

Functional Changes and Threats to Hyperseasonal Neotropical Savannas After Australian *Acacia* Invasion

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Abstract

The hyperseasonal savanna experiences regular flooding and drought stresses and is a neotropical vegetation type threatened by global change including *Acacia* spp. invasion. To deepen the understanding of hyperseasonal savannas after *Acacia* invasion in a climate change scenario, we aimed to answer if: i) the plants of the studied hyperseasonal savanna are separated into C3, C4 or CAM species; ii) *Acacia* invasion can change the hyperseasonal savanna functioning for C3, C4 and CAM plants; iii) how invasive *Acacia* uptake water compared to native species in this hyperseasonal savanna. We detected both C3 and C4 metabolic groups of plants but two C3 species are possibly CAM facultative. The functioning of C3 plants as a group was not affected by the *Acacia* invasion, but this result does not exclude a species turnover between C3 herbs and C3 trees. The C4 plants of invaded Mussununga lost their response of increasing water use efficiency to the increasing Leaf N%. Plants of hyperseasonal savannas depend on the same water source as the soil water from recent rains. There are differences in $d^{18}O$ among species because some grow mostly during the rainy season with the ^{18}O -enriched water meanwhile the invader *Acacia mangium* grows throughout the year whenever it rains. According to our results, the threat to C4 plants is high and they can be excluded from Mussunungas and from hyperseasonal savannas. However, hyperseasonal savannas are threatened as a vegetation. Therefore, hyperseasonal savannas should be considered critically endangered because of global change, especially because *Acacia* invasions. Initiatives for conservation of hyperseasonal savannas could save these remarkable ecosystems.

Introduction

Along with land use change and climate change, alien plant invasion are considered a major threat globally to biodiversity and ecosystem functioning (Strayer 2012; Ens et al. 2015). In most terrestrial ecosystems the climate is predominantly shifting to one with more heavy precipitation, more hot extremes and more consecutive dry days (IPCC 2021a, b). Consequently, the climate is changing towards more extreme seasonal climate in most of the tropical regions. In addition, biological invasion as one of the main factors of loss of biodiversity and ecosystem services, also contributes to global change (Simberloff and Holle 1999; Rundel et al. 2014; Rouget et al. 2016). For instance, Australian *Acacia* are among the most invasive plants that are changing the composition of native vegetation and the function of non-forested terrestrial ecosystems, including hyperseasonal tropical savannas. (Richardson et al. 2011; Aguiar Jr et al. 2014; Meira-Neto et al. 2018b). The hyperseasonal savannas have extreme environmental conditions that shape plant species that grow and persist with annual drought and flooding stresses and high water deficit (Sarmiento 1984) which are expected to deepen with the changing climate. Besides climate change, a better understanding of species coexistence and functioning of hyperseasonal savannas in the context of Australian *Acacia* spp. invasion is crucial for its conservation.

The tropical hyperseasonal savanna is associated with flat plains of poorly structured soil with layers of water-carried deposits that slow down drainage at the point that the vegetation is stressed by

months of flooding during rainy season. Few months after the rainy season the soil becomes completely dry and the vegetation is stressed by months of drought. This extreme seasonality creates an open vegetation dominated by grasses and xeromorphic sedges with few woody plants or palms (Sarmiento and Monasterio 1975; Sarmiento 1984). Mussununga is a hyperseasonal sandy savanna which occurs in small areas under the Brazilian Atlantic Rainforest distribution with Spodosol soils evolved from Tertiary sandstones with a cementation layer of complexed water-carried deposits at a variable depth (Meira-Neto et al. 2005, 2015; Saporetti-Junior et al. 2012). The double stress of flooding and drought interacts with soil texture and depth of cementation layer and drives species richness and predominance of grassy or woody plants, respectively (Saporetti-Junior et al. 2012). The Mussunungas are similar to the much larger Campinaranas ecosystems with more than a hundred thousand km² that occur amid Amazon Tropical Rainforests (Monteiro-Filho 2012; Adeney et al. 2016; Mendonça et al. 2017). Mussunungas and Campinaranas are also similar concerning the *Acacia* spp. invasion that threatens hyperseasonal sandy savannas in South America (Aguiar Jr et al. 2014; Heringer et al. 2019b).

Alien plant invasion can determine irreversible biodiversity decline due to the disappearance of native species, and subsequent ecosystem instability through drastic changes in vegetation composition and function (Hooper et al. 2004, 2005). Successful alien plants are considered to possess a high resource-use efficiency (e.g. water, nutrients) with improved performance facilitated by high resource availability resulting from disturbance or low resource uptake by the native plant community (Funk and Vitousek 2007; Funk 2013). Thus, competition for a limiting resource can induce the disappearance of native species (Valliere et al. 2017). Australian *Acacia* spp. invasions (hereafter *Acacia* invasion) are part of the global change that threaten biodiversity and ecosystem functioning in different continents (Richardson et al. 2011; Rascher et al. 2011a; Heringer et al. 2019a) especially because they are nitrogen-fixing invaders that alter the water balance (Rascher et al. 2011b), change the way the terrestrial ecosystems respond to nitrogen and light (Rascher et al. 2012; Meira-Neto et al. 2018b) and benefit from anthropogenic activities (Aguiar Jr et al. 2014; Heringer et al. 2020). In addition, *Acacia* invasions seem to respond readily to changing climate challenging actions to monitor and control these invaders (Vicente et al. 2020). Therefore, the plants resource-use (i.e., nutrients, water) in a global change context is important to understand *Acacia* invasion and coexistence mechanisms.

Nitrogen is the most common limiting nutrient for terrestrial plants (Vitousek et al. 2010), and the ability of invasive plants to use N is important for the invasiveness (González et al. 2010). Natural N isotopes (i.e. $\delta^{15}\text{N}$ values) in plants have been suggested as an effective tool to understand plant N acquisition and utilization under different soil N status for plant species in invaded ecosystems (Rascher et al. 2012; Hu et al. 2019). Stable isotopes have helped to understand the assembly rules of Mussunungas and the effects of *Acacia* invasion concerning the relations between d^{15}N and nitrogen overflow from the nitrogen-fixing invader to neighboring plants influencing the plant responses to N and light and, consequently, influencing the whole hyperseasonal savanna functioning (Meira-Neto et al. 2018b). The d^{15}N is useful to track the N transference from different sources to living plants, for instance from nitrogen fixers that have levels of d^{15}N more similar to the atmosphere or from termites mounds

that have higher $d^{15}\text{N}$ because that N comes from a higher trophic level (Ji and Brune 2006). Tracking $d^{15}\text{N}$ have indicated that Mussununga is deeply influenced by *Acacia* invasion that not only enriches the ecosystem with N but also triggers shading that threatens the herbaceous flora of this hyperseasonal savanna. However, the $d^{13}\text{C}$ and $d^{18}\text{O}$ have not been explored to understand the hyperseasonal savannas functioning, the *Acacia* invasion or the plant metabolism of their flora (eg., C3, C4 and CAM plants), (Meira-Neto et al. 2018a, b).

