OUTCOMES AND UNDERLYING MECHANISMS OF GRASSLAND RESPONSES TO LEGUME INTRODUCTION AND MANIPULATION OF DEFOLIATION INTENSITY

By

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To Maria Elena and my family

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Bahiagrass (*Paspalum notatum* Flügge) is the most important forage species in Florida grazing systems, adapted to high grazing intensity, low soil fertility, and continuous stocking. However, it has production, economic and environmental limitations associated with inadequate soil nitrogen (N) and poor nutritive value. Alternatives to overcome these limitations include introducing rhizoma peanut (*Arachis glabrata* Benth.), a C3-perennial legume, into bahiagrass, and improving grazing management. In this context, the objectives were to (1) identify legume proportion maximizing plant and animal responses, and (2) study potential effects of short-term increases in defoliation intensity on bahiagrass leaf characteristics and digestibility. For two years, a grazing trial assessed N dynamics, radiation use efficiency (RUE), biomass accumulation and animal responses along a rhizoma peanut proportion gradient in rhizoma peanut-bahiagrass mixtures (0-60% legume; Objective 1); and a clipping trial quantified seasonal changes in bahiagrass leaf tissue proportions and fiber concentration in response to different defoliation intensities (Objective 2). Overall, moderate legume proportions (~30-40%) optimized N dynamics and plant and animal responses. Within this optimal range, atmospheric-derived N yield was 27-50% greater than in legume-dominated sites (~60% legume), while soil-derived N yield was 225-285% and 73-134% greater than a grass monoculture and legume-dominated sites, respectively. Consequently, RUE and biomass accumulation increased quadratically with increasing legume proportion up to ~40% legume, and RUE was 86-110% greater at this proportion than for legume-dominated sites. Responses suggest RUE is N-limited below 30% legume, and physiologically-limited above 30% legume, where C4-grasses are replaced by C3-legumes. At 40% legume, cattle actively selected legume (60% diet vs. 40% pasture), maximizing gain per animal (604 vs. 290 g day^{-1}) and gain per hectare (350 vs. 154 kg LW ha^{-1}) compared with the bahiagrass monoculture. In the clipping trial, longer bahiagrass leaves, in response to lower defoliation intensity, increased the proportion of non-degraded vascular bundle and sclerenchyma tissues, which increased cell wall concentration and decreased digestibility. Legume proportions of ~30-40% represent a reasonable target for optimizing plant and animal responses, while short-term increases in defoliation intensity during late summer may overcome increases in non-degraded tissues, the presence of which reduces nutritive value and animal production.

CHAPTER 1
OVERVIEW

Grasslands cover about 40% of the global terrestrial area (O’Mara, 2012; Bardgett et al., 2021) and deliver many ecosystem services (Sollenberger et al., 2019). Approximately half of the global grasslands lie within the tropics and subtropics (between 30°S and 30°N), where one-third of global beef is produced. Most Florida grasslands lie within this range and include native and perennial planted grasslands, occupying approximately 2.1 M ha and feeding 1.6 M beef cattle per year (USDA, 2024). Native grasslands in the state, i.e., rangelands, are dominated by C4 native bunch grass species (Boughton et al., 2018) and account for about 30% of Florida’s grasslands. The remaining 70% of the area is planted primarily to introduced perennial C4 grass species, including bahiagrass (*Paspalum notatum* Flügge), bermudagrass (*Cynodon dactylon* [L.] Pers), limpograss (*Hemarthria altissima* [Poir.] Stapf & C.E. Hubb.) and stargrass (*Cynodon nlemfuensis* Vanderyst).

Bahiagrass has become the most important grass species in Florida after its introduction from South America in the early 1900s, and it has been widely used in grazing systems (Gates et al., 2004). Some of the reasons explaining its relevance in the state are its adaptation to subtropical climates, specifically to the southeastern United States, and its tolerance of high grazing intensity, low soil fertility, and continuous stocking (Sollenberger et al., 1988), the traditional management of many beef operations. Despite these benefits and the potential of bahiagrass, it has production, economic, and environmental limitations compared with other species.

The production limitations are relatively low biomass accumulation and animal performance of cattle grazing bahiagrass pastures due in part to nitrogen (N) limitations to plant growth and decreased nutritive value during the summer months (Stewart et al., 2007; Kohmann et al., 2022). Consequently, economic and environmental impacts arise because bahiagrass requires inorganic N fertilizer to overcome the N limitation (Stewart et al., 2007). This practice increases the cost of production and the potential for negative environmental effects (Herridge et al., 2008). Therefore, overcoming these limitations through adopting different management practices will have positive production, economic and environmental impacts in the region.

One possible solution for addressing the negative impacts of N fertilization is the incorporation of rhizoma peanut (*Arachis glabrata* Benth.), a perennial legume that is adapted to moderately to well-drained soils in Florida (Mullenix et al., 2014; Castillo et al., 2015; Jaramillo et al., 2018). Its introduction into grass monoculture pastures can increase N cycling in the system (Kohmann et al., 2018; Garcia et al., 2021), biomass production and nutritive value (Kohmann et al., 2022), and animal performance and animal gain (Sollenberger et al., 1988; Williams et al., 1991; Jaramillo et al., 2021). However, the adoption of rhizoma peanut-bahiagrass mixtures in grazing systems is constrained by high establishment costs and complex grazing management during the legume establishment period (Aryal et al., 2021).

One alternative to reduce establishment costs is to introduce the legume in strips into existing bahiagrass (Castillo et al., 2014; Mullenix et al., 2014), reducing the area planted to legume. Rhizoma peanut is propagated vegetatively by rhizomes, and over time it can spread into surrounding grass and increase its proportion in the pasture (Castillo et al., 2014). The scientific evidence is conclusive about the benefits of introducing legumes into only-grass systems, and particularly introducing rhizoma peanut into bahiagrass, but the optimum proportion of this legume in bahiagrass, and more generally of legumes in warm-climate grasslands, remains to be determined (Sollenberger et al., 2019). Empirical evidence indicates that the legume proportion in pastures can affect the biomass production (Kohmann et al., 2022), biological N fixation (BNF) by the legume (e.g., Nyfeler et al., 2011; Suter et al., 2015) and animal performance (e.g., Watson and Whiteman; 1981; Pereira et al., 2020). Therefore, identifying the optimum rhizoma peanut proportion is relevant to maximize productive, economic and environmental outcomes of this warm-climate grassland.

Another potential alternative to overcome limitations of bahiagrass is through grazing management. For example, the lower animal performance, i.e., “summer slump”, of cattle grazing bahiagrass compared with other C4 species [e.g., ‘Mott’ dwarf elephantgrass (*Pennisetum purpureum* Schum.)] has been attributed to abundance of sclerenchyma fibers in bahiagrass leaf tissue and reduced digestibility during the summer (Flores et al., 1993). However, these inherent attributes of bahiagrass leaves associated with rapid growth and maturation during summer may be modified with grazing intensity (Boggiano et al., 2001; Pakiding and Hirata, 2002; Gastal and Lemaire, 2015). These studies show that short-term increases in defoliation intensity may reduce grass leaf length and increase leaf digestibility, without affecting tiller mass and appearance rate (Interrante et al., 2010). Therefore, adopting appropriate and specific grazing management practices could help to overcome productive limitations on bahiagrass pastures.

The objectives of this project were to address gaps in the knowledge of 1) the impact of legume proportion in grass-legume mixtures on plant and animal responses and 2) the effect of short-term increases in defoliation intensity on grass leaf characteristics and digestibility, with the goal to contribute information for the design of more efficient grazing systems in tropical and subtropical environments.

CHAPTER 2
LITERATURE REVIEW

Grassland ecosystems cover approximately 40% of the terrestrial land area (O'Mara, 2012; Bardgett et al., 2021) and are responsible for the delivery of multiple ecosystem services (Sollenberger et al., 2019). These ecosystems provide food; support nutrient cycling, N fixation and pollination; regulate soil organic C, greenhouse gas emissions and water quality; and they provide a range of cultural services (Sollenberger et al., 2019). These biomes are generally composed of grasses, legumes and other forbs, and occasionally woody species (Allen et al., 2011), varying in proportion depending on the environment and management practices (Milchunas et al., 1988).

Grasslands are the source of most cattle feed in many parts of the world (Herrero et al., 2013) and, therefore, are directly responsible for a significant proportion of global beef production (O'Mara, 2012, Boval and Dixon, 2012). However, these ecosystems are threatened by land conversion and degradation (Bardgett et al., 2021). Meanwhile, there is a rising global demand for livestock-derived foods (Komarek et al., 2021), coinciding with a global call to sustain the delivery of other ecosystem services that are vital to climate change mitigation, biodiversity conservation, and food security (Herrero et al., 2013). Within this context, understanding mechanisms and outcomes in response to specific management practices at different spatial and temporal scales is needed to preserve grasslands, maximize resource-use efficiency and returns, pathways toward the sustainable intensification of these ecosystems (Soussana and Lemaire, 2014; Sollenberger et al., 2019).

Tropical Grasslands

A significant proportion of the world's grasslands lie within the tropics and subtropics (30°N and 30°S; Boval and Dixon, 2012). These tropical native and improved perennial grasslands, i.e., warm-climate grasslands, are dominated by C4 grass species, which are better adapted than C3 species to the higher temperatures in the tropics and subtropics (Sollenberger et al., 2020). Additionally, the C4 species are typically more efficient than C3 species in terms of water, radiation, and nutrient use (Sinclair and Muchow, 1999; Wedin, 2004; Stöckle and Kemanian, 2009). Despite these advantages, the C4 species have typically lesser nutritive value than C3 species, associated with anatomical, morphological, and physiological differences (Akin and Burdick, 1975; Wilson et al., 1989; Wilson and Mertens, 1995; Sollenberger et al., 2020). Thus, under similar environmental conditions, the C4 species may produce more biomass per unit of resource used (e.g., radiation, water, N; Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009) but provide herbage with lesser energy concentration than C3 species (Minson, 1990).

The lower inherent energy concentration and nutritive value, defined through dry matter (DM) digestibility and N concentration, result in lesser gain and efficiency of animals grazing pastures dominated by C4 species (Watson and Whiteman, 1981; Pereira et al., 2020). This explains that while approximately half of the global cattle stock is in warm climates, this region accounts for only one third of the global beef produced annually (FAO, 2023). Thus, the current global call to reduce greenhouse gas emissions from the livestock sector (Cusack et al., 2021) while increasing global livestock-derived food production (Komarek et al., 2021) and soil organic carbon sequestration (Bai and Cotrufo, 2022), requires an increase in primary and secondary production in grasslands worldwide, and particularly in warm-climate regions (Herrero and Thornton, 2013). This reinforces the need for identifying practices that increase plant and animal production and resource-use efficiency while maintaining threatened ecosystems and achieving the sustainable intensification of food production systems in tropical environments.

Florida Grasslands

Florida native and planted grasslands are a component of the global tropical and subtropical grasslands, and they are dominated by perennial C4 grass species (Sollenberger et al., 2020). These ecosystems occupy approximately 2.1 M ha of the state, feeding more than 1.6 M beef cattle per year (USDA, 2024). These stock numbers position Florida as having the ninth largest beef cattle census among US states (USDA, 2024) and as one of the major providers of animals for the feedlot component of the beef industry in the Midwest. These data are evidence of the productive, economic and social relevance of Florida native and improved grasslands to the beef sector of the state and US.

Approximately 70% of the 2.1 M ha of Florida grassland are perennial improved pastures (~1.5 M ha) of bahiagrass, bermudagrass, limpograss and stargrass. Among these species, bahiagrass is the most important grass species in Florida. It is widely used owing to its adaptation to the traditional grazing management of grasslands, employing high grazing intensity and continuous stocking (Sollenberger et al., 1988). In general, bahiagrass, bermudagrass, and stargrass concentrate their biomass production between May to October and require N fertilization to sustain production over time (Vendramini, 2010). Nitrogen fertilizer inputs often support greater plant production and stocking rates (Stewart et al., 2007; Jaramillo et al., 2021), but they seldom translate into greater daily gain per animal (Sollenberger et al., 1988; Williams et al., 1991). As a result, the increase in beef production per hectare results mostly from increases in stocking rate but not from increases in individual animal performance (Jaramillo et al., 2021). Additionally, N fertilizer inputs may have negative implications to the environment, driving N losses via leaching and denitrification (Dubeux et al., 2007), while increasing production costs and carbon footprint.

Alternatives to N fertilizer inputs to warm-climate grasslands include, primarily, the introduction of legumes into grass-only grazing systems in combination with improvements in grazing management (Sollenberger et al., 2019; Sollenberger and Dubeux, 2022). For example, studies carried out throughout the state have reported that legumes in mixture with grasses, and particularly rhizoma peanut in mixture with bahiagrass, increased the amount of N cycling in the system (Kohmann et al., 2018; Garcia et al., 2021), biomass production and animal gain (Sollenberger et al., 1987; Williams et al., 1991; Jaramillo et al., 2021; Kohmann et al., 2022). However, one of the limitations of studies assessing the impacts of presence vs. absence of legumes is that they generally do not provide information about the optimum or minimum legume proportion in pastures needed to maximize soil, plant, and animal responses (Sollenberger et al., 2019).

In general, identifying the optimum legume proportion is considered to be important in temperate grasslands (Nyfeler et al., 2009; Nyfeler et al., 2011; Suter et al., 2015), and especially relevant in tropical grasslands, where there is limited availability of legume seed and planting material (Schultze-Kraft et al., 2018), legume establishment can be expensive and complex (Castillo et al., 2014), and legumes typically show poor persistence in competition with vigorous C4 grasses (Sollenberger and Dubeux, 2022). In this regard, the benefits of legumes are often compared with a grass-only condition, but there is evidence that excessive legume proportions might reduce biomass production (Nyfeler et al., 2009; Kohmann et al., 2022) and BNF (Nyfeler et al., 2011) in C4 grass-based systems. This suggests that efforts to achieve a very large legume proportion, which often require intensive and sometimes costly inputs, may not provide the anticipated production and economic benefits. This likewise reinforces the need to identify optimum legume proportions that maximize production, environmental and economic returns. This approach could contribute a way forward toward the management intensification required to achieve greater grassland production with fewer ecosystem disservices, while incentivizing producers to incorporate legumes in a manner leading to greater resource-use efficiency.

In addition to legume introduction, improvements in grazing management can positively impact plant and animal responses in bahiagrass pastures. Animals grazing bahiagrass typically show a summer slump in animal performance during the summer (Stewart et al., 2007), likely associated with inherent attributes of bahiagrass, including sclerenchyma fibers in leaf tissue and reduced digestibility during the summer (Flores et al., 1993; Stewart et al., 2007; Interrante et al., 2009). However, leaf length and tissue composition, and maturation can be modified by grazing intensity (Boggiano et al., 2001; Pakiding and Hirata, 2002; Gastal and Lemaire, 2015). In this context, strategic short-term increases in defoliation intensity may reduce leaf length and size, likely reducing sclerenchyma concentration in leaves and increasing DM digestibility of bahiagrass (Hayes et al., 2023). While this practice may give rise to persistence concerns for some species, Interrante et al. (2010) demonstrated that short-term increases in defoliation intensity did not decrease tiller mass and appearance rate of bahiagrass. Therefore, this suggests that short-term periods of heavy grazing intensity could increase forage digestibility, without compromising stand persistence. This zero-cost strategy for increasing bahiagrass digestibility, however, requires additional investigation.

Further improvements in the understanding of plant and animal responses to practices such as legume introduction and proportion in pasture, and strategic short-term increases in defoliation intensity can provide helpful information for Florida grassland management. Most importantly, understanding the underlying mechanisms can broaden the impacts of these practices to other species and regions, allowing for more generalizable inferences. In this context, the effect of legume introduction and proportion in pasture on plant and animal responses and the effect of strategic short-term increases in defoliation intensity on leaf and canopy attributes will be described following causal relationships.

Legume Proportion in Grass-Legume Systems

Legume introduction affects the soil-plant-animal interaction in different ways, linearly and non-linearly, directly and indirectly, with positive and negative effects (Figure 2-1). In general, the introduction of legumes into grass-only systems, i.e., rhizoma peanut into bahiagrass pastures, first affects the N cycling dynamics within the grassland ecosystem (Kohmann et al., 2018; Garcia et al., 2021). The increases in the amount of N cycling in the system are translated into increases in plant N concentration, and, consequently, in biomass production and the nutritive value of the diet (Kohmann et al., 2022). These changes further affect animal behavior through diet selection, individual animal performance, and gain per area (Jaramillo et al., 2021). Thus, the first overall goal of this dissertation includes assessing the effect of legume proportion in pastures on plant and animal responses. The first specific objective within this overall goal is to study the effect of legume proportion in bahiagrass pastures on BNF and N facilitation between the grass and legume, which are relevant mechanisms for overcoming the N limitation in grasslands. The second specific objective is to assess the effect of legume proportion on canopy attributes and radiation use efficiency, a key metric of ecosystems productivity, which is relevant in grasslands composed by species of different photosynthetic pathway (C3 vs. C4) and functional groups (grass vs. legume). The third specific objective aims to quantify the effect of rhizoma peanut proportion in bahiagrass pastures on animal diet selection and daily weight gain and gain per area.

Biological N Fixation And N Facilitation Of Grass-Legume Mixtures

The co-existence of grass and legume species is relevant for the productivity and sustainability of temperate and tropical grassland ecosystems (Suter et al., 2015; Sollenberger and Dubeux, 2022). The grass-legume co-existence is typically driven by niche partitioning and abiotic and biotic feedback among species (Nyfeler et al., 2009; Barry et al., 2019; Churchill et al., 2024). Niche partitioning occurs when grass and legume species differ in the resources that they use (Barry et al., 2019), reducing the competition among species and, therefore, facilitating their co-existence. In addition, the abiotic and biotic feedback include the benefits associated with greater biodiversity, hydraulic lift, and other factors (Barry et al., 2019).

Nitrogen is the main resource limiting the productivity of grassland ecosystems (Fay et al., 2015). Under N-limited environments, legume species benefit from their symbiosis with bacteria (Figure 2-1-arrow 1 [A1]), acquiring atmospheric N through BNF (Peoples et al., 1995; Carlsson and Huss-Danell, 2003; Jensen et al., 2012; Dubeux et al., 2017). When legumes are mixed with grasses, the latter often benefit from the increase in availability of N fixed by the legume which is transferred to the grass through different belowground (Thilakarathna et al., 2016) and aboveground pathways (Dubeux et al., 2007; García et al., 2021).

In addition, the BNF is stimulated not only when soils have low N content but also by grasses in the mixture (Figure 2-1-A2-3; Nyfeler et al., 2011). Grasses often outcompete legumes for soil N (Mallarino et al., 1990), depleting that resource and, consequently, stimulating the BNF by legumes (Nyfeler et al., 2011). As a result, this positive feedback, where grasses take up N from soil and legumes fix N from the atmosphere, drives an overall increase in N uptake and yield by the mixture compared with the species in monoculture (Nyfeler et al., 2011). When the mixture outperforms the grass and legume components in monoculture in N uptake and fixation it is referred to as N overyielding (Nyfeler et al., 2011; Santos et al., 2018a). Thus, the amount of N cycling in the system increases, having positive benefits on plant and animal responses (Kohmann et al., 2018; Garcia et al., 2021).

In this regard, two types of studies can be distinguished in assessing the effect of legumes on responses such as BNF and N uptake, namely qualitative and quantitative studies. Qualitative studies, assessing only the absence or presence of legume in mixture with grass species, have demonstrated in temperate and tropical environments that the binary mixture of grass and legume increases the N uptake compared with the species in monoculture (Santos et al., 2018a; Churchill et al., 2024; Louarn et al., 2024). However, this type of study does not define optimum legume proportions for maximizing N uptake relative to that of the species in monoculture.

Quantitative studies assessing the effect of temperate legumes (*Trifolium pratense* L. and *T. repens* L.) proportion in mixture with temperate grasses (*Lolium perenne* L., *Dactylis glomerata* L.), have demonstrated that N yield and BNF were maximized with moderate legume proportion in the mixture (~40%; Nyfeler et al., 2011; Suter et al., 2015). Similarly, a study with binary mixtures of *F. arundinacea* and *T. pratense*, *T. repens* and *Lotus corniculatus* L. showed that the proportion of N derived from the atmosphere relative to total legume N decreased quadratically with increasing legume proportion in the mixture (Mallarino et al., 1990). This evidence indicates that at high legume proportion in the mixture in temperate environments, the competition by grasses for soil N decreases, and consequently, the BNF is not stimulated (Fig 1; A3; Mallarino et al., 1990; Nyfeler et al., 2011). However, while studied qualitatively (Santos et al., 2018a; Churchill et al., 2024), the N yield and N dynamics for grass and legume species when legumes occur at varying proportions in the pasture remains little studied in warm climates, with rhizoma peanut and bahiagrass mixtures in Florida environments representing one example.

This information is relevant because N overyielding associated with greater BNF and N uptake from soil increases the N concentration in plants (Figure 2-1-A4-5; Nyfeler et al., 2011; Kohmann et al., 2022). A greater N concentration in plants associated with the presence of the legume has numerous biotic (e.g., biodiversity; Tilman et al., 2001; Fornara and Tilman, 2009) and abiotic effects (e.g., hydraulic lift; Liste and White, 2008; Figure 2-1-A9), including increasing photosynthesis rate (Sinclair and Horie, 1989), and therefore, biomass accumulation and nutritive value (Figure 2-1-A6-7; Nyfeler et al., 2009; Kohmann et al., 2022). As a result, greater biomass production is typically observed in temperate and tropical grass-legume mixtures compared with the species components in monocultures (Nyfeler et al., 2009; Kohmann et al., 2022), without the need for N fertilizer (Carlsson and Huss-Danell, 2003; Herridge et al., 2008).

In this regard, moderate legume proportions have been observed maximizing biomass production in temperate climate grass-legume mixtures (Mallarino and Wedin, 1990; Thomas 1992; Nyfeler et al., 2009; Nyfeler et al., 2011; Lüscher et al., 2014; Helgadóttir et al., 2018). Yet, moderate legume proportions maximizing biomass production of grass-legume mixtures has been implied only from theoretical models in warm climates (Thomas, 1995). Hence, empirical evidence is needed from field experiments studying N dynamics and biomass accumulation in response to legume proportion in these environments (Sollenberger et al., 2019). Particularly for N-limited grasslands in Florida, understanding N facilitation and BNF of bahiagrass and rhizoma peanut mixtures is relevant for designing more efficient grazing systems, increasing productivity while mitigating negative environmental impacts associated with N fertilization practices (Carlsson and Huss-Danell, 2003; Herridge et al., 2008; Lüscher et al., 2014; Fay et al., 2015).

Radiation Use Efficiency Of Grass-Legume Mixtures

The biomass productivity of grassland ecosystems is affected by interactions occurring at soil-plant-animal-environment levels (Moore et al., 2004). The 'potential' productivity results from inherent attributes of species in interaction with different environmental resources, including solar radiation, temperature, and CO_2 concentration (Sinclair and Horie, 1989; Sinclair and Muchow, 1999; Moore et al., 2004). This potential productivity is further limited by water and nutrient availability, defining the 'actual' primary, i.e., biomass, productivity of grasslands. Within the context of Florida grasslands, and, in particular, for bahiagrass and rhizoma peanut pastures, the potential primary productivity will be defined by the botanical composition of the mixture (i.e., the proportion of grass and legume) and the environmental conditions during the growing season. Additionally, this potential productivity will be limited by water and N availability and grazing management. In this case, the ability of species to fix N from the atmosphere will play a significant role in overcoming potential N limitations under unfertilized conditions (Figure 2-1-A5-A7).

A key ecophysiological concept integrating limiting factors defining the grassland's primary productivity is the 'radiation-use efficiency' (RUE; Monteith, 1972; Monteith 1977). The RUE is an integrative metric, defined as the biomass produced or net primary production per unit of solar radiation or photosynthetically active radiation (iPAR) intercepted or absorbed by the canopy in a given period of time (Monteith 1977; Sinclair and Muchow, 1999). The RUE can be reported in terms of aboveground RUE, which only includes the aboveground biomass production, or in terms of total RUE, including above and belowground biomass production (Bélanger et al., 1994; Cristiano et al., 2015). The standardization of biomass production in response to iPAR through RUE and its different ways of quantification enables greater understanding of species growth and efficiency under different practices and environments (Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009). In addition, its quantification can be used to predict and simulate net carbon assimilation under potential environments and theoretical scenarios associated with climate change (Sinclair and Horie, 1989; Peng et al., 2020).

The RUE varies between species of different photosynthetic pathways and functional groups (Sinclair and Horie, 1989; Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009; Druille et al., 2019). For example, C4 species typically show greater RUE than C3 species (Stöckle and Kemanian, 2009). The RUE of C4 species ranges between 1.0 and 2.0 g MJ^{-1} iPAR under field conditions (Sinclair and Muchow, 1999; Kiniry et al., 2007; Stöckle and Kemanian, 2009), and above 2.0 g MJ^{-1} iPAR under unlimited conditions of N and light availability (e.g., Bélanger et al., 1994; Cruz, 1997; Gómez et al., 2012).

In general, lower RUE values can be expected for C3 grass species, averaging approximately 1.4 g MJ^{-1} iPAR (Monteith, 1977; Stöckle and Kemanian, 2009). Within C3 species, grasses have on average, greater RUE than legumes (Stöckle and Kemanian, 2009). In addition, when grasses are in mixture with legumes, such as alfalfa (*Medicago sativa* L.) with tall fescue (*Lolium arundinaceum* [Schreb.] S.J. Darbyshire), RUE can vary from 0.9 to 1.4 g MJ^{-1} iPAR under different N and defoliation treatments (Ojeda et al., 2018; Grigera and Oesterheld, 2021). The lowest RUE is expected in native grasslands, i.e., rangelands, where RUE can range between 0.2 to 1.0 g MJ^{-1}

iPAR (Piñeiro et al., 2006), likely associated with N limitation (Boggiano et al., 2001) and inherent limitations of species components of the pasture.

The general differences in RUE between C3 and C4 photosynthetic pathway species and grass and legume functional groups suggest that while incorporating legumes into C4 grass-only pastures can help overcome the N limitation to primary productivity (Tilman et al., 2001; Fornara and Tilman, 2009; Kohmann et al., 2018; Garcia et al., 2021; Jaramillo et al., 2021), a high proportion of legume may reduce the overall RUE of the pasture. This is supported by empirical evidence in bahiagrass and rhizoma peanut mixtures in Florida, where primary productivity of rhizoma peanut is less than that of heavily fertilized bahiagrass (Santos et al., 2018a), and it decreases when legume proportion is high (Figure 2-1-A9; Kohmann et al., 2022). This indicates that RUE is expected to decrease with increases in legume proportion in the mixture, associated with inherent differences between C3 legumes and C4 grasses, an effect likely exacerbated under high temperatures in warm climates. However, other factors, such as N availability, contribute to defining the benefits that can accrue from legumes (Santos et al., 2018a) and play a significant role in defining their optimum proportion in pasture.

Additionally, the identification of optimum legume proportion maximizing RUE and primary productivity requires the study of canopy attributes of the mixture. The canopy architecture may affect the primary productivity (Sinclair and Muchow, 1999), as in general, legumes have more horizontal leaves than grasses (Brougham, 1958), meaning that legumes can intercept a greater amount of solar radiation with similar leaf area index (LAI) than grasses. This may determine different rates of gross photosynthesis between species under similar defoliation management (e.g., Monteith, 1972) and different rates of net biomass accumulation after similar intervals post-defoliation (Parsons et al., 1983). Moreover, this can affect the succession of the mixture, defined by grass and legume proportion, in response to different grazing intensities affecting light competition among species (Tilman, 1985). For example, in cases where legumes intercept greater solar radiation at similar LAI or similar canopy height, heavier grazing intensities would favor the legume over the grass (Figure 2-1-A10; Spasiani et al., 2023), and therefore, the mixture would tend toward legume dominance.

Radiation use efficiency and primary productivity can be affected by environmental conditions and management-related factors, such as canopy architecture, temperature, photoperiod, and most importantly, soil moisture and N nutrition (Sinclair and Muchow, 1999). These factors also interact, often non-linearly, adding complexity to identifying the main factors limiting RUE and management practices for maximizing pasture RUE and, consequently, primary productivity. One option for identifying limiting factors under complex interactions and environments is quantile regressions and statistical learning methods (Cade et al., 1999; Breiman, 2001; Cade and Noon, 2003). In the current study, where the goal is to identify the optimum legume proportion maximizing RUE and primary productivity of Florida grasslands, the use of quantile regressions may help to identify the shape of response in RUE and primary productivity on rhizoma peanut proportion in pasture, and the identification of optimum proportions under potential non-linear responses.

Plant And Animal Responses In Tropical Grass-Legume Mixtures

The introduction of legumes into warm-climate, grass-based systems increases the amount of N cycling (Figure 2-1-A1-2), the biomass production and forage nutritive value (Figure 2-1-A4-9), and animal performance while reducing or avoiding inputs of N fertilizer (Sollenberger et al., 2019; Sollenberger and Dubeux, 2022). These positive benefits have significant relevance in enhancing the delivery of ecosystem services (Peoples et al., 2019; Sollenberger et al., 2019). Particularly for bahiagrass and rhizoma peanut, studies carried out throughout Florida have conclusively demonstrated that cattle grazing bahiagrass monocultures gain about 300 g day^{-1} during the summer months (Sollenberger et al., 1988; Stewart et al., 2007; Jaramillo et al., 2021). However, when rhizoma peanut is introduced into bahiagrass pastures, the animal gain generally increases to ~600 g day^{-1} (Figure 2-1-A8-13; Valencia et al., 2001; Jaramillo et al., 2021). Animal gain per area for the mixture increased by approximately 100% compared with unfertilized bahiagrass, from ~130 to ~280 kg LW ha^{-1} (Figure 2-1-A16; Jaramillo et al., 2021; Pereira-Neto et al., 2024).

Similar gains per area can be achieved through N fertilization of bahiagrass associated with increases in stocking rate in response to greater bahiagrass production (Figure 2-1-A14); however, N fertilization seldom increases individual animal performance (Williams et al., 1991; Stewart et al., 2007; Jaramillo et al., 2021; Pereira-Neto et al., 2024). Introducing rhizoma peanut into bahiagrass pasture replaces N fertilizer inputs and reduces greenhouse gas emissions, avoiding or decreasing emissions associated with production of N fertilizer (Peoples et al., 2019) and with field N losses from volatilization and denitrification (Sollenberger et al., 2019). In addition, introducing rhizoma peanut into bahiagrass pasture increases beef production per area with fewer but more efficient animals (Figure 2-1-A17; Williams et al., 1991; Jaramillo et al., 2021), reducing environmental impacts. This practice, where gain per area is underpinned with greater animal performance (not stocking rate), is an effective strategy for reducing enteric methane emissions from livestock systems (Herrero and Thornton, 2013).

Despite the positive direct and indirect effects of legume introduction into C4 grass species pastures, its adoption by producers has been limited by seed or planting material availability. Additionally, the high establishment costs, complex management during the establishment period, and poor persistence have aggravated the problem in tropical environments (Castillo et al., 2014; Schultze-Kraft et al., 2018; Aryal et al., 2021). Another constraint to legume use by producers may be the perception that returns from legume introduction are positively and linearly correlated with its proportion in pasture. However, previous studies have shown that to achieve high legume proportions is costly and may not always provide advantages from production, environmental, or economic perspectives (Nyfeler et al., 2009; Lüscher et al., 2014; Kohmann et al., 2022).

