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RESEARCH ARTICLE

Carbon Isotope Composition of Nighttime Leaf-Respired CO_2 in the Agricultural-Pastoral Zone of the Songnen Plain, Northeast China

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Abstract

Variations in the carbon isotope signature of leaf dark-respired CO_2 ($\delta^{13}C_R$) within a single night is a widely observed phenomenon. However, it is unclear whether there are plant functional type differences with regard to the amplitude of the nighttime variation in $\delta^{13}C_R$. These differences, if present, would be important for interpreting the short-term variations in the stable carbon signature of ecosystem respiration and the partitioning of carbon fluxes. To assess the plant functional type differences relating to the magnitude of the nighttime variation in $\delta^{13}C_R$ and the respiratory apparent fractionation, we measured the $\delta^{13}C_R$, the leaf gas exchange, and the $\delta^{13}C$ of the respiratory substrates of 22 species present in the agricultural-pastoral zone of the Songnen Plain, northeast China. The species studied were grouped into C_3 and C_4 plants, trees, grasses, and herbs. A significant nocturnal shift in $\delta^{13}C_R$ was detected in 20 of the studied species, with the magnitude of the shift ranging from 1‰ to 5.8‰. The magnitude of the nighttime variation in $\delta^{13}C_R$ was strongly correlated with the daytime cumulative carbon assimilation, which suggests that variation in $\delta^{13}C_R$ were influenced, to some extent, by changes in the contribution of malate decarboxylation to total respiratory CO_2 flux. There were no differences in the magnitude of the nighttime variation in $\delta^{13}C_R$ between the C_3 and C_4 plants, as well as among the woody plants, herbs and graminoids. Leaf respired CO_2 was enriched in ^{13}C compared to biomass, soluble carbohydrates and lipids; however the magnitude of enrichment differed between 8 pm and 4 am, which were mainly caused by the changes in $\delta^{13}C_R$. We also detected the plant functional type differences in respiratory apparent fractionation relative to biomass at 4 am, which suggests that caution should be exercised when using the $\delta^{13}C$ of bulk leaf material as a proxy for the $\delta^{13}C$ of leaf-respired CO_2.

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Introduction

The stable C isotope composition ($\delta^{13}C$) has been widely used to trace the carbon flow within ecosystem components or between the ecosystem and the atmosphere [1–7]. The applications of stable carbon isotope technique require a mechanistic understanding of the temporal and spatial variation in the $\delta^{13}C$ signature of component fluxes [8–10]. As an important component of ecosystem carbon fluxes, leaf respired CO_2 has been reported to vary substantially in its carbon isotope composition at diurnal timescale [11–15]. Unfortunately, we still lack a comprehensive understanding of the processes controlling the dynamics in the $\delta^{13}C$ signature of leaf respired CO_2 [9, 16].

It has been extensively reported that $\delta^{13}C_R$ varied substantially, up to 14.8‰, on a diurnal timescale [12, 13, 17, 18]. Several mechanisms have been developed to explain short-term variation in $\delta^{13}C_R$. Firstly, intramolecular ^{13}C distribution is not homogeneous in hexose molecules, which combined with changes in the relative contribution of the metabolic pathways to the respiration could lead to variation in $\delta^{13}C_R$ [14, 19–22]. Secondly, shifts in $\delta^{13}C_R$ can be attributed to the changes in the contribution of malate (^{13}C-enriched) decarboxylation to the overall respiratory flux [8, 23]. Thirdly, changes in the use of the respiratory substrates having different $\delta^{13}C$ may subsequently affect $\delta^{13}C_R$ [16, 18]. Finally, short variation in carbohydrate pool size may also influence $\delta^{13}C_R$ through affecting the allocation of respiratory intermediates [24]. The results of previous study showed substantial intraspecific and interspecific differences in the amplitude of the short-term variation in $\delta^{13}C_R$ [14, 18, 25]. Intraspecific differences in the range of the diurnal variation in $\delta^{13}C_l$ are caused mainly by the availability of resources associated changes in the substrate availability and the allocation of the respiratory intermediates [25]. The findings of previous studies by Werner et al. (2007) and Priault et al. (2009) indicated large diurnal variations in the $\delta^{13}C_R$ of slow-growing aromatic plants, whereas no apparent diurnal shift was found in the $\delta^{13}C_R$ in temperate trees and fast-growing herbs. These results highlight the potential plant functional type differences relating to the extent of the variation in $\delta^{13}C_R$ on a diurnal timescale [11, 14, 22]. However, plant functional type differences in the magnitude of short-term variation in $\delta^{13}C_R$ need to be further explored, especially for C_3 and C_4 species which differed substantially in the magnitude of heterogeneous ^{13}C distribution within hexose molecules [21, 26].

Leaf dark-respired CO_2 is often enriched in ^{13}C compared with leaf bulk tissue or other potential respiratory substrates, such as starch, soluble carbohydrates, and others [27–29]. This phenomenon is attributed mainly to non-homogeneous ^{13}C distribution among the carbon atoms within the hexose molecules and the incomplete oxidation of hexoses. However, the phenomenon is also attributed partially to the utilization of isotopically different respiratory substrates [21, 24, 30]. Because of the intramolecular $^{13}C/^{12}C$ differences, CO_2 that evolved from pyruvate decarboxylation contains more ^{13}C relative to that derived from acetyl-CoA oxidation [31, 32]. Depending on substrate availability, ^{13}C-depleted acetyl-CoA could be used for the biosynthesis of lipids and secondary compounds, or decarboxylation in the TCA cycle to generate adenosine triphosphate (ATP). Therefore, the incomplete oxidation of acetyl-CoA could lead to ^{13}C enrichment in leaf dark-respired CO_2 relative to respiratory substrates. The plant functional types differed substantially with regard to the allocation of ^{13}C-depleted compounds. Trees, for instance, allocated proportionally more acetyl-CoA to the synthesis of lipids and lignin than did grasses [14, 26]. This could lead to the leaf-respired CO_2 in trees being more enriched in ^{13}C in comparison with the putative respiratory substrates or the bulk leaf material. Indeed, plant functional type differences in respiratory apparent fractionation have been reported between C_3 and C_4 species, but not between woody plants and C_3 herbs [15, 16]. However, these findings were derived from meta-analysis of results obtained under various

growing habitats and using different methods, which makes the conclusions unreliable. Therefore, the existence of plant functional type differences with regard to respiratory apparent fractionation needs to be further verified and, if they do exist, we need to incorporate these into the flux partitioning.

The vegetation of the agricultural-pastoral zone of the Songnen Plain is characterized as mosaics of grassland and cultivated land, dominated mainly by C_3 species and C_4 species, respectively. We measured the $\delta^{13}C_R$ and $\delta^{13}C$ of the putative primary respiratory substrates of 22 species at 8 pm and 4 am. The leaf gas-exchange parameters, including net assimilation rate, stomatal conductance, and nighttime respiration rate were also measured. The plant species were grouped into C_3 and C_4 species, trees, graminoids, and herbs. The objectives of the present study were to assess the plant functional type differences in the range of nighttime variation in $\delta^{13}C_R$ and the respiratory apparent fractionation relative to the bulk leaf material and the putative respiratory substrates.

