The response of grassland mycorrhizal fungal abundance to a range of long-term grazing intensities

Maede Faghihinia[1,2], Yi Zou[1], Zheng Chen[1], Yongfei Bai[3], Wenhuai Li[3], Rob Marrs[2], Philip L. Staddon[1,4,5]

[1] Department of Health and Environmental Sciences, Xi'an Jiaotong-Liverpool University, Suzhou, Jiangsu, 215123, China;

[2] School of Environmental Sciences, University of Liverpool, Liverpool L69 3GP, UK;

[3] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China;

[4] Countryside and Community Research Institute, University of Gloucestershire, Cheltenham, GL50 4AZ, UK;

[5] School for Agriculture, Food and the Environment, Royal Agricultural University, Cirencester, GL7 6JS, UK.

Abstract

Keystone root symbiotic arbuscular mycorrhizal fungi play a major role in maintaining plant biodiversity, increasing plant productivity and enhancing storage of carbon in soil. AM fungi are ubiquitous and found in most ecosystems including grasslands currently experiencing increasing pressures form human activity. Grazing is known to impact AM fungi but very little is known about how AM fungi are affected by different levels of grazing intensity. Here we report on results from a long-term experimental site in a typical steppe in the north of China, containing seven levels of field-manipulated grazing intensities maintained for over 13 years. We assessed arbuscular mycorrhizal fungal abundance, represented by soil hyphal length density and mycorrhizal root colonization (mycorrhizal root frequency, intensity and arbuscule intensity) within the farm-scale field experiment. We also measured environmental variables to explain the responses of mycorrhizal fungi to grazing intensity. Our results showed that with an increase in grazing intensity, soil hyphal length density linearly decreased. There was, however, no significant trend for mycorrhizal root colonization variables in relation to grazing intensity. Mycorrhizal root frequency was negatively correlated with topographic-induced changes in soil nitrogen and phosphorus, while arbuscule intensity was marginally negatively correlated with soil available phosphorus. Further, we found a possible hump-shaped relationship between the ratio of external to internal AM fungal structures and grazing intensity. Our finding showed that external AM fungal structure was clearly impacted by grazing intensity but that this was not the case for internal mycorrhizal structures. This indicated that mycorrhizal functioning was impacted by the intensity of grazing as the mycorrhizal structures responded differently. Indeed the ratio of the foraging extra-radical mycorrhizal hyphae to intra-radical mycorrhizal structures was highest at moderate grazing intensity but strongly decreased by high grazing intensity. Our study suggests that the impacts of grazing intensity on the plant-AMF association could lead to further knock-on effects on the plant-soil system via the feedbacks that exist between plant and AMF communities.

1. Introduction

Grasslands play a crucial role in global ecosystem functioning and human well-being (O'Mara, 2012; Steinfeld et al, 2006). However, many grasslands are currently facing great pressures, of which over-grazing is one of the major drivers reducing grassland productivity and sustainability (Conant, 2010; O'Mara, 2012). Continuous excessive grazing for prolonged periods of time leads to the removal of plant biomass, changes plant community composition and increases soil erosion, resulting in a loss of grassland ecosystems productivity and the impoverishment of soil carbon stocks (Conant, 2010; McSherry & Ritchie, 2013). In order to maintain the sustainability of these ecosystems and optimize grazing management, a better understanding of ecological factors underlying below-ground processes under grazing pressures is crucial, as the above- and below-ground parts of terrestrial ecosystems are strongly interconnected (Yang et al, 2018).

Root symbiotic mycorrhizal fungi are key soil micro-organisms that play a vital role in maintaining grassland ecosystem productivity and stability (Asmelash et al, 2016; Moora & Zobel, 2010). Approximately 72% of all vascular plant species are associated with the mutualist arbuscular mycorrhizal fungi (AM fungi) (Brundrett & Tedersoo, 2018). The fungal symbiont relies on carbon obtained from the plant roots in return for providing nutrients, in particular phosphorus, to the plant (Moora & Zobel, 2010; van der Heijden et al, 2006). Therefore, AM fungi can enhance plant grazing-tolerance by improving nutritional status and thereby improve plant productivity (Moora & Zobel, 2010; Walling & Zabinski, 2006).

