

# Historic Grazing Enhances Root-Foraging Plasticity and Assimilation But Not Absorbability For Nitrogen of Clonal Offspring in *Leymus Chinensis*

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# Abstract

**Aims** Plants with a history of overgrazing show trait-mediated legacy effects. These legacy effects strongly influence growth dynamics and stress tolerance of grassland plants, thus impacting ecosystem functioning. Long-term overgrazing has strong effects on plant growth and carbon assimilation via asexual propagation. However, the links between nitrogen (N) cycling and grazing-induced plant legacy effects are largely unknown.

**Methods** We tested the strength of legacy effects of long-term overgrazing on N metabolism in the clonal plant *Leymus chinensis*, and its associated changes at the physiological and molecular levels. These tests were conducted in both field and greenhouse experiments.

**Results** The clonal offspring of overgrazed *L. chinensis* were significantly smaller than the control offspring, with lower individual N uptake and N utilization efficiency, indicating that the N dynamics were impacted by plant legacy effects. The response ratios of root traits to N patches in the clonal offspring of overgrazed *L. chinensis* were significantly higher than those of the control, indicating that root nutrient foraging plasticity increased to cope with grazing-induced N heterogeneity. Moreover, the observed plant legacy effects slightly decreased N absorbability in roots but significantly increased N assimilation capacity, by increasing N resorption efficiency in particular, with biotic stress memory activated at the enzymatic and transcriptional levels.

**Conclusions** We propose that multigenerational exposure of perennial plants to herbivore foraging can produce a legacy effect on nutrient uptake, which offers insights into the potential resilience of grasslands to overgrazing.

## Introduction

Livestock grazing, an ancient method of grassland utilization worldwide, has long played a central role in maintaining ecosystem structures and functions as well as achieving land sustainability (Wang et al. 2019; Wu et al. 2015). Owing to widespread overgrazing over the past century, however, grasslands have undergone unprecedented degradation globally (Dlamini et al. 2016), but especially in the eastern Eurasian temperate steppe (Wu et al. 2015), the world's largest remaining grassland. A large number of studies have revealed the mechanisms underlying the observed degradation distinctly from the perspective of plant responses (Diaz et al. 2007; Rotundo and Aguiar 2008). Grazing avoidance, resistance, and resilience of grassland plants have attracted global attention from plant behavioral ecologists. One group of studies has demonstrated that plant species have some defensive anti-herbivore mechanisms that enable them to adapt to repeated high-intensive grazing via increasing polyphenolic allelochemicals and physical strategies (e.g., spines) (Robbins et al. 1987; Zheng et al. 2014). However, some evidence has shown these chemical or physical defenses have relatively minor effects on plant avoidance of or resistance to large herbivores (e.g., sheep and cattle) (Liu et al. 2019), contrasting with results from plant–insect systems (Zhu et al. 2014). Recently, the significance of legacy effects mediated

by traits plasticity has been recognized as one of the key mechanisms for grassland plants responding to long term grazing (Ren et al. 2017) because of its potential influences on plant tolerance, population dynamics, and ultimately, ecosystem multifunctionality (Herman and Sultan 2011; Latzel et al. 2013; Walter et al. 2016).

Plant trait-mediated legacy effects reflected by the plasticity of the offspring of plants exposed to substantial and long-term stress (De Long et al. 2019; Mousseau et al. 2009) are receiving increasing attention as an indirect response of plants to abiotic and biotic stresses (Yin et al. 2019). These trans-generational impacts of stress on plant performance can be expressed in the form of morphological, biochemical, or physiological responses in the offspring (Rendina González et al. 2018). Among them, trait-mediated legacy effects such as plant selection (Wurst and Ohgushi 2015), propagule modification (Valencia-Díaz and Montaña 2005) and epigenetic variation (Portela et al. 2020b; Rendina González et al. 2018) were found to be more important. Firstly, the intraspecific selection of genotypes adapted to the external environment can increase the plant fitness and ecosystem functioning (Zupping-Dingley et al. 2016). Moreover, stressors in the maternal environment, such as drought, salinity, insect herbivory, and livestock grazing, can dramatically affect the quality of plant seeds or buds, which can thus significantly influence the growth and stress resistance of offspring (Hatzig et al. 2018; Walter et al. 2016). In addition, a growing number of studies provide evidence that molecular ecological processes play key roles in regulating trans-generational adaptation in plants (Herman and Sultan 2011). For example, after multigenerational exposure to elevated CO<sub>2</sub> levels, seed-grown offspring of *Plantago lanceolata* exhibited enhanced growth and transcriptome reprogramming of photosynthesis despite no significant genetic difference (Watson-Lazowski et al. 2016).

The direction and strength of plant legacy effects in response to stress tends to be associated with exposure time (from several months to multiple generations) (Li et al. 2019a) and life history traits (annual/perennial or seed producing/clonal plant) (González et al. 2017). However, most existing studies related to plant legacy effects have been performed on annual species or on sexually produced offspring (Portela et al. 2020a). A small but growing body of evidence suggests that stresses, such as drought and herbivory, can also trigger significant clonal plasticity in offspring growth (Latzel and Klimešová 2010). Recently, our research group has provided experimental evidence that long-term livestock grazing imposes legacy effects that negatively affected plant growth rate, biomass allocation and the photosynthetic capacity of *Leymus chinensis*, the dominant perennial clonal plant species in the eastern Eurasian steppe (Li et al. 2015; Ren et al. 2017). Indeed, more than 80% of the intergenerational turnover of plant individuals depends on clonal reproduction of the bud bank in semiarid grasslands (Benson and Hartnett 2006). Therefore, as a key dimension of the observed plant legacy effect, clonal offspring may play a stronger role in regulating grassland productivity than seeds in semiarid grassland regions.

Plant strategies to withstand grazing are strongly linked with the feedbacks associated with changing soil properties, particularly nutrient supply and availability (da Silveira Pontes et al. 2010; Lü et al. 2015). In some previous studies, soil nutrients have been shown to have a large effect on plant trait plasticity (Lü et al. 2012; Siebenkäs et al. 2015), leading to a strong influence on community productivity and the

stability of grassland ecosystems, and this may be largely shaped by herbivore (Laliberté et al. 2012). In field tests of the maternal environment of plant growth, some experimental studies have shown that long-term overgrazing decreased the soil nitrogen (N) pool and N availability, which could possibly enhance N resorption efficiency and the metabolism of grassland plants (Lü et al. 2015; Millett and Edmondson 2015). Grazing-induced plant legacy effects may thus be linked with plant nutrient strategies. Similarly, Walter et al. (2016) found that offspring of *Genista tinctoria* developed from parents exposed to drought conditions showed higher leaf C:N ratios than those of offspring developed from well-watered parents, thereby sustaining adaptive changes in growth rate across generations. However, there are no empirical studies that have explicitly examined the potential linkages between N metabolism and plant legacy effects induced by livestock overgrazing.