Materials and Methods

Ethics Statement

No specific permissions were required for the field studies described, because the Songnen Grassland Ecological Research Station is a department of the Northeast Normal University. No specific permissions were required for the study either, as it was conducted in accordance with the guidelines set by the Northeast Normal University. No specific permissions were required for the locations or the activities. No location was privately owned or protected in any way, and the field studies did not involve endangered or protected species.

Study site

The study was conducted in the agricultural-pastoral zone of the Songnen Plain (44°40′–44° 44′N, 123°44′–123°47′E, elevation 138–144 m) in northeast China. The study area has a semi-arid continental climate, with a mean annual temperature of 6.4°C. The mean annual rainfall is 471 mm, with over 70% of the precipitation occurs from June to August (Fig 1A). Drought, especially spring drought, occurs frequently in the studied area during the growing season; however there is no fixed drought period. The duration of the frost-free season is approximately 150 days. Detailed information on daily precipitation and daily temperature in 2012 is provided in Fig 1B. The main soil type of the study area is chernozem, with a soil organic carbon content of 2.0% and a soil total nitrogen content of 0.15% [33]. The vegetation in the study area is characterized as mosaics of grassland and cultivated land. The grassland is dominated by *Leymus chinensis*, while *Phragmites australis* and *Chloris virgata* are present in abundance [34]. The major crops on the cultivated land are *Zea mays*, *Setaria italica*, *Helianthus annuus*, and *Sorghum bicolor*. We studied 22 plant species, representative of the major species present on the grassland and the cultivated land. The details of the species studied are provided in Table 1. The field studies were conducted from July 25th to August 5th in 2012, which is the peak biomass season for the study area. None of the studied species showed signs of senescence. Information on the development stage of the studied species is provided in Table 1.

Leaf gas-exchange measurement

Leaf photosynthesis was measured every three hours, from 6 am to 6 pm, using a LI-6400 infrared gas-exchange analyzer (LI-COR Biosciences Inc., Lincoln, NE, USA). We selected fully expanded leaves from the sunny side for leaf photosynthetic rate measurements. Before each measurement, the leaf chamber conditions were set to match the environmental conditions,

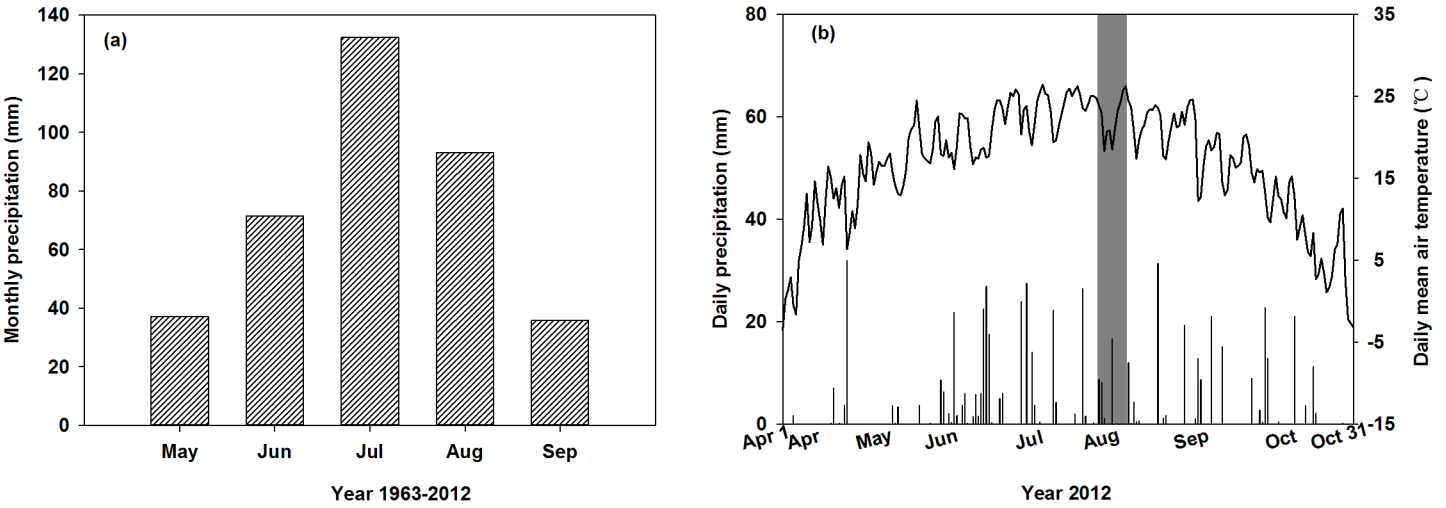

Fig 1. Growing season average monthly precipitation (mm) from 1963 to 2012 (a), and daily precipitation (mm) and daily mean air temperature (°C) from April 1th to October 31th in 2012 (b). The shaded area in panel b denotes the field sampling period.

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including the photosynthetically active radiation, air temperature, and relative humidity. Leaf respiration rates at 8 pm and 4 am were measured under zero light intensity. The leaf photosynthesis and respiration measurements for each species were conducted on five randomly selected plants.

Table 1. Information on species studied. List of plant species studied, and their photosynthetic pathway, classification and phenological phase.

Species	Photosynthetic pathway	Functional type	Class	Phenological phase
Leymus chinensis	C_3	Graminoids	Monocotyledoneae	Fructescence
Phragmites australis	C_3	Graminoids	Monocotyledoneae	Florescence
Lespedeza bicolor	C_3	Trees	Dicotyledoneae	Florescence
Malus asiatica	C_3	Trees	Dicotyledoneae	Fructescence
Populus simonii	C_3	Trees	Dicotyledoneae	Post-fruiting vegetative stage
Prunus salicina	C_3	Trees	Dicotyledoneae	Fructescence
Chenopodium glaucum	C_3	Herbs	Dicotyledoneae	Florescence
Glycine max	C_3	Herbs	Dicotyledoneae	Flowering and pod formation stage
Helianthus annuus	C_3	Herbs	Dicotyledoneae	Florescence
Saussurea amara	C_3	Herbs	Dicotyledoneae	Florescence
Vigna radiata	C_3	Herbs	Dicotyledoneae	Flowering and pod formation stage
Vigna unguiculata	C_3	Herbs	Dicotyledoneae	Flowering and pod formation stage
Xanthium sibiricum	C_3	Herbs	Dicotyledoneae	Florescence
Chloris virgata	C_4	Graminoids	Monocotyledoneae	Florescence
Echinochloa crusgalli	C_4	Graminoids	Monocotyledoneae	Florescence
Hemarthria altissima	C_4	Graminoids	Monocotyledoneae	Heading stage
Panicum miliaceum	C_4	Graminoids	Monocotyledoneae	Florescence
Setaria italica	C_4	Graminoids	Monocotyledoneae	Florescence
Setaria viridis	C_4	Graminoids	Monocotyledoneae	Florescence
Sorghum bicolor	C_4	Graminoids	Monocotyledoneae	Elongation stage
Zea mays	C_4	Graminoids	Monocotyledoneae	Tasseling stage
Amaranthus retroflexus	C_4	Herbs	Dicotyledoneae	Florescence