Grazing can alter AM fungal communities and function through changes to the mycorrhizal environment including plant and soil conditions (Ba et al, 2012; Guo et al, 2016). Long-term grazing reduces plant productivity and biodiversity through eliminating photosynthetic plant tissues and removing grazing-

sensitive rare species or palatable dominant species (Schönbach et al, 2011; Shelton et al, 2014; Wang et al, 2014). Herbivory can alter nutrient dynamics positively through the addition of dung and urine to the soil, and negatively through the reduction of plant biomass production and litter accumulation (Metera et al, 2010; Vertès et al, 2019). Both plant composition and soil conditions affect AM fungal communities. Therefore, it is reasonable to assume that the extent of the grazing impact on AM fungal function and community structure is largely dependent on the number of livestock per unit area as this will have different levels of impact on above- and below-ground productivity and diversity (Ba et al, 2012; Yan et al, 2013).

While overgrazing has destructive and irreversible negative impacts on plant community and soil properties, under-grazing can also be harmful to grassland biodiversity and functioning through less stimulation of plant growth and loss of grazing-dependent legumes and grasses (Metera et al, 2010). Moderate grazing has been shown to benefit grassland ecosystem conditions by the enhancement of natural fertilization, seed distribution, creating favorable conditions for annual and bi-annual species and inducing periodic defoliation (Metera et al, 2010). However, the effects of different grazing intensities on AM fungi is still contentious. Most studies compared the effects of grazing on AM fungal abundance in grazed and un-grazed plots (Guo et al, 2016; Murray et al, 2010; van der Heyde et al, 2017), with very few assessing impacts along a gradient of grazing intensity such as that ranging from light to overgrazing (Ba et al, 2012; Mendoza et al, 2011b; Ren et al, 2018).

Moreover, AM fungi exist in the two media of roots and soil, but most published studies focus either on AM fungal abundance within root by measuring mycorrhizal root colonization (Ba et al, 2012) or assessing the abundance in soil by determining the length of hyphae in the soil (Ren et al, 2018), with few studies examining both simultaneously (van der Heyde et al, 2017). However, as different AM fungal structures

vary in their response to grazing (van der Heyde et al, 2017), the various responses of different AM fungal parameters to environmental stresses are important since they may reveal mechanisms underlying those responses (Smith & Read, 2008).

Additionally, the effects of grazing on AM fungi is through grazing-induced changes in the environment experienced by the mycorrhizal fungi and this includes plant and soil-related factors (Guo et al, 2016; van der Heyde et al, 2017). Significant correlation between AM fungal variables and edaphic conditions such as soil organic carbon (Ren et al, 2018; Soudzilovskaia et al, 2015), nitrogen (Bai et al, 2013; Soudzilovskaia et al, 2015), phosphorus (Guo et al, 2016; Johnson et al, 2015), pH (Guo et al, 2016; Mendoza et al, 2011a), soil water content (Murray et al, 2010; van der Heijden et al, 2006) and soil bulk density (Augé, 2004; Simard & Austin, 2010) has been reported. Accordingly, a strong relationship between AM fungi and host plants has been documented for above-ground biomass (Ba et al, 2012; Hiiesalu et al, 2014), plant species richness (Ba et al, 2012; Chen et al, 2018) and diversity (Lekberg & Waller, 2016; Prober et al, 2015). It is, therefore, important to study not only the changes in AM fungal community in response to grazing, which requires long-term monitoring to be able to detect changes robustly, but also the environmental conditions, which may be altered by grazing and mediate many aspects of plant-mycorrhizal interactions (Mendoza et al, 2011a; van der Heyde et al, 2017).

Topography may also mediate grazing effects on AM fungal and mycorrhizal environment by altering resource availability (e.g. soil moisture, soil organic carbon and total nitrogen stocks) and plant community structure (Kölbl et al, 2011; Murray et al, 2010). Given that the plant-AM fungi association is fundamentally a symbiotic relationship based on nutrients exchange (Johnson et al, 2015; Powell & Rillig, 2018), topographic gradients of moisture and nutrient availability may interact with grazing to influence AM fungi variables. This interaction under natural environments requires further investigation.

Here we undertook a study in a long-term farm-scale field experiment where seven levels of field-manipulated grazing intensities have been maintained over 13 years within two topographic locations in a typical steppe in northern China. We aimed to assess (1) how AM fungal abundance changed in response to seven grazing intensities, (2) whether the impact of grazing was mediated by topography, and (3) which grazing- or topographic-induced changes in the mycorrhizal environment were associated with a change in AM fungal abundance.

