

## Nitrogen and water addition reduce leaf longevity of steppe species

Haiyan Ren<sup>1,2</sup>, Zhuwen Xu<sup>3</sup>, Jianhui Huang<sup>1,\*</sup>, Christopher Clark<sup>4</sup>, Shiping Chen<sup>1</sup> and Xingguo Han<sup>1</sup>

<sup>1</sup>State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Beijing 100093, China, <sup>2</sup>Graduate University of the Chinese Academy of Sciences, Yuquanlu, Beijing 100049, China, <sup>3</sup>Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, 110016, China and <sup>4</sup>School of Life Sciences and Global Institute of Sustainability, Arizona State University, Tempe, AZ 85287–4501, USA

\*For correspondence. E-mail [jhhuang@ibcas.ac.cn](mailto:jhhuang@ibcas.ac.cn)

Received: 11 June 2010 Returned for revision: 30 September 2010 Accepted: 4 October 2010 Published electronically: 17 November 2010

- **Background and aims** Changes in supplies of resources will modify plant functional traits. However, few experimental studies have addressed the effects of nitrogen and water variations, either singly or in combination, on functional traits.
- **Methods** A 2-year field experiment was conducted to test the effects of nitrogen and water addition on leaf longevity and other functional traits of the two dominant (*Agropyron cristatum* and *Stipa krylovii*) and three most common species (*Cleistogenes squarrosa*, *Melilotoides ruthenica* and *Potentilla tanacetifolia*) in a temperate steppe in northern China.
- **Key Results** Additional nitrogen and water increased leaf nitrogen content and net photosynthetic rate, and changed other measured functional traits. Leaf longevity decreased significantly with both nitrogen addition (–6 days in 2007 and –5.4 days in 2008; both  $P < 0.001$ ) and watering (–13 days in 2007 and –9.9 days in 2008; both  $P < 0.001$ ), and significant differences in leaf longevity were also found among species. Nitrogen and water interacted to affect leaf longevity and other functional traits. Soil water content explained approx. 70% of the shifts in leaf longevity. Biomass at both species and community level increased under water and nitrogen addition because of the increase in leaf biomass production per individual plant.
- **Conclusions** The results suggest that additional nitrogen and water supplies reduce plant leaf longevity. Soil water availability might play a fundamental role in determining leaf longevity and other leaf functional traits, and its effects can be modified by soil nitrogen availability in semi-arid areas. The different responses of species to resource alterations may cause different global change ramifications under future climate change scenarios.

**Key words:** *Agropyron cristatum*, *Cleistogenes squarrosa*, *Melilotoides ruthenica*, *Potentilla tanacetifolia*, *Stipa krylovii*, grassland, Inner Mongolia, leaf functional traits, leaf longevity, nitrogen, steppe, water.

### INTRODUCTION

Nitrogen (N) deposition and changes in precipitation are two important components of global change. It has been demonstrated that N supply is enhancing globally due to ever-increasing N deposition caused by increasing use of N fertilizers and combustion of fossil fuels (Vitousek *et al.*, 1997). Precipitation patterns are predicted to change at both the regional and the global scales (Dore, 2005; Cui *et al.*, 2009), which will have fundamental significance in arid or semi-arid areas where water supply is limited (Hooper and Johnson, 1999). Changes in N and water supplies in the context of global change can profoundly affect ecosystem processes and functioning.

Increasing N and water availability can change plant uptake of nutrients, and cause further changes in leaf longevity and other plant functional traits (Chapin *et al.*, 2002; Lambers *et al.*, 2008). Leaf longevity largely determines the duration of photosynthesis of an individual leaf (Chabot and Hicks, 1982), and thus plays an important role in linking ecophysiology of each individual leaf to the growth of the whole plant (Kikuzawa and Ackerly, 1999; Craine and Reich, 2001). Leaf longevity has been documented to correlate with other

leaf functional traits, such as photosynthetic rate, leaf N content and leaf mass per area (LMA) (Reich *et al.*, 1997, 1999; Cordell *et al.*, 2001; Shipley *et al.*, 2006; Abrahamson, 2007). It also reflects the strategies of plants in their adaptation to variable resource conditions and plant resource use (Chapin, 1980; Eckstein *et al.*, 1999). Alterations in resource use strategies of species indicated by changed leaf longevity could cause further shifts in community composition under changing resource availability. However, it remains unclear how variations in N and water supplies and their interaction affect leaf longevity and other leaf functional traits, and how different species respond to resource variations.

The determinant of the longevity of an individual leaf is largely considered to be the result of a balance between leaf construction and maintenance costs and carbon gain (Chabot and Hicks, 1982; Kikuzawa, 1995; Cordell *et al.*, 2001). Longer leaf longevity has been observed under conditions of N and water shortages (Sandquist and Ehleringer, 1998; Wright *et al.*, 2002). But most of these studies focused on meta-data analysis, and there are only a few manipulatively experimental studies on the effects of either adding N (Shaver, 1981, 1983; Craine and Reich, 2001) or drought treatment (Casper *et al.*, 2001) on leaf longevity and other plant

leaf functional traits, and especially fewer on the combined effects of the two factors.

The typical steppe of Inner Mongolia in northern China is the dominant vegetation type in semi-arid regions of Eurasia. It is also extremely sensitive to climate change (Christensen *et al.*, 2004; Kang *et al.*, 2007) because N and water are each limiting factors for plant growth in this area (Xia *et al.*, 2009; Xu *et al.*, 2010). As summer precipitation in northern China is predicted to increase in the future (Cholaw *et al.*, 2003) as is N deposition, understanding how increased supplies of N and water, and their combination, alter leaf longevity and other leaf functional traits in this extensive grassland ecosystem is urgently required. As increasing N availability may enhance leaf N content, stimulate photosynthetic activity (Field and Mooney, 1983; Pons and Anten, 2004) and then lead to rapid leaf turnover, we hypothesize that leaf longevity will decline with increasing N supply. As water addition may also promote leaf metabolic activity (Lajtha and Whitford, 1989; Patrick *et al.*, 2007) and hence accelerate leaf ageing, we also hypothesize that leaf longevity will be reduced under increased water supply.

## MATERIALS AND METHODS

### Study site and experimental design

This study was carried out in a typical steppe ecosystem in Duolun county (116°17'E, 42°02'N, elevation 1324 m a.s.l.), Inner Mongolia, China. It has a temperate continental monsoon climate with cold winters and warm summers (mean January and July temperatures of  $-17.5$  and  $18.9$  °C, respectively). Mean annual temperature is  $2.1$  °C and mean annual precipitation is around 380 mm. More than 80 % of the precipitation occurs between June and September. Chestnut soil is a predominant type in this area with mean soil bulk density of  $1.21$  g cm<sup>-3</sup> and pH of 7.29. The plant community at the study site is dominated by two grasses (*Stipa krylovii* and *Agropyron cristatum*) and one forb species (*Artemisia frigida*).

