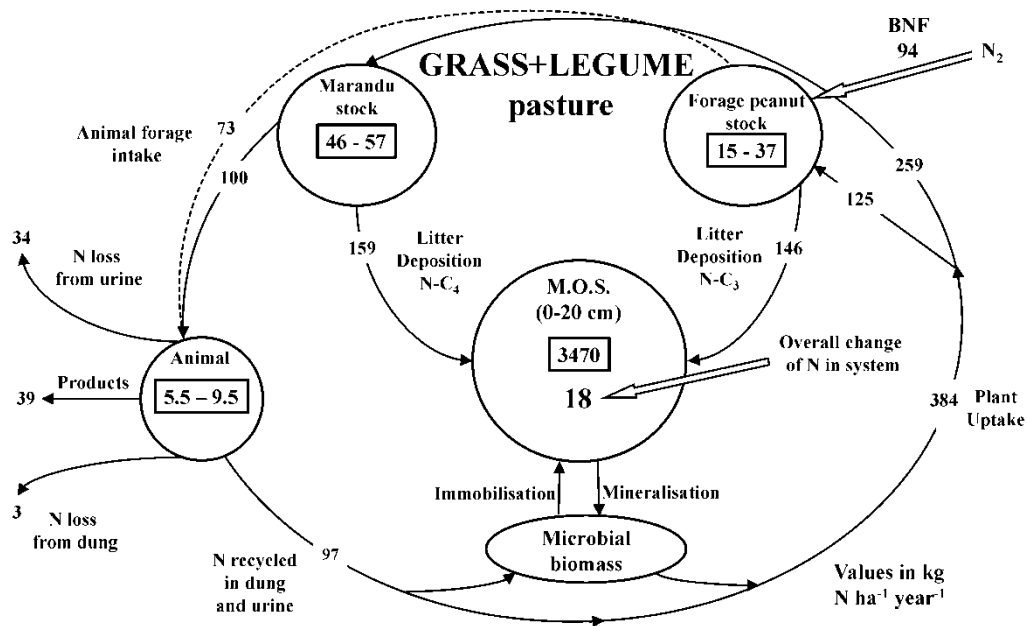
Item	Pasture types (PT)			Seasons (S)				SEM <sup>1</sup>	P-value		
	GRASS+N	GRASS+LEGUME	GRASS	Summer	Fall	Winter	Spring		PT	S	PT*S
Stocking rate, animals ha <sup>-1</sup>	4.8a	3.6b	3.1b	6.1A	4.3B	1.7D	3.3C	0.6	<0.001	<0.001	0.638
Total N intake, kg N ha <sup>-1</sup> season <sup>-1</sup>	69.5a	43.1b	26.7c	75.8A	39.8C	11.8D	58.3B	7.5	<0.001	<0.001	0.106
Grass N intake, kg N ha <sup>-1</sup> season <sup>-1</sup>	69.5a	24.9b	26.7b	65.8A	35.8C	10.5D	49.2B	9.1	<0.001	<0.001	0.180
Legume N intake, kg N ha <sup>-1</sup> season <sup>-1</sup>	-	18.3	-	10.0	3.9	1.3	9.1	2.5	-	-	-
Total N excretion, kg N ha <sup>-1</sup> season <sup>-1</sup>	52.2a	33.4b	22.0c	57.6A	34.3B	12.2C	40.0B	5.3	<0.001	<0.001	0.117
Faecal N excretion, kg N ha <sup>-1</sup> season <sup>-1</sup>	21.7a	16.5b	11.2b	28.5A	13.2B	7.1C	16.8B	3.0	0.002	<0.001	0.630
Urinary N excretion, kg N ha <sup>-1</sup> season <sup>-1</sup>	32.0a	16.9b	11.5c	29.0A	23.2B	5.1C	23.2B	2.4	<0.001	<0.001	0.244
N retained, kg N ha <sup>-1</sup> season <sup>-1</sup>	17.2a	9.7ab	4.7b	18.2A	5.5B	-0.3B	18.3A	5.8	0.059	<0.001	0.465

<sup>a-d</sup>Least squares mean within a row with different lowercase, and uppercase letters differ at  $p \leq .10$ ;