Results from qualitative studies carried out in Florida on rhizoma peanut and bahiagrass pastures suggest that moderate legume proportions are sufficient to increase animal performance markedly (e.g., Williams et al., 1991; Jaramillo et al., 2021). Similarly, Watson and Whiteman (1981) found for other tropical grass-legume mixtures that low to moderate legume proportion was sufficient to increase animal performance, and did not find responses of animal gain to legume above 30%. Despite the linear relationship between animal gain and legume proportion reported by Pereira et al. (2020), most of the limited scientific evidence suggests that moderate proportions appear to maximize animal gain. This range has also been identified in temperate grass-legume mixtures as the optimum to maximize soil and plant responses and was suggested to maximize animal responses in temperate grass-legume mixtures (e.g., Nyfeler et al., 2009; Nyfeler et al., 2011; Lüscher et al., 2014).

Kohmann et al. (2022) reported that while biomass production of bahiagrass-rhizoma peanut mixtures decreases with increasing legume proportion, forage nutritive value increases up to 45-50% legume. Thus, this trade-off between biomass production and nutritive value (Figure 2-1-A11) implies an optimum proportion below 50% legume in this warm-climate pasture. Moreover, in spite of the high individual animal performance at high legume proportion, the decrease in biomass production at this level might compromise the stocking rate, and therefore, the gain per area (Figure 2-1-A14-A17). This suggests that the optimum legume proportion could be defined as the range at which gain per area and individual animal performance are maximized (Figure 2-1-A15-17), as revealed by Mott (1960) and Mott and Moore (1985) for a grazing intensity gradient. Interestingly, information from qualitative studies indicates this optimum is between 30-50% legume, proportions that can be achieved with strip-planting methods where the establishment cost is reduced by 50%, as only half or less of the pasture area is planted with legume (Castillo et al., 2014; Jaramillo et al., 2021). In this scenario, quantitative studies are needed to appropriately validate this moderate range as optimum in warm climates.

Interestingly, grazing selectivity of cattle may be a relevant mechanism to compensate for the low to moderate legume proportion in mixtures in grazing systems (Lascano, 2000; Valencia et al., 2001; Jaramillo et al., 2021). It has been conclusively reported that cattle and sheep prefer and select legumes over grass in temperate and some tropical grass-legume mixtures (Figure 2-1-A8-A12-13; Parsons et al., 1994; Lascano, 2000; Valencia et al., 2001; Rutter, 2006; Jaramillo et al., 2021; Kohmann et al., 2022). For example, Jaramillo et al. (2021) reported that cattle consumed a diet of 44% rhizoma peanut when only 32% was offered in pasture. This reveals that under appropriate grazing management (Sollenberger et al., 1987) cattle selectivity could be a zero-cost tool compensating for lesser rhizoma peanut proportion and other legumes in mixture with tropical grasses.

One important aspect to consider is that this general foraging selectivity toward legumes can be altered when animals graze less palatable, high-tannin legumes (Lascano, 2000), and when cattle actively select for grasses and against legumes. Similarly, cattle can actively select grasses and against legumes when the latter species are in high proportion in pasture (Kohmann et al., 2022). In the latter scenario where legumes dominate, animals can actively search and select grasses (Parsons et al., 1994), a response likely attributable to ingestive and post-ingestive feedback processes (Provenza et al., 2007). In this case, the significant and costly management efforts to achieve high legume proportion are counterbalanced by animals, limiting the *a priori* benefits of a legume-dominated condition, in both temperate (Parsons et al., 1994) and tropical pastures (Kohmann et al., 2022).

Defoliation Intensity And Leaf Tissue Composition And Digestibility

The adaptation of bahiagrass to Florida environments and grazing management (Sollenberger et al., 1988) and its relevance for the beef sector emphasize the need to study alternative practices to overcome current limitations. One of the most important weaknesses of the species is the decline in digestibility from spring to autumn (Interrante et al., 2009). As a result, the average daily gain of cattle grazing bahiagrass can decrease from ~0.7 kg animal^{-1} in spring to ~0.3 kg animal^{-1} during the summer (Sollenberger et al., 1988; Williams et al., 1991). This decline in animal performance is more pronounced in bahiagrass compared to other C4 species (e.g., Mott dwarf elephantgrass; Flores et al. 1993), which suggests inherent attributes of bahiagrass limiting animal performance. Some of the specific attributes of bahiagrass that may explain this summer slump in animal gain are the abundance of sclerenchyma fibers in leaf tissue, which reduces digestibility (Flores et al., 1993), in addition to increased forage maturation during periods of high biomass production (Stewart et al., 2007).

Hence, the second overall goal of this dissertation is to study the potential effect of short-term increases in defoliation intensity on leaf tissue composition and fiber concentration and how these differences explain changes in leaf nutritive value as a potential driver of animal performance. Understanding the impacts of short-term increases in defoliation intensity on attributes at leaf scale may provide insights and further hypotheses to be tested at larger scales, focused on strategic changes in grazing intensity to overcome specific limitations, such as the summer slump in animals grazing bahiagrass.

In this scenario, short-term increases in grazing intensity have potential positive impacts on tissue composition and digestibility (Figure 2-2). These causal relationships are driven by the defoliation intensity, which first affects the canopy structure, through the leaf length and leaf and tiller size (Figure 2-2-A1-2; Chapman and Lemaire, 1993; Boggiano et al., 2001; Pakiding and Hirata, 2002). The leaf length and size are typically positively correlated with each other (Figure 2-2-A3) and with sheath length (Gastal and Lemaire, 2015). Leaf length and size are affected by the grazing intensity (Pakiding and Hirata, 2002), where the length of the fully expanded leaf decreases with high grazing intensity (Boggiano et al., 2001) and determines the tissue composition of the leaf (Figure 2-2-A4).

Previous studies have demonstrated leaf length and size are determinants of leaf digestibility in temperate grasses (Insua et al., 2017; Insua et al., 2018). In general, as the leaf length increases, the leaf and total forage digestibility decrease (Insua et al., 2017). This relationship is mediated by the tissue composition of leaves, where the proportion of cell wall and tissue composition (Figure 2-2-A4-5), such as sclerenchyma fibers, increased with leaf length and size. The cell wall and sclerenchyma fibers have lower digestibility than other tissue or cell structures, such as mesophyll and epidermis (Wilson and Mertens, 1995). Therefore, reducing the leaf length by increasing the grazing intensity during the summer could reduce the leaf sclerenchyma and cell wall concentrations, tissue types associated with support functions, and lesser digestibility. This mechanism possibly reduces the pasture height and can affect the bite mass, suggesting an offset relationship between canopy structure and nutritive value, which is beyond the objective of this project but should be addressed in further research on an appropriate scale, including animal gain responses.

Therefore, capitalizing on the potential of bahiagrass and rhizoma peanut pastures in Florida and improving the resource-use efficiency in grazing systems while reducing environmental impacts associated with N fertilization and high greenhouse gas emissions are critical for the sustainability of the beef sector of the state and other tropical regions. Achieving the goals proposed in this project will help the understanding of mechanisms and processes to overcome specific limitations and provide insights of general underlying mechanisms in tropical grasses and legumes.

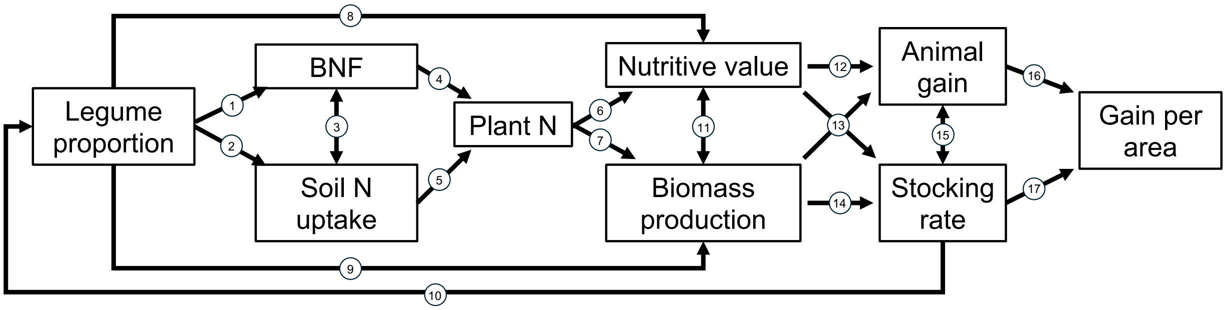

Figure 2-1. Conceptual model for legume introduction. Relationships between legume introduction and proportion in pasture on soil-plant-animal interaction levels.

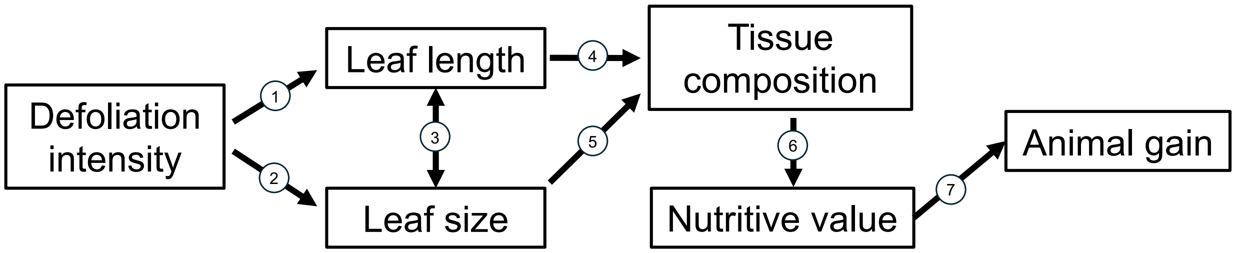

Figure 2-2. Conceptual model for defoliation intensity. Relationships between defoliation intensity and leaf and canopy attributes, tissue composition and nutritive value and animal gain.

CHAPTER 3

NITROGEN NICHE DIFFERENTIATION AND OVERYIELDING ALONG A LEGUME PROPORTION GRADIENT IN A TROPICAL GRASS-LEGUME MIXTURE

Introduction

The association of legumes with grasses is relevant for enhancing the productivity and sustainability of grassland ecosystems (Suter et al., 2015). The grass-legume co-existence is typically driven by niche partitioning and facilitation (Nyfeler et al., 2009; Churchill et al., 2024), where grass and legume components of the mixture benefit from using different portions of a given available resource, resulting in a more complete and efficient use of the resource (Schwinning and Parsons, 1996; Barry et al., 2018).

One of the main resources limiting ecosystem productivity is nitrogen (N; Fay et al., 2015). In N-limited environments, legume species benefit from their symbiosis with bacteria, using atmospheric N through biological N fixation (BNF; Peoples et al., 1995; Carlsson and Huss-Danell, 2003; Jensen et al., 2012). However, high soil N levels inhibit BNF in legumes. In mixed grass-legume swards, grasses uptake soil N, thereby reducing its availability and enhancing the proportion of N derived from the atmosphere (%NDFA) in legume and likely, BNF (Nyfeler et al., 2011). In this scenario, there is a complementarity effect between grasses and legumes (Barry et al., 2018), and an overall increase in N uptake in grass-legume mixtures compared with the monocultures (Nyfeler et al., 2011). Moreover, grasses often benefit from the transfer of BNF from the legume through different belowground (e.g., N transfer through senescence and decomposition of roots and nodules, root exudates and mycorrhiza; Thilakarathna et al., 2016) and aboveground pathways (e.g., N transfer through excreta and litter; Dubeux et al., 2007; Garcia et al., 2021). Thus, understanding N niche differentiation or resource partitioning in grass-legume mixtures is critical in N-limited grassland ecosystems to increase productivity while mitigating negative environmental impacts associated with N fertilization (Carlsson and Huss-Danell, 2003; Herridge et al., 2008; Lüscher et al., 2014).

The N niche differentiation of grass-legume mixtures and abiotic facilitation and biotic feedback (Barry et al., 2018) typically drive N and biomass overyielding of mixtures (Nyfeler et al., 2011). Nitrogen and biomass overyielding occurs when grass-legume mixtures outperform monocultures of the same species in N yield and biomass accumulation (Nyfeler et al., 2011; Santos et al., 2018a; Churchill et al., 2024). In this case, the presence of grass in a mixture with legumes increases total N uptake from soil and BNF (Nyfeler et al., 2011), increasing the amount of N cycling in the system (Kohmann et al., 2018; Garcia et al., 2021).

Qualitative studies comparing the presence or absence of legumes in mixtures with grass species have demonstrated N overyielding of temperate and tropical grass-legume mixtures compared with the grass and legume components in monoculture (e.g., Santos et al., 2018a; Churchill et al., 2024; Louarn et al., 2024). Yet, given their qualitative nature, these studies do not identify the optimum proportion of each species for maximizing N overyielding, and studies of mixtures with varying proportions are needed. Some quantitative studies assessing responses to a legume gradient are available from temperate climates. They show N yield and BNF are maximized with moderate legume proportion in the mixture (~40%; Nyfeler et al., 2011; Suter et al., 2015), and the proportion of N fixed by the legume relative to total shoot N decreases as the proportion of legumes in the mixture increases (Mallarino et al., 1990; Nyfeler et al., 2011). High legume proportion also reduces the depletion of soil N by grasses, and consequently, BNF is less stimulated (Mallarino et al., 1990; Nyfeler et al., 2011). While studied qualitatively (e.g., Santos et al., 2018a; Churchill et al., 2024), there is limited information on N niche differentiation and N overyielding in warm-climate, grass-legume mixtures across a gradient of legume proportion.

The N niche differentiation stimulating overall N uptake from soil and atmosphere in grass-legume mixtures increases the amount of N cycling in the system (Nyfeler et al., 2011; Kohmann et al., 2018), overcoming the N limitation to grassland productivity (Fay et al., 2015). Thus, temperate and tropical grass-legume mixtures that increase the amount of N cycling in the system are likely to increase biomass production (Nyfeler et al., 2009; Kohmann et al., 2022). Within this context, a biomass overyielding is achieved without the need for N fertilizer inputs (Carlsson and Huss-Danell, 2003; Herridge et al., 2008), with accompanying increases in animal production and economic efficiency while minimizing environmental impact (Lüscher et al., 2014; Sollenberger et al., 2019; Sollenberger and Dubeux, 2022).

Based on these findings, moderate legume proportion has been proposed for temperate climate grass-legume mixtures to maximize biomass overyielding (Mallarino and Wedin, 1990; Thomas 1992; Nyfeler et al., 2009; Nyfeler et al., 2011; Lüscher et al., 2014; Helgadóttir et al., 2018). However, while a similar range has been proposed for maximizing biomass accumulation in warm-climate, grass-legume mixtures (Thomas, 1995), empirical evidence of the optimum legume proportion in pasture remains scant (Sollenberger et al., 2019, Kohmann et al., 2022).

The goal of this study was to quantify the N niche differentiation and N and biomass overyielding of a warm-climate, grass-legume mixture along a legume gradient in a field experiment, with the specific aim of identifying optimum legume proportions for maximizing N and biomass overyielding. Specifically, the objective was to quantify the concentration of shoot N derived from soil and from the atmosphere in grass monocultures and in grass and legume components of the mixture varying in legume proportion in the field. In addition, the biomass accumulation, BNF and soil-derived N yield of the grass monoculture and in mixture with legume were measured, and N and biomass overyielding in response to legume proportion were estimated. This novel field study allows to empirically test models and responses, already reported for temperate grass-legume mixtures, in a warm-climate context with mixtures across a wide legume gradient. Identifying optimum or, alternatively, minimum legume proportion in grass-legume mixtures that maximize N and biomass overyielding is relevant in warm climates, where seed availability and planting material of legumes are limited and the persistence of legumes is often low and their establishment is complex and expensive (Castillo et al., 2014; Schultze-Kraft et al., 2018; Sollenberger and Dubeux, 2022).

Materials and Methods

Site

The study was conducted at the Beef Research Unit of the University of Florida, in Gainesville, Florida (29.74 N, 82.27 W) during 2022 and 2023. Pastures evaluated were long-term ‘Pensacola’ bahiagrass monocultures, receiving no N fertilizer, and mixtures of bahiagrass with ‘Florigraze’ rhizoma peanut, where legume proportion in the mixture varied among pastures. The soils in the experimental area are Chipley sand soils, somewhat poorly drained to rapidly permeable. Soils samples were collected in March of 2022 and 2023 and analyzed for nutrient availability and soil pH. Soil pH across pastures averaged 6.3 and 5.9 for 2022 and 2023, and no lime was recommended. Mehlich-3 soil P, K, and Mg averaged 59, 24, and 54 mg kg^{-1}, respectively, in 2022, and 66, 40, and 50 mg kg^{-1}, respectively, in 2023. Based on the nutrient availability and fertilization recommendations, all pastures received 240 kg ha^{-1} $year^{-1}$ of 0-18-36 (19 kg P and 72 kg K ha^{-1}), applied in April 2022 and 2023.

The historical (2000-2021) daily temperature from May to October averaged 24.4° C (Figure 3-1), while the average rainfall accumulated during the same period was 833 mm. The average daily temperature was greater than the long-term average during the early-growing season of 2022, but below the historical average during the late-growing season. In addition, rainfall during 2022 was below the historical average throughout the season. The 2023 daily temperature was similar to the historical average throughout the early and late-season, and above the historical average during the middle part of the growing season. The rainfall was similar to the historical average, except for September, where it was less than the average.

Experimental Design

The goal for the field experimental design was to quantitatively assess plant responses across a wide range of legume proportion. Thus, to represent a wide range of grass and legume proportion in the mixture, different sites were selected in the bahiagrass monoculture, i.e., 0% legume, receiving no N fertilization, and in bahiagrass-rhizoma peanut mixtures. The grass-legume mixtures resulted from pure stands of rhizoma peanut established in 1983 (Ortega et. al., 1992), which was then colonized to varying by Pensacola bahiagrass.

Twenty-four, 1-m^{-2} sites were assessed simultaneously in the fields during four, 4-week periods in each of two years: six ‘control’ sites of only grass were selected every period in two 0.5-ha bahiagrass pastures; and 18 grass-legume sites were chosen in six, 0.5-ha mixed pastures to represent a wide range of grass and legume proportions. After every 4-week regrowth period, new sites were selected and assessed throughout the following regrowth period for a total across years of 8 regrowth periods, each containing 24 sites (n=192). Pastures were continuously stocked with cattle during the growing seasons of 2022 and 2023 and maintained at the same grazing intensity, defined by a herbage allowance of 1.5 kg DM^{-1} kg^{-1} animal live weight (LW; Sollenberger et al., 2005). Exclusion cages were used to prevent grazing in the sampling sites, and each site was considered as an experimental unit containing their respective legume proportion in the total biomass.

Sampling Procedures

Sites were selected and cages placed on 23 June 2022 and 7 June 2023. After a 4-week period of regrowth, an area of 0.25-m^2 in the center of each caged site was clipped to 2 cm. The clipped samples were hand-separated into grass and legume fractions and dried at 60 °C to determine the legume component proportion in total shoot biomass (%). Immediately after clipping, new cage sites were selected for the following period.

The initial and final biomass within the cages was measured using the double sampling technique (Haydock and Shaw, 1975), with a rising plate meter as the indirect measure (adapted from Rayburn and Rayburn, 1998). Direct assessments of shoot biomass were conducted at five, 0.25 m^{-2} uncaged sites in each of 8 pastures. Before clipping these uncaged sites, the disk height was measured with the rising plate meter. The uncaged sites were clipped at 2 cm, samples were dried at 60 °C, and they were weighed to determine dry biomass. Regression equations were used to relate the direct measures of biomass (y-axis) to the disk height (x-axis) for every period and pasture. The coefficient of determination (R^2) of the equations averaged 82.2 ±8.6% and 85.3% ±9.8% for 2022 and 2023, respectively.

Measured And Estimated Responses

Shoot biomass

The shoot biomass is defined as the standing grass and legume biomass above a 2-cm height, expressed as g DM m^{-2} (grass + legume). Grass and legume biomass were calculated as the proportion of grass and legume times the total shoot biomass, based on hand separation of botanical fractions (g m^{-2}).

Shoot N

To determine the proportion of total N in shoot grass and legume components, and %NDFA relative to total N, grass and legume samples were ground to pass a 1-mm screen and ball milled at 25 Hz for 9 min, and then analyzed using a CHNS analyzer (Dumas dry combustion method). The proportion of N derived from atmosphere in each component was estimated using the natural abundance technique (δ^{15}N; Dubeux et al., 2017), and the equation developed by Shearer and Kohl (1986), as:

$$\%\mathrm{NDFA}_{\mathrm{grass/legume}} = \frac{\delta^{15}\mathrm{N\ reference\ species} - \delta^{15}\mathrm{N\ N_2\ fixing\ species}}{\delta^{15}\mathrm{N\ reference\ species} - \mathrm{B}} \times 100 \qquad (3\text{-}1)$$

where %NDFA in the grass or legume component represents the proportion of N derived from the atmosphere and δ^{15}N reference species is the δ^{15}N of a non-fixing and unfertilized reference species. In this case, bermudagrass was selected as the reference species to estimate the %NDFA, because bahiagrass can fix N from the atmosphere (Kass et al., 1971). Samples of bermudagrass were taken from neighboring areas the same day as when cages were clipped and dried at 60 °C. The δ^{15}N of the fixing species is the δ^{15}N of bahiagrass and rhizoma peanut, and B is the value of a fixing species in the absence of inorganic N, which was -1.41%, as reported for *Arachis hypogaea* L. (Okito et al., 2004) for estimating the proportion of N derived from the atmosphere in legume. For estimating the N derived from the atmosphere in grass, the lowest ^{15}N measured in bahiagrass for each year was used (Frankow-Lindberg and Dahlin, 2013; Santos et al., 2019; Garzon et al., 2023), which corresponded to -1.21 and -1.17% for 2022 and 2023, respectively. In particular for bahiagrass, the %NDFA represents the ability of the species to acquire N from the atmosphere in association with *Azotobacter paspali* (Kass et al., 1971) when grown in monoculture (0% legume), while in mixture represents its ability to acquire N from the atmosphere through *A. paspali* and NDFA transferred from the legume.

Having the total, grass, and legume shoot biomass, the total N concentration, and the %NDFA, the proportion of total N derived from soil was then estimated (%NDFS), as the difference between total N in grass and legume shoot biomass and the %NDFA in each component (%NDFS = 100 - %NDFA). To express these proportions in total shoot biomass, the average of grass and legume N concentration were weighted according to their proportion in shoot biomass (%). The %NDFA and

%NDFS were then converted and expressed as concentration in total, grass and legume shoots (g kg^{-1}). Lastly, the concentrations of total N, NDFA and NDFS in total, grass and legume shoots were converted to N content (g m^{-2}), as the product of biomass content (total, grass, and legume shoots; g m^{-2}) and the respective concentration (g kg^{-1}).

Biomass accumulation and N yield

Biomass accumulation was estimated as the difference between final and initial biomass for a given site and regrowth period. Having quantified biomass accumulation, it was possible to quantify N yield and partition it into NDFA yield (the direct BNF and internal recycling of NDFA) and N uptake from soil by plants (soil-derived N yield and internal recycling). The NDFA yield was estimated as the biomass accumulation in each 4-week period times the shoot N concentration times the %NDFA in each sample, as:

$$\text{NDFA yield}_i\ (\text{g m}^{-2}) = \text{biomass accumulation}_i\ (\text{g m}^{-2}) \times \text{shoot}_i\ \text{N}\ (\text{g kg}^{-1}) \times \%\text{NDFA}_i \qquad (3\text{-}2)$$

where NDFA yield is the BNF for the i^{th} site, the internal recycling of NDFA and the transfer from legume to grass , and the shoot N (g kg^{-1}) is the N concentration of the sample, adjusted by the proportion of N derived from the atmosphere (%NDFA) measured in the i^{th} sample. Similarly, the soil-derived N yield during the 4-week period was estimated as the biomass accumulation in each 4-week period times the shoot N concentration times (%NDFS) in each sample, as:

$$\text{soil N yield}_i\ (\text{g m}^{-2}) = \text{biomass accumulation}_i\ (\text{g m}^{-2}) \times \text{shoot}_i\text{N g kg}^{-1}) \times (\%\text{NDFS}) \qquad (3\text{-}3)$$

where soil-derived N yield estimates the amount of N taken up by plants from soil and the internal recycling in plants in the i^{th} site, and the shoot N (g kg^{-1}) is the N concentration of the sample, adjusted by the proportion of N derived from soil (%NDFS

= 100-%NDFA) measured in the i^{th} sample. One important aspect is that this approach does not consider potential internal N recycling, which could be particularly important in these perennial species that have a large amount of rhizome biomass and N reserves.

Statistical Analyses

The overyielding effect, defined as the outperformance of mixtures relative to the individual components of the mixture, can be tested in qualitative studies as the increased productivity of the mixture relative to the standardized productivity of the individual components of the mixture (Churchill et al., 2024). In this quantitative study, the overyielding of the mixture along the legume proportion gradient was tested relative to a grass-only condition (0% legume) or a legume-dominated condition (+ 60% legume) using a regression approach (Nyfeler et al., 2011). In this case, a statistical significance of the quadratic response included in a second-order regression indicates overyielding of the mixture relative to the grass-only or legume-dominated conditions. Additionally, this approach allows identification of optimum legume proportions for maximizing plant responses, including shoot (grass + legume), grass, and legume total N concentration and content, and the partitioned N derived from the atmosphere and from soil. In addition, the effect was tested of legume proportion in pasture on %NDFA and on biomass accumulation and N yield, decomposed in NDFA yield and soil-derived N yield during the 4-week periods.

Linear mixed models were developed for each response. The full model included the initial shoot biomass as a covariate, year, a linear effect of the legume in pasture within years and the quadratic effect of legume in pasture within years as fixed effects, while period event within year was included as random effect, as:

$$y_i = \beta_0(shoot_{initial}) + \beta_1(yr) + \beta_2(yr) \times legume_i + \beta_3(yr) \times legume_i^2 + period_j$$

where y is the response variable for i^{th} site, $\beta_0(shoot_{initial})$ is the initial shoot biomass included as covariate effect, $\beta_1(yr)$ is the intercept of the regression for each year, $\beta_2(yr)$ is the linear parameter (β_2) of the legume proportion in pasture ($legume$) within years (yr), $\beta_3(yr)$ is the quadratic parameter (β_3) of the legume proportion in the pasture ($legume^2$) within years (yr), and $period_j$ is the random effect of j^{th} period events within years.

To test the significance of each parameter ($\beta_0(shoot_{initial}), \beta_1(yr), \beta_2(yr)$ and $\beta_3(yr)$), a sequential test was performed, from the simplest null model to the full model, including each parameter, one at a time. First, the covariate effect of initial shoot biomass was included, then the year effect, as the intercept of the regression, then the linear effect of legume proportion in pasture, and lastly, the quadratic effect of the legume in the pasture. In this way, it is possible to sequentially test significant effects of linear response once the intercept (i.e., year) already accounted for variability, and then test significant effects of quadratic response, when the intercept and linear effect already accounted for variability. Parameters that did not improve the model fit for a given response, according to a χ^2 test ($P > 0.05$), were excluded from the model. Additionally, each parameter was contrasted between years, such as intercept, the linear parameter and the quadratic parameter, to test whether parameters and the overall model differ among years. The linear mixed models were performed using the *glmmTMB* package (Books et al., 2017). Residuals were studied using DHARMa package (Hartig and Hartig, 2017). All analyses were performed in R 4.4.0 (R Development Core Team).

Results

Shoot Biomass And Nitrogen

The total shoot biomass increased quadratically with legume proportion in the pasture, being maximized at 18 and 26% legume, for 2022 and 2023, respectively (Figure 3-2). As expected, grass shoot biomass decreased with increasing legume proportion, while legume shoot biomass increased. Relative to N concentration, the shoot N increased quadratically with the legume proportion in pasture (Table A-1, Figure A-1). Similarly, the N concentration in grass increased with legume in pasture; during 2022, it did not achieve the maximum in the legume range analyzed, while during 2023 it was maximized at 39% legume. At this level, grasses had an average N concentration of 16 g kg^{-1}, overyielding by 40% the base level of the grass monoculture. The legume shoot N concentration differed between years (29 vs. 31 g kg^{-1} for 2022 and 2023, respectively), but it was not affected by legume proportion in the pasture.

The shoot N content, the product of N concentration and shoot biomass, increased quadratically with increasing legume proportion, reaching a maximum at a legume proportion of 38% during both years (Figure 3-2; Table 3-1). At this proportion, the overyielding relative to the unfertilized control with 0% legume was 67 and 128% for 2022 and 2023, respectively. The grass shoot N content peaked when legume proportion was 9 and 20% during 2022 (3.4 g m^{-2} N) and 2023 (3.3 g m^{-2} N), suggesting an overyielding relative to the 0% legume of only 3% during 2022, but of 28% during 2023. The legume N content increased quadratically with increasing legume proportion in the pasture, suggesting diminishing returns beyond the range analyzed.

Nitrogen Derived From The Atmosphere

The proportion of N derived from the atmosphere (%NDFA) in total, grass and legume shoot components was not affected by legume proportion in the pasture (Table A-1). The %NDFA in total shoot biomass averaged 64.9%; it was 62.3% in the grass component and 74.7% in the legume component. However, the concentration of NDFA in total shoot increased quadratically with increasing legume proportion (Table A-2; Figure A-1). Similarly, the concentration of NDFA in grass increased quadratically with legume in the pasture and reached a maximum when legume proportion was 36% in 2022 and 35% in 2023. At these levels of legume participation, it overyielded the 0% legume by 32% in 2022 (9.5 vs. 7.2 g kg^{-1} NDFA) and by 35% in 2023 (9.6 vs. 7.1 g kg^{-1} NDFA). The concentration of NDFA in legume was not affected by the proportion of legume in the pasture, averaging 22.5 g kg^{-1} N derived from the atmosphere.

The NDFA content in total shoot biomass was maximized at 38 (2022) and 40% legume (2023), reaching an average of 3.6 and 3.8 g m^{-2} NDFA, respectively (Figure 3-2; Table 3-2). At this ‘optimum’ range, the legume proportion overyielded the 0% legume by 84 and 148% during 2022 and 2023, respectively. The NDFA content in the grass component, as a result of BNF when in monoculture (direct BNF or indirect BNF through internal N recycling), and BNF and NDFA transfer from legume when in mixture, was maximized at 13 and 19% legume in 2022 and 2023, overyielding the 0% legume by 7 and 27%, respectively. The NDFA content in legume showed a linear increase with increasing legume proportion that did not differ between years.

Soil-derived N

The concentration of N derived from soil (NDFS) in total and grass shoot fractions, as a result of direct plant N uptake or indirect through internal N recycling, increased linearly in response to legume in the pasture (Table A-2; Figure A-1). The concentration of soil-derived N in legumes tended to show a quadratic response to its proportion in pasture (*P* = 0.087; Table A-2), achieving a minimum soil-derived N concentration at 17 and 34% legume in 2022 and 2023. At these legume proportions in pasture, the concentration of NDFS in legume was 37 and 88% less than achieved at the maximum legume proportion (60%), revealing the biotic feedback between BNF and N uptake from soil in grass-legume mixtures varying in legume proportion.

The total shoot N content derived from soil increased quadratically with increasing legume proportion (Figure 3-3) and was maximized at 41 and 34% legume during 2022 and 2023. Mixtures at these legume proportions overyielded the grass-only pasture in soil-derived N content by 79 and 75%, respectively. For grass shoots, the N content derived from soil was maximized at 16 (2022) and 21% legume (2023). At this legume proportion, the grass soil-derived N content was 14 (2022) and 42% (2023) greater than for the unfertilized grass monoculture. Similarly, legumes showed a quadratic increase in shoot N content derived from soil in response to increases in legume proportion in pasture.