The experiment used a randomized block design, with five replicate blocks, each containing four  $8 \times 8$ -m<sup>2</sup> plots. Within each block, each of the four plots was randomly assigned to one of the following four treatments: control, N addition, water addition, and combined N and water addition. The plots with N addition received  $10$  g N m<sup>-2</sup> yr<sup>-1</sup> in the form of urea applied twice, half in early May and the other half in late June. From June to August, the watered plots received 15 mm of precipitation weekly by sprinkling irrigation. A total 180 mm of precipitation was added in the growing season (12 weeks of  $15$  mm week<sup>-1</sup>), which was approx. 50 % of mean annual precipitation in this study site.

Five perennial herbs (*A. cristatum*, *S. krylovii*, *Cleistogenes squarrosa*, *Melilotoides ruthenica* and *Potentilla tanacetifolia*), which occurred in each plot, were selected to determine the responses of leaf longevity and other leaf functional traits to N and water addition. The two co-dominant species, *S. krylovii* and *A. cristatum*, are C<sub>3</sub> grasses that are widespread in the studied ecosystem. *C. squarrosa* is the exclusive C<sub>4</sub> perennial grass in the research area. *M. ruthenica* is a typical C<sub>3</sub> legume, and *P. tanacetifolia* is a common perennial forb in the study site.

### Measurements of soil water content, inorganic N and total N contents

In 2007 and 2008, soil water content (SWC, fresh weight basis) was determined by taking a 10-cm-deep soil core randomly from each plot every week from late June to late September and then dried for 48 h at 105 °C. Soil samples were collected by mixing three 3-cm-diameter cores from 10-cm-deep soil randomly taken in each plot at the peak of growing season for measuring total N (Kjtek™ 2300, Hillerød, Denmark) and inorganic N (FIAstar 5000 Analyzer, Foss Tecator, Denmark).

Added water significantly raised SWC by 39.8 % ( $P < 0.001$ ) while N addition weakly enhanced SWC by 2.3 % ( $P > 0.05$ ) across the two study years. Soil inorganic N content (SIN) increased by 85 % in N added plots compared with no N added plots ( $P < 0.001$ ), but decreased by 19.4 % in water-added plots compared with no water added plots ( $P < 0.05$ ). Soil total N content (STN) showed no notable responses to N and water addition.

### Measurements of leaf longevity

We employed the census protocol and calculations of Craine *et al.* (1999) and Craine and Reich (2001) with small modifications to determine the average leaf longevity of the five steppe species. Three randomly chosen individuals per species in each plot were monitored. Three approximately fully expanded leaves per individual plant were marked by tying a small tag to the leaf base. Thus, 180 leaves per plant species was monitored each year. Censuses of the tagged leaves were made every 10 d from late June to mid-October in each year. When a leaf was judged to be fully expanded, the time was defined as its birth date. When a leaf or at least two-thirds of its area turned yellow or brown, it was considered senesced.

At each census date, for three grasses (*C. squarrosa*, *A. cristatum* and *S. krylovii*), length of total leaf and the senesced portion of the leaf were measured. For two forbs (*M. ruthenica* and *P. tanacetifolia*), the maximum width, and total and senesced length of the compound leaf were recorded and leaf area was predicted by establishing regression equations. For *M. ruthenica*, the equation was: Leaf area =  $0.17 + 0.38$  Length  $\times$  Width ( $r^2 = 0.82$  in 2007,  $P < 0.001$ ); Leaf area =  $0.06 + 0.52$  Length  $\times$  Width ( $r^2 = 0.95$  in 2008,  $P < 0.001$ ). For *P. tanacetifolia*, the equation was: Leaf area =  $1.29 + 0.46$  Length  $\times$  Width ( $r^2 = 0.93$  in 2007,  $P < 0.001$ ); Leaf area =  $0.68 + 0.41$  Length  $\times$  Width ( $r^2 = 0.94$  in 2008,  $P < 0.001$ ).

The average longevity of the leaves was estimated from division of the leaf-days of all the leaves sampled in a plot by the length or area of these leaves that were born and senesced over the census period using the following model:

$$\text{Leaf longevity} = (A - B - C - D)/(S_j - L_i) \text{ or } (A - B - C - D)/(S_j - A_i)$$

$$\left\{ \sum_{n=i}^j \left( \frac{L_n + L_{n+1}}{2} \right) K_n \right\} \text{ or } \left\{ \sum_{n=i}^j \left( \frac{A_n + A_{n+1}}{2} \right) K_n \right\} \quad (1a)$$

$$\sum_{n=i}^j \left( \frac{S_n + S_{n+1}}{2} \right) K_n \quad (1b)$$

$$\left\{ (n_s - n_i) L_i - \left[ \sum_{n=i}^{n_s} \left( \frac{S_n + S_{n+1}}{2} \right) K_n \right] \right\} \text{ or} \\ \left\{ (n_s - n_i) A_i - \left[ \sum_{n=i}^{n_s} \left( \frac{S_n + S_{n+1}}{2} \right) K_n \right] \right\} \quad (1c)$$

$$\left\{ \left[ \sum_{n=n_l}^j \left( \frac{L_n + L_{n+1}}{2} \right) K_n \right] - [(n_j - n_l) S_j] \right\} \text{ or} \\ \left\{ \left[ \sum_{n=n_l}^j \left( \frac{A_n + A_{n+1}}{2} \right) K_n \right] - [(n_j - n_l) S_j] \right\} \quad (1d)$$

where  $i$  is the starting census date;  $j$  is the ending census date;  $L_n$  and  $A_n$  are cumulative length or area of leaves produced by the census date  $n$ , and  $S_n$  is the corresponding cumulative length or area of leaves senesced by the census date;  $K_n$  is the time interval between census  $n$  and  $n + 1$ ;  $n_s$  is the date at which the amount of leaf length or leaf area senesced is equivalent to the amount of leaf length or leaf area at the first census ( $S = L_i$ , or  $S = A_i$ ); and  $n_l$  is the date at which the cumulative leaf length or leaf area produced is equal to the amount of leaf length or leaf area senesced by the last census date ( $L = S_j$ , or  $A = S_j$ );  $n_l$  and  $n_s$  were calculated by linear interpolation.

Equations (1a) and (1b) represent the cumulative amount of leaf-days for both green and senesced leaves and for senesced leaves, respectively, and their difference is the total un-senesced leaf-days over the entire census period. Equation (1c) represents the amount of leaf-days for leaves present at the beginning of the census period, and eqn (1d) represents the leaf-days of leaves that had not senesced by the end of the census period.

#### Measurements of leaf functional traits

Leaf net photosynthetic rate based on area ( $P_{\text{net}}$ ) and stomatal conductance ( $g_s$ ) were measured every 2 weeks from early July to late September, by using a Li-Cor 6400 photosynthesis system (Li-Cor, Lincoln, NE, USA) at a saturated photosynthetic photon flux density of  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  using an LED light source, for the two dominant species (*S. krylovii* and *A. cristatum*) in this study site. The SPAD value, which indicates leaf chlorophyll concentration, was determined with a portable SPAD-502 meter (Minolta, Tokyo, Japan).

