Nitrogen control of ^{13}C enrichment in heterotrophic organs relative to leaves in a landscape-building desert plant species

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Abstract

A longstanding puzzle in isotope studies of C_3 plant species is that heterotrophic plant organs (e.g., stems, roots, seeds, and fruits) tend to be enriched in ^{13}C compared to the autotrophic organ (leaves) that provides them with photosynthate. Our inability to explain this puzzle suggests key deficiencies in understanding post-photosynthetic metabolic processes. It also limits the effectiveness of applications of stable carbon isotope analyses in a variety of scientific disciplines ranging from plant physiology to global carbon cycle studies. To gain insight into this puzzle, we excavated whole plant architectures of *Nitraria tangutorum* Bobrov, a C_3 species that has an exceptional capability of fixing sands and building sand dunes, in two deserts in northwestern China. We systematically and simultaneously measured carbon isotope ratios and nitrogen and phosphorous contents of different parts of the excavated plants. We also determined the seasonal variations in leaf carbon isotope ratios on nearby intact plants of *N. tangutorum*. We found, for the first time, that higher nitrogen contents in heterotrophic organs were significantly correlated with increased heterotrophic ^{13}C enrichment compared to leaves. However, phosphorous contents had no effect on the enrichment. In addition, new leaves had carbon isotope ratios similar to roots but were progressively depleted in ^{13}C as they matured. We concluded that a nitrogen-mediated process, hypothesized to be the refixation of respiratory CO_2 by phosphoenolpyruvate (PEP) carboxylase, was responsible for the differences in ^{13}C enrichment among different heterotrophic organs while processes such as fractionating foliar metabolism and preferentially loading into phloem of ^{13}C enriched sugars may contribute to the overall autotrophic – heterotrophic difference in carbon isotope compositions.

Key words: carbon isotope fractionation, post-photosynthetic discrimination, nitrogen, phosphorous, phosphoenolpyruvate carboxylase

INTRODUCTION

The natural abundance analysis of stable carbon isotopes in plants has become an essential tool for studying plant-environmental interactions, plant metabolism, carbon allocation, and biosphere-atmosphere exchanges of carbon fluxes (Dawson *et al*. 2002; Bowling *et al.* 2008; Tcherkez *et al.* 2011; Cernusak *et al.* 2013). Understanding processes and factors controlling carbon isotope compositions in different plant organs, which are not homogenous (Leavitt and Long 1986), is crucial to the successful applications of this tool (Hobbie and Werner 2004). The primary determinant of plant carbon isotope compositions is the photosynthetic discrimination against the heavier carbon isotope ^{13}C. This primary discrimination process has been relatively well understood and detailed theoretical models relating the discrimination to environmental forcing conditions and leaf physiology and biochemistry have been developed (Farquhar *et al*. 1982; Farquhar and Cernusak 2012; Gu and Sun 2014). However, other processes must also influence plant carbon isotope compositions as heterotrophic plant organs (e.g., stems, roots, seeds and fruits) in C_3 plant species have been found to be generally enriched in ^{13}C as compared to the autotrophic organ (leaves) that supplies them with carbohydrates (Craig 1953; Leavitt and Long 1982; Ehleringer *et al*. 1987; Hobbie and Werner 2004; Badeck *et al*. 2005; Cernusak *et al*. 2009). In contrast to the relatively well-understood photosynthetic carbon isotope discrimination, processes controlling the observed heterotrophic ^{13}C enrichment in C_3 plant species remain unclear even though the phenomenon was first reported sixty years ago (Craig 1953).

Cernusak *et al*. (2009) and Ghashghaie and Badeck (2014) summarized more than half a dozen of nonexclusive processes that may explain the heterotrophic ^{13}C enrichment in C_3 plant species. These processes generally belong to two broad groups. Group I processes involve the occurrence of contrasting biochemical and metabolic fractionations between autotrophic and heterotrophic organs, for example, ^{13}C-enriched autotrophic vs. ^{13}C-depleted heterotrophic mitochondrial respirations, low autotrophic vs. high heterotrophic CO_2 fixation by phosphoenolpyruvate (PEP) carboxylase, and low autotrophic vs. high heterotrophic loss rates of ^{13}C-depleted volatile organic compounds, surface waxes and other products from secondary plant metabolism. Group II processes involve the utilization of contrasting organ-building photoassimilates, which in turn may be a result of a number of processes, including preferential export of ^{13}C-enriched nighttime sucrose to heterotrophic organs, reduced photosynthetic discrimination against ^{13}C due to developmental shifts in exporting mature

leaves, and asynchronous growth of autotrophic vs. heterotrophic organs in contrasting environmental conditions. Although the term post-photosynthetic discrimination or post-carboxylation discrimination has been often used to refer the processes included in both groups, some of the processes in Group II cannot be strictly considered as occurring post photosynthesis or carboxylation. Nearly all processes outlined above have supporting as well as opposing evidences from observational and experimental studies (Cernusak *et al*. 2009). Thus it remains a challenge to identify cause(s) for the sixty-year old puzzle of heterotrophic ^{13}C enrichment.

It is important to overcome this challenge as many fundamental issues in a variety of scientific disciplines ranging from plant physiology to global carbon cycle studies depend on a precise knowledge of plant carbon isotope compositions. Towards this goal, we have identified two areas that require strengthening in the studies of heterotrophic ^{13}C enrichment. First, there is a need for systemic, whole-plant studies. Although heterotrophic ^{13}C enrichment in C_3 plant species has been reported widely, most previous studies have been done by comparing heterotrophic organs independently and on a piecemeal basis with leaves. This lack of systemic, whole-plant studies is not conducive to understanding the mechanism of heterotrophic ^{13}C enrichment because to achieve this understanding, one must first have a comprehensive picture of the enrichment (or depletion) across all organs of the same plant.

Second, whether and how nutrients affect heterotrophic ^{13}C enrichment needs to be investigated. Nutrients, particularly nitrogen (N) and phosphorous (P), control leaf photosynthetic capacity (Field and Mooney 1986; Domingues et al. 2010), which in turn affects the drawdown of CO_2 along stomatal and mesophyll diffusional pathways. It has been shown that leaf N content is positively (negatively) correlated with leaf $\delta^{13}C$ (carbon isotope discrimination) (Sparks and Ehleringer 1997; Livingston *et al*. 1999; Duursma and Marshall 2006; Cernusak et al. 2007). This relationship is consistent with the expectation that higher leaf photosynthetic capacity associated with higher leaf N leads to a sharper drawdown of CO_2 along the diffusional pathways (Cernusak et al. 2007, 2013), resulting in an expected pattern according to the photosynthetic isotope discrimination equations (Farquhar *et al*. 1982; Farquhar and Cernusak 2012; Gu and Sun 2014). To our knowledge, hitherto there has been no effort to systematically investigate how plant nutrients might affect heterotrophic ^{13}C enrichment compared to leaves. A lack of such an effort is not justifiable because plant nutrients play important roles in many of the processes discussed in Cernusak *et al*. (2009) and Ghashghaie

and Badeck (2014). Thus it would not be surprising if certain relationships exist between plant nutrients and heterotrophic ^{13}C enrichment. An identification of such relationships will greatly assist the illumination of the underlining cause(s) of heterotrophic ^{13}C enrichment.

Therefore, the objective of the present study was to gain insight into the longstanding puzzle of heterotrophic ^{13}C enrichment by jointly addressing the two deficiencies identified above. We conducted systematic and simultaneous analyses of carbon isotope ratios and N and P contents with excavated whole architectures of *Nitraria tangutorum* Bobrov, a C_3 shrub species endemic to northwestern deserts in China. These analyses were complemented with investigations of seasonal variations in leaf carbon isotope ratios on intact plants of the same species, thus enabling the analyses of carbon isotope compositions of different heterotrophic organs in a dynamic reference framework. *N. tangutorum* is interesting because it has an exceptional capability of controlling landscape evolution by fixing sands and building sand dunes known as nebkha or coppice dunes around its extensive shoot and root systems (Baas and Nield 2007; Lang *et al*. 2013; Li *et al*. 2013). This characteristic makes it relatively easy to excavate the whole plant including roots for isotope and nutrient analyses, although to our knowledge, this species has never been investigated for heterotrophic ^{13}C enrichment.

We will report, for the first time, that variations in ^{13}C enrichment in different heterotrophic organs strongly depend on their N contents, indicating a role of a within-organ N-mediated process in heterotrophic ^{13}C enrichment. We will also show that the observed N – heterotrophic ^{13}C enrichment relationship is most parsimoniously explained through the respiratory CO_2 refixation by PEP carboxylase. Future studies on heterotrophic ^{13}C enrichment should investigate isotopic effects of N content and CO_2 refixation in different plant organs. Direct measurements of PEP carboxylase activity will be essential.