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Collection of leaf-respired CO_2

Leaf-respired CO_2 was collected in the field using the gas-tight syringe incubation method [11]. In brief, young and fully expanded leaves (comparable to those used to measure the photosynthesis and the respiration rates), were detached and placed inside a gas-tight syringe barrel. The syringe barrel was subsequently flushed with CO_2-free air to remove the background CO_2. The syringe barrel was then sealed and incubated for 15 min to allow for the buildup of leaf-respired CO_2. After the incubation, 5 ml of air, containing leaf-respired CO_2, was injected into a 12 ml vial. The vial was filled with helium and fitted with septum caps. For each studied species, the leaf-respired CO_2 was collected twice (8 pm and 4 am) during a single night. For the period of field sampling, sunset time ranged from 7:17 pm to 7:04 pm, therefore the collection of leaf-respired CO_2 at 8 pm was conducted at least 40 min after sunset and in the darkness. The leaf-respired CO_2 was sampled each time on five randomly selected plants or populations.

Lipids, soluble carbohydrates, and starch extractions

Simultaneously with the leaf-respired CO_2 sampling, we collected leaves for measuring the carbon isotope composition of leaf bulk materials and potential respiratory substrates. The collected leaves were comparable (age and canopy position, etc.) with those used for the leaf photosynthesis measurements. The collected leaves were immersed in liquid nitrogen to stop the metabolic activities. They were subsequently stored at -80°C in a deep freezer before being freeze-dried with a Labconco freeze drier (Labconco Kansas City, MO, USA). A ball mill (MM 400 Retsch, Haan, Germany) was used to grind the freeze-dried leaves into fine powder.

We used the protocols described by Wanek et al. (2001) and Göttlicher et al. (2006) [35, 36] for the extraction of lipids, soluble carbohydrates, and starch. In brief, the powdered leaf material was extracted with methanol/chloroform/water (MCW; 12:5:3, v/v/v). After centrifugation, the chloroform and the deionized water were added to the supernatant for phase separation. The chloroform phase (containing lipids) was dried in a ventilation device and subsequently analyzed for the carbon isotope composition.

The upper water phase (containing soluble carbohydrates) was transferred into a reaction vial and oven dried for carbohydrate extraction. The residue was re-dissolved in deionized water. After centrifugation, the phase containing the soluble carbohydrates was purified by an ion-exchange column, including both anion- and cation-exchange resin. The eluent was collected and oven dried, and the carbon isotope composition was subsequently analyzed, using an isotope ratio mass spectrometer.

The plant materials were re-extracted with MCW to remove the residual lipids and the soluble carbohydrates. After the starch in the plant materials had been gelatinized and hydrolyzed, the aqueous phase was separated from the pellet by centrifugation. The upper aqueous phase was subsequently purified by a centrifugal filter unit (Microcon YM-10; Millipore, Billerica, MA, USA). The filtrate containing glucose originated from starch was oven dried in a tin capsule and then analyzed for the carbon isotope composition.

Carbon isotope ratio analysis

All carbon isotope ratio analyses were performed using an isotope ratio mass spectrometer (Isoprime 100, Isoprime Ltd., Manchester, UK). The precision of repeated $\delta^{13}C$ measurements on solid and gaseous working standards was $< 0.1‰$. The C isotope ratios are reported in

parts per thousand relative to Vienna Pee Dee Belemnite (VPDB) as

$$\delta^{13}\mathrm{C}(‰) = (R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1,000$$

Respiratory apparent $^{13}C/^{12}C$ fractionation

Respiratory apparent $^{13}C/^{12}C$ fractionation, relative to potential respiratory substrates, was calculated as:

$$\Delta_{R,X} = \frac{\delta^{13}\mathrm{C}_X - \delta^{13}\mathrm{C}_R}{1 + \delta^{13}\mathrm{C}_R} \quad (1)$$

where $\Delta_{R,X}$ represents the respiratory apparent $^{13}C/^{12}C$ fractionation, relative to substrate X, and $\delta^{13}C_X$ represents the carbon isotope composition of substrate X. X represents the leaf bulk materials, lipids, starch, or soluble carbohydrates, while $\delta^{13}C_R$ represents $\delta^{13}C$ of the leaf-respired CO_2.

Statistical analysis

We used one-way analysis of variance (ANOVA) to assess the differences between the photosynthetic pathways in the range of the nighttime variation of $\delta^{13}C_R$, the leaf net CO_2 assimilation rate (A), stomatal conductance (g_s), C_i/C_a, and the respiratory rate (R). Linear regression analysis was employed to assess the dependence of the magnitude of the nighttime variation in $\delta^{13}C_R$ on the cumulative carbon assimilation. One-way analysis of variance was conducted to assess the plant functional type differences with regard to the leaf gas-exchange parameters, the magnitude of the nocturnal shifts in $\delta^{13}C_R$, and the nighttime respiratory apparent fractionation. The Statistical Package for the Social Science (SPSS) (version 13.0, IBM, Armonk, NY, USA) was used for all statistical analyses. Data are reported as mean ± 1 standard error.

Results

Leaf gas exchange

For the species studied, the maximum leaf net CO_2 assimilation rate (Amax) in the C_3 species varied from 15.1±1.2 to 38.4±5.8 μmol m^{-2} s^{-1}; whereas it ranged from 22.3±1.7 to 33.7±1.6 μmol m^{-2} s^{-1} in the C_4 plants (Table 2). The average Amax value in the C_4 plants (28.1 μmol m^{-2} s^{-1}) was greater than it was in the C_3 plants (22.4 μmol m^{-2} s^{-1}) (Table 3). The mean leaf net CO_2 assimilation rate (Mean A) varied substantially between 9.6±0.4 and 24.0±0.5 μmol m^{-2} s^{-1} (Table 2). There were no statistically significant differences in Mean A between the C_3 and C_4 species (Table 3). Moreover, no apparent differences in Amax and Mean A were detected among the different plant functional types (Table 3).

The maximum daily stomatal conductance ($g_s$max) varied from 0.10±0.01 to 0.94±0.05 mol m^{-2} s^{-1}. Moreover, the apparent differences in $g_s$max were detected between the C_3 and the C_4 species (Tables 2 & 3). In addition, we detected significant differences in $g_s$max among the woody plants, forbs, and graminoids (Table 3). The mean respiration rate (Mean R) varied from 0.51±0.06 to 2.22±0.17 μmol m^{-2} s^{-1} (Table 2). There was no significant difference in the mean R among the functional groups, and it did not differ between the C_3 and the C_4 species either (Table 3). The mean ratio of the leaf intercellular air space to the ambient CO_2 concentration (Mean C_i/C_a) varied from 0.20±0.03 to 0.81±0.01 and 0.27±0.02 to 0.59±0.03 for the C_3 and C_4 species, respectively. The mean C_i/C_a in the C_3 plants was significantly greater than it

Table 2. Leaf gas-exchange data. Maximum leaf net CO_2 assimilation rate (*A*max, μmol m^{-2} s^{-1}), mean leaf net CO_2 assimilation rate (Mean *A*, μmol m^{-2} s^{-1}), maximum stomatal conductance ($g_s$max, mol m^{-2} s^{-1}), mean ratio of intercellular air space to ambient CO_2 concentration (Mean C_i/C_a), and nighttime mean respiratory rate (Mean *R*, μmol m^{-2} s^{-1}) of the species studied. Data are reported as mean ± 1 SE.