Area, weight and nutrient contents of leaves were measured biweekly for the five species during the period from mid-June to mid-October. For each species at each sampling time, 10–60 representative leaves were harvested from at least three individual plants in each plot and then kept in a cooler. After immediate measurements of leaf fresh weight and leaf area in the laboratory, leaves were dried at  $65^\circ\text{C}$  for

48 h and then weighed to determine leaf dry mass. The dried leaf samples were used to determine total N (LNC), phosphorus (LPC) and carbon concentrations (C) with the Auto-Kjeldahl method (Kjektec System 1026 distilling unit, Sweden), molybdenum stibium anti-spectrophotometry and  $\text{K}_2\text{CrO}_7\text{-H}_2\text{SO}_4$  oxidation, respectively. Leaf mass per area was determined as the leaf dry mass per unit area. Dry matter content (DMC) was the ratio of dry mass to fresh mass. Nitrogen use efficiency (NUE) was estimated from the inverse of N content in dead leaves.

Leaf samples collected at the peak of the growing season were used to analyse carbon isotopic composition ( $\delta^{13}\text{C}$ ), via a Thermo Finnigan MAT Delta<sup>plus</sup> XP isotope ratio mass spectrometer coupled to a Flash elemental analyser (Thermo Finnigan, Bremen, Germany).

#### Measurements of biomass at leaf, species and community levels

In late August 2007 and 2008, above-ground living tissues were harvested by species in a  $0.15 \times 2\text{-m}$  randomly sampled quadrat in each plot. Plant tissues of each species were weighed after drying to constant weight to determine the species biomass and community above-ground net primary productivity (ANPP). Leaf biomass per individual was also measured by dividing leaves from each individual so as to determine leaf biomass production in 2008. We employed the root in-growth core method to measure below-ground net primary productivity (BNPP) (Xu and Wan, 2008).

#### Data analysis

Data for SWC and leaf functional traits except leaf longevity for statistical analysis were mean values taken across all the measurement dates. Multiple-way ANOVA was performed to test the effects of year, species, N and water addition on leaf longevity, other leaf functional traits and soil attributes. Pearson's correlation coefficient was used to investigate correlations between variables. Stepwise multiple regression analysis (forward) was conducted to examine the contribution of leaf functional traits and environmental factors to the variation of leaf longevity. Standardized major axis (SMA) was run to test the differences in SMA slopes and intercepts among groups (Falster *et al.*, 2006). The SMA relationships between leaf longevity and net photosynthetic rate based on mass ( $P_{\text{net-mass}}$ ), LNC and LMA in the present study were analysed in comparison with the Cedar Creek data set (Tjoelker *et al.*, 2005) and Glopnet data set (Wright *et al.*, 2004). Furthermore, the species in this study were categorized into four plant functional groups, i.e. C<sub>3</sub> grass, C<sub>4</sub> grass, forb and legume, to compare with grasses or sedges and herbs chosen from the Glopnet data set and the corresponding plant functional groups of the Cedar Creek data set, respectively. All statistical analyses were performed using SAS software (version 8.2).

## RESULTS

#### Patterns of leaf longevity with varying N and water availabilities

Nitrogen addition decreased leaf longevity by 6 d in 2007 and by 5.4 d in 2008 across all the studied species ( $P < 0.001$ ,

Table 1). Water addition also reduced leaf longevity by 13.0 d in 2007 and by 9.9 d in 2008 ( $P < 0.001$ , Table 1). There was a significant interaction between species and N in 2008 on leaf longevity, with the highest decrease in *M. ruthenica* (17.7%), followed by *A. cristatum* (14.4%), *C. squarrosa* (10.4%), *S. krylovii* (7.6%) and the lowest decrease in *P. tanacetifolia* (only 1.8%) (Table 1, Fig. 1A–E). Interaction between species and water was also prominent in both 2007 and 2008 with *S. krylovii* exhibiting the highest reduction (37.4 and 30.0%, respectively), *C. squarrosa* the lowest reduction (14.1 and 8.2%), and *A. cristatum*, *M. ruthenica* and *P. tanacetifolia* moderate reductions (24.9 and 13.3%, 21.4 and 19.1%, and 16.6 and 16.5%, respectively) in leaf longevity (Table 1, Fig. 1A–E). Significant interactive effects of N and water addition on leaf longevity were also found in 2008 ( $P < 0.001$ , Table 1). Compared with the ambient N control, N addition decreased leaf longevity by 13.3% in the unwatered plots but only by 5.6% in the watered plots across all the studied species in 2008 (Fig. 1A–E). Water addition caused a 20.3% decrease in leaf longevity without N addition but a 13.8% reduction with N addition in 2008 (Fig. 1A–E).

#### Responses of leaf photosynthetic parameters, LNC, DMC, C/N ratio, $\delta^{13}C$ value to N and water addition

Marginally significant differences were found between the two dominant species, *S. krylovii* and *A. cristatum*, for  $P_{net}$  and  $g_s$  across the two sampling years ( $P < 0.10$ , Table 2). Nitrogen addition significantly increased  $P_{net}$  by 8.4% ( $P < 0.05$ , Table 2), but had no significant effects on  $g_s$ . Water addition enhanced  $P_{net}$  and  $g_s$  by 51.5 and 69.5%, respectively ( $P < 0.001$ , Table 2). The interactive effects of N with water addition were also significant on  $P_{net}$  ( $P < 0.05$ ), but not significant on  $g_s$ .  $P_{net}$  and  $g_s$  decreased, respectively, by 119.2 and 161.4% in 2008 than in 2007.

Nitrogen and water addition increased SPAD values by 3.7 and 9.8%, respectively, and significant interactions of N with water addition on SPAD were observed ( $P < 0.001$ , Table 2). SPAD in 2008 was 5.9% higher than in 2007 ( $P < 0.001$ ).

Leaf N content showed marked increases of 22.3 and 19.0%, respectively, in response to N and water addition,

and responded significantly to the interaction between N and water addition ( $P < 0.001$ , Table 2, Fig. 1F–J). In addition, leaf N content was 14.1% higher in 2008 than in 2007 ( $P < 0.001$ ).

Nitrogen addition and supplemental water decreased DMC by 4.9 and 6.9%, respectively, and their interaction had a marked effect on DMC ( $P < 0.001$ , Table 2). DMC was 18.6% lower in 2008 than in 2007 ( $P < 0.001$ ).

C/N ratio decreased with N and water addition by 20.7 and 21.0%, respectively, and there was a notable response to the interaction of N with water addition ( $P < 0.001$ , Table 2). C/N ratio in 2008 was 20.5% lower than in 2007 ( $P < 0.001$ ).

Leaf  $\delta^{13}C$  value was lower in plots with N addition than in those without N addition ( $P < 0.10$ ), and was significantly lower in watered plots than in non-watered plots ( $P < 0.01$ ) (Table 2). Interactive effects of N and water addition on  $\delta^{13}C$  were not significant. Leaf  $\delta^{13}C$  was 2.3% lower in 2008 than in 2007 ( $P < 0.001$ ).