Spatial heterogeneity of soil nutrients is a ubiquitous feature of semi-arid grasslands (Fridley et al. 2011). Numerous experiments have demonstrated that grazing may enhance the natural spatial heterogeneity of soil N owing to selective foraging on plants and the presence of dung and urine patches from livestock (Liu et al. 2016; Yoshihara et al. 2010). Root foraging behavior may thus increase the nutrient uptake of plants under grazing by adapting to heterogeneous nutrient environments. Additionally, ecological stoichiometric analysis has shown that plant growth and community productivity of grasslands are always limited by N compared to other soil nutrients in semiarid regions of eastern Eurasia (Yang et al. 2017), and this may be dramatically aggravated by long-term overgrazing (Bai et al. 2012). Under these circumstances, historic grazing can be hypothesized to produce a legacy on plant N strategy utilized by the offspring, potentially leading to an enhancement in root foraging ability to effectively adapt to grazing-impacted N-deficient and N-heterogeneous habitats.

In this study, we tested the possible links between plant legacy effects after more than three decades of continuous overgrazing and plant N uptake, metabolism, and root N foraging strategy. Using the clonal perennial *L. chinensis* as a target species, we aimed to detect the direct response of grazing in the maternal environment and the indirect legacy effects on *L. chinensis* clonal offspring that occurred in field and growth chamber tests, respectively. Our experiment investigated the following: (a) whether long-term overgrazing-induced plant legacy effects generally influence clonal offspring N absorption and utilization processes in subsequent benign conditions; (b) the extent to which this legacy effect is linked to plant root foraging plasticity in N-heterogeneous patches; and (c) the potential role of physiological and molecular changes of key metabolic processes related to N in maintaining the observed plant legacy effect.

## Methods

### Plant species

In this research, we used *L. chinensis* as the study species, as it is highly abundant in important grazing ecosystems. *L. chinensis* is a perennial species in the family Gramineae, and it is distributed in the eastern region of the Eurasian steppe (Li et al. 2015; Xu and Zhou 2006), including the outer Baikal area

of Russia, the northern and eastern parts of the People's Republic of Mongolia, the Northeast China Plain, the Northern China Plain, and the Inner Mongolia Plateau of China. The plant is highly drought tolerant and can withstand both low fertility and high pH conditions, while still producing high yields. Owing to its excellent stress tolerance, *L. chinensis* is found throughout a broad gradient of temperature and precipitation conditions. It is highly palatable for grazing livestock and frequently used as hay. Therefore, it is often exposed to both grazing and mowing. *L. chinensis* has a rhizomatous rooting system and produces clonal buds, leading to its extensive spread, through which it often forms large patches of monoculture (Bai et al. 2009). Its highly branched rhizomes lie horizontally about 10 cm under the surface of the ground. From an ecological perspective, *L. chinensis* is a desirable plant for use in the restoration of degraded grassland because its rapid propagation and rhizomatous net can reduce soil erosion and desertification in arid areas, such as those of northern China (Liu and Han 2008).

#### Field study: plant and soil effects of grazing

The field site of our study is located at the Xilingol Grassland Ecosystem Research Station (XGERS) in the Xilin River catchment (43°38' N, 116°42' E) of the Inner Mongolia Autonomous Region, P.R. China, which has a semiarid continental climate (Yao et al. 2010). According to long-term monitoring (1982–2016), the climate is characterized by an annual precipitation of approximately 320 mm, with more than 70% falling from May to August, which coincides with the highest temperatures (Li et al. 2015). The mean annual temperature is 1.01 °C, ranging from – 18.8 °C in January to 21.6 °C in July. The major soil types of this region are calcic chestnuts and calcic chernozems (Gong et al. 2008). The vegetation is a temperate steppe, and the dominant species are *L. chinensis* and *Stipa grandis* (Li et al. 2015).

In this study, the field experiment consisted of two treatments: grazing exclusion and heavy grazing by livestock. The grazing plot, which was about 200 ha in area, has experienced year-round grazing by sheep and goats for more than 40 years. The stocking rate of approximately 3.0 sheep units ha<sup>-1</sup> is substantially higher than the recommended stocking rate of 1.5 sheep units ha<sup>-1</sup> (Li et al. 2015; Ren et al. 2017). Since 1983, the grazing exclusion plot adjacent to the grazing area has been fenced off for the purpose of long-term ecological observation and research by XGERS. Using the paired sampling method, five 10 m × 20 m replicated subplots were established in treatments of both grazing exclusion and heavy grazing along the pasture fence. The paired subplots were randomly allocated within 30 m of each other along the pasture fence.

The plant and soil sampling was conducted in the ten selected subplots of grazing exclusion and heavy grazing on August 15, 2016, corresponding to the peak growing season of *L. chinensis*. In each subplot, three 1 m × 1 m quadrats were randomly established for sampling. Each quadrat was divided into three equal parts. In the first part of the quadrats, the green leaves of *L. chinensis* individuals were collected in order to estimate leaf N resorption efficiency (Lü et al. 2012). To analyze the potential influence of grazing on leaf N resorption, we estimated N concentrations from relatively young (upper) and relatively old (lower) leaves in the second part of each quadrat. At the same time, we sampled soil associated with *L. chinensis* following the same plant sampling scheme using a soil auger (diameter = 7 cm, depth =

20 cm). In the third part of the quadrats, we collected about 15 senesced leaves of *L. chinensis* on November 15, 2016, corresponding to the non-growing season.