MATERIALS AND METHODS

Biological and environmental characteristics of *Nitraria tangutorum* Bobrov

Nitraria tangutorum Bobrov (Fig. 1) is a spiny shrub species in the *Nitraria* genus of the Zygophyllaceae family. Species in the *Nitraria* genus are generally xerophytes, widely distributed in the Middle East, Central Asia, and northwestern regions of China. *N. tangutorum*, however, is endemic to the northwestern regions of China, including northeastern Tibet, Gansu, Qinghai,

Xinjiang, western Inner Mongolia, western Ningxia, and northern Shaanxi. It is a pioneer species and has high tolerance for drought, heat, and salts. *N. tangutorum* plays an important ecological role in combating desertification due to its exceptional capabilities in forming phytogenic nebkha dunes which prevent or slow down the movement of sands. According to Li and Jiang (2011) and Li *et al*. (2013), the process of forming a nebkha typically starts when occasional ample moisture allows a seed to germinate inside clay cracks in dried-up flat beds of previous rivers or lakes. As the resulting ortet grows, it intercepts aeolian sands and the plant enters into a clonal reproductive stage. When branches are buried by sands, layering occurs and adventitious roots are formed. Under appropriate sand burial depth and sufficient moisture, ramets are developed from axillary buds in the layering and a clonal colony is formed. If aeolian sand supply is not interrupted, repetitive layering and ramet development will enlarge the colony and further increases its capacity to intercept aeolian sands and a phytogenic nebkha dune is formed (Fig. 1c).

The height of a *N. tangutorum* nebkha ranges from 1 to 3 m and some can reach 5 m. The base of a nebkha often has the shape of an ellipse with the major axis parallel to the local prevailing wind direction. The formation of nebkhas alters local microenvironments and provides habitats for other desert species. Li and Jiang (2011) described in detail the biological and environmental characteristics of species in the *Nitraria* genus with a focus on *N. tangutorum*.

Study sites

The field work was carried out at two desert locations. The first study site was within an experimental area (40°24′ N, 106°43′ E) managed by the Experimental Center of Desert Forestry of the Chinese Academy of Forestry. This site is located in Dengkou County, Inner Mongolia Autonomous Region, China. Dengkou County is at the junction between the Hetao Plain and Ulan Buh Desert of the Mongolian Plateau in the middle reaches of the Yellow River. The mean annual temperature is 8.84℃ and the mean annual precipitation is 147 mm with 77.5% of annual rainfall occurring from June to September (1983-2012 averages). The mean annual potential evaporation is 2381 mm (Li *et al*. 2013). The soil in the study region in general is sandy soil and gray-brown desert soil (Cambic Arenosols and Luvic Gypsisols in FAO taxonomy). The *N. tangutorum* nebkhas at the study site are formed on clay soils deposited by the Yellow River. Although the plant community is dominated by *N. tangutorum*, xerophytic species such as semi-shrub *Artemisia ordosica*, perennial

grass *Psammochloa villosa*, and annual species *Agriophyllum squarrosum* and *Corispermum mongolicum* can also be found.

The second study site was the Gansu Minqin Desert Ecosystem Research Station (38°34′ N, 102°58′ E), Minqin County, Gansu Province, China. Minqin County is located in the lower reaches of Shiyang River, surrounded by the Badain Jaran Desert in the west and north and the Tengger Desert in the east. The mean annual temperature is 8.87℃ and the mean annual precipitation is 117 mm with 73.1% of annual rainfall occurring from June to September (1983-2012 averages). The mean annual potential evaporation is 2643 mm (Du *et al*. 2010). Thus the second study site is somewhat drier than the first site but with similar annual mean temperatures. The soil at the Minqin site is similar to that at the Dengkou site with sandy soil in the nebkhas and gray-brown desert soil between nebkhas. The native vegetation in the study area is usually dominated by shrubs and semi-shrubs with species such as *N. tangutorum* and *Calligonum mongolicum*. Experimental plots used in this study contained semi-fixed nebkha dunes developed by the growth of *N. tangutorum*. Typically in dry years, *N. tangutorum* is the only species growing in the nebkhas although in wet years, annual species such as *Agriophyllum squarrosum* and *Corispermum mongolicum* can also be found. Because the Minqin site is drier than the DengKou site, the nebkhas at the Minqin site are generally smaller and less populated with plants than at the Dengkou site. The rooting depth is deeper at the Minqin site than at the Dengkou site (Table 1).

Excavation of *Nitraria tangutorum* nebkhas

In August 2012, we excavated three nebkhas at each study site. The geometrical and biometrical characteristics of the six nebkhas were summarized in Table 1. At the Dengkou site, the three nebkhas were excavated in a sampling area of 40m×40m. At the Minqin site, nebkhas were generally much smaller. To ensure availability for analyses of sufficient biomass materials at this site, particularly the fine roots (see below), three sampling areas each with a dimension of 30m×30m were established and three nebkhas from each sampling area were tentatively excavated. Two nebkhas from one sampling area and one from another were determined to have sufficient amount of fine roots for analyses and were therefore excavated fully.

We excavated the nebkhas by carefully teasing away the sands from the mounds to expose the root architecture of *N. tangutorum* with particular attention paid to the preservation of fine roots. The

roots of a *N. tangutorum* can be found inside the sand mounds as well as inside the clay layer that generally forms a plain on which the sand mounds rest. We therefore also excavated any roots inside the clay layer to a depth until no more roots could be found.

We separated the whole plant biomass into leaves, stems, in-sand roots and below-plain roots. The in-sand roots, which were roots found inside the nebkha sands but above the plain formed by the underlying clay layer, were further separated into in-sand fine roots (diameter $\leqslant$ 2mm) and in-sand coarse roots (diameter > 2mm). The same root diameter threshold was used to separate the below-plain roots, which were found inside the clay layer under the nebkha sands. Furthermore, the below-plain fine and coarse roots were grouped in a 20cm depth increment from the plain surface. We did not separate the in-sand fine and coarse roots into layers because a nebkha has a cone shape on top, making a layer hard to define. Also we did not use a simple 'below-ground' group because 'ground' is not well defined in a nebkha-populated landscape and because there are large physical and chemical differences between sands and clay which may affect the isotope compositions of roots growing in them. Litter was rarely found on the nebkhas, presumably because strong winds at the study sites can easily blow away any litter produced. However, woody debris from dead ramets was present inside the sand mounds and was collected during excavation. Thus for each nebkha, we differentiated the following categories of *N. tangutorum* biomass: the autotrophic organ of leaves, the heterotrophic organs of stems, in-sand fine roots (ISFR), in-sand coarse roots (ISCR), below-plain fine roots (BPFR) in 20 cm depth increments, and below-plain coarse roots (BPCR) in 20cm increments, and the heterotrophic woody debris (WD). Nutrient contents and carbon isotope compositions were measured separately for each category.

Measurements of nutrient contents and carbon isotope compositions with excavated biomass

All categories of *N. tangutorum* biomass (leaves, stems, ISFR, ISCR, BPFR in 20cm increments, BPCR in 20cm increments, and WD) from each excavated nebkha were dried to constant weight (60℃, 48 hours). The dry weight of biomass was determined with 0.01 g accuracy on an analytical scale. The biomass carbon stocks were expressed relative to the base area of the nebkha which was assumed to be an ellipse. The fraction of each component was also calculated.

Dried materials were randomly selected from each biomass category and ground to 80 mesh. The resultant powder was separated into six duplicates. Three duplicates were analyzed for carbon

(C), nitrogen (N) and phosphorous (P) contents and the remaining three for isotope compositions. The C, N and P contents were measured in the Environmental Chemistry Analysis Laboratory in the Institute of Geographic Sciences and Natural Resources Research, the Chinese Academy of Sciences, Beijing, China. Total sample carbon and N were measured with the vario MACRO cube (Elementar Company, Germany). The analytical precision was better than 0.5% Relative Standard Deviation (RSD). Total P was measured with the ICP-OES OPTIMA 5300DV (PE, USA). The analytical precision was better than 2% RSD.

The carbon isotope compositions were analyzed at the Stable Isotope Ratio Mass Spectrometer Laboratory of the Chinese Academy of Forestry (SIRMSL, CAF), Beijing, China. The instrument used was a Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific, Inc., USA) coupled with an elemental analyzer (FlashEA 1112; HT Instruments, Inc., USA) in the continuous flow mode. Isotope compositions were expressed using the delta notation (δ) in parts per thousand (‰): $\delta^{13}C$ (‰) = $[(R_{sample})/(R_{standard}) -1] \times 1000$, where R is the ratio of ^{13}C to ^{12}C. The measurement applied the IAEA-600 standard (Caffeine) relative to V-PDB (Vienna PeeDee Formation Belemnite Limestone). The analytical precision was better than 0.1‰ based on replicate measurements of the reference standard.