There were strong species differences for the above-mentioned functional traits ( $P < 0.001$ , Table 2). Leaf N content and SPAD were higher but leaf DMC, C/N ratio and  $\delta^{13}C$  were lower for *M. ruthenica* and *P. tanacetifolia* than for *S. krylovii*, *A. cristatum* and *C. squarrosa*.

#### Effects of N and water addition on biomass at the leaf, species and community levels

Nitrogen addition and water addition enhanced leaf biomass production by 60.6 and 76.5%, respectively (both  $P < 0.001$ , Table 1). Interaction between species and N was also significant in 2008 on leaf biomass production ( $P < 0.01$ , Table 1), with *P. tanacetifolia* showing the highest increase (164.9%), followed by *S. krylovii* (99.3%), *A. cristatum* (80.0%) and *C. squarrosa* (62.2%); *M. ruthenica* showed the lowest increase (4.2%). There was a marginally significant interaction between species and water in 2008 on leaf biomass production ( $P < 0.10$ , Table 1).

As the dominant species of the community, the above-ground biomass of *S. krylovii* and *A. cristatum* increased by 171.9 and 56.5%, respectively, with N addition and by 66.7 and 61.4%, respectively, with watering over the two years ( $P < 0.001$ , Fig. 2A, B). Water addition notably increased the above-ground biomass of *C. squarrosa* and *P. tanacetifolia* ( $P < 0.05$ , Fig. 2C, D), whereas N addition showed no such significant effects ( $P > 0.10$ , Fig. 2C, D). For *M. ruthenica*, N addition decreased its biomass by 42.2% ( $P < 0.05$ ), while supplemental water enhanced its biomass by 199.9% in 2007 ( $P < 0.05$ ), but had no significant effects in 2008 ( $P > 0.10$ ) (Fig. 2E). The above-ground biomass of *S. krylovii*, *A. cristatum* and *M. ruthenica* was significantly higher in 2007 than in 2008 ( $P < 0.05$ , Fig. 2A, B, E), while insignificant interannual differences existed for *C. squarrosa* and *P. tanacetifolia* ( $P > 0.10$ , Fig. 2C, D).

Additional N and water supplies notably increased community ANPP by 27.8 and 42.2% ( $P < 0.001$ , Fig. 3A), respectively, over the two years. BNPP was 36.9% higher under watering than the control in natural and increased N plots, but elevated N significantly decreased BNPP by 14.9% across the natural and increased water plots in 2007 (both  $P < 0.01$ , Fig. 3B). Neither N nor water addition, however,

TABLE 1. Results (F values) of three-way ANOVA of responses of leaf longevity and biomass production to N and water addition

	Leaf longevity		Leaf biomass production 2008
	2007	2008	
Species (Sp)	70.5***	173.65***	43.63***
Water (W)	128.93***	263.21***	55.69***
Nitrogen (N)	27.11***	77.22***	39.32***
W × N	0.33	16.87***	0.21
Sp × W	6.13***	14.73***	2.32
Sp × N	1.31	6.38***	5.18**
Sp × W × N	0.66	1.31	3.45*

Asterisks indicate statistically significant differences at \* $P < 0.05$ , \*\* $0.01$  and \*\*\* $0.001$ .

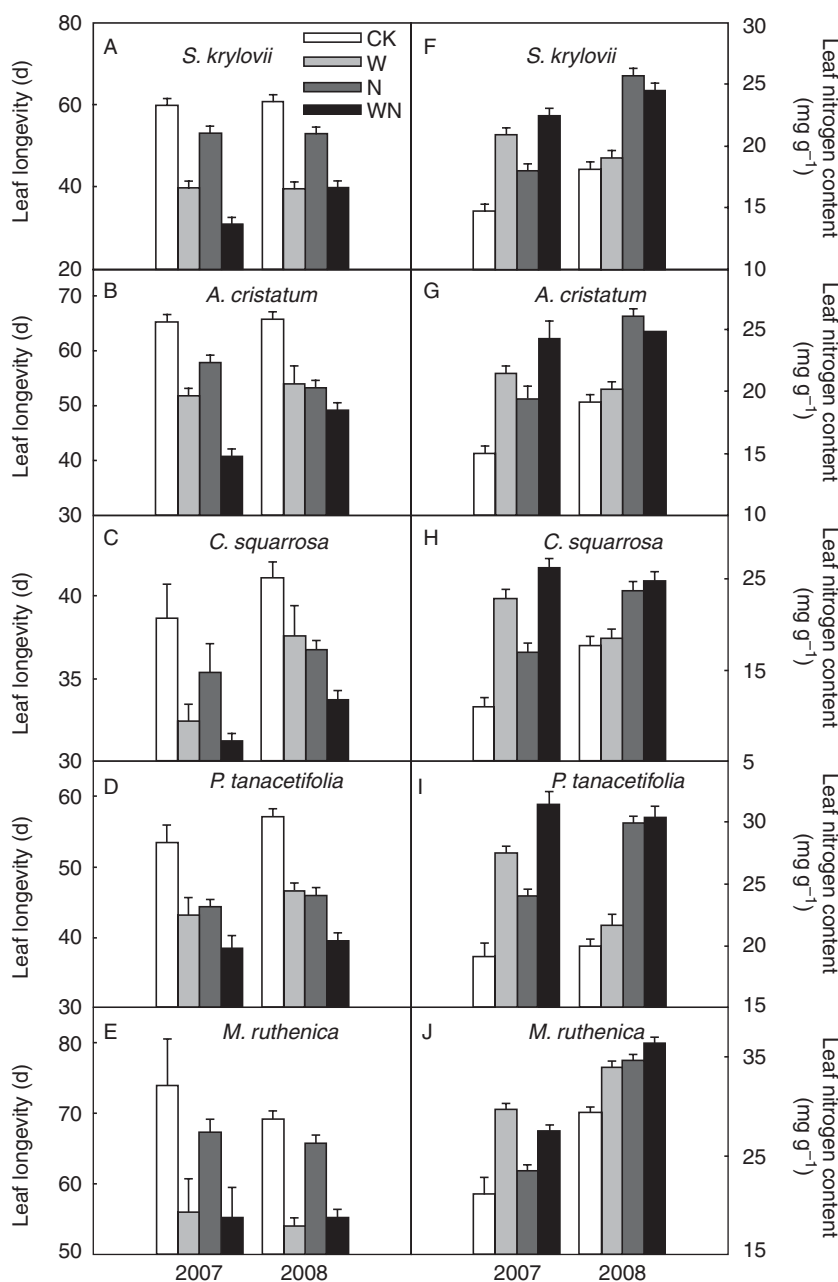


FIG. 1. Effects of N and water addition on longevity and N content in leaves for the five steppe species. CK, control; W, water addition; N, N addition; WN, combined addition of N and water.

resulted in marked changes in BNPP in 2008 (Fig. 3B). Nevertheless, ANPP was significantly higher in 2008 than in 2007, whereas BNPP was lower in 2008 than in 2007 (both  $P < 0.001$ , Fig. 3).

