A single early-life herbivory event increases nodule investment in African savanna woody legumes

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1. Abstract

Objective

The impact of browsing herbivory on woody legumes is well understood in savanna landscapes. However, it is relatively unknown whether browsing impacts the rhizobia-legume mutualism with savanna trees. Our study investigated how early-life herbivory (simulated by clipping), influences nodulation and traits associated with nitrogen (N_2) fixation in saplings of three African savanna tree species (*Senegalia nigrescens*, *Vachellia exuvialis*, and *V. sieberiana*). We examined both species- and age-specific responses that may facilitate survival in savanna environments with high herbivore pressure.

Methods

Plants were germinated and grown in a semi-arid savanna experimental plot and subjected to simulated browsing via clipping at 3, 4 or 5 months old. Nodulation was assessed using nodule biomass (total nodule mass per plant, reflecting overall nodulation) and nodule mass fraction (NMF; representing the proportion of belowground biomass allocated to nodules). Leaf $\delta^{15}N$ was measured as an indirect proxy for N_2 fixation. Plants were harvested after 22 months of growth to assess longer-term effects of early-life herbivory.

Results

Clipping increased nodule biomass and NMF, with the strongest responses following clipping at four months. Nodule biomass and NMF were largest in *V. sieberiana* and lowest in *S. nigrescens*. Leaf $\delta^{15}N$ did not differ among clipping treatments or species.

Conclusion

We suggest that early-life herbivory alters nodule investment in savanna saplings. However, experiments incorporating repeated herbivory would also help determine whether the responses observed following a single clipping event persist under the repeated browsing experienced by savanna woody plants.

Keywords - savanna; biological nitrogen fixation; nodulation; plant–herbivore interactions; herbivory; resource allocation; woody legumes

2. Introduction

African savannas are home to a diversity of large mammal herbivores (Cumming, 1982; Scogings & Mopipi, 2008; Sebata, 2013). Browsing of woody plants by these herbivores exerts a major control on savanna vegetation structure and function (Archibald & Hempson, 2016; Bond, 2008; Osborne et al., 2018). Millions of years of plant-herbivore co-evolution have shaped browsing strategies and the defensive responses of woody plants (Charles-Dominique et al., 2016). In savanna regions dominated by browsers, woody plants have evolved both structural and chemical defences to resist and tolerate herbivory. However, seedlings to saplings are vulnerable herbivory events (Belsky, 1986; Staver et al., 2009; Staver & Bond, 2014). Browsers consume leaves; damaging plants, altering plant architecture, and suppressing the transition of saplings to adult size classes, a process known as the "browse trap" (Staver et al., 2009). Unlike more predictable disturbances such as fire, browsing is highly stochastic in both space and time and can occur repeatedly during the early stages of plant establishment (Archibald et al., 2021). This unpredictability means that herbivory not only acts as a filter through mortality but may also generate strong and variable selection on phenotypic traits in surviving individuals. The extent to which early browsing shapes subsequent plant physiology and resource allocation remains poorly understood. Little is known about whether herbivory experienced during early establishment alters investment in belowground rhizobial mutualisms that underpin nutrient acquisition.

Fabaceae (legumes) are a dominant plant family in African arid and semi-arid savanna (Osborne et al., 2018). Legume tree density positively corresponds to browser biomass (Archibald & Hempson, 2016). Due to the high leaf protein content legume trees typically constitute a major component of browser diets (Milewski et al., 1991; Osborne et al., 2018; Sauer, 1983; Zinn et al., 2007). *Senegalia* and *Vachellia* species have evolved structural defences such as spinescence and a cage-like architecture to minimise loss of aboveground biomass to herbivory events (Charles-Dominique et al., 2016; Hanley et al., 2007; Sebata, 2013). Additionally, some *Senegalia* and *Vachellia* species respond to herbivory by producing smaller, tannin-rich leaves that reduce palatability and subsequent consumption (Fornara & Du Toit, 2007; Scogings, 2014; Zinn et al., 2007). Consequently, browsers may preferentially consume trees with larger leaves and/or smaller spines, where more nutrition is available per bite and unit of time (Owen-Smith & Novellie, 1982; Zinn et al., 2007). *Senegalia* and *Vachellia* species also employ tolerance strategies in response to browsing pressure by rapidly regrowing damaged shoots and from protected meristems (Scogings et al., 2011). Regrown leaves often have a greater photosynthetic capacity than mature leaves, increasing regrowth capacity (Sebata, 2013). Tolerance strategies allow plants to compensate for biomass loss following herbivory and are characteristic of plants occurring in nutrient-rich environments (Rosenthal & Kotanen, 1994; Sebata, 2017; Skarpe &

Hester, 2008). However, herbivore avoidance and tolerance strategies impose substantial nutrient costs on *Senegalia* and *Vachellia* species under browsing pressure in savanna ecosystems (Tomlinson et al., 2016; Wigley et al., 2018).