Measurements of seasonal variations in leaf $\delta^{13}C$ and C_i/C_a ratio

Photosynthetic carbon isotope discrimination depends on environmental conditions (Farquhar *et al*. 1982; Farquhar and Cernusak 2012; Gu and Sun 2014); consequently, leaf carbon isotope ratio $\delta^{13}C$ may change seasonally, potentially making the autotrophic - heterotrophic differences in carbon isotope compositions time dependent. Thus in addition to the isotopic and nutrient analyses for samples from the excavated plant materials, we also measured seasonal variations in leaf carbon isotope compositions and ratios of leaf intercellular airspace (C_i) to ambient (C_a) CO_2 concentrations on nearby un-excavated nebkhas at both the Dengkou and Minqin study sites. Four samples of leaves were taken in each month from May to September of 2012 at both sites and analyzed for carbon isotope ratios at the SIRMSL of CAF. The seasonal variations in C_i/C_a ratios were measured with a Li-6400 portable photosynthetic system (LiCor Environmental Sciences, Lincoln, NE, USA) each month from June to September of 2012 at the Dengkou site with 24 – 28 samples per month and from July to September of 2011 at the Minqin site with 16 samples per month. The chamber

environment (temperature, light, and relative humidity) was kept close to ambient conditions at the time of measurement. Seasonal variations in leaf nutrient contents were not measured. The measurements of seasonal variations in leaf $\delta^{13}C$ provide a dynamic reference framework for examining the $\delta^{13}C$ values of heterotrophic organs while the independent measurements of seasonal variations in C_i/C_a ratios allow us to determine whether the seasonal patterns in leaf $\delta^{13}C$ are consistent with our current understanding of the photosynthetic carbon isotope discrimination (Farquhar *et al*. 1982).

Quantification of heterotrophic ^{13}C enrichment and statistical analyses

We quantified the difference in carbon isotope composition between the leaves (autotrophic) and a heterotrophic organ with the following expression:

$$\Delta^{13}C_{organ} = \left(\frac{R_{leaf}}{R_{organ}} - 1 \right) \times 1000 = \left(\frac{\delta^{13}C_{leaf}/1000 + 1}{\delta^{13}C_{organ}/1000 + 1} - 1 \right) \times 1000 = \frac{\delta^{13}C_{leaf} - \delta^{13}C_{organ}}{1 + \delta^{13}C_{organ}/1000}. \quad (1)$$

Thus a value of $\Delta^{13}C_{organ} < 0$ indicates an enrichment of ^{13}C in a heterotrophic organ relative to the leaves while $\Delta^{13}C_{organ} > 0$ indicates heterotrophic depletion. The values of $\delta^{13}C_{Leaf}$ used to calculate $\Delta^{13}C_{organ}$came from leaves harvested from *N. tangutorum* of the excavated nebkhas, not from those for seasonal patterns. The use of Δ in Eq. (1) makes the relationship between autotrophic and heterotrophic organs analogous to that between reactants and products (Farquhar et al. 1989), which is appropriate for the purpose of this study. A great advantage of introducing $\Delta^{13}C_{organ}$is that heterotrophic ^{13}C enrichment can be compared not only among the organs of the same plant but also across different plants at the same site or at different sites which may differ in autotrophic isotopic signatures. Thus the use of $\Delta^{13}C_{organ}$facilitates the identification of general patterns.

Two-way ANOVA analyses (organ by site) were performed with SPSS (Ver.17.0). C, N, and P contents, $\delta^{13}C$, $\Delta^{13}C_{organ}$, C/N ratios, N/P ratios and C/P ratios were analyzed for differences between organs and between study sites. Tukey post-hoc tests were used to determine pairwise differences for significant effects ($P < 0.05$). Regression analyses were used to determine the relationship between the heterotrophic ^{13}C enrichment and nutrient contents.

RESULTS

Variations in $\Delta^{13}C_{organ}$ among plant organs and between study sites

At both the Dengkou and Minqin study sites, the values of $\Delta^{13}C_{organ}$ for all heterotrophic organs examined were significantly smaller than zero, indicating that without any exception, the heterotrophic organs were enriched in ^{13}C compared to the leaves (Fig. 2). However, there were considerable variations in $\Delta^{13}C_{organ}$ among the heterotrophic organs at both study sites and between the heterotrophic organs across the study sites. Stems were less enriched (closer to zero) than roots at both sites. At the Dengkou site, the most enriched organ was the coarse roots inside the nebkha sands. At the Minqin site, the most enriched part was the fine roots inside the negkha sands although the difference between the coarse and fine roots inside the sands was not significant. At the Dengkou site, the coarse roots were consistently more enriched than the corresponding fine roots both inside the nebkha sands and below the plains. In contrast at the Minqin site, the coarse roots were less enriched than the corresponding fine roots except for the roots deep into the plains (40 – 80 cm) where the coarse roots were more enriched. However at both sites, the statistical power of the coarse – fine root isotope differences were low as they were not significant at the significance level of 0.05. At the Denkou site, the woody debris was more enriched than the stems but less enriched than the roots while at the Minqin site, it was less enriched than either the stems or the roots. In all biomass categories investigated, the Dengkou site was more enriched than the Minqin site, particularly in below-plain roots and in woody debris.

Variations in nutrient concentrations among plant organs and between sites

There are considerable variations in nutrient contents among plant organs and between sites (Fig. 3). At both the Dengkou and Minqin sites, leaves appeared to have the lowest C (Fig. 3a) but highest N (Fig. 3b) and P (Fig. 3c) contents. At both sites, stems tended to have lower N contents than roots either inside the sand dunes or below the plains under the sand dunes; in contrast, P contents in stems were within the variations of P contents in roots. At the Denkou site, roots inside the sand dunes had lower N contents than roots below the plain; at the Minqin site, the coarse roots inside the sand dunes had lower N than either coarse or fine roots below the plain while the fine roots inside the sand dunes had N within the variations of those of coarse and fine roots below the plain. At the Dengkou site, the

fine roots appeared to have higher P than coarse roots but the differences diminished from inside sands to below plain. There were no clear patterns on root P at the Minqin site. Woody debris had N contents similar to stems at both sites and tended to have significantly less P contents than leaves, stems or roots. Between the two study sites, the leaves had lower C but higher N and P contents at the Dengkou site than at the Minqin site, but the difference is not significant at the significance level of 0.05. In contrast, heterotrophic organs at the Dengkou site tended to have significantly higher N and P contents than at the Minqin site. This contrast suggests that *N. tangutorum* may be able to maintain nutrient contents in leaves for photosynthesis at the expense of stems and roots.

Consistent with the variations in C, N and P contents, there were also substantial variations in the ratios of C/N (Fig. 4a), N/P (Fig. 4b) and C/P (Fig. 4c) among plant organs and between sites. For the live biomass (leaves, stems, and roots), the ratios of C/N ranged from about 11 to 30, N/P from 20 to 40 and C/P from 300 to 700. As expected, leaves had the lowest C/N and C/P ratios at both sites. Leaves also had the lowest N/P ratios except for stems. Overall, the Dengkou site had lower ratios of C/N and C/P but higher ratios of N/P than the Minqin site, particularly for roots below the plain.

Relationships between ^{13}C enrichment and nutrient contents

The observed large variations in ^{13}C enrichment and nutrient contents among heterotrophic organs and between study sites give us an opportunity to examine whether ^{13}C enrichment in heterotrophic organs relative to leaves could be affected by their nutrient contents. We found that across the two study sites and across the heterotrophic organs, $\Delta^{13}C_{organ}$ was significantly correlated with the N content (Fig. 5b), the C/N ratio (Fig. 5d), and the N/P ratio (Fig. 5e) in the heterotrophic organs. The correlation was negative for N content and N/P ratio but positive for C/N ratio, indicating that higher heterotrophic N contents resulted in larger heterotrophic ^{13}C enrichment relative to leaves. The C/N ratio explained a higher percentage (52%) of variance in $\Delta^{13}C_{organ}$ than did the N content or the N/P ratio (44 and 42%, respectively). No significant effect of heterotrophic organ C content (Fig. 5a), P content (Fig. 5c), or C/P ratio (Fig. 5f) on $\Delta^{13}C_{organ}$ were found.

We did not have enough independent samples to look at how leaf N contents might affect the

heterotrophic ^{13}C enrichment. However, we examined the relationship between $\Delta^{13}C_{organ}$ and organ nutrient contents normalized by the corresponding leaf nutrient contents (i.e., the ratio of heterotrophic to corresponding leaf nutrient values). The normalized heterotrophic N contents explained somewhat less variance with reduced statistical power compared to the un-normalized values (Compare Fig. S1 to Fig. 5), suggesting that it is the absolute N contents of the heterotrophic organs, not their relative departure from the corresponding leaf N contents, that affect the heterotrophic ^{13}C enrichment.