#### Relationships between leaf longevity and environmental factors

For all the species studied, SWC exhibited a close negative correlation with leaf longevity in 2008 ( $P < 0.10$  for *C. squarrosa*,  $P < 0.01$  for the other species) (Table 3). There was no significant relationship between STN and leaf longevity ( $P > 0.10$ ), except a weak negative correlation for

*M. ruthenica* in 2007 ( $P < 0.10$ ) (Table 3). The negative relationship between SIN and leaf longevity was significant for *A. cristatum* in 2008 ( $P < 0.05$ ), and marginally significant for *P. tanacetifolia* ( $P < 0.10$ ) (Table 3).

Stepwise multiple regression analysis indicated that SWC explained 77.4 and 63.4% of the shifts in leaf longevity (LL) in 2007 ( $LL = -3.55SWC + 77.84$ ) and 2008 ( $LL = -3.05SWC - 0.12SIN + 85.84$ ), respectively, across all the studied species (both  $P < 0.001$ ,  $n = 20$ ). In addition, SIN accounted for 7.9% of the variation in leaf longevity in 2008 ( $P < 0.05$ ). Soil total N content was excluded from the regression model.

TABLE 2. ANOVA results (F values) of leaf functional traits in response to N and water addition

	$P_{\text{net}}$	$g_s$	SPAD	LNC	DMC	C/N ratio	$\delta^{13}\text{C}$
Year (Yr)	450.27****	125.14****	93.25****	173.32****	1942.24****	124.36****	27.56****
Species (Sp)	2.27*	3.94*	1307.72****	238.24****	822.01****	49.72****	2050****
Water (W)	135.21****	41.94****	248.82****	300.15****	234****	131.22****	6.37***
Nitrogen (N)	5.27**	1.14	37.54****	400.6****	114.96****	126.55****	2.63*
W × N	6.17**	1.66	39.21****	17.41****	29.34****	26.14****	0.03
Yr × N	1.41	1.24	0.47	50.77****	11.26****	0.44	0.86
Yr × W	10.15***	1.24	43.77****	176.07****	48.64****	99.15****	0.34
Yr × Sp	4.43**	5.24**	7.59****	34.2****	25.64****	8.91****	1.52
Sp × W	0.05	1.54	4.08****	6.54****	8.11****	1.4	1.24
Sp × N	0	0.04	1.57	11.33****	4.73***	5.24****	1.5
Sp × W × N	0.53	0	2.87**	0.96	0.87	0.46	1.09
Yr × Sp × W × N	0.50	1.03	1.53*	2.36***	1.43	3.23****	0.77

$P_{\text{net}}$ , net photosynthetic rate based on area;  $g_s$ , stomatal conductance; SPAD, estimated leaf chlorophyll concentration; LNC, leaf nitrogen content; DMC, leaf dry matter content; C/N ratio, ratio of leaf carbon to nitrogen contents;  $\delta^{13}\text{C}$ , leaf carbon isotopic composition.

Asterisks indicate statistically significant differences at \* $P < 0.10$ , \*\* $0.05$ , \*\*\* $0.01$ , and \*\*\*\* $0.001$ .

#### Relationships between leaf longevity and other leaf functional traits

Leaf longevity showed significantly negative correlations with  $P_{\text{net}}$  and  $g_s$  for *S. krylovii* over the two years ( $P < 0.01$ , Table 3). For *A. cristatum*, leaf longevity was significantly negatively correlated with  $P_{\text{net}}$  ( $P < 0.001$ ) and  $g_s$  ( $P < 0.05$ ) in 2007, and marginally significantly correlated with  $P_{\text{net}}$  ( $P < 0.10$ ) but insignificantly with  $g_s$  ( $P > 0.10$ ) in 2008 (Table 3). Negative correlations between leaf longevity and SPAD values were marginally significant at least for all the species over the two years ( $P < 0.10$  for *S. krylovii* and *C. squarrosa* in 2008;  $P < 0.05$  for the other species, Table 3). There were markedly negative relationships between LNC and leaf longevity for the studied species ( $P < 0.01$ ) except *S. krylovii* in 2008 ( $P > 0.10$ ) (Table 3). The negative correlations between LNC and leaf longevity were significant for *S. krylovii* in 2007 and *P. tanacetifolia* in 2008 (both  $P < 0.05$ , Table 3), and marginally significant for *C. squarrosa* and *M. ruthenica* in 2008 (both  $P < 0.10$ , Table 3). Leaf C/N ratio and DMC were both highly positively associated with leaf longevity for all the studied species ( $P < 0.05$ ) except for *S. krylovii* in 2008 ( $P > 0.10$ ) (Table 3). Positive correlations of leaf longevity with leaf mass per area were found (for *M. ruthenica*,  $P < 0.10$  in 2007; for the other species,  $P < 0.05$ ), although the effects were not statistically significant for *C. squarrosa* in 2007 ( $P > 0.10$ ) or for *S. krylovii* in 2008 ( $P > 0.10$ ) (Table 3). There were marginally significant positive correlations between  $\delta^{13}\text{C}$  value and leaf longevity for *C. squarrosa* and *M. ruthenica* in 2007 ( $P < 0.10$ ), and significantly positive correlations for *S. krylovii* in 2007 ( $P < 0.05$ ), *P. tanacetifolia* in 2008 ( $P < 0.05$ ) and *M. ruthenica* in 2008 ( $P < 0.01$ ) (Table 3). NUE was positively correlated with leaf longevity for the studied species ( $P < 0.10$ ) except for *S. krylovii* and *C. squarrosa* in 2008 ( $P > 0.10$ ) (Table 3).

Stepwise multiple regression analysis revealed that leaf photosynthetic capacity including  $P_{\text{net}}$ ,  $g_s$  and SPAD, and LNC could explain between 17.1 and 94.3 % of the variances in leaf longevity for the different species (all  $P < 0.01$ , Appendix). Leaf C/N ratio, DMC and  $\delta^{13}\text{C}$  value were also strongly positively associated with leaf longevity (all

$P < 0.01$ , Appendix). Other leaf functional traits were rejected from the regression equation.

## DISCUSSION

The results of the present study indicate that additional N and water supply can, either singly or in combination, reduce the leaf longevity of all the studied perennials in the temperate typical steppe ecosystem, although significant differences exist among the different plant species.

Leaf longevity of all the five herbaceous perennials declined significantly with addition of N, in agreement with the results of previous studies which showed that leaf longevity decreased with N fertilization (Shaver, 1983; Cordell *et al.*, 2001; Craine and Reich, 2001). We suggested that additional N supply resulted in high LNC, high chlorophyll content and consequently high  $P_{\text{net}}$  (Table 2), and further led to high metabolic activity and leaf turnover, and therefore shortened leaf longevity. Such negative relationships between photosynthetic rates and leaf longevity have also been reported previously (Mooney and Gulmon, 1982; Reich *et al.*, 1992; Wright *et al.*, 2004; Escudero *et al.*, 2008).