In a hyperseasonal savanna, such as Mussununga, plant metabolism can be a good indicator of the type of stress the plants are going through. In general, most plants have C3 metabolism, but C4 and CAM plants have alternate metabolisms to conciliate the need of water with CO_2 acquisition. The C_3 and C_4 photosynthetic pathways show differences between carboxylation reactions which induce the disparate photosynthetic ^{13}C fractionation (Marshall et al. 2007). The conflict between the need to get CO_2 by opening stomata and the need to save water by closing the stomata during the day is a selective pressure for C3 plants that gave rise to the C4 and CAM metabolisms that can fix CO_2 into metabolites of leaves to release it for photosynthesis with an improved water use (Lüttge 2004; Osborne and Sack 2012). Thus, natural C proportion of stable isotopes (i.e. $\delta^{13}\text{C}$ values) is primarily related to intrinsic water-use efficiency, which depends on the ratio of intercellular to ambient CO_2 concentrations and is influenced by both stomatal conductance and the rate of CO_2 fixation by Rubisco (Farquhar et al. 1989). During the photosynthesis, the Rubisco prefers largely ^{12}C that lowers in C3 plants the natural proportion of ^{13}C stable isotope found in atmosphere (Marshall et al. 2007). However, improved Water Use Efficiency (WUE) compel plants to use a higher proportion of ^{13}C (Ehleringer et al. 1991; Marshall et al. 2007) such that the CO_2 is depleted in leaves during photosynthesis in C4 and CAM plants, but not to the same extent in C3 plants (Werner and Máguas 2010). C4 and CAM can be separated from C3 plants because of their improved WUE and, as a consequence, their quite higher values of $d^{13}\text{C}$. While the C4 and CAM plants are similar in $d^{13}\text{C}$ levels, they can be separated from each other by the deuterium proportion (dD) and $d^{18}\text{O}$ proportion closely and positively related in the Global Meteoric Water Line (GMWL) that separates C3 and C4 plants with lower dD and lower $d^{18}\text{O}$ from CAM plants with higher dD and higher $d^{18}\text{O}$. As a result, $d^{18}\text{O}$ can also vary depending on the growing season and on the soil depth that roots uptake water, being the summer rains from clouds of low altitude, as well as the water from soil surface, enriched in ^{18}O compared to the winter rains from high altitude clouds and water from deep soil (Ehleringer et al. 1991; Marshall et al. 2007).

Considering the threat to hyperseasonal savannas in the context of global change and to deepen the understanding of the functioning of hyperseasonal savannas before and during *Acacia* invasion, we aimed to answer the following questions:

- i. Are the plants of the invaded hyperseasonal savanna separated into C3, C4 and CAM species?
- ii. Can *Acacia* invasion change the hyperseasonal savanna functioning for C3, C4 and CAM plants?

iii. How does invasive *Acacia* uptake water compared to native species in this hyperseasonal savanna?

Material And Methods

Vegetation sampling

The vegetation plots were set in three different Mussunungas divided into two series of plots arrangements: *Marcetia* centered plots and *Acacia* centered plots. Each circular plot with 6m of diameter was centered in different shrubs or treelets of *Marcetia taxifolia* or *Acacia mangium*. These two focal species were chosen because the former was abundant, non N-fixer and easily found and the latter because is the N-fixing invader. All plants of all life forms were sampled and most plots had one or few small termite mounds. The *Acacia* plants were less abundant despite usually larger than *Marcetia* plants. There were settled five pairs of plots in each Mussununga (Figure S1). At the sampling time, *Acacia mangium* was the only species of *Acacia* invaders, but *Acacia dealbata* and *Acacia auriculiformis* have been also reported in Mussunungas of the region.

Stable isotopes

Five leaves from each plant were collected during the rainy season (March 2013) and dried in an oven at 70-80 °C, excluding the petiole and evident veins. The dry material was ground in a ball mill to reduce it to particles of no more than 40 µm. For an elemental analysis of C and N, 5 µg of samples were weighed on a precision balance (XM 1000 P, Sartorius) and deposited in 5x9 tin capsules (EuroVector, Milano).

The C/N and the stable isotope ratio $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ and $^{16}\text{O}/^{18}\text{O}$ were determined by stable isotope ratio mass spectrometry in continuous flow mode (CF-IRMS) in an Isoprime mass spectrometer (GV, United Kingdom), coupled to a EuroEA Elementary Analyzer (EuroVector, Italy), which prepares the combustion forms of Dumas. For C, the reference materials IAEA-CH6 and IAEA-CH7 were used. For N, the reference materials IAEA-CN1 and USGS-35 were used. Combustion was carried out in excess of oxygen ($\text{H}_2\text{O} < 3\text{ppm}$, $\text{CnHm} < 0.5\text{ppm}$) (Airliquide, Portugal) and chromatographic gas loaded with helium (He) ($\text{H}_2\text{O} < 3\text{ppm}$, $\text{O}_2 < 2\text{ppm}$, $\text{CnHm} < 0.5\text{ppm}$) (Airliquide, Portugal)

Values of $\delta^{13}\text{C}$ are presented on the scale provided by PeeDee Belemnite (PDB), while values of $\delta^{15}\text{N}$ refer to atmospheric air. All δ values were determined according to the following equation:

$$\delta^{xx} E = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where xx is the atomic mass of the heaviest isotope of the ratio and R expresses the relationship between the rare isotope and the most abundant isotope in the sample and in the source of the element of interest (E). Values of R were notated in delta per thousand (‰) (Farquhar et al. 1989). The analyzes were performed at the SIIAF (Stable Isotopes and Instrumental Analysis Facility, Lisbon, Portugal).

Statistical analysis

Generalized Linear Models (GLMs) were used to explain the variation of leaf elements concentrations, such as C/N, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ and the relationship between these variables. The most suitable data distribution based on family error and link function was evaluated for all GLM tested using the lme4 package with the 'lmer' function (Bates et al. 2014) using the multi-model inference approach with the dredge function of the MuMIn package (Bartón 2018) all possible fixed variables included in the models were tested. The general adjustment of all models was using the information-theoretical approach based on the Akaike Information Criteria (AICc), which indicates the best model by the lowest AICc value (Burnham et al. 2010). The predictors' coefficients to interpret parameter estimates on a comparable scale were estimated using the "jtools" package (Long 2021). For graphical illustration, we used the 'ggplot2' package (Wickham 2016). All analyses were performed on the R platform version 4.0.5 (R Development Core Team 2021).

Results

In the studied hyperseasonal savannas, we detected the C3 and C4 metabolic groups of plants among the species according to the $\delta^{13}\text{C}$ values found for the sampled species. Two species are possibly C3 and CAM facultative.