Seasonal variations in leaf $\delta^{13}C$ and C_i/C_a ratios

At both Dengkou and Minqin sites, leaf $\delta^{13}C$ of *N. tangutorum* decreased from May to September (Fig. 6a), indicating progressive depletion in the heavier carbon isotope in leaves as the season progressed. Meanwhile, the C_i/C_a ratio increased from the early to late growing season (Fig. 6b). Thus the relationship between the seasonal patterns in leaf $\delta^{13}C$ and C_i/C_a ratios is consistent with the prediction by the leaf photosynthetic carbon isotope discrimination models (Farquhar *et al.* 1982; Farquhar and Cernusak 2012; Gu and Sun 2014). However, the differences in leaf $\delta^{13}C$ between the two sites cannot be entirely explained by the differences in the C_i/C_a ratios. In all months examined, the C_i/C_a ratios at the Dengkou site were consistently higher than at the Minqin site. If the C_i/C_a ratios were the only factor controlling the leaf $\delta^{13}C$, then the Dengkou site should have consistently lower leaf $\delta^{13}C$ (higher C_i/C_a ratios increase discrimination against ^{13}C during photosynthesis). To the contrary, the Dengkou site had higher leaf $\delta^{13}C$ than the Minqin site in May, June and July; only in August and September, the difference in leaf $\delta^{13}C$ was consistent with the effect of the difference in C_i/C_a ratios between the two sites (although the difference in leaf $\delta^{13}C$ between the two sites were still not significant).

Interestingly, the leaf $\delta^{13}C$ in May and June was close to the biomass-weighted average of root $\delta^{13}C$ at both study sites, suggesting that the initial building materials of new leaves might have largely come from stored carbon in roots.

DISCUSSION

A major finding from this study is that the N content of a heterotrophic organ, expressed either as a

fraction of total dry biomass or as a ratio of C to N or N to P, is strongly correlated with this organ's enrichment in ^{13}C relative to leaves with higher N concentrations corresponding to larger enrichment. Because this relationship is caused by variations among heterotrophic organs and because normalizing the heterotrophic N content by the corresponding leaf N content did not improve or even worsened this relationship, the process responsible for it must reside inside the heterotrophic organs themselves. Further, this process must be mediated by N.

What N-mediated process could be responsible for the positive N - ^{13}C enrichment relationship among heterotrophic organs? A parsimonious candidate is the respiratory CO_2 refixation by PEP carboxylase. CO_2 from the respiration of heterotrophic organs may dissolve into water and be hydrated into HCO_3^- which is then fixed by PEP carboxylase into oxaloacetate. Both the dissolution of CO_2 into water and the fixation of HCO_3^- by PEP carboxylase discriminate slightly against ^{13}C. However, the hydration process fractionates strongly in favor of ^{13}C and causes it to concentrate in HCO_3^-. Consequently, the CO_2 refixation by PEP carboxylase has a net fractionation of 5.7‰ in favor of ^{13}C relative to the gaseous CO_2 (Farquhar 1983; Melzer and O'Leary 1987; Farquhar *et al*. 1989). Thus the respiratory CO_2 refixation by PEP carboxylase should lead to a depletion of ^{13}C in CO_2 escaped to outside compared to the original substrates for respiration while heterotrophic organs should be ^{13}C-enriched due to the addition of organic materials from PEP carboxylase activities. Previous studies have reported high PEP carboxylase activities in heterotrophic organs of a variety of C_3 plant species (Melzer and O'Leary 1987; Berveiller and Damesin 2008; Gessler et al. 2009; Gessler et al. 2014). If increased N content increases the respiratory CO_2 refixation in heterotrophic organs, then it should also increase ^{13}C enrichment in these organs. Berveiller *et al*. (2010) showed that CO_2 refixation rates of *Fagus sylvatica* stems increased as stem N content increased, which provides a direct support for the hypothesis that CO_2 refixation by PEP carboxylase is a process responsible for our observed positive relationship between N and ^{13}C enrichment in heterotrophic organs.

Observed respiration rates of leaves, stems and roots tend to increase with increased N contents (Reich *et al*. 2008). This does not necessarily contradict the PEP carboxylase hypothesis suggested above. The actual respiration rates of these organs may increase so much with increased N contents that the increase cannot be offset by the increased refixation rates by PEP carboxylase. Consequently,

415 the observed rates of CO_2 evolved from heterotrophic organs may still increase even though the
416 refixation rates have increased with increased N contents.
417 The PEP carboxylase hypothesis does imply that the CO_2 escaped to outside from the
418 heterotrophic organs are depleted in ^{13}C compared to the substrates utilized for respiration. As
419 summarized in the review of Ghashghaie and Badeck (2014), most isotopic studies on root
420 respiration have found that CO_2 evolved from roots are depleted in ^{13}C compared with bulk root
421 material, in contrast to leaf dark respiration which is generally enriched. For stem respiration,
422 however, more contradictory results have been reported. Wingate *et al*. (2010) showed that CO_2
423 evolved from stems of *Pinus pinaster* was depleted in ^{13}C compared with the currently measured net
424 CO_2 flux by photosynthetic branches or with the phloem water-soluble organic matter and wood
425 cellulose. Gessler *et al*. (2009) also found that the respiration of stems as well as roots of *Ricinus*
426 *communis* was depleted in ^{13}C relative to the assumed respiratory substrates. This latter study was
427 particularly relevant to this present study because the authors determined that the depletion was
428 caused by a strong refixation of respiratory CO_2 catalyzed by PEP carboxylase. In contrast to these
429 studies, Damesin and Lelarge (2003) reported that stem respiration of *Fagus sylvatica* was enriched
430 in ^{13}C compared with the total organic matter while Kodama *et al*. (2008) showed that CO_2 evolved
431 from the stem of *Pinus sylvestris* had higher or similar $\delta^{13}C$ values compared to that of phloem
432 exudate organic matter, depending on respiration rates. More studies are needed to determine
433 whether carbon isotope fractionations of stem respiration depend on species, ages, or environments.
434 Also, the dissolution and hydration of respiratory CO_2 may decouple in location from the fixation
435 of HCO_3^- by PEP carboxylase if there is a strong transpiration stream in xylem, with isotopic
436 consequences. For example, respiratory CO_2 can be dissolved and hydrated in roots and stems but
437 the HCO_3^- molecules formed can be carried up in xylem transpiration streams (Aubrey & Teskey
438 2009; Angert & Sherer 2011; Bloemen *et al*. 2013, Trumbore *et al* 2013) and fixed by PEP
439 carboxylase in branches, which will serve to redistribute isotope signatures among different parts of
440 the plant body.
441 Additional studies are also needed to determine whether there are other causes for the observed
442 heterotrophic N – ^{13}C enrichment relationship. For example, if different organ N contents are
443 associated with chemical compounds with different isotope signatures or different ‘fragmentation

fractionation' (enzymatic reaction of substrate molecules with heterogeneous ^{13}C distribution; Tcherkez *et al*. 2004; Hobbie and Werner 2004), one may expect organ N contents to be correlated with organ isotope signatures, potentially leading to the observed relationship. Another possibility to consider is that atmospheric $\delta^{13}C$ has been decreasing since the Industrial Revolution due to the emission of ^{13}C-depleted fossil CO_2. If a heterotrophic organ contains a higher fraction of carbon with an old age, then its bulk $\delta^{13}C$ would be higher. Stems and roots should contain more old carbon than leaves do. We do not have data to quantify this possibility. However, a qualitative reasoning led us to doubt that a general decreasing trend in atmospheric $\delta^{13}C$ can explain the observed heterotrophic N – ^{13}C enrichment relationship. Although we do not know the ages of the six nebkhas excavated, atmospheric N deposition has probably been increasing during the life time of these nebkhas. Therefore younger tissues should contain lower $\delta^{13}C$ and higher N, which would imply a negative N – ^{13}C enrichment relationship, opposite to what we observed. Therefore the positive heterotrophic N – ^{13}C enrichment relationship most likely has a phytogenic, rather than an atmospheric, origin.