2. Methods

2.1. Study Site

The study was set up at the Sino-German grazing experimental site in Xilin River Basin (116° 42′ E; 43° 38′ N), Inner Mongolia, China, which is a steppe grassland ecosystem with a semi-arid, continental climate. We set up our experiment in 14 plots located in two topographic blocks, flat and slope blocks, with each block containing seven levels of grazing intensities (GI). Each plot contained an area of 2 ha. The "sloped block" had a topographical slope of about 8 degrees, and the "flat block" had no noticeable slope. Each experimental plot was subjected to one level of grazing intensities, from 0 to 9 ewes per ha. Hereafter we define the GI by the number of grazers per hectare as 0 (no grazing), 1.5 (very light), 3 (light), 4.5 (light-moderate), 6 (moderate), 7.5 (heavy) and 9 (overgrazing). Grazers were young female sheep (ewes) of about 35 kg live-weight. Ewes were put in plots for 90 days throughout the growing season from June and to September each year. Until we took samples in 2018, the grazing experiment had been run continuously for 13 years. A detailed description of the climate, vegetation cover, soil characteristics and the design of the experimental site can be found in previously published papers (Schönbach et al, 2011; Wan et al, 2011) and in the supplementary information (SI-1).

2.2. Soil sampling

Soil samples were taken in mid July 2018. In each plot, five evenly distributed double soil core samples (2 cm diameter × 20 cm height) were collected for mycorrhizal and soil properties measurement. Immediately after collecting, samples were kept in an ice box with a temperature of around 0°C, and then stored at -20°C within 24 hours, and kept until analysis. In addition, five undisturbed cores (5 cm diameter and 5 cm deep) next to the sampling cores were collected to measure soil bulk density.

2.3. AM fungal responses

2.3.1. AM root colonization

Roots were collected from five soil cores, comprising multiple plant species, in each plot. The roots were rinsed carefully with distilled water and a sonicator was used to remove the soil particles adhering to the root surface. Roots were cut into pieces *ca*. 1 cm long and then around 5 g of fine roots of each sample was rinsed in 2% KOH (w/v) at 90°C for 60 min and rinsed thoroughly in water using a fine sieve and then acidified in 2% HCl (v/v) for 30 min and stained in 0.05% (w/v) trypan blue: glycerol: lactic acid (1:2:1) for 30 min at 90 °C. Root segments of each sub-sample were rinsed with lactic acid: glycerol: dH_2O (1:2:1), selected randomly and mounted onto slides in 50% glycerol. Thirty pieces of roots from each root sub-sample were observed under a compound microscope (Nikon eclipse Ci-L) at ×200 and ×400 magnification. Mycorrhizal root frequency (F%) (ratio of the number of colonized root fragments to the total number of analyzed root fragments), mycorrhizal colonization intensity in the root system (M%) (percentage of total segment length colonized) and arbuscule intensity (A%) (arbuscular abundance in the root system) were assessed according to the five-class system of Trouvelot (1986). We selected the Trouvelot (1986) method because it has been shown to provide more detailed information compared to

the other commonly used method developed by McGonigle et al, (1990) (see Füzy et al (2015); Kokkoris et al (2019)).

2.3.2. Hyphal length density (HLD)

Soil hyphae were extracted from two sub-samples of 5 g soil from each soil core (140 samples in total) in 500 ml of deionized water (dH_2O) following a modified membrane filter technique from Jakobsen *et al* (1992) and Boddington *et al* (1999). The hyphae of AM fungi were identified based on microscopic features, namely angular, aseptate in appearance, and 1.0–13.4 µm in diameter (Boddington et al, 1999; Shen et al, 2016). The total length of hyphae (mm) was measured for a minimum 60 fields of view for each filter paper at × 100 magnification. The developed modified GIM (Gridline Intersect Method) equations based on (Tennant, 1975) were used for calculating the total length of hyphae (mm) per gram of soil (m g^{-1}) (Shen et al, 2016) (SI-2).

2.4. Soil properties

Soil water content, pH, soil bulk density, organic carbon, available nitrogen and phosphorus were measured. Soil water content was measured using a Soil Moisture Measurement System (HS2 HydroSense® II, Campbell Scientific, Inc. USA) during soil sampling at each sampling point. Soil pH was gauged in a soil suspension of 1:1 soil-water ratio using an ion meter. Soil bulk density (g cm^{-3}) was measured by drying the undisturbed soil cores for 12 hours at 105 °C before being weighed. Soil organic carbon was determined by the potassium dichromate method according to NY/T 1121.6-2006 (Standards of the agricultural industry of the PRC, 2006). Soil available phosphorus (Olsen-P) was extracted with NH 4 F-HCl and determined by spectrophotometry following NY/T 1121.7-2014 (Standards of the agricultural industry of the PRC, 2014) and soil available nitrogen was measured according to DB/T 843-2007 (Recommended local agricultural standards, 2007).