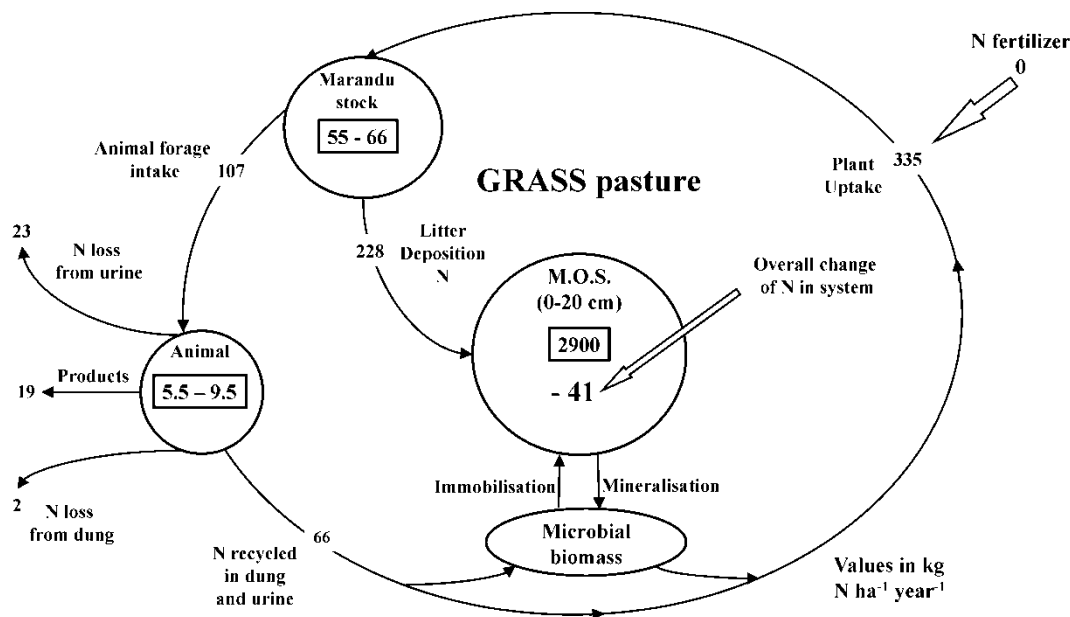
<sup>1</sup>Standard error of the means.



**Fig. 6.** Schematic of the nitrogen annual cycle in a Marandu palisadegrass pasture fertilised with 150 kg N ha<sup>-1</sup> and continuously stocked by Nellore heifers.



**Fig. 7.** Schematic of the nitrogen annual cycle in a mixed Marandu palisadegrass and forage peanut pasture continuously stocked by Nellore heifers.



**Fig. 8.** Schematic of the nitrogen annual cycle in a Marandu palisadegrass pasture without N fertiliser application continuously stocked by Nellore heifers.

#### 4. Discussion

The experimental area is within a region with a Cw climate type (Köppen-Geiger climate classification), which are found in several countries worldwide (Peel *et al.*, 2007). Regions with this climate type have well-defined rainy and dry seasons, which radically alter forage growth rates. Winter is a dry season in regions with Cw climate type with lower rainfall and temperatures (Fig. 1). Seasons markedly affected N cycling for the treatments since the greatest nutrient fluxes occurred during the rainy season (spring, summer, and fall). In winter, litter deposition rate was greatest (an increase of 18.0, 16.9, and 13.7% compared to summer, fall, and spring, respectively; Table 3). Thus, owing to a decrease in rainfall and temperature during the dry season (winter), there was probably an increase in the senescence rate (Fagundes *et al.*, 2006). However, even with the greatest litter deposition rate during the dry season, N cycling decreased due to the lower litter decomposition rate (Table 3), which resulted in increased values of existing litter at that time of the year (Table 2). Concerning livestock excretion, just 8.5% of excreta N was recycled during the dry season (Table 6). Favourable temperature and rainfall conditions in the spring, summer, and fall promote faster plant growth, which demands greater stocking rates to maintain management target and increases N cycling (Rezende *et al.*, 1999; Boddey *et al.*, 2004; Gomes *et al.*, 2020). Furthermore, microbial activity

in the soil is related to temperature and water availability, affecting a litter decomposition as a result (Kohmann *et al.*, 2018).