After the field sampling, plant traits, such as plant height, dry matter content (DMC), and aboveground biomass, were measured at the individual scale in the laboratory. The fresh weights of *L. chinensis* leaves were measured and were then oven-dried at 65 °C for 48 h to determine the DMC. Using a mechanical micromill, the green and senesced *L. chinensis* leaves were then ground. The ground samples were passed through a 0.5-mm mesh sieve. Soil samples were air-dried at room temperature for 20 days. The soil samples were then ground, homogenized, and passed through a 2-mm mesh sieve after removing fine roots and stones. Using the Kjeldahl method, total concentrations of plant and soil N were measured (Wang et al. 2018). The soil organic matter content was analyzed using dichromate oxidation (Li et al. 2019b). In addition, available soil N soil was photometrically measured by a continuous flow analyser (SAN Plus, Skalar, Netherlands) from on-site KCl extractions using the fresh soil (Geng et al. 2017).

#### Growth chamber study 1: test for grazing-related plant legacy effects

The growth chamber study was a continuation of our field grazing experiment. The method of asexual reproduction by rhizome buds of *L. chinensis* was used to determine how long-term heavy grazing influences plant legacy effects on N processing. In each quadrat of our field site, we collected more than five dormant buds of *L. chinensis* in March 2017 during peak production of rhizome buds (from November 2016 to March 2017). In total, more than 15 buds (more than five buds from each of three replicated quadrats) were prepared corresponding to each of the ten field subplots (five grazing subplots and five ungrazing subplots). At this stage, all the buds were at the initial development state. In the laboratory, the associated rhizomes with buds were cut into 2-cm lengths to ensure the offspring had similarly proportioned sources of nutrients from maternal rhizomes. The clonal offspring were cultivated in a hydroponic container with Hoagland nutrient solution (15 cm × 15 cm × 12 cm) (Ren et al. 2017). In offspring groups consisting of samples from both grazing exclusion and heavy grazing, we had four replicates (four randomly selected from the five field sampling subplots) for each experimental container. In each of the hydroponic containers, 12 individuals of *L. chinensis* clonal offspring were retained after excluding other dead buds or plants that grew inconsistently. Therefore, 96 experimental plant individuals were grown (2 maternal treatments × 4 containers × 12 buds). In our plant growth chamber (Percival Scientific, Inc., Perry, IA, USA), the lights remained on for 14 h day<sup>-1</sup> with a 25 °C daytime temperature, 15 °C nighttime temperature, and relative humidity of 70–80%. We changed the Hoagland nutrient solution every five days. During the experiment, we measured plant height every seven days, which was used as the indicator of clonal plasticity induced by plant legacy effects. After 65 days of growth, half of the plants were sampled for the purpose of measuring phenotypic traits, aboveground biomass, and N concentrations after they were oven-dried at 65 °C for 48 h.

#### Growth chamber study 2: plant legacy effects on N metabolism

Using the plant materials of growth chamber study 1, plant samples for the analysis of N-related enzyme activity and gene expression were sampled after 65 days of cultivation. The mature plants were immediately frozen in liquid N<sub>2</sub> and then stored in an Ultra-low Temperature Freezer (Thermo Fisher, Waltham, MA, USA) at -80 °C. The activities of nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase of leaves and roots were determined with a Nitrate Reductase Kit (NR-1-Y, Comin), Nitrite Reductase Kit (NIR-1-G, Comin), Glutamine Synthetase Kit (GS-1-Y, Comin), and Glutamate Synthase Kit (GOGAT-1-Y, Comin) following the manufacturer's instructions. In addition, the expression levels of three N-related genes (*NRT1.1*, *NRT1.2*, and *NRT1.7*) in leaves, which had been preliminarily confirmed by our transcriptome data, were measured using real-time quantitative PCR (qPCR). Total RNA levels were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Full-length cDNA was then reverse transcribed using a cDNA synthesis kit (RR047A, TaKaRa, Dalian, China). For each gene, qPCR was performed according to the manufacturer's instructions (4472908, Applied Biosystems, Foster City, CA, USA), and all the genes were assayed with three independent biological replicates. The *Actin* gene (GenBank accession number, HM623326.1) was used as an internal reference. The reaction conditions were 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The primer sequences are provided in Supplementary Table S1.

### Growth chamber study 3: plant legacy effects on root foraging

In order to investigate the potential mechanism of root foraging behavior underlying N uptake, we studied the foraging behavior of grazed and ungrazed plant offspring roots in a mesocosm study. Plants from two maternal source treatments (OG+, clonal offspring of plants that had experienced long-term herbivore grazing; OG-, clonal offspring of plants that had not experienced grazing) exposed to a soil N patch were distributed in a randomized, complete block design with eight replicates. The background soil was collected from natural grassland near XGERS and passed through a 5-mm mesh sieve to remove the fine roots and stones. The pots (15-cm diameter and 18-cm deep) were split into two parts by polyethylene barriers. In order to create N heterogeneity, half of each pot was filled with N rich soil by adding 150 mg NH<sub>4</sub>NO<sub>3</sub>, whereas the other half was filled with original background soil. After 10 days of pre-cultivation in hydroponic nutrient solution, the asexual offspring of *L. chinensis* were transplanted into pots using the root-splitting method (Sun et al. 2016). After 45 days of asexual offspring establishment, the root length and root biomass were measured both in the N-rich and N-poor parts to assess the sensitivity of clonal offspring of grazed and ungrazed *L. chinensis* to soil N patches.

### Calculation and statistical analysis

Significant differences in plant phenotypic traits, physiological traits, and gene expression levels between the experimental groups were assessed by one-way ANOVA. Before the analyses, the phenotypic traits, including plant height, DMC, and individual biomass, were averaged from all the *L. chinensis* plants of each quadrat in the field grazing experiment and of one hydroponic container in the growth chamber study. In addition, the relative levels of the three N-related genes from the qPCR analysis were log-transformed before analysis.

Leaf N resorption efficiency (NRE) was defined as the proportion of N in mature leaves that was resorbed during senescence (Lü et al. 2015). In the field experiment, NRE was calculated as

$$\text{NRE} = (1 - N_{\text{senesced}} / N_{\text{green}}) \times 100\%, (1)$$

where  $N_{\text{senesced}}$  and  $N_{\text{green}}$  are the N concentrations of senesced *L. chinensis* leaves in November and green *L. chinensis* leaves in August, respectively. The N concentrations of *L. chinensis* leaves were expressed on a dry mass basis.