2.5. Data analysis

We conducted three analyses. First, we assessed grazing and topography effects on AM fungal variables by generalized linear mixed effect models. Response variables included (i) soil hyphal length density (ii) mycorrhizal root frequency (iii) mycorrhizal root intensity and (iv) arbuscule intensity. Explanatory variables were grazing intensity with interaction with topography, and random variables were study plot (nested by topography and grazing intensity). We run a full model first, then the best model was selected in conformity with Akaike's information criterion (AIC) (Burnham & Anderson, 2004).

In the second analyses, we assessed the relationship between AM fungal hyphal length density, mycorrhizal root colonization and environmental variables. Environmental variables included (i) soil available nitrogen, (ii) soil organic carbon, (iii) soil available phosphorus, (iv) pH, (v) soil bulk density, (vi) soil water content. As the effect of environmental conditions on AM fungal responses might not be independent within our soil cores, but could be homogeneous within the plot, we pooled data from the same plot, and analyzed the relationship between AM fungal measures and environmental variables by the mean of each plot using linear regression (see Crawley (2012) and Zuur et al (2009)).

Finally, we examined environmental variables in response to different grazing intensity in different topographical blocks by linear mixed effect models. Linear mixed effect models were applied to available nitrogen, carbon, phosphorus, pH, soil bulk density and soil water content with grazing intensity nested in topography and topography nested in site. Full model and best model (based on AIC) were both presented.

All statistical analyses were conducted using R, version 3.5.2 (R Core Team, 2018). Linear and generalized linear mixed effect models were applied using "nlme" (Pinheiro et al, 2018) and "glmer" (Bates et al, 2015) packages respectively. Model selections were carried out in "MuMIn" package (Barton, 2018). All models were validated by checking the distribution of residuals following Zuur et al (2009).

3. Results

3.1. AM fungal responses to grazing intensity and topography

Soil hyphal length density was strongly negatively related to grazing intensity (β=-0.43±0.08, P<0.001), and this grazing impact was consistent for both topographical blocks (Figure 1.a, SI-3). Conversely, no relationship between mycorrhizal root frequency (Figure 1.b), mycorrhizal root intensity and arbuscule intensity with grazing intensity were detected (Figure 1.c). However, the sloped block had significantly higher mycorrhizal root frequency (β=-0.68±0.25, P=0.006), mycorrhizal root intensity (β=4.27±2.05, P=0.059) and arbuscule intensity (β=2.39± 0.86, P=0.017) than the flat block. The interaction between grazing intensity and topography was not significant for these AM fungal variables. Additionally, we found a hump relationship between the ratio of external (hyphal length density in soil) to intra-radical AM fungal structures (mycorrhizal root intensity) and grazing intensity, and this appeared stronger in the flat area (Figure 2). The results of the linear model with moderate grazing (4.5) as a base shows that the ratio of external to intra-radical AM fungal structure in response to grazing intensity is higher in moderate grazing (4.5) than the other grazing intensities in the flat site. In the sloped site, significance might not be detected because of the overall lower values observed (Figure 2).

