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Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups

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Summary

• The first broad species survey of diurnal variation in carbon (C) isotope signatures of leaf dark-respired CO_2 ($\delta^{13}C_{res}$) is presented here and functional differences and diurnal dynamics are linked to fractionation in different respiratory pathways, based on ^{13}C-labelling experiments.

• $\delta^{13}C_{res}$ was analysed with a rapid in-tube incubation technique in 16 species.

• A large diurnal increase in $\delta^{13}C_{res}$ (4–8‰) occurred in evergreen, slow-growing and aromatic species and correlated significantly with cumulative photosynthesis, whereas no variation occurred in herbaceous, fast-growing plants or temperate trees. The diurnal increase in $\delta^{13}C_{res}$ declined almost proportionally to reductions in cumulative light and was reduced in growing compared with mature leaves.

• Pyruvate positional labelling provided direct evidence that functional groups differ in C allocation between respiratory pathways owing to different metabolic demands for growth, maintenance and secondary metabolism. Diurnal increase in C flux through pyruvate dehydrogenase (for investment in, for example, isoprene or aromatic compounds) combined with consistently low Krebs cycle activity resulted in pronounced increase in $\delta^{13}C_{res}$ in evergreen and aromatic species. By contrast, fast growing herbs with high respiratory demand exhibited no diurnal changes since C was fully respired. Hence, diurnal $\delta^{13}C_{res}$ pattern may provide information for C allocation in plants.

Introduction

Increasing atmospheric carbon dioxide concentrations and corresponding climate change have increased the demand for a better process-based understanding of carbon (C) exchange processes (i.e. photosynthesis and respiration) at individual plant and ecosystem scales. Stable C isotopes are a sensitive tool for disentangling C fluxes from the leaf to the ecosystem level (Yakir & Sternberg, 2000; Bowling *et al.*, 2008) and for analysing biophysical and biochemical processes in photosynthetic pathways. The marked discrimination against the heavier isotope (^{13}C) during photosynthesis has been well characterized (Farquhar *et al.*, 1982, 1989). Similarly, fractionation during photorespiration is thought to be significant (Sharkey, 1988; Gillon & Griffiths, 1997), while apparent fractionation during dark respiration has long been considered negligible (Lin & Ehleringer, 1997). However, there is now evidence for substantial apparent fractionation leading to differences between the C isotope composition of leaf dark-respired CO_2 ($\delta^{13}C_{res}$) and its putative substrates in many C_3 species (Ghashghaie *et al.*, 2003). Therefore, $\delta^{13}C_{res}$ has become the subject of several recent studies (Schnyder *et al.*, 2003; Tcherkez *et al.*, 2003; Nogués *et al.*, 2004; Hymus *et al.*, 2005; Klumpp *et al.*, 2005; Prater *et al.*, 2006; Bathellier *et al.*, 2008). In general, foliar $\delta^{13}C_{res}$ has been found to be ^{13}C-enriched compared with a wide variety of metabolites, for example, an enrichment of 9‰ in dark-respired CO_2 relative to plant organic material was found in *Nicotiana sylvestris*

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(Ghashghaie *et al.*, 2001). This apparent fractionation effect is highly variable, changing with species and environmental factors (for a review, see Ghashghaie *et al.*, 2003). Based on these laboratory experiments there is now increasing knowledge on the mechanisms accounting for fractionation occurring during dark respiration at the leaf level (Tcherkez *et al.*, 2003; for recent reviews see Ghashghaie *et al.*, 2003; Werner *et al.*, 2007a). Indeed, based on the heterogeneous C isotope distribution in hexose molecules (DeNiro & Epstein, 1977; Rossmann *et al.*, 1991; Gleixner *et al.*, 1998), Ghashghaie *et al.* (2001) indicated two metabolic origins for the respired CO_2 (oxidation of pyruvate releases ^{13}C-enriched CO_2 relative to substrate while the acetyl-CoA decarboxylated through the Krebs cycle is depleted) possibly accounting for the ^{13}C-enrichment of the overall respired CO_2 compared with respiratory substrates. Recent work has also shown that CO_2 respired by tree trunks is in general ^{13}C-enriched, while that of roots is ^{13}C-depleted compared with their respective bulk organic matter or carbohydrates (Badeck *et al.*, 2005; Klumpp *et al.*, 2005; Gessler *et al.*, 2007; Maunoury *et al.*, 2007; Bathellier *et al.*, 2008).

However, only a few studies have focused on potential diurnal short-term variations of $\delta^{13}C$ of dark-respired CO_2. Indeed, assuming that the pool of fresh assimilates carries C of variable isotope composition resulting from changes in photosynthetic discrimination, it can be expected that the isotope ratio of dark-respired CO_2 may also change during the light period, even without involving any fractionation by the process of respiration itself. In field studies, Hymus *et al.* (2005) and Prater *et al.* (2006) found a pronounced enrichment of respired CO_2 along a light period up to 5–10‰ when compared with the respired $\delta^{13}CO_2$ measured during the dark period. This ^{13}C enrichment was correlated with the concomitant cumulative CO_2 assimilation (Prater *et al.*, 2006). Similarly, rapid dynamics, though with smaller magnitudes, have been shown in other ecosystem compartments, for example, at the trunk and soil levels (Maunoury *et al.*, 2007; Kodama *et al.*, 2008).

The isotopic signature of ecosystem-respired CO_2 ($\delta^{13}C_R$) is a complex response of different respiratory sources, including respiration by autotrophic and heterotrophic organisms. Ecosystem respiration is still poorly understood even though it is a major component of the global C balance (Valentini *et al.*, 2000; Reichstein *et al.*, 2002; Davidson *et al.*, 2006). Understanding the driving environmental factors of $\delta^{13}C_R$ is therefore important for applications of isotope-based models of the global C budget. So far, the short-term dynamics of the C isotopic composition of respired CO_2 have been disregarded in most studies despite their potential implications, for example, for the sampling protocols used to collect nocturnal Keeling plots. There is increasing evidence of rapid dynamics (minutes to hours) of $\delta^{13}C_R$ (i.e. 4‰ and 6‰ during one night; Bowling *et al.*, 2003; Werner *et al.*, 2006, respectively). However, data on this