The regrowth of leaves, spinescence, chemical and physical defences can increase plant nitrogen demand (N; Pellegrini, 2016; Santi et al., 2013). Many legumes acquire additional N through symbiotic N_2 fixation, whereby rhizobia housed within root nodules convert atmospheric N_2 into plant-usable NH_4^+ in exchange for photosynthetically derived C (Cohn et al., 1998; Sprent, 2009). Supplemental N acquisition can confer a competitive advantage, given the crucial role of N in plant growth and performance (Fathi, 2022; Ye et al., 2022). Interactions between plant fitness and N_2 fixation depend on soil N availability and form (West et al., 2002), the biotic and abiotic environment (Denison, 2000; Heath & Tiffin, 2007), the genotypes of the interacting plant and bacteria (Heath, 2010; Heath & Tiffin, 2007), and species plant traits (Heath, 2010; Parker, 1999). It remains unclear whether herbivory alters allocation to rhizobial symbiosis in woody savanna legumes, and whether any such effects persist beyond the immediate response to tissue loss.

Biological N_2 fixation could be a key functional trait enabling *Senegalia* and *Vachellia* species to survive herbivore pressure. However, investment in rhizobial symbioses presents a resource allocation trade-off because nodules require photosynthetically derived C (Cohn et al., 1998; Sprent, 2009). This trade-off is particularly acute during early establishment, when seedlings possess limited C reserves and browsing can remove photosynthetic tissue (Holland et al., 2017). At the same time, rapid regrowth following herbivory increases plant N demand (Du Toit et al., 1990). Previous work has shown that generalist insect herbivores trigger nodulation in *Medicago truncatula* (Heath & Lau, 2011), *M. lupulin* and soybean (*Glycine max*; Dean et al., 2009). Increases in nodulation due to insect herbivory have been attributed to alterations in the root:shoot C ratio, via increased belowground C allocation and shared hormonal signalling networks (Machado et al., 2013). Jasmonic acid is a plant hormone released in response to tissue injury, and elevated levels are associated with increased nodulation (Hause & Schaarschmidt, 2009; Oka-Kira & Kawaguchi, 2006; Sun et al., 2006). To date there has been minimal examination in savanna woody legumes of the impacts of browsing on nodulation and N_2 fixation. We suggest that the capacity to adjust rhizobial symbioses in response to browsing could represent a functional mechanism enabling savanna woody legumes to tolerate herbivory .

Here, we aimed to determine whether **a single early-life herbivory event can alter subsequent belowground rhizobial investment in African savanna woody legumes.** The seedling life phase for

savanna woody plants represents a critical period of plant development, when C reserves are limited and the loss of photosynthetic tissue may fundamentally alter resource allocation. We investigated this interaction in legumes occurring in regions of high (*Senegalia nigrescens; Vachellia exuvialis*) and low (*V. sieberiana*) herbivore densities**.** We measured individual plant nodule biomass and nodule mass fraction (NMF) as indicators of nodulation and a proxy for belowground allocation to nodules, along with the natural abundance of stable N isotopes ($\delta^{15}N$) in leaf tissues as an indirect proxy for N_2 fixation (Table 1). We hypothesised that **browsing in early-life stages alters investment in nodulation and N_2 fixation in three species of *Vachellia* and *Senegalia,*** reflecting increased demand for N following tissue loss.

144 **Table 1.** An overview of the traits measured as proxies for nodulation and N_2 fixation in this experiment. The traits include nodule biomass, nodule mass
145 fraction (NMF), and leaf $\delta^{15}N$ values.

Trait	Theoretical basis	Method	Strengths	Limitations	References
Nodule biomass and Nodule mass fraction (NMF)	- Nodule biomass is an absolute measure of the total mass of nodules per plant, reflecting the overall extent of nodulation - NMF is a biomass allocation trait that represents the proportion of belowground biomass invested in nodules	- Destructive harvesting - Biomass collection	- Useful for result interpretation when combined with other measurements - Indicative of long-term N allocation patterns	- Requires destructive sampling - Does not capture seasonal variability	(Alon et al., 2021; Batterman et al., 2013; Hardarson & Danso, 1993; Telford et al., 2023)
Leaf $\delta^{15}N$	- Reflects the ratio of ^{15}N:^{14}N in leaf tissue - During N_2 fixation, biological processes discriminate against heavier ^{15}N (fractionation) - N_2-fixing plants have lower leaf $\delta^{15}N$ due to preferential uptake of ^{14}N	- Mass spectrometry	- Widely used as indirect proxy for N_2 fixation - Indicative of short term N allocation patterns	- Requires destructive harvesting - Does not capture seasonal variability - Can be affected by soil N sources and fractionation	(Ariz et al., 2015; Chalk et al., 2016; Delwiche & Steyn, 1970; Hardarson & Danso, 1993; Peoples et al., 2002; Unkovich et al., 2008)