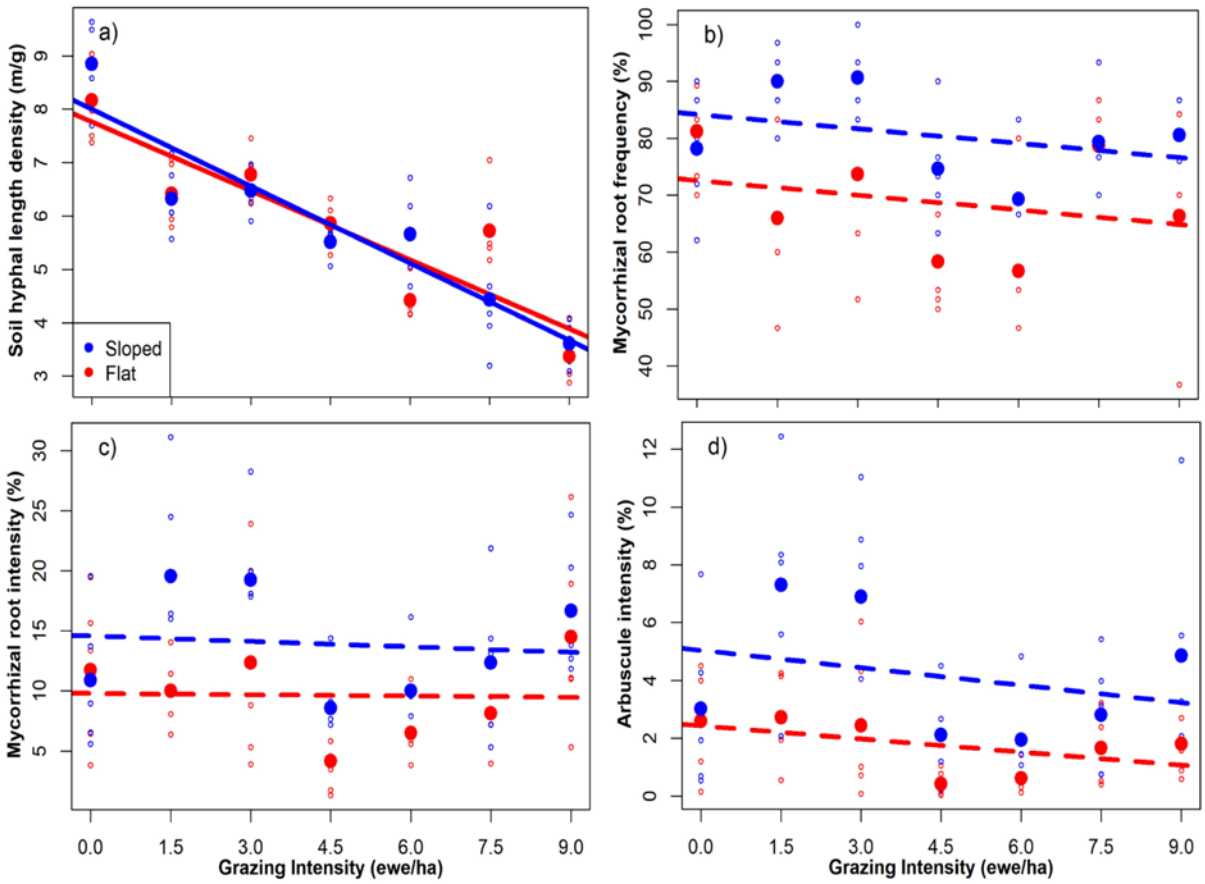

Figure 1. Soil mycorrhizal hyphal length density (a) mycorrhizal root frequency (b), mycorrhizal root intensity (c) and arbuscule intensity (d) in response to grazing gradient along two topographic conditions. Solid and hollow circles indicate mean and individual observations at each grazing intensity respectively. Lines are fitted regression lines from linear mixed-effects models (Table 1), where solid and dashed lines indicate significant ($P<0.05$) and non-significant ($P>0.05$) relationships respectively.

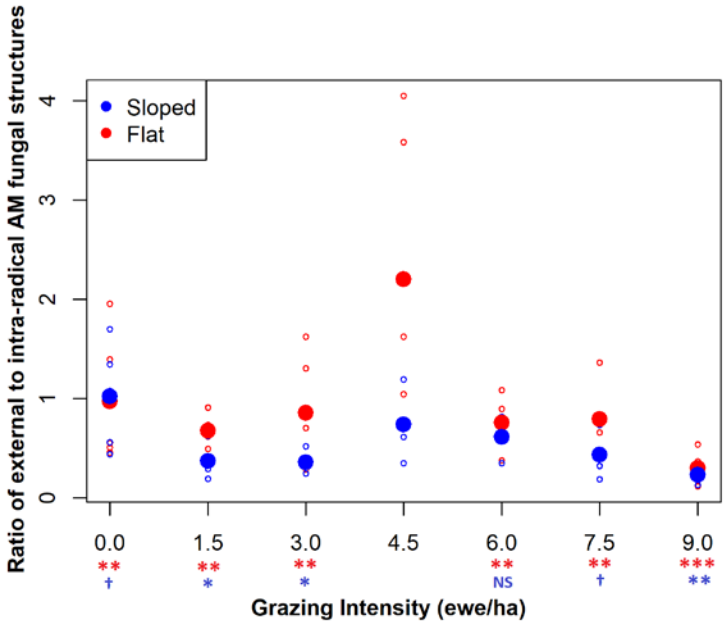

Figure 2. Ratio of soil hyphal length density (external AM fungal structure) to mycorrhizal root intensity (intra-radical AM fungal structure) in response to grazing gradient along two topographic conditions. Solid and hollow circles indicate mean and individual observations at each grazing intensity respectively. Asterisks represent significance level obtained from the linear model with moderate grazing (4.5) as base ($p < .001$, "***", $p < .01$, "**", $p < .05$, "*", $p < 0.1$, "†", NS: non-significant).

