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Faghihinia, Maede, Zou, Yi, Bai, Yongfei, Pourbakhtiar, Alireza, Marrs, Rob and Staddon, Philip L. ORCID logoORCID: https://orcid.org/0000-0002-7968-3179 (2022) Long-Term Grazing Intensity Impacts Belowground Carbon Allocation and Mycorrhizas Revealed by 13CO2 Pulse Labeling. Rangeland Ecology and Management, 86. pp. 64-72. doi:10.1016/j.rama.2022.11.001

Official URL: https://doi.org/10.1016/j.rama.2022.11.001
DOI: http://dx.doi.org/10.1016/j.rama.2022.11.001
EPrint URI: https://eprints.glos.ac.uk/id/eprint/11914

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Long-term grazing intensity impacts below-ground carbon allocation and mycorrhizas revealed by $^{13}CO_2$ pulse labelling

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Abstract

Despite the importance of grasslands for carbon storage and climate regulation, there is uncertainty about the effect of livestock grazing intensity on above-ground carbon assimilation and below-ground carbon partitioning. Furthermore, the relationship between below-ground carbon allocation and arbuscular mycorrhizal fungi, which serve as a conduit for carbon movement through the plant and soil, is unclear. To investigate this, we used an *in situ* ^{13}C stable isotope pulse-chase labelling approach in plots under seven rates of sheep grazing intensity in a steppe grassland in northern China. We quantified the allocation of carbon to plants, soil, and soil-respired CO_2 along with measurements of mycorrhizal hyphal density in the soil. With increasing grazing intensity, carbon assimilation per unit shoot biomass significantly increased, whereas carbon allocation to roots marginally decreased. Soil-respired CO_2 appeared to be independent of grazing intensity.

Mycorrhizal hyphal density decreased with increasing grazing intensity and was correlated significantly with new carbon input to roots two days after labelling, and marginally related to that of soil one day after the $^{13}C-CO_2$ pulse. Our study suggests that grazing intensity alters the distribution of carbon among different carbon pools within the plant-soil system. The results also underscored the key role of mycorrhizas as a fast route for carbon transfer from plant to soil.

Keywords

Grassland, carbon flux, stable isotope labelling, grazing density, respiration, AM fungi

1. Introduction

Grasslands play a remarkable role in mitigating climate change by storing carbon in their soils. Their importance in mitigating global climate change lies in their presence over a large extent of the Earth’s land area (about 37%) (Conant 2010, O'Mara 2012) and their great capacity for carbon sequestration and carbon storage in the soil (Lal 2004, Conant 2010). The global soil carbon stock of grasslands has been estimated at about 343 billion tonnes of carbon, which is nearly 50 percent more than the carbon stored in all the world's forests (Conant 2010). Nonetheless, grasslands have become one of the most degraded biomes in the world over the past 50 years, largely due to intensive livestock grazing. This degradation has led to widespread declines in soil organic carbon, plant productivity and biodiversity, and loss of ecosystem functions and services (Conant 2010, McSherry and Ritchie 2013). Given that grazing is a common practice on grasslands worldwide, it likely plays an important role in carbon storage and distribution in this ecosystem (Conant 2010, McSherry and Ritchie 2013, Eze et al. 2018). However, the effect of livestock grazing intensity on carbon allocation to above- and below-ground carbon pools is less clear (Wu et al. 2010, Wilson et al. 2018). This could be due to a variety of different biotic and abiotic factors that affect the soil carbon pool, including precipitation, plant community composition, soil structure, and the type of grazing animals (O'Mara 2012, McSherry and Ritchie 2013).

The magnitude of the impact of grazing on carbon sequestration and storage in grasslands depends strongly on livestock density, which is known to affect both the above- and below-ground parts of the ecosystem (Yan et al. 2013). Since long-term intensive livestock grazing reduces plant productivity (Bagchi and Ritchie 2010, Bai et al. 2013), litter fall and litter mass (Liu et al. 2015), root elongation and biomass (Bagchi and Ritchie 2010) and soil microbial biomass (Hosseini Bai et al. 2015, Zhou et al. 2017), it could, therefore, be postulated that below-ground carbon allocation and soil carbon storage should decrease with increasing grazing intensity. The effects of different grazing intensities on below-ground root biomass (Yan et al. 2013, Chen et al. 2015, Zhou et al. 2017), soil carbon stock (Medina-Roldán et al. 2008, McSherry and Ritchie 2013, Zhang et al. 2018, Harvey et al. 2019) and sequestration (Bagchi and Ritchie 2010, Schönbach et al. 2012, Chen et al. 2015) have been reported previously. However, we are not aware of any study that has examined the effects of different grazing intensities on carbon allocation patterns of recently fixed carbon. An assessment of the allocation pattern of carbon flux from plants to below-ground pools is required to determine the contribution of newly photosynthetically fixed carbon to the soil carbon cycle. Recent studies have reported the effects of grazing itself, but not grazing intensity, on below-ground carbon allocation by comparing grazed and ungrazed plots (Ingrisch et al. 2015, Wilson et al. 2018). This clear knowledge gap is hindering the development of an evidence-based approach to soil carbon management. Developing a strategy to reduce intensive livestock grazing to a more sustainable level should be a key objective of sustainable grassland management to potentially restore agriculture-induced soil carbon losses (Conant 2010).

Isotope tracer technology and pulse labelling of plants with ^{13}C- (or ^{14}C-labelled) CO_2 enables analysis of the dynamics of carbon allocation via the plant-root-soil route and quantification of carbon movement in terrestrial ecosystems *in situ* (Pausch and Kuzyakov 2018). Using this approach, partitioning of assimilated carbon in response to grazing in grasslands has been documented in only a few studies (Zou et al. 2014, Ingrisch et al. 2015, Wilson et al. 2018, Bai et al. 2021). These studies have produced contradictory findings suggesting that carbon transfer to above- and below-ground pools differs substantially across plant communities and ecosystems. For example, Wilson et al. (2018) reported positive long-term grazing effects on below-ground carbon allocation in a C4-dominated subtropical pasture in the USA, whereas Ingrisch et al. (2015) reported no grazing effects on root and soil carbon allocation in a C3/C4 co-occurring grassland in the Chinese Tibetan Plateau. Further research is needed to understand how livestock grazing intensity affects below-ground carbon allocation and ecosystem carbon cycling, and the underlying mechanisms of these grazing intensity effects on carbon cycling.