It is important to clarify that our suggestion that the process responsible for the positive heterotrophic N - ^{13}C enrichment relationship resides in heterotrophic organs does not imply that the cause(s) for heterotrophic enrichment of ^{13}C relative to leaves resides entirely in heterotrophic organs. In fact, to explain the full magnitude of the observed heterotrophic enrichment (2‰), about 35% (100 × 2/5.7) of the carbon of heterotrophic organs has to have cycled through PEP carboxylase once, which appears to be surprisingly large for C_3 plants (Hobbie et al. 2003). Also, our finding that the $\delta^{13}C$ of leaves in the early growing season was close to the mean isotope ratio of roots but decreased as the season progressed indicates that processes inside leaves must also contribute to the overall isotope differences between leaves and heterotrophic organs if the leaf samples for reference are from middle to late growing seasons. The reference leaf samples in our calculation of $\Delta^{13}C_{organ}$were from middle growing seasons (August). Therefore, the progressive seasonal depletion in foliar ^{13}C increased the magnitude of the obtained $\Delta^{13}C_{organ}$. Furthermore, processes such as preferential loading into phloem of the heavier isotope and loss of depleted outer bark materials should also affect the overall autotrophic – heterotrophic isotope differences (Cernusak *et al*. 2009; Ghashghaie and Badeck 2014). While these processes may boost the overall magnitude of heterotrophic ^{13}C

enrichment, they cannot explain its relationship with N content among heterotrophic organs.

It is likely that leaf N also plays an important role in determining ^{13}C enrichment in heterotrophic organs relative to leaves. We do not have enough leaf-level data to examine this issue in depth but findings from previous studies allow us to speculate about what this role might be. As discussed early, leaf N content is positively correlated with leaf $\delta^{13}C$ because higher leaf N increases leaf photosynthetic capacity, which results in decreased C_i/C_a ratios and thus reduced discrimination against ^{13}C during photosynthesis (Sparks and Ehleringer 1997; Livingston *et al*. 1999; Duursma and Marshall 2006; Cernusak *et al*. 2007, 2013). However, a positive relationship between leaf N and leaf $\delta^{13}C$ does not necessarily mean that higher leaf N will reduce the degree of heterotrophic enrichment in ^{13}C compared to leaves as heterotrophic organs use photosynthetic products from leaves. An interesting pathway for leaf N to influence heterotrophic ^{13}C enrichment may lie in the relationship between leaf N and dark respiration. It is known that leaf dark respiration scales with leaf N (Reich *et al*. 2008). It is also known that leaf dark respiration is enriched in ^{13}C, contrary to respirations of stems and roots (Ghashghaie and Badeck 2014). Thus higher leaf N may actually increase the depletion of ^{13}C in leaves relative to heterotrophic organs. Consequently one may expect that N in autotrophic and heterotrophic organs of plants contributes to the isotope difference between these two types of organs in the same direction but through fundamentally different mechanisms.

Our analyses benefitted from the large variations in nutrient contents and heterotrophic ^{13}C enrichment both across plant organs and between sites, allowing any relationship (if exists) between these two sets of variables to be seen clearly. The large variations across plant organs are a validation of our systemic, whole-plant sampling strategy. The large between-site differences in organ nutrient contents likely reflect a site difference in soil fertility. The soil of vegetated area at Dengkou contained 0.024±0.006% N (Jia 2010) while at Minqin the value was 0.01±0.001% (Song et al. 2012), explaining the generally higher plant organ N contents at Dengkou than at Minqin. Soil P contents have not been measured at either site. However, we suspect that soil at Dengkou was also richer in P than at Minqin as plant organs generally contained higher P contents at the former than latter site. The cross-organ variations in nutrient contents were larger at Dengkou than at Minqin, possibly because poorer soil nutrient availability limited organ nutrient content variations at the latter site. Correspondingly, the range of heterotrophic ^{13}C enrichment was also wider at Dengkou than at Minqin. Both the cross-organ and between-site variations contributed the observed relationship

between the N content and heterotrophic ^{13}C enrichment. However, even within the same site, a pattern between N content and heterotrophic ^{13}C enrichment can be clearly seen, particularly at the Dengkou site. Further, the patterns of the two sites appear to be consistent with each other and form a single relationship. This consistency suggests that the same mechanism operates at the two sites to generate a unified dependence of ^{13}C enrichment on N content across heterotrophic plant organs.

The lack of a clear relationship between P content and heterotrophic ^{13}C enrichment (Fig. 5c and Fig. S1c) is interesting. In plants, proteins, which are rich in N, must be maintained with an allocation of a certain fraction of total body P to ribosomal ribonucleic acid (rRNA) (Niklas et al. 2005; Elser et al. 2010). Thus the N and P contents are generally positively correlated and the measurements from Minqin and Dengkou are no exception (Fig. S2). So why is there is a clear dependence of heterotrophic ^{13}C enrichment on N but not on P? It could be that the relationship of heterotrophic ^{13}C enrichment with P is considerably weaker than that with N and our data were not sensitive enough to detect it.

The relationship of heterotrophic ^{13}C enrichment with the N/P ratio (Fig. 5e and S1e) is broadly similar to that with N (Fig. 5b and S1b), suggesting that the relationship of heterotrophic ^{13}C enrichment with the N/P ratio is largely due to the effect of N rather than to the ratio itself. However, some level of direct dependence of the enrichment on the N/P ratio cannot be ruled out. Niklas et al. (2005) and Elser et al. (2010) integrated biological stoichiometry and metabolic scaling theories, which led them to suggest that growth rates and plant sizes should be related to N/P ratios. These authors' analyses focused on individual plants while our study is on plant organs. However, if the N/P ratio affects fractionating metabolic processes of plant organs, it is conceivable that the N/P ratio can also affect the ^{13}C enrichment (or depletion) of this organ relative to leaves.

CONCLUSION

We conclude that heterotrophic ^{13}C enrichment is affected jointly by fractionation processes occurring within heterotrophic organs and within leaves. Processes taking place between heterotrophic organs and leaves (e.g., preferential phloem loading of ^{13}C enriched sugars) may also contribute to this phenomenon. A nitrogen-mediated process, hypothesized to be the CO_2 refixation by PEP carboxylase, may be responsible for variations in ^{13}C enrichment within heterotrophic organs while processes within leaves or between leaves and heterotrophic organs may determine the overall

magnitude of heterotrophic ^{13}C enrichment. We suggest that future efforts should focus on the roles of nitrogen and refixation of respiratory CO_2 by PEP carboxylase in carbon isotope fractionation processes both within leaves and within heterotrophic organs as well as in between them. The findings of this study may have implications beyond isotope ecology. There has been a general lack of studies of refixation of respiratory CO_2 by PEP carboxylase in C_3 plant species. To our knowledge, no current terrestrial carbon cycle models consider this post-photosynthetic process. If PEP carboxylase can significantly affect carbon isotope compositions in heterotrophic organs of C_3 plant species, it may very well have strong influence on post-photosynthetic plant carbon budget and therefore terrestrial ecosystem carbon balance.

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Table 1. Main geometrical and biometrical characteristics of the nebkhas excavated in this study.

Nebkha	Dengkou-1	Dengkou-2	Dengkou-3	Minqin-1	Minqin-2	Minqin-3
Major axis (m)	13.6	9.9	3.65	4	4.6	6.4
Minor axis (m)	8.38	5.9	3.24	3.5	2.9	4.6
Height (m)	2.02	1.38	0.57	0.35	0.44	0.8
Plant cover (%)	80	70	80	11	15	7
Below-plain rooting depth (cm)	< 60	< 40	< 40	< 80	< 80	< 80
Leaf biomass (g C m^{-2} & %)	62.9 (10)	93.7 (12)	85.1 (11)	12.7 (6)	23.0 (11)	11.0 (9)
Stem biomass (g C m^{-2} & %)	159.7 (25)	169.3 (22)	213.3 (28)	35.2 (16)	70.0 (34)	22.2 (19)
In-sand root biomass (g C m^{-2} & %)	289.9 (45)	370.6 (47)	214.7 (28)	92.0 (41)	34.9 (17)	51.9 (44)
Blow-plain root biomass (g C m^{-2} & %)	137.7 (21)	148.7 (19)	260.8 (34)	84.5 (38)	80.5 (39)	32.5 (28)
Total biomass (g C m^{-2} & %)	650.2 (100)	782.3 (100)	773.9 (100)	224.4 (100)	208.3 (100)	117.6 (100)

Figure Captions

Figure 1. Flowers (top, 10 June 2009, Minqin), fruits (middle, 18 July 2009, Minqin) and nebkha (bottom, 3 August 2010, Dengkou) of *Nitraria tangutorum* Bobrov. Pictures courtesy of Jianmin Chu, Research Institute of Forestry, Chinese Academy of Forestry.

Figure 2. The difference in carbon isotope compositions between leaves and heterotrophic organs of *Nitraria tangutorum* Bobrov, which is measured by $\Delta^{13}C_{organ}$ in Eq (1) and averaged across the nebkhas excavated at the same study site (Dengkou or Minqin). Negative values indicate ^{13}C enrichment in heterotrophic organs compared to leaves. Upper-case letters denote ANOVA results within a study site (i.e., comparing $\Delta^{13}C_{organ}$ among different organs at the same site) and lower case letters between the two sites (i.e., comparing $\Delta^{13}C_{organ}$ of the same organ between the two sites). IS stands for in-sand, FR fine root and CR coarse root. 1, 2, 3 and 4 in front of FR or CR stand for 0 - 20, 20 – 40, 40 – 60 and 60 – 80 cm below the plains on which nebkhas rest. Woody debris (WD) from dead ramets is also included in the figure. No ANOVA results for 3FR and 3CR at the Dengkou site as there was only one nebkha having roots between 40 to 60 cm. No roots were found below 60 cm at the Dengkou site.