146

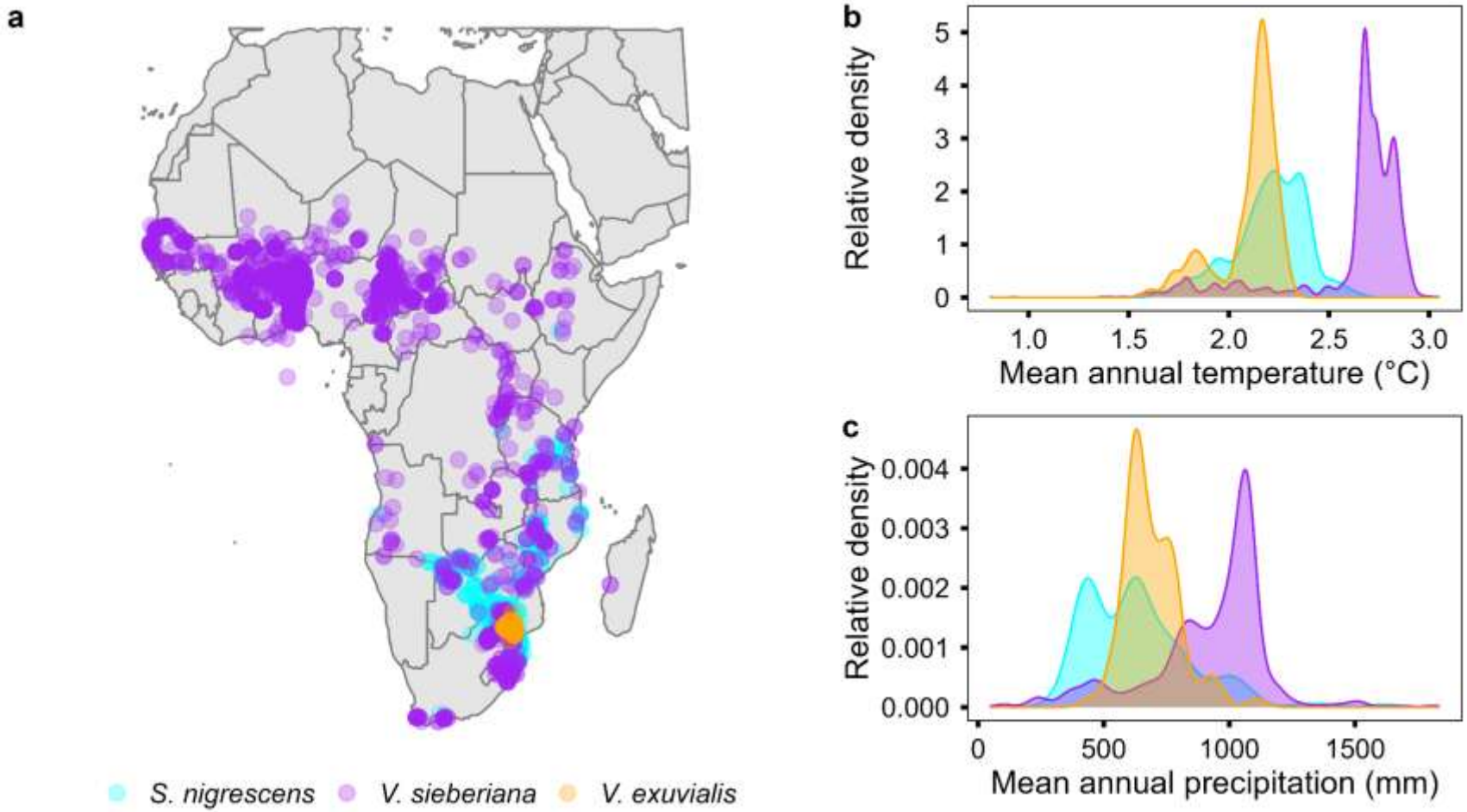
3. Materials and methods

3.1 Study species

***Senegalia nigrescens* (Oliv.) P.J.H.Hurter** is predominantly found in semi-arid African savannas with a mean annual precipitation of 400 mm (Fig. 1a & 1b) and associated with a high herbivore biomass (>2,000 kg km^{-2}; Archibald & Hempson, 2016). It is regularly browsed by giraffe (*Giraffa camelopardalis*), kudu (*Tragelaphus strepsiceros*) and elephant (*Loxodonta Africana;* Fornara & Du Toit, 2008; Ruwanza, 2019). *Senegalia nigrescens* develops spines and prickles, and herbivory has been shown to increase spinescence in response to damage (Du Toit et al., 1990; Fornara & Du Toit, 2008; Scogings, 2014).

***Vachellia exuvialis* (I.Verd.) Kyal. & Boatwr.** is typically found in semi-arid African savannas with a mean annual precipitation of 600 mm (Fig. 1a & 1b) and is associated with a high herbivore biomass (>2,000 kg km^{-2}; Archibald & Hempson, 2016). Leaves and pods of *V. exuvialis* are palatable and typically browsed by impala (*Aepyceros melampus)* and duiker (Cephalophinae; Grant & Thomas, 2005; Hattas et al., 2011). *Vachellia exuvialis* employs herbivore tolerance strategies by rapidly regrowing leaves and shoots following browsing damage (Hean & Ward, 2012; Lama et al., 2019; Skarpe & Hester, 2008).