Biomass Accumulation And N Yield

The biomass accumulation increased quadratically in response to increasing legume proportion in the pasture (Figure 3-4; Table 3-3). It was maximized at 33% legume in 2022, where the production reached 216 g m^{-2} during a 4-week period, and at

31% legume in 2023, achieving 264 g m^{-2} during a 4-week period. At these optimum levels of legume proportion, the overyielding compared to 0% legume was 145 (2022) and 96% (2023). In addition, the overyielding compared to the maximum legume proportion achieved in this study, i.e., 60%, was 65 (2022) and 66% (2023).

The NDFA yield, including direct BNF during the experimental period and internal NDFA recycling and potential transfer from legume to grass, followed a similar pattern, being maximized at 38% legume in 2022, where the mixture 'fixed' 2.50 g m^{-2} NDFA during a 4-week period, and at 34% legume in 2023, where it achieved 3.46 g m^{-2} NDFA during a 4-week period. In this case, the overyielding in NDFA yield compared to the maximum legume proportion in pasture (i.e., 60%) reached 27 and 50% for 2022 and 2023, respectively. The soil-derived N yield by plants during the 4-week period was maximized at a legume proportion of 35 and 33% during 2022 and 2023, respectively. At this legume proportion, the amount of N taken up from soil was 225 and 285% greater than for 0% legume, and also, 73 and 135% greater than at 60% legume, demonstrating the overyielding response of moderate legume proportion in N cycling in the system.

Discussion

Understanding of N niche differentiation and resource partitioning, biotic feedback and abiotic facilitation between grass and legume species is relevant for improving ecosystem productivity while minimizing environmental impacts (Herridge et al., 2008; Suter et al., 2015; Barry et al., 2019). Studies in temperate regions assessing the effect of legume proportion in pastures on biomass and N overyielding have suggested that moderate legume proportions maximize BNF and soil N uptake by plants

(Nyfeler et al., 2011; Sutter et al., 2015). These increases in the amount of N cycling in the system typically translate into greater plant production, without the need of N fertilizer (Nyfeler et al., 2009). Qualitative studies in tropical regions assessing discrete treatments of grass and legume monoculture vs. the binary mixture, have demonstrated the benefits of grass-legume mixtures on N niche differentiation and overyielding (Santos et al., 2018a; Churchill et al., 2024). However, the effect of legume proportion in pastures on these responses are seldom considered in tropical grass-legume mixtures.

In this field experiment studying N niche differentiation and overyielding along a C4 grass-C3 legume mixture gradient, it was demonstrated that moderate legume proportion, i.e., 30-40%, maximized NDFA yield, as direct BNF and indirect BNF through internal recycling and transfer of NDFA, as well as the amount of soil-derived N yield by plants (Figure 3-4). The N overyielding achieved with moderate legume proportion in pastures increases the amount of N in shoot and is typically translated into greater biomass accumulation compared with grass-only or legume-dominated pastures (Figure 3-4; Kohmann et al., 2022). Therefore, under scenarios where ecosystem productivity is limited by N (Fay et al., 2015), moderate legume proportions can maximize N yield and plant production in warm climates (Figure 3-4) as well as in temperate regions (Nyfeler et al., 2009; Suter et al., 2015).

In general, studies assessing overyielding of grass-legume mixtures have contrasted the relative increase of responses under the grass-legume mixture compared with the grass and legume components in monoculture (e.g., Santos et al., 2018a; Churchill et al., 2024). In this study, however, the overyielding of the grass-legume mixture was either relativized to the grass monoculture (0% legume) or to sites with the maximum legume proportion, i.e., 60% legume, but not to a legume monoculture. In this scenario, it would be appropriate to relativize the overyielding of the mixture to a legume monoculture; hence, the interpretation of these results should consider this limitation. However, even with this limitation of having only 60% legume as the most extreme case of legume participation, the range allowed detection of a quadratic relationship between legume proportion and most of the responses (Figures 3-2, 3-3, and 3-4).

Further, the typical poor persistence of legumes in warm climates and the high establishment costs and complex management of pure stands (Castillo et al., 2014; Schultze-Kraft et al., 2018; Sollenberger and Dubeux, 2022), determine that low to moderate legume proportions are more likely attainable than legume-dominated pastures (e.g., 80% legume as in Nyfeler et al., 2011). In addition to this consideration, the decreases in biomass accumulation and N yield observed beyond 40% legume (Figure 3-4; Kohmann et al., 2022) do not support assessments under very high legume proportions. Moreover, the quadratic response found in most of the variables suggest that the overyielding of the mixture relativized to the 60% legume-dominated sites would be underestimated compared to a scenario of 100% legume. Hence, the robustness of the quadratic response observed in most variables combined with the realistic scenario of a low to medium realized legume proportion in grass-legume mixtures, support the findings relativizing N and biomass overyielding to grass monoculture and legume-dominated sites (60%), rather than to legume monoculture sites (100%).

In addition to the aforementioned limitation, the measured NDFA and soil-derived N in plants might correspond to not only direct BNF of the grass and legume for the case of NDFA, but also to NDFA fixed prior the start of the experiment, which was stored in rhizomes and roots of these perennial species, and then recycled internally to the shoot (Dubeux et al., 2017). Also, the NDFA in the grass component might be the result of direct BNF via association with *A. paspali* and indirect BNF through internal recycling when grown in monoculture, and when grown in mixture with legume, part of the NDFA might be transferred from the legume (Santos et al., 2018b). Similarly, the amount of NDFS in plants can be direct N uptake by plants during the experimental period and internal recycling of N already taken up prior to the experiment. As a result, while this can lead to overestimations of soil-derived N and NDFA yield, the differences are likely to be attenuated under steady-state conditions during the growing season, where part of the N derived from soil and fixed from the atmosphere during the experimental period might be stored in root and rhizomes, and therefore, not accounted for during these assessments of shoots.

The results revealed a similar pattern of N niche differentiation and N overyielding under moderate legume proportion to that reported for temperate grass-legume mixtures (Nyfeler et al., 2011; Suter et al., 2015). Overall, there was a strong quadratic response to legume proportion in the pasture ($P < 0.001$ for most responses; Tables 3-1, 3-2, 3-3; Tables A-1, A-2). Moreover, differences in parameters among years were mostly detected for the intercept (β_1), but not for the linear (β_2) and quadratic responses (β_3), indicating a consistency in the shape of relationships across years. Beyond the statistical significances, the models explained a significant proportion of total variability in most cases.

The %NDFA did not vary with legume proportion in pasture (Table A-1) as demonstrated by Carlsson and Huss-Danell (2003). Conversely, Mallarino et al. (1990) and Nyfeler et al. (2011) reported a decrease in %NDFA in response to increasing legume proportion in pastures with short-term mixtures (i.e., one to three years). However, the amount of NDFA (g m^{-2}) in total shoot was maximized at 38 and 40% legume, and then increased quadratically with greater legume proportions. Specifically in grass shoots, the NDFA content peaked between 13-19% legume, which determined an overyielding of 7 and 27% relative to the grass monoculture during 2022 and 2023. This amount of NDFA, the concentration of NDFA (Figure A-1) for grasses in mixture with legumes, and the overyielding compared with the grass monoculture indicate a transfer of NDFA fixed by legumes (Thilakarathna et al., 2016; Barry et al., 2019) and/or a stimulation of N fixation by the *A. paspali* in association with the bahiagrass. Interestingly, the observed BNF of the grass in monoculture (in association with *A. paspali*) averaged 0.73 g m^{-2} NDFA during a 4-week period, slightly lower than the predicted intercept of the regression (Figure 3-4). Overall, the NDFA yield, as direct BNF during the experimental period and internal NDFA recycling and potential transfer from legume to grass, was maximized at 38 and 34% legume during 2022 and 2023, ranging from 100 to 138 kg N ha^{-1} fixed in the growing season (2.50 and 3.46 g m^{-2} NDFA), similar to that reported by Santos et al. (2018a) for bahiagrass-rhizoma peanut mixtures.

Legumes present in moderate proportion in the grass-legume mixture tended to show a lesser proportion of N derived from soil compared to when they dominated the pasture (~60% proportion). Conversely, under moderate legume presence in the pasture, grass showed the greatest amount of N derived from soil, likely outcompeting legumes for soil N (Mallarino et al., 1990; Nyfeler et al., 2011) and stimulating BNF, which was maximized at this range of legume proportion. A seldom-considered aspect of N overyielding and resource partitioning in mixtures is the stimulation of BNF due to the presence of grasses competing for soil N (Nyfeler et al., 2011; Churchill et al., 2024), which typically drives greater biomass accumulation. Thus, under moderate legume proportions, the BNF is not only stimulated by the presence of grasses, but it is also the optimum legume proportion for maximizing biomass accumulation in temperate (Thomas, 1992; Nyfeler et al., 2009) and warm-climate grass-legume mixtures (Kohmann et al., 2022; Figure 3-4). This suggests positive feedback between N uptake and fixation and photosynthesis, where the processes benefit each other. For example, the increase in plant N content derived from soil in grasses observed when legume proportion increased from 0 to 20% suggests that the greater presence of legumes may facilitate greater capture of N from the atmosphere, which in turn, increases grass growth, exploring more soil area, and therefore, increasing soil-N uptake. Further research is needed to clarify these relationships which are relevant for understanding grass-legume competition and facilitation.

In conclusion, in this novel field study in warm-climate tropical grass-legume mixtures, moderate legume proportion was identified as maximizing N niche differentiation and overyielding compared with grass monocultures and legume-dominated grasslands. This indicates that efforts to achieve legume proportion greater than 60% may not stimulate greater NDFA, soil-derived N yield, and biomass accumulation in this mixture. These findings could be relevant for warm-climate environments, where seed availability and planting material of legumes are limited and expensive. This empirical evidence demonstrates that low to moderate legume proportions maximize N uptake and biomass accumulation, a finding that can be used to incentivize producers to incorporate legumes into grasslands where efforts to achieve high legume proportions have been too costly or unsuccessful.

Table 3-1. Sequential tests for simple to more complex regression models for different responses. The sequential test first included the year effect (Y, i.e., the intercept of the regression), followed by a linear response of the dependent variable to the legume proportion in pasture (first order regression, L) and then by a quadratic response (second order regression, Q). The contrast tested the intercept, slope, and quadratic parameters between 2022 and 2023 when the effect was significant at $P < 0.10$.

Response	Effect	χ^2	*P*	Contrast	Z-value	*P*
Total shoot biomass (g m^{-2})	Y	35.0	<0.001	β_1	-3.81	<0.001
	L	4.2	0.122	β_2	-	-
	Q	6.2	0.045	β_3	0.81	0.665
Grass shoot biomass (g m^{-2})	Y	43.3	<0.001	β_1	-3.81	<0.001
	L	85.9	<0.001	β_2	-1.53	0.240
	Q	14.2	<0.001	β_3	0.84	0.644
Legume shoot biomass (g m^{-2})	Y	-	-	β_1	-	-
	L	272.6	<0.001	β_2	0.38	0.786
	Q	10.6	0.005	β_3	-0.85	0.457
Total shoot N content (g m^{-2})	Y	15.2	<0.001	β_1	3.66	<0.001
	L	91.3	<0.001	β_2	- 2.33	0.042
	Q	27.4	<0.001	β_3	1.52	0.242
Grass shoot N content (g m^{-2})	Y	18.6	<0.001	β_1	3.47	0.001
	L	25.1	<0.001	β_2	-2.46	0.030
	Q	33.2	<0.001	β_3	1.75	0.157
Legume shoot N content (g m^{-2})	Y	-	<0.001	β_1	-	-
	L	308.6	<0.001	β_2	0.28	0.862
	Q	7.9	<0.001	β_3	- 0.81	0.481

Table 3-2. Sequential tests for simple to more complex regression models for different responses. The sequential test first included the year effect (Y, i.e., the intercept of the regression), followed by a linear response of the dependent variable to the legume proportion in pasture (first order regression, L) and then by a quadratic response (second order regression, Q). The contrast tested the intercept, slope, and quadratic parameters between 2022 and 2023 when the effect was significant at $P < 0.10$.

Response	Effect	χ^2	*P*	Contrast	Z-value	*P*
Total shoot NDFA content (g m^{-2})	Y	0.2	0.647	β_1	-	-
	L	67.4	<0.001	β_2	-0.79	0.674
	Q	13.3	<0.001	β_3	0.33	0.943
Grass NDFA content (g m^{-2})	Y	11.0	<0.001	β_1	2.47	0.029
	L	13.2	0.001	β_2	-1.02	0.519
	Q	17.5	<0.001	β_3	0.67	0.760
Legume NDFA content (g m^{-2})	Y	-	-	β_1	-	-
	L	132.9	<0.001	β_2	-1.54	0.123
	Q	1.7	0.420	β_3	-	-
Total shoot NDFS content (g m^{-2})	Y	0.5	0.469	β_1	-	-
	L	14.3	<0.001	β_2	-0.65	0.762
	Q	5.1	0.078	β_3	0.65	0.761
Grass NDFS content (g m^{-2})	Y	0.2	0.650	β_1	-	-
	L	2.0	0.375	β_2	-	-
	Q	8.4	0.015	β_3	0.71	0.721
Legume NDFS content (g m^{-2})	Y	12.8	0.002	β_1	-	-
	L	47.7	<0.001	β_2	0.06	0.992
	Q	6.9	0.031	β_3	0.31	0.837

Abbreviations: NDFA: N derived from the atmosphere; NDFS: N derived from soil

Table 3-3. Sequential tests for simple to more complex regression models for different responses. The sequential test first included the year effect (Y, i.e., the intercept of the regression), followed by a linear response of the dependent variable to the legume proportion in pasture (first order regression, L) and then by a quadratic response (second order regression, Q). The contrast tested the intercept, slope, and quadratic parameters between 2022 and 2023 when the effect was significant at $P < 0.10$.

Response	Effect	χ^2	*P*	Contrast	Z-value	*P*
Biomass accumulation (g m^{-2})	Y	21.9	<0.001	β_1	0.87	0.654
	L	35.9	<0.001	β_2	-0.13	0.996
	Q	16.6	<0.001	β_3	-0.23	0.982
NDFA yield (g m^{-2})	Y	11.1	<0.001	β_1	-0.96	0.550
	L	76.0	<0.001	β_2	-0.99	0.533
	Q	19.9	<0.001	β_3	0.57	0.815
Soil-derived N yield (g m^{-2})	Y	2.6	<0.001	β_1	-0.77	0.685
	L	26.8	<0.001	β_2	-1.24	0.380
	Q	6.6	0.036	β_3	1.44	0.278

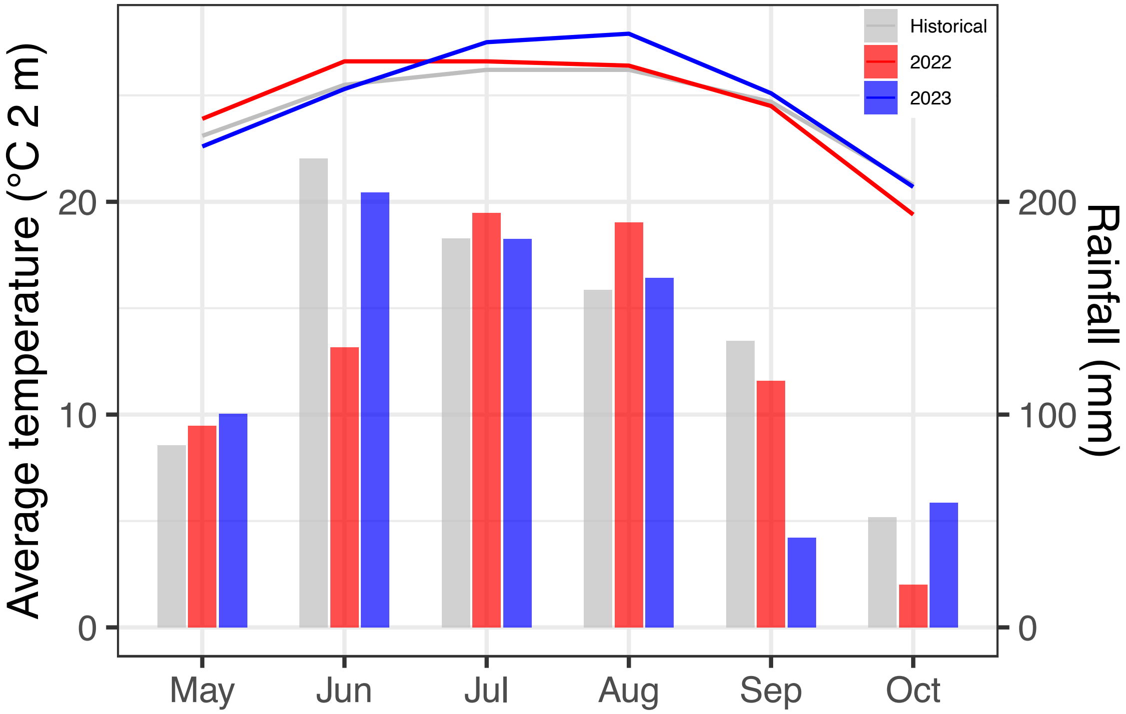

Figure 3-1. Weather data for the growing season of 2022 and 2023 and the historical data (2000-2021). The data were obtained from the weather station of Alachua County, located at 15.4 km from the Beef Research Unit of the University of Florida, Gainesville (https://fawn.ifas.ufl.edu/data/reports).

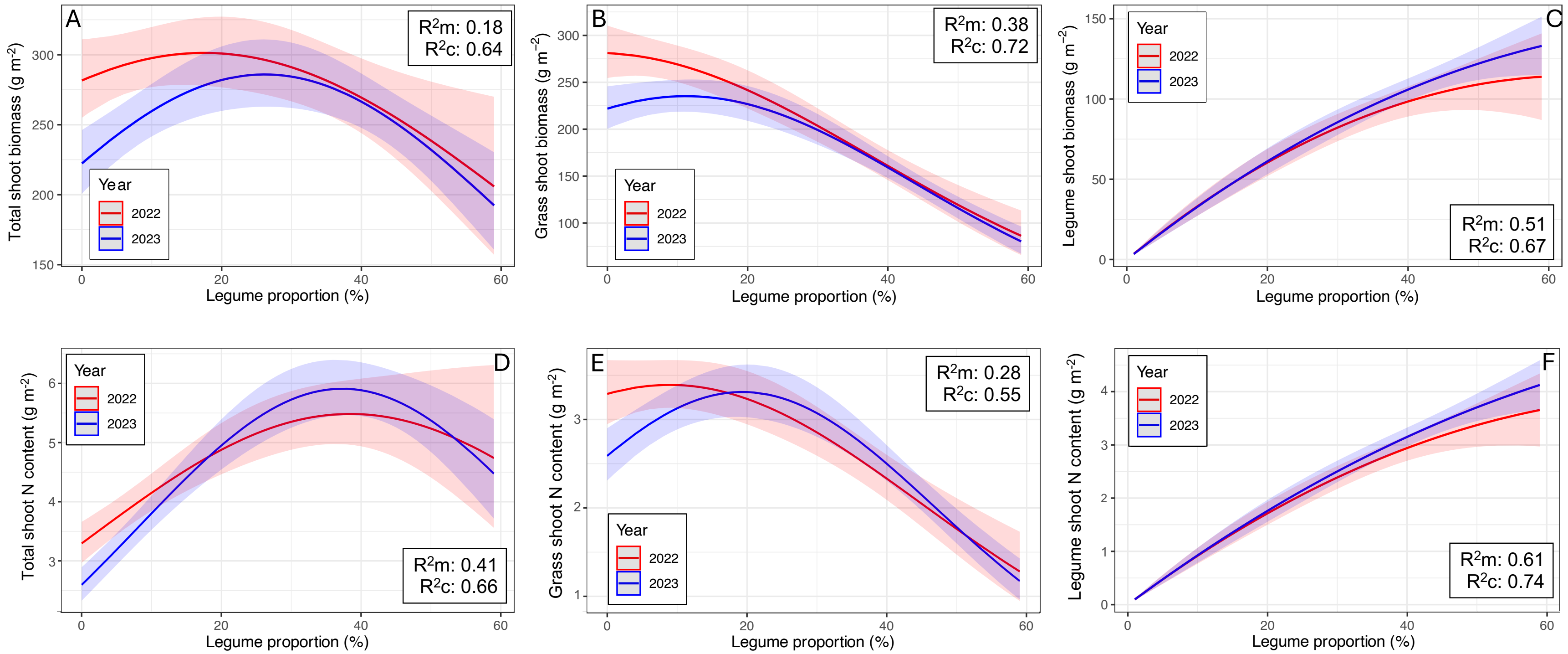

Figure 3-2. Biomass and nitrogen content in response to legume proportion. Shoot biomass (grass + legume; A), grass shoot biomass (B) and legume shoot biomass (C) in response to legume proportion in pasture during 2022 and 2023. Total shoot N content (grass + legume; D), grass shoot N content (E) and legume shoot N content (F) in response to legume proportion in pasture during 2022 and 2023. R^{2}m denotes the marginal R^2 of the model, corresponding to the fixed effect component model, and the R^{2}c denotes the conditional R^2 of the model, accounting for the fixed and random effects components of the model.

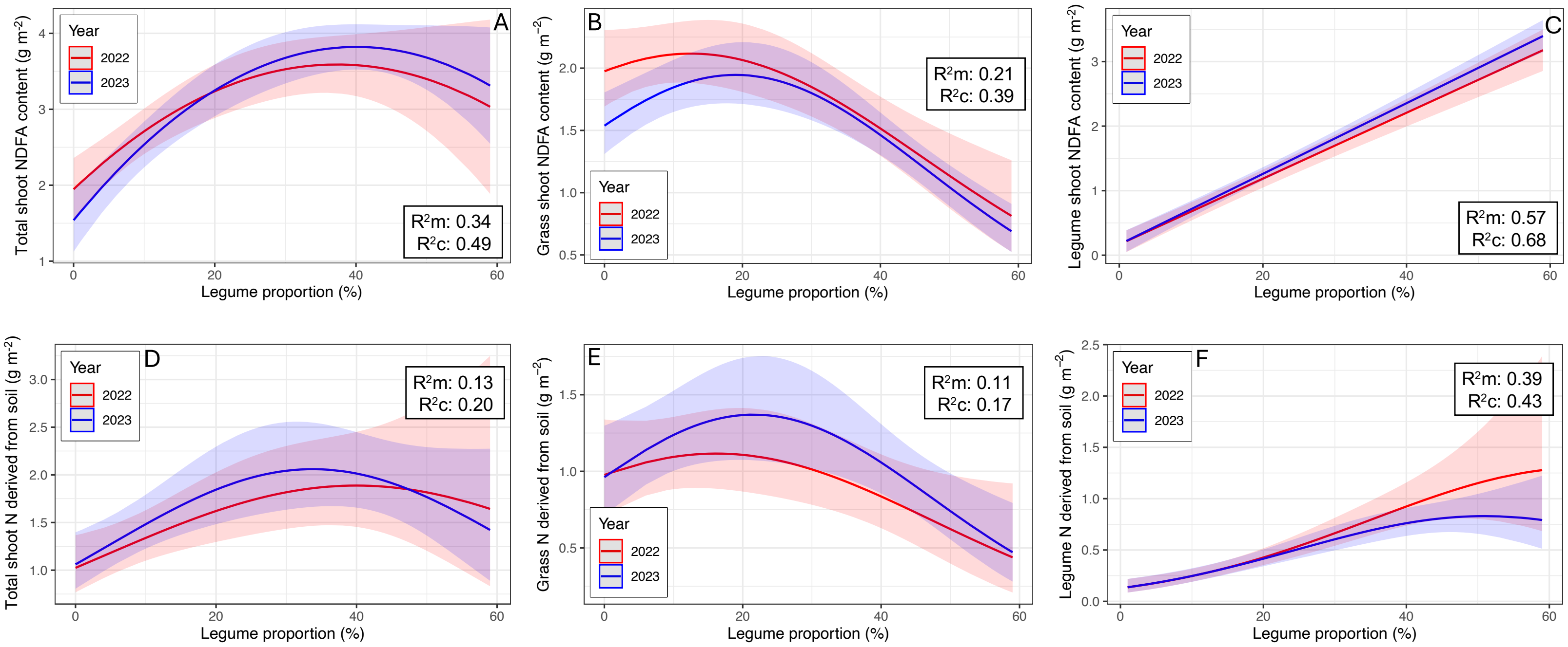

Figure 3-3. Shoot nitrogen from atmosphere and soil. Total shoot N derived from the atmosphere content (NDFA; grass + legume; A), grass shoot NDFA content (B) and legume shoot NDFA content (C) in response to legume proportion in pasture during 2022 and 2023. Total shoot N derived from soil content (grass + legume; D), grass shoot N derived from soil content (E) and legume shoot N derived from soil content (F) in response to legume proportion in pasture during 2022 and 2023. R^2m denotes the marginal R^2 of the model, corresponding to the fixed effect component model, and the R^2c denotes the conditional R^2 of the model, accounting for the fixed and random effects components of the model.

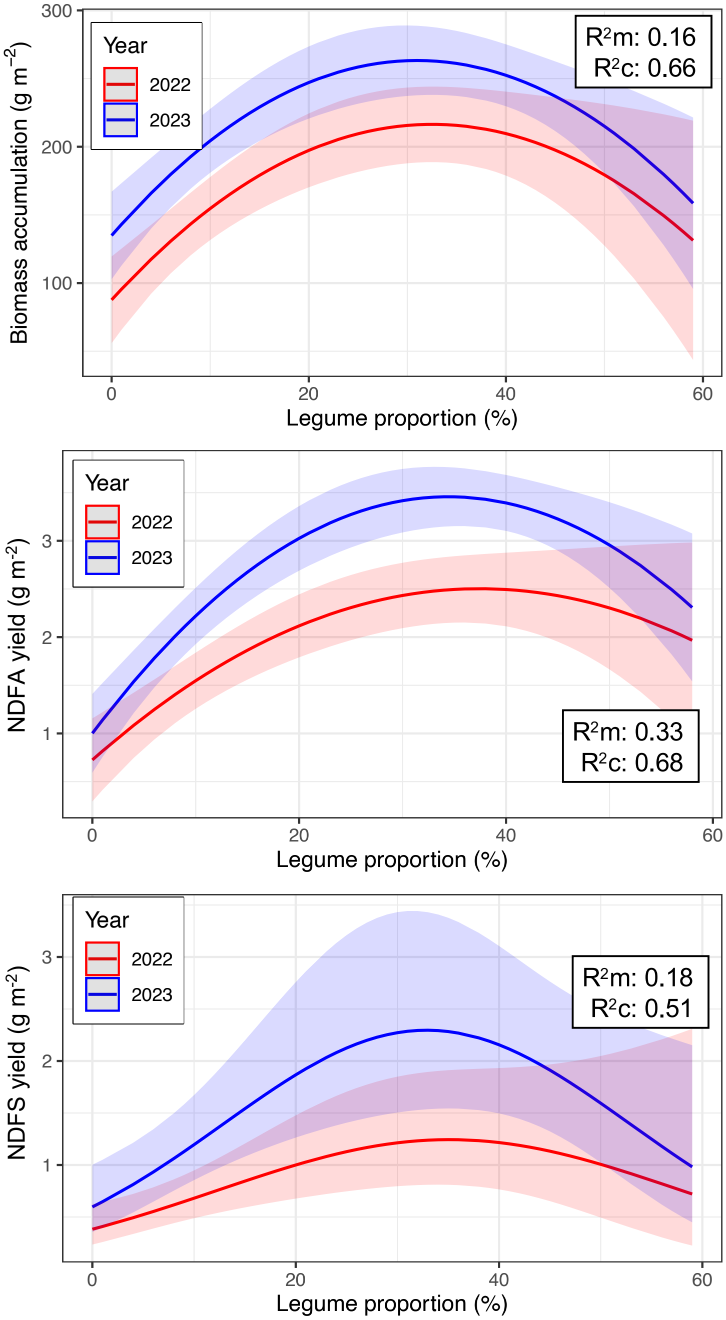

Figure 3-4. Biomass accumulation and atmospheric and soil N yield in response to legume proportion. Biomass accumulation, N derived from the atmosphere (NDFA) and N derived from soil (NDFS) yield are in response to legume proportion in the pasture for 2022 and 2023. R^2m denotes the marginal R^2 of the model, corresponding to the fixed effect component model, and the R^2c denotes the conditional R^2 of the model, accounting for the fixed and random effects components of the model.

CHAPTER 4
NUTRITIONAL AND PHYSIOLOGICAL LIMITATIONS SHAPE THE RADIATION-USE EFFICIENCY RESPONSE OF GRASS-LEGUME MIXTURES IN WARM CLIMATES

Introduction

Global grazing lands occupy about 37% of terrestrial land area (O'Mara, 2012), store a similar proportion of the global soil carbon (Bardgett et al., 2021; Bai and Cotrufo, 2022) and play critical roles in global agricultural systems (O'Mara 2012; Sollenberger et al., 2019). However, with rising pressures from global population and economic expansion, and worsening climate change, there is a need to sustainably intensify the use of grasslands to ensure the delivery of critical ecosystem services, biodiversity conservation and food security (Soussana and Lemaire, 2014; Sollenberger et al., 2019).

Although a variety of practices contribute to the sustainable intensification of grasslands, legume introduction is one of the most promising for boosting productivity, enhancing soil carbon, and reducing fertilizer inputs and associated emissions, particularly for C4-grass dominated subtropical and tropical grasslands (Muir et al., 2011; Sollenberger et al. 2019). Despite the widely recognized value of legume incorporation in both ecological (Tilman et al., 2001; Fornara and Tilman, 2009) and agronomic systems (Jaramillo et al., 2021; Sollenberger and Dubeux, 2022), there is still lack of a strong theoretical and practical basis for identifying a target legume proportion to optimize productivity and other ecosystem services, or for prescribing management interventions to move systems toward that optimum. Here, a key ecophysiological concept, the so-called 'radiation-use efficiency' (RUE, Monteith, 1972; Monteith, 1977), is discussed and applied to the case of identifying optimum legume proportion in a subtropical pasture system, using data from two field experiments linked to a conceptual model.

RUE is an integrative metric, defined as the biomass produced per unit of solar radiation intercepted or absorbed by the canopy in a given period of time (Monteith, 1972; Monteith, 1977). More specifically, RUE can be defined as the net primary production per absorbed or intercepted photosynthetically active radiation (iPAR) and can be reported relative to the proportion of carbon allocated to different organs (i.e., aboveground RUE and total RUE; Bélanger et al., 1994). Measurement of RUE enables greater understanding of species growth and efficiency under different practices and environments (Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009). In addition, RUE can be used to predict and simulate carbon assimilation under potential environments and theoretical scenarios associated with climate change (Peng et al., 2020). Thus, identifying management practices maximizing species and pasture RUE is critical for achieving sustainable intensification of grassland ecosystems and mitigating climate change (Soussana and Lemaire, 2014). For example, while incorporating legumes alongside C4 grasses can help overcome the N limitation on primary productivity (Tilman et al., 2001; Fornara and Tilman, 2009; Kohmann et al., 2018; Garcia et al., 2021; Jaramillo et al., 2021), empirical evidence also indicates that primary productivity, and likely RUE, decreases when legume proportions are too high (Kohmann et al., 2022).