A significant proportion of fixed carbon translocated below-ground is allocated to root-associated soil biota, in particular arbuscular mycorrhizal fungi (AMF), which can receive between 3% and 20% of photosynthate via plant roots in return for acquiring additional nutrients (Smith and Read 2008, Jansa and Treseder 2017). Therefore, AMF are a potential pathway for translocating atmospheric C into the soil, although much of the carbon is likely returned to the atmosphere due to the high turnover rate (few days) of fungal hyphae (Staddon et al. 2003b, Lekberg et al. 2013, Jansa and Treseder 2017). In addition, AMF facilitate soil organic matter turnover (Paterson et al. 2016), supply plant-derived carbon to other groups of living soil microorganisms (Jansa et al. 2013, Nottingham et al. 2013), and stabilize carbon in highly recalcitrant organic compounds (e.g., glomalin) that have a slow turnover rate (Rillig 2004). Thus, AMF play a key role in determining the terrestrial carbon balance of the global carbon cycle (Staddon 2005, van der Heijden et al. 2015, Jansa and Treseder 2017). Of the total AMF biomass, external fungal hyphae are the largest component and these mycorrhizal hyphal networks are largely responsible for lateral soil carbon fluxes (Godbold et al. 2006, Treseder and Cross 2006, Talbot et al. 2008, Kaiser et al. 2015). However, these thin, delicate networks of mycorrhizal hyphae are easily damaged by herbivores' trampling and treading resulting in reduced fungal colonization capacity, reduced uptake of nutrients by associated plants (van der Heyde et al. 2017, Ren et al. 2018, Faghihinia et al. 2020c), and reduced redistribution of newly-fixed carbon in the soil (Gui et al. 2018). Nonetheless, the association between the abundance of fungal hyphae and below-ground carbon flow under multiple long-term grazing intensities is less well understood (Faghihinia et al. 2020b). A deeper understanding of how grazing intensity drives AMF abundance in soil is needed and would help predict the impact of grazing intensity on carbon dynamics of grassland soils.

Much of the current knowledge on the effects of grazing on below-ground carbon allocation and mycorrhizas comes from short-term experiments, and the long-term effects are not well understood (Pausch and Kuzyakov 2018, Faghihinia et al. 2020b). It is clear that results could be quite different on longer time scales, as organisms in natural environments need time to respond to disturbances (McSherry and Ritchie 2013). For instance, the effects of grazing on below-ground carbon allocation in roots and soil may not be detected in the short term due to the rapid recovery of plant photosynthetic tissue (Ingrisch et al. 2015).

Here, we conducted a study in a long-term experimental site with a gradient of seven grazing intensities applied for 13 years. In ecological experiments, such gradient designs have been shown to perform better than replicated designs in identifying underlying patterns of response to continuous environmental drivers, as they focus mainly on patterns of response variables along gradients of environmental drivers, rather than examining variation among experimental treatments (Kreyling et al. 2018). Ecological studies, however, still rely mainly on replicated experimental designs with a small number of sampling sites (typically 2 or 3) along the gradient of environmental factors. Here, we applied a ^{13}C pulse labelling approach to track the flux of newly fixed photosynthate from plants into below-ground pools to study carbon assimilation and below-ground carbon allocation along the grazing intensity gradient. We also measured the hyphal length density of mycorrhizas in soil at sites with different grazing intensities. We addressed the following research questions: (1) how does the pattern of above-ground carbon assimilation and below-ground carbon allocation vary along the grazing intensity gradient? (2) how rapidly is carbon distributed to the different carbon pools? and (3) how is recently fixed carbon in the soil related to the abundance of external mycorrhizal fungal hyphae in the soil? We hypothesized that above-ground carbon assimilation and below-ground carbon allocation would decrease along the grazing gradient due to the negative effects of livestock on plants. We also hypothesized that carbon allocation to different carbon pools would be a rapid process and that fresh carbon input would be related positively to soil fungal hyphae abundance.

2. Methods

2.1. Experimental site

The study was conducted in a steppe grassland in a semi-arid zone with a continental climate at the Sino-German Inner Mongolia Grassland Ecosystem Research Station (IMGERS) in Xilin River Basin, Inner Mongolia, China. The study area is characterized by an average annual temperature of 0.9°C and an average annual precipitation of 329 mm, with over 70% of the annual precipitation occurring during the growing season from April to September (Wan et al. 2011). For more details, see S-1.

Since 2005, plots in the study site have been maintained with different grazing intensity treatments. We sampled in 2018, when the treatments had been applied and maintained continuously for 13 years prior to our study, therefore the effects of different grazing intensities should have stabilized (Li et al. 2017). Here, we selected seven plots, each covering a flat area of two hectares (ha). Each plot was exposed to a level of grazing intensity (GI) ranging from 0 to 9 ewes ha^{-1} with interval increments of 1.5 ewe ha^{-1}: GI(0) (no grazing), GI(1.5) (very light), GI(3) (light), GI(4.5) (light-moderate), GI(6) (moderate), GI(7.5) (heavy) and GI(9) (overgrazing). The plots were continuously grazed by young female sheep (ewes) of approximately 35 kg live weight since 2005. The plots were grazed for 90 days from June to September each year.

Two perennial dominant plant species, rhizome grass *Leymus chinensis* (Trin.) Tzvel. and bunchgrass *Stipa grandis* P. Smirn. represent more than 75% of the total above-ground standing biomass (Li et al. 2017). Long-term grazing intensity decreased plant above- and below-ground net primary production (ANPP and BNPP), plant height, plant species diversity and community composition and plant specific leaf area (SLA) along the grazing gradient (Hoffmann et al. 2016, Li et al. 2017) (Fig S-3). A detailed overview of the vegetation cover, climatic conditions, soil properties and experimental design of the study plot was presented in Wan et al. (2011), Schönbach et al. (2011) and supplementary materials (S-1 and S-4).