In this study, plant N accumulation ( $N_{\text{accumulation}}$ ), N uptake efficiency (NTE), N utilization efficiency (NUE), and N utilization index (NUI) were calculated to assess the N use strategies. These calculations were as follows:

$$N_{\text{accumulation}} = \text{BM} \times N_{\text{concentration}}, (2)$$

$$\text{NTE} = N_{\text{accumulation}} / N_{\text{supply}} \times 100\%, (3)$$

$$\text{NUE} = \text{BM} / N_{\text{accumulation}}, (4)$$

$$\text{NUI} = \text{BM} \times \text{NUE}. (5)$$

Above, BM is the total biomass of one hydroponic container, and  $N_{\text{supply}}$  is the total quantity of N supply in Hoagland nutrient solution.

The plasticity index (PI), defined as the responses of plant height to grazing induced plant legacy effects (Li et al. 2015), were calculated as

$$\text{PI} = (H_{\text{OG-}} - H_{\text{OG+}}) / H_{\text{OG-}} \times 100\%, (6)$$

where  $H_{\text{OG-}}$  is the plant height of the clonal offspring of plants that had not experienced grazing and  $H_{\text{OG+}}$  is the clonal offspring of plants that had experienced long-term livestock grazing.

The response ratios (RRs) of *L. chinensis* root traits, including root length and biomass, to soil N heterogeneity in the root-splitting experiment were calculated as

$$\text{RR} = \ln(T_{\text{N+}} / T_{\text{N-}}), (7)$$

where  $T_{\text{N+}}$  and  $T_{\text{N-}}$  are the root traits of plants from the N-rich and N-poor patches, respectively.

## Results

In the field experiment, we observed significant phenotypic plasticity of *L. chinensis* after long-term overgrazing, with a decrease in plant height ( $P < 0.01$ ) and individual biomass ( $P < 0.01$ ) and a slight increase in DMC (Table 1, Fig. S1). With regard to topsoil nutrients, total N content, plant available N content, and organic C content were significantly decreased in the grazed plots (all  $P < 0.05$ , Table 1).

However, grazing increased the green leaf N concentration of individual *L. chinensis* plants by 23.65% at the peak of the growing season ( $P < 0.01$ , Fig. 1a). Additionally, in our field experiment, senesced leaf N concentrations of *L. chinensis* were not significantly affected by grazing at the end of growing season (by -0.08%,  $P > 0.05$ , Fig. 1b). Consequently, foliar N resorption efficiency was significantly increased by long-term overgrazing by large herbivores at the peak of the growing season (by 33.81%,  $P < 0.05$ , Fig. 1c) mainly driven by green leaf N concentration ( $P < 0.01$ , Fig. 1c–e). Specifically, throughout the aging processes of leaves, the N concentrations of young leaves were more sensitive to grazing relative to old leaves ( $P < 0.05$ , Fig. S2).

Table 1

Size of *Leymus chinensis* individuals and topsoil nutrient properties (0–10 cm soil layer) affected by long-term overgrazing by large herbivores in a typical steppe.

Category	Response variable	Ungrazed		Grazed		Difference ( $P$ -value)
		Value	SE	Value	SE	
Plant properties	Plant height (cm)	83.01	1.40	23.89	0.85	< 0.01
	Aboveground biomass (g/individual)	1.53	0.08	0.31	0.02	< 0.01
	Dry matter content (%)	34.62	0.61	36.18	0.95	> 0.05
Soil properties	Total nitrogen (g/kg)	2.36	0.11	1.94	0.10	< 0.01
	Available nitrogen (mg/kg)	3.74	0.37	2.45	0.13	< 0.01
	Organic carbon (g/kg)	20.85	0.96	18.92	0.38	< 0.05

Then, we conducted a growth chamber experiment to determine how plant N processing is associated with grazing-induced plant legacy effects on plant plasticity; the clonal offspring of grazed *L. chinensis* had significantly lower biomass than the ungrazed control group ( $P < 0.05$ , Fig. S1). Moreover, grazed *L. chinensis* offspring had stronger responses to N patches in terms of root length ( $P < 0.01$ , Fig. 2a) and root biomass ( $P < 0.01$ , Fig. 2b). However, individual N uptake and N utilization index were significantly lower in the offspring of grazed *L. chinensis* ( $P < 0.05$ , Fig. 3a, Table 2). However, this change was not related to the variation in shoot N concentration ( $P > 0.05$ , Fig. 3b), including the N components  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N ( $P > 0.05$ , Fig. 3c). To elucidate the underlying biochemical and molecular mechanisms of grazing-induced plant legacy effects on plant N metabolism, we analyzed expression patterns of a series of key enzymes and related genes (i.e., *NRT1.1*, *NRT1.2*, and *NRT1.7*). Nitrate reductase activity was lower in the roots of offspring from grazed plants ( $P < 0.01$ , Fig. 4a). There was no change in nitrite reductase activity in roots and leaves ( $P > 0.05$ , Fig. 4b). However, grazed plant offspring had higher glutamine synthetase and glutamate synthase activity levels ( $P < 0.01$ , Fig. 4c, d). Meanwhile, for the three key genes associated with nitrogen metabolism, *NRT1.1* showed no change ( $P > 0.05$ ), *NRT1.2* was down-regulated ( $P < 0.01$ ), and *NRT1.7* was up-regulated ( $P < 0.01$ ; Fig. 5).

Table 2

Plant legacy effects of long-term overgrazing on *Leymus chinensis* N uptake and utilization in growth chamber study. OG+, clonal offspring of plants that had experienced herbivore grazing; OG-, clonal offspring of plants that had not experienced grazing.

Parameter	Average		Range		Significance
	OG-	OG+	OG-	OG+	
Plant N uptake efficiency (%)	2.76	2.25	2.07–3.57	1.91–2.83	$P < 0.05$
Plant N utilization efficiency (g·g <sup>-1</sup> ) <sup>1)</sup>	32.45	29.64	27.20–36.55	26.30–32.69	$P > 0.05$
N utilization index (g <sup>2</sup> /g)	32.81	24.7	17.54–48.20	19.69–28.92	$P < 0.05$

## Discussion

Overgrazing is a serious and widespread threat to sustainable development and the livelihoods of local people in grassland regions (Tälle et al. 2016); consequently, grazing-induced plant legacy effects and their cascading effects on phenotypic plasticity to nutrient cycling and ecosystem productivity conditions offer many fascinating scientific questions for current research. This study is the first to quantify the strength of overgrazing-induced plant legacy effects on N cycling in a clonal and perennial grassland plant species at the physiological and molecular levels, and the important impact of N utilization efficiency was also examined in this context (Fig. 6). A key finding of our study was that multiyear exposure of perennial *L. chinensis* to livestock grazing impacts nutrient utilization of their clonal offspring, leading to legacy effects on plant growth and thus potentially impacting population dynamics. The clonal offspring produced by maternal plants experiencing grazing exhibited a decrease in N uptake efficiency and increased root nutrient foraging plasticity and N assimilation, and these responses were underpinned by changes at the physiological and molecular levels.