***Vachellia sieberiana* (DC.) Kyal. & Boatwr.** is prevalent in mesic savannas, where mean annual precipitation exceeds 1000 mm (Fig. 1a & 1b), and where herbivore biomass is relatively low (>1,000 kg km-2; Archibald & Hempson, 2016). *Vachellia sieberiana* experiences infrequent herbivory by giraffe (*G. camelopardalis*). In response *V. sieberiana* foliage contains high levels of tannins, while increasing spine length, and a decrease in leaf size, form important inducible defences (Zinn et al., 2007).



171

172 **Fig. 1.** *Geographic distributions and climatic niches of Senegalia nigrescens, Vachellia exuvialis and V. sieberiana.* **(a)** Occurrence records across Africa.
173 Occurrence data were obtained from (GBIF.org, 2020). Kernel density distributions of **(b)** mean annual temperature (MAT, °C) and **(c)** mean annual
174 precipitation (MAP, mm). Climatic variables were extracted from WorldClim v2.

175 **3.2 Study site**

176 The study site was located on the western boundary of Kruger National Park at the Wits Rural Facility
177 (WRF; 24.57° S, 31.09° E). The WRF is a protected area, and is a field station owned by the University
178 of Witwatersrand. WRF is situated on weathered granite with occasional dolerite intrusions
179 (Shackleton, 1993, 2002). The site is a semi-arid savanna with a mean annual rainfall of 651 ± 123 mm
180 and at an altitude of ~550 m (Fisher et al., 2015). Rainfall is seasonal and falls from October to March,
181 in the form of convectional thundershowers (Fisher et al., 2015; Shackleton, 1993, 2002). The savanna
182 woody plant community is dominated by species from the Combretaceae and Mimosoideae clades
183 (Mucina & Rutherford, 2006).

184

185 **3.3 Experimental design**

186 *Senegalia nigrescens, V. exuvialis*, and *V. sieberiana* were germinated from seed. Initially, plants were
187 established and grown under a shade cloth at WRF for two weeks in August 2016. In September 2016,
188 seedlings were transplanted into a 10 m × 10 m experimental plot, spaced 0.5 m apart. An irrigation
189 system was installed to ensure consistent moisture and prevent water limitation. The soil was
190 inoculated with rhizobia using a mixture of river sand, potting soil, and soil from WRF, where a variety
191 of N_2-fixing legumes, including *Dichrostachys, Senegalia*, and *Vachellia* species, naturally grow (Fisher
192 et al., 2015). The experimental plot was fenced to exclude herbivores, and grass competition was kept
193 to a minimum.

194

195 Herbivory treatments involved simulated browsing (here after referred to as clipping). All
196 aboveground tissue >1 cm above the cotyledon was removed as a single event at either 2, 3, 4, or 5
197 months old, or were not clipped as a control. Due to lack of survival in the herbivory treatment where
198 plants were 2-month-old this treatment was excluded from all analysis. Herbivory treatment was
199 applied as a randomised block design across 16 subplots (excluding plants in the 2 month herbivory
200 treatment) within the experimental plot (SI Fig. 1). Each herbivory treatment was replicated across
201 four subplots, with four individuals of each species planted within each subplot. Survival varied among
202 species and herbivory treatments; final replication is provided in SI Table 1.

203

204 Following herbivory treatments, the plants continued to grow for two additional seasons, maturing
205 into saplings. This experiment was part of a larger study, for a full description see Archibald et al.,
206 (2021).

207

208 **3.4 Harvest**

Plants were harvested at 22 months old in a random order during June 2018. Aboveground plant parts were separated by cutting the stem at the soil surface, oven-dried at 70 °C for 48 hours and weighed using a three-point balance.

Root systems were excavated and coarse roots extracted using methods adapted from Castellanos et al., (1991). At the point of excavation (width and length; 25 cm x 25 cm), the soil surrounding the length of the coarse root system was placed directly into two one litre plastic containers. The soil containers were emptied onto a white 2 x 2 m board. Roots were sorted for a standardised search effort of nine person-minutes per sample, a method adapted from Metcalfe et al., (2007). In this investigation nine minutes (three people for three minutes) per one litre of soil was sufficient root sorting time.

Preliminary sampling of the soil surrounding 100 cm of root profile did not find nodules deeper than 20 cm. Nodules were separated from roots, cleaned using a fine brush, and washed using a 1 mm fine mesh sieve. Dead and decaying nodules were discarded and remaining nodules were used as an indicator of active/recent nodulation. Nodules were oven dried at 70 °C in paper bags for 48 hours and weighed using a three-point balance (Telford et al., 2023).