2.2. Pulse-chase experiment with ^{13}C-CO2

In July 2018, a pulse-chase labelling experiment was conducted with seven individual labelling units using a field-labelling approach (one labelling unit in each plot) (Ostle et al. 2000, Staddon et al. 2003a, Staddon et al. 2014). The labelling units were installed in the middle of each plot at the peak of the growing season in July, when carbon fixation would be near maximal and transfer below-ground would be occurring. Care was taken to include the two dominant plant species (*L. chinensis and S. grandis)* in all labelled areas to ensure that the vegetation cover was as uniform as possible in all systems.

99 Atom% $^{13}CO_2$ was mixed with CO_2-free air in a Tedlar bag (400 L) to achieve the desired concentration (400 μL per liter ±20%). The labelled air was pumped into the transparent acrylic labelling chamber (40 cm diameter, 50 cm height, and 5 mm thickness) at a rate of 1.0 liter per minute. Care was taken not to trap vegetation under the steel chamber base to avoid disturbance. The chambers were placed on the soil surface and depressed only a few millimeters to achieve a seal while minimizing disturbance. A hole was drilled in one side of the chamber to supply the labelled air, and another hole was drilled in the opposite side of the chamber to allow air movement to the outside (Fig.1). The exhaust air from the chamber was expelled at least 10 m away from the labelling area. After four hours, the tops of the chambers were removed, but the bases of the chambers were left in place as markers of the labelled areas.

2.3. Soil, root, shoot and gas sampling

After ^{13}C delivery, samples were collected within the chamber areas at different times and at 5 cm from the outer edge of the labelled chamber. Plant samples were taken before labelling, 4 hours (h), 1 and 2 days (d) after labelling. Soil cores (diameter of 2 cm and depth of 20 cm) followed by root samples (taken from soil cores) were also taken in all labelled areas before labelling, 4h, 1d, 2d, 4d, 1 week (w), 2w and 4 w after the ^{13}CO2 labelled pulse. Soil core locations were backfilled with soil from adjacent areas of the field. To track the relative ^{13}C signature in above-ground plant tissue, shoot samples were collected only from the two dominant native grasses, *L. chinensis* and *S. grandis*, to ensure uniform vegetation composition in the samples. Roots were collected by hand from soil cores. Dry and black roots were characterized as dead roots and removed from the analysis; all others were included in the analysis as live roots.

Gas samples (soil-respired CO_2) were collected before labelling, 4 h, and 1, 2, and 4 days after $^{13}CO_2$ pulse labelling. To collect soil-respired CO_2, 10 ml pre-evacuated Exetainer glass vials (Labco Ltd., High Wycombe, UK) were purged with ambient air to ensure that they all had the same initial conditions, and they were then gently pressed upside down with the lid removed slightly into the soil surface and allowed to stand for 4 h, after which the Exetainers were carefully lifted from the soil surface and quickly recapped. No evidence was observed of soil being trapped when the exetainers were resealed. Care was taken to exclude any respiration caused by shoot biomass within the trapped soil CO_2 efflux. The Exetainers filled with soil-respired CO_2 were kept at ambient temperature, and the plant, soil, and root samples were frozen at -20°C until subsequent $^{13}C/^{12}C$ analysis.

A total of 175 samples were obtained, including 35 gas, 28 shoot, 56 soil and 56 root samples. In addition, five soil core samples (diameter of 2 cm and depth of 20 cm) evenly-distributed in each plot (70 in total) were collected in mid-July 2018 to measure hyphal length density in the soil.

2.4. $^{13}C/^{12}C$ analysis and isotope quantification

Carbon isotope analysis was performed by Zhongding Testing Technology Co. China, following the standard protocol for isotope ratio mass spectrometry (IRMS) (Muccio and Jackson 2009). The $^{13}C/^{12}C$ content of the samples was measured using the Dumas combustion method on a Vario ISOTOPE Cube elemental analyzer with continuous flow to an IsoPrime100 mass spectrometer (Isoprime Ltd, UK). Analysis of solid samples was carried out on 300 µg of finely ground dried samples weighed into tin capsules for elemental combustion. The ^{13}C content of gas samples (soil respiration) was analyzed in subsamples in alumina tubes with copper wires at 600 °C. The analytical standard (OAS) consisted of a homogenous batch of a urea standard used as a routine laboratory standard in the determination of carbon isotopes ^{13}C (Elemental Microanalysis Ltd, UK).

Carbon isotope enrichment in the samples was expressed as $\Delta^{13}C$ (%) and calculated as follows;

$\Delta^{13}C\ (\%) = \delta^{13}C_{sample} - \delta^{13}C_{control}$

Where $\delta^{13}C_{sample}$ and $\delta^{13}C_{control}$ are the $^{13}C/^{12}C$ ratio of the samples after and before the $^{13}C\text{-}CO_2$ pulse respectively. All samples of shoot, root, soil and soil-respired CO_2 were collected from all labeling units before starting the $^{13}C\text{-}CO_2$ pulse labeling ($\delta^{13}C_{control}$).

$\delta^{13}C$ expresses the sample's ^{13}C content relative to the reference standard Vienna-PeeDee Belemnite (V-PDB) with a $^{13}C/^{12}C$ abundance ratio of 1.1237×10^{-2}. $\delta^{13}C$ was determined using the following equation:

$\delta^{13}C = 1000 \times (R_{sample} - R_{standard})/(R_{standard})$

where $R_{standard}$ and R_{sample} represent the $^{13}C/^{12}C$ abundance ratios of the V-PDB reference standard and the sample, respectively. $\delta^{13}C$ is expressed in enrichment per mil (‰). Additionally, carbon isotope enrichment was also calculated using ^{13}C atom (%) (Fig S-4).

2.5. Soil hyphal extraction

A modified membrane filter technique was used to extract soil hyphae (Jakobsen et al. 1992, Boddington et al. 1999). Soil hyphae were extracted from 140 soil subsamples (two soil subsamples of 5 g were collected from each soil core). The total length of hyphae (mm) was then quantified at 100× magnification with at least sixty microscope fields of view for each nitrocellulose membrane filter paper sample. AMF hyphae were detected based on the microscopic features that were aseptate, angular in appearance, and 1-13.4 µm in diameter (Shen et al. 2016). The modified gridline intersect method equation based on Tennant (1975) and Shen et al. (2016) was used to measure the total length of hyphae (m) per g of soil dry weight (SI-2).