3.2. The association between AM fungi and environmental variables

Soil hyphal length density was positively related to pH (Table 1). Mycorrhizal root frequency was positively related with soil water content and organic carbon but negatively related with available nitrogen and available phosphorus. Mycorrhizal root intensity and arbuscule intensity were positively related with organic carbon and soil water content (Table 1).

Table 1. Relationship between environmental variables and AM fungi.

Environmental variables	HLD	F%	M%	A%
Organic Carbon (g/kg)	2.42±1.28 **(0.084)**	18.69±8.04 **(0.039)**	8.03±3.55 **(0.043)**	4.58±1.42 **(0.007)**
Available Nitrogen (g/kg)	0.01±0.09 (0.95)	-1.16± 0.49 **(0.036)**	-0.30±0.24 (0.247)	-0.16±0.11 (0.167)
Available Phosphorus (g/kg)	-0.14±0.36 (0.69)	-5.21±1.85 **(0.016)**	-1.37±0.97 (0.180)	-0.81±0.42 **(0.076)**
pH	3.71±1.35 **(0.018)**	-14.82±10.55 (0.186)	-8.47±4.34 **(0.075)**	-3.32±2.07 (0.134)
Soil Water Content (%)	0.03±0.10 (0.803)	1.33±0.56 **(0.034)**	0.53±0.25 **(0.056)**	0.29 ±0.11 **(0.021)**
Bulk Density (g/cm3)	-6.92±6.91 (0.336)	-3.78±47.64 (0.938)	-12.83±20.51 (0.543)	-0.91±9.53 (0.925)

Regression coefficients and relative p-value were estimated by linear regression model. Significant relationships are indicated in bold font. Abbreviation: HLD: soil hyphal length density (m/g), F%: mycorrhizal root frequency (%), M%: mycorrhizal root Intensity (%), A%: arbuscule intensity.

3.3. Responses of environmental variables to grazing intensity and topography

Among soil variables, pH was negatively affected by grazing intensity while soil bulk density significantly increased with grazing intensity (Table 2) (SI-3-b and c). No relationships between soil organic carbon, soil water content, available nitrogen and phosphorus with grazing intensity were detected (SI-3-b and c).

Comparing the two topographical blocks, the flat block had higher soil nitrogen, phosphorus and pH, but lower soil water content and soil organic carbon than the sloped block (Table 2). In addition, significant interactions between grazing intensity and topography were only observed for soil organic carbon and bulk density (Table 2).

Table 2. Linear mixed-effects model of the effects of grazing intensity and topography on environmental variables.

Response variables	Model No.	Grazing Intensity	Topography	Grazing Intensity x Topography Interaction	AIC
Organic Carbon (mg/kg)	1	0.00±0.03(0.859)	0.73±0.16 **(0.001)**	-0.10±0.03 **(0.006)**	45.1
Available Nitrogen	1	-0.56±0.33 (0.113)	-10.52± 2.45 **(0.002)**	0.56± 0.45 (0.245)	434.8
(mg/kg)	2	-	-8.00±1.49**(0.000)**	-	432.7
Available Phosphorus	1	0.06±0.07 (0.461)	-1.79±0.55**(0.008)**	-0.07±0.10 (0.485)	211.0
(mg/kg)	2	-	-2.12±0.31**(0.000)**	-	227.8
pH	1	-0.05±0.02**(0.013)**	-0.30±0.14(0.054)	0.00±0.03(0.923)	38.5
	2	-0.05±0.01 **(0.002)**	-0.28±0.08**(0.003)**		35.9
Soil Water Content (%)	1	-0.35±0.21(0.127)	6.79±1.59 **(0.002)**	0.20±0.30(0.511)	363.0
	2	-0.25±0.15(0.126)	7.70±0.89 **(0.000)**	-	360.9
Bulk Density (g/cm3)	1	0.02±0.00 **(0.005)**	0.10±0.03 **(0.008)**	-0.02±0.01**(0.009)**	-155.4

The full model (model No. 1) and the best model selected according Akaike's information criteria (AIC) (model No. 2) are presented; dashes (-) indicate variables that were not included in the model.