Figure 3. Carbon (C) (a), nitrogen (N) (b) and phosphorous (P) content (c) of different organs of *Nitraria tangutorum* Bobrov, at the Dengkou and Minqin study sites. Symbols and letters denoting ANOVA results are explained in Figure 2.

Figure 4. Carbon (C) to nitrogen (N) (a), N to phosphorous (P) (b) and C to P mass ratios (c) of different organs of *Nitraria tangutorum* Bobrov, at the Dengkou and Minqin study sites. Symbols and letters denoting ANOVA results are explained in Figure 2.

Figure 5. Nutrient dependence of the difference in carbon isotope compositions between leaves and heterotrophic organs of *Nitraria tangutorum* Bobrov, which is measured by $\Delta^{13}C_{organ}$ in Eq (1) and

averaged across the nebkhas excavated at the same study site. Negative values indicate ^{13}C enrichment in heterotrophic organs compared to leaves. Changes of $\Delta^{13}C_{organ}$ as a function of organ contents of carbon (C) (a), nitrogen (N) (b) and phosphorous (P) (c) and of organ mass ratios of C to N (d), N to P (e), and C to P (f). The two arrows in (b) indicate values for woody debris from dead ramets at each study site while in (d) indicates an outlier caused by measurements in phosphorous content (see the outlier in c and f).

Figure 6. Seasonal changes in the ratios of leaf carbon isotopes (a) and intercellular (C_i) to ambient (C_a) CO_2 concentrations of *Nitraria tangutorum* Bobrov at the Dengkou and Minqin study sites. For comparison, the biomass-averaged isotope ratios of roots from the excavated nebkhas are also shown in (a).

Figure 1

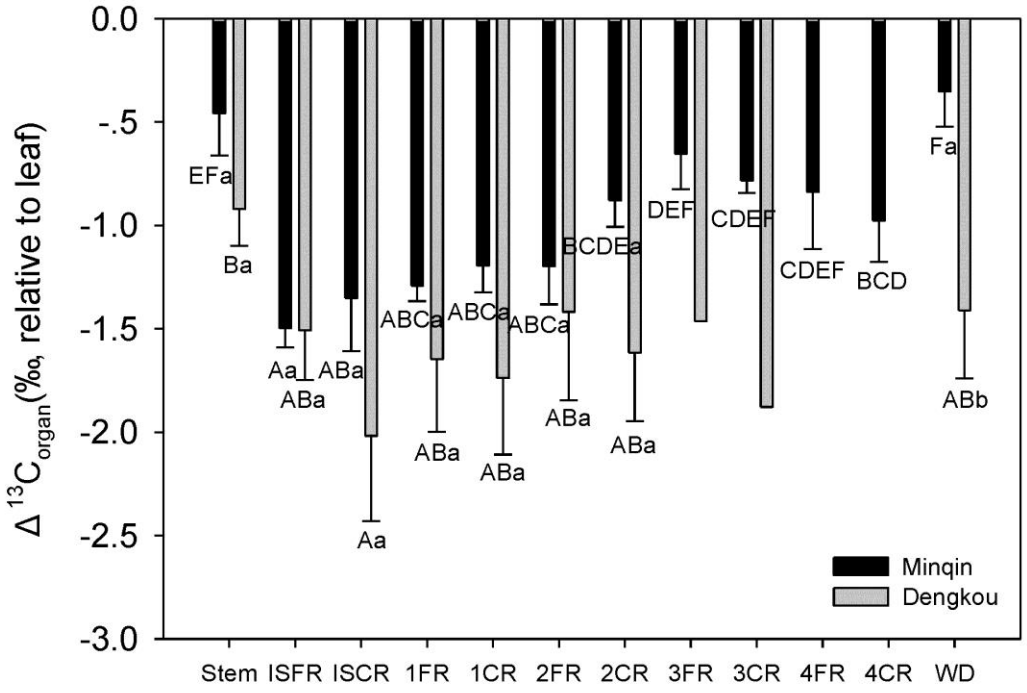
0.0
-.5
-1.0
-1.5
-2.0
-2.5
-3.0
Δ 13Corgan(‰, relative to leaf)
EFa
Ba
Aa
ABa
ABa
Aa
ABCa
ABa
ABCa
ABa
ABCa
ABa
BCDEa
ABa
DEF
CDEF
CDEF
BCD
Fa
ABb
Minqin
Dengkou
Stem
ISFR
ISCR
1FR
1CR
2FR
2CR
3FR
3CR
4FR
4CR
WD

Figure 2

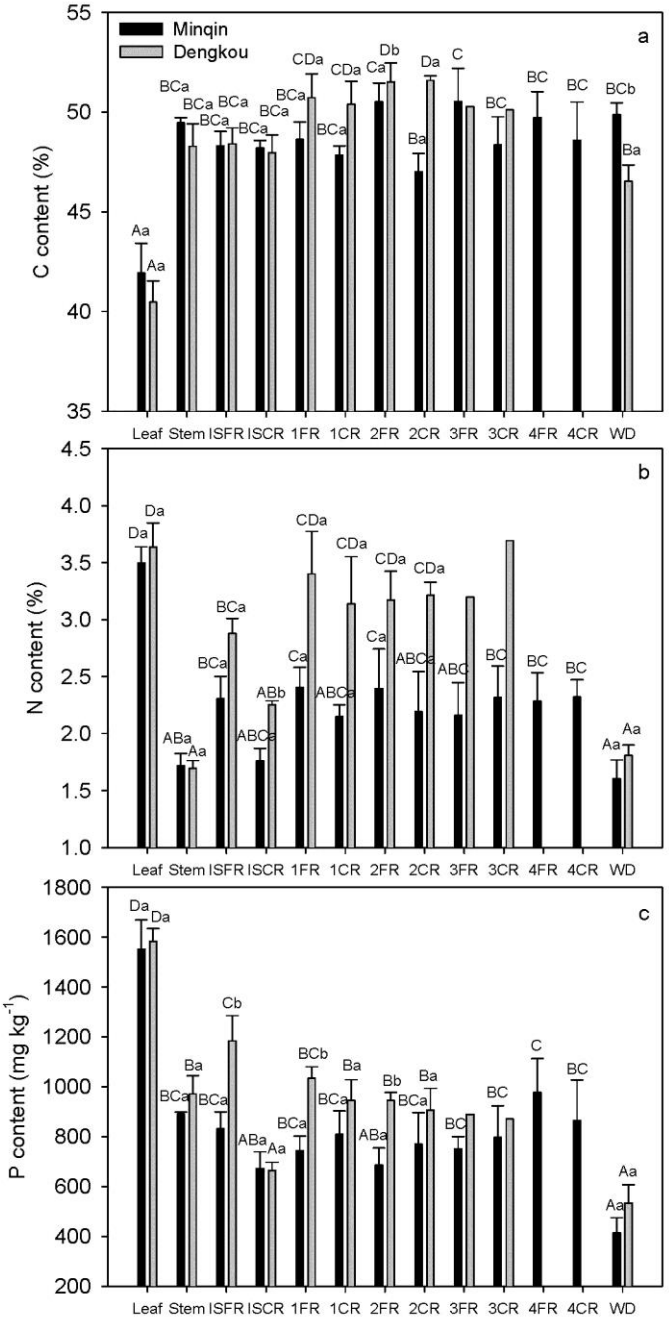
Minqin
Dengkou
a
C content (%)
b
N content (%)
c
P content (mg kg-1)
Leaf Stem ISFR ISCR 1FR 1CR 2FR 2CR 3FR 3CR 4FR 4CR WD