2.6. Statistical analysis

We used a gradient design with seven ranges of grazing intensity to examine the relationship between recently fixed carbon in different carbon pools and mycorrhizal abundance with grazing intensity. We recognize that without true replication, this approach is not as powerful as a replicated experimental design because it does not allow quantification of treatment variance. However, a gradient test in conjunction with a regression analysis provides a first approximation of the overall trends to be evaluated. Having replicates at the plot level would be ideal, but is usually unrealistic. It has been suggested that establishing limited experimental units along an environmental gradient, rather than increasing the number of replicates at two arbitrarily selected points, would contribute to new insights in a variety of ecological studies (Kreyling et al. 2018).

Within a given resource and sufficient number of sampling points along the grazing intensity gradient, we believe our study contributed to the power of the chosen regression approach.

Linear regression models were applied to evaluate the effect of grazing intensity on above- and below-ground carbon pools. Response variables included ^{13}C enrichment in (i) above-ground plant tissue (shoot) (ii) root (iii) soil and (iv) soil-respired CO_2. Explanatory variables were grazing intensity and post-labelling time and their interaction. Grazing intensity was included as a continuous variable in the analysis due to the large-scale design of this long-term experimental site. We first fitted a model with all terms as well as their interactions (grazing, time, and grazing × time). Then, automatic model selection was performed using Akaike's Information Criterion (AIC) (Burnham and Anderson 2004) to find the Minimal Adequate Model (Crawley 2012).

To analyze the effect of grazing intensity on soil hyphal length density (m/g) (HLD), a linear regression model was applied with HLD as the response variable and grazing intensity as the explanatory continuous variable. Additionally, linear regression models were applied to evaluate the relationship between HLD and recently fixed carbon in roots and soils. We pooled data from HLD at the plot level to more conservatively estimate significant differences and reduce the likelihood of presenting type I errors as recommended by Crawley (2012) and Zuur et al. (2009).

All data analyses were performed in R, version 3.5.2 (R Core Team, 2018). Model selection was performed using the R package "MuMIn" (Barton 2018). All models were checked by assessing the distribution of residuals and standard model validation graphs to verify homogeneity and normality as well as identifying any influential observations following Zuur et al. (2009). Visual inspection of the residual plots did not reveal any obvious deviations from normality or homoscedasticity.

3. Results

3.1. Shoot carbon

After 4 h, above-ground plant tissue (shoot) samples were highly enriched in ^{13}C at all grazing intensities, with the greatest concentration observed at the highest grazing intensity, GI (9). One day after pulse labelling, ^{13}C in the shoot decreased slightly in all grazing treatments, but remained most enriched in GI (9) compared to the other treatments (Fig.2-a). The ^{13}C in the shoot then gradually decreased between one and two days after labelling along the grazing gradient in all treatments, and it decreased sharply in GI (9) (Fig.2-a).

The ^{13}C concentration in the shoots increased significantly along the grazing intensity gradient at all time points: four hours, one day, and two days after pulse labelling (Fig. S-1, Table 1).

3.2. Root carbon

The ^{13}C in roots increased slightly between four hours and one day after labelling, then increased rapidly after two days in all grazing treatments except the most intensive, GI (9) (based on one observation at each grazing intensity). Root ^{13}C increased slowly at GI (9) between four hours and one day after labelling, after which it remained unchanged, and then it decreased between two to four days after labelling. The greatest ^{13}C label concentrations in root samples were observed two days after labelling for all grazing intensities, in decreasing order GI (0), GI (4.5), GI (3), GI (1.5), GI (7.5), GI (6), and GI (9). Root ^{13}C label content decreased from two days post-labelling to four weeks post-labelling, where it was close to initial concentrations at all grazing intensities (Fig.2-

b). Root ^{13}C concentration decreased with increasing grazing intensity. Root ^{13}C concentration reached a significant peak two days after labelling (Fig.S-1, Table 1).

3.3. Soil carbon

The bulk soil ^{13}C concentration increased immediately after labelling in all grazing treatments. It peaked dramatically one day after labelling in GI(0) to GI(4.5) but peaked two days after labelling when GI was ≥6. The ^{13}C concentration of the soil samples then declined until four weeks after labelling in all grazing intensities (Fig.2-c). However, linear model analysis showed no significant relationship between grazing intensity and below-ground carbon allocation in soil (Fig.S-1, Table 1).

3.4. Soil-respired carbon

The ^{13}C concentration in soil-respired CO_2 increased significantly four hours after labelling and peaked after one day (Fig.2-d), then decreased consistently in all grazing treatments. As for carbon allocation to soil, no significant correlation was found between grazing intensity and ^{13}C concentration of soil-respired CO_2 (Table 1).

3.5. Soil hyphal length density and recently-fixed carbon

A strong negative relationship was found between hyphal length density in soil (HLD) and grazing intensity (GI). HLD significantly declined with increasing grazing intensity (β=-0.43±7.76, P=0.001) (Fig.3-a). A strong positive association was observed between HLD and ^{13}C content of roots (β=0.18±4.28, P=0.008) and ^{13}C content of soil (β=0.74±0.30, P=0.058) at their peak values, two days and one day after labelling respectively (Fig.3-b and c).

4. Discussion

Our experiment is the first field research to examine the relationship between a range of grazing intensities and above- and below-ground allocation patterns of recently fixed carbon. First of all, we would like to acknowledge that our study lack of true replicates, which comes from two levels: the grazing plot level and chamber experimental level. True replicates of grazing intensity on each plot would have improved the statistical rigor of the work and allowed detection of differences among grazing treatments. However, due to the resource limitation, conducting a replicate study would not have been feasible in a large-scale study of this type where plot sizes must be relatively large, here 2 ha each to accommodate grazing animals. Our use of a gradient approach together with regression analysis allows a first approximation of response patterns along the gradient of grazing intensity (Kreyling et al. 2018), which was the aim of our study. The lack of experimental level replicates does not allow us to evaluate within plot variance, which can also be seen that we were not able to show the standard error in our results. To capture some of the variance caused by differences in plant community composition within chambers, care was taken to select and collect a uniform vegetation cover in terms of plant species involved; two dominant plant species, *L. chinensis* and *S. grandis,* were included in all chambers.