Nitrogen stored in herbage and grass mass was greatest in the GRASS+N pasture (Fig. 5) due to greater values of herbage mass and nitrogen concentration (Fig. 3 and 4). Nitrogen application has an impact on tiller population density due to faster generation of new leaves from several axillary buds, which may result in new tillers and increase tillers population density, with a positive impact on herbage mass (Lafarge, 2006; Paiva *et al.*, 2012; Paiva *et al.*, 2015). Furthermore, the impact of N application on the increase of N concentration in the herbage mass is associated with the increases of the non-protein N forms (nitrates, amides, amino acids) and small molecules such as peptides (Johnson *et al.*, 2001). During summer and spring, the GRASS+LEGUME pasture showed intermediate values of N stored in the herbage mass (Fig. 5). However, the grass N stock was similar for the GRASS+LEGUME and GRASS pastures due to their similar grass mass and N concentration (Figs. 3 and 4). Forage peanut increased total N stock in the GRASS+LEGUME pasture since this specie had the greatest N concentration. Thus, even though forage peanut was able to fix 94 kg ha<sup>-1</sup> of N, transfer of this N to grass was likely slower, since it is dependent on the biological process of mineralization (i.e., litter decomposition) or transfer from livestock excreta. This would explain the similar grass N concentration on both pastures (Fig. 4).

Nitrogen input may affect litter dynamics (Sollenberger *et al.*, 2019). The GRASS pasture had the greatest pool of existing litter (Table 2). The amount of existing litter in the pasture is the net result between litter deposition and decomposition rate (Dubeux Jr. *et al.*, 2006a; Dubeux Jr. *et al.*, 2006b; Gomes *et al.*, 2020). Thus, the greatest pool of existing litter in the GRASS pasture occurred because of the greater deposition rate and lower decomposition rate, as well as the longer litter half-life time relative to GRASS+N and GRASS+LEGUME pastures (Table 3). Litter deposition rate in pastures is linked to harvest efficiency by animals and senescence rate of plant tissues (Boddey *et al.*, 2004; Apolinário *et al.*, 2013). Nitrogen input in pastoral systems increases defoliation frequency and intensity of tillers when managing the canopy under similar target height, resulting in greater harvested forage and less senescence (Sbrissia *et al.*, 2018). This is the likely reason for the lower litter deposition rate in the GRASS+N and GRASS+LEGUME pastures (Table 3). Thus, N input may enhance harvest efficiency due to enhanced pasture growth rate and consequent necessary increase in stocking rate to maintain the management target (Homem *et al.*, 2021a). The GRASS pasture most likely

had the smallest harvest efficiency of all treatments, which contributed to the increased litter deposition.

The GRASS+N and GRASS+LEGUME pastures had the greatest litter decomposition rate (Table 3). Litter decomposition rate and litter half-life are related to litter quality (Dubeux Jr. *et al.*, 2006a; Dubeux Jr. *et al.*, 2006b; Kohmann *et al.*, 2018; Gomes *et al.*, 2020). In this study, litter decomposition rate increased with increasing N application, a result in line with other studies that demonstrated the positive effect of N addition on litter decomposition (Dubeux Jr. *et al.*, 2006a; Liu *et al.*, 2011; Kohmann *et al.*, 2018). This is probably because of increased decomposition of cellulose and other soluble compounds (Berg and Matzner, 1997). Litter chemical composition is a major factor controlling factor in litter decomposition and nutrient release (Liu *et al.*, 2011). Critical characteristics of litter quality include its physical properties and chemical composition, especially concentrations and ratios of N, P, C, lignin, and polyphenols (Thomas and Asakawa, 1993). Nitrogen application promoted a 70% increase in N concentration in the deposited grass litter relative to the GRASS pasture (Table 3). Initial N concentration in leaf litter greater than 10 g kg<sup>-1</sup> has little or no net N immobilization in the soil, releasing N to the system. Another important point, the increase of N concentration in the litter in the GRASS+N pasture may have caused reduction in the C/N ratio and probably also the lignin/N ratio of the deposited litter (Dubeux Jr. *et al.*, 2006b; Liu *et al.*, 2011). Litter N mineralization predominates when C/N ratio is <20, whereas C/N ratio >30 favours immobilization (Wolf and Wagner, 2005).