4. Discussion

In this study, we investigated AM fungal abundance in grassland along a range of long-term grazing intensities. Our first finding is that soil hyphal length density significantly decreased as grazing intensity increased. This is explained by the impact of grazing on plant community and soil conditions. Indeed it has been observed in previous studies that long-term livestock grazing reduced soil fungal hyphal length density in grassland ecosystems (Ren et al, 2018; van der Heyde et al, 2017). Generally, long-term livestock grazing decreases plant diversity via loss of grazing-sensitive rare species or removal of palatable dominant or sub-dominant plant species from species pool (Schönbach et al, 2011; Shelton et al, 2014; Wang et al, 2014). It leads to a decline in the range of below-ground plant root types and root exudates and consequently decreases the variability of root exudates and soil resources for soil microorganisms including AM root-associated fungi (Ba et al, 2012; Epelde et al, 2017; Wan et al, 2011). For example, hyphal extension and germination of AM fungal spores preferentially takes place in the presence of roots and root exudates (Smith & Read, 2008; Tahat et al, 2010). Consistent with this view, a positive significant relationship between plant diversity and soil hyphal length density has been reported recently in the same

site (Ren et al, 2018). In addition, livestock trampling and treading disrupts the hyphal networks in the soil via increasing soil compaction and soil bulk density (Hao & He, 2019; van der Heyde et al, 2017). Along with this expectation, we observed soil bulk density was significantly negatively related to grazing intensity. Moreover, long-term grazing exerts a negative impact on soil pH (Guo et al, 2016; van der Heyde et al, 2017), while lower pH and soil acidification suppresses microbial growth and activities through lower nutrient use efficiency (Zhang et al, 2008). This phenomenon agrees with our finding of positive relationship between pH and soil hyphal length.

We did not observe an association between mycorrhizal root colonization (frequency and intensity) and grazing intensity. These results are consistent with the findings of a meta-analysis of 99 experiments which showed that actual herbivory or simulated grazing decreased mycorrhizal colonization by considerable amounts in only a limited number of studies (Barto & Rillig, 2010). Similarly, van der Heyde et al (2017) reported no grazing effect on mycorrhizal root colonization in grazed sites compared to non-grazed ones in nine grasslands in Canada. However, both positive (Eom et al, 2001; Wearn & Gange, 2007), and negative (Ba et al, 2012; Birhane et al, 2017; Cavagnaro et al, 2018) effects of large herbivores on root colonization have also been documented. It is worth considering that total length of root colonized may decrease following herbivory but percent root colonization, as a relative measure, may remain unchanged (van der Heyde et al, 2017). Although microscopic classical approaches for estimating percent root length colonization provide greater resolution of AM fungal structures, these approaches fail to describe the amount of AM fungi in a whole root system due to not accounting for the total root length (Hart & Reader, 2002). In addition, percent root length colonization doesn't account for number of structures that were observed at each intersection, which means that AM fungal biomass cannot be easily deduced.

Apart from these limitations in the assessment of mycorrhizal root colonization measurement, conflicting

results are also attributed to the context-dependent nature of the symbiotic association (Alzarhani et al, 2019; Hoeksema et al, 2010; Smith et al, 2010; Tao et al, 2016) and to the mycorrhizal environment itself (Ba et al, 2012; van der Heyde et al, 2017). We found significant positive relationships between mycorrhizal root frequency and soil water content and significant negative relations between mycorrhizal root frequency, soil available nitrogen and phosphorus as reported at other sites (Binet et al, 2017; Birgander et al, 2014; Soudzilovskaia et al, 2015).

The two topographic locations in our study site, flat and sloped, were significantly distinct in terms of soil water content and soil resource availability. Soil available nitrogen and phosphorus were significantly higher in the flat area compared with the sloped area, therefore, the sloped areas were more nutrient limited. Indeed, our data showed AM fungal root frequency, intensity and arbuscule intensity were lower in the more nutrient limited sloped area. This result is consistent with the theory that plants benefit most from their mutualistic symbiotic fungi in nutrient limited soils while benefit least in highly fertile soils (Hoeksema et al, 2010; Johnson et al, 2015). Additionally, we found a higher soil organic carbon in the sloped area than in the flat area and higher mycorrhizal frequency and intensity in the sloped area than in the flat one. Given that arbuscular mycorrhizal symbiosis is a carbon and nutrients tradeoff between plant and fungal partner (Hodge et al, 2010), it is likely that plants are more dependent on mycorrhizal fungi for obtaining nutrients in the sloped area, which is more nutrient limited. In this case plants would allocate more carbon below-ground in exchange for these additional nutrients provided by their AM fungi symbionts.