Our first finding is that ^{13}C enrichment in different carbon pools was differentially related to grazing intensity. Grazing intensity was positively associated with above-ground carbon assimilation (P=0.000) and marginally negatively associated with below-ground carbon allocation to roots (P=0.065). However, carbon allocation to soil and soil-respired CO_2 were not correlated with grazing intensity.

With increasing grazing intensity, we observed an increase in carbon assimilation per unit shoot biomass by vegetation. The higher concentration of ^{13}C in shoot biomass in the plots with the greatest grazing intensity is likely due to a faster rate of photosynthesis by the plants, particularly in the younger leaves that grew in response to defoliation. These results agree with those of Shen et al. (2019) who showed a significant increase in photosynthetic capability of most common plant species in both alpine steppe and alpine meadow under high grazing intensity. Higher photosynthetic rates in response to grazing have been associated with increased light use efficiency, increased stomatal opening leading to increased CO_2 uptake and improved water use efficiency (Liu et al. 2016, Shen et al. 2019). In addition, as the vegetation cover was relatively uniform in each chamber in terms of plant community composition, the trend is largely independent of differences in the rate of photo-assimilation among the plant species present. Differences in carbon assimilation between chambers can be attributed to plant size, leaf age, and overall canopy cover. Canopy and leaf size were smaller in heavily-grazed plots than in plots with no- or light-grazing, which may be attributed to high defoliation rates and subsequent regrowth. Canopy photosynthesis depends on the total amount of leaf area of the plant (Roscher et al. 2019). A decrease in plant biomass and plant leaf area in response to high grazing pressure in heavily-grazed plots was reported at the same experimental site (Fan et al. 2009, Li et al. 2017). Therefore, the greater concentration of ^{13}C in shoot biomass in heavily-grazed plots may be attributed to faster photosynthetic rates per unit leaf area in younger leaves.

Our results showed that carbon allocation to roots decreased with increasing grazing intensity. This can be explained by the adverse effects of grazing on the production of standing shoot and root biomass (Gao et al. 2008, Hoffmann et al. 2016, Zhou et al. 2017) (see also Figure S-3-f). For example, Gao et al. (2008) reported belowground net primary production at the same experimental site decreased from 2677 gm^{-2} under low grazing to 1487 gm^{-2} under heavy grazing . Accordingly, Chen et al. (2015) found a significant negative effect of grazing intensity on below-ground root production and turnover rate, and a positive relationship between these variables and soil carbon change in a steppe grassland in China. The lowest root production and limited substrate availability in plots with high grazing pressure in this study were associated with reduced carbon allocation to roots due to large removal of photosynthetic plant tissue by herbivory (Chen et al. 2015). The decreasing allocation of below-ground carbon to roots along the grazing gradient suggests a phenotypic shift in plant community carbon allocation priorities in response to grazing intensity; in other words, plants allocate more of their available carbon to shoot growth in heavily-grazed systems than they otherwise would. There also appears to be a lower demand for nutrient uptake by the root system in ungrazed and low grazed areas compared to high grazed areas. Whether the effect of grazing intensity on plant carbon allocation strategy is attributable to soil nutrient availability cannot be corroborated in our study. It can be assessed by complementary studies through measurements of nutrient concentration in soil and plant tissues under different conditions of nutrient supply. For example, Kafle et al. (2019) reported greater below-ground carbon allocation to the fungal partner in *Medicago truncatula* when nitrogen was readily available, but presumably phosphorus was limiting.

We observed an abrupt discontinuity between GI (7.5) and GI (9) in our results (Fig 2). The very heavily grazed plot of GI (9) reflected an extreme overgrazing scenario in previous studies. For example, Schönbach et al. (2011) reported reductions in litter accumulation and soil coverage from GI (0) to GI (9) of 83% and 43%, respectively. End-of-season standing biomass (ESSB) of grazing plots was also significantly ($P < 0.001$) affected by grazing intensity, decreasing consistently from 162 g DM m^{-2} at GI (0) to 2 g DM m^{-2} at GI (9). Relative to controls, average standing biomass removal in three study years was 18, 42, 60, 61, 80, and 99% for GI (1.5), GI (3), GI (4.5), GI (6), GI (7.5), and GI (9), respectively (Schoenbach et al., 2011). It appears that GI (9) may be a threshold for grazing intensity at this study site, beyond which plant community biomass and extent of bare soil is irreparably damaged.

Contrary to our hypothesis, we found no relationship between grazing intensity and below-ground carbon input into the soil. The direction of this relationship is still controversial. The conflicting results in different ecosystems emphasize the context-dependence of grazing effects on the below-ground carbon pool (Pausch and Kuzyakov 2018). For instance, McSherry and Ritchie (2013) indicated that increasing grazing intensity decreased soil organic carbon (SOC) by 18% in C3-dominated pastures, while SOC increased by 6–7% in C4/C3 co-occurring and C4-dominated grasslands. In subtropical C4-dominated grasslands in Florida, USA, Wilson et al. (2018) reported lower below-ground carbon partitioning and microbial biomass in grazed exclosures compared to grazed areas, mainly due to faster rates of grazing-stimulated fine root exudation, promoting greater root system production and turnover in grazed areas. They suggested that fine root exudation is associated with fine root biomass rather than grazing pressure within long-term timeframes (Wilson et al. 2018).