Nodule mass fraction (NMF: Table 1) was quantified through dry belowground plant biomass and dry nodule biomass data were used to calculate NMF using the following equation:

$$NMF\ (\%\ biomass) = \left(\frac{dry\ nodule\ biomass\ (g)}{dry\ belowground\ biomass\ (g)}\right) x\ 100$$

Dried leaves were analysed for $\delta^{15}N$ and separately ground to a powder using a tissue lyser (Qiagen, Hilden, Germany), 3 mg of milled material was weighed into tin capsules (Electrical Microanalysis Ltd., Okehampton, UK) for mass spectrometry at the University of Edinburgh. Elemental analysis was performed using a Elementar vario PYRO cube Elemental Analyser (Elementar-Straße 1, 63505 Langenselbold, Germany) and a Elementar PRECISION stable isotope ratio mass spectrometer (Elementar-Straße 1, 63505 Langenselbold, Germany). Internal standards with known isotope ratios were used to calibrate the mass spectrometer. The $\delta^{15}N$ values for each sample were calculated using the formula:

$$\delta 15N = \left(\frac{R\ sample}{R\ standard} - 1\right)\ x\ 1000$$

where R_{sample} is the ratio of ^{15}N:^{14}N in the sample, and $R_{standard}$ is the ^{15}N:^{14}N ratio in the standard (atmospheric N = 0‰). Due to funding constraints $\delta^{15}N$ analysis was carried out on a subset of saplings (SI Table 1).

3.5 Statistical analysis

All statistical analyses were conducted in R (R Core Team, 2024), and graphs were made using the 'ggplot2' package (Wickham, 2016).

The proportion of individuals that developed nodules (nodulated; not nodulated) was modelled using a binomial generalised linear mixed-effects model, with species (3 species), herbivory treatment (4 herbivory treatments) and their interaction as fixed effects and subplot (16 subplots) as a random effect, to account for the experimental design and non-independence among individuals within subplots ('lme4' package; Bates et al., 2015). Candidate models containing the species, herbivory treatment, and interaction, along with an intercept-only model were compared using Akaike's Information Criterion (AIC). Model convergence and diagnostics were assessed prior to interpretation.

Nodule biomass and NMF were analysed using generalised linear mixed-effects models ('glmmTMB' package; Brooks et al., 2017). Both responses contained substantial numbers of true zero values, corresponding to individuals that had not developed nodules, and positive values were strongly right-skewed. A Tweedie error distribution with a log link was used to accommodate continuous positive responses with exact zero values. Species (3 species), herbivory treatment (4 treatments) and their interaction were included as fixed effects, with subplot (16 subplots) included as a random effect. For nodule biomass and NMF, candidate models included the full species × herbivory treatment interaction, additive effects of species and herbivory treatment, models containing either species or herbivory treatment alone, and an intercept-only model. Candidate models were compared using AIC, with lower AIC values indicating greater support. Where the best-supported model contained significant fixed effects, Type II tests of fixed effects were conducted ('car' package; (Fox & Weisberg, 2019), followed by estimated marginal means and Tukey-adjusted pairwise comparisons ('emmeans' package; Lenth, 2023). Model estimates for nodule biomass and NMF were presented on the response scale.

Leaf $\delta^{15}N$ was measured on a randomly selected subset of individuals independently, resulting in insufficient replication within subplots to estimate subplot-level variance reliably. Consequently,

subplot was not included as a random effect in analyses of leaf $\delta^{15}N$ due to replication numbers (SI Table S1). Linear models included species (3 species), herbivory treatment (4 herbivory treatments) and their interaction as fixed effects, with candidate models compared using AIC ('stats' package; R Core Team, 2024).

4. Results.

The proportion of saplings that developed nodules differed significantly among species (df = 2, χ^2 = 7.72, $p < 0.05$). Nodule development was lowest in *S. nigrescens* (27–78%), intermediate in *V. exuvialis* (60–78%), and highest in *V. sieberiana* (83–100%; Fig. 2a & SI Table 2). There was the strongest model support for the species-only model (AIC = 122.70), with less support for models including herbivory treatment (AIC = 129.38) or the treatment × species interaction (AIC = 129.20; SI Table 3).

Nodule biomass was significantly affected by herbivory treatment (χ^2 = 9.83, df = 3, p = 0.020), and differed among species (χ^2 = 20.29, df = 2, $p < 0.001$). The model estimated mean nodule biomass across species as 0.03 g in control plants and increased to 0.04 g when clipped at 3 months, 0.20 g when clipped at 4 months, and 0.12 g when clipped at 5 months. Pairwise comparisons indicated that nodule biomass was significantly greater following clipping at 4 months than in control plants (~7-fold; Tukey-adjusted $p < 0.05$), while the remaining treatment contrasts were not significant (Fig. 2b). Estimated mean nodule biomass was 0.09 g in *S. nigrescens*, 0.03 g in *V. exuvialis*, and 0.15 g in *V. sieberiana*. Nodule biomass was significantly greater in *S. nigrescens* than *V. exuvialis* (~3-fold; Tukey-adjusted $p < 0.05$) and in *V. sieberiana* than *V. exuvialis* (~5-fold; Tukey-adjusted $p < 0.001$), whereas *S. nigrescens* and *V. sieberiana* did not differ significantly ($p > 0.05$). Nodule biomass was best described by additive effects of herbivory treatment and species (AIC = −13.37). This model received greater support than the corresponding interaction model (AIC = −5.40), species-only model (AIC = −12.24), treatment-only model (AIC = 0.80), and null model (AIC = 1.56; SI Table 3).