### Patterns of N uptake and assimilation in offspring of grazed plants

Plant legacy effects have been proposed to explain rapid adaptation of higher plants to cope with potentially recurring biotic and abiotic stresses (Rottstock et al. 2017; Walter et al. 2016). The present study indicated more than three decades of continuous overgrazing led to significant legacy effects on plant growth and the N utilization efficiency of *L. chinensis* clonal offspring, which confirmed the previous findings of grazing-induced plant legacy effects (Ren et al. 2017). The difference in phenotypic plasticity between grazed and ungrazed *L. chinensis* clonal offspring gradually increased with plant growth time, indicating grazing-induced plant legacy effects had a significant cumulative effect throughout their growth and development. We demonstrated that overgrazing-induced plant legacy effects in clonal offspring limited N absorption at the individual scale but also promoted nutrient assimilation efficiency and reabsorption from aging to young tissues (Fig.6). This could be attributed to

the adaptive regulation of N balance by plants themselves, both intra- and inter-generationally under N-limited conditions.

At the individual scale, we found that plant N uptake significantly declined by 18.3% in the offspring of grazed *L. chinensis* compared with offspring of ungrazed plants. However, our field experiment showed that the decrease in individual N uptake by maternal *L. chinensis* plants was 74.3% after long-term overgrazing. This clearly reflected that the change in N absorbability induced by plant legacy effects was only partially (by approximately a quarter) maintained by the plant clonal offspring compared with the *in-situ* plasticity index of N uptake (Fig.6). A possible explanation is that *in situ* effects of disturbances on plant growth and nutrient absorption were caused by multiple mechanisms, including plant legacy effects, soil microbial feedback, and nutrient availability (Bell et al. 2014; Robson et al. 2010; Sun et al. 2017). However, this change was not caused by the variation in N concentration between our two experimental groups. We did not find a significant change in N components, such as  $\text{NO}_3^-$ -N,  $\text{NH}_4^+$ -N, and soluble protein, in spite of the significant morphological plasticity of offspring of grazed plants. Therefore, strong N homeostasis under grazing-induced plant legacy effects might be a preferable strategy. Furthermore, we found that nitrate reductase activity significantly declined in roots of offspring of grazed plants. This indirectly implied that root absorption limitation has a potential linkage to grazing-induced plant legacy effects, thus maintaining plant N balance. However, our finding that grazing legacy effects significantly increased the enzymatic activity and gene expression level of N assimilation provided results contrasting with the N absorbability findings. Indeed, N assimilation is a very important biological process involved in transforming inorganic N into organic N for grassland plants (Lü et al. 2014; Xu et al. 2012). We found that grazing-induced plant legacy effects significantly increased the activity of glutamine synthetase and glutamate synthase in leaves. Moreover, plant legacy effects also significantly increased the N utilization index. Hence, the dynamics of N metabolism enzymes may explain the influence of grazing on clonally trans-generational adaptation of asexual grassland plants.

Root foraging plasticity helped plants cope with grazed N-heterogeneous habitat

It is valuable to determine why offspring of long-term overgrazed *L. chinensis* had a lower N uptake but higher N concentration compared with those of offspring of ungrazed plants at the individual scale. We found a dramatic decrease in soil available N caused by long-term overgrazing. Consequently, grassland plants are exposed to N-heterogeneous habitats. It is known that the plants in our study region of the Inner Mongolia grasslands of the eastern Eurasian steppe are N-limited (Sun et al. 2018; Zhang et al. 2019). Specifically, long-term overgrazing has caused soil deterioration, especially in term of its available N. Under these conditions, root foraging behavior of grassland plants is particularly important in responding to grazing. In this paper, furthermore, a root-splitting method was used to interrogate foraging ability (measured as the ln-transformed response ratio of root biomass or length between the N-rich and N-poor patches) of grazed and ungrazed *L. chinensis* clonal offspring under N heterogeneity. Interestingly, we found that the response ratios of root length and root biomass of clonal offspring of previously grazed *L. chinensis* were significantly higher than those of previously ungrazed *L. chinensis* clonal offspring. This reflected that grazing-induced plant legacy effects obviously enhanced sensitivity to N-

enriched areas surrounding individual plants. Thus, root nutrient foraging plasticity is an important signal predicting adaptation to grazing-induced N-heterogeneous habitats. According to root foraging theory, there had been a trade-off between foraging scale and foraging ability to some extent, contributing to coexistence of all species in the same population (Cahill Jr and McNickle 2011; Campbell et al. 1991; Kembel et al. 2008). The observed increase in N foraging ability of offspring of grazed *L. chinensis* implies that root foraging strategies would shift from far-ranging foraging to precise foraging. This could be ascribed to the shallow distribution and biomass reduction of roots in response to long-term overgrazing. Therefore, the potential abilities of far-ranging foraging by plants were partially lost in degraded grasslands. Another possible explanation is N-rich urine and dung produced by livestock increasing N heterogeneity of grassland soil (Hirobe et al. 2013; Xi et al. 2014). Soil nutrient heterogeneity can promote plants to develop precise N-foraging behavior rather than far-ranging foraging behavior.