The results of previous studies in the same experimental site have shown that SOC was significantly affected by grazing intensity (Steffens et al. 2008, Hoffmann et al. 2016). In fact, grazing may decrease SOC through reducing above-ground plant biomass, infiltration rates, enhancing soil compaction and SOC decomposition (McSherry and Ritchie, 2013; Hao and He,2019). However, we didn’t find a significant negative effect of grazing intensity on soil organic carbon (p=0.75) (Fig. S-3-c). This can be explained by the fact that grazing may increase SOC via adding dung and urine to the soil, enhancing soil nitrogen stocks (SON), plant tissue deposition, and belowground root litter deposition (Faghihinia et al. 2020b). This can also be confirmed by the observation that were made in our previous study (Faghihinia et al. 2020a) conducted in the same site showing that SOC significantly decreased along the grazing gradient in spring and autumn when grazers were not allowed to graze in the plot. However, during grazing season in summer, no significant results of grazing intensity on SOC was observed. In line with our findings, no relationship between grazing intensity and SOC was found in 22 saltmarshes in the UK (Harvey et al. 2019). They showed that the wider environmental context determined most of the spatial variation in soil carbon stocks in these saltmarshes, while no grazing effect was detected (Harvey et al. 2019). In addition, two recent global scale meta-analyses show that the inconsistent responses of below-ground carbon pools to grazing may be related to differences in grazing intensity, grazing duration, or environmental conditions such as climate (McSherry and Ritchie 2013, Zhou et al. 2017). However, none of the experimental studies employed a range of livestock intensities to investigate the response of recently-fixed carbon allocation to different carbon pools, as we have done here. Further confirmatory work at a range of sites is needed to improve our understanding of the mechanisms behind the response of soil carbon fluxes to grazing intensity. Our results may indicate that differences in short term carbon fluxes (inputs and outputs) result in relatively stable carbon sequestration and stocks over the longer term across a range of grazing intensity in our study site.

Soil $^{13}C\text{-}CO_2$ efflux did not change with grazing intensity over the entire surveyed time period. Similar results have been reported, for example, Schmitt et al. (2013) found no effect of stimulated clipping on $^{13}C\text{-}CO_2$ efflux from soil in a pot experiment with two perennial grasses. Soil-derived

CO_2 comes from plant root and microbial respiration. The separation of root and microbial respiration is extremely challenging as both are similarly labelled and located in the same place. Tang et al. (2019) suggested that shifts in soil derived-CO_2 are rarely influenced by microbial decomposition of organic matter in the short period of time, but are mainly influenced by root respiration in grazed systems.

Below-ground carbon allocation is a fast process; assimilated CO_2 is allocated to roots and soil very quickly, mostly on the first and second day (Wu et al. 2010, Pausch and Kuzyakov 2018). Consistently, carbon input to roots in our study increased one day after labelling, peaked on the second day, and then steadily decreased until the end of the sampling period. Carbon allocation to the soil peaked one day after labelling under ungrazed, light and medium grazing intensity, while it peaked two days after the ^{13}C pulse under heavy, very heavy and very heavy grazing intensity. This result suggests that grazing intensity may negatively affect the rate of carbon transfer to the soil. This makes sense in that heavily-grazed plants invest a significant proportion of their photosynthate to replace their lost photosynthetic capacity. Given that the relationship between grazing intensity and below-ground carbon allocation has not been reported previously and the current study has not been fully replicated due to its large scale, further research is needed to test whether these hypotheses hold. In the short term, carbon allocation to roots was greater than to soil in our study. This finding is consistent with the results of a recent meta-analysis of 258 studies, which indicated greater carbon allocation to roots than to net rhizodeposition in more than 80% of studies at sampling times greater than one day after labelling (Pausch and Kuzyakov 2018).

Soil hyphal length density (HLD) showed a significant negative linear relationship with grazing intensity, with the same trend reported in grassland ecosystems (van der Heyde et al. 2017, Vowles et al. 2018, Faghihinia et al. 2020c), while a significant positive correlation was found between

HLD and recently fixed carbon in roots and soil, suggesting that HLD is promoted by significant below-ground carbon partitioning. Moreover, the positive linear relationship between HLD and recently fixed carbon can be interpreted as a positive effect of HLD on soil organic carbon, followed by additional benefits to the plants in the forms of nutrient inflows, especially phosphorus. There is some evidence that AMF stimulate microbial degradation of soil organic matter by providing photosynthetic carbon to soil microbes to facilitate the release of nutrients from organic matter (Jansa et al. 2013, Kaiser et al. 2015). This, rapid and significant movement of fixed ^{13}C from plants through the myco-rhizosphere and hyphae has been shown previously in isotope pulse studies (Johnson et al. 2002, Heinemeyer et al. 2006, Kaiser et al. 2015). Direct evidence for the transfer of photo-assimilated carbon from the plant to HLD in the field is still lacking due to the lack of efficient methods for *in situ* tracking and quantification of cellular and subcellular carbon dynamics (Kaiser et al. 2015). Nevertheless, our findings provide field evidence that HLD is positively and linearly associated with availability of fresh plant carbon, highlighting the crucial role of mycorrhiza as a rapid pathway for carbon movement in temperate grasslands. Future studies are needed to elucidate the role of mycorrhizal fungi as a potential pathway for significant plant-derived carbon translocation and sequestration in grasslands.

5. Conclusions

Our results showed that there was an increase in above-ground carbon assimilation with increasing grazing intensity, while there was a decrease in below-ground carbon translocation to roots with increasing grazing intensity. We found no relationship between below-ground carbon allocation to soil and soil-respired CO_2 with grazing intensity. These results suggest that grazing intensity alters carbon allocation to different carbon pools differently. The decreasing below-ground carbon allocation to roots along the grazing gradient reflects different allocation strategies of plants under different grazing intensities. Moreover, the positive correlations between HLD and recently fixed carbon in roots and soil indicate the key role of mycorrhizas as a fast route for carbon translocation from plants to soil in grasslands. Therefore, any environmental perturbation such as excessive grazing, that affects mycorrhizal abundance and function has the potential to have a critical impact on net carbon fluxes in grassland ecosystems. These results have implications for both the translocation of carbon into the bulk soil and the ability of mycorrhizas to absorb nutrients. Furthermore, to address humanity's greatest challenges of food security and climate change mitigation, much more effort is needed to better understand the effects of grazing intensity on grassland ecosystem function and carbon cycling. Consequently, policy makers, landowners, and farmers need more evidence-based information on optimal grazing options to ensure the best long-term and sustainable outcomes for ecosystem services under a range of circumstances, including those arising from climate change. Nonetheless, caution is warranted in extrapolating our conclusions to another system, and we therefore recommend that further studies be conducted on the effects of grazing intensity on the soil carbon cycle.