Increasing water supply also shortened leaf longevity. This was in agreement with results of a previous study which showed that plants that lived in sites where water was abundant had shorter leaf longevity as photosynthetic rates can be high at such sites (Kikuzawa, 1995). Results from regression analysis indicated that SWC could explain a large proportion of this variation in leaf longevity. We thus consider that water addition can trigger an increase of LNC (Table 2, Fig 1F–J) as high water availability can enhance both the transformation from organic to inorganic N and the mobility of inorganic N in soils. Thus, increased LNC as a result of water addition could promote plant photosynthetic activity and leaf senescence, and accordingly reduce leaf longevity. However, we also noted that the higher natural precipitation in 2008 (357 mm, close to the long-term mean) than in 2007 (208 mm, 46.7 % lower than the mean) did not change leaf longevity in most studied species, although the manipulative water addition did shorten leaf longevity of all studied species (Fig. 1A–

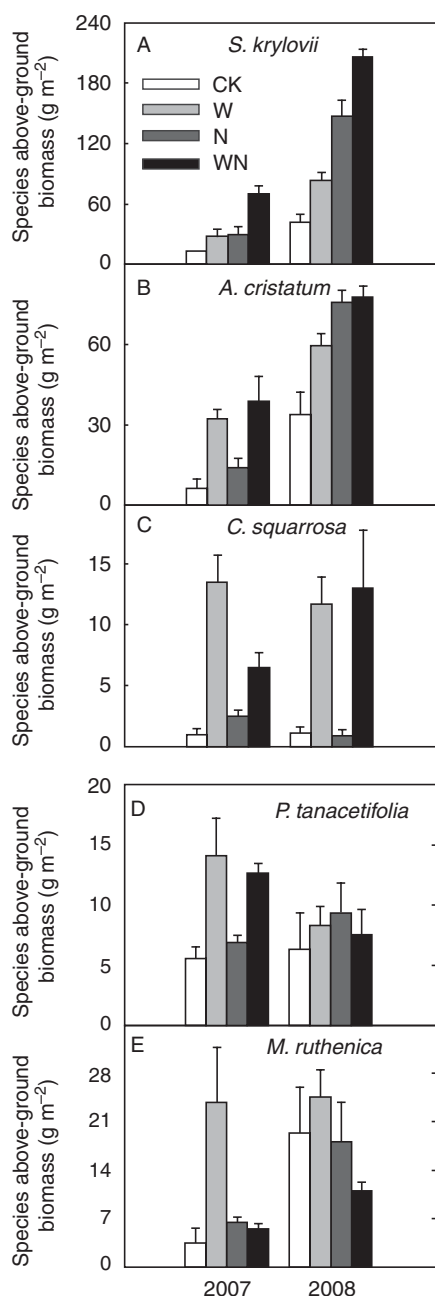


FIG. 2. Impacts of N and water addition on species above-ground biomass. CK, control; W, water addition; N, N addition; WN, combined addition of N and water.

E). We speculate that artificial increases of water supply in the growing season may exert stronger effects on leaf longevity than any natural increase in precipitation, which is distributed throughout the year and may not be efficient for plant use especially in the non-growing season.

Few studies have addressed the interactive effects of N and water addition on leaf longevity. In the present study, a significant interaction between N and water addition was found in 2008, but not in 2007. This interannual difference in interactive effects of the two factors could mainly be

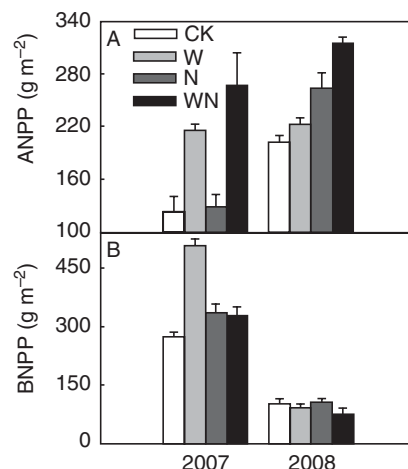


FIG. 3. Responses of above-ground net primary productivity (ANPP) and below-ground net primary productivity (BNPP) at community level to N and water addition. CK, control; W, water addition; N, N addition; WN, combined addition of N and water.

attributed to the remarkable interannual variation in natural precipitation in the study area. Leaf N content significantly increased with additional water supply in 2007 but changed little in 2008 except for the legume *M. ruthenica* (Fig. 1F–J). As a consequence, the effect of N on leaf longevity might be influenced significantly by water availability in this semi-arid typical steppe.

Most previous studies reported close relationships between leaf longevity and other leaf functional traits (Reich *et al.*, 1997, 1999; Cordell *et al.*, 2001; Shipley *et al.*, 2006; Abrahamson, 2007). Leaf  $\delta^{13}\text{C}$  may reflect the water use efficiency and metabolism of plants (Livingston *et al.*, 1999). C/N ratio can indicate total carbon fixed per unit N and carbon use efficiency (Chapin and Van Cleve, 1989; Patterson *et al.*, 1997). Dry matter content also implies a trade-off between rapid biomass production and efficient nutrient conservation (Poorter and De Jong, 1999; Garnier *et al.*, 2001; Saura-Mas and Lloret, 2007). There are close inherent relationships among these leaf functional traits. Shorter leaf longevity under increased N and water supplies could imply lower use efficiency of both N and water by plants. Different strategies of resource use by different species under changing resource conditions may reflect how a community might respond to variable resource supplies, and will thus contribute greatly to ecosystem stability in both structure and function. Furthermore, the different responses of species to alterations in water and N availability may have different global change ramifications as different areas are dominated by different species and global change drivers.

Placing our results into a quantitative context with regard to previous studies, we found that mean values of leaf longevity in this study (48.71 d) were close to the results for Cedar Creek (63.36 d,  $P > 0.05$ ) but different from those for Glopnet (133.39 d,  $P < 0.05$ ). This indicated that higher discrepancies might occur among different biomes as the Glopnet data set included more species from a variety of ecosystems while the Cedar Creek data set included species only from grassland,

TABLE 3. Correlations between leaf longevity and environmental factors and other leaf functional traits for the five steppe species