Higher proportions of legume might lead to reduced RUE because species of different photosynthetic pathways and functional groups vary in RUE (Sinclair and Horie, 1989; Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009; Druille et al., 2019). For example, C4 species have greater RUE than C3 species, ranging between 1.0 and 2.0 g MJ^{-1} iPAR under field conditions (Sinclair and Muchow, 1999; Kiniry et al., 2007; Stöckle and Kemanian, 2009), and above 2.0 g MJ^{-1} iPAR under conditions of abundant N and light (Bélanger et al., 1994; Cruz, 1997; Gómez et al., 2012). In general, C3 grass monocultures average 1.4 g MJ^{-1} iPAR (Monteith, 1977; Stöckle and Kemanian, 2009), and when mixed with legumes, RUE ranges from 0.9 to 1.4 g MJ^{-1} iPAR according to N and defoliation treatments (Ojeda et al., 2018; Grigera and Oesterheld, 2021). Typically, the lowest RUE values are seen in native grassland ecosystems, i.e., rangelands, where RUE ranges from 0.2 to 1.0 g MJ^{-1} iPAR (Piñeiro et al., 2006), likely associated with N limitation (Boggiano et al., 2001). Therefore, one of the potential reasons for the reduced RUE with higher legume proportions is the generally lower inherent RUE of legumes compared to C4 grasses (Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009), an effect likely exacerbated under high temperatures in warm climates. Based on this evidence, the emergence of an optimum legume proportion is theoretically illustrated (Figure 4-1), resulting from a decline in resource limitation with higher proportions of legumes (e.g., N) trading off with an increase in physiological limitations (i.e. lower RUE).

Applying the ecophysiological theoretical model to a management practice requires the identification of the optimum legume proportion that overcomes the N limitation without compromising RUE, i.e., the intersection of the curves in Figure 4-1. However, those curves are not merely determined by genetics (species-inherent), as RUE is highly sensitive to environmental conditions and management-related factors, including canopy architecture, temperature, photoperiod, and most importantly, soil moisture and N nutrition (Sinclair and Muchow, 1999). As a matter of fact, legumes can show more horizontal leaves than grasses (Brougham, 1958), intercepting greater solar radiation with similar LAI. This might drive different rates of gross photosynthesis under similar defoliation/grazing managements (Monteith, 1972), change plant successions and species composition (Tilman, 1985), and result in different rates of net biomass accumulation (Parsons et al., 1983). For instance, grasses and legumes might reach an 'optimum' LAI, i.e., intercepting 90-95% of incident light which maximizes rates of net biomass accumulation (Brougham, 1958), at different intervals post-defoliation/grazing. In addition, these factors also interact with each other, often non-linearly, adding complexity to identification of factors limiting RUE and management practices for maximizing pasture RUE, and consequently, primary productivity. Thus, photosynthetic pathway (C3 vs. C4), plant functional group (grass vs. legume), and soil N availability can have conflicting effects on RUE, especially in warm-climate grasslands where N is typically the main limiting resource defining primary productivity (Figure 4-1).

Therefore, the application of the ecophysiological theoretical model (Figure 4-1) in any given environment and management context, requires a quantitative assessment of RUE in response to a legume gradient under different N availabilities. In this context, the use of quantile linear and non-linear regressions and statistical learning methods, such as random forest, are powerful strategies for identifying the limiting effect of legume proportion and N availability on RUE and candidate shape responses (Cade et al., 1999; Cade and Noon, 2003), and for distinguishing and ranking other management and environmental factors limiting RUE (Breiman et al., 2001). Addressing this knowledge gap will increase the understanding of mechanisms affecting RUE and primary productivity of grass-legume mixtures under a range of conditions, guiding selection of management practices that maximize net primary production and efficiency of grassland-based systems. This is particularly important in warm climates where legume adoption has been constrained by poor persistence, limited access to planting material, complex management during establishment, and high costs (Muir et al., 2011; Castillo et al., 2014; Schultze-Kraft et al., 2018; Sollenberger and Dubeux, 2022).

Here, the effects of legume introduction on aboveground RUE (i.e. biomass production) were qualitatively (i.e., presence vs. absence) and quantitatively (i.e., proportion of legume) assessed, with the overall goal of identifying an optimal legume proportion for maximizing RUE in a warm-climate grassland under different N nutrition levels, testing the conceptual model proposed in Figure 4-1. To achieve this goal, two experiments were conducted in long-term C4-bahiagrass monocultures and mixtures of bahiagrass in different proportions with the C3 perennial legume, rhizoma peanut, during three years in Florida, USA. The specific objectives were to (1) quantify canopy characteristics and relationships among canopy height, light interception, and LAI of grass monocultures and mixtures with legume under different defoliation and N fertilization regimes, as determinants of biomass accumulation and plant successions; (2) assess the qualitative effect of legume introduction (grass vs. grass-legume) and the quantitative effect of the relative proportion of legume on aboveground RUE; (3) investigate the potential limiting effect of legume proportion on aboveground RUE under different N and defoliation treatments and according to an ecophysiological theoretical model (Figure 4-1) and distinguish shapes of these responses and potential optimum legume proportions using quantile regressions of linear models (first and second order regression) and non-linear models (plateau-linear and plateau-quadratic regressions); and (4) quantify the relative effect of different management and environmental explanatory variables on aboveground RUE using statistical learning methods.

Materials And Methods

Experimental Site

Two studies were carried out at the Beef Research Unit of the University of Florida, Gainesville, FL (29.74 N, 82.27 W), during the growing seasons of 2021, 2022, and 2023. The experimental area is dominated by Chipley sand soils that are rapidly permeable. Experiment 1 was conducted in small plots under clipping defoliation management from May to October 2021 and 2022, while Experiment 2 was conducted in grazed pastures from June to October 2022 and 2023.

Treatments And Experimental Design

Both experiments were conducted in long-term Pensacola bahiagrass monocultures and mixtures with ‘Ecoturf’ (Experiment 1) or Florigraze (Experiment 2) rhizoma peanut. Experiment 1 plots were arranged in a semi-factorial, split-plot design with four replicates. Bahiagrass monoculture and bahiagrass-rhizoma peanut mixtures, hereafter referred to as “canopy types”, were the whole-plot treatments. The 1-m^2 sub-plot treatments were (1) an undefoliated control receiving no N fertilizer, (2) clipped to 5 cm when LAI > 3 and receiving no N fertilizer, and (3) clipped to 5 cm when LAI > 3 and fertilized immediately after harvest with 20 kg N ha^{-1} as ammonium nitrate. The LAI > 3 threshold was chosen in an attempt to competitively balance the bahiagrass component against competition from the lower-growing, decumbent ‘Ecoturf’ rhizoma peanut cultivar. The rationale was that a longer regrowth interval, as indexed by an LAI ~3, would allow the grass to partially shade out the peanut.

Experiment 2 pastures were planted as rhizoma peanut monocultures in 1983 (Ortega et al., 1992), and afterwards were colonized by bahiagrass creating mixtures with different proportions of each species. The control areas were two pastures of 0.5 ha each that were dominated by bahiagrass (> 95 % of biomass) and did not receive N fertilization. The grass-legume mixtures were represented by 6 pastures of 0.5 ha varying in legume proportion. The eight pastures were continuously stocked during the growing seasons of 2022 and 2023. Throughout the 16 weeks of the grazing period in each year, the grazing intensity was the same among pastures. Grazing intensity was defined based on herbage allowance (Sollenberger et al., 2005), which relates the biomass on offer, in kg DM, and the animal LW. The target herbage allowance in all pastures was 1.5 kg DM kg^{-1} LW (Jaramillo et al., 2021). The realized herbage allowance averaged 1.7 and 1.6 kg DM kg^{-1} LW during 2022 and 2023.

Sampling Procedures And Measurements

Light interception (also referred to as fractional photosynthetically active radiation, fPAR) and LAI were estimated weekly in each 1-m^2 experimental unit of Experiment 1 using a LiCOR LAI-2200C. Simultaneously, the canopy height was measured in each experimental unit using an electronic rising plate meter (Platemeters, NZ). The experimental units were clipped, except for the undefoliated control, when canopies achieved a LAI greater than 3, resulting in four clipping events in 2021 (23 June, 2 August, 7 September, and 25 October) and five in 2022 (31 May, 6 July, 1 August, 15 September, and 26 October). The undefoliated control was clipped on the final sampling day each year, 25 Oct. 2021 and 26 Oct. 2022. The proportion of bahiagrass and rhizoma peanut in total biomass was measured in each experimental unit in June 2021 and July 2022. Species were hand separated and dried at 60°C for 72 hours to determine dry mass and legume proportion (%) in the canopy.

For Experiment 2, three 0.25-m^2 exclusion cages were placed in each of the eight pastures, totaling 24 cages. Sites were selected to represent a wide range of legume proportion in pasture (0 to 60%). The LAI and canopy light interception were estimated weekly in each caged site using a LiCOR LAI-2200C. Simultaneously, the canopy height was measured using a rising plate meter (adapted from Rayburn and Rayburn, 1998). In each cage, herbage was clipped to 1 cm after a four-week regrowth period, resulting in four sampling events during the growing season of 2022 (21 July, 19 August, 16 September, and 14 October) and 2023 (6 July, 3 August, 1 and 29 September). Species were hand separated and dried at 60°C until constant weight to determine plant dry mass and legume proportion (%). After clipping, cages were then relocated to a different site within the 0.5-ha pasture to represent the range in legume proportion; thus, sites caged throughout the growing season were assumed to be independent. All clipped samples were dried, weighed, ground to pass a 1-mm stainless steel screen using a Wiley mill (Model 4 Thomas-Wiley Laboratory Mill, Thomas Scientific), and analyzed for N concentration (g kg^{-1}) as an indication of the N nutritional status of canopies.

The paired data of light interception and LAI were used to fit the Beer-Lambert Law equation (Monsi and Saeki, 1953), as follows:

$$fPAR = 1 - e^{-k*LAI} \tag{4-1}$$

where the light interception (i.e., fPAR) exponentially increases in response to the LAI and the light extinction coefficient (*k*) determines the shape of the curve. Canopies with more horizontal leaves will have greater *k* and may compromise the RUE, as upper leaves may light saturate (Sinclair and Horie, 1989). Using the canopy height paired data, it is possible to assess the canopy density (LAI cm^{-1}), and canopy height needed to intercept a targeted fraction of the PAR. Density of canopies and heights to intercept a target incident PAR have implications for light competition among species under different grazing intensities, which would lead to diverse plant successions.

Solar radiation data (MJ m^2) for different growing periods in each experiment were acquired from the Florida Automated Weather Network (https://fawn.ifas.ufl.edu) for Alachua County. Solar radiation was then converted to PAR, using a factor of 0.48. The aboveground RUE of a given canopy was estimated by solving the following equation:

$$\frac{\Delta\text{biomass}}{\Delta\text{ time}} = \text{PAR} * \text{fPAR} * \text{RUE} \tag{4-2}$$

where Δ biomass / Δ time is the aboveground biomass accumulation in a certain period of time, PAR is the photosynthetically active radiation, estimated as a fraction (0.48) of the solar radiation (MJ m^2), fPAR is the fraction of the PAR intercepted by the canopy, and aboveground RUE is the radiation-use efficiency (g MJ^{-1}). The cumulative iPAR (PAR * fPAR) for a given clipping event was estimated as the sum of weekly assessments of fPAR and cumulative PAR during that week. Therefore, aboveground RUE was estimated as:

$$\text{RUE} = \frac{\left(\frac{\Delta\text{biomass}}{\Delta\text{ time}}\right)}{\sum(\text{PAR} * \text{fPAR})} \tag{4-3}$$

This equation (eq. 4-3) does not consider potential senescence and herbivory losses, which were likely to be negligible, as most clipping events were within a four week period (except for the undefoliated control in Experiment 1). In this scenario, clipping events within a four week period minimize senescence, with sites in the clipped Experiment 1 ungrazed and those in the grazing Experiment 2 caged to avoid grazing events.

The biomass accumulated in each clipping event in Experiment 1 (4 in 2021 and 5 in 2022), was considered as the DM harvested above 5 cm. Potential biomass produced below 5 cm was not considered. The biomass accumulated in each cage in Experiment 2 was estimated as the difference between final and initial biomass for each clipping event (4 in 2022 and 2023). Initial and final biomass were estimated using the double sampling technique (Haydock and Shaw, 1975). Equations of disk height and biomass for each pasture at initial and final clippings were built using direct measurements of biomass and canopy height with the rising plate meter. The equations were then used to predict the biomass in the caged site. The coefficient of determination R^2 averaged 83.2% (± 8.6%) during 2022 and 85.3% (± 9.8%) during 2023.

Data Analysis

To address Objective 1 of quantifying canopy characteristics of grass-only canopies compared with the grass-legume mixtures, as determinants of plant succession and biomass accumulation, differences in *k*, i.e., the light extinction coefficient, were tested between canopy types and treatments fitting non-linear mixed models (Beer-Lambert Law, eq [4-1]) using the *nlme* function of the 'nlme' package (Pinheiro et al., 2021). The response variable was the light interception (fPAR) relative to the canopy LAI. For Experiment 1, differences in *k* between canopy types were tested within treatments (control, clipped with no added N, and clipped plus N fertilization). For Experiment 2, differences in *k* were tested between canopy types. Canopy types and treatments, when present, were included as fixed effects, while plots and sub-plots within plots (Experiment 1) and sites within pastures (Experiment 2) were included as random effects to model the correlation in time within clipping events.

In addition, differences in canopy density were tested, regressing LAI against canopy height, between canopy types within treatments, when present, for each experiment, fitting linear mixed models using the *lme* function of the ‘nlme’ package (Pinheiro et al., 2021). For both experiments, canopy height, pasture, treatment, and their interactions were included as fixed effects, and similarly, plots and sub-plots within plots (Experiment 1) and sites within pastures (Experiment 2) were included as a random effect to model the correlation in time. The slope of the LAI on canopy height, i.e., canopy density (LAI cm^{-1}), was tested between treatments and canopy types using the *emtrends* function of the ‘emmeans’ package.

To address Objectives 2 and 3 of assessing the effect of legume presence and proportion on aboveground RUE under different N and defoliation treatments, the qualitative effects of canopy types and treatments were first assessed on the growing season RUE, performing linear mixed models using *lme* (Pinheiro et al., 2021). Canopy types, treatment (only for Experiment 2), year, and the interactions were included as fixed effects, and experimental unit as a random effect to model the correlation in time. The assumptions of residuals for all models were assessed graphically as suggested by Kozak and Piepho (2018). One of the limitations of qualitative assessments of grass-legume mixtures is that the species proportion can vary along a continuous axis (in an extreme case from 1 to 99%) and may have implications for RUE and biomass accumulation (e.g., Nyfeler et al., 2009; Grigera and Oesterheld, 2021). Therefore, the effect of legume proportion on RUE for both experiments was assessed using regression analysis. This analysis provides a better understanding of the effect of legume proportion on RUE and allows identification of the optimum species proportion maximizing canopy responses. All observations for each experiment were considered, including years and clipping events.

RUE was regressed on legume proportion separately for each experiment pooling Year 1 and Year 2 data (overall response), and then individually for each year. In addition, quantile regressions were performed, which allows testing of the median response of RUE vs. legume proportion (50th quantile), the frontier response of RUE vs. legume proportion where, theoretically, the legume proportion is the only limiting factor of RUE (95th quantile, i.e., under no limiting conditions; Cade et al., 1999; Cade and Noon, 2003), and the scenario where resources other than legume proportion limit RUE (10th quantile). To distinguish the ‘best’ candidate model, and therefore, shape of RUE in response to legume proportion which would allow identification of potential optimum legume proportions, linear and non-linear models were performed. The candidate models were first and second order linear regressions and plateau-linear and plateau-quadratic non-linear regressions. These four candidate models were performed for each experiment, for the Year 1 and Year 2 pooled data (overall response) and for each year within experiments, and for each quantile (10th, 50th and 95th). The ‘best’ candidate quantile regression model in each case defining the shape of the response was selected based on the lowest Akaike Information Criteria (AIC), the corrected AIC (AICc; Burnham and Anderson, 2002) and root mean square error (RMSE; Table A-3, A-4).

To study predictors of RUE other than canopy type and treatments (Objective 4), a Random Forest regression model was performed, including management- and environmental-related predictors, using the *randomForest* function of the 'randomForest' package (Breiman, 2001; Liaw and Wiener, 2002). This non-parametric machine learning algorithm does not assume specific data distributions and is suitable for large numbers of predictors, building hundreds of regression trees based on random samples of the data set and random selection of predictors included in the model in each node of the tree. The average of all trees built then conform the predictor of the trained data (~70% of the data), which is tested using the remaining data (~30%). This machine learning tool allows ranking of predictor variables based on the prediction accuracy of the model when it is permuted, where the larger the increase in the mean square error of the model, the more important the variable. One model for both experiments was performed, pooling all data to have more robust predictors of the RUE in these tropical environments. Before pooling, RUE of each experiment was scaled to the same mean (0) and standard deviation (1).

The final model included legume proportion (% of rhizoma peanut in present biomass), N concentration (g N kg^{-1} DM), and average LAI as management practice predictors, and average maximum temperature, extreme degree hours (hours during the growing period above 30°C per day of regrowth), vapor pressure deficit (kPa) and rainfall (mm per day of regrowth) during the growing period, as environmental predictors. In addition, experiment was included as a factor to control other factors inherent to each experiment not accounted for with the explanatory predictors included in the model. The number of trees in the forest (ntree) was set at 500, and the number of predictors available during the growing process was defined as 3 after a 10-fold cross-validation. The model was trained with a 70% random sample of the data and then tested with the remaining 30%.

Results

Canopy Attributes

Studying determinants of biomass production and botanical composition and plant succession under different defoliation treatments showed that canopies of both experiments and types intercepted 90% of PAR at LAI between 3.8 and 4.1 (Figure 4-2 and 4-3). In all cases, the light extinction coefficient (*k*) was statistically greater in grass-only canopies compared with the grass-legume mixtures. Despite having similar LAI intercepting a target 'optimum' 90% of PAR, the canopy density, expressed as LAI units per canopy cm (LAI cm^{-1}), differed between canopy types (Figure 4-2 and 4-3). Data are not comparable between experiments, but except for the N-fertilized, clipped treatment in Experiment 1, mixtures showed a 50-60% denser canopy than the grass-only canopies. As a result, the same target LAI is achieved with shorter canopy heights in mixtures than in grass-only canopies.

Aboveground Radiation Use Efficiency

RUE was greatest in clipped N-fertilized treatments (0.68 ±0.05 g MJ^{-1}), intermediate in unfertilized, clipped (0.41 ±0.05 g MJ^{-1}), and least in the undefoliated control (0.32 ±0.05 g MJ^{-1}; Figure 4-4, $P < 0.001$). By contrast, RUE differed minimally among canopy types (0.46 vs 0.47 ±0.05 g MJ^{-1}; for grass-legume mixtures and grass-only canopies, respectively; $P = 0.495$), and there is no evidence of meaningful interaction between treatment and canopy type. For Experiment 2, the RUE was greater under grass-legume mixtures (0.97 ±0.12 g MJ^{-1}) than under grass-only canopies (0.43 ±0.12 g MJ^{-1}; $P < 0.001$).

Quantitatively assessing the RUE in response to legume proportion in pasture across Experiments 1 and 2 (Figure 4-5 and 4-6), it was consistently found that legume proportions greater than ~30% of biomass may compromise canopy RUE. Assessing the results by experiment, on average for 2021 and 2022, the median (50th quantile) of RUE in Experiment 1 showed an apparent linear plateau response to legume proportion in pasture (Table A-3). This model showed the lowest AIC and RMSE compared with the other candidate models. According to this model, RUE was not affected by the proportion of legume up to 26% (averaging 0.60 g MJ^{-1} across treatments). However, further increases in legume proportion resulted in a linear decrease in RUE at a rate of -0.009 g MJ^{-1} per unit increase in legume, suggesting a limitation in RUE associated with the increase in legume proportion in the canopy. For example, increases from 30 to 50% in legume proportion determined decreases in RUE of 0.18 g MJ^{-1}. For 2021, considering the plateau-linear model, RUE averaged 0.70 g MJ^{-1} until 30% legume and then decreased at a rate of -0.009 g MJ^{-1} per percentage unit increase in legume. If other plausible models are considered, such as the quadratic regression, the model indicated that RUE was maximized at 23% legume, and quadratically decreased beyond this optimum. For 2022, the best model was the plateau-linear model, where RUE averaged 0.50 g MJ^{-1} until 18% legume, and then decreased at a rate of -0.006 g MJ^{-1} per percentage of legume.

The frontier response , i.e., 95th quantile, indicated a similar pattern to the median response (Figure 4-5; Table A-3). Averaging both years, RUE showed a plateau-linear response. According to this candidate model, RUE plateaued at 1.17 g MJ^{-1} below 26% legume; above this level, it decreased at 0.016 g MJ^{-1} per percentage unit of legume. This suggests that under non-limiting conditions, where theoretically legume is the only limiting factor, RUE also decreased beyond a critical proportion of 26% legume. In this case, the rate of decrease in RUE was accentuated compared to the median response (-0.016 vs. -0.009 g MJ^{-1}, respectively). For 2021, and based on an apparent plateau-linear regression, RUE plateaued at 1.29 g MJ^{-1} until 43% legume, and then decreased at 0.038 g MJ^{-1} per percentage unit of legume. The other plausible model in this case, which minimized the RMSE, was a plateau-quadratic regression (Table A-3). According to this model, RUE plateaued until 48% legume, and quadratically decreased above this level. For 2022, the candidate plateau-linear model indicated that RUE was 0.94 g MJ^{-1} until 28% legume, and then decreased at a rate of -0.012 g MJ^{-1} per percentage unit increase in legume. On average for both years, the 10th quantile, i.e., when RUE is limited by factors other than legume proportion, showed a linear decrease in RUE at a rate of -0.002 g MJ^{-1} per each unit increase in legume from a level of 0.23 g MJ^{-1} at 0% legume. Considering this linear model, RUE decreased from 0.24 (2021) and 0.22 g MJ^{-1} (2022) at a rate of 0.001 (2021) and 0.002 g MJ^{-1} (2022) per one unit increase in legume proportion.

For Experiment 2 and on average for 2022 and 2023, the median RUE (50th quantile) showed an apparent quadratic response to legume proportion (Figure 4-6; Table A-4). This quadratic response was also found as the 'best' model in each year, and for the 95th quantile (Table A-4). The RUE averaged 0.53 g MJ^{-1} at 0% legume (i.e., intercept of the regression; 0.45 and 0.57 g MJ^{-1} for 2022 and 2023, respectively), similar to the intercept in Experiment 1. On average, RUE peaked at 31% legume in pasture (33 and 28% for 2022 and 2023, respectively) and then quadratically decreased. The RUE at the optimum legume proportion of 31% legume was 1.10 g MJ^{-1}, overyielding the 0% legume and 60% legume pastures by 110 and 86%, respectively. Under unlimited conditions during the growing season, i.e., 95th quantile, the RUE response was similar to the median, averaging 1.23 g MJ^{-1} at 0% legume, similar to the 95th quantile for Experiment 1 and 116% greater than the median response (1.31 and 1.12 g MJ^{-1} for 2022 and 2023, respectively). The RUE of the 95th quantile peaked at 27% legume (33 and 27% for 2022 and 2023, respectively), and then decreased at a greater rate than for the median. Lastly, when limiting resources included those not associated with the legume proportion, i.e., 10th quantile, based on the model fit metrics, the RUE quadratically increased with legume proportion until 30%.

Management And Environmental Explanatory Variables

The Random Forest model accounted for a moderate 67.5% of total RUE variability. The RMSE of the train and test data were 0.57 and 0.59, suggesting no overfitting. The most important variables explaining RUE variability were rainfall, N concentration, legume proportion, and average maximum temperature (Table 4-1). Exploring relationships between predictors and RUE, it was found that, as expected, RUE increased with rainfall up to a level of 5 mm day^{-1} (Figure 4-7), while RUE decreased with increases in maximum temperature and increases in legume proportion beyond ~30%, confirming previous results. Interestingly, RUE increased with plant N concentration up to ~17-18 g kg^{-1} and quadratically increased with average LAI, maximizing between 3 and 4.

Based on these findings, and considering the theoretical model of RUE for grass-legume mixtures in warm climates which integrates N levels and environmental factors (Figure 4-1), ~30% was identified as an ‘optimum’ legume proportion where RUE is maximized for canopies of bahiagrass and rhizoma peanut receiving no N (Figure 4-6). Below this optimum, the RUE of this warm-climate pastures with low proportion or no legumes is limited by a nutritional N deficiency, which is overcome with N fertilization (as shown by the difference in shape response between N-fertilized and unfertilized pastures, Figures 4-5 and 4-6). However, despite the N fertilization treatment, the RUE decreased beyond 30%, (Figures 4-5, 4-6, and 4-7), suggesting that as proportion of C3 legumes exceeds 30%, RUE in this warm-climate pasture will decrease, likely associated with a physiological limitation of the RUE (Figure 4-1).

Discussion

The current demand to achieve sustainable intensification in agricultural systems requires the identification of practices optimizing resources use efficiency (Soussana and Lemaire, 2014). In this context, RUE is a uniquely integrative metric to assess management practices, encompassing the net response to environmental conditions and management practices. In the case of subtropical pastures, previous work has strongly supported that introducing legumes into grass-only systems enhances primary and secondary production, and other ecosystem services (Jensen et al., 2012; Sollenberger et al., 2019; Sollenberger and Dubeux, 2022). An important next step in the sequence of intensification levels is to identify whether there is an optimal range of legume proportion to aim for, a currently unanswered question in warm-climate grasslands (Sollenberger et al., 2019). In this study, approximately 30% legume proportion appears to maximize aboveground RUE across two distinct experiments within the study system, adding robustness to the findings. Consistent with the theoretical model introduced in Figure 4-1, approximately 30% legume proportion substantially alleviates N limitation, without the need of N fertilizer inputs, while the RUE is not compromised due to an excessive proportion of C3 species in these subtropical grasslands of bahiagrass and two different cultivars of rhizoma peanut. Thus, the study suggests that practical management should target moderate legume proportions to maximize primary productivity of this warm-climate grassland ecosystems. The proposed ecophysiological theoretical model should be tested in other warm-climate regions including different tropical grass and legume species, which highlights the contribution of this research and its generalizable practical and theoretical findings.

While qualitative comparison of canopy types does not provide insight into the effects of legume proportion on RUE, quantitative analyses testing linear and non-linear regressions of responses to legume proportion consistently showed a decrease in canopy RUE when legume proportion was above ~30% (Figure 4-5 and 4-6). The difference in the shape of the response, according to model fit metrics, between experiments was likely due to N nutrition level at low legume proportion (Figure 4-7). In Experiment 1, the N concentration did not vary along the plateau of the median and 95th quantile response (Figure 4-5). Therefore, greater N concentrations associated with the clipped, N-fertilized treatment at very low legume proportion maintain the RUE along the initial legume gradient. In Experiment 2, the N concentration increased with initial increases in legume proportion (Figure 4-6), likely explaining the increases in RUE in both 50th and 95th quantile regressions until ~30% legume. Beyond this point and in both experiments and for the two years, the RUE declined either linearly or quadratically in spite of the N concentration.

Here, this reduction in aboveground RUE can be inferred as a physiological limitation of the canopy (Figure 4-1). In other words, an excessive proportion of C3 species (i.e., legume) in this warm-climate environment compromises the RUE of the canopy. Thus, proportions of about 30% legume are sufficient to maximize RUE and therefore, primary production in these pastures. A similar optimum proportion has been identified for temperate grass-legume mixtures (Nyfeler et al., 2009; Lüscher et al., 2014). In this case, and in particular for rhizoma peanut, this optimum range supports and validates the recently proposed strip-planting method (Castillo et al., 2014; Shepard et al., 2022), which aims to achieve a meaningful but far from dominant legume presence. Despite the perception that the more legume the better, targeting this realized optimum proportion will reduce the high establishment costs of this species as lesser area needs to be planted within a pasture, i.e., 50% of the area, encouraging producers to incorporate the legume into more area of bahiagrass pastures in warm-climate regions. Further research is needed to clarify whether this optimum target of realized legume proportion differs with different N levels and age of stand.

One of the main contrasting findings in the two experiments was that aboveground RUE did not statistically differ between canopy types in the clipping trial (Experiment 1; Figure 4-4), but it did in the grazing study (Experiment 2). Likewise, there was no positive effect of introducing legumes on RUE in the clipping trial, likely associated with a lower amount of biologically fixed N that was cycling in the unfertilized control and in the clipped mixtures. In contrast, there was a statistically significant increase in RUE when legumes were introduced into the grass-only canopy type in the grazing trial. One potential explanation is that in Experiment 1, the plots were clipped, and the biomass was removed, reducing the amount of N fixed by the legume that was returned through litter decomposition (Kohmann et al., 2018). In contrast, return of livestock excreta and litter decomposition in the grazing study likely contributed significantly to N cycling (Garcia et al., 2021).

Studying canopy attributes of the grass-only and grass-legume mixed canopies (Figure 4-2 and 4-3) revealed similar LAI was needed in both canopy types to intercept the same fraction of PAR (~4). These results differ from the classical study of Brougham (1958), where pastures including legumes intercepted the same fPAR with lower LAI, associated with more horizontal leaves of legumes (white clover [*Trifolium repens* L.] in this case). Results indicate that, both grass-only and grass-legume swards of very similar light coefficient extinction may approach photosynthetic light saturation at similar LAI (Sinclair and Horie, 1989). Therefore, grass and grass-legume LAI would not be a covariate affecting RUE.

Despite the similar ‘optimum’ LAI between canopy types on intercepting PAR, mixtures showed denser canopies than only bahiagrass in both experiments. This information derived from first order linear regressions can also be tested with power law models, likely indicating very similar results. The fact that canopy types have similar LAI when intercepting the same fPAR but achieve the same LAI at different canopy heights, and the observed curvilinear response of RUE on LAI (Figure 4-7) have implications for botanical composition, plant succession and grazing management. Similar to what was empirically reported by Spasiani et al. (2023), the results suggest that greater grazing intensity (i.e., shorter post-grazing canopy height) favor legume proportion in pastures due to light competition (greater LAI and consequently RUE, at same height). Moreover, this advantage for the legume would be intensified under N-limited conditions because of the ability of rhizoma peanut to fix a significant amount of atmospheric N (Dubeux et al., 2017; Jaramillo et al., 2021). In these conditions, it is expected that legumes will outcompete grasses for light and N, two main resources affecting plant succession (Tilman, 1985).

Exploring relationships between environmental-related predictors of RUE with the Random Forest regression, showed that RUE increases with water availability (i.e., rainfall; Sinclair and Muchow, 1999). In addition, RUE increases with average maximum temperature (Newman et al., 2001); however, maximum temperatures above 33-35°C decrease net CO_2 assimilation rate (Sage and Kubien, 2007), likely affecting RUE. This suggests that in the current climate change scenario, increasing legume biomass production with increasing atmospheric CO_2 concentration is diminished by the increases in temperature (Newman et al., 2001; Sollenberger and Kohmann, 2024), compromising RUE in grass-legume mixtures. Exploring management-related predictors of RUE, the findings (Figure 4-5 and 4-6) are confirmed that RUE decreases with legume proportion above 30%. In terms of N nutrition level, RUE plateaus above 20 g kg^{-1} N concentration, suggesting no productive, environmental, and economic benefits beyond this point. Lastly, RUE in response to average LAI shows the classical sigmoidal curve (Saul and Chapman, 2002; Sollenberger et al., 2012), considering biomass and time (here RUE and LAI).

On average, the observed aboveground RUEs are lower than the average reported for other pastures (Ojeda et al., 2018; Druille et al., 2019; Grigera and Oesterheld, 2021). This difference can be attributed to an N limitation, in particular for the unfertilized bahiagrass and mixture in Experiment 1 and the unfertilized bahiagrass in Experiment 2. In addition, differences in methodology could have affected the findings. For example, Bélanger et al. (1994) and Cristiano et al. (2015) reported that 16-55% of the total biomass produced is ignored when assessing RUE only with the aboveground biomass accumulation. This could be important in the long-term pastures (+40 years; Ortega et al., 1992), which differ from studies of short-term perennial pastures assessed for RUE (2 to 3 years), where plants prioritize shoot biomass allocation. Regardless of this limitation, the results were consistent across different experiments and years and identified ~30% as the optimum in legume proportion in this warm-climate grassland. In addition, the analysis assumes a constant energy concentration for each g of aboveground DM produced per MJ (e.g., same digestibility and crude protein). However, increasing the proportion of legumes in pasture increases the energy concentration of each g of aboveground DM produced, as measured by digestibility and crude protein, up to a certain legume proportion (e.g., ~45% in Kohmann et al., 2022). Hence, adjusting the RUE based on energy concentration would increase the optimum legume proportion from 30% to approximately 40%. Beyond this point, it is not expected that energy concentration compensates for the lower RUE, as both digestibility and crude protein tend to plateau at higher legume proportions (Kohmann et al., 2022).