#### Elevated N resorption: from maternal plants to clonal offspring

In the maternal environment, long-term overgrazing significantly elevated foliar N resorption efficiency, mainly induced by transporting the nitrogen from old to young leaves. Furthermore, our growth chamber experiment showed that maternal experience of overgrazing significantly up-regulated the relative expression of transcripts of *NRT1.7*, which encodes a protein responsible for N resorption, in *L. chinensis* clonal offspring. This dynamic may help explain the influence of grazing-induced plant legacy effects on N resorption at the molecular level. We speculated that the increase in N resorption induced by grazing can be ascribed to an adaptive strategy to nutrient depletion and plant-soil N decoupling in a degraded grassland (Liu et al. 2017; Pan et al. 2018). It has been widely recognized that N content in plant tissues is highly affected by soil N availability at a large spatial scale (Craine et al. 2010; Heyburn et al. 2017; Tang et al. 2018). However, in our case, long-term overgrazing significantly increased N concentrations in plant tissues in the maternal environment, leading to a tradeoff between N concentration and individual N uptake. This clearly reflected that there had been a decoupling of plant and soil N processes to grazing at the site scale, which contrasts with mesoscale and large-scale estimates (Li et al. 2016; Tang et al. 2018). Therefore, the prevailing paradigm of plant–soil N coupling needs to be reconsidered under intensive external stress at a small spatial scale. Another possible explanation for the increase in N resorption is that grazing promoted synthesis of nitrogenous secondary metabolites to cope with livestock forage. In this situation, high N resorption efficiency is a key mechanism by which plants reduce their dependence on soil nutrients (Lü et al. 2015; Luo et al. 2018). Plants can also increase the biosynthesis of unpalatable secondary metabolites that are rich in N-based molecules to protect themselves from repeated animal feeding, which is supported by theory on growth–defense tradeoffs in plant–herbivore interaction systems (Liu et al. 2019).

Similarly, ecological homeostasis of chemical elements in plant tissues may explain this pattern. Ecological homeostasis is the ability of plants to maintain relative stability of chemical elements, despite variation in the corresponding elemental content of soils (Ma et al. 2019). Together, these results suggest that plants can adjust their N uptake, resorption, and homeostasis through phenotypic plasticity and plant legacy effects resulting from long-term overgrazing.

# Conclusions

Extending our previous studies, we have shown that maternal grazing experience significantly suppressed the growth ability, individual N uptake, and N utilization efficiency of the clonal offspring of *L. chinensis* compared with the control group. In addition, the responses to N heterogeneity of the clonal offspring of previously grazed *L. chinensis* were significantly higher than those of ungrazed *L. chinensis*. This reflected that root nutrient foraging plasticity would increase to cope with grazing-induced N-heterogeneous habitats. Moreover, physiological and molecular evidence showed that plant legacy effects decreased enzymatic activity levels and expression of genes related to N absorbability in roots but also significantly increased N assimilation capacity to some extent, especially in increasing N resorption efficiency, exhibiting a form of activated biotic stress memory at the enzymatic and transcriptional levels. In short, multigenerational exposure of perennial plants to herbivore foraging can produce legacy effects on the nutrient uptake and utilization of their clonal offspring. This phenomenon may provide a better explanation of long-term overgrazing-induced plant legacy effects.

# Declarations

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## Authors' contributions

X.L. and W.R. conceived the ideas and designed methodology; X.L., N.H. and J.Y. collected the data; X.L. and W.R. analyzed the data; X.L. W.R. and E.F. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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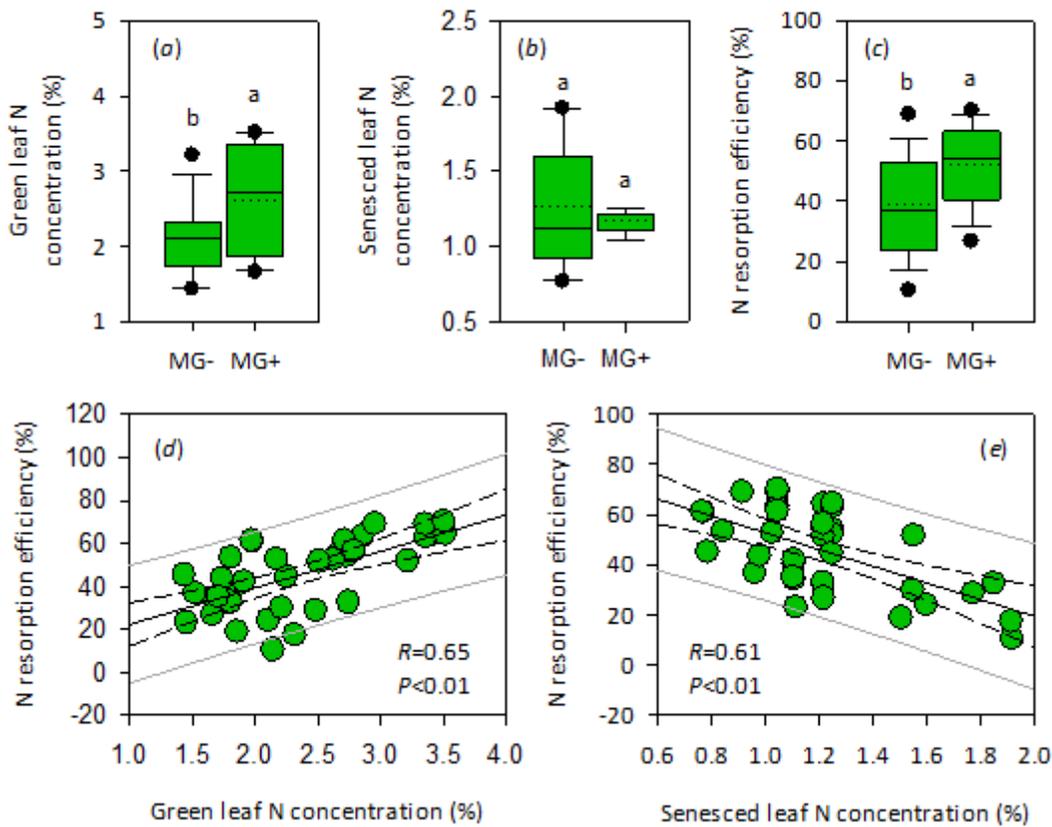
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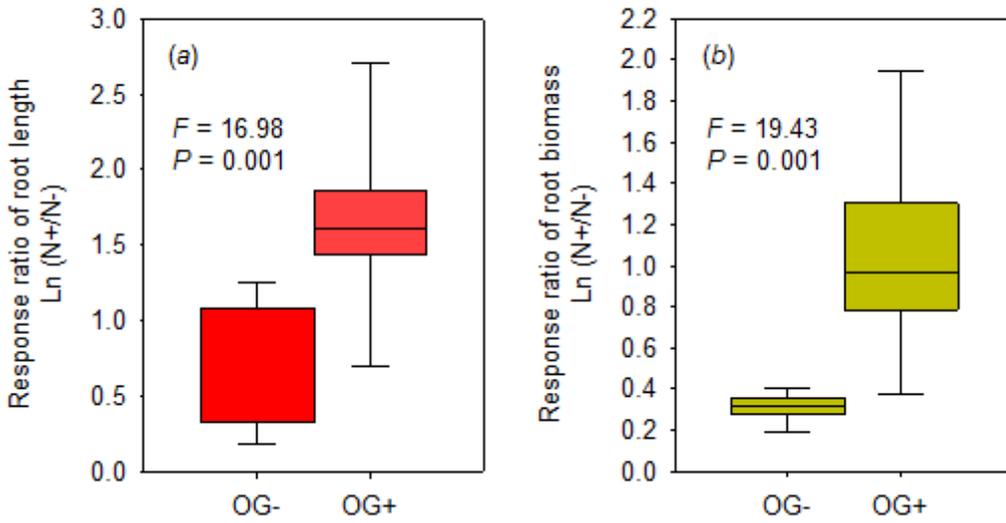
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## Figures