Species	*A*max	Mean *A*	$g_s$max	Mean C_i/C_a	Mean *R*
L. chinensis	19.9±3.99	11.8±0.82	0.295±0.022	0.75±0.01	1.32±0.16
P. australis	17.2±0.35	11.8±0.35	0.268±0.011	0.71 ±0.01	0.71 ±0.08
L. bicolor	22.6±1.87	12.2±0.67	0.586±0.047	0.78±0.01	2.07 ±0.26
M. asiatica	22.3±0.90	17.0±0.63	0.446±0.017	0.69±0.01	0.74 ±0.09
P. simonii	23.2±0.32	17.1±0.52	0.511±0.041	0.73±0.03	1.22 ±0.11
P. salicina	15.3±1.65	11.2±0.75	0.531±0.057	0.65±0.01	0.51 ±0.06
C. glaucum	28.8±1.04	21.1±0.43	0.943±0.051	0.80 ±0.01	1.28 ±0.13
G. max	17.0±0.54	10.8±0.49	0.098±0.005	0.20±0.03	1.45±0.11
H. annuus	21.1±0.13	13.1±0.28	0.128±0.002	0.44 ±0.01	1.10 ±0.10
S. amara	15.1±1.20	9.6 ±0.36	0.443±0.022	0.82 ±0.01	0.73 ±0.17
V. radiata	26.3±2.98	14.8±1.64	0.247±0.009	0.81 ±0.01	2.22±0.17
V. unguiculata	23.9±4.90	15.2±2.66	0.712±0.072	0.59 ±0.14	1.54 ±0.17
X. sibiricum	38.4±5.80	21.2±1.20	0.814±0.083	0.79 ±0.02	2.12 ±0.13
C. virgata	28.1±1.71	15.5±1.14	0.179±0.005	0.42 ±0.03	0.91 ±0.13
E. crusgalli	22.6±1.34	13.1±0.20	0.212±0.017	0.49 ±0.03	0.87 ±0.08
H. altissima	22.3±1.68	13.8±1.57	0.146±0.015	0.49 ±0.03	1.36 ±0.24
P. miliaceum	27.9±0.31	18.1±0.28	0.204±0.019	0.41 ±0.03	1.54 ±0.07
S. italica	30.8±2.27	18.0±0.63	0.247±0.013	0.41 ±0.03	0.82 ±0.40
S. viridis	27.6±1.88	15.3±0.57	0.199±0.007	0.49 ±0.01	1.15 ±0.11
S. bicolor	26.2±1.34	17.0±0.47	0.183±0.034	0.27 ±0.02	1.45 ±0.16
Z. mays	33.7±1.62	23.6 ±0.93	0.367±0.051	0.54 ±0.06	0.82 ±0.05
A. retroflexus	33.3±0.53	24.0±0.49	0.520±0.040	0.59 ±0.03	1.80 ±0.10

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was in the C_4 plants (Tables 2 & 3). Furthermore, no apparent differences in the Mean C_i/C_a were detected among the different functional types (Table 3).

$\delta^{13}C$ of nighttime leaf-respired CO_2

We observed a significant nocturnal shift of the $\delta^{13}C$ of leaf-respired CO_2 ($\delta^{13}C_R$) in 20 of the 22 species studied, the exceptions being *Chenopodium glaucum* and *Saussurea amara* (Table 4). The leaf-respired CO_2 at 8 pm was enriched in ^{13}C compared with that at 4 am. However, the C_3 and C_4 plants showed no statistical significant difference in the amplitude of the nighttime variation in $\delta^{13}C_R$ (Table 3). Moreover, no significant differences in the nocturnal shift in $\delta^{13}C_R$ were detected among the plant functional types (Table 3).

Correlations between the nocturnal shift in $\delta^{13}C_R$ and cumulative carbon assimilation

We calculated the daytime cumulative carbon assimilation, using gas-exchange data to explore the potential effects of the pool size of the daytime cumulative photosynthates on the magnitude of the nighttime variation in $\delta^{13}C_R$. A strong positive correlation was found between the amplitude of the nighttime variation in $\delta^{13}C_R$ and the cumulative carbon assimilation (Fig 2).

Table 3. Statistical data. The *df* and *P* values from the photosynthetic pathway and the plant functional type differences in the maximum leaf net CO_2 assimilation rate (*A*max, μmol m^{-2} s^{-1}), the mean leaf net CO_2 assimilation rate (Mean *A*, μmol m^{-2} s^{-1}), maximum stomatal conductance ($g_s$max, mol m^{-2} s^{-1}), mean ratio of leaf internal to ambient CO_2 concentration (Mean C_i/C_a), nighttime mean respiration rate (Mean *R*, μmol m^{-2} s^{-1}), magnitude of nocturnal shift in $\delta^{13}C_R$ (Variation in $\delta^{13}C_R$), and respiratory apparent $^{13}C/^{12}C$ fractionation (‰) comparative to biomass ($\Delta_{R,\ biomass}$), soluble carbohydrates ($\Delta_{R,\ sugar}$), starch ($\Delta_{R,\ starch}$) and lipids ($\Delta_{R,\ lipid}$) at 8 pm and 4 am, respectively.

	Photosynthetic pathway		Functional type	
	df	*P*	*df*	*P*
*A*max	1	**0.03**	2	0.40
Mean *A*	1	0.39	2	0.77
g_s max	1	**0.02**	2	**0.02**
Mean C_i/C_a	1	**<0.01**	2	0.09
Mean R	1	0.59	2	0.14
Variation in $\delta^{13}C_R$	1	0.07	2	0.29
$\Delta_{R,\ biomass\text{-}8pm}$	1	0.90	2	0.78
$\Delta_{R,\ biomass\text{-}4am}$	1	**0.02**	2	0.52
$\Delta_{R,\ sugar\text{-}8pm}$	1	**<0.01**	2	0.09
$\Delta_{R,\ sugar\text{-}4am}$	1	0.22	2	0.27
$\Delta_{R,\ starch\text{-}8pm}$	1	0.89	2	0.60
$\Delta_{R,\ starch\text{-}4am}$	1	0.11	2	0.33
$\Delta_{R,\ lipid\text{-}8pm}$	1	**<0.01**	2	**<0.01**
$\Delta_{R,\ lipid\text{-}4am}$	1	**<0.01**	2	**<0.01**

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Respiratory apparent $^{13}C/^{12}C$ fractionation