Species	Environmental factors					Other leaf functional traits							
	SWC	SIN	STN	LNC	LPC	C/N	DMC	LMA	$\delta^{13}\text{C}$	NUE	SPAD	$P_{\text{net}}$	$g_s$
2007													
<i>S. krylovii</i>	-0.85****	0.09	-0.33	-0.89****	-0.57**	0.86****	0.89****	0.65**	0.60**	0.75***	-0.69****	-0.90****	-0.78***
<i>A. cristatum</i>	-0.86****	-0.02	-0.17	-0.88****	-0.3	0.85****	0.84****	0.65**	0.07	0.77***	-0.82****	-0.84****	-0.64**
<i>C. squarrosa</i>	-0.57****	0.13	0.04	-0.89****	-0.28	0.82****	0.82****	0.35	0.44*	0.76***	-0.88****	-	-
<i>P. tanacetifolia</i>	-0.69****	-0.17	-0.23	-0.83****	-0.03	0.74****	0.75****	0.75****	0.27	0.71***	-0.84****	-	-
<i>M. ruthenica</i>	-0.62****	0.15	-0.39*	-0.88****	0.14	0.84****	0.72****	0.55*	0.52*	0.71***	-0.91****	-	-
2008													
<i>S. krylovii</i>	-0.76****	-0.01	0.03	-0.06	-0.21	0.16	0.44	-0.13	0.43	0.1	-0.46*	-0.83****	-0.75***
<i>A. cristatum</i>	-0.57****	-0.44**	-0.16	-0.82****	0.07	0.85****	0.81**	0.78****	-0.1	0.69***	-0.61**	-0.48*	-0.25
<i>C. squarrosa</i>	-0.42*	-0.26	0.25	-0.92****	0.47*	0.88****	0.86****	0.83****	-0.18	0.39	-0.48*	-	-
<i>P. tanacetifolia</i>	-0.64****	-0.42*	0.01	-0.79****	-0.63**	0.83****	0.82****	0.67**	0.61*	0.54*	-0.84****	-	-
<i>M. ruthenica</i>	-0.93****	0.12	0.14	-0.71****	-0.48*	0.66**	0.74****	0.58**	0.75**	0.45*	-0.72****	-	-

SWC, soil water content; SIN, soil inorganic nitrogen; STN, soil total nitrogen; LNC, leaf nitrogen content; LPC, leaf phosphorus content; C/N ratio, ratio of leaf carbon to nitrogen contents; DMC, leaf dry matter content; LMA, leaf mass per area;  $\delta^{13}\text{C}$ , leaf carbon isotopic composition; NUE, nitrogen use efficiency; SPAD, estimated leaf chlorophyll concentration;  $P_{\text{net}}$ , net photosynthetic rate based on area;  $g_s$ , stomatal conductance.

Asterisks indicate statistically significant differences at \* $P < 0.10$ , \*\* $0.05$ , \*\*\* $0.01$  and \*\*\*\* $0.001$ .

similar to those of the present study. LMA might be a relatively stable leaf trait as its values in this study ( $86.11 \text{ g m}^{-2}$ ) were close to the means from both the Glopnet ( $83.78 \text{ g m}^{-2}$ ) and the Cedar Creek ( $86.63 \text{ g m}^{-2}$ ) data sets. The higher LNC values in this study ( $23.34 \text{ mg g}^{-1}$ ) than in Cedar Creek ( $19.81 \text{ mg g}^{-1}$ ) might be explained by the N addition in this study ( $10 \text{ g m}^{-2} \text{ yr}^{-1}$ ) whereas no N was applied in Cedar Creek. The differences in  $P_{\text{net}}$ -mass values (Glopnet,  $192.24 \text{ nmol g}^{-1} \text{ s}^{-1}$ ; Cedar Creek,  $258.14 \text{ nmol g}^{-1} \text{ s}^{-1}$ ; present study,  $110.36 \text{ nmol g}^{-1} \text{ s}^{-1}$ ) among the three data sets were presumably caused by the different resource availability, especially for water. At Cedar Creek, precipitation was around 660 mm, much higher than the 380 mm for Duolun county, and water stress was largely ignored by watering all the plots during the growing season (Tjoelker *et al.*, 2005). In the present study, water was the major limiting factor for plant growth (Xu *et al.*, 2010), although it was added in half of the experimental plots.

The overall relationships between leaf longevity and  $P_{\text{net}}$ -mass, LMA and LNC in the present study are largely in agreement with previous findings although the coefficients of determination might be different between studies; in particular, the relationships between leaf longevity and LMA have identical slopes in these three data sets (common slopes of 0.70 for  $\text{C}_3$  grasses, 0.73 for forbs and 0.75 for legumes; Fig. 4A–E). This suggests there are globally consistent patterns of relationships among leaf functional traits despite the fact that different biomes and environmental factors among study sites may influence their slopes.

Although increased N input and water supply shortened leaf longevity, species biomass production was nevertheless increased in the N-fertilized and watered plots. According to the cost–benefit model proposed by Kikuzawa (1991, 1995), leaf longevity was adjusted to maximize carbon gain, and plants would produce more new leaves to replace the old leaves when leaf longevity was reduced. Our results corroborated the model in that N and water addition increased leaf biomass production of individual plants for all the five studied species although their leaf longevity decreased (Table 1). Thus, plant above-ground biomass, especially for the two dominant species, *S. krylovii* and *A. cristatum*, increased with enhanced N and water supply (Fig. 2A, B), and consequently increased ANPP at the community level (Fig. 3A). Increasing N and water availability enhanced LNC and leaf photosynthetic capacity, and hence more carbon was transferred to the plant root. As a result, BNPP also showed a positive response to water addition (Fig. 3B).

In summary, future changes in N deposition and precipitation in temperate typical steppe and similar terrestrial ecosystems may significantly alter leaf longevity and other leaf functional traits. Soil water supply may be a central factor in controlling leaf longevity and other leaf functional traits, and its effects could be mediated by soil N supply. As molecular biological studies have shown that leaf senescence is programmed by cell death and is genetically controlled (Hensel *et al.*, 1993; Jiang *et al.*, 1993; Chandlee, 2001; Buchanan-Wollaston *et al.*, 2003; Lambers *et al.*, 2008), further work, either genetically, physiologically or ecologically, is required to determine in more detail the mechanism of variations in plant functional traits.