Figure 3

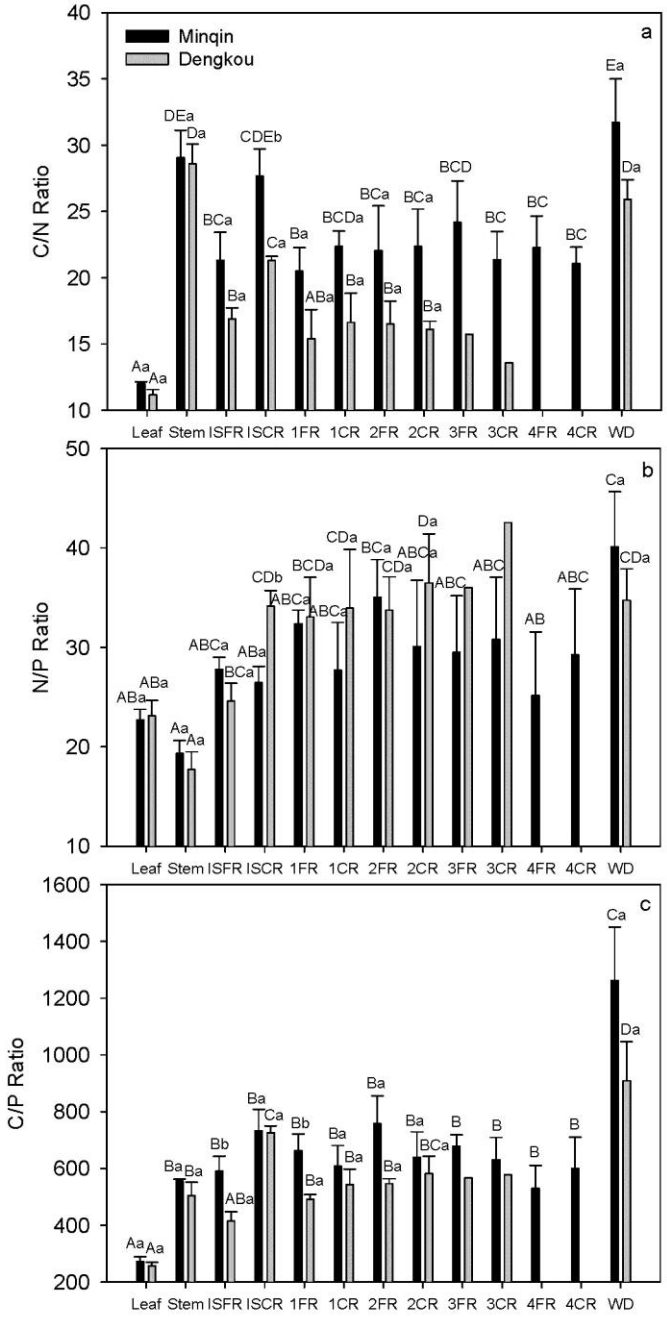
a
Minqin
Dengkou
C/N Ratio
40
35
30
25
20
15
10
Aa Aa
DEa Da
BCa Ba
CDEb Ca
Ba ABa
BCDa Ba
BCa Ba
BCa Ba
BCD
BC
BC
BC
Ea Da
Leaf Stem ISFR ISCR 1FR 1CR 2FR 2CR 3FR 3CR 4FR 4CR WD
b
N/P Ratio
50
40
30
20
10
ABa ABa
Aa Aa
ABCa BCa
ABa
CDb ABCa
BCDa ABCa
CDa
BCa CDa
ABCa Da
ABC
ABC
AB
ABC
Ca CDa
Leaf Stem ISFR ISCR 1FR 1CR 2FR 2CR 3FR 3CR 4FR 4CR WD
c
C/P Ratio
1600
1400
1200
1000
800
600
400
200
Aa Aa
Ba Ba
Bb ABa
Ba Ca
Bb Ba
Ba Ba
Ba Ba
Ba BCa
B
B
B
B
Ca Da
Leaf Stem ISFR ISCR 1FR 1CR 2FR 2CR 3FR 3CR 4FR 4CR WD

Figure 4

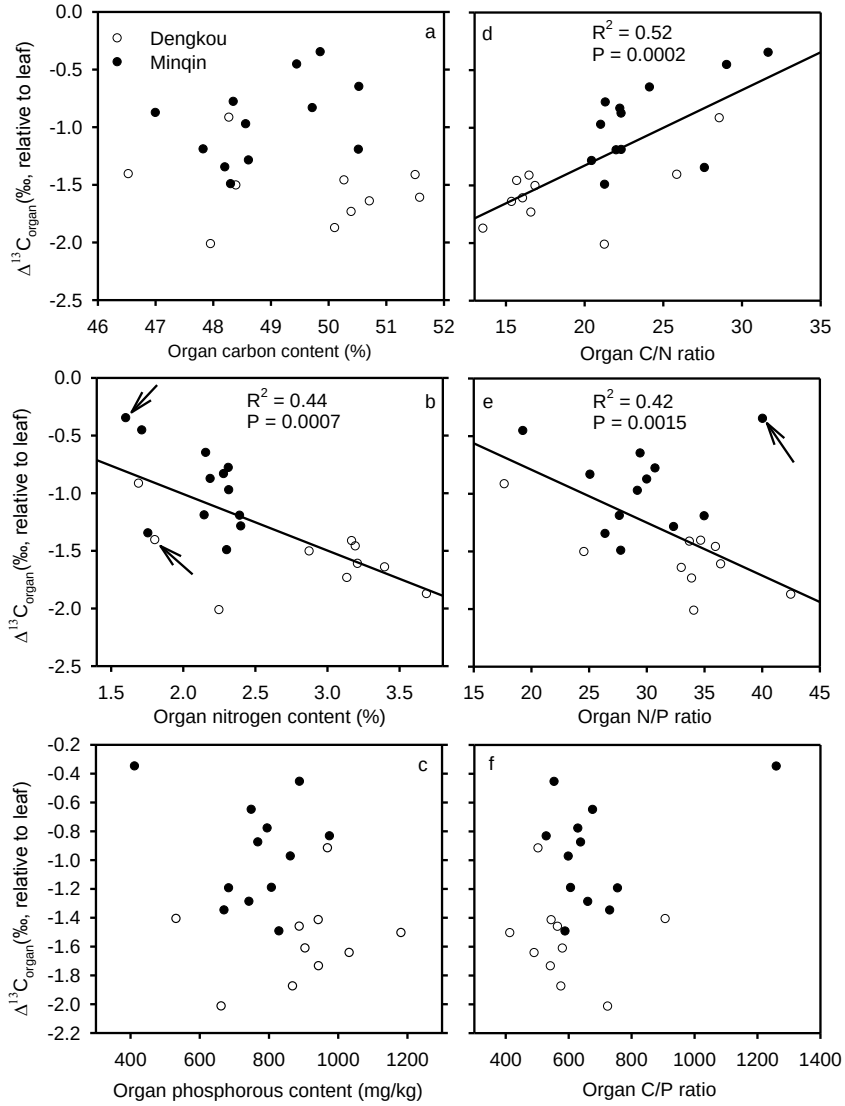
Dengkou
Minqin
a
b
c
d
e
f
R2 = 0.52
P = 0.0002
R2 = 0.44
P = 0.0007
R2 = 0.42
P = 0.0015
Δ13Corgan(‰, relative to leaf)
Organ carbon content (%)
Organ C/N ratio
Organ nitrogen content (%)
Organ N/P ratio
Organ phosphorous content (mg/kg)
Organ C/P ratio