Interestingly, we found a hump-shaped relationship between the ratio of external (hyphal length density in soil) to internal AM fungal structures (mycorrhizal root intensity) and grazing intensity, particularly in flat area. Compared to the control, this ratio first decreased (less external hyphae per unit internal

hyphae) at low grazing intensities (1.5 and 3 ewe/ha), then increased at moderate grazing intensity (4.5 ewe/ha) before decreasing as grazing intensity increased to the higher values (9 ewe/ha). It is important to note that the values for internal colonization by AM fungal structures are in a similar range and relatively constant throughout the range of grazing intensities while that of external mycorrhizal hyphae varies with grazing intensity. Given that this relationship has not been reported previously, the observation of a potential hump-shape relationship could, if real, have large implications for grazing management.

The exact mechanism by which grazing intensity is impacting the various mycorrhizal structures is not known. It is possible that the initial decrease at low grazing intensity is due to selective grazing (Wan et al, 2015), resulting in more palatable (Ren et al, 2012; Wan et al, 2015) and more mycorrhizal dependent plant species being removed which are associated with larger mycorrhizal hyphal networks. Therefore, reduction in the abundance of more mycorrhizal dependent plants would lead to less external hyphal density. The increase in this ratio of external hyphae to internal colonisation at moderate grazing intensity could be due to moderately grazed plants needing more nutrients to fund shoot regrowth (Harvey et al, 2019; van der Heyde et al, 2019), thus investing in AM fungi with larger external hyphal networks to search for more nutrients, particularly phosphorus. It is more cost effective for plants to invest in exploring increased soil volume via their mutualistic AM fungi partners than by expanding their root system (Jansa et al, 2013). The final reduction in this ratio at high grazing intensity could be due to the excessive grazing imposing carbon stress on plants (Ba et al, 2012) via large removal of above-ground biomass and thus decreasing below-ground carbon allocation to AM fungal root colonizers. In this case, less carbon is available for external hyphal growth despite good levels of root colonization. Further research would be needed to test whether these hypotheses are correct.

5. Conclusion

Overall, our study provides new insights on the effects of the intensity of long-term grazing on AM fungal abundance driven by changes in environmental variables. Whilst we acknowledge that a fully replicated block design with multiple plots under the same grazing intensity could strengthen the study, to repeat such a large-scale experiment with multiple large plots (in this case a total 14 plots of 2 hectares each) is extremely expensive and unrealistic. Nonetheless, our results clearly showed that, in the study site, soil hyphal length density was negatively related with grazing intensity irrespective of topographic location. Our main finding suggests that it's the grazing intensity rather than grazing *per se* that determines the impact of grazing on mycorrhizas. This is novel and of clear importance to soil management approaches. While further research is essential to better understand how grazing intensity impacts the belowground ecosystem, changes in mycorrhizal hyphal density along a range of grazing intensities could be significant for soil carbon sequestration, which is critical in the face of accelerating climate change. That mycorrhizal root colonization variables were not related to grazing intensity requires further work to confirm the reasons as many confounding factors exist. For example, it is possible that effects exist but were masked by differential plant or fungal species responses.

The fact that one measure (external hyphal density) of the mycorrhizal community was clearly impacted by grazing intensity, but not other measures (mycorrhizal root colonization), means that mycorrhizal functioning was impacted. This is supported by the observation that the ratio of the foraging extra-radical mycorrhizal hyphae to intra-radical mycorrhizal structures was altered. This impact of grazing intensity on the ratio of external to internal mycorrhizal structures does require further testing. Nonetheless, in time, the impacts of grazing intensity on mycorrhizal fungi reported in this study would lead to further knock-on effects on the plant-soil system via altered interspecific competition within both plants and AM fungi communities. Indeed, consequences for ecosystem functioning could be significant as plant and AM fungi

communities are intimately linked and diversity aboveground can drive diversity belowground and *vice versa*. Altered nutrient uptake capacity by the AM fungal community would lead to some plant species benefitting at the expense of others (e.g. less mycorrhizal dependent species) altering plant community structure. The potential impacts of this change in mycorrhizal hyphal density is also significant for soil carbon sequestration as AM fungi can account for up to 20 % of host plant photosynthate (Smith & Read, 2008) and are a rapid pathway of carbon flow to the soil (Staddon et al, 2014). This implication for the soil carbon cycle in grasslands clearly deserves further investigation. By increasing our understanding of the impacts of land management regimes on belowground ecology we will approach the goal of sustainable plant and livestock production. Managing grasslands with an aim of maintaining soil biodiversity and soil ecosystem processes is fundamental to the sustainability of grazed grasslands worldwide and crucial to food security in the face of accelerating climate change.

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