C3 plants

Among C3 plants, there were no differences between non-invaded (*Marquetia* centered plots) and invaded (*Acacia* centered plots) Mussunungas. The explained $\delta^{13}\text{C}$ increases as $\delta^{15}\text{N}$ increases and as leaf C% decreases, with $\delta^{15}\text{N}$ explaining more than 60% of the global model (Figure 1 A, B and C and 2 A, B and C). Among C3 monocots the $\delta^{15}\text{N}$ is high, especially in *Actinocephalus ramosus* and *Lagenocarpus rigidus*, the same monocots that predominate near termite mounds (Tables 2-4 and S1-S3, see Figure S2). Leaf C% increases as C/N increases and as $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ decrease with C/N and $\delta^{15}\text{N}$ as the main variables that explain together more than 70% of the global model (Figure 1 D-F and 2 D-F). The Leaf N% increases as C/N decreases and as $\delta^{15}\text{N}$ decreases, with the C/N as the main variable explaining more than 80% of the global model (Figure 1 G-I, and Figure 2 G-I). The C/N, an indicator of plant N-nutritional status, increases as Leaf N% decreases and as $\delta^{15}\text{N}$ and leaf C% increases, but Leaf N% is the main explaining variable explaining more than 80% of the global model (Figure 1 J-L, Figure 2 J-L).

C4 plants

Among C4 plants, there were contrasting differences between responses in non-invaded and invaded plots. The $\delta^{13}\text{C}$ only responded to variables in the non-invaded plots increasing as Leaf N% increases and as C/N decreases with the Leaf N% and C/N as the most important variables and explaining almost equally the global model (Figure 3 A, B and C). Leaf C% also responded to variables only in non-invaded plots, increasing as $\delta^{15}\text{N}$ decreases (Figure 3 D-F). C4 plants responded to Leaf N% in non-invaded and

invaded plots in different ways: in non-invaded plots Leaf N% increases as $d^{13}C$ increases and as C/N decreases (Figure 3 G-I); in invaded plots Leaf N% lost response to $d^{13}C$ and only responds to C/N (Figure 4 A-C). The C/N of C4 plants also responded differently in non-invaded and invaded plots: in non-invaded plots the C/N increases as $d^{13}C$, $d^{15}N$ and Leaf N% decrease; in invaded plots the C/N lost the responses to $d^{13}C$ and $d^{15}N$ and increases with decreasing Leaf N% (Figure 4 D – F).

C3 monocots and other important C3 species

Concerning the stable isotope ^{18}O , *Actinocephalus ramosus* presented the highest $d^{18}O$ values among the five species sampled for $d^{18}O$ analyses. *Marcetia taxifolia* presented intermediate values for $d^{18}O$, lower than *Actinocephalus ramosus* and higher than *Acacia mangium* and *Gaylussacia brasiliensis* (Table 1). *Actinocephalus ramosus* presented higher $d^{13}C$ than 14 species out of 20 other species, including *Marcetia taxifolia*. *Marcetia taxifolia* presented $d^{13}C$ lower than 5 species and not different from 14 species (Table S1).

The monocots *Lagenocarpus rigidus* and *Actinocephalus ramosus* as well as *Blechnum serrulatum*, a pteridophyte species, are ^{15}N -enriched with low N-nutritional status and with high $d^{13}C$ (Tables 2-4 and S1-S3, see Figure S2). *Syngonanthus nitens* and *Panicum trinii* are also C3 monocots that have high $d^{15}N$, high $d^{13}C$ and low C/N (Tables 2-4 and S1-S3).

Only two species are C4, *Urochloa* sp and Cyperaceae sp1 with $d^{13}C$ values varying between -12.9 and -11.7 (Table S4). All other species have $d^{13}C$ between -42.7 and -24 and are C3 or could be CAM facultatives as is likely the case of *Marcetia taxifolia* and *Actinocephalus ramosus* (Table S5).

Discussion

C3 plants

For C3 plants, the *Acacia* invasion did not induce changes in the Mussununga functioning. In non-invaded and invaded plots, the $d^{13}C$ increases as $d^{15}N$ increases and because high values of $d^{13}C$ are associated with high WUE (Werner and Máguas 2010), increasing WUE is also associated with increasing $d^{15}N$. As hyperseasonal savannas have pulses of flooding and nitrogen is more available during the rainy season in the wet soil than in dry soil (see Chen et al. 1996), pulses of ^{15}N -enriched nitrogen from higher trophic levels as termite mounds, dungs and urine deposition during rainy season possibly allow C3 plants to uptake a lot of nitrogen to grow with low nitrogen use efficiency (NUE) but with high WUE (high $d^{13}C$) since WUE and NUE are negatively related (Field et al. 1983). Possibly, plants species with high $d^{15}N$ and $d^{13}C$, especially small monocots plants with superficial roots, can grow in harsh sites of these hyperseasonal savannas getting and using N during pulses with low NUE but with high WUE as the soil surface dries.

In C3 plants, Leaf C% responds negatively to $d^{13}C$ and $d^{15}N$, suggesting that plants with more structural C in leaves are not benefitting from N pulses during rainy season growing with high NUE and low WUE. Moreover, the plants with higher Leaf N% possibly do not use pulses of N availability during rainy season (low $d^{15}N$) while maintaining high N-nutritional status (low C/N).

C4 plants

For C4 plants, the Mussununga functioning is not the same after invasion by *Acacia*. In non-invaded plots, C4 plants have $d^{13}C$ positively related to Leaf N% and negatively related to C/N. Therefore, the low $d^{13}C$ (low WUE) is a response to low Leaf N% and low N-nutritional status (high C/N) with an expected high NUE (Field et al. 1983). However, in invaded plots the $d^{13}C$ in C4 plants respond neither to Leaf N% nor to C/N indicating a loss of WUE response in an environment with N outflow from *Acacia* invaders (Meira-Neto et al. 2018b) that, possibly, enables C4 plants grow with low NUE. Congruently, the Leaf N% of C4 plants in invaded plots also lost the response to $d^{13}C$ suggesting that Leaf N% variation in invaded Mussunungas is related with N outflow into the invaded ecosystem (Rascher et al. 2012). Leaf C% also lost the response to $d^{15}N$ variation due to lack of response to $d^{15}N$ possibly because the invaded ecosystem is enriched with N with lower $d^{15}N$ from N-fixing invaders. The C/N (N-nutritional status) also lost responses to $d^{13}C$ and $d^{15}N$, possibly influenced by N outflow from the *Acacia* invaders.

Hyperseasonal savanna functioning and consequences of *Acacia* invasion

In the current scenario of global change, not only the increasing concentration of CO_2 and increasing temperatures can benefit C3 plants over C4 plants (Chen et al. 1996) in hyperseasonal savannas, but also the nitrogen-fixer invaders that cause loss of influence of N on $d^{13}C$ (WUE) in C4 plants with unpredictable consequences. Only two species are C4 in studied Mussunungas with most of the plants from only one sedge species. If the *Acacia* invasion is going to increase shading with increasing N contents in the Mussununga ecosystem (Meira-Neto et al. 2018b), the C4 plants will be possibly excluded from invaded Mussunungas since neotropical C4 plants do not tolerate shading (Klink and Joly 1989). However, the shading is not going to be caused by C3 herbs or monocots that are also shade intolerant. Instead, *Acacia* invaders and shade-tolerant C3 woody species are likely to shift hyperseasonal savannas into dense woodlands as the N outflow from *Acacia* invaders enriches the ecosystem with N (Meira-Neto et al. 2018b), especially if fire disturbance that boosts the invaders is frequent and the recruitment of *Acacia* invaders surpasses the recruitment of native species from hyperseasonal savannas (Meira-Neto et al. 2005; Le Maitre et al. 2011).