We calculated respiratory apparent $^{13}C/^{12}C$ fractionation (Δ_R) relative to the $\delta^{13}C$ values of the bulk leaf material, soluble carbohydrates ($\Delta_{R,\ sugar}$), starch ($\Delta_{R,\ starch}$), and lipids ($\Delta_{R,\ lipid}$) at 8 pm and 4 am, respectively (Table 5). Differences in $\Delta_{R,\ biomass}$ between the C_3 and the C_4 species were detected at 4 am, but not at 8 pm (Table 3; Fig 3A and 3B). There were no nighttime $\Delta_{R,\ biomass}$ differences among the trees, graminoids and herbs at either 8 pm or 4 am (Table 3; Fig 3A and 3B). Significant differences were found in the $\Delta_{R,\ sugar}$ between the C_3 and the C_4 species at 8 pm (Table 3). Neither photosynthetic pathway nor plant functional type differences in $\Delta_{R,\ starch}$ were detected (Table 3). The C_3 and C_4 plants differed significantly in the $\Delta_{R,\ lipid}$ at both 8 pm and 4 am (Table 3; Fig 3G and 3H). We also detected plant functional type differences in the $\Delta_{R,\ lipid}$ (Table 3), with the $\Delta_{R,\ lipid}$ values in the graminoids being more negative than they were in the trees or herbs (Fig 3G and 3H).

Discussion

Variation in $\delta^{13}C$ of leaf-respired CO_2

For most of the species studied, leaf-respired CO_2 collected at 8 pm and 4 am differed significant in its carbon isotope composition, with the leaf-respired CO_2 at 8 pm was enriched in ^{13}C compared with the evolved CO_2 at 4 am (Table 4). This result is consistent with the findings of previous studies [12, 18, 30]. No significant variation in $\delta^{13}C_R$ were detected in two C_3 herbs (*Chenopodium glaucum* and *Saussurea amara*), which is in agreement with the finding of Priault et al. (2009). However, we observed a significant variation in $\delta^{13}C_R$ in the other three herbs (Table 4) with the magnitude of the nighttime shift was up to 4.7±0.4‰ in a C_4 herb *Amaranthus retroflexus*. In contrast with the results of Priault et al. (2009), the three tree species we studied showed significant nocturnal shifts in $\delta^{13}C_R$. Large nocturnal shifts in $\delta^{13}C_R$

Table 4. Carbon isotope composition. The C isotope composition (‰) of leaf-respired CO_2 ($\delta^{13}C_R$), leaf soluble carbohydrates ($\delta^{13}C_{sugar}$), leaf starch ($\delta^{13}C_{starch}$) and leaf lipids ($\delta^{13}C_{lipid}$) at 8 pm and 4 am, respectively. The carbon isotope composition of leaf bulk tissue ($\delta^{13}C_{biomass}$) was measured once at 8 pm. The amplitude of the nighttime variation in $\delta^{13}C_R$ (Variation in $\delta^{13}C_R$) was estimated as $\delta^{13}C_{R\text{-}8pm}$—$\delta^{13}C_{R\text{-}4am}$. Differences in the $\delta^{13}C_R$ between the samples collected at 8 pm and 4 am were assessed by One-way analysis of variance (ANOVA) and the *P* values are presented.

Species	$\delta^{13}C_{R\text{-}8pm}$ (‰)	$\delta^{13}C_{R\text{-}4am}$ (‰)	Variation in $\delta^{13}C_R$	*P*	$\delta^{13}C_{biomass}$ (‰)	$\delta^{13}C_{sugar\text{-}8pm}$ (‰)	$\delta^{13}C_{sugar\text{-}4am}$ (‰)	$\delta^{13}C_{starch\text{-}8pm}$ (‰)	$\delta^{13}C_{starch\text{-}4am}$ (‰)	$\delta^{13}C_{lipid\text{-}8pm}$ (‰)	$\delta^{13}C_{lipid\text{-}4am}$ (‰)
L. chinensis	-22.7±0.17	-24.5±0.16	1.8±0.15	<0.01	-26.6±0.21	-27.4±0.55	-27.9±0.23	-24.7±0.17	-25.3±0.38	-29.9±0.36	-29.3±0.47
P. australis	-21.7±0.09	-23.5±0.10	1.8±0.12	<0.01	-25.8±0.13	-29.0±0.17	-29.5±0.10	-22.9±0.32	-24.2±0.10	-30.2±0.31	-29.5±0.32
L. bicolor	-24.9±0.13	-26.6±0.16	1.7±0.26	<0.01	-28.5±0.09	-30.1±0.20	-30.9±0.30	-29.6±0.06	-30.0±0.10	-30.0±0.22	-30.5±0.25
M. asiatica	-21.2±0.17	-24.7±0.16	3.5±0.32	<0.01	-26.8±0.16	-25.5±0.37	-25.6±0.30	-24.0±0.08	-24.5±0.14	-31.3±0.12	-30.6±0.15
P. simonii	-22.9±0.24	-25.6±0.17	2.7±0.26	<0.01	-27.8±0.15	-27.4±0.17	-28.5±0.04	-26.1±0.25	-28.5±0.10	-30.6±0.32	-31.8±0.33
P. salicina	-22.6±0.15	-25.5±0.22	3.0±0.31	<0.01	-26.9±0.18	-25.9±0.26	-25.6±0.08	-25.9±0.21	-25.6±0.16	-30.5±0.13	-29.9±0.17
C. glaucum	-26.0±0.32	-26.1±0.28	0.2±0.47	0.72	-28.5±0.18	-32.6±0.36	-33.3±0.26	-26.6±0.39	-25.4±0.36	-30.9±0.25	-30.3±0.15
G. max	-22.6±0.16	-24.7±0.10	2.2±0.25	<0.01	-27.8±0.07	-28.6±0.35	-28.5±0.35	-26.8±0.12	-27.7±0.10	-32.4±0.24	-32.2±0.25
H. annuus	-26.3±0.31	-28.1±0.26	1.9±0.73	<0.01	-28.2±0.36	-27.7±0.26	-26.8±0.18	-27.0±0.25	-27.6±0.11	-32.3±0.42	-32.1±0.16
S. amara	-25.5±0.16	-25.0±0.30	0.5±0.32	0.17	-28.4±0.12	-29.1±0.05	-29.4±0.06	-27.0±0.12	-26.8±0.23	-31.4±0.09	-31.8±0.23
V. radiata	-19.9±0.19	-23.4±0.07	3.5±0.24	<0.01	-26.5±0.09	-27.5±0.04	-27.6±0.15	-26.4±0.07	-26.7±0.11	-33.0±0.32	-30.6±1.06
V. unguiculata	-23.5±0.32	-27.0±0.41	3.5±0.41	<0.01	-29.3±0.22	-29.3±0.60	-28.1±0.23	-27.4±0.18	-28.8±0.21	-31.1±0.16	-30.8±0.24
X. sibiricum	-23.7±0.22	-24.6±0.26	1.0±0.41	0.02	-26.2±0.27	-25.5±0.14	-25.3±0.29	-24.9±0.29	-24.4±0.26	-29.5±0.20	-29.1±0.20
C. virgata	-8.3±0.17	-10.2±0.10	2.0±0.16	<0.01	-12.8±0.26	-15.5±0.24	-15.6±0.32	-11.8±0.07	-12.1±0.38	-21.7±0.28	-21.9±0.36
E. crusgalli	-9.6±0.12	-11.3±0.08	1.8±0.18	<0.01	-13.2±0.35	-15.7±0.28	-14.1±0.36	-11.6±0.41	-11.2±0.13	-20.1±0.34	-17.9±0.70
H. altissima	-9.4±0.10	-11.4±0.14	2.0±0.21	<0.01	-12.8±0.31	-15.3±0.27	-15.0±0.21	-11.7±0.16	-12.2±0.13	-21.3±0.18	-21.3±0.28
P. miliaceum	-8.9±0.33	-13.1±0.14	4.1±0.33	<0.01	-13.3±0.13	-16.0±0.46	-18.1±0.58	-11.4±0.12	-11.9±0.13	-21.3±0.16	-21.1±0.61
S. italica	-8.6±0.19	-11.9±0.30	3.2±0.39	<0.01	-12.1±0.14	-17.7±0.16	-16.9±0.46	-11.2±0.21	-11.1±0.13	-22.2±0.51	-20.8±0.39
S. viridis	-7.3±0.14	-9.5±0.17	2.2±0.27	<0.01	-12.2±0.07	-13.5±0.19	-19.4±0.24	-11.4±0.04	-11.8±0.29	-21.1±0.52	-20.5±0.64
S. bicolor	-9.5±0.13	-12.2±0.20	2.7±0.18	<0.01	-12.3±0.19	-13.2±0.20	-12.9±0.32	-10.8±0.19	-11.5±0.59	-22.0±0.24	-21.2±0.40
Z. mays	-5.3±0.22	-11.1±0.28	5.8±0.24	<0.01	-12.4±0.19	-14.9±0.71	-13.6±0.28	-10.3±0.09	-11.0±0.44	-22.0±0.68	-21.9±0.35
A. retroflexus	-7.6±0.26	-12.3±0.31	4.7±0.36	<0.01	-12.2±0.13	-16.6±0.74	-16.5±0.58	-10.8±0.45	-12.4±0.39	-21.0±0.50	-20.9±0.45