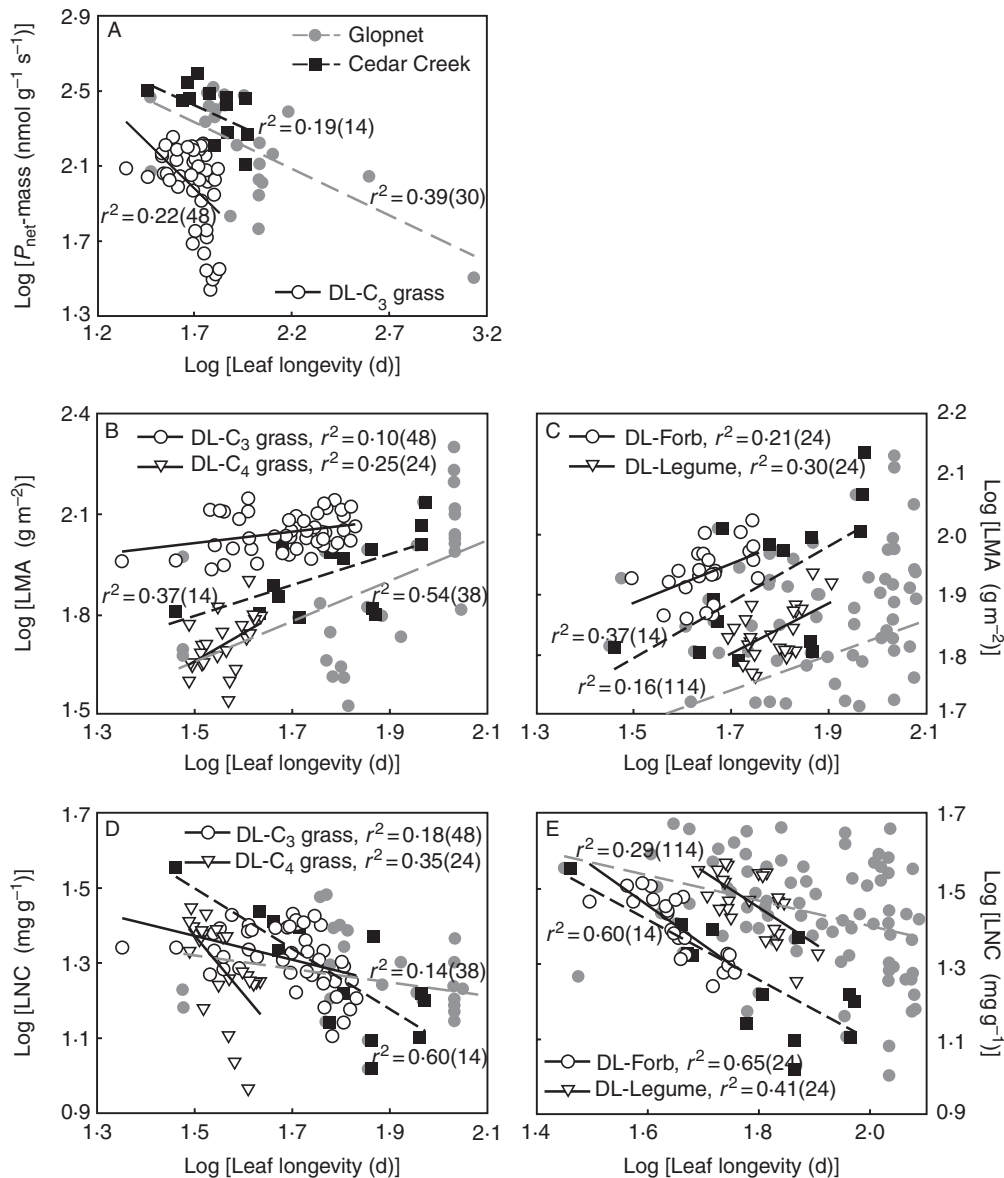


FIG. 4. Standardized major axis (SMA) relationships between leaf longevity (LL) and mass-based net photosynthetic rate ( $P_{\text{net-mass}}$ ), leaf mass per area (LMA) and leaf nitrogen content (LNC) for Duolun (DL) steppe species in comparison with the Cedar Creek data set (CC) and the Glopnet data set (G). LL versus  $P_{\text{net-mass}}$ ; (A) slopes: DL  $-2.22$ , CC  $-1.02$ , G  $-0.80$ . LL versus LMA: (B) common fitted slope among three sites was  $0.70$  for  $C_3$  grasses; slopes: DL  $1.71$ , CC  $0.77$ , G  $0.82$  for  $C_4$  grasses. (C) Common slopes fitted within each functional group: forb  $0.73$ , legume  $0.75$ . LL versus LNC; (D) slopes: DL for  $C_3$  grasses  $-0.75$ , DL for  $C_4$  grasses  $-2.57$ , CC  $-1.03$ , G  $-0.49$ . (E) Slopes: DL for forb  $-1.31$ , DL for legume  $-1.50$ , CC  $-1.03$ , G  $-0.62$ . All relationships were significant,  $P < 0.05$ , except the correlation between longevity and  $P_{\text{net-mass}}$  in Cedar Creek ( $P = 0.191$ ). Sample sizes are given in parentheses. The Glopnet data set is not shown in totality, but the statistical results were obtained via the whole data set.

#### ACKNOWLEDGEMENTS

We thank Xiaoyong Cui and Guohong Wang for helpful suggestions regarding the experiment; Zhenqing Li, Haishan Niu and Jianxiong Huang for statistical assistance; Wenming Bai and Osvaldo Sala for constructive comments on a previous version of the manuscript; and Bill Shipley and two anonymous referees for their valuable comments on previous versions of the manuscript. This research was financially supported by the National Basic Research Program of China (973 Program) (2007CB106801) and the National Natural Science Foundation of China (30821062). We also thank the

Duolun Restoration Ecology Research Station for access to the study site and technical assistance.

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## APPENDIX

Results of stepwise multiple regression analysis between leaf longevity and other leaf functional traits in five steppe species

Species	Regression equations	Step no.	Variable entered	$R^2$	Change in $R^2$ (%)	$P$ value
2007						
<i>S. krylovii</i>	$LL = -3.39P_{\text{net}} + 70.89$	1	$P_{\text{net}}$	0.805	80.5	<0.001
<i>A. cristatum</i>	$LL = -1.11LNC - 3.12P_{\text{net}} + 103.65g_s + 86.69$	1	LNC	0.779	77.9	<0.001
		2	$P_{\text{net}}$	0.883	10.4	<0.001
		3	$g_s$	0.943	6.0	<0.001
<i>C. squarrosa</i>	$LL = -0.49LNC + 43.67$	1	LNC	0.790	79.0	<0.001
<i>P. tanacetifolia</i>	$LL = -1.84SPAD + 121.27$	1	SPAD	0.713	71.3	<0.001
<i>M. ruthenica</i>	$LL = -2.63SPAD + 191.18$	1	SPAD	0.830	83.0	<0.001
2008						
<i>S. krylovii</i>	$LL = -3.43P_{\text{net}} + 109.27$	1	$P_{\text{net}}$	0.683	68.3	<0.001
<i>A. cristatum</i>	$LL = 1.64C/N - 24.84g_s + 0.19DMC - 54.59$	1	C/N	0.722	72.2	<0.001
		2	$g_s$	0.913	19.1	<0.001
		3	DMC	0.955	4.2	<0.001
<i>C. squarrosa</i>	$LL = -0.74LNC + 0.06DMC + 28.58$	1	LNC	0.855	85.5	<0.001
<i>P. tanacetifolia</i>	$LL = -1.76SPAD + 0.15DMC + 73.84$	2	DMC	0.927	7.2	<0.001
		1	SPAD	0.701	70.1	<0.001
<i>M. ruthenica</i>	$LL = 7.08\delta^{13}C - 1.19SPAD + 314.61$	2	DMC	0.835	13.4	<0.001
		1	$\delta^{13}C$	0.564	56.4	<0.01
		2	SPAD	0.735	17.1	<0.01

LL, leaf longevity;  $P_{\text{net}}$ , net photosynthetic rate based on area;  $g_s$ , stomatal conductance; LNC, leaf nitrogen content; SPAD, estimated leaf chlorophyll concentration; C/N ratio, ratio of leaf carbon to nitrogen contents; DMC, leaf dry matter content;  $\delta^{13}C$ , leaf carbon isotopic composition.