In the GRASS+LEGUME pasture, the greater decomposition rate and lower litter half-life were associated with the integration of the legume, as long as N concentration in grass deposited litter was similar between the GRASS+LEGUME and GRASS pastures (Table 3). Nitrogen deposited in legume litter corresponded to 50% of N cycled via litter in the GRASS+LEGUME pasture. Legumes have greater nutritional value than grasses (Gomes *et al.*, 2018), which directly interfere with the quality of the deposited litter (Gomes *et al.*, 2020). Thus, legume litter has greater N concentration (Table 3), lesser neutral and acid detergent fibre concentrations, and lower C/N ratio (Kohmann *et al.*, 2018). Another potential benefit of including legumes with grasses in mixed pastures is the greater microbial diversity in mixed-species litter and greater abundance and variety of enzymes to mineralize litter material, an attribute that may significantly affect the rate and the extent of litter decomposition (Chapman *et al.*, 2013; Chuan *et al.*, 2020). Thus, even with the smaller litter deposition rate, the

GRASS+N and GRASS+LEGUME pastures had the greatest N cycling via deposited litter per hectare.

Total DM intake was similar between all pasture types (Table 4). In forage diets, the NDF concentration is one of the determinants of forage intake (Baumont *et al.*, 2004). All pasture types had NDF intake close to 1.3% of BW/d (Homem *et al.*, 2021a). This probably indicates that non-nutritional factors did not affect forage intake. Canopy structural characteristics of tropical forages are relatively more important than nutritional factors in terms of regulation of forage intake (Poppi *et al.*, 1987). Thus, pastures with similar management targets, as in the current experiment, would have the same forage intake due to similar canopy structure (Forbes, 1988). However, differences between pasture types were obtained in terms of total N intake (Table 4). The N intake by heifers was the result of balancing forage nutritive value and forage intake. As previously mentioned, there were no differences between pasture types for forage intake (Table 4). Thus, the increased total N intake in the GRASS+N and GRASS+LEGUME pastures was consequence of the greater nutritive value of diet. As previously mentioned, N application in the GRASS+N pasture increases non-protein N in the grass (nitrates, amides, amino acids) and small molecules such as peptides, improving diet nutritive value (Johnson *et al.*, 2001). In the GRASS+LEGUME pasture, forage peanut had the greatest N concentration (Table 3). Thus, the presence of legume in the diet of grazing animals increased the nutritive value of the ingested forage.

The greater N intake by heifers in the GRASS+N and GRASS+LEGUME pastures resulted in increased N excretion, since adult beef cattle retain less than 15-20% of the ingested N (Detmann *et al.*, 2014). Thus, there was an increase of 34.7 and 19.1% in total N excretion for the GRASS+N and GRASS+LEGUME pastures compared to the GRASS pasture (Table 5). In this respect, N excretion may be greater if protein ingestion greater than the required level to balance the energy component of the diet, resulting in increased ratio of urinary-N to faecal-N in the excreta (Scholefield *et al.*, 1991; Detmann *et al.*, 2014). Urinary N excretion is mainly in the form of urea, which hydrolyses rapidly after reaching the soil and liberates  $\text{NH}_4^+$  in the affected area at levels comparable to high fertiliser N application rates ( $>300 \text{ kg N ha}^{-1}$ ; Lessa *et al.*, 2014). On the other hand, faecal N excretion is mainly in organic forms that resisted degradation in the animal digestive tract and is mostly unavailable in the short term for cattle feeding on pastures (MacDiarmid and Watkin, 1972; Boddey *et al.*, 2020).