Data are reported as the mean ±1 SE (n = 5)

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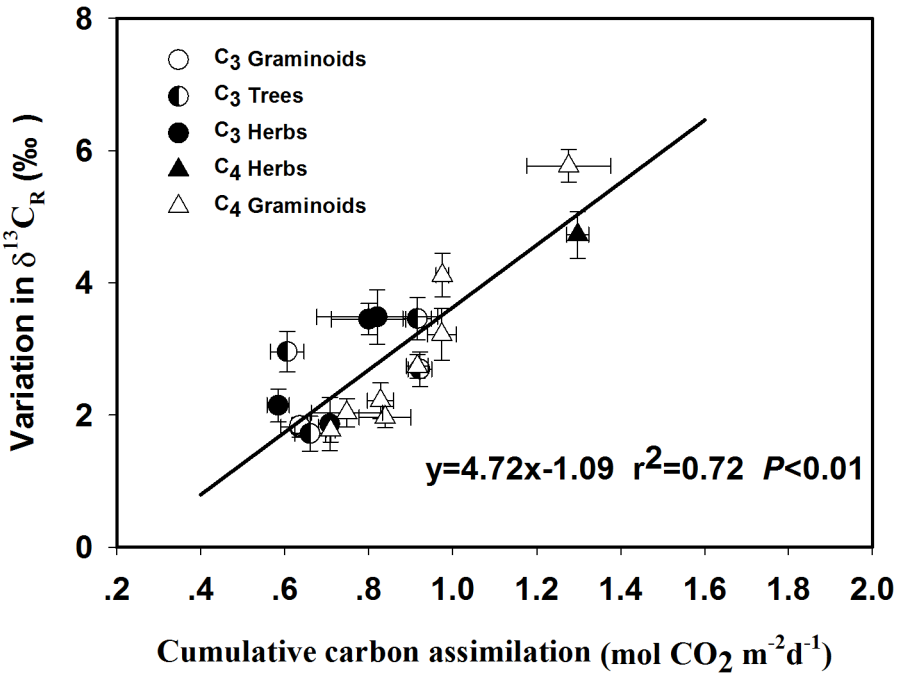

Fig 2. Dependence of the amplitude of the variations in $\delta^{13}C$ of leaf-respired CO_2 ($\delta^{13}C_R$, ‰) on the amount of cumulative carbon assimilation (mol CO_2 m^{-2} d^{-1}). The data contains 19 of the 22 studied species, the exceptions being *Chenopodium glaucum*, *Saussurea amara* and *Xanthium sibiricum*. The r^2 and *P* values are provided. The data are reported as mean ±1 standard error (n = 5).

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have also been reported in other tree species, such as *Prosopis velutina* and *Celtis reticulata* [25]. To assess potential plant functional type differences in the magnitude of variation in $\delta^{13}C_R$, we grouped the studied species with significant variation in $\delta^{13}C_R$ according to their differences in photosynthetic pathway and growth form. However, there were no differences in the magnitude of variation in $\delta^{13}C_R$ between the C_3 and C_4 plants, as well as among the woody plants, herbs and graminoids (Table 3).

The amplitude of the nighttime variation in $\delta^{13}C_R$ was strongly correlated with the daytime cumulative carbon assimilation (Fig 2). Similar phenomenon has also been reported previously and was mainly attributed to changes in the contribution of light-enhanced dark respiration (LEDR) associated malate decarboxylation to the total respiratory CO_2 flux [8, 14]. In the light, malate, fixed by phosphoenolpyruvate carboxylase (PEPc), accumulates because of the inhibition of the key respiratory enzymes, such as the mitochondrial isocitrate dehydrogenase, the succinate dehydrogenase, and the 2-oxoglutarate dehydrogenase [16, 37]. In darkness, the mitochondrial malic enzyme and the mitochondrial malate dehydrogenase catalyzed decarboxylation of the ^{13}C-enriched malate pool caused the respired CO_2 to be ^{13}C enriched [38]. In a study by Barbour et al. (2011), they reported the effects of LEDR associated malate decarboxylation on ^{13}C-enrichment in leaf-respired CO_2 can last up to 100 min after sunset. For the present study, ^{13}C enrichment in leaf-respired CO_2 at 8 pm (40–50 min after sunset) may partially be attributed to the decarboxylation of malate.