In conclusion, results from two different experiments indicate that aboveground RUE is limited by N when legume proportion is below 30% in these unfertilized canopies. When legume proportion exceeds 30%, a physiological limitation to RUE is imposed due to replacement of the C4 grass (bahiagrass) with a C3 legume (rhizoma peanut). This suggests that within the optimum range, rhizoma peanut proportion overcomes the N limitation, without the need of N fertilizer, while not compromising RUE due to a physiological limitation. Therefore, based on the empirical evidence, moderate legume proportion could be used as a target to maximize productive, environmental and economic outcomes in these subtropical grasslands of bahiagrass and rhizoma peanut. Further research is needed to determine if this optimum holds across different environments and species. Nonetheless, the proposed ecophysiological theoretical model could serve as a reference for other warm-climate regions.

Table 4-1. Relative importance of environmental- and management-related predictors included in the final regression model.

Predictor		% Increase in mean square error
Environment	Rainfall (mm day^{-1})	37.0
	Average Maximum T (C)	22.8
	Extreme degree hours	21.9
	Vapor pressure deficit (kPa)	17.3
Management	N concentration (g kg^{-1})	28.6
	Legume proportion (%)	23.2
	Average LAI	16.6
	Experiment	13.5

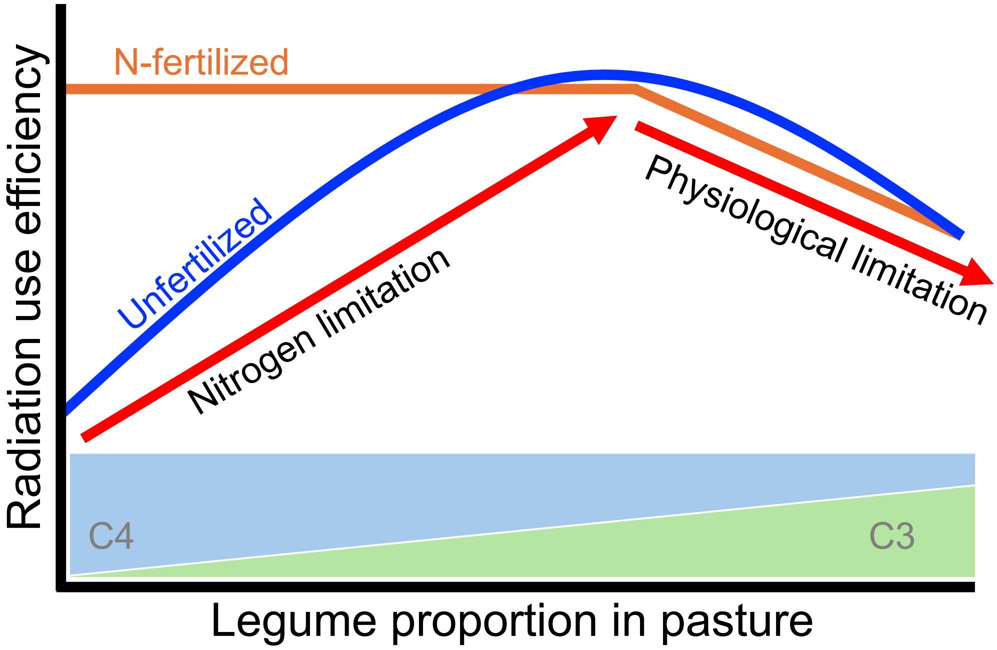

Figure 4-1. Theoretical model of factors limiting radiation-use efficiency (RUE) for bahiagrass-rhizoma peanut mixtures in a subtropical environment. The N-fertilizer RUE is the RUE for bahiagrass in monoculture (at 0% legume in x-axis) and in mixture with Florigraze or Ecoturf rhizoma peanut. The unfertilized RUE corresponds to the RUE for bahiagrass in monoculture (at 0% legume in x-axis) and in mixture with Florigraze or Ecoturf rhizoma peanut along their proportion in the pasture.

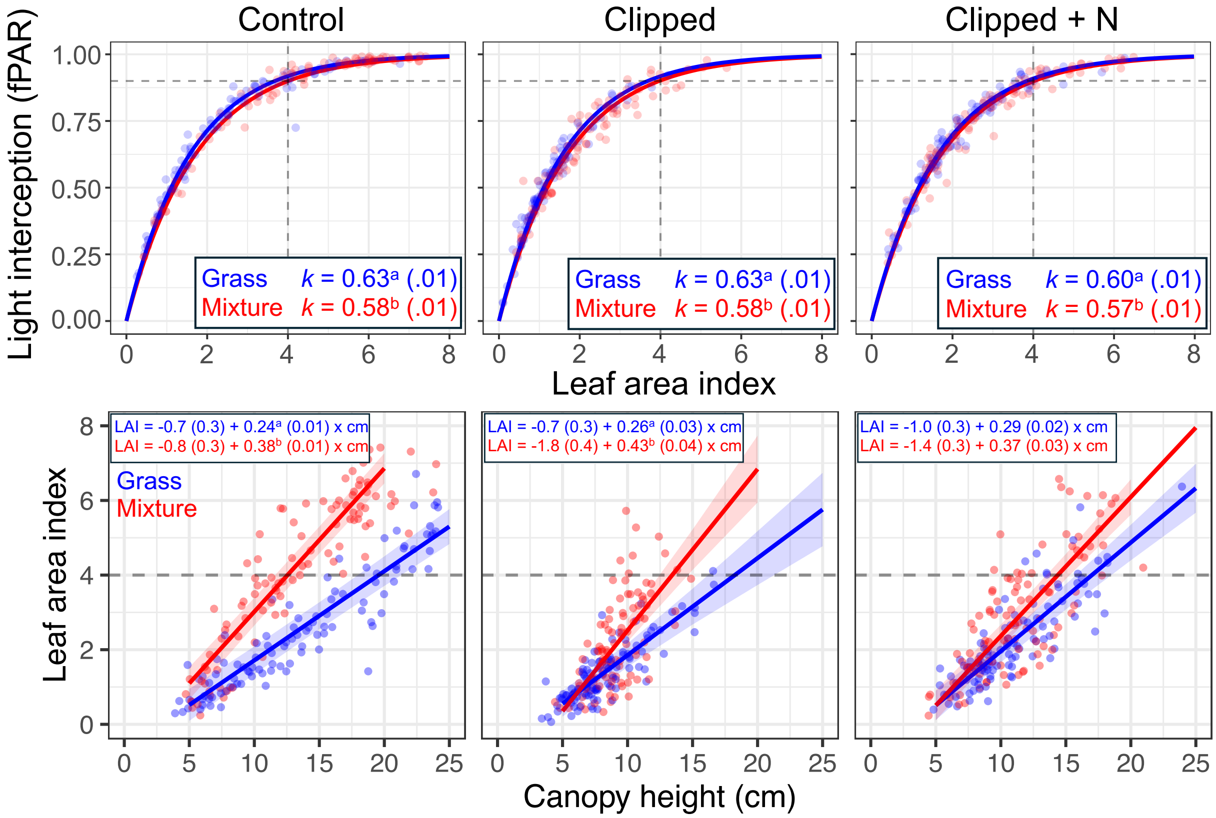

Figure 4-2. Canopy attributes in the clipping study (Experiment 1). The upper panel shows the light interception response to leaf area index (LAI; as a fraction of photosynthetically active radiation [fPAR]) for each defoliation and fertilization treatment and canopy type. Different letters following k (SE) indicate differences between canopy types within treatments at $P < 0.05$. The lower panel shows the canopy density for each defoliation and fertilization treatment and canopy type, expressed as units of leaf area index per unit of canopy height (LAI cm^{-1}). Different letters following the slope (SE) of the regression indicate differences between canopy types within treatments at $P < 0.01$.

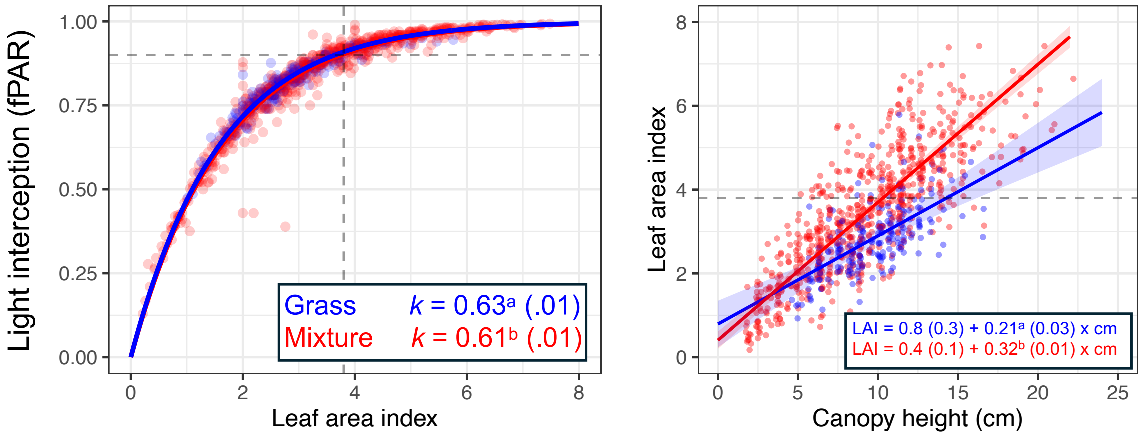

Figure 4-3. Canopy attributes in the grazing study (Experiment 2). The left panel shows light interception response to leaf area index (LAI; as fraction of photosynthetically active radiation [fPAR]) for each canopy type. Different letters following k (SE) indicate differences between canopy types at *P* < 0.01. The right panel shows the canopy density for each canopy type, expressed as units of leaf area index per unit of canopy height (LAI cm^{-1}). Different letters following the slope (SE) of the regression indicate differences between canopy types at *P* < 0.01.

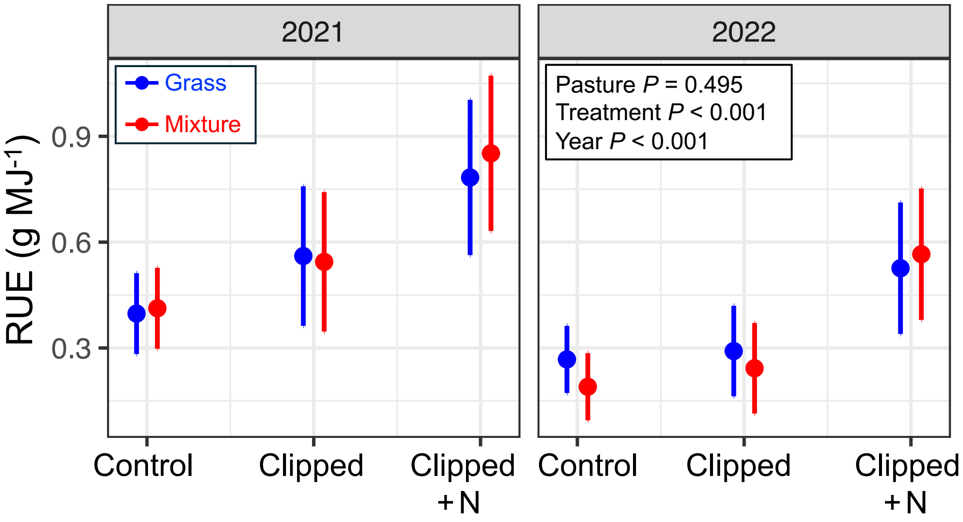

Figure 4-4. Canopy radiation use efficiency (RUE) in response to treatments and canopy type for the clipping study during 2021 and 2022. Treatments are the undefoliated control, unfertilized clipped and clipped + N, and canopy types include the only grass and the grass-legume mixture. The *P* values correspond to the significance of main effects. Vertical lines indicate ± 95% confidence intervals.

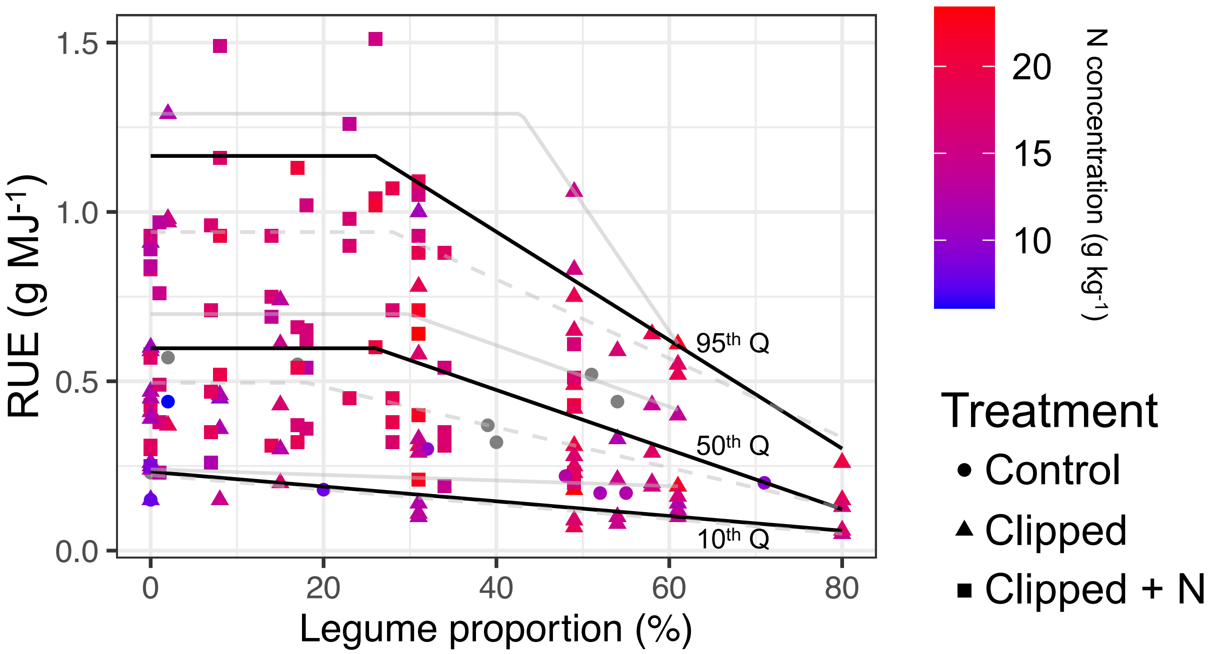

Figure 4-5. Canopy RUE response to legume proportion in the clipping study (Experiment 1) during 2021 and 2022. The N concentration indicates the g kg^{-1} N of the aboveground biomass. The three quantile regressions report the median response in RUE to legume proportion (50th), the frontier response (95th) and the limited response (10th). Solid (2021) and dashed (2022) grey lines indicate the best fitted regression for each year.

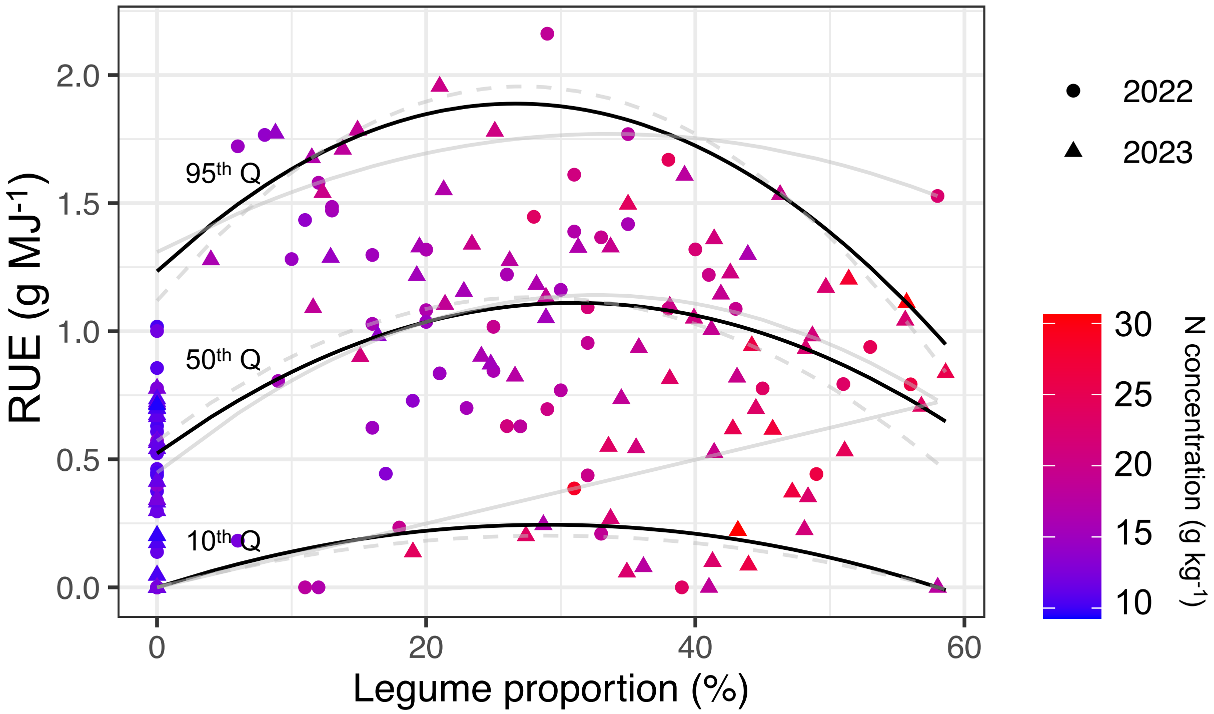

Figure 4-6. Canopy RUE response to legume proportion for the grazing study (Experiment 2) during 2022 and 2023. The N concentration indicates the g kg^{-1} N of the aboveground biomass. The three quantile regressions report the median response in RUE to legume proportion (50th), the frontier response (95th) and the limited response (10th). Solid (2022) and dashed (2023) grey lines indicate the best fitted regression for each year.

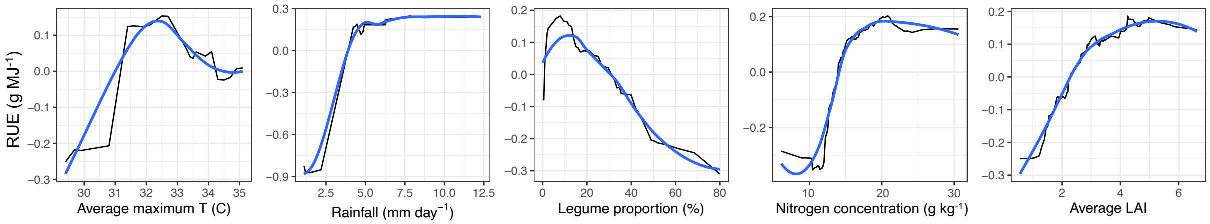

Figure 4-7. Partial-dependence plots of scaled RUE in response to the most important environmental and management explanatory variables. Most important environmental-related predictors included in the model include average maximum temperature and rainfall, while management-related predictors include legume proportion, N concentration and average LAI.

CHAPTER 5
OPTIMUM LEGUME PROPORTION FOR PLANT AND ANIMAL PRODUCTION IN A WARM-CLIMATE GRASSLAND

Introduction

Integrating legumes into warm-climate, grass-based systems may increase the amount of N cycling, forage nutritive value, and animal performance, while reducing or avoiding inputs of N fertilizer (Sollenberger and Dubeux, 2022). These positive benefits have significant relevance in enhancing delivery of a range of ecosystem services (Peoples et al., 2019; Sollenberger et al., 2019). For example, cattle grazing bahiagrass gained about 350 g day^{-1} (Sollenberger et al., 1988; Stewart et al., 2007; Jaramillo et al., 2021), but when rhizoma peanut was introduced into bahiagrass pastures, animal performance increased 70% to ~600 g day^{-1} (Valencia et al., 2001; Jaramillo et al., 2021). Although N fertilization increases grass forage accumulation and pasture stocking rate, greater animal daily gain due to adding legumes may result in similar or even greater liveweight gain per hectare than for the fertilized bahiagrass (Jaramillo et al., 2021; Pereira-Neto et al., 2024). This outcome can contribute to reductions in greenhouse gas emissions by avoiding or decreasing emissions associated with production of N fertilizer (Peoples et al., 2019) and with field N losses from volatilization and denitrification (Sollenberger et al., 2019). Moreover, increasing beef production per hectare with fewer but more efficient animals is an effective strategy for reducing enteric methane emissions from livestock systems (Herrero and Thornton, 2013).

Limited availability of seed and planting material, high establishment costs, more complex management, and poor persistence have limited legume adoption by producers in warm-climate regions (Castillo et al., 2014; Schultze-Kraft et al., 2018; Aryal et al., 2021). Another more subtle constraint to legume use may be the perception that legume contribution is dependent upon maximizing legume proportion in pastures. Yet, previous studies have shown this goal is costly and may not always provide advantages from production, environmental, or economic perspectives (Nyfeler et al., 2009; Lüscher et al., 2014; Kohmann et al., 2022).

Quantitative studies on temperate grass-legume mixtures identified 30-50% legume as an optimum range to maximize several soil-plant-animal-atmosphere outcomes (Nyfeler et al., 2009; Nyfeler et al., 2011; Lüscher et al., 2014). Kohmann et al. (2022) found that biomass accumulation of bahiagrass-rhizoma peanut mixtures in the subtropics decreases as legume proportion increases, but nutritive value increases up to 45-50% legume. Hence, the trade-off between biomass accumulation and nutritive value suggests an optimum proportion of less than 50% legume in warm-climate pastures. Interestingly, moderate rhizoma peanut proportions can be achieved with strip-planting methods that reduce establishment cost by planting 50% or less of the pasture area to the legume (Castillo et al., 2014; Jaramillo et al., 2021).

Moderate legume proportion in pastures can be partially compensated for by animal selection, in some cases without compromising animal gain (Valencia et al., 2001; Jaramillo et al., 2021). In general, cattle and sheep prefer legumes over grass in temperate and tropical grass-legume mixtures (Parsons et al., 1994; Lascano, 2000; Valencia et al., 2001; Rutter, 2006; Jaramillo et al., 2021; Kohmann et al., 2022). However, some exceptions exist for less palatable high-tannin legumes (Lascano, 2000), or when legumes are present in high proportions in pastures (Kohmann et al., 2022). In the latter scenario, animals may actively seek out and select grasses (Parsons et al., 1994), a response likely attributable to ingestive and post-ingestive feedback processes, limiting the benefit of a high proportion of legume in the pasture (Provenza et al., 2007). Thus, under appropriate grazing management (Sollenberger et al., 1987), cattle selectivity could well be a zero-cost tool compensating for lesser legume proportion in pastures or overcoming the limitations associated with a high proportion of legumes.

Most scientific evidence supports legume introduction into grass-based pastures, but quantitative analyses of different legume proportions in pasture are needed to identify the optimum or minimum legume proportion in pastures to achieve particular productivity and economic outcomes (Sollenberger et al., 2019). Here, the aim is to quantitatively identify the optimum legume proportion where biomass and animal production of a warm-climate pasture are maximized. To address this goal, a two-year grazing trial was carried out in long-term bahiagrass monoculture pastures receiving no N fertilizer or bahiagrass-rhizoma peanut mixtures with different proportions of rhizoma peanut. According to previous studies, the hypotheses are that herbage accumulation will show a quadratic response in relation to legume proportion, while the forage nutritive value will show a non-linear increase with legume proportion. In this case, the diet selection toward legumes could potentially compensate for their low proportion in pasture, which may maximize animal gain at lower (i.e., moderate) legume proportions (Jaramillo et al., 2021). These mechanisms will likely determine an overlap in optimum legume proportions maximizing plant and animal production. Thus, the objectives were to quantitatively study the effect of legume proportion on (1) biomass accumulation and forage nutritive value, (2) cattle selectivity, and (3) animal daily gain and gain per hectare, incorporating uncertainty using a Bayesian probabilistic approach.

Materials And Methods

Study Area

The experiment was carried out at the Beef Research Unit of the University of Florida, Gainesville (29.74 N, 82.27 W), from June to October (112 days) of 2022 and 2023. The experimental area is dominated by Chipley sand soils, ranging from poorly drained to rapidly permeable conditions. The historical (2000-2021) average temperature for Alachua County (Florida Automated Weather Network, located 15.4 km from the experimental area, https://fawn.ifas.ufl.edu) is 24.4 °C from May to October (Table 5-1). The rainfall at this location averages 833 mm for the same period. For 2022 (24.6 °C) and 2023 (24.9 °C), the average temperature was above the historical average and rainfall was less than average (748 and 753 mm in 2022 and 2023), with seasonal variation between years. The cumulative incident solar radiation was less than the historical average during 2022 but 15% greater than the historical average during 2023.

Soil was sampled prior to each growing season (March 2022 and 2023). Mehlich-3 soil P, K, and Mg were 59, 24, and 54 mg kg^{-1}, respectively, in 2022, and 66, 40, and 50 g kg^{-1}, respectively, in 2023. Soil pH averaged 6.3 in 2022 and 5.9 in 2023. Based on soil test results, all pastures were fertilized with 19 kg P and 72 kg K ha^{-1} in April 2022 and 2023. No lime was recommended in either year.

Pastures and experimental design and management

To address the hypothesis and objectives, different pastures that visually (*a priori*) varied in legume proportion were selected, representing a significant range in *treatment* levels (i.e., pastures having a unique legume proportion). A limitation of this design is that it is difficult to precisely impose and replicate a specific percentage of legumes on a given experimental unit, resulting in a lack of true spatial and potentially temporal replicates for the legume percentages. However, while qualitative studies assessing the presence or absence of legumes in mixture with grasses, i.e., only grass vs. grass-legume mixtures, have true spatial replicates, they would not address the objective to identify the optimum legume proportion in the pasture maximizing plant and animal production.

Thus, to address this objective, two 0.5-ha ‘Pensacola’ bahiagrass monocultures experimental units with no N fertilization and no legumes were selected as the ‘control’ *treatment* (*a posteriori* varying between 0 to 2% legume). An additional six 0.5-ha experimental units of Pensacola bahiagrass in mixture with ‘Florigraze’ rhizoma peanut varying in legume proportion of total biomass were selected (*a posteriori* varying between 16.5 to 49.2% legume). The rhizoma peanut on these latter pastures was planted in monoculture in 1983 (Ortega et al., 1992) and afterwards was colonized by bahiagrass to varying degrees, creating pastures with different proportions of each species. Hence, eight 0.5-ha experimental units varying in legume proportion between 0% and 49.2%, became the *treatments* in this experiment, having a unique legume proportion. While the legume proportion in the experimental units, i.e., *treatments*, varied between years, the range achieved during 2022 and 2023 was similar, which allowed the analyses across years. The control without legumes and without N fertilization was selected to quantify the sole effect of legume introduction associated with N fixation, without any potential interaction with N fertilization. This control establishes a clear baseline that emphasizes the contribution of legumes under unfertilized conditions.

The eight experimental units were continuously stocked for 112 days of 2022 and 2023. In 2022, the grazing trial started 23 June and finished on 13 October. Beginning and ending dates in 2023 were 13 June and 3 October. Throughout the experiment, the grazing intensity for each experimental unit was expressed as herbage allowance (Sollenberger et al., 2005), with a target allowance of 1.5 kg forage DM kg^{-1} animal live weight (LW; Jaramillo et al., 2021). Herbage allowance was quantified weekly and adjusted using the “put-and-take” method (Mott and Lucas, 1952) by adding and removing additional (i.e., non-testers) open heifers based on current herbage allowance. Weeds in the experimental units (e.g., *Eupatorium capillifolium* [Lam.] Small, *Rubus cuneifolius* Pursh and *Solanum viarum* Dunal) were mechanically and chemically controlled prior to and during the experimental period with glyphosate (41% of active ingredient at 1% solution in a backpack sprayer).

Herbage Responses

Herbage mass was determined using the double sampling technique (Haydock and Shaw, 1975). Indirect (rising plate meter) and direct (clipping with hand shears to 2 cm) measurements were made monthly at five 0.25-m^{-2} sites within each experimental unit. Herbage was dried to constant weight at 60 °C and weighed. The data were used to build equations relating plate meter height with herbage mass. On average, the intercepts of the regressions were 430 (±350) and 800 (±560) kg ha^{-1} DM, while the slopes were 760 (±190) and 860 (±150) kg ha^{-1} DM for 2022 and 2023, respectively. The coefficient of determination for prediction of herbage mass averaged 83.2%

(±8.6%) during 2022 and 85.3% (±9.8%). Weekly indirect measures of herbage mass occurred at 30 sites per experimental unit using the rising plate meter (Rayburn and Rayburn, 1998). Herbage mass was predicted weekly in each experimental unit to quantify herbage allowance.

The grass and legume proportions in pastures were measured three times each year. Five sites of 0.25 m^{-2} were clipped at 2 cm in each experimental unit. Samples were hand separated and dried during 72 hours at 60 °C and then weighed to report the legume proportion in total dry mass. These 15 observations per experimental unit across a growing season were averaged and reported as the legume proportion in each experimental unit for that year (n = 8).

Three exclusion cages were placed in each experimental unit to estimate biomass accumulation during 4-week periods using the plate meter. The disk height was measured in each cage immediately after placement and every week during the 4-week period, totaling 5 disk height measurements per 4-week period. To reduce potential sampling error during initial and final samplings, simple linear regressions were developed using the five observations within a given cage to estimate initial and final disk height. Using the equations developed for predicting herbage mass in conjunction with these estimated disk heights, initial and final herbage mass were determined. The biomass accumulation in each cage was estimated as the difference between final and initial biomass. Data from the three cages per experimental unit were averaged for every 4-week period. The biomass accumulation of the experimental unit during each growing season (i.e., 2022 and 2023) was then estimated as the sum of these four period averages, totaling 16 observations for the two growing seasons (n = 16).

The forage nutritive value was assessed using the botanical composition samples clipped in each experimental unit. The five samples clipped in each of the three periods were averaged within experimental units, and reported for the three sampling periods, totaling 48 observations for the two growing seasons. Once dried at 60 °C for 72 hours, samples were ground to pass a 1-mm screen using a Wiley mill (Model 4 Thomas-Wiley Laboratory Mill, Thomas Scientific). Then, a 10-g subsample was recreated according to the % of grass and legume in the sample and analyzed for *in vitro* digestible organic matter (IVDOM; g kg^{-1}) using a modification of the two-stage technique (Moore and Mott, 1974). These samples were also analyzed for N concentration and multiplied by 6.25 to estimate CP, using a modification of the aluminum block digestion technique (Gallaher et al., 1975), and N was determined by semiautomated colorimetry (Hambleton, 1977).

Animal Responses

Full (unshrunk) weights were taken on both tester and put and take animals 1 week before starting the experiment and animals were allocated to experimental units to establish the target forage allowance and minimize any pre-experiment variation in gut fill. After this week of adjustment and prior to starting the experiment, animals were fasted overnight and weighed to record shrunk weights. Groups of two heifers were then assigned to experimental units as testers, balancing breed (one Brangus heifer and one Brahman heifer per unit) and average initial LW. Put and take heifers were used throughout the study as needed to maintain the target forage allowance (Mott and Lucas, 1952). These animals were kept in adjacent mixed pastures of bahiagrass and rhizoma peanut. The average daily gain of each experimental unit corresponded to the average daily gain of tester animals. The model included the weighing events (*n* = 5) as the independent variables, and the LW of each tester as a dependent variable (*n* = 2). The average daily gain of testers on each experimental unit during each growing season was estimated as the slope of the regressions (*n* = 16).