The plants with high $d^{15}N$ and $d^{13}C$ that use water efficiently and can grow in the harsh sites of Mussunungas often occur near termite mounds that are nitrogen sources (see Ji and Brune 2006) from a higher trophic level and with a higher $d^{15}N$ than leguminous N-fixers (Marshall et al. 2007).

There is a difference between C3 and C4, especially in invaded plots. In C3 plants, the higher $d^{13}C$, the higher $d^{15}N$ and the lower Leaf C%. Also, the greater the need of C3 plants in N, the greater will be the

$d^{15}\text{N}$. Therefore, on the one hand the C3 plants that have low N-nutritional status depend on ^{15}N -enriched sources of N to succeed in sites with strong N-limitation because of high WUE and high $d^{13}\text{C}$, especially small monocots like *Lagenocarpus rigidus* and *Actinocephalus ramosus*. On the other hand, the C4 plants do not depend on ^{15}N -enriched N, do not present significant relations between $d^{13}\text{C}$ (WUE) and $d^{15}\text{N}$ and, in invaded Mussunungas, the N-nutritional status (C/N) do not relate with $d^{13}\text{C}$ and with $d^{15}\text{N}$.

The association between termite mounds and certain species with high $d^{13}\text{C}$ and high WUE in hyperseasonal savannas deserves attention because can shed light in an old controversy about the process in hyperseasonal savannas that originates *Murundus*, mounds attributed to termites or differential erosion, possibly with a facilitative influence of termite mounds according to our results (see Marimon et al. 2015). Moreover, apparently C3 plants that are ^{15}N -enriched tolerate harsh sites that they would not tolerate without the N mineralization from termite mounds. But the origin of ^{15}N -enriched N from higher trophic levels is not restricted to termites. Facilitative processes in African savannas associated to N-enriched dung of giraffes (high N:P ratio) or herbivores urine benefited grasses more than tree and shrub seedlings (Sitters and Venterink 2021) and termite mounds potentially cause similar effects on small monocots as *Actinocephalus ramosus* and *Lagenocarpus rigidus* especially because Mussunungas are used as pasture for extensive livestock in the region (Meira-Neto et al. 2005). Likely, the C3 monocots of the harshest sites of these hyperseasonal savannas, as *Actinocephalus ramosus* and *Lagenocarpus rigidus*, are dependent on ^{15}N -enriched N from termite mounds, dungs and urine of herbivores (see Marshall et al. 2007; see also Sitters and Venterink 2021).

Actinocephalus ramosus presented the highest $d^{18}\text{O}$ possibly because of its lifeform, an annual plant, which could explain the high $d^{18}\text{O}$ value as its growth season coincides with the rainy season where the heaviest rains come from low altitude clouds enriched with ^{18}O while rain of other seasons tends to be originated from higher clouds with lower $d^{18}\text{O}$ (see Marshall et al. 2007). The $d^{18}\text{O}$ profile of *Blechnum serrulatum*, a geophyte that resists without leaves in the soil during droughts and grows during rainy season, is similar to that of *Actinocephalus ramosus*. *Marcetia taxifolia* is a perennial shrub that presented intermediate values of $d^{18}\text{O}$ even though it can grow every time it rains. *Gaylussacia brasiliensis* and *Acacia mangium* have lower values of $d^{18}\text{O}$ as they are perennial and can also grow every time it rains. These results help to partially explain the remarkable growth of *Acacia mangium* in hyperseasonal savannas.

Mussununga, as a hyperseasonal savanna, does not accumulate water in the soil. Therefore, the entire volume of water is depleted by evapotranspiration or drainage in streams and does not drain water into aquifers available to plants. As a consequence, soil aquifers are not the cause of $d^{18}\text{O}$ variation in Mussununga plant species. *Actinocephalus ramosus* is an annual species, a C3 species with improved WUE as its $d^{13}\text{C}$ is higher (Werner and Máguas 2010) than most other C3 species but higher $d^{13}\text{C}$ can also be a result of a CAM facultative metabolism; however, $d^{13}\text{C}$ is not as high as the expected for CAM plants (Marshall et al. 2007). The high $d^{18}\text{O}$ of *Actinocephalus ramosus* could also be caused by the ^{18}O -

enriched rainwater that falls during its growing season, a $d^{18}\text{O}$ possibly boosted by a CAM facultative metabolism (Marshall et al. 2007). *Actinocephalus ramosus* can be a good indicator of hyperseasonal savannas as well as *Marcetia taxifolia*, another well-adapted species to hyperseasonal savanna that could also be CAM facultative because of intermediate values of $d^{13}\text{C}$ and high values of $d^{18}\text{O}$.

Some C3 monocots as *Lagenocarpus rigidus* and *Actinocephalus ramosus* are ^{15}N -enriched possibly because they are commonly associated to termite mounds, dungs and urine but they have low N-nutritional status, especially *Lagenocarpus rigidus* that is amongst the species with the highest C/N. Possibly, their high $d^{13}\text{C}$ means high WUE that depends on ^{15}N -enriched N that is better mineralized and mostly available during pulses of N availability during rainy season for small C3 monocots. *Blechnum serrulatum*, a pteridophyte, is another species that benefits from the ^{15}N -enriched in the same way of the small monocots *Lagenocarpus rigidus* and *Actinocephalus ramosus*.

Conclusions

Are the plants of the studied hyperseasonal savanna separated into C3, C4 or CAM species?

Most individuals and plant species in the studied Mussunungas are C3. *Actinocephalus ramosus* and *Marcetia taxifolia*, besides C3 could also be CAM facultative and deserve further attention as they can be indicative of hyperseasonal savannas. Two plant species are probably C4: some individuals of an unidentified species of Cyperaceae, a native sedge, and few individuals of *Urochloa decumbens*, an african grass species commonly used in pastures.

Can *Acacia* invasion change the hyperseasonal savanna functioning for C3, C4 and CAM plants?

Among C3 plants, the more N-limited the plants (higher C/N), the more dependent on ^{15}N -enriched N (possibly from termite mounds, dungs or urine) and the more dependent on nitrogen pulses. Thus, the higher $d^{15}\text{N}$, the higher the $d^{13}\text{C}$ (the higher WUE), indistinctly if the Mussunungas are invaded or not. Therefore, the responses of the C3 plants were not affected by the *Acacia* invasion, but this result does not exclude a composition shift caused by a turnover among C3 species from small monocots/herbs to C3 trees that compete for light as *Acacia* invasion outflows N into the ecosystem and shifts the hyperseasonal savanna ecosystem towards a dense woodland.