Large diel variation in photosynthetic discrimination (Δ_P) has also been hypothesized to affect $\delta^{13}C_R$ by changing the $\delta^{13}C$ of the primary respiratory substrates [18, 28]. We observed no significant differences in the primary respiratory sources (soluble carbohydrates, starch, and lipids) between 8 pm and 4 am. A previous report [24] has also found no apparent variation in the $\delta^{13}C$ of the leaf primary respiratory substrates. However, we could not discount the

Table 5. Respiratory apparent fractionation. Respiratory apparent $^{13}C/^{12}C$ fractionation (‰) of the nighttime leaf-respired CO_2, comparative to biomass ($\Delta_{R,\ biomass}$), soluble carbohydrates ($\Delta_{R,\ sugar}$), starch ($\Delta_{R,\ starch}$) and lipids ($\Delta_{R,\ lipid}$) at 8 pm and 4 am, respectively.

Species	$\Delta_{R,\ biomass\text{-}8pm}$ (‰)	$\Delta_{R,\ biomass\text{-}4am}$ (‰)	$\Delta_{R,\ sugar\text{-}8pm}$ (‰)	$\Delta_{R,sugar\text{-}4am}$ (‰)	$\Delta_{R,\ starch\text{-}8pm}$ (‰)	$\Delta_{R,starch\text{-}4am}$ (‰)	$\Delta_{R,\ lipid\text{-}8pm}$ (‰)	$\Delta_{R,lipid\text{-}4am}$ (‰)
L. chinensis	-4.00±0.28	-2.12±0.27	-4.80±0.69	-3.52±0.28	-2.07±0.48	-0.77±0.52	-7.36±0.51	-4.93±0.60
P. australis	-4.23±0.63	-2.35±0.23	-7.49±0.20	-6.10±0.12	-1.31±0.33	-0.68±0.16	-8.72±0.34	-6.19±0.31
L. bicolor	-3.67±0.31	-1.91±0.26	-5.49±0.21	-4.40±0.43	-4.81±0.15	-3.37±0.21	-5.22±0.27	-4.00±0.34
M. asiatica	-5.72±0.33	-2.19±0.29	-4.42±0.32	-0.97±0.22	-2.90±0.16	0.19±0.23	-10.35±0.20	-6.13±0.13
P. simonii	-5.02±0.43	-2.67±0.33	-4.62±0.39	-3.05±0.18	-3.27±0.40	-3.02±0.25	-7.90±0.55	-6.41±0.29
P. salicina	-4.41±0.41	-1.38±0.33	-3.40±0.40	-0.07±0.20	-3.46±0.34	-0.05±0.27	-8.15±0.27	-4.51±0.12
C. glaucum	-2.60±0.83	-2.44±0.44	-6.86±0.61	-7.41±0.45	-0.70±0.43	0.70±0.37	-5.11±0.45	-4.33±0.40
G. max	-5.35±0.57	-3.16±0.33	-6.14±0.34	-3.85±0.36	-4.28±0.11	-3.08±0.15	-10.06±0.24	-7.65±0.30
H. annuus	-1.99±0.57	-0.08±0.48	-1.49±-0.86	-1.35±0.27	-0.77±0.41	0.55±0.35	-6.21±0.59	-4.06±0.11
S. amara	-3.03±0.52	-3.56±0.96	-3.71±0.14	-4.50±0.32	-1.54±0.23	-1.86±0.10	-6.05±0.20	-7.03±0.41
V. radiata	-6.73±1.03	-3.22±0.52	-7.76±0.20	-4.31±0.11	-6.75±0.29	-3.36±0.13	-13.3±0.51	-7.39±1.08
V. unguiculata	-5.90±0.23	-2.34±0.45	-5.93±0.70	-1.09±0.56	-3.99±0.26	-1.84±0.59	-7.74±0.26	-3.92±0.55
X. sibiricum	-2.60±0.36	-1.62±0.45	-1.92±0.28	-0.73±0.44	-1.25±0.98	0.24±0.37	-5.98±0.39	-4.65±0.36
C. virgata	-4.42±0.26	-2.47±0.29	-7.21±0.19	-5.32±0.35	-3.49±0.11	-1.78±0.38	-13.49±0.20	-11.70±0.35
E. crusgalli	-3.68±0.38	-1.89±0.50	-6.21±0.28	-2.78±0.34	-2.02±0.38	0.12±0.06	-10.6±0.33	-6.68±0.68
H. altissima	-3.44±0.32	-1.39±0.35	-5.94±0.30	-3.58±0.31	-2.31±0.20	-0.72±0.16	-11.97±0.18	-9.93±0.23
P. miliaceum	-4.37±0.49	-0.22±0.30	-7.13±0.41	-5.12±0.65	-2.46±0.34	1.19±0.21	-12.48±0.26	-8.20±0.74
S. italica	-3.49±0.87	-0.24±0.39	-8.92±0.49	-5.09±0.64	-2.59±0.23	0.74±0.34	-13.64±0.38	-9.02±0.45
S. viridis	-4.91±0.20	-2.68±0.19	-10.46±0.21	-9.99±0.19	-4.14±0.12	-2.30±0.18	-13.91±0.50	-11.09±0.59
S. bicolor	-2.81±0.55	-0.04±0.67	-4.00±0.25	-0.71±0.34	-1.30±0.29	0.74±0.43	-12.6±0.22	-9.11±0.38
Z. mays	-7.07±0.61	-1.28±0.38	-9.58±0.75	-2.56±0.40	-4.97±0.14	0.11±0.66	-16.8±0.80	-10.88±0.34
A. retroflexus	-4.72±0.40	-0.04±0.54	-9.07±0.95	-4.31±0.40	-3.24±0.58	-0.08±0.51	-13.53±0.65	-8.69±0.56

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effects of the changes in the use of respiratory substrates having different $\delta^{13}C$, because we, along with most other studies, measured the $\delta^{13}C$ of the entire leaf respiratory substrate pools. It is difficult to identify the pool size and the isotopic signature of the fast- and the slow-turnover pools.

Other mechanisms, such as heterogeneous ^{13}C distribution within hexose molecules and nighttime variation in the utilization of respiratory intermediates [14, 21], short term variation in carbohydrate pool size and respiration rate, have also been employed to explain nighttime variation in $\delta^{13}C_R$ [15, 16]. However, with the current data set we are unable to test these hypotheses.