The carrying capacity (350 kg LW steer days ha^{-1}) of each experimental unit was estimated as the sum of the average LW of the animals times the number of grazing days on the experimental unit, divided by 350 to standardize the carrying capacity based on an animal unit (AU) of 350 kg LW (AU = 350 LW). Then, the gain per area of each experimental unit during each year was estimated as the carrying capacity times the average daily gain of tester heifers (*n* = 16).

The diet selection of heifers was estimated using $\delta^{13}C$ technique on fecal samples. Samples were collected three times in each year, which corresponded to the same day when pastures were clipped to estimate the botanical composition. With this approach, it was possible to compare the legume proportion in the diet with that in the pasture based on changes to isotopic signature (*n* = 48). Fecal samples from the two testers were collected early in the morning immediately after defecation from the center of dung piles, avoiding soil or herbage contamination (Kohmann et al., 2022). Composite samples across testers within experimental unit and sampling event were dried at 60 °C until dry, and then ground and ball milled for isotopic analysis. Similarly, the samples of the botanical composition (i.e., grass and legume) taken the same day as the fecal samples, were dried at 60 °C until dry, ground and ball milled for isotopic analysis. The proportion of legume in feces was estimated as:

$$\%\ \text{legume in feces} = 100 - \left(100 \times \frac{\delta^{13}\text{C fecal samples} - \delta^{13}\text{C rhizoma peanut}}{\delta^{13}\text{C bahiagrass} - \delta^{13}\text{C rhizoma peanut}}\right) \quad (5\text{-}1)$$

where, the legume in feces is in response to the δ^{13}C of fecal samples on rhizoma peanut and on bahiagrass pastures. Then, to correct the proportion in the feces to proportion in diet, the equation proposed by Jaramillo et al. (2022) is solved for estimating the proportion of C3 species in feces in response to the proportion in diet in a period of 8 days, as:

$$\%\ \text{legume in diet} = \frac{\%\ \text{legume feces} + 5.718}{0.98} \quad (5\text{-}2)$$

where, the legume in diet is estimated according to the legume proportion in feces (eq. 5-1), -5.718 is the intercept and 0.98 is the slope of the regression proposed by Jaramillo et al. (2022).

Statistical Analyses

To address the effect of legume proportion in a pasture on herbage (objective 1), plant-animal (objective 2), and animal responses (objective 3), first- and second-order regressions were performed. While non-linear models, such as linear and quadratic plateau models, can be used to test whether plant or animal responses plateau beyond a critical breakpoint of legume in pasture, they would not detect potential decreases in the response after the optimum proportion. For example, linear and quadratic plateau models would not detect potential decreases in biomass accumulation with increasing legume proportion, as demonstrated for bahiagrass-rhizoma peanut mixtures (Kohmann et al., 2022), or potential decreases in gain per area in response to legume proportion, associated with decreases in biomass accumulation and stocking rate.

In addition, responses to legume proportion in pastures were analyzed within a Bayesian probabilistic approach (Lambert and Harris, 1993), which helps to probabilistically describe and discuss the results. Also, an overall analysis was performed for each response, including both years (i.e., average response), and then, to test whether the response was similar across years, the same analysis was performed by year (i.e., 2022 and 2023). Thus, to quantify the effect of pasture legume proportion on biomass accumulation, animal daily gain, and gain per hectare, 16 observations were considered, corresponding to the 8 experimental units in each of 2 years. Responses that take only positive values, such as biomass accumulation and gain per hectare (e.g., biomass accumulation cannot be negative) were modeled within a gamma distribution (Bolker, 2008), as:

$$y_i \sim Gamma(\alpha_i, \beta) \tag{5-3}$$

$$E[y_i] = \frac{\alpha_i}{\beta} = \exp(\beta_0 + \beta_1 legume_i + \beta_2 legume_i^2) \tag{5-4}$$

where, the mean of responses $E[y_i]$, is the second order regression, defined by the ratio between the shape α and the rate β of the gamma distribution. Equation 5-4 is the average response across years. To model responses by year and capture specific effect of years, each parameter, intercept β_0, slope β_1 and quadratic term β_2 was estimated separately for 2022 and 2023.

The average daily gain, which can take positive or negative values, was modeled within a normal distribution, as:

$$y_i \sim N(\mu_i, \sigma^2) \tag{5-5}$$

$$E[y_i] = \mu_i = \beta_0 + \beta_1 legume + \beta_2 legume^2 \tag{5-6}$$

where, the mean μ of the average daily gain is the second order regression. Equation 5-6 is the average response across 2022 and 2023. Further, each parameter was then estimated separately for 2022 and 2023.

Nutritive value (CP and IVDOM) and diet selection responses, which included 48 observations, given 8 experimental units, 2 years and 3 assessments within each year and experimental unit, were modeled with a gamma distribution (Eq. 5-3) and second order regression (Eq. 5-4). Here, the experimental unit was included as a random effect to incorporate the variability within experimental units across assessments. As for all responses, each parameter was then estimated individually for 2022 and 2023. Finally, vague uninformative priors [e.g., $N(0, 10)$] were specified for all parameters to allow the observed data to drive the inference. 'Optimum' legume proportions maximizing different responses that showed a quadratic response were estimated as $-\beta_1 legume/(2 * \beta_2 legume^2)$. The R^2 was estimated in each case as the ratio between explained variance and the total variance (Gelman et al., 2018).

All models were analyzed in R (R version 4.4.0; R Core Development Team 2013) using the JAGS function of the 'jagsUI' package (Kellner and Meredith, 2019). The model selection between first and second order regressions was defined based on the lowest Deviance Information Criterion (DIC; Hooten and Hobbs, 2015). Five Markov chains of 20,000 iterations were run with 5,000 burn-in iterations, and a thinning interval of 30 iterations. The r-hat criteria (< 1.1) and visual assessment of chains were used to check model convergence. For each response, the posterior predictive check was plotted with the regression line along with bands representing the 50th, 80th and 95th percentile values of the distribution.

Results

Herbage Mass, Carrying Capacity And Herbage Allowance

The herbage mass across sampling dates averaged 2.7 and 2.8 Mg DM ha^{-1} during 2022 and 2023 (Table 5-2), being similar across experimental units varying in legume proportion. Similarly, the realized herbage allowance averaged 1.7 and 1.6 kg DM kg^{-1} BW during 2022 and 2023, very close to the target herbage allowance of 1.5 kg DM kg^{-1} BW. The overall carrying capacity averaged 1,620 and 1,760 kg LW ha^{-1} during 2022 and 2023. In general, the carrying capacity of the grass-legume mixtures was greater than the bahiagrass monoculture (5.0 vs. 4.5 , AU ha^{-1}), with the difference accentuated during 2023.

Biomass Accumulation And Nutritive Value

Annual biomass accumulation increased quadratically with increasing legume proportion in pastures during both years and, on average, was maximized at 39% legume (R^2 = 0.69). At this legume proportion, the predicted biomass accumulation was 9.0 Mg ha^{-1} (Figure 5-1A), 173% greater than the bahiagrass monoculture receiving no N fertilization (9.0 vs. 3.3 Mg ha^{-1} DM). Over the years, the biomass accumulation of bahiagrass monocultures was 36% greater during 2023 than 2022 (β_0 = 3.8 vs. 2.8 Mg DM ha^{-1}). During 2022, the biomass accumulation was maximized at 32% legume, while during 2023, it was maximized at 46% legume. At these optimum values, the predicted biomass accumulation was 7.6 and 10.2 Mg ha^{-1} DM, for 2022 and 2023, respectively. This difference translates into a 34% greater biomass accumulation during 2023, similar to what was observed for the bahiagrass monocultures. Overall, these results suggest that legume proportions above 40-50% are not likely to increase biomass accumulation.

Similarly, IVDOM and CP increased non-linearly with legume proportion in the pasture, showing diminishing returns with increases in legume proportion. The estimates of IVDOM did not differ between years, which suggests a consistency in the response across years. The average IVDOM increased from 381 g kg^{-1} in bahiagrass pastures and was maximized at 58% legume, where it reached 563 g kg^{-1} (Figure 5-1B, $R^2 = 0.72$). The CP of bahiagrass monocultures averaged 74 g kg^{-1} and increased non-linearly up to 145 g kg^{-1} at 57% legume (Figure 5-1C; $R^2 = 0.89$). The estimates for CP did not differ between years and showed a lower uncertainty compared with the biomass accumulation and IVDOM predictions. Remarkably, the predictions for IVDOM and CP suggest forage nutritive value is maximized at ~60% legume, likely plateauing beyond this optimum.

Cattle Selectivity

Cattle actively selected legume in both years, given the quadratic response of legume in the diet to legume in the pasture (Figure 5-2; $R^2 = 0.84$). This suggests that cattle composed a diet with greater legume than was offered in the pasture until there was ~70-80% legume in the pasture. At 30-40% legume in the pasture, the model predicted a legume proportion in the diet about 20 percentage units greater than that offered in the pasture (50 and 60%, respectively). Further, averaging across years, the prediction indicated with a 90% probability that cattle actively selected legume until there was 60% legume in the pasture. This indicates that under appropriate levels of herbage mass and herbage allowance, cattle can compensate for lesser proportions of legume in the pasture through selection.

Animal Responses

Animal daily gain (R^2 = 0.72) and gain per hectare (R^2 = 0.67) increased non-linearly with legume proportion in the pasture (Figure 5-3). The average daily gain of cattle grazing only bahiagrass did not differ between years, averaging 290 g day^{-1} at 0% legume. The non-linear response indicates that animal performance was maximized at 36% legume, achieving 604 g day^{-1}, 108% greater than for the unfertilized bahiagrass. On average for the two growing seasons, the predicted gain per hectare in the unfertilized bahiagrass was 154 kg LW, not differing between years. Averaging the response of two years, the gain per hectare was maximized at 36% legume, achieving 350 kg LW, 127% greater than the bahiagrass monoculture without N fertilization. In addition, at this legume proportion, there is a 75% probability of producing at least 300 kg LW ha^{-1}. Thus, given the high certainty of a quadratic response in gain per hectare, similar to herbage accumulation, and the diet selection compensating for moderate presence of legume in pasture, proportions of ~40% would be productive and likely near an economic optimum.

Discussion

Integrating legumes into grass-only pastures is an effective alternative to N fertilizer for improving productive, economic and environmental performance of grazing systems (Jensen et al., 2012; Lüscher et al., 2014; Sollenberger et al., 2019; Sollenberger and Dubeux, 2022). However, identification of the optimum legume proportion in pastures for maximizing resource-use efficiency remains little studied (Watson and Whiteman, 1981; Nyfeler et al., 2009; Nyfeler et al., 2011; Pereira et al., 2020; Kohmann et al., 2022). Here, plant and animal responses to rhizoma peanut proportion in bahiagrass pasture were quantified using a probabilistic approach. Results revealed that biomass accumulation, animal daily gain, and gain per hectare were maximized at ~40% legume in herbage mass, varying to some extent between years. The results, accounting for uncertainty, support previous conclusions that moderate legume proportions between 30 and 50% are optimum in grasslands (Lüscher et al., 2014). Above this level, benefits lessen or disappear, even for forage nutritive value responses (Figure 5-1), which likely plateau above the range assessed in this study (60% legume), as demonstrated by Kohmann et al. (2022).

A limitation of this study is the lack of spatial replicates. This was unavoidable because replicating legume proportion without error in grazed pastures cannot be achieved. However, regressing plant and animal responses to legume proportion in the pasture allows identification of optimum legume proportions for maximizing pasture and animal responses, information which is not provided in qualitative studies comparing only the presence or absence of legume growing with a grass. In addition, these non-spatially replicated studies improve inferences of the effect of legumes on plant and animal responses (Bransby et al., 1988). Moreover, performing the analysis within a Bayesian probabilistic approach describes the degree of uncertainty of responses within years, and probabilistically discusses results in a manner relevant to on-farm decision making (Lambert and Harris, 1993). In cases where the benefits of introducing legumes into grass-only systems are clear based on scientific support from qualitative studies, the next step is identification of optimum or, preferably, minimum legume proportions which maximize productive and environmental outcomes. In these cases, appropriate experimental designs which allow these questions to be addressed are critical.

Results predict an average biomass accumulation of 3.3 Mg ha^{-1} for bahiagrass monoculture receiving no N fertilizer, similar to that reported by Stewart et al. (2007) for low and moderately N-fertilized bahiagrass pastures and by Kohmann et al. (2022) for unfertilized bahiagrass. Comparing years, a 36% greater biomass accumulation in 2023 was found compared with 2022 (Figure 5-1A), likely due in part to a 28% greater solar radiation during the grazing period of 2023 compared with 2022 (2,230 vs. 1,740 MJ m^{-2}). Biomass accumulation was maximized at 39% legume proportion, which explained almost 70% of the variability, and determined a forage accumulation of 9.0 Mg ha^{-1} yr^{-1} at this optimum level (Figure 5-1A). Kohmann et al. (2022) showed a decrease in biomass accumulation as rhizoma peanut increased in the pasture, a response which was detected in this study only above 32 and 46% legume, for years 1 and 2. Considering the additional evidence reported for the same grass-legume mixture (Kohmann et al., 2022), legume proportions above 40% likely compromise biomass accumulation. This makes efforts to achieve a greater legume proportion of questionable productive and economic benefit.

Similar responses were found for IVDOM and CP, which increased non-linearly with legume proportion, which explained a significant proportion of total variability (72 and 89%, respectively). Herbage IVDOM and CP achieved a maximum at 58% legume, slightly greater than the optimum legume proportions reported in Kohmann et al. (2022) for IVDOM (49%) and CP (43%). Despite these differences, the results indicate that even for nutritive value, proportion of legume above 60% would not be beneficial, as these nutritive value parameters very likely plateau with high legume proportions. At 39% legume, where biomass accumulation is maximized, the models predict IVDOM of

543 g kg^{-1} and CP of 136 g kg^{-1}. At these levels, animal gains of 600 g day^{-1} were observed, comparable to that predicted for pastures within this range of nutritive value and unlimited herbage allowance by Sollenberger and Vanzant (2011) and by Woli et al. (2023). The lower nutritive value reported for the bahiagrass monoculture pasture in this study compared with other studies (e.g., Santos et al., 2018a; Kohmann et al., 2022) can be explained by differences in stubble height. In this study, samples for nutritive value were clipped at 2 cm, which likely decreased the nutritive value of the whole sample by increasing proportion of older leaves and flowering stems (Sollenberger and Burns, 2001).

An important finding of this work which aligns with previous reports (e.g., Parsons et al., 1994; Valencia et al., 2001; Jaramillo et al., 2021) is that cattle can compensate for low legume proportion in pastures (Figure 5-2). In this study, cattle selected a diet of ~20 percentage units greater legume than that offered in the pasture, e.g., when 40% legume was offered in pasture, cattle composed a diet of 60% legume. The probabilistic approach indicates that there is a 90% probability that cattle actively select legume up to 60% legume in the pasture. This high probability of active selection should be considered in the context of herbage allowance and herbage mass in the experiment. In this study, herbage mass was above 1.5 Mg ha^{-1} and herbage allowance above 1.2 kg DM kg^{-1} LW. These values are generally thought not to limit cattle selectivity, but even if they did, the likely response to lesser grazing intensities would be a greater proportion of legume in diet (Piaggio et al., 1995). Therefore, cattle selectivity is a zero-cost tool that can compensate for low legume proportion in appropriately stocked grass-legume mixed pastures provided that the legume lacks antinutritional factors reducing selection (Lascano, 2000).

In general, the effect of legume introduction into grass-only systems on animal daily gain and gain per hectare has been assessed qualitatively, with some exceptions. For example, Watson and Whiteman (1981) found a curvilinear response of animal daily gain and carrying capacity to legume proportion in pasture, while Pereira et al. (2020) showed a linear increase in animal daily gain in response to legume proportion until 60% legume. Here, not only was the effect of legume proportion on animal daily gain assessed, but the gain per hectare response was also measured and both were maximized at about 40% legume (Figure 5-3). This evidence confirms that moderate legume proportion not only maximizes plant production (e.g., Nyfeler et al., 2009; Kohmann et al., 2022) but also animal production (Figure 5-3), where legume proportion above 50% would not yield additional benefits.

With few exceptions (e.g., Williams et al., 1991; Pereira Neto et al., 2024), the animal daily gain (290 g day^{-1}) on bahiagrass monoculture pastures without N fertilization was consistent with previous findings (Sollenberger et al., 1989; Valencia et al., 2001; Stewart et al., 2007; Jaramillo et al., 2021). Further, the results indicated a 50% probability of finding a gain of 250-350 g animal^{-1} day^{-1} for cattle grazing only bahiagrass, regardless of the N fertilization. Likewise, the animal daily gain at ~40% legume, which explained 72% of animal performance variability, is consistent with those previously reported for bahiagrass pastures in mixture with rhizoma peanut (Valencia et al., 2001). Similar gains were also reported by Hernandez-Garay et al. (2004) in bahiagrass-rhizoma peanut mixtures ranging in legume proportion between 70 and

90%, and by Jaramillo et al. (2021) for mixtures having 32% legume. The analysis indicated a 90% probability of obtaining an animal daily gain above 500 g day^{-1} at 40% legume. In addition, mechanistic models studying the relationship between herbage mass, nutritive value, and average daily gain predict daily gains of 600 g on pastures with IVDOM of 550 g kg^{-1} and herbage mass greater than 1.5 Mg DM ha^{-1} (Sollenberger and Vanzant, 2011) or herbage allowance greater than 1.5 kg DM kg^{-1} LW (Woli et al., 2023). The results are aligned with these models, where animal daily gain averages 600 g in mixtures with IVDOM of 550 g kg^{-1} and herbage allowance of 1.5 kg DM kg^{-1} LW and herbage mass above 1.5 Mg DM ha^{-1} (Figure 5-1B and 5-1C; Figure 5-3). One important consideration of the animal performance findings is the quadratic response suggests a decrease in average daily gain beyond the optimum of 40% legume (Figure 5-3A). However, the predicted decreases in animal gain is considered unlikely and associated with the low number of animals used in this study. In this case, the two low animal gains observed at ~45-50% legume, likely attributable to outliers, would be the drivers of the quadratic response. Thus, 40% legume would be considered the minimum legume proportion rather than the optimum, for achieving the highest animal performance.

Gain per hectare showed a similar response which was maximized, on average, at 36% legume, which explained 67% of total variability. At this proportion, it is possible to expect with 90% probability, gain per hectare of at least 240 kg LW ha^{-1} during a 112-d period (Figure 5-3), as reported by Pereira-Neto et al. (2024). Further, the predicted gain per hectare at the optimum legume proportion is 116% greater than the unfertilized bahiagrass monoculture, which averaged 158 kg. Pereira-Neto et al. (2024) found gain per hectare of unfertilized bahiagrass of 127 kg, while Stewart et al. (2007) reported gains of 101 and 208 kg LW ha^{-1} for bahiagrass monocultures receiving low or moderate amount of N fertilizer, respectively. These values are within the range of the prediction for gain per hectare for under-fertilized bahiagrass. Lastly, assessing the efficiency of production and averaging across pastures and years,, 23.3 kg DM produced were needed to produce 1 kg LW, which assuming a grazing efficiency of 50%, would represent 12 kg DM kg^{-1} LW, values that can be used as a reference for other studies.

To summarize, in this study the under-evaluated effect of legume proportion on plant and animal performance was assessed in a warm-climate grassland. Results demonstrated that legume proportions of ~30-40% maximized plant and animal production and represent a reasonable target for these warm-climate, grass-legume mixtures. This optimum could be potentially achieved with the strip-planting method, as demonstrated by Jaramillo et al. (2021), where strip plantings in 50% of the area determined 30-40% legume years later. While further research is needed, once established, the legume proportion in the mixture could be managed with strategic fertilizations (e.g., N; Valencia et al., 1999), and grazing management practices that affect light competition between species (Spasiani et al., 2023). This optimum range of legume in pasture, relevant to maximize resource-use efficiency in grasslands ecosystems (Sollenberger et al. 2019), cannot be determined from studies comparing simply the presence vs. absence of legume introduction, revealing the strength of this study. This work contradicts the paradigm that more legume is always better, as there appear to be productive, economic and environmental advantages of having moderate legume proportions, at least in some cases. In practice, the additional cost associated with managements that maximize legume proportion may not be recouped in greater production. Lastly, including uncertainty within a probabilistic approach may aid decision-making processes at a farm scale, providing tools for analyzing potential outcomes under different scenarios.

Table 5-1. Monthly weather data during the experimental periods of 2022 and 2023 and the historical average (2000-2021).

		May	Jun	Jul	Aug	Sep	Oct
Temperature (°C)	2000-2021	23.1	25.5	26.2	26.2	24.7	20.8
	2022	23.9	26.6	26.6	26.4	24.5	19.4
	2023	22.6	25.3	27.5	27.9	25.1	20.7
Rainfall (mm)	2000-2021	86	220	183	159	135	53
	2022	95	132	195	190	116	20
	2023	100	205	183	164	42	59
Radiation (MJ m^{-2})	2000-2021	616	542	555	523	455	425
	2022	615	589	530	488	372	422
	2023	676	627	627	629	550	483

Table 5-2. Experimental unit legume proportion, herbage mass, carrying capacity and herbage allowance. This includes the average (standard deviation, ±SD) for each experimental unit within the growing season for each year (2022 and 2023).

		Experimental unit							
		1	2	3	4	5	6	7	8
Legume proportion (%)	2022	26	18	2	2	22	16	46	44
	2023	38	39	0	1	34	23	49	45
Herbage mass (Mg ha^{-1})	2022	3.0 (0.4)	2.9 (0.4)	2.5 (0.4	2.8 (0.4)	2.7 (0.5)	2.8 (0.5)	2.5 (0.5)	2.5 (0.4)
	2023	3.2 (0.4)	3.1 (0.4)	2.0 (0.2)	2.3 (0.3)	2.7 (0.4)	2.8 (0.3)	3.1 (0.3)	3.4 (0.4)
Carrying capacity (AU§ ha^{-1})	2022	5.2 (0.8)	5.0 (0.8)	4.4 (0.1)	4.4 (0.1)	4.8 (0.6)	4.4 (0.4)	4.8 (0.2)	4.1 (0.2)
	2023	5.7 (1.2)	5.6 (1.1)	4.5 (0.1)	4.6 (0.1)	4.8 (0.6)	4.7 (0.1)	4.8 (0.2)	5.6 (1.2)
Herbage allowance (kg DM kg^{-1} BW)	2022	1.7 (0.2)	1.7 (0.2)	1.6 (0.2)	1.8 (0.3)	1.6 (0.2)	1.8 (0.3)	1.5 (0.3)	1.7 (0.3)
	2023	1.6 (0.3)	1.6 (0.3)	1.2 (0.1)	1.5 (0.1)	1.6 (0.2)	1.7 (0.1)	1.9 (0.2)	1.8 (0.3)

§ Animal unit (AU): animal of 350 kg LW.

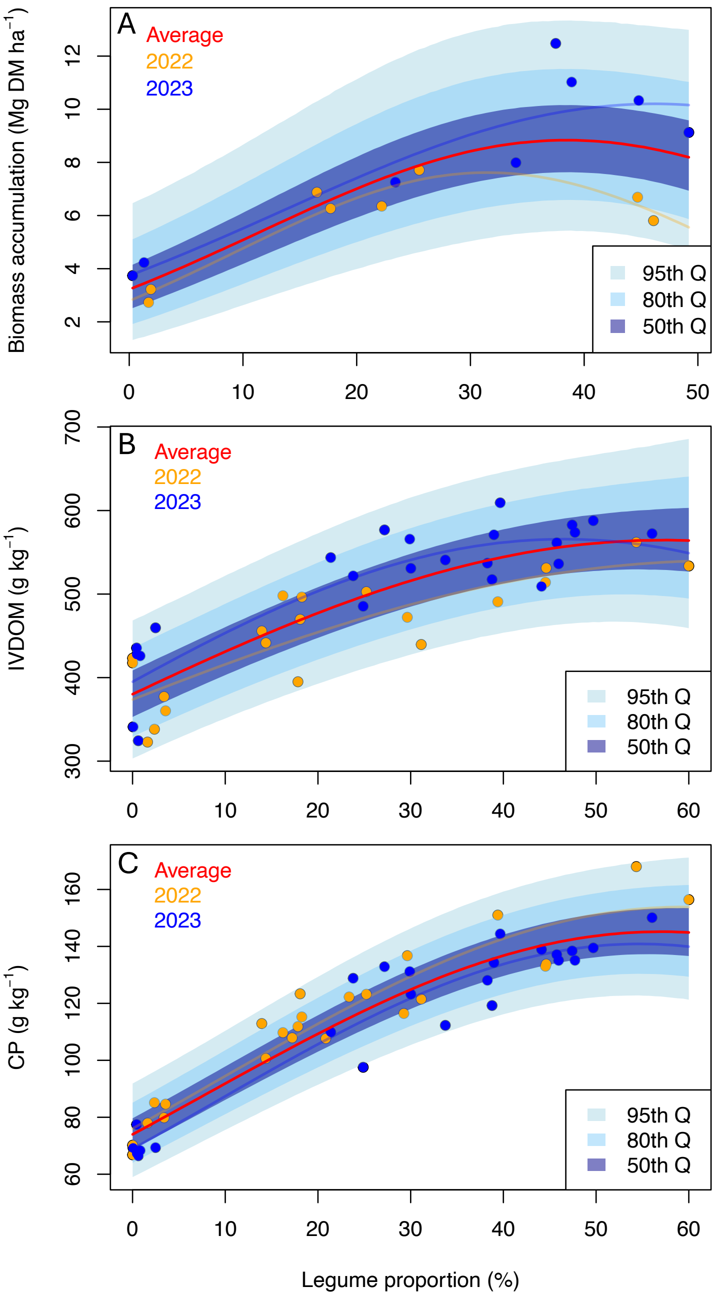

Figure 5-1. Biomass accumulation and nutritive value in response to legume proportion. Biomass accumulation (A), *in vitro* digestible organic matter concentration (IVDOM; B) and crude protein concentration (CP; C) are in response to legume proportion in pasture, where orange and blue lines indicate the regression for 2022 and 2023, respectively, while the red line indicates the average regression for both years. Bands indicate credible intervals for the 50th, 80th and 95th percentile values of the predictive posterior distribution.

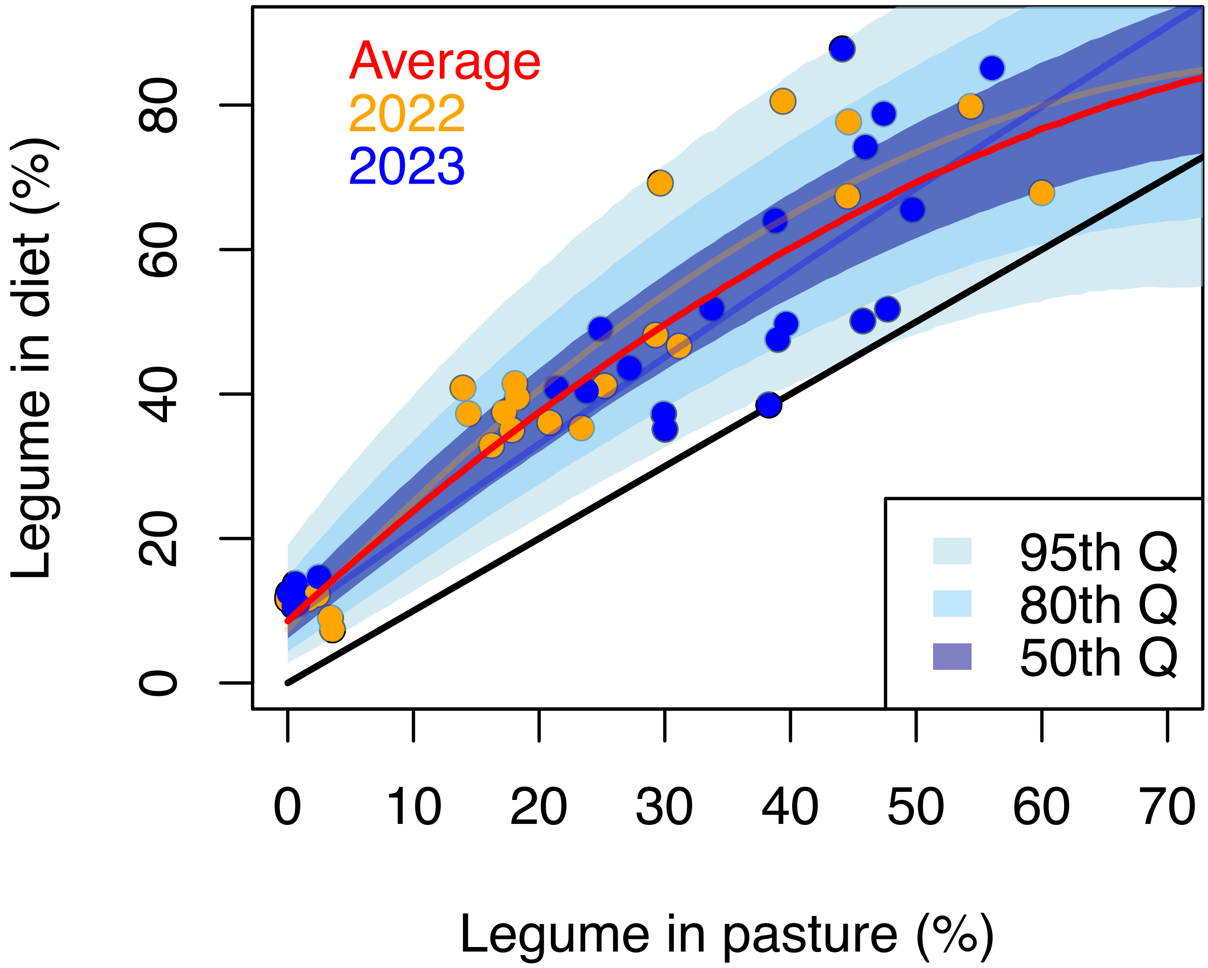

Figure 5-2. Cattle selectivity expressed through the proportion of legume in diet in response to legume proportion in pasture. Orange and blue lines indicate the regression for 2022 and 2023, respectively, while the red line indicates the average regression for both years. Bands indicate credible intervals for the 50th, 80th and 95th percentile values of the predictive posterior distribution. The black line indicates the 1:1 relationship between legume in diet and in pasture, where cattle do not select actively or against legumes.