Nodule mass fraction (NMF) was significantly affected by herbivory treatment (χ^2 = 22.66, df = 3, $p < 0.001$), and differed among species (χ^2 = 6.62, df = 2, $p < 0.05$). The model estimated mean NMF across species as 0.11% in control plants, increasing to 0.25% when plants were clipped at 3 months, 1.04% when clipped at 4 months, and 0.64% when clipped at 5 months. Pairwise comparisons indicated that NMF was significantly greater following clipping at 4 months than in control plants (~9-fold; Tukey-adjusted $p < 0.001$) and following clipping at 5 months than in control plants (~6-fold; Tukey-adjusted $p < 0.01$), while the remaining treatment contrasts were not significant (Fig. 2c). Estimated mean NMF was 0.39% in *S. nigrescens*, 0.22% in *V. exuvialis*, and 0.58% in *V. sieberiana.* NMF was significantly

greater in *V. sieberiana* than *V. exuvialis* (~3-fold; Tukey-adjusted $p < 0.05$), whereas *S. nigrescens* did not differ significantly from either species ($p > 0.05$). NMF was best described by the additive model containing herbivory treatment and species (AIC = 208.28). This model received greater support than the corresponding interaction model (AIC = 214.58), treatment-only model (AIC = 210.69), species-only model (AIC = 215.12), and null model (AIC = 216.56; SI Table 3).

Leaf $\delta^{15}N$ values were consistently low across treatments and species (means ranging from 1.25‰ to 2.94‰).The best-supported model included herbivory treatment alone (AIC = 161.47; SI Table 3). However, we found no evidence that leaf $\delta^{15}N$ differed between herbivory treatments ($df = 3$, $F = 2.33$, $p > 0.05$), species ($df = 2$, $F = 0.74$, $p > 0.05$), and there was no interaction ($df = 6$, $F = 0.34$, $p > 0.05$; Fig. 2d).