Acknowledgment

We thank the Xian-Jiatong-Liverpool University Research Development Fund (RDF-15-02-13) for financial support and the kind cooperation of the staff in the Inner Mongolia Grassland Ecosystem Research Station (IMGERS, Chinese Academy of Sciences) and their provision of equipment and facilities. Particular thanks to our research team Chuanyu Zhou, Zirui Hu, Ye Wang, Hanyu Jian, Shasha Zou and Fanjing Liu for their collaborative effort during data collection, and Yuwei He and Yuxiang Ren for setting up the experiments.

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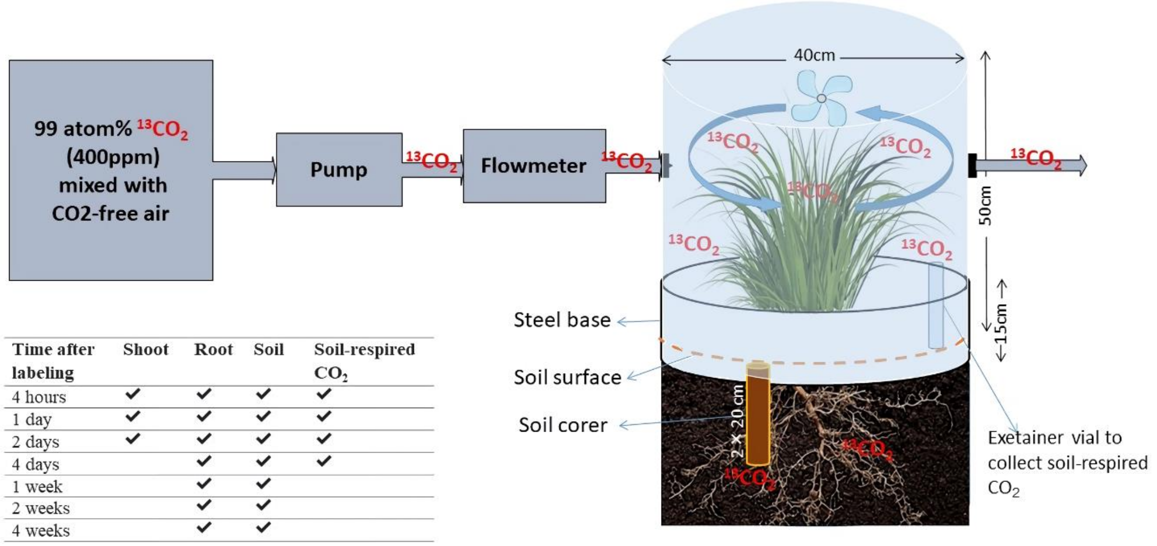
Table 1. Linear model of the relationship between grazing intensity and time after labelling and different carbon pools. The full model (No. 1) and the best model (No. 2) selected based on Akaike's information criteria (AIC). Dashes (-) represent variables which have not been included in the best model. Significant relationships have been highlighted in bold format and P-values have been presented in parenthesis.

Response variables	**Model No.**	**Time after labelling**	**Grazing Intensity**	**Grazing Intensity × Time after labelling**	**AIC**
Carbon pools					
Shoot	1	-11.26±19.92(0.579)	19.94±4.77**(0.001)**	-6.24±3.68(0.108)	221.8
	2	-39.35±11.61**(0.003)**	13.35±2.89**(0.000)**	-	221.6
Root	1	-0.39±0.20(0.054)	-0.86±0.45(0.060)	0.03±0.04(0.444)	338.4
	2	-0.26±0.11**(0.019)**	-0.63±0.34(0.065)	-	336.6
Soil	1	-0.09±0.031**(0.005)**	-0.07±0.07(0.300)	0.00±0.01(0.544)	157.7
	2	-0.08±0.02**(0.000)**	-	-	154.1
Soil-respired CO_2	1	-1.59±1.02(0.134)	-0.43±0.42(0.322)	0.12±0.18(0.522)	145.8
	2	-1.06±0.58(0.084)	-	-	140.1



Time after labeling	**Shoot**	**Root**	**Soil**	**Soil-respired CO_2**
4 hours	✓	✓	✓	✓
1 day	✓	✓	✓	✓
2 days	✓	✓	✓	✓
4 days		✓	✓	✓
1 week		✓	✓	
2 weeks		✓	✓	
4 weeks		✓	✓	

Fig.1. Schematic of pulse chase labelling experiment using ^{13}C-CO_2. Schematic shows the pulse chase labelling experiment through delivering 99 atom% $^{13}CO_2$ to the transparent acrylic labelling chamber. The table illustrates the time of sample collection for different carbon pools represented with different colors.