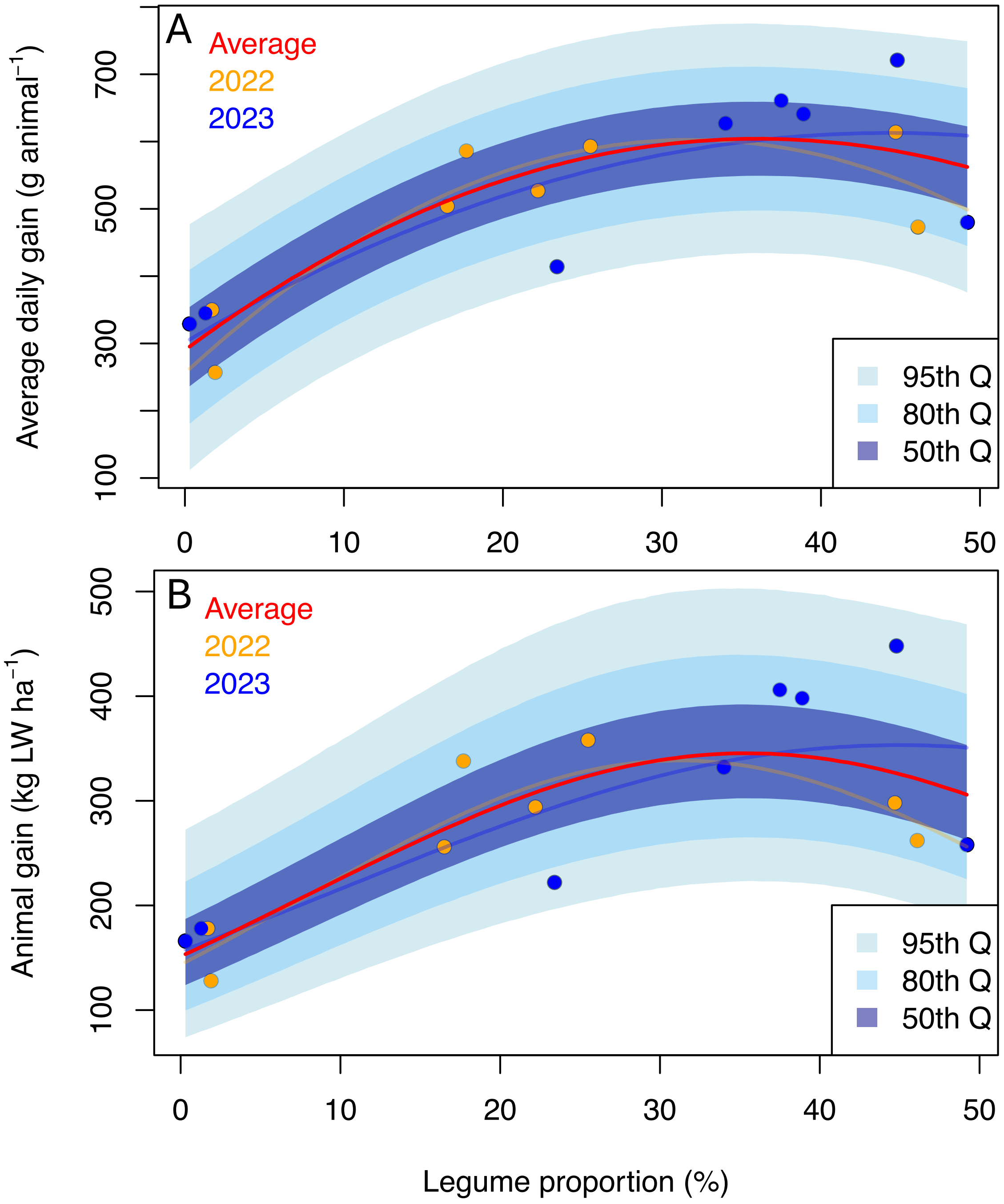

Figure 5-3. Average daily gain and gain per hectare in response to legume proportion in pasture. Orange and blue lines indicate the regression for 2022 and 2023, respectively, while the red line indicates the average regression for both years for average daily gain (A) and gain per hectare (B). Bands indicate credible intervals for the 50th, 80th and 95th percentile values of the predictive posterior distribution.

CHAPTER 6
DEFOLIATION INTENSITY AFFECTS BAHIAGRASS NUTRITIVE VALUE THROUGH CHANGES IN PROPORTION OF NON-DEGRADED LEAF TISSUE COMPONENTS

Introduction

A significant proportion of grasslands of the world lie within the tropics and subtropics (30°N and 30°S; Boval and Dixon, 2012). These tropical native and improved perennial grasslands are dominated by C4 grass species, which are better adapted than C3 species to the high temperatures of these regions (Sollenberger et al., 2020). Despite the advantages of C4 grasses associated with higher resource-use efficiency (Moore et al., 2004; Stöckle and Kemanian, 2009), these species have typically lesser nutritive value than C3 species, associated with anatomical, morphological, and physiological differences (Akin and Burdick, 1975; Akin, 1989; Wilson et al., 1989; Wilson and Mertens, 1995; Sollenberger et al., 2020). In this context, the C4 species may produce more biomass per unit of resource used than C3 species growing under similar conditions (e.g., radiation, water, N; Sinclair and Muchow, 1999; Stöckle and Kemanian, 2009), but provide herbage with lesser energy concentration (Minson, 1990). The lower inherent energy concentration and nutritive value, defined through dry matter (DM) digestibility and N concentration, result generally in lesser gain and efficiency of animals grazing pastures dominated by C4 species (Moore and Mott, 1973; Watson and Whiteman, 1981).

Bahiagrass is an important C4 perennial grass in southeastern US grazing systems (Gates et al., 2004), with widespread use attributable to its adaptation and persistence in subtropical environments under high grazing intensities and continuous stocking (Sollenberger et al., 1988). However, one of the most important limitations of this species is the decline in digestibility from spring to autumn (Stewart et al., 2007;

Interrante et al., 2009). As a result, the average daily gain of cattle grazing bahiagrass can decrease from ~0.7 kg animal^{-1} in spring to ~0.3 kg animal^{-1} during the summer (Sollenberger et al., 1988; Williams et al., 1991; Stewart et al., 2007). Despite its high leaf proportion in herbage mass, the decline in animal performance is more pronounced in bahiagrass compared with other C4 species (e.g., Mott dwarf elephantgrass, Flores et al. 1993), which suggests inherent attributes of bahiagrass that may not be generalizable to other C4 grasses. Specific traits of this species that may explain the summer slump in animal gain are the abundance and organization of sclerenchyma fibers in leaf tissue which reduces digestibility (Flores et al., 1993), and the increase in rate of forage maturation during periods of high biomass production (Stewart et al., 2007).

In general, sclerenchyma and vascular tissue (i.e., the vascular bundle) are non- to slowly-degraded tissues (Akin and Burdick, 1975). While these groups of cells represent only about 15-20% of C4-grass leaves (Akin and Burdick, 1975; Flores et al., 1993), in some species they form a firmly-linked ‘girder structure’ (Wilson et al., 1989), which reduces the degradability of forage leaves during chewing and in the rumen. The girder structure of Pensacola bahiagrass slowed leaf particle size reduction and passage rate, constraining forage daily intake and therefore animal performance (Flores et al., 1993). Additionally, these lignified tissues limit preference and acceptability by animals (García-Favre et al., 2023; Marcon et al., 2023), with implications for nutritional and behavioral responses.

Beyond sclerenchyma and vascular bundle cells, the remaining proportion of the leaf is composed of epidermis and bundle sheath, tissues of slow degradability, and mesophyll and phloem, classified as rapidly degraded tissues (Akin, 1989; Wilson and Mertens, 1995). In this context, management practices that reduce the proportion of non- and slowly-degraded tissues, such as sclerenchyma and vascular bundle, may help to overcome the short-term limitations of bahiagrass on animal performance, through nutritional and behavioral improvements.

Previous studies with temperate grasses (e.g., tall fescue) have demonstrated that leaf length and size are determinants of leaf digestibility (Insua et al., 2018). In general, as leaf length increases, the leaf and total forage digestibility decrease (Insua et al., 2017). This negative relationship is mediated by leaf composition, where the proportion of cell wall and likely of sclerenchyma fibers, increased with leaf length and size. Therefore, reducing the leaf length through short-term increases in defoliation intensity during the summer (Chapman and Lemaire, 1993; Boggiano et al., 2001; Pakiding and Hirata, 2002; Gastal and Lemaire, 2015) could reduce the proportion of non- and slowly-degraded sclerenchyma and cell wall. Thus, studying the causal relationships between canopy attributes, leaf tissue composition and nutritive value is important to predict plant and animal responses under different management practices. In addition, improving understanding of the underlying mechanisms could help to identify new target traits in bahiagrass to modify through plant breeding and genetic modification (e.g., Hopkins et al., 2009; Jung et al., 2012).

The objective was to study the effect of short-term increases in defoliation intensity on leaf tissue composition and fiber concentration and to quantify the relationships between defoliation intensity, leaf attributes and nutritive value, as potential drivers of animal performance. To assess causal relationships, the effect of defoliation intensity on canopy height and leaf length of two contrasting bahiagrass genotypes was quantified during three different periods of the growing seasons. Second, associations between leaf length and tissue composition were investigated. Third, indirect effects of defoliation intensity and leaf length were explored by quantifying tissue composition relationships with leaf digestibility, N concentration, and proportion of cell wall estimated through neutral detergent fiber (NDF) and acid detergent fiber (ADF). Identifying the effects of short-term increases in defoliation intensity on attributes at leaf scale may provide insights and further hypotheses to be tested on larger scales, incorporating other plant and animal responses.

Materials and Methods

Site And Experimental Design

The study was carried out at the Beef Research Unit of the University of Florida, Gainesville, Florida (29.74 N, 82.27 W) between 18 July and 19 September 2022 and 2023. The soils of the experimental area are Chipley sands with rapid permeability. The weather data, including average maximum and minimum temperature and rainfall since 2000, were obtained from the Alachua County station of the Florida Automated Weather Network (FAWN; https://fawn.ifas.ufl.edu), located about 15 km from the experimental area. The average maximum and minimum temperatures during the experimental period were, in general, above the historical average (2000-2021; Table 6-1). The overall rainfall during 2022 was above the historical average while it was below the 2000-2021 average during 2023.

Treatments consisted of two bahiagrass entries, ‘3Fpen8’ and ‘Hybrid 93’, selected by the forage breeding program of the University of Florida (Hayes et al., 2023), managed under three defoliation treatments. These two genotypes were selected from among 10 genotypes with the goal of representing contrasting growth habits and levels of forage accumulation (Hayes et al., 2023). Hybrid 93 was selected as an upright-growing more productive genotype, while 3Fpen8 was selected as a decumbent-growing genotype with lower productivity.

The defoliation intensity treatments consisted of three stubble heights of 4, 8 and 12 cm with the goal of achieving different canopy heights and leaf lengths across intensity treatments and genotypes. The experimental design was a randomized complete block design (RCBD) with four replicates. The arrangement of treatments (genotype and defoliation intensity) was a split-plot, where genotypes were the main plots (3 x 3-m, Hayes et al., 2023), and defoliation intensities were the subplots (1 x 3-m). The experimental units were mowed and fertilized with 40 kg N ha^{-1} a month prior to the first clipping each year. The genotypes were clipped to the stubble heights of 4, 8 and 12 cm on 18 July, 8 August and 29 August of 2022 and 2023. The assessments were done 3 weeks after clipping, which corresponds to the date when the next clipping event was scheduled, and the defoliation intensity treatments were reset. This 3-week regrowth interval corresponds to a moderate frequency of defoliation between high defoliation frequency (2-week interval) and low defoliation frequency (4-week interval) intervals typically tested in Florida (Vendramini et al., 2013; Hayes et al., 2023).

Responses

All responses were measured at the end of three, 3-week regrowth periods in 2022 and 2023. The three periods correspond to mid-summer (18 July – 8 August), late summer (8 August – 29 August) and early fall (29 August – 19 September). The canopy height was measured with a ruler in 10 random sites within each experimental unit. Additionally, a site of 0.1 m^2 within each subplot was clipped at 1-cm for nutritive value analysis. These samples were hand separated and only leaf blades were retained. A subsample of 40 leaf blades within each subplot was selected to measure leaf blade length and analyzed for nutritive value.

Samples were dried at 60 °C until constant weight, and once dried, were ground using a Wiley mill (Model 4 Thomas-Wiley Laboratory Mill, Thomas Scientific) to reduce particle size to pass a 1-mm screen. These samples were analyzed for N concentration which was multiplied by 6.25 to estimate crude protein (CP). In addition, samples were analyzed for *in vitro* digestible organic matter (IVDOM) using a modification of the two-stage technique (Moore and Mott, 1974). Lastly, each sample was analyzed for NDF and ADF, using the modified sequential method of detergent fiber analysis (Van Soest et al., 1991), with the ANKOM fiber analyzer (ANKOM 2000 Fiber Analyzer, ANKOM Technology Corp., Fairport, NY).

Concurrent to leaf blade sampling for length and nutritive value, three to five fully expanded leaves without disease presence were randomly selected from each subplot to assess the proportion of different tissues in the leaves. The collected leaves were placed in a Ziploc® plastic bag containing a damp paper towel to prevent water loss through transpiration during transport to the lab. Once in the lab, the leaves were rehydrated overnight in deionized water. After the hydration period, each leaf blade length was measured with a ruler and then hand sectioned with disposable feather stainless blades at approximately the mid-point of the blade’s length. These cross sections were stained with 0.1% Toluidine Blue O on temporal slides.

Anatomical micrographs were captured using a LEICA DM2500 LED microscope equipped with a LEICA DMC4500 camera. The images of each cross section were then processed using ImageJ software (Schneider et al., 2012). The proportion of different tissues was estimated in ImageJ software by manually tracing their proportion between two secondary veins, i.e., the interveinal distance (Dengler et al., 1994). The tissues measured included vascular bundle (phloem and xylem), bundle sheath, epidermis (upper and lower epidermis and bulliforms cells), and sclerenchyma. Mesophyll (including intercellular airspace) was estimated by difference. The girder structure was estimated by adding the proportion of vascular bundle and sclerenchyma.

Statistical Analysis

Following a sequential relationship, the effect of defoliation intensity treatment and genotype on canopy height and leaf length were first analyzed (Objective 1). Linear mixed models were performed for canopy height and leaf length responses, including defoliation intensity, genotype, period and year as fixed effects, and block as a random effect. Linear mixed models were then performed to test the effect of defoliation intensity through leaf blade length on leaf tissue composition (Objective 2). Vascular bundle, sclerenchyma, girder structure (vascular bundle + sclerenchyma), bundle sheath, epidermis and mesophyll proportion were the responses, while the covariate effect of leaf length (as an indirect effect of defoliation intensity), genotype, period and year were included as fixed effects and block was included as a random effect.

Lastly, to explore effect of leaf length and tissue composition on leaf digestibility (IVDOM), N concentration (CP), and cell wall concentration through NDF and ADF (Objective 3), linear mixed models were used. In this case, and following the sequential relationships, genotype, period, year and the covariate effects of leaf length and tissue composition were included as fixed effects and block as a random effect. The covariates, i.e., leaf length, vascular bundle, sclerenchyma, girder structure, bundle sheath, epidermis and mesophyll, were included one at a time in the model. In this way, it is possible to estimate the direct and individual effect of a particular tissue proportion and leaf length on leaf nutritive value and cell wall, and the indirect effect of defoliation intensity through these covariates. All linear mixed models were performed using the *glmmTMB* package (Books et al., 2017).

In all cases, the correlation in time through periods and the appropriate error terms were modeled to account for the split-plot arrangement. In cases where covariates were included (e.g., leaf length or a given tissue proportion), the slope of the relationship was estimated across genotype and periods on average for both years, and it was considered statistically significant if the upper or lower confidence intervals did not overlap 0. Means and slopes were tested using ‘emmeans’ and ‘emtredns’ functions, respectively, of the *emmeans* package (Lenth et al., 2018). The residuals were assessed using *DHARMa* package (Hartig and Hartig, 2017). All analyses were performed in R 4.4.0 (R Development Core Team).

Results

Canopy Height And Leaf Length

The average canopy height after 3 weeks of regrowth differed among defoliation treatments and between genotypes during the three periods and two years. In all defoliation intensities, Hybrid 93 achieved greater height than 3Fpen8 (Figure 6-2A; *P* <

0.001). As expected, low, moderate and high defoliation intensities resulted in tall, intermediate and short canopies for both genotypes (*P* < 0.001).

Following the same pattern of canopy height, leaf blade length differed between genotypes and among defoliation treatments across periods and years. Leaves of Hybrid 93 were longer than 3Fpen8 in all defoliation treatments (Figure 6-2B; *P* < 0.010). As anticipated, blades were longer under low defoliation intensity, intermediate under moderate intensity, and shorter under high defoliation intensity for both genotypes. These results confirm that different defoliation intensities effectively manipulated the canopy height and leaf length of both genotypes.

Tissue Composition

The proportion of vascular bundle in leaf blades was greater during mid- and late summer in Hybrid 93 and during late summer in 3Fpen8 (Table 6-2). Both genotypes showed the lowest vascular bundle proportion during early fall. The proportion of sclerenchyma reached a maximum during late summer in both genotypes, and then declined by 1.1 and 1.4 percentage units during early fall in 3Fpen8 and Hybrid 93, respectively.

The proportion of girder structure, composed of non-degraded vascular bundle and sclerenchyma tissues, achieved a maximum during mid-summer in Hybrid 93 and during late summer in 3Fpen8. In both cases, the girder proportion decreased by early fall. The bundle sheath did not differ within genotypes across periods (Table 6-2), and only differed between genotypes during late summer, where 3Fpen8 had a 1.2 percentage unit greater bundle sheath proportion than Hybrid 93. The epidermis differed only within Hybrid 93, increasing from summer to fall, while mesophyll differed only within 3Fpen8, achieving its maximum in mid-summer and minimum in late summer.

The non-degraded tissues, i.e., vascular bundle and sclerenchyma, were indirectly affected by defoliation intensity through the leaf blade length (Table 6-3). Overall, the proportion of vascular bundle and girder structure (i.e., vascular bundle + sclerenchyma) increased with leaf length (i.e., low grazing intensity), in particular during late summer and early fall for both genotypes (positive slope, $+\beta_1$), suggesting that increases in defoliation intensity reduce the proportion of non-degraded tissues. Slowly-degraded tissues, i.e., bundle sheath and epidermis, were affected by increasing leaf length only during mid-summer in 3Fpen8, when the proportion of bundle sheath increased while epidermis decreased with leaf length. Epidermis was also negatively affected by increasing leaf length in Hybrid 93 during late summer. Lastly, the rapidly degraded mesophyll was only negatively affected by increasing leaf length during mid-summer in 3Fpen8, and this was likely associated with the increases in the proportion of non-degraded tissues.

Nutritive Value

The IVDOM and CP of 3Fpen8 did not differ across periods and averaged 455 and 90 g kg^{-1}, respectively. In contrast, the IVDOM and CP of Hybrid 93 were less during late summer, when NDF and ADF were greater, compared with mid-summer and early fall (Table 6-4). Studying the effect of covariates in explaining leaf nutritive value and cell wall components revealed a stronger effect of tissue components, i.e., proportion of non- and slowly-degraded tissues (Table 6-5, 6-6, 6-7), than leaf blade *per se* given the number of statistically significant relationships (Table A-5). Leaf length explained only the NDF and ADF variability in Hybrid 93 and NDF variability in 3Fpen8 during early fall (Table A-5). In contrast, the individual effects of vascular bundle (Table 6-5) and sclerenchyma (non-degraded tissues; Table 6-6), and when considered together as girder structure (Table 6-7), explained more of the variability in IVDOM and CP and of NDF and ADF during summer and fall in both genotypes. The relationships indicate that as leaf length increased, there was an increase in NDF and ADF during late summer and early fall (Table A-5, i.e., positive slope). More importantly, as the proportion of non-degraded tissues increased, there was a decrease in IVDOM and an increase in the proportion of NDF and ADF in leaf (Tables 6-5, 6-6, 6-7).

Compared with non-degraded tissues, weaker relationships were found for slowly- (Table A-6, A-7) to rapidly-degraded tissue components in explaining nutritive value and cell wall components (Table A-8). Significant relationships between bundle sheath proportion and nutritive value indicate that the IVDOM decreased with increases in bundle sheath proportion during late summer and early fall in 3Fpen8 and Hybrid 93, respectively. Conversely, the NDF and ADF increased during fall in Hybrid 93 with increases in bundle sheath proportion in leaves (Table A-6). The only significant relationships including the proportion of mesophyll show that as it increased there was an increase in IVDOM and CP (Table A-8), likely associated with its negative correlation with non- to slowly-degraded tissues.

Discussion

Studying relationships between defoliation management, canopy attributes, leaf tissue composition and nutritive value helps to elucidate direct and indirect relationships affecting plant and animal responses. In addition, it aids in identifying management practices to overcome current limitations and to target potential traits for genetic modification to improve nutritive value of forages (Wilson, 1993; Hopkins et al., 2009; Jung et al., 2012). In this small-plot clipping study, it was demonstrated that defoliation intensity affects nutritive value through its effect on proportions of non- and slowly-degraded tissue components. On the one hand, this suggests that short-term increases in defoliation intensity could partially overcome increases in non-degraded tissues which constrain animal intake and performance during late summer (Flores et al., 1993). On the other hand, plant breeding programs can use this information to target specific traits to attenuate negative nutritional and behavioral impacts constraining animal responses (Jung et al., 2012), an effort that could have particular importance in bahiagrass. These data provide justification for further exploration and exploitation of the genotype by environment by management interaction to overcome current limitations of bahiagrass.

The observed summer slump in performance of animals grazing bahiagrass is associated with various factors including the abundance and organization of non-degraded leaf tissue and the seasonal increase in forage maturation rate (Flores et al., 1993; Stewart et al., 2007). One of the strengths of this experimental design is potential effects of leaf maturation within periods are avoided by assessing responses at fixed regrowth intervals. It is acknowledged that the rate of maturation can be affected by factors other than regrowth interval, i.e., temperature, having different implications across periods (Table 6-1). Yet, differences in leaf components and nutritive value within periods are most likely associated solely with the effect of defoliation intensity, through leaf length, and not with leaf maturation which increases cell wall concentration and reduces bahiagrass digestibility (Interrante et al., 2009; Hayes et al., 2023).

In this regard, the results indicate that increases in leaf length, in response to low defoliation intensity (Figure 6-2B; Chapman and Lemaire, 1993), increase the proportion of non- and slowly-degraded tissues (Table 6-3), such as sclerenchyma and vascular bundle. These tissues compose the girder structure (Wilson et al., 1989), which when present, increases structural integrity of the leaf and decreases particle size reduction and bacteria accessibility in the rumen, with consequent negative impacts on animal behavior and daily intake (Flores et al., 1993). However, increases in defoliation intensity (i.e., reduction in post-grazing canopy height from 12 to 4 cm) reduced the subsequent pre-grazing leaf length by about 15 cm (Figure 6-2B). On average, shorter leaves (15 cm) had lesser presence of girder structure than longer leaves (30 cm) during late summer, the period of lesser IVDOM (Table 6-4). For example, within genotypes, the predicted reduction in girder structure by decreasing leaf length from 30 to 15 cm would be 5 and 2 percentage units in 3Fpen8 (17 vs. 12%) and Hybrid 93 (16 vs. 14%), respectively (Table 6-3; $\beta_{1\ \mathrm{3Fpen8}}$= 0.34, $\beta_{1\ \mathrm{Hybrid\ 93}}$= 0.12).

Exploring the relationships through direct and indirect pathway effects showed a stronger effect of non-degraded tissues, i.e., vascular bundle and sclerenchyma (Akin, 1989), on nutritive value measures than the effect of rapidly-degraded tissues or the direct effect of leaf length. In other words, vascular bundle, sclerenchyma and bundle sheath (Table 6-5, 6-6, 6-7) played a more important role in determining nutritive value parameters than epidermis, mesophyll and leaf length *per se* (Table A-5 to A-8). This suggests that the reported increase in NDF concentration and decrease in total and NDF digestibility in response to longer leaf length of tall fescue (Insua et al., 2017; Insua et al., 2018) is likely mediated by increases in non-degraded tissues of thicker cell walls.

In the current study, direct increases in leaf length increased NDF and ADF concentration, mainly during late summer in both genotypes (Table A-5). However, there was a stronger indirect effect of leaf length on NDF and ADF concentration driven by increases in proportion of vascular bundle, sclerenchyma, and girder structure in leaf (Table 6-7).

The negative correlation between tissue components (*data not shown*) might suggest similar impacts on nutritive value across components because as one group of cells increase another should decrease. However, there was a greater (significant at $P < 0.05$) effect of non- and slowly-degraded tissues (e.g., bundle sheath) on nutritive value parameters than of rapidly degraded tissues (Table 6-3). Although bundle sheath cells contain potentially rapidly degraded protein and carbohydrates (Wilson, 1993), they are classified as slowly degraded cell types, associated with a relatively thick cell wall and the suberized lamella (Wilson, 1993). Aligning with these previous results (Akin and Burdick, 1975; Akin, 1989; Wilson, 1993), current data indicate that increases in bundle sheath proportion increased the proportion of NDF and ADF concentration, while reducing the digestibility of both genotypes (Table A-6).

Interestingly, the tissue composition of bahiagrass leaf cross sections for the bahiagrass genotypes evaluated in this study aligns with previous findings for ‘Pensacola’ bahiagrass (Flores et al., 1993). One important contrasting finding between the studies is that seasonal differences in leaf tissue composition were detected within genotypes, while such differences were not detected in Flores et al. (1993). This reflects the inherent variability in tissue composition of bahiagrass, likely associated with environmental determinants of tissue proportion and cell wall deposition, such as daily temperature (Wilson et al., 1991).

The findings revealed that the inherent attributes of bahiagrass leaf, i.e., a relatively large proportion of non- and slow-degraded tissues which negatively affect nutritive value and animal gain, could be partially modified with changes in defoliation intensity. While long-term increases in defoliation intensity may compromise plant persistence and animal intake, Interrante et al. (2010) demonstrated that short-term increases in defoliation intensity did not decrease tiller mass and appearance rate of bahiagrass. This suggests that short-term increases in defoliation intensity could be adopted strategically to reduce anti-quality components of bahiagrass. This, however, should not overlook potential impacts of excessive heat and humidity that could limit cattle intake, and therefore, contribute to the summer slump beyond the effects of forage anti-quality components. In this context, studies at larger scale would provide valuable information regarding the potential effects of increases in defoliation intensity on plant persistence and animal performance, but also, proving information of cattle behavioral responses to heat stress, responses that were not possible to measure in this small-plot study. Overall, this study contributes to the understanding of relationships between defoliation intensity, tissue composition and nutritive value, and helps to generate new hypotheses to be tested at larger scales. Further, plant breeding programs and genetic modifications which target specific traits, such as sclerenchyma and vascular bundle proportion, could have significant impacts on animal responses.

Table 6-1. Weather characterization for the experimental area. This includes the observed maximum and minimum temperature and rainfall during the experimental periods of 2022 and 2023 and the historical average during the same periods (2000-2021).

	Period	18 July – 8 August	8 August – 29 August	29 August – 19 September
Maximum temperature (°C)	2000-21	32.2	32.3	31.1
	2022	33.4	32.6	30.9
	2023	33.9	34.5	32.3
Minimum temperature (°C)	2000-21	22.0	21.7	21.0
	2022	22.3	21.9	22.4
	2023	22.8	23.4	21.1
Rainfall (mm)	2000-21	129	98	118
	2022	144	169	108
	2023	105	55	79

Table 6-2. Tissue proportion across genotypes and periods. The proportion of tissues in cross section of leaf blades (%) for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) include the vascular bundle and sclerenchyma (non-degraded tissues), bundle sheath and epidermis (including bulliform cells; slowly-degraded tissues), and mesophyll (rapidly-degraded tissue) across two years. Girder structure (%) is the sum of vascular bundle (%) and sclerenchyma tissues (%), thus this category groups the non-degraded tissues.

	3Fpen8			Hybrid 93			
	Period*			Period			
Tissue	Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall	SE
Vascular bundle (%)	$8.3^{Bab\S}$	9.1^{Aa}	7.8^{b}	9.1^{Aa}	7.9^{Bb}	7.5^{b}	0.3
Sclerenchyma (%)	6.1^{ab}	7.0^{a}	5.9^{b}	7.1^{ab}	7.6^{a}	6.2^{b}	0.3
Girder (%)	14.4^{b}	16.1^{a}	13.7^{b}	16.2^{a}	15.5^{a}	13.7^{b}	0.5
Bundle sheath (%)	10.8	11.9^{A}	11.5	11.5	10.7^{B}	11.3	0.4
Epidermis (%)	25.5	24.8	25.3	24.2^{b}	23.9^{b}	26.1^{a}	0.5
Mesophyll (%)	49.3^{a}	47.2^{b}	49.5^{a}	48.1	49.9	48.9	0.5

§ Different uppercase letters following means indicate statistical difference between genotypes within period ($P < 0.05$). Different lowercase letters following means indicate statistical difference within genotypes across periods ($P < 0.05$).

*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table 6-3. Effect of leaf length on the proportion of various tissues in bahiagrass leaf blade cross sections (%). The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Tissue		Mid-summer	Late summer	Early Fall	Mid-summer	Late summer	Early Fall
Vascular bundle	β_1	0.03	**0.27**	**0.15**	**-0.18**	**0.11**	**0.08**
	CI 95%	[-0.10 - 0.16]	[0.11 - 0.44]	[0.05 - 0.26]	[-0.34 - -0.03]	[0.01 - 0.22]	[0.01 - 0.15]
Sclerenchyma	β_1	0.01	**0.14**	**0.09**	0.01	0.01	0.01
	CI 95%	[-0.09 - 0.10]	[0.01 - 0.26]	[0.03 - 0.15]	[-0.17 - 0.18]	[-0.08 - 0.11]	[-0.05 - 0.05]
Girder	β_1	0.07	**0.34**	**0.23**	-0.10	**0.12**	**0.12**
	CI 95%	[-0.09 - 0.22]	[0.16 - 0.52]	[0.05 - 0.40]	[-0.26 - 0.06]	[0.00 - 0.24]	[0.02 - 0.22]
Bundle sheath	β_1	**0.25**	0.07	-0.05	-0.18	-0.07	-0.02
	CI 95%	[0.04 - 0.46]	[-0.03 - 0.16]	[-0.30 - 0.21]	[-0.39 - 0.03]	[-0.16 - 0.02]	[-0.20 - 0.17]
Epidermis	β_1	**-0.47**	0.03	-0.16	0.04	**-0.16**	0.04
	CI 95%	[-0.67 - -0.26]	[-0.19 - 0.26]	[-0.35 - 0.03]	[-0.15 - 0.22]	[-0.32 - -0.01]	[-0.08 - 0.17]
Mesophyll	β_1	0.33	**-0.43**	0.07	0.34	-0.11	0.08
	CI 95%	[-0.04 - 0.69]	[-0.70 - -0.16]	[-0.41 - 0.26]	[-0.04 - 0.71]	[-0.30 - 0.08]	[-0.16 - 0.31]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).

*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table 6-4. Nutritive value and cell wall parameters across genotypes and period. In vitro digestible organic matter (IVDOM), crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

	3Fpen8			Hybrid 93			
	Period*			Period			
Nutritive value	Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall	SE
IVDOM (g kg^{-1})	466	445$^{\mathrm{A}}$§	454	463$^{\mathrm{a}}$	412$^{\mathrm{Bb}}$	460$^{\mathrm{a}}$	9
CP (g kg^{-1})	87	91	93	87$^{\mathrm{ab}}$	82$^{\mathrm{b}}$	94$^{\mathrm{a}}$	5
NDF (g kg^{-1})	744$^{\mathrm{Bb}}$	784$^{\mathrm{a}}$	775$^{\mathrm{a}}$	776$^{\mathrm{Ab}}$	804$^{\mathrm{a}}$	784$^{\mathrm{b}}$	6
ADF (g kg^{-1})	393$^{\mathrm{Bb}}$	406$^{\mathrm{ab}}$	413$^{\mathrm{a}}$	412$^{\mathrm{Ab}}$	422$^{\mathrm{a}}$	413$^{\mathrm{b}}$	4

§ Different uppercase letters following means indicate statistical difference between genotypes within period ($P < 0.01$). Different lowercase letters following means indicate statistical difference within genotypes across periods ($P < 0.05$).