The C4 plants of invaded Mussunungas lost their response of increasing $d^{13}\text{C}$ (WUE) to the increasing N. The C/N is negatively related to the $d^{15}\text{N}$ and $d^{13}\text{C}$ (WUE) in non-invaded plots and shows that N-nutritional status and WUE likely depend on the ^{15}N -enriched termite mounds, dungs and urine of herbivores; in invaded Mussunungas the C/N lost its response to $d^{15}\text{N}$ and $d^{13}\text{C}$ (WUE). Thus, termite mounds and dungs and urine of herbivores lose their influence on C4 plants in invaded Mussunungas, possibly because of the N outflow from the N-fixing invader into the ecosystem that can alter the N cycling and the N-nutritional status of plants. Therefore, C4 plants are the most affected plants by the *Acacia* invasion as they are the group of plants that lost the $d^{13}\text{C}$ responses to the N variation as well as

to the C/N variation. If we consider that this effect can be associated with shading promoted by the *Acacia* invasion, a rapid exclusion of C4 plants in Mussunungas is conceivable. This effect could be general for hyperseasonal savannas, at least for the hyperseasonal savannas invaded by *Acacia*.

How do invasive *Acacia* uptake water compared to native species in this hyperseasonal savanna?

Mussununga plants depend on the same water source as the soil water from recent rains. This is because during the dry season the soil water is completely depleted. However, there are differences in $d^{18}O$ among species because some are annuals and geophytes that grow mostly during the rainy season with the ^{18}O -enriched water of heavy rains, sometimes with high $d^{18}O$ possibly boosted by CAM facultative metabolism, meanwhile other species grow throughout the year whenever it rains, such as the invader *Acacia mangium*. The perennial *Marcetia taxifolia* plants can also grow throughout the year, but its high $d^{18}O$ can also indicate that *Marcetia taxifolia* is CAM facultative. The high $d^{18}O$ of *Marcetia taxifolia* plants means that they use proportionally more water from heavy rains and/or they grow during shorter periods than *Acacia mangium* and for longer periods than annuals like *Actinocephalus ramosus* or geophytes like *Blechnum serrulatum*. This is a clear leverage for the biomass production of *Acacia* invaders, as they grow throughout the year every time it rains.

Concluding remarks

According to our results, the threat to C4 plants is high and this metabolic group of plants can be excluded from Mussunungas very soon and possibly can be excluded from hyperseasonal savannas in general as *Acacia* invasion advances. However, not only C4 plants are threatened by *Acacia* invasions with the formation of dense woodlands, but also the hyperseasonal savannas as a whole, since most of their plant species are shading intolerant. Therefore, hyperseasonal savannas should be considered critically endangered because of global change including *Acacia* invasions. An IUCN initiative (see IUCN 2015) could be an eye-opener for researchers to deepen the understanding of this remarkable type of ecosystem that potentially occurs worldwide in tropics but it is not even mentioned outside Neotropics.

Declarations

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Availability of data and material: the data will be share as demanded

Code availability: codes will be shared as demanded.

Authors' contributions: JAAMN, FU and CM conceived and designed the survey. FU, MCNAS and GST performed the survey. JAAMN, GST, PMV, NS and CM analyzed the data. JAAMN wrote the first version of the manuscript and PMV, NS, TB and CM provided editorial advice.

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Consent to participate: All authors consent to participate and publish this manuscript. (include appropriate statements)

Consent for publication: All authors consent to participate and publish this manuscript.

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Tables

Table 1. Z-scaled $d^{18}O$ differences between sampled species of Mussununga, a hyperseasonal neotropical savanna. Negative or positive values indicate that the rows' labels are lower or higher in $d^{18}O$ than the columns labels, respectively. The numbers at the bottom in each cell is the probability and asterisks indicate significance if lower than 0.025 since the test is bilateral ($\alpha=0.05$)

Col Mean- Row Mean	<i>Acacia mangium</i>	<i>Actinocephalus ramosus</i>	<i>Baccharis dracunculifolia</i>	<i>Gaylussacia brasiliensis</i>	<i>Marcetia sp1.</i>
<i>Actinocephalus ramosus</i>	-4.229 0.000*				
<i>Baccharis dracunculifolia</i>	-1.790 0.036	2.586 0.004*			
<i>Gaylussacia brasiliensis</i>	0.341 0.366	3.549 0.000*	1.723 0.042		
<i>Marcetia sp1.</i>	-0.950 0.171	3.425 0.000*	0.868 0.193	-1.083 0.139	
<i>Marcetia taxifolia</i>	-2.670 0.003*	2.012 0.022*	-0.807 0.210	-2.343 0.010*	-1.732 0.042

Table 2 - Z-scaled $d^{13}C$ differences between sampled species of Mussununga, a hyperseasonal neotropical savanna. Negative or positive values indicate that the rows' labels are lower or higher in $d^{13}C$ than the columns labels, respectively. The numbers at the bottom in each cell is the probability and asterisks indicate significance if lower than 0.025 since the test is bilateral ($\alpha=0.05$)

Col Mean- Row Mean	<i>Acacia mangium</i>	<i>Actinocephalus ramosus</i>	<i>Blechnum serrulatum</i>	<i>Lagenocarpus rigidus</i>	<i>Syngonanthus nitens</i>	<i>Gaylussacia brasiliensis</i>
<i>Actinocephalus ramosus</i>	4.545 0.000*					
<i>Blechnum serrulatum</i>	-4.799 0.000*	-0.964 0.167				
<i>Lagenocarpus rigidus</i>	-3.059 0.001*	1.836 0.033	2.481 0.006*			
<i>Syngonanthus nitens</i>	-2.680 0.004*	0.959 0.169	1.613 0.053	-0.346 0.365		
<i>Gaylussacia brasiliensis</i>	1.650 0.049	4.746 0.000*	5.044 0.000*	3.768 0.000*	3.5544 0.000*	
<i>Marcetia taxifolia</i>	0.348437 0.3638	5.877 0.000*	5.825 0.000*	4.106 0.000*	3.272 0.000*	-1.527 0.063

Table 3 - Z-scaled $d^{15}N$ differences between sampled species of Mussununga, a hyperseasonal neotropical savanna. Negative or positive values indicate that the rows' labels are lower or higher in $d^{15}N$ than the columns labels, respectively. The numbers at the bottom in each cell is the probability and asterisks indicate significance if lower than 0.025 since the test is bilateral ($\alpha=0.05$)

Col Mean- Row Mean	<i>Acacia mangium</i>	<i>Actinocephalus ramosus</i>	<i>Blechnum serrulatum</i>	<i>Lagenocarpus rigidus</i>	<i>Syngonanthus nitens</i>	<i>Gaylussacia brasiliensis</i>
<i>Actinocephalus ramosus</i>	-3.032 0.001*					
<i>Blechnum serrulatum</i>	-1.354 0.088	1.476 0.070				
<i>Lagenocarpus rigidus</i>	-2.607 0.0046*	0.537 0.296	-1.043 0.148			
<i>Syngonanthus nitens</i>	-0.521 0.301	2.049 0.020*	0.690 0.245	1.674 0.047		
<i>Gaylussacia brasiliensis</i>	2.856 0.002*	5.037 0.000*	3.814 0.000*	4.760 0.000*	3.082 0.001*	
<i>Marcetia taxifolia</i>	3.351 0.000*	7.686 0.0000*	4.897 0.000*	7.217 0.000*	3.488 0.000*	-0.830 0.203

Table 4 - Z-scaled C/N differences between sampled species of Mussununga, a hyperseasonal neotropical savanna. Negative or positive values indicate that the rows' labels are lower or higher in C/N than the columns labels, respectively. The numbers at the bottom in each cell is the probability and asterisks indicate significance if lower than 0.025 since the test is bilateral ($\alpha=0.05$)

Col Mean- Row Mean	<i>Acacia mangium</i>	<i>Actinocephalus ramosus</i>	<i>Blechnum serrulatum</i>	<i>Lagenocarpus rigidus</i>	<i>Syngonanthus nitens</i>	<i>Gaylussacia brasiliensis</i>
<i>Actinocephalus ramosus</i>	-2.743 0.003*					
<i>Blechnum serrulatum</i>	-2.742 0.003*	-0.406 0.342				
<i>Lagenocarpus rigidus</i>	-7.038 0.000*	-5.196 0.000*	-3.870 0.000*			
<i>Syngonanthus nitens</i>	-2.290 0.011*	-0.170 0.432	0.163 0.435	3.535 0.000*		
<i>Gaylussacia brasiliensis</i>	-4.106 0.000*	-2.577 0.005*	-2.167 0.015*	0.218 0.414	-2.165 0.015*	
<i>Marcetia taxifolia</i>	0.132 0.447	3.452 0.000*	3.262 0.001*	8.630 0.000*	2.652 0.004*	4.454 0.000*

Figures

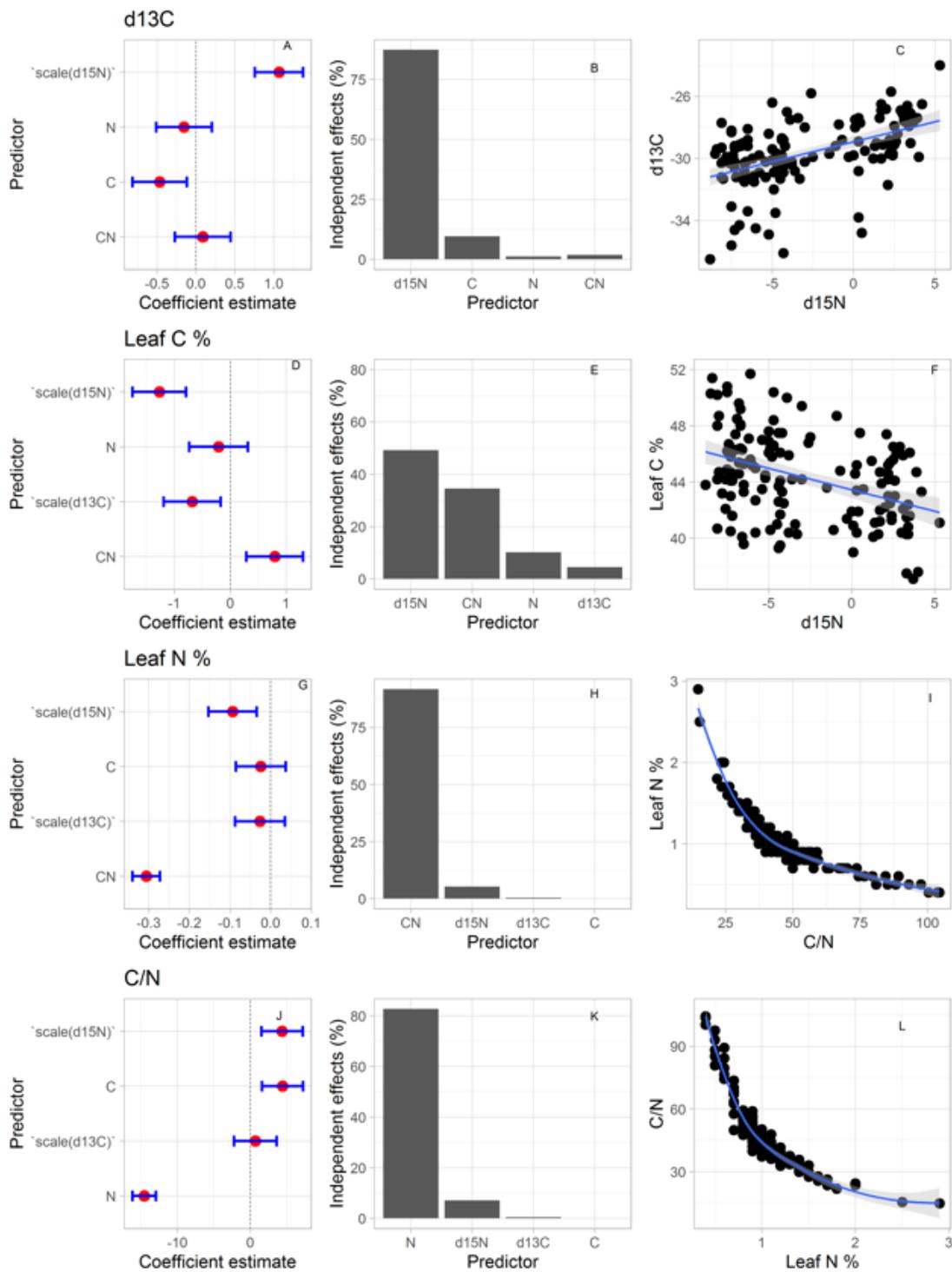


Figure 1

Coefficients of GLMs (A-F) and non-linear models (G-L) of C3 plants in non-invaded plots of Mussunungas, a type of hyperseasonal sandy savanna in Caravelas Municipality, Brazil. A-C: A, coefficients of $\delta^{13}\text{C}$ ($d^{13}\text{C}$) responding to Z-scaled $\delta^{15}\text{N}$ (scaled $d^{15}\text{N}$), leaf nitrogen % (N), leaf carbon % (C), C/N (CN); B, explaining percentages of the $\delta^{13}\text{C}$ responses by predictor variables; C, biplot of $\delta^{13}\text{C}$ responding to $\delta^{15}\text{N}$. D-F: D, coefficients of Leaf C% responding to explaining variables; E, explaining

percentages of Leaf C% responses by predictor variables; F, biplot of Leaf C% responding to $\delta^{15}\text{N}$. G-I: G, coefficients of Leaf N% responding to explaining variables; H, explaining percentages of Leaf N% responses by predictor variables; I, biplot of Leaf N% responding to C/N. J-L: J, coefficients of C/N responding to explaining variables; K, explaining percentages of C/N responses by predictor variables; L, biplot of C/N responding to Leaf N%.

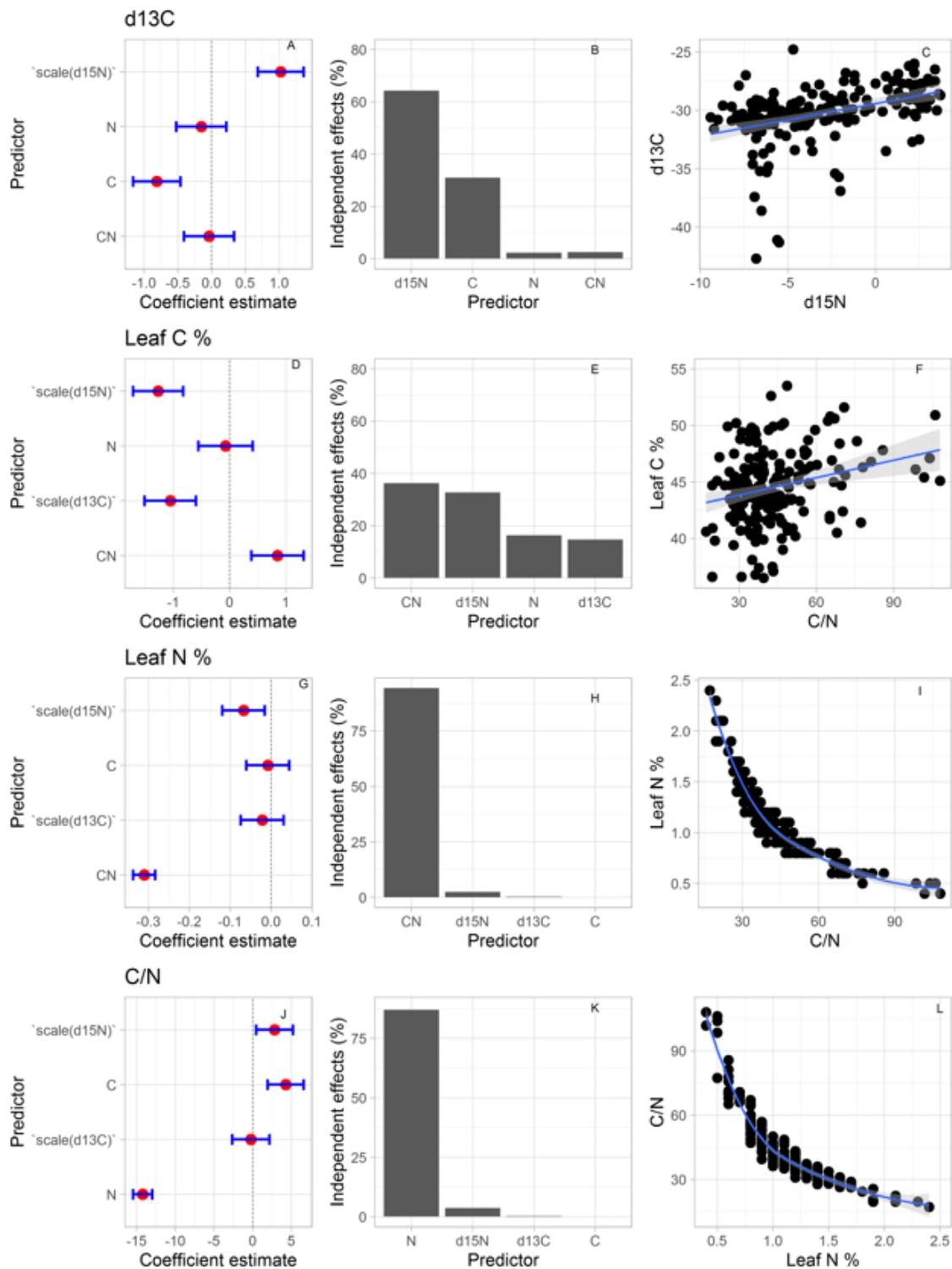


Figure 2

Coefficients of GLMs (A-F) and non-linear models (G-L) of C3 plants in invaded plots of Mussunungas, a type of hyperseasonal sandy savanna in Caravelas Municipality, Brazil. A-C: A, coefficients of $\delta^{13}\text{C}$ (d13C) responding to Z-scaled $\delta^{15}\text{N}$ (scaled d15N), leaf nitrogen % (N), leaf carbon % (C), C/N (CN); B, explaining percentages of the $\delta^{13}\text{C}$ responses by predictor variables; C, biplot of Leaf C% responding to $\delta^{15}\text{N}$. D-F: D, coefficients of Leaf C% responding to explaining variables; E, explaining percentages of Leaf C% responses by predictor variables; F, biplot of Leaf C% responding to C/N. G-I: G, coefficients of Leaf N% responding to Z-scaled $\delta^{15}\text{N}$ (scaled d15N), leaf carbon % (C), Z-scaled $\delta^{13}\text{C}$ (scaled d13C), C/N (CN); H, explaining percentages of Leaf N% responses by predictor variables; I, biplot of Leaf N% responding to C/N. J-L: J, coefficients of C/N responding to explaining variables; K, explaining percentages of C/N responses by predictor variables; L, biplot of C/N responding to Leaf N%.

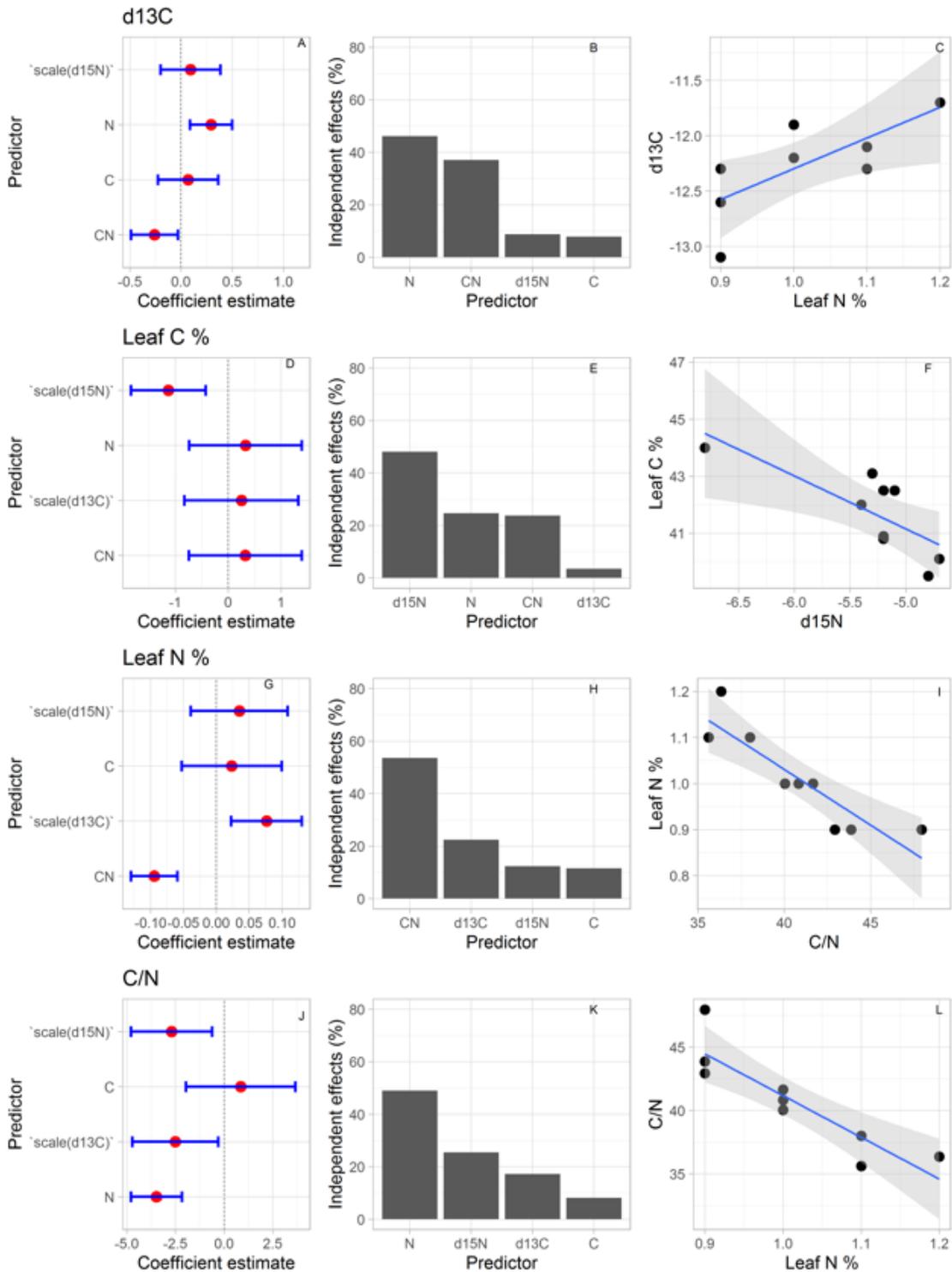


Figure 3

Coefficients of GLMs of C4 plants in non-invaded plots of Mussunungas, a type of hyperseasonal sandy savanna in Caravelas Municipality, Brazil. A-C: A, coefficients of $\delta^{13}\text{C}$ (d13C) responding to Z-scaled $\delta^{15}\text{N}$ (scaled d15N), leaf nitrogen % (N), leaf carbon % (C), C/N (CN); B, explaining percentages of the $\delta^{13}\text{C}$ responses by predictor variables; C, biplot of $\delta^{13}\text{C}$ responding Leaf N%. D-F: D, coefficients of Leaf C% responding to explaining variables; E, explaining percentages of Leaf C% responses by predictor

variables; F, biplot of Leaf C% responding to $\delta^{15}\text{N}$. G-I: G, coefficients of Leaf N% responding to responding to explaining variables; H, explaining percentages of Leaf N% responses by predictor variables; I, biplot of Leaf N% responding to C/N. J-L: J, coefficients of C/N responding to explaining variables; K, explaining percentages of C/N responses by predictor variables; L, biplot of C/N responding to Leaf N%.

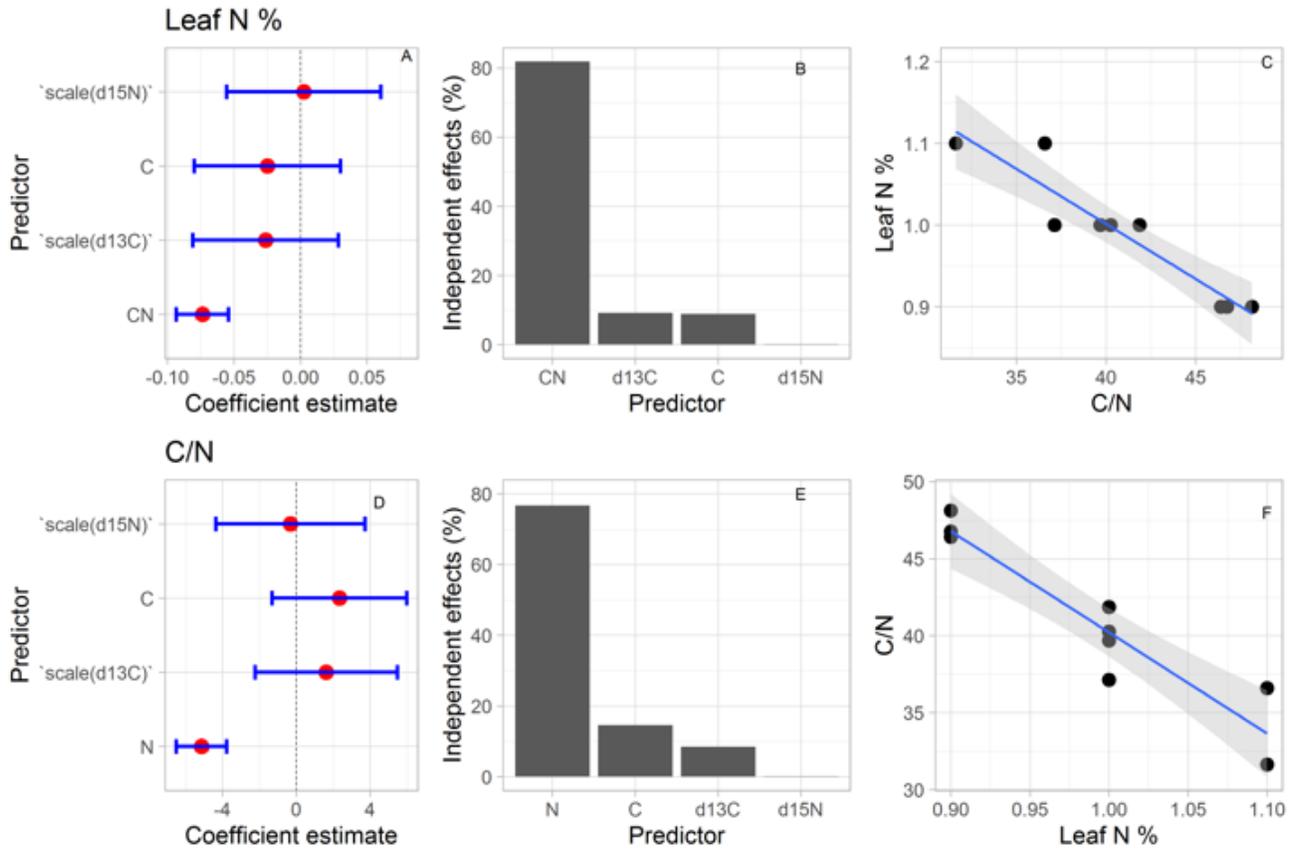


Figure 4

Coefficients of GLMs of C4 plants in invaded plots of Mussunungas, a type of hyperseasonal sandy savanna in Caravelas Municipality, Brazil. A-C: A, coefficients of Leaf N% responding to Z-scaled $\delta^{15}\text{N}$ (scaled d15N), leaf carbon % (C), Z-scaled $\delta^{13}\text{C}$ (scaled d13C), C/N (CN); B, explaining percentages of Leaf N% responses by predictor variables; C, biplot of Leaf N% responding to C/N. D-F: D, coefficients of C/N responding to explaining variables; E, explaining percentages of C/N responses by predictor variables; F biplot of C/N responding to Leaf N%.

Supplementary Files

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- [Supplementarymaterial.docx](#)