Figure 5

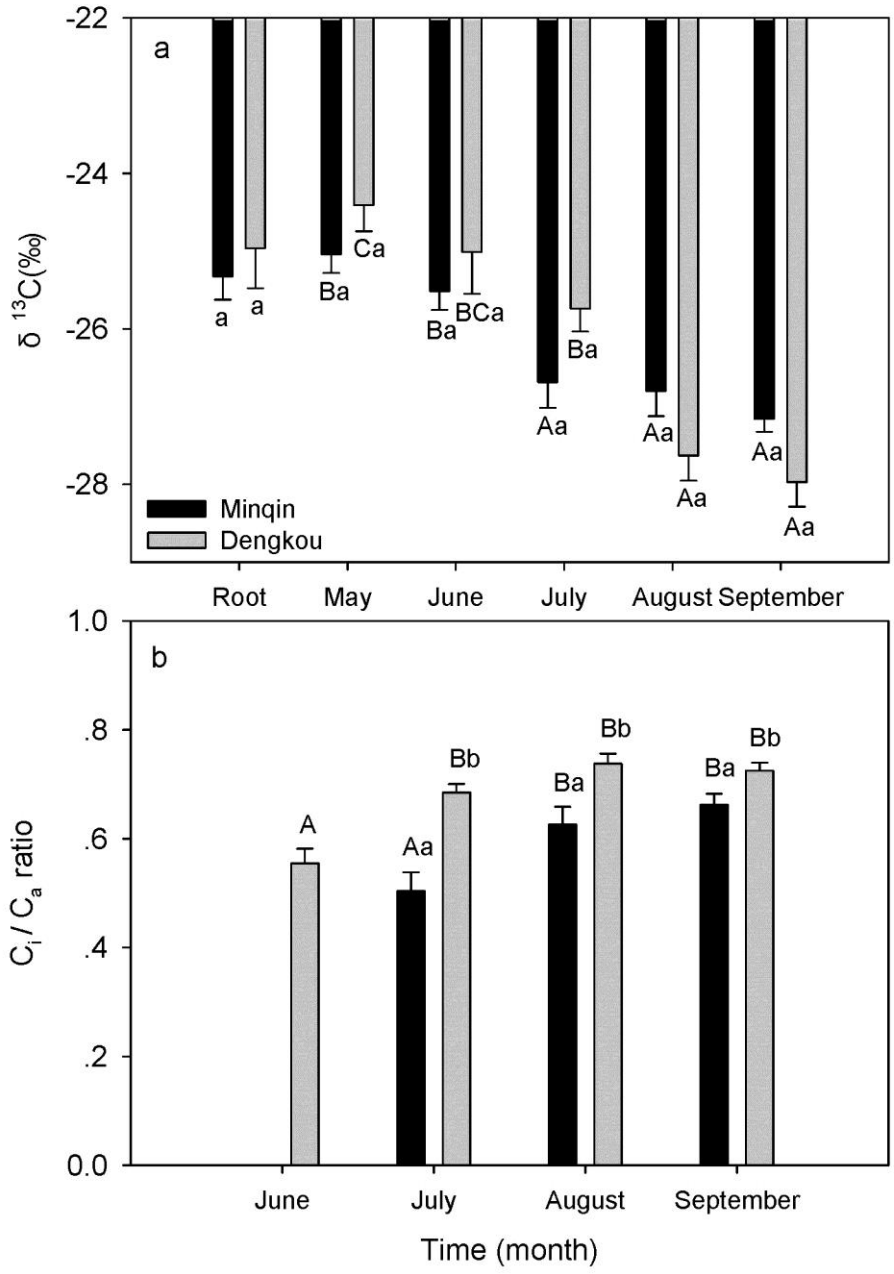
a
-22
-24
-26
-28
δ 13C(‰)
a
a
Ba
Ca
Ba
BCa
Aa
Ba
Aa
Aa
Aa
Aa
Minqin
Dengkou
Root
May
June
July
August
September
b
1.0
.8
.6
.4
.2
0.0
Ci / Ca ratio
A
Aa
Bb
Ba
Bb
Ba
Bb
June
July
August
September
Time (month)

Figure 6

Figure S1. Nutrient dependence of the difference in carbon isotope compositions between leaves and heterotrophic organs of *Nitraria tangutorum* Bobrov, which is measured by $\Delta^{13}C_{organ}$ in Eq (1) and averaged across the nebkhas excavated at the same study site. Negative values indicate ^{13}C enrichment in heterotrophic organs compared to leaves. Changes of $\Delta^{13}C_{organ}$ as a function of organ contents of carbon (C) (a), nitrogen (N) (b) and phosphorous (P) (c) and of organ mass ratios of C to N (d), N to P (e), and C to P (f). The two arrows in (b) indicate values for woody debris from dead ramets at each study site while in (d) indicates an outlier caused by measurements in phosphorous content (see the outlier in c and f). All nutrient values are normalized (divided) by their corresponding values in the leaves.

Figure S2. The relationship between organ nitrogen (N) and phosphorus (P) contents at the Dengkou (open circles) and Minqin (dots) sites.

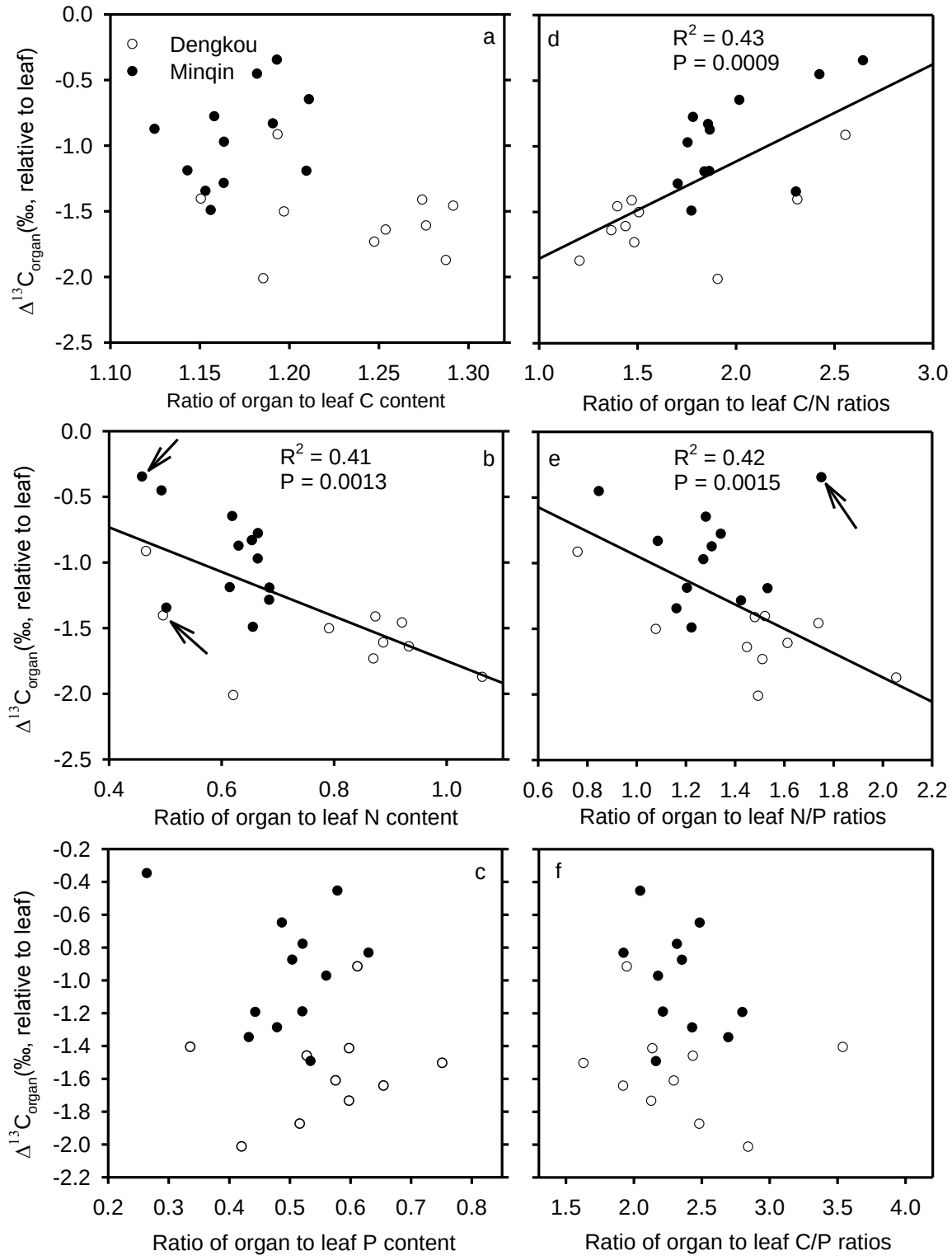
Dengkou
Minqin
a
b
c
d
e
f
R^2 = 0.43
P = 0.0009
R^2 = 0.41
P = 0.0013
R^2 = 0.42
P = 0.0015
$\Delta^{13}C_{organ}$(‰, relative to leaf)
Ratio of organ to leaf C content
Ratio of organ to leaf C/N ratios
Ratio of organ to leaf N content
Ratio of organ to leaf N/P ratios
Ratio of organ to leaf P content
Ratio of organ to leaf C/P ratios

Figure S1

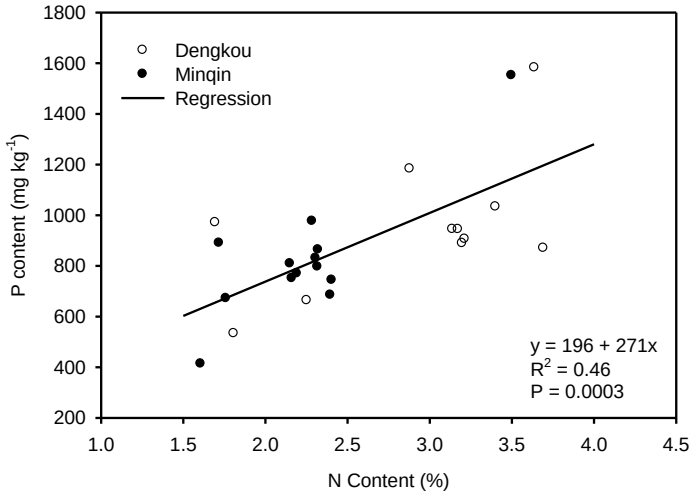
Dengkou
Minqin
Regression
y = 196 + 271x
R2 = 0.46
P = 0.0003
P content (mg kg-1)
N Content (%)
1800
1600
1400
1200
1000
800
600
400
200
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

Figure S2