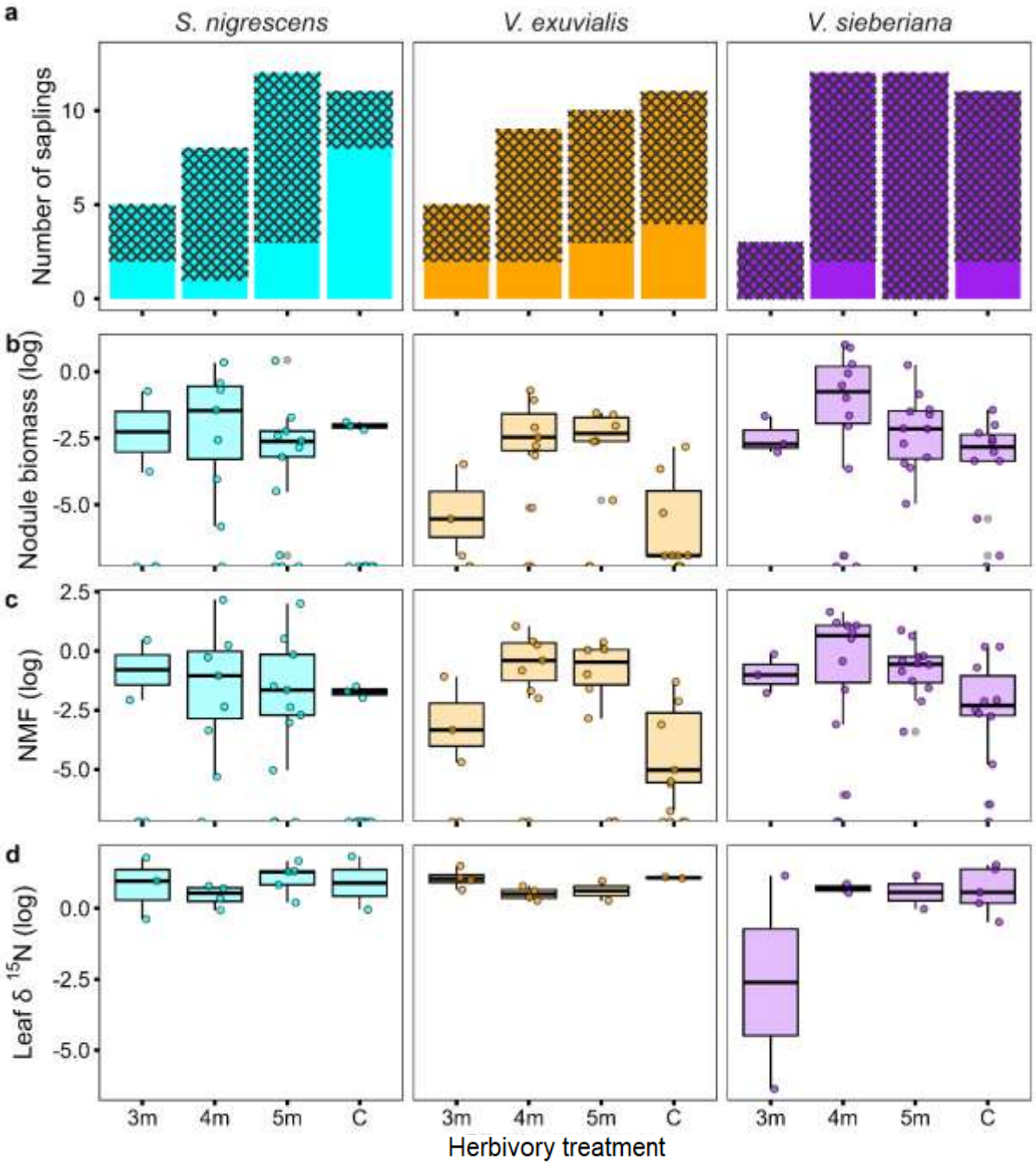


Fig. 2. *Nodulation and N_2 fixation related traits for Senegalia nigrescens, Vachellia exuvialis, and V. sieberiana subjected to clipping at different ages*. **(a)** a stacked bar chart proportion of saplings developing nodules (crosshatched bars indicate nodules present), boxplots showing **(b)** nodule biomass (g), **(c)** nodule mass fraction (%), and **(d)** leaf $\delta^{15}N$ values. Herbivory treatment corresponds to the age when plants were clipped (3 months (3m), 4 months (4m), 5 months (5m), and an unclipped control (C)). The Y-axes for **(b-d)** were log-transformed to improve visibility and comparison of distributions.

5. Discussion

A single early-life herbivory event altered long-term patterns of nodule biomass and belowground allocation to nodules in *S. nigrescens, V. exuvialis,* and *V. sieberiana* saplings, but did not affect the probability of nodule development or leaf $\delta^{15}N$. Thus, clipping potentially triggers a greater investment in nodules rather than an increase in the likelihood of establishing the rhizobial symbiosis. The data provides partial support for our hypothesis that early-life herbivory would alter investment in nodulation and N_2 fixation. Our results suggest that herbivory can modify nodule resource allocation in *Senegalia* and *Vachellia* saplings, with potentially important consequences for nutrient acquisition and growth during subsequent developmental stages.

Higher proportions of nodulated saplings in *V. sieberiana* and *V. exuvialis* than in *S. nigrescens* likely reflect inherent species-specific nodulation strategies rather than typical herbivory levels in their native ranges. This interpretation is supported by the contrasting nodulation patterns of *V. exuvialis* and *S. nigrescens*, despite both occurring in regions with high herbivore densities. This pattern is consistent with a previous pot-based study, which found that one-year-old *Vachellia* species have higher levels of nodulation relative to *Senegalia* (Telford et al., 2025). A potential explanation could be that despite lower nodulation *S. nigrescens* exhibited greater regrowth and branching following herbivory (Archibald et al., 2021). Therefore, *S. nigrescens* may prioritise structural defences like branching growth generating a "cage-like architecture" that reduces herbivore access (Cooper & Owen-Smith, 1985; Rohner & Ward, 1997). In contrast, *Vachellia* species may favour N-acquisitive strategies, as *V. exuvialis* and *V. sieberiana* regained biomass following herbivory (Archibald et al., 2021). Regrowth was less pronounced in *Vachellia* species in comparison to *S. nigrescens*, suggesting that increased N acquisition in *Vachellia* may not be allocated primarily to compensatory regrowth. The higher nodule investment observed in the two *Vachellia* species may indicate greater allocation to N acquisition, although whether this reflects a broader genus-level strategy requires testing across a larger number of species.

In this experiment, a single herbivory event altered investment in nodulation across all three species. Saplings clipped at four months exhibiting the largest nodule biomass and NMF, although developmental stage and time since clipping cannot be separated in this design. However, increased nodulation following herbivory during early life stages could be explained through seedling root traits. Juvenile plants can exhibit greater plasticity in root hair development, potentially facilitating penetration by rhizobial infection threads (Bhuvaneswari et al., 1980; Remmler et al., 2014; Tominaga et al., 2009). However, nodules are dynamic structures that undergo continuous development and