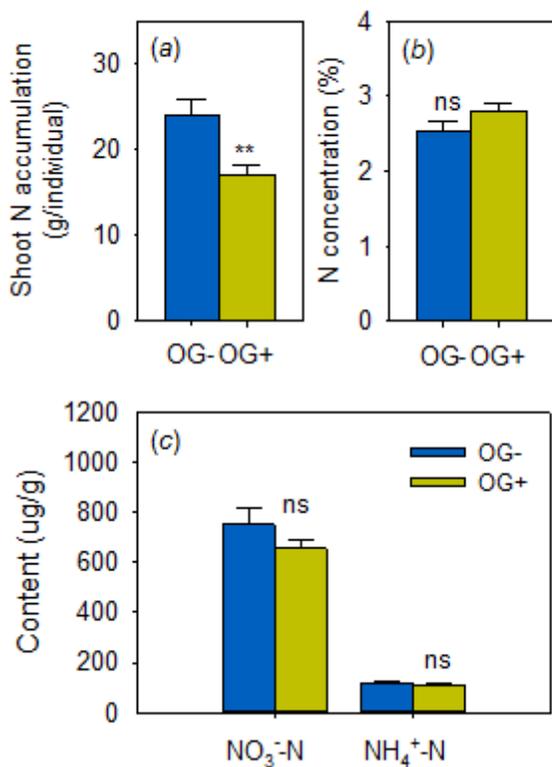
**Figure 1**

Effects of long-term overgrazing by large herbivores on leaf N resorption efficiency of *Leymus chinensis* at the peak of the growing season in field study. Boxplots show the changes in N concentrations in green (a) and senesced (b) leaves and in N resorption efficiency (c) of *L. chinensis* affected by grazing. Values with the same letter are not significantly different between the two treatments ( $P > 0.05$ ). The relationships of N resorption efficiency with N concentration in green (d) and senesced (e) leaves. In panels (d) and (e), dashed and gray lines indicate the 95% confidence bands and 95% prediction bands, respectively. Abbreviations: MG+, maternal plants from populations that had experienced herbivore grazing; MG-, maternal plants from populations that had not experienced grazing.



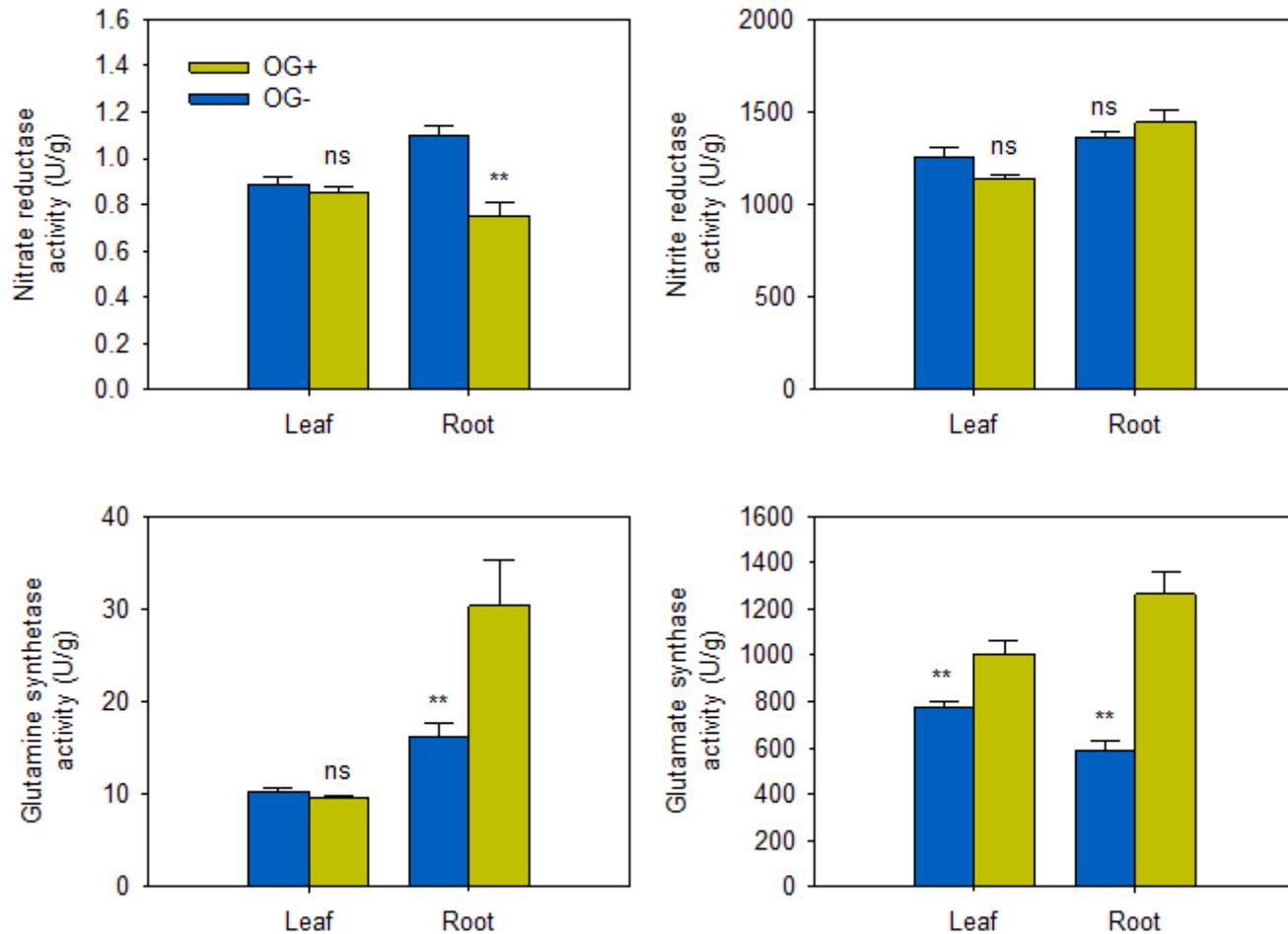
**Figure 2**

Effects of soil N heterogeneity on the response ratios of root length (a) and root biomass (b) of *Leymus chinensis* clonal offspring after long-term overgrazing in growth chamber study. Abbreviations: RR, response ratio; OG+, clonal offspring of plants that had experienced herbivore grazing; OG-, clonal offspring of plants that had not experienced grazing.