*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table 6-5. Effect of vascular bundle proportion (%) on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall
IVDOM (g kg^{-1})	β_1	**8.6**	-1.1	-0.1	2.4	-9.8	0.5
	CI 95%	[1.5 - 15.8]	[-10.1 - 7.8]	[-15.4 - 15.3]	[-4.7 - 9.5]	[-22.3 - 2.6]	[-18.8 - 19.9]
CP (g kg^{-1})	β_1	-0.9	-0.7	**-6.1**	-0.4	-3.0	**-5.7**
	CI 95%	[-3.8 - 2.0]	[-2.5 - 1.2]	[-10.2 - 2.1]	[-3.8 - 2.9]	[-7.0 - 1.0]	[-11.4 - -0.1]
NDF (g kg^{-1})	β_1	-1.8	1.3	8.2	**-9.2**	6.9	7.6
	CI 95%	[-7.9 - 4.4]	[-2.9 - 5.5]	[-3.0 - 19.4]	[-17.8 - -0.5]	[-2.8 - 16.6]	[-6.4 - 21.7]
ADF (g kg^{-1})	β_1	**-7.7**	-1.8	**9.2**	-0.1	**10.1**	**9.8**
	CI 95%	[-14.6 - -0.8]	[-4.6 - 0.9]	[4.1 - 14.2]	[-7.4 - 7.3]	[4.8 - 15.3]	[4.4 - 15.2]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).

*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table 6-6. Effect of sclerenchyma proportion (%) on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall
IVDOM	β_1	3.3	-6.5	10.2	-8.9	-7.9	-17.5
(g kg^{-1})	CI 95%	[-7.1 - 13.7]	[-20.9 - 7.9]	[-23.6 - 44.0]	[-17.9 - 0.1]	[-19.4 - 3.7]	[-36.9 - 1.9]
CP	β_1	**8.0**	-1.1	-4.5	-2.4	3.1	**-5.7**
(g kg^{-1})	CI 95%	[5.2 - 10.8]	[-6.1 - 3.8]	[-10.9 - 1.8]	[-5.7 - 1.0]	[-0.4 - 6.7]	[-10.8 - -0.6]
NDF (	β_1	-6.3	0.7	6.9	-1.9	**7.6**	**14.6**
g kg^{-1})	CI 95%	[-17.7 - 5.1]	[-8.7 - 10.0]	[-13.9 - 27.7]	[-9.7 - 5.8]	[1.7 - 13.4]	[3.0 - 26.3]
ADF	β_1	**-9.9**	-4.0	5.7	5.0	**9.6**	11.5
(g kg^{-1})	CI 95%	[-17.8 - -2.0]	[-11.9 - 3.9]	[-13.2 - 24.5]	[-2.4 - 12.5]	[4.1 - 15.1]	[-2.2 - 25.2]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).
*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table 6-7. Effect of girder proportion (%) on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall
IVDOM	β_1	5.0	-1.4	-0.8	-0.4	**-7.5**	-9.0
(g kg^{-1})	CI 95%	[-0.2 - 10.2]	[-8.5 - 5.7]	[-13.7 – 12.0]	[-5.1 - 4.3]	[-14.9 - -0.2]	[-20.1 - 2.2]
CP	β_1	**3.9**	0.1	-2.9	0.3	0.1	-3.9
(g kg^{-1})	CI 95%	[2.3 - 5.5]	[-1.9 - 2.2]	[-6.6 - 0.8]	[-1.7 - 2.3]	[-2.3 - 2.5]	[-8.2 - 0.4]
NDF	β_1	-4.7	1.7	4.7	-3.0	**5.8**	**8.9**
(g kg^{-1})	CI 95%	[-9.7 - 0.3]	[-0.9 - 4.3]	[-3.0 - 12.3]	[-9.7 - 5.8]	[2.1 - 9.6]	[2.3 - 15.6]
ADF	β_1	**-6.6**	-0.4	0.5	2.0	**4.7**	**9.7**
(g kg^{-1})	CI 95%	[-11.5 - -1.7]	[-3.3 - 2.6]	[-5.4 - 6.3]	[-1.9 - 5.8]	[1.2 - 8.2]	[3.3 - 16.2]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).

*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

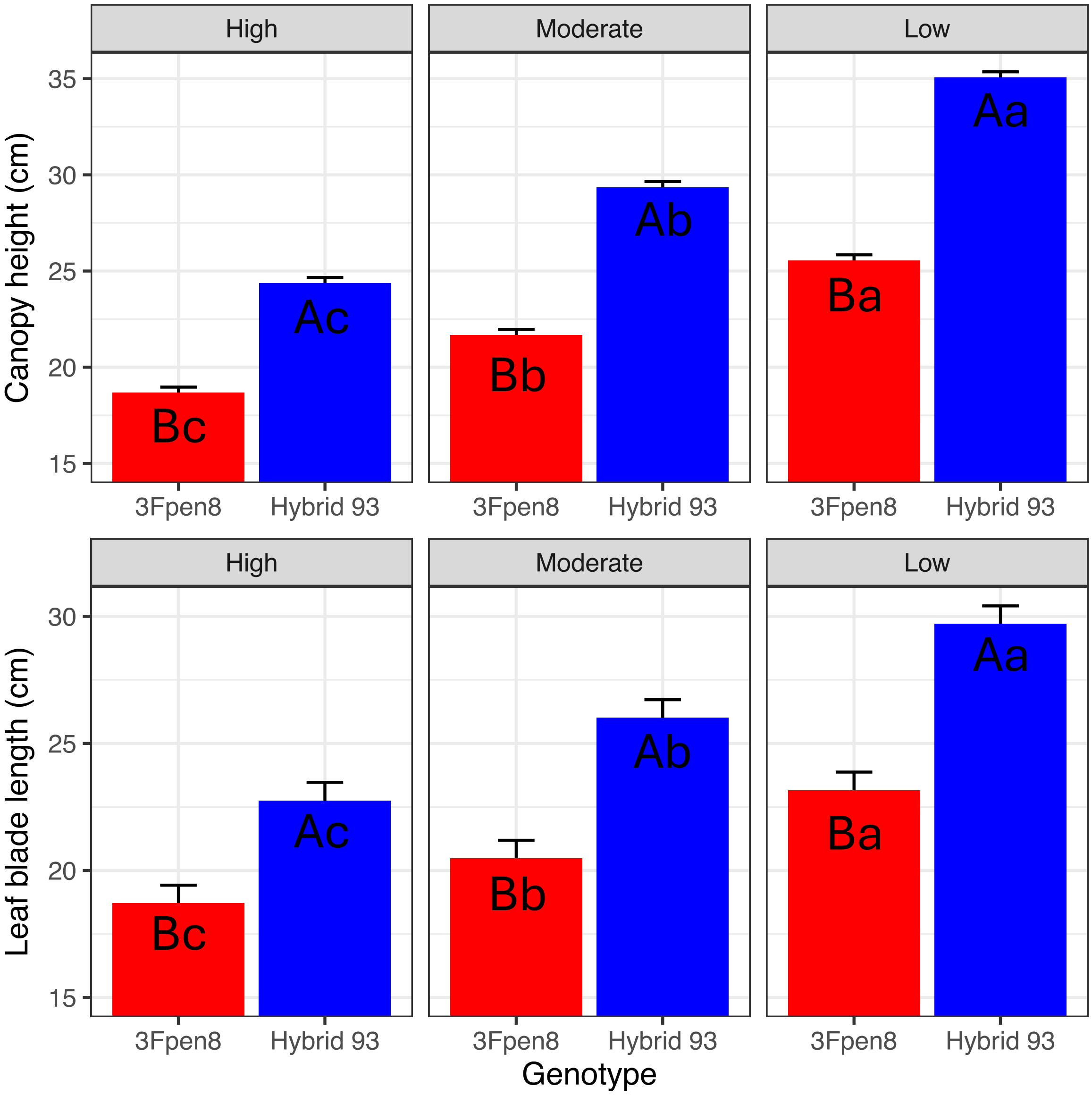

Figure 6-1. Canopy attributes in response to genotype and defoliation intensity treatment. Attributes include canopy height (A) and leaf blade length (B) in response to defoliation treatments (high = 4 cm, moderate = 8 cm and low = 12 cm) and genotype (3Fpen8 and Hybrid 93) across two years. Vertical bars indicate SE. Different uppercase letters within defoliation intensity indicate statistical difference between genotypes ($P < 0.05$). Different lowercase letters within genotypes indicate statistical difference across defoliation intensity treatments ($P < 0.05$).

CHAPTER 7
SUMMARY

Bahiagrass is the most important grass species in Florida grazing systems (Gates et al., 2004; Vendramini, 2010), associated with its adaptation to subtropical climates and tolerance of high grazing intensity, low soil fertility, and continuous stocking (Sollenberger et al., 1988). Despite these benefits and the potential of bahiagrass, it has production, economic and environmental limitations compared with other species, which merit further investigation. Alternatives to overcome these limitations include introducing rhizoma peanut (*Arachis glabrata* Benth.), a C3-perennial legume, into bahiagrass, and improvements in grazing management.

The introduction of rhizoma peanut into bahiagrass pastures increases N cycling in the system (Kohmann et al., 2018; Garcia et al., 2021), forage biomass production and nutritive value (Kohmann et al., 2022), and animal performance on an individual animal or per unit land area basis (Sollenberger et al., 1988; Williams et al., 1991; Jaramillo et al., 2021). However, its adoption in grazing systems is constrained by high establishment costs and complex grazing management during the legume establishment period (Aryal et al., 2021). In this context, where achieving a high proportion of rhizoma peanut in a pasture is difficult and expensive, an important research question is how much legume is needed to optimally overcome the limitations of bahiagrass monocultures.

Alternatively, improvements in grazing management could overcome limitations of bahiagrass, specifically those associated with rapid maturation and lesser nutritive value during summer (Stewart et al., 2007; Interrante et al., 2009). The decreases in bahiagrass nutritive value have been attributed to the abundance and organization of non-degraded vascular bundle and sclerenchyma tissues in leaf blades. An important research question is whether these inherent attributes of bahiagrass can be modified with short-term increases in grazing intensity, a management practice associated with reduced leaf length and greater digestibility of other species (Boggiano et al., 2001; Pakiding and Hirata, 2002; Gastal and Lemaire, 2015).

This project was designed to assess two alternatives for overcoming the shortcomings of bahiagrass-based pasture systems in Florida. These include the integration of rhizoma peanut into bahiagrass pastures or implementation of alternative defoliation management practices of bahiagrass pastures, with the ultimate goal of improving the resource-use efficiency while reducing environmental impacts. Additionally, understanding the underlying mechanisms driving responses to these alternative practices can broaden their impact, allowing for more generalizable inferences.

In this context, the objectives of this project were to (1) identify optimum legume proportion maximizing plant and animal responses and (2) study potential effects of short-term increases in defoliation intensity on grass leaf characteristics and digestibility. To address these objectives, two experiments were developed during two years in Gainesville, Florida. A grazing trial was carried out assessing N dynamics, radiation use efficiency (RUE), biomass accumulation, and nutritive value and animal responses along a gradient of rhizoma peanut proportion in mixture with bahiagrass (0% to 60% legume; Objective 1). Simultaneously, a small-plot clipping trial was carried out to quantify leaf tissues proportion and fiber concentration, drivers of bahiagrass nutritive value, in response to different defoliation intensities of two bahiagrass genotypes (Objective 2).

Overall, moderate legume proportions (~30-40%) optimized N dynamics and plant and animal responses. Within this optimum range of legume participation, shoot N content derived from the atmosphere and soil overyielded that of a bahiagrass monoculture by 84-148% and 75-79% in the two years of assessment. Likewise, within the 30-40% range of legume presence, the NDFA yield averaged 100-138 kg N ha^{-1} during 112 days in both years, overyielding sites with maximum legume proportion (60%) by 27 to 50%. Also, at this level of legume participation, the soil-derived N yield was 225-285% and 73-134% greater than for either grass monocultures or legume-dominated sites, respectively. In addition, the aboveground shoot RUE (1.10 g MJ^{-1}) was maximized at 26-30% legume contribution across experiments, overyielding the grass-only and the 60% legume swards by 110 and 86%, respectively. Consequently, the herbage accumulation was maximized at 39% legume, being 173% greater than the bahiagrass monoculture (9.0 vs. 3.3 Mg ha^{-1} DM).

These responses suggest a simple conceptual model where mixture RUE and biomass accumulation are limited by N when legume presence is below 30% in unfertilized bahiagrass canopies, while legume above 30% imposes a physiological limitation to RUE due to replacement of the C4 grass with a less productive C3 legume. At 30-40% legume, the herbage in vitro digestible organic matter and crude protein concentrations were 543 and 136 g kg^{-1}, respectively, 43 and 84% greater than at 0% legume (381 and 74 g kg^{-1}, respectively), and legume in cattle diets was 20 percentage units greater than offered in the pasture. Average daily gain (ADG) and gain per hectare

(GPH) were maximized at 36% legume, with ADG being 108% greater (604 vs. 290 g day^{-1}) and GPH 127% greater (350 vs. 154 kg LW ha^{-1}) than for animals grazing the bahiagrass monoculture.

When exploring the effect of defoliation intensity on canopy attributes and tissue components, i.e., the drivers of bahiagrass nutritive value and animal response, increasing defoliation intensity (i.e., defoliation to a shorter height) from a 12- to 4-cm stubble height, decreased the bahiagrass monoculture canopy height and leaf length. Shorter leaves had a lesser proportion of non-degraded vascular bundle and sclerenchyma tissues, components of the firmly linked and rigid girder structure. In most cases, lesser presence of non-degraded tissues decreased leaf NDF and ADF, while increasing leaf digestibility.

In conclusion, the results from this project indicate that (1) legume proportions of ~30-40% represent a reasonable target for optimizing plant and animal responses. Knowledge that relatively modest levels of legume participation can produce a meaningful and beneficial response may increase interest in, and adoption of legume technology. Greater use of legumes provides economic and environmental benefits, reducing the requirement for N fertilizer which is costly to producers and associated with ecosystem disservices such as nitrate leaching to ground water and greenhouse gas emissions. Additionally, (2) the lowest bahiagrass herbage digestibility was observed during late summer, a period when greater leaf length strongly increased non-degraded tissue proportion. This suggests that strategic short-term increases in defoliation intensity during this season may aid in overcoming rapid increases in non-degraded tissues which constrain animal intake and performance. Further research at larger scales, including animal responses, is needed to test the merit of seasonal increases in bahiagrass grazing intensity.

APPENDIX
SUPPLEMENTARY INFORMATION

Table A-1. Sequential tests for simple to more complex regression models for N concentration responses. The sequential test first included the year effect (Y, i.e., the intercept of the regression), followed by a linear response of the dependent variable to the legume proportion in pasture (first order regression, L) and then by a quadratic response (second order regression, Q). The contrast tested the intercept, slope, and quadratic parameters between 2022 and 2023 when the effect was significant at $P < 0.10$.

Response	Effect	χ^2	*P*	Contrast	Z-value	*P*
Shoot N concentration (g kg^{-1})	Y	30.4	<0.001	β_1	0.16	0.991
	L	275.1	<0.001	β_2	- 2.28	0.048
	Q	26.5	<0.001	β_3	1.80	0.143
Grass N concentration (g kg^{-1})	Y	306.9	<0.001	β_1	0.33	0.944
	L	63.5	<0.001	β_2	- 2.56	0.021
	Q	16.3	<0.001	β_3	2.30	0.046
Legume N concentration (g kg^{-1})	Y	20.6	<0.001	β_1	- 2.62	0.015
	L	2.5	0.286	β_2	-	-
	Q	1.5	0.473	β_3	-	-
Shoot %NDFA	Y	28.7	<0.001	β_1	0.10	0.917
	L	1.0	0.619	β_2	-	-
	Q	1.1	0.582	β_3	-	-
Grass %NDFA	Y	0.8	0.357	β_1	-	-
	L	0.9	0.640	β_2	-	-
	Q	0.3	0.844	β_3	-	-
Legume %NDFA	Y	7.2	<0.001	β_1	0.43	0.827
	L	3.4	0.179	β_2	-	-
	Q	5.0	0.084	β_3	0.04	0.999

Table A-2. Sequential tests for simple to more complex regression models for N concentration responses. The sequential test first included the year effect (Y, i.e., the intercept of the regression), followed by a linear response of the dependent variable to the legume proportion in pasture (first order regression, L) and then by a quadratic response (second order regression, Q). The contrast tested the intercept, slope, and quadratic parameters between 2022 and 2023 when the effect was significant at $P < 0.10$.

Response	Effect	χ^2	*P*	Contrast	Z-value	*P*
Shoot NDFA concentration (g kg^{-1})	Y	6.2	0.013	β_1	-0.09	0.999
	L	101.9	<0.001	β_2	-0.27	0.966
	Q	8.2	0.016	β_3	-0.20	0.983
Grass NDFA Concentration (g kg^{-1})	Y	0.2	0.619	β_1	-	-
	L	12.0	0.003	β_2	-0.31	0.950
	Q	7.8	0.020	β_3	0.27	0.964
Legume %NDFA concentration (g kg^{-1})	Y	16.3	<0.001	β_1	-1.69	0.120
	L	0.5	0.768	β_2	-	-
	Q	3.7	0.153	β_3	-	-
Shoot soil-derived N concentration (g kg^{-1})	Y	1.5	0.222	β_1	-	-
	L	24.6	<0.001	β_2	0.16	0.875
	Q	2.0	0.365	β_3	-	-
Grass soil-derived N concentration (g kg^{-1})	Y	21.9	<0.001	β_1	-0.35	0.893
	L	8.2	0.016	β_2	-0.61	0.726
	Q	1.8	0.407	β_3	-	-
Legume soil-derived N concentration (g kg^{-1})	Y	1.8	0.176	β_1	-	-
	L	4.9	0.088	β_2	0.63	0.685
	Q	4.9	0.087	β_3	-0.07	0.997

Table A-3. Fit statistics for candidate models for each quantile for Experiment 1. This includes the Akaike information criteria (AIC), corrected AIC (AICc) and root mean square error (RMSE) for the four candidate models of RUE in response to legume proportion for the pooled data (overall response including year 1 and year 2), and for each year. Each model was fitted for the median response (50th), the frontier response (95th) and the limited response (10th).

		10th			50th			95th		
	Candidate models	AIC	AICc	RMSE	AIC	AICc	RMSE	AIC	AICc	RMSE
Overall	Linear	**43.6**	**43.6**	**0.455**	77.3	77.4	0.302	224.2	224.3	0.626
	Quadratic	45.0	45.1	0.442	70.9	71.2	0.295	204.5	204.7	0.573
	Plateau linear	44.1	44.3	0.444	**69.1**	**69.4**	**0.293**	**199.9**	**200.1**	**0.580**
	Plateau quadratic	47.1	47.4	0.443	71.3	71.6	0.293	201.4	201.6	0.580
2021	Linear	**57.9**	**58.1**	**0.545**	50.2	50.5	0.329	107.3	108.2	0.633
	Quadratic	**55.9**	**56.3**	**0.513**	**45.7**	**46.3**	**0.314**	**96.3**	**96.6**	**0.704**
	Plateau linear	60.0	60.4	0.545	**48.1**	**48.7**	**0.319**	**101.0**	**101.4**	**0.653**
	Plateau quadratic	62.3	62.9	0.534	49.9	50.8	0.319	**101.0**	**101.6**	**0.649**
2022	Linear	**-31.1**	**-31.0**	**0.334**	-1.7	-1.4	0.232	86.8	86.9	0.565
	Quadratic	-29.7	-29.4	0.334	-1.6	-1.1	0.229	74.2	74.5	0.499
	Plateau linear	-29.2	-28.9	0.340	**-3.3**	**-2.8**	**0.227**	**77.2**	**77.5**	**0.474**
	Plateau quadratic	-19.9	-19.4	0.333	-1.3	-0.6	0.227	**74.0**	**74.4**	**0.510**

Note: 'Best' candidate models are in bold according to the model metrics (lowest AIC/AICc and/or RMSE).

Table A-4. Fit statistics for candidate models for each quantile for Experiment 2. This includes the Akaike information criteria (AIC), corrected AIC (AICc) and root mean square error (RMSE) of the four candidate models of RUE in response to legume proportion for the pooled data (overall response including year 1 and year 2), and for each year. Each model was fitted for the median response (50th), the frontier response (95th) and the limited response (10th).

		10th			50th			95th		
	Candidate models	AIC	AICc	RMSE	AIC	AICc	RMSE	AIC	AICc	RMSE
Overall	Linear	298.7	298.8	0.862	254.8	255.0	0.499	366.3	366.4	1.008
	Quadratic	**293.3**	**293.4**	**0.834**	**233.3**	**233.5**	**0.466**	**343.1**	**343.3**	**0.888**
	Plateau linear	308.6	308.9	0.892	295.0	295.1	0.513	362.1	362.2	1.012
	Plateau quadratic	355.5	355.7	0.842	297.1	297.3	0.513	369.5	369.7	0.993
2022	Linear	**128.0**	**128.1**	**0.763**	111.9	112.2	0.481	170.3	170.4	1.046
	Quadratic	128.5	128.9	0.781	**107.9**	**108.4**	**0.463**	**165.2**	**165.5**	**0.876**
	Plateau linear	147.5	147.8	0.979	136.9	137.2	0.521	172.5	172.8	1.032
	Plateau quadratic	146.9	147.2	0.768	123.9	124.5	0.514	174.5	175.0	1.033
2023	Linear	165.7	165.8	0.887	144.4	144.6	0.501	194.2	194.3	1.009
	Quadratic	**163.2**	**163.4**	**0.845**	**130.6**	**131.1**	**0.461**	**165.3**	**165.6**	**0.884**
	Plateau linear	169.1	169.4	0.883	147.6	148.0	0.504	186.1	186.4	0.987
	Plateau quadratic	162.7	163.1	0.864	147.9	148.3	0.505	210.6	211.0	1.016

Note: ‘Best’ candidate models are in bold according to the model metrics (lowest AIC/AICc and/or RMSE).

Table A-5. Effect of leaf length on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early Fall	Mid-summer	Late summer	Early Fall
IVDOM (g kg^{-1})	β_1	1.2	-3.9	-0.9	-2.4	-3.1	0.8
	CI 95%	[-3.2 - 5.5]	[-9.4 - 1.6]	[-4.6 - 2.9]	[-6.5 - 1.7]	[-6.5 - 0.3]	[-2.8 - 4.5]
CP (g kg^{-1})	β_1	0.3	2.6	0.1	0.1	0.6	0.3
	CI 95%	[-1.4 - 2.1]	[-0.6 - 5.8]	[-1.2 - 1.1]	[-1.4 - 1.4]	[-1.1 - 2.2]	[-0.6 - 1.3]
NDF (g kg^{-1})	β_1	-0.5	1.9	**3.7**	1.8	1.2	**4.2**
	CI 95%	[-4.1 - 3.2]	[-1.9 - 5.7]	[1.8 - 5.6]	[-2.0 - 5.6]	[-1.0 - 3.5]	[2.4 - 5.9]
ADF (g kg^{-1})	β_1	1.4	0.8	0.7	-1.4	0.5	**3.9**
	CI 95%	[-3.1 - 5.9]	[-3.4 - 5.1]	[-0.8 - 2.3]	[-5.8 - 3.0]	[-1.8 - 2.8]	[2.4 - 5.3]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).
*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table A-6. Effect of bundle sheath proportion (%) on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall
IVDOM (g kg^{-1})	β_1	0.8	-1.8	**-8.9**	-0.1	**-14.7**	4.5
	CI 95%	[-3.1 - 4.6]	[-10.3 - 6.7]	[-15.2 - -2.5]	[-3.5 - 3.5]	[-25.6--3.9]	[-0.8 - 9.8]
CP (g kg^{-1})	β_1	-0.7	0.3	-1.2	-0.9	1.5	-0.9
	CI 95%	[-2.1 - 0.8]	[-2.3 - 2.9]	[-2.9 - 0.4]	[-2.2 - 0.3]	[-1.9 - 5.0]	[-2.4 - 0.7]
NDF (g kg^{-1})	β_1	1.8	-0.3	-0.2	1.1	**6.4**	-2.0
	CI 95%	[-2.6 - 6.1]	[-2.9 - 2.3]	[-4.2 - 3.7]	[-3.4 -5.7]	[2.4 - 10.4]	[-5.2 - 1.3]
ADF (g kg^{-1})	β_1	0.9	1.8	-0.8	-0.1	**6.0**	-2.2
	CI 95%	[-2.9 - 4.8]	[-1.9 - 5.4]	[-4.6 - 3.0]	[-4.0 - 3.9]	[1.1 - 11.0]	[-5.6 - 1.1]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).
*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table A-7. Effect of epidermis proportion (%) on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall
IVDOM (g kg^{-1})	β_1	-1.2	1.0	-6.0	2.4	1.9	5.0
	CI 95%	[-5.0 - 2.5]	[-4.3 - 6.2]	[-14.5 - 2.6]	[-1.8 - 6.7]	[-5.4-9.1]	[-1.8 - 11.8]
CP (g kg^{-1})	β_1	-1.4	1.2	-0.9	-0.2	-0.8	0.6
	CI 95%	[-2.7 - 0.1]	[-0.7 - 3.0]	[-3.1 - 1.3]	[-1.9 - 1.6]	[-2.8 - 1.1]	[-1.4 - 2.7]
NDF (g kg^{-1})	β_1	2.6	-1.1	3.9	3.8	-2.9	-2.4
	CI 95%	[-1.0 - 6.2]	[-4.8 - 2.6]	[-1.1 - 9.0]	[-0.4 -8.0]	[-7.2 - 1.5]	[-6.8 - 2.0]
ADF (g kg^{-1})	β_1	**5.0**	-1.1	-5.1	0.7	-2.2	-1.6
	CI 95%	[0.7 - 9.4]	[-3.3 - 1.2]	[-11.2 - 1.0]	[-3.8 - 5.2]	[-5.3 - 0.9]	[-5.3 - 2.1]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).
*Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

Table A-8. Effect of mesophyll proportion (%) on nutritive value and cell wall parameters. The effect is described in terms of the slope of the relationship (β_1) and the confidence interval of the slope ([CI 95%]), for each genotype (3Fpen8 and Hybrid 93) and period (mid- and late summer, and early fall) across two years.

		3Fpen8			Hybrid 93		
		Period*			Period		
Nutritive value		Mid-summer	Late summer	Early fall	Mid-summer	Late summer	Early fall
IVDOM (g kg^{-1})	β_1	-1.5	2.1	**7.9**	-1.0	**6.0**	-2.0
	CI 95%	[-6.2 - 3.2]	[-1.0 - 5.2]	[3.7 - 12.1]	[-3.8 - 1.8]	[1.7- 10.4]	[-5.8 - 1.7]
CP (g kg^{-1})	β_1	0.5	0.2	**2.4**	0.4	0.3	0.7
	CI 95%	[-1.2 - 2.2]	[-1.0 - 1.3]	[0.9 - 4.0]	[-0.6 - 1.4]	[-1.4 - 1.9]	[-0.7 - 2.0]
NDF (g kg^{-1})	β_1	-0.4	-0.4	-1.9	-1.6	-2.0	0.5
	CI 95%	[-3.3 - 2.6]	[-2.2 - 1.4]	[-5.4 - 1.6]	[-3.3 -0.1]	[-4.5 - 0.6]	[-2.1 - 3.1]
ADF (g kg^{-1})	β_1	0.0	0.0	0.0	0.3	-1.6	1.6
	CI 95%	[-3.1 - 3.0]	[-1.6 - 1.5]	[-2.6 - 2.6]	[-1.5 - 2.2]	[-3.9 - 0.6]	[-0.7 - 4.0]

Note: Slopes in bold are statistically significant (i.e., the upper or lower 95% confidence interval does not overlap 0).
* Mid-summer = 18 July – 8 August; Late summer = 8 August – 29 August; Early fall = 29 August – 19 September.

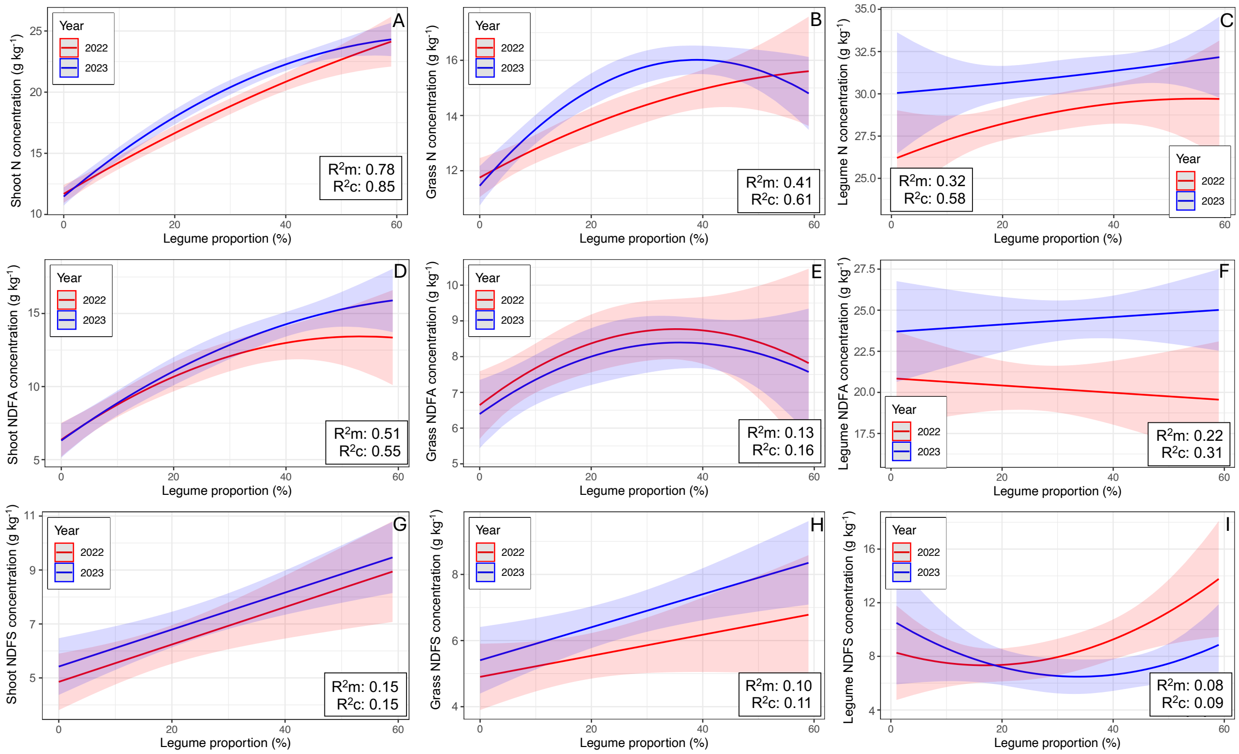

Figure A-1. Nitrogen concentration and its partitioning between atmospheric and soil concentration in response to legume proportion. Total N concentration for shoot (A, grass + legume), grass (B) and legume component (C). Nitrogen derived from the atmosphere (NDFA) concentration in shoot (D), grass (E) and legume component (F), and N derived from soil (NDFS) concentration in shoot (G), grass (H) and legume component (I), during 2022 and 2023. R^{2}m denotes the marginal R^2 of the model, corresponding to the fixed effect component model, and the R^{2}c denotes the conditional R^2 of the model, accounting for the fixed and random effects components of the model.

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BIOGRAPHICAL SKETCH

Nicolas Caram received his Ph.D. in agronomy from the University of Florida Agronomy Department, where he started his program in Fall 2021. He is from Uruguay, where he received his bachelor's in agronomy and Master of Science in agricultural sciences degrees from the National University and obtained a Fulbright scholarship for studies in the United States. Nicolas is a trained grassland ecologist with a research background in functional diversity of native grasslands, foraging behavior and grazing ecology and management. He has been serving as a Research and Teaching Assistant at the Faculty of Agronomy in Uruguay since 2015 and at the University of Florida since August 2021.