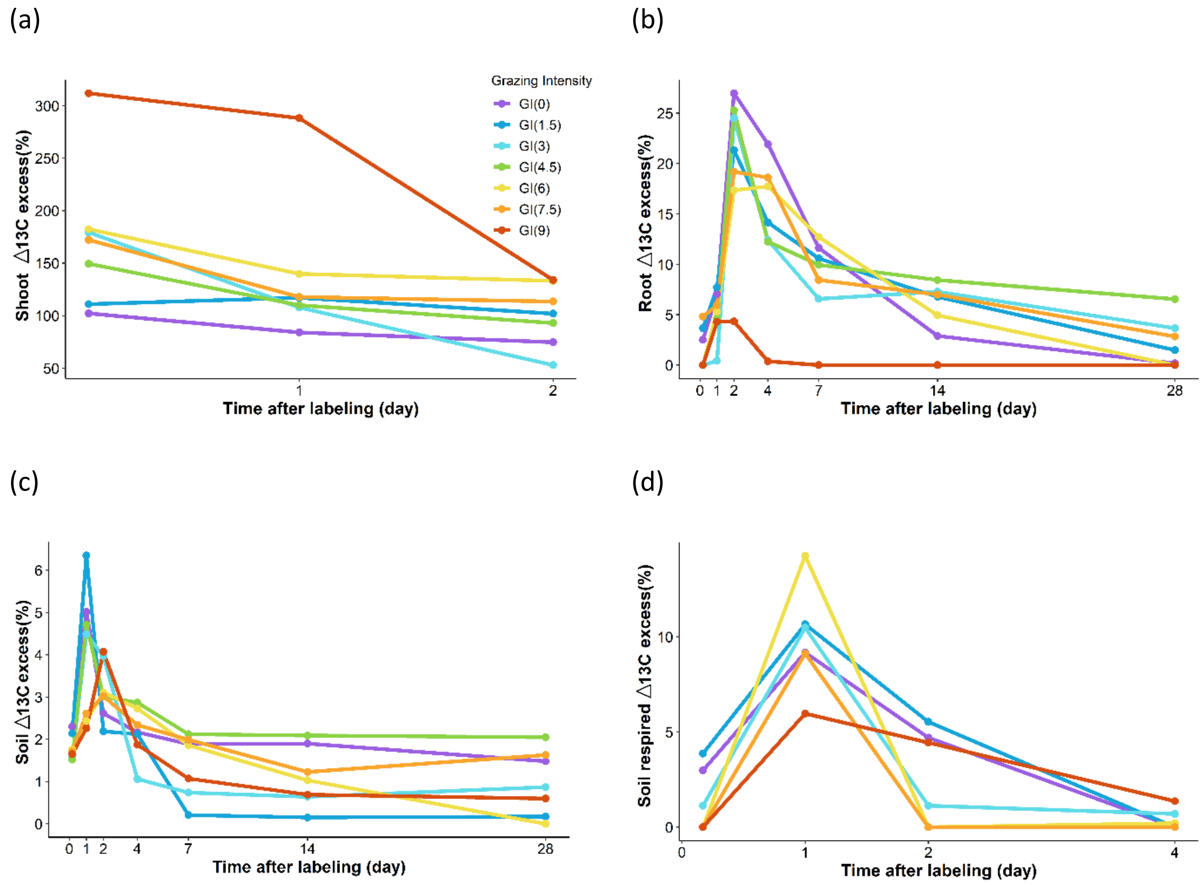


Fig. 2. Carbon isotopic enrichment in samples expressed as $\Delta^{13}C$ (%) in shoot (a), root (b), soil (c) and soil-respired CO_2 (d) in seven grazing intensities (GI) (ewe/ha) after four hours, one day, two days, four days, one week, two weeks, and four weeks after labelling.

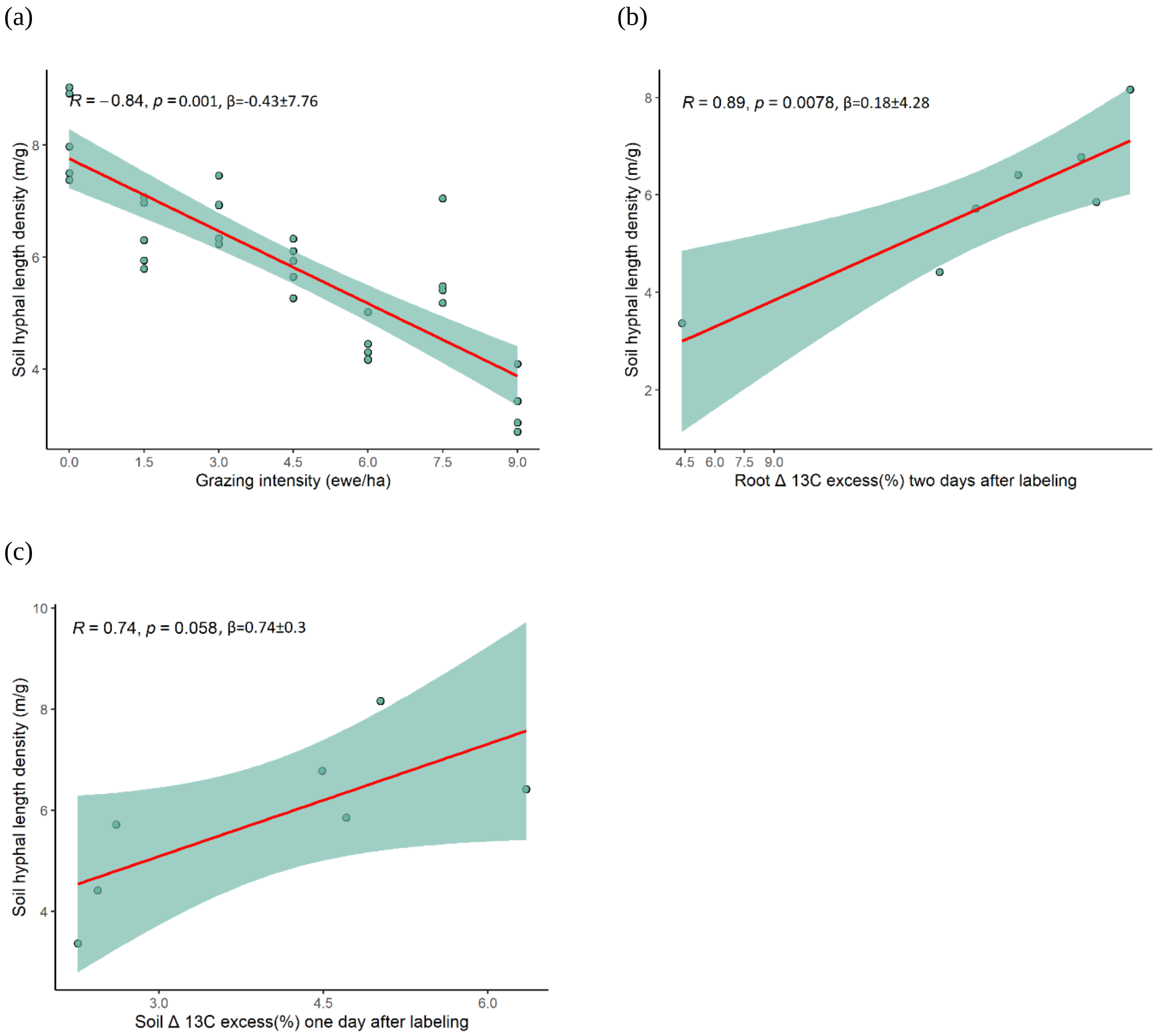


Fig. 3. The relation between soil hyphal length density and (a) grazing intensity, (b) root $\Delta^{13}C$ excess and (c) soil $\Delta^{13}C$ excess. Solid and hollow circles in "a" represent mean and individual observations at each level of grazing intensity respectively. Solid circles in "b" and "c" indicate individual observations. Lines are fitted the regression lines from the linear model; shade areas represent 95% CI from the estimation.