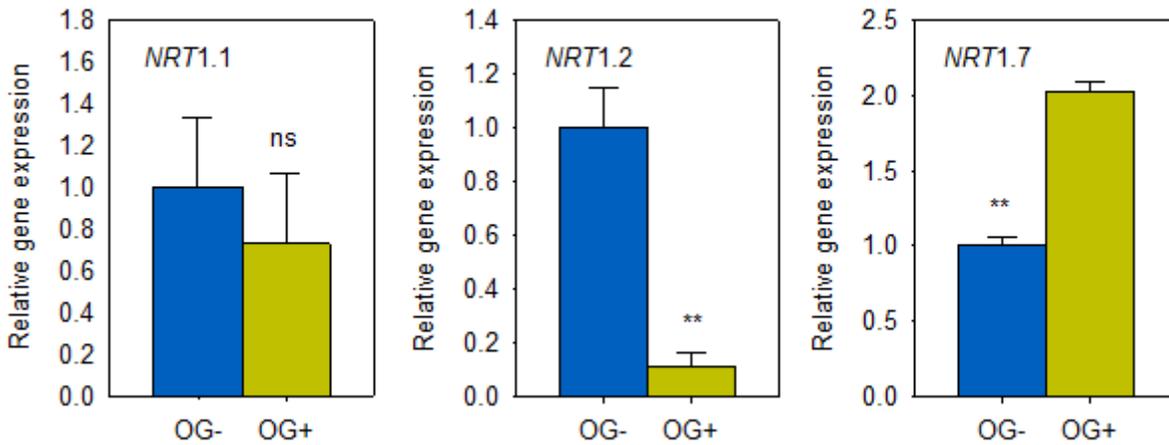
**Figure 3**

Plant legacy effects of long-term overgrazing on *Leymus chinensis* leaf N characteristics in growth chamber study. (a) individual N accumulation; (b) N concentration; (c) different N forms. Abbreviations: OG+, clonal offspring of plants that had experienced herbivore grazing; OG-, clonal offspring of plants that had not experienced grazing. Symbols: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ .



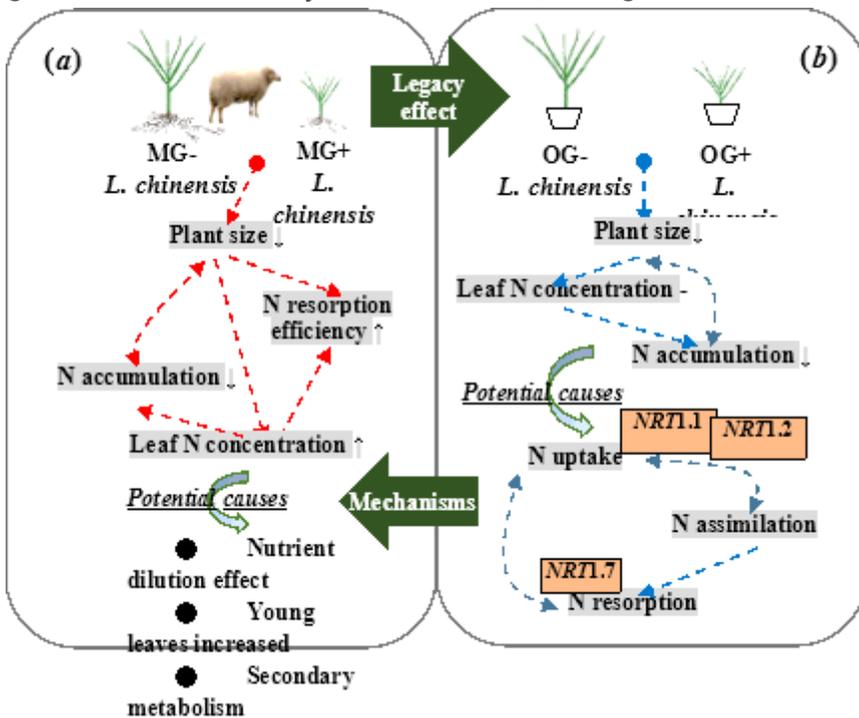
**Figure 4**

Plant legacy effects of long-term overgrazing on activity levels of *Leymus chinensis* enzymes related to nitrogenous metabolism in leaves in growth chamber study. Abbreviations: OG+, clonal offspring of plants that had experienced herbivore grazing; OG-, clonal offspring of plants that had not experienced grazing. Symbols: \*\*,  $P < 0.01$ ; ns,  $P > 0.05$ .



**Figure 5**

Relative expression levels of genes related to N metabolism from clonal offspring of *Leymus chinensis* that had and had not experienced herbivore grazing (OG+ and OG-, respectively) by bud cultivation in growth chamber study. \*\*,  $P < 0.01$ ; ns, no significant difference.



**Figure 6**

Schematic diagram illustrating the potential mechanisms underlying the linkages between nitrogen dynamics and plant phenotypic plasticity and plant legacy effect of *Leymus chinensis* after long-term overgrazing. (a) Field experiments; (b) greenhouse experiments. Here, the results of growth chamber study were continuations of the findings of field grazing experiment, suggesting assimilation rather than absorbability for nitrogen of *L. chinensis* clonal offspring are linked with grazing-induced plant legacy

effects. Abbreviations: ↑ up-regulation of a metabolic process; ↓ down-regulation of a metabolic process; - no change. MG+, maternal plants from populations that had experienced herbivore grazing; MG-, maternal plants from populations that had not experienced grazing; OG+, clonal offspring of plants that had experienced herbivore grazing; OG-, clonal offspring of plants that had not experienced grazing.

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