Respiratory apparent $^{13}C/^{12}C$ fractionation

Leaf-respired CO_2 was enriched in ^{13}C compared with the biomass, starch, soluble carbohydrates, and lipids (Table 5). This finding is in agreement with the results of previous studies [16, 19, 27, 28, 39, 40]. ^{13}C enrichment in leaf-respired CO_2, relative to the potential respiratory substrates, could be caused by the incomplete oxidation of hexose molecules, which cause a greater ratio of C-3 and C-4 atoms (^{13}C enriched compared with other C atoms) being converted to CO_2 [30, 37]. However, there were some differences in the magnitude of the respiratory apparent fractionation between the C_3 and C_4 species, and among the different plant functional types (Fig 3, Table 3). Differences in $\Delta_{R,\ biomass}$ were detected between C_3 and C_4 species at 4 am, but not at 8 pm, which were caused primarily by different magnitude of

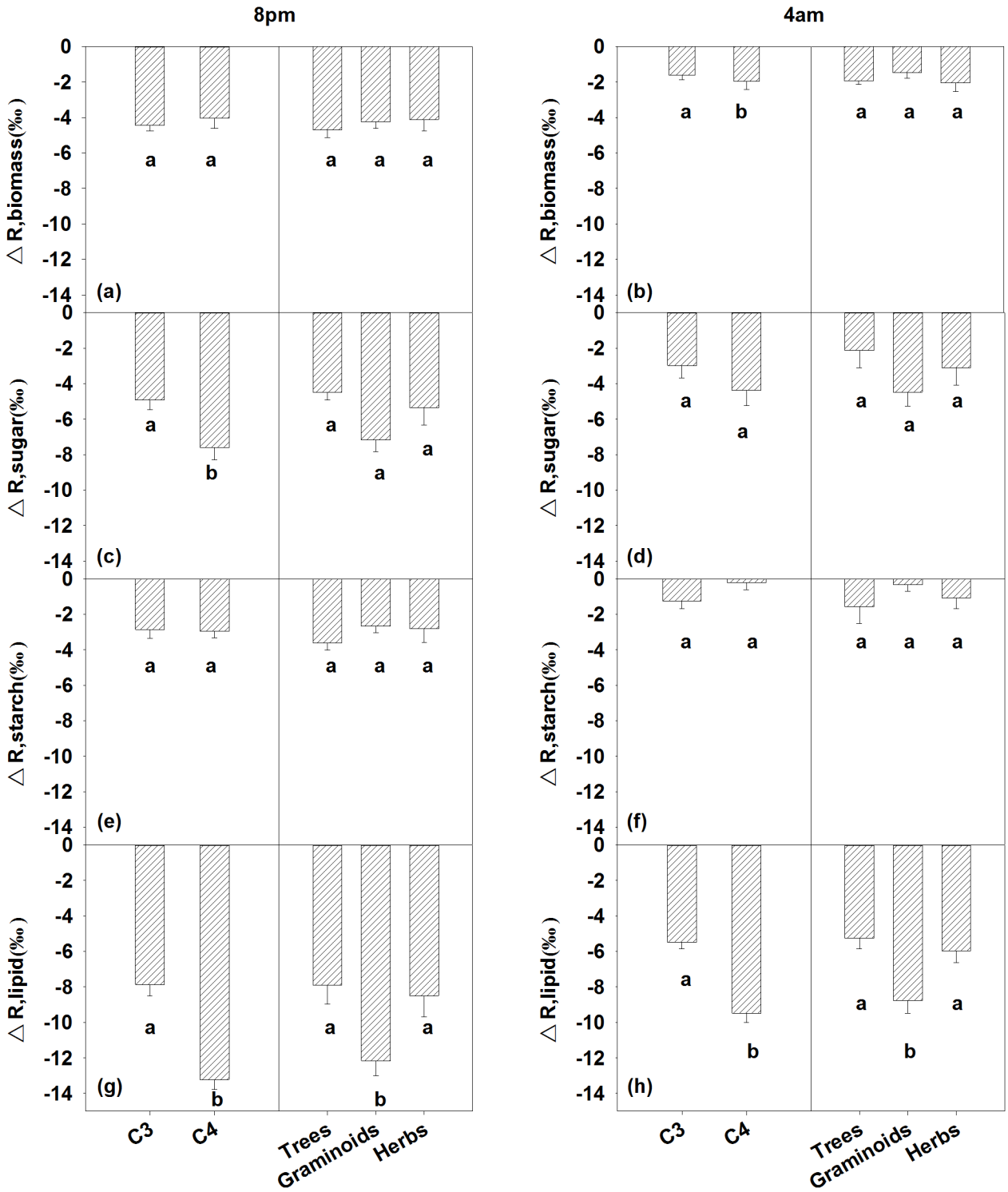

Fig 3. Plant functional type differences in respiratory apparent fractionation comparative to leaf bulk materials ($\Delta_{R,\ biomass}$), soluble carbohydrates ($\Delta_{R,\ sugar}$), starch ($\Delta_{R,\ starch}$) and lipids ($\Delta_{R,\ lipid}$) at 8 pm and 4 am, respectively. Data are reported as mean ± 1 standard error (n = 5). The different letters within each panel indicate the significant differences (P <0.05) among the plant functional types.

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variation in $\delta^{13}C_R$ between the two photosynthetic types. In a recent review paper, Ghashghaie & Badeck (2014) reported that C_3 and C_4 species differed in $\Delta_{R,\ biomass}$. There were no differences in the $\Delta_{R,\ biomass}$ among the trees, the herbs, and the graminoids (Fig 3A), which is in line with the results of Ghashghaie & Badeck (2014). The detected differences in $\Delta_{R,\ biomass}$ between C_3 and C_4 species needs to be incorporated into the partitioning of the CO_2 exchange between the ecosystem and the atmosphere when leaf bulk materials are used as a proxy for $\delta^{13}C_R$ [25].

$\Delta_{R,\ lipid}$ significantly differed between the C_3 and the C_4 plants, as well as among the plant functional types (Table 3), which could have resulted primarily from the carbon allocation differences associated with the plant functional group types. Compared with the woody plants or the herbs, the grasses allocated a smaller proportion of acetyl CoA to the synthesis of lipids. This leads to grass lipids being generally more depleted compared with the bulk tissue [26, 41].

Soluble carbohydrates are often found to be ^{13}C-enriched compared to bulk leaf material [9, 15, 28]. However, we observed that soluble carbohydrates in 16 of the 22 studied species were depleted in ^{13}C relative to the leaf biomass (Table 4). Similar results with up to 4‰ depletion in soluble carbohydrates compared to bulk materials have also been reported previously in various C_3 and C_4 species [3535, 36, 42]. This discrepancy may be attributed to both differences in the extraction method and timing of leaf sample collection.

Conclusions

For both the C_3 and the C_4 species (except *Chenopodium glaucum* and *Saussurea amara*), the $\delta^{13}C$ of leaf-respired CO_2 ($\delta^{13}C_R$) showed a significant nocturnal shift. The leaf-respired CO_2 at 8 pm was enriched in ^{13}C, relative to what it was at 4 am. The amplitude of the nighttime variation in $\delta^{13}C_R$ was strongly correlated with the daytime cumulative carbon assimilation, which suggests that variation in $\delta^{13}C_R$ were influenced, to some extent, by changes in the contribution of malate decarboxylation to total respiratory CO_2 flux. There were no differences in the magnitude of the nocturnal shift in $\delta^{13}C_R$ between the C_3 and C_4 plants, as well as among the woody plants, herbs and graminoids. The plant functional group differences in respiratory apparent fractionation relative to biomass indicate that caution should be exercised when the $\delta^{13}C$ of bulk leaf material is used as a proxy for the $\delta^{13}C$ of leaf-respired CO_2.

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Author Contributions

Conceived and designed the experiments: WS HC SC. Performed the experiments: HC YW J-YM QJ. Analyzed the data: HC WS. Contributed reagents/materials/analysis tools: J-YM SC. Wrote the paper: HC WS. Proofread before submission: WS J-YM SC.

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