Nitrogen application on the GRASS+N resulted in an increase in stocking rate of 33.3 and 54.8% relative to GRASS+LEGUME and GRASS pasture, respectively (Table 6). The use



of N fertiliser on pastures increases the morphogenetic rhythm, tiller population density, and tiller appearance rate, which culminates in greater forage accumulation rate (Lafarge, 2006; Paiva *et al.*, 2012; Paiva *et al.*, 2015). Thus, for canopies under similar management targets, greater forage accumulation results in the enhancement of the herbage mass (Fig. 3) and, ultimately, the stocking rate (Table 6). The greater stocking rate in the GRASS+N pasture led to an increase in total N intake (Table 6). On the other hand, even without differences in stocking rate between the GRASS+LEGUME and GRASS pastures, the greater N intake in the first resulted in increased total N intake in GRASS+LEGUME pasture (Table 6). Therefore, N application or legume integration increased the N use efficiency (NUE; kg N ingested kg N in plant uptake<sup>-1</sup>) by heifers, which were 45.9, 45.0, and 31.9% for the GRASS+N, GRASS+LEGUME, and GRASS pastures, respectively (Figs. 6, 7, and 8). As previously explained, even with similar forage intake between pasture types, N input enhanced harvest efficiency and NUE due to the increase in pasture growth rate and consequent necessary increase in stocking rate. Greater harvest efficiency and NUE on pastures may allow for increments in animal production and productivity in pastoral system.

The differences in harvest efficiency between pasture types impacted changes in routes of N cycling. Nitrogen cycling via litter corresponded to 54.1, 63.8, and 68.1% of the total N cycling in the GRASS+N, GRASS+LEGUME, and GRASS pastures, respectively. Therefore, greater harvest efficiency, such as observed in GRASS+N pasture, led to proportionally lesser N cycling via litter input and greater N cycling via excreta, compared to GRASS+LEGUME and GRASS pastures (Figs. 6, 7, and 8). Differences in routes of N cycling affect N losses, mainly N cycling via excreta (Boddey *et al.*, 2004; Dubeux Jr. *et al.*, 2007). Nitrogen deposited in the soil as urine is much more susceptible to losses via ammonia volatilization, leaching, or denitrification (Lessa *et al.*, 2014). Losses of N to the atmosphere or leached below the rooting depth of the grasses from cattle urine and faeces may be approximately 50 and 5% of the deposited N, respectively. Furthermore, N cycling via faeces and urine is generally deposited to a greater extent in limited areas of pasture (Dubeux Jr. *et al.*, 2007). Studies have shown that across a range of environments and grazing methods have shown nutrient accumulation occurs near shade and water points (Dubeux Jr. *et al.*, 2007). On the other hand, the distribution of litter is more uniform. Furthermore, as pastures have perennial dense rooting system, N slowly released from decomposing litter is almost certainly recycled more efficiently (Haynes and Williams, 1993).

The magnitudes of the total N change in the systems (Figs. 6, 7, and 8) are based on the supposition that N losses from N mineralised from decaying plant litter or roots were negligible (or approximately balanced by small inputs from rainfall and biological N<sub>2</sub> fixation associated with *Brachiaria*; Boddey *et al.*, 2004). Therefore, the pasture with no N input, such as the GRASS pasture treatment in the current experiment, had the lowest forage nutritive value (Homem *et al.*, 2021a), the lowest animal production (Homem *et al.*, 2021b), and the largest negative impact on soil N (Fig. 8). In addition to the low economic viability of this pasture type, over the years, the system will reach more severe degrees of pasture degradation. On the other hand, N input via N fertiliser application or legume integration improved conservation of soil N reserves, enhancing systems' potential for soil carbon input (Figs. 6 and 7). Thus, N input is a way to enhance the income of grass-fed beef operations in Brazil (Homem *et al.*, 2021b) beyond increase soil fertility and pasture sustainability through N cycling.

## 5. Conclusions

Over the years, pastures with no N input could reach severe degrees of pasture degradation. Nitrogen application or the integration of forage peanut in a grass pasture increased conservation of soil N reserves by enhancing the total N recycled through litter and livestock excretion. Thus, N application or introduction of a legume on the cycling of N is essential for achieving productive and sustainable grassland systems.

The efficiency of N utilization by heifers was greater with N input on pastures subjected to similar targets of continuous stocking management. However, greater harvest efficiency in a pasture with N application led to proportionally lesser N cycling via litter input and greater N cycling via livestock excreta.

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