365 senescence (Puppo et al., 2005), and our measurements were taken 17-20 months after clipping. We
366 therefore interpret the higher nodule biomass and NMF observed following clipping at four months
367 as a seedling response to early-life disturbance. We suggest that the physiological consequences of
368 herbivory may depend on the plant life stage, with early damage potentially altering belowground
369 investment in rhizobial symbiosis. Experiments measuring root development, rhizobial infection and
370 C allocation at multiple time points following herbivory would help determine the mechanisms
371 underlying any possible stage-dependent responses.
372
373 Although we did not measure the mechanisms underlying why herbivory increased nodule
374 investment, several processes could potentially explain this response. We theorise that herbivore
375 damage may increase nodulation by triggering the reallocation of C in the form of non-structural
376 carbohydrates to belowground tissues (Babst et al., 2005; Heath & Lau, 2011; Heath & McGhee, 2012;
377 Henkes et al., 2008). Surplus root C promotes root exudation (Babst et al., 2005; Henkes et al., 2008;
378 Holland et al., 1996), providing chemical cues that attract rhizobia, facilitate infection, and boost
379 nodulation (Cohn et al., 1998). Given the physiological overlap between plant hormones active in
380 defence and symbiotic signalling, it is also possible that induced plant defences may disrupt the
381 regulation of nodulation (Heath & Lau, 2011). Tissue damage increases plant jasmonic acid levels
382 modulating nodule development (Hause & Schaarschmidt, 2009; Oka-Kira & Kawaguchi, 2006; Sun et
383 al., 2006). Elevated shoot jasmonic acid levels promote nodulation through interaction with ethylene
384 signalling, and increases root calcium levels in preparation for nodule development (Hause &
385 Schaarschmidt, 2009; Sun et al., 2006). An example of this mechanism is the supernodulator soybean
386 *nt* mutant which contains high concentrations of jasmonic acid in its shoots (Kinkema & Gresshoff,
387 2008; Seo et al., 2007). Herbivory could stimulate nodulation through C reallocation and hormone-
388 mediated signalling, due to interactions between plant defence responses and rhizobia. Distinguishing
389 between these mechanisms requires measurements of root C allocation, rhizobial infection and
390 nodule development immediately following herbivory.
391
392 We found no evidence that early-life clipping altered leaf $\delta^{15}N$ between species and across treatments
393 at harvest. However, leaf $\delta^{15}N$ values were relatively low (1.25–2.94 ‰), consistent with contribution
394 of N_2 fixation to meet N requirement in these legumes (Cramer & Bond, 2013; Kambatuku et al., 2011,
395 2013). We propose that the lack of isotopic differences between species and herbivory treatment does
396 not necessarily indicate that these factors had no effect on N acquisition at any point in the plant's life
397 cycle. The absence of differences 17-20 months after clipping suggests that any effects of early-life
398 herbivory on N_2 fixation may not persist to this developmental stage. Biological N_2 fixation is

responsive to environmental conditions such as drought, atmospheric CO_2 levels, and increased available soil N, rather than fixed (Kambatuku et al., 2013; Serraj, 2003; Telford et al., 2023). As N_2 fixation is energetically costly (Sprent, 1989; Vitousek & Howarth, 1991), saplings regulate N_2 fixation once established (Isaac et al., 2011), but in the seedling life phase N_2 fixation may be necessary to support rapid growth and escape from the "browse trap" (Staver et al., 2009). It is important to note that $\delta^{15}N$ was measured at the end of the experiment. Sampling leaf tissues during early-life stages and immediately after clipping may have better captured the short-term effects of herbivory on leaf $\delta^{15}N$.

Our study has several limitations that should be considered when interpreting these findings. First, herbivory was simulated as a single clipping event during early plant development, whereas browsing in natural savanna ecosystems is often repeated, variable in intensity, and occurs over multiple growing seasons. Consequently, our experiment highlights the long-term effects of an isolated early-life browsing event rather than chronic herbivory. Second, plants were harvested 17-20 months after treatment, providing insight into persistent responses but limiting our ability to assess short-term dynamics in nodulation and N_2 fixation immediately following damage. Third, different clipping ages necessarily resulted in different durations between clipping and harvest, preventing separation of developmental stage from time-since-herbivory effects. Fourth, due to funding constraints leaf $\delta^{15}N$ analysis had limited replication and therefore potentially limited power to detect treatment effects. Finally, our excavation and root sorting may underestimate data relating to nodulation. Future studies combining repeated herbivory treatments with $\delta^{15}N$ and nodule sampling would help further clarify herbivore-induced changes in the rhizobial symbiosis and N acquisition.

6. Conclusion

Our study provides evidence that early-life herbivory can increase nodule biomass and alter nodule allocation in savanna legumes. Leaf $\delta^{15}N$, used as an indirect proxy for N_2 fixation, did not differ between species and was not affected by clipping. Therefore, we suggest that early-life browsing can increase a plant's investment in belowground rhizobial mutualisms without necessarily producing detectable long-term changes in its isotopic N signature.

Future research should quantify nodulation and N_2 fixation at shorter time intervals following herbivory to resolve the short-term dynamics of the response, as well as at longer intervals to determine how persistent herbivory-induced changes in nodulation and N_2 fixation are. Further, experiments incorporating repeated and more realistic patterns of herbivory would provide insight

into whether the responses observed following a single clipping event persist under the repeated browsing experienced by juvenile savanna woody plants.

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authorship contributions

All authors contributed to the study conception and design. The wider experiment was conceptualised by Sally Archibald, Nicola Stevens and Wayne Twine. Material preparation, data collection and analysis were performed by Elizabeth Telford. The first draft of the manuscript was written by Elizabeth Telford. Caroline Lehman and Nicola Stevens commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

The dataset and code used in this study is available at the following repository (https://doi.org/10.5061/dryad.x95x69q1r)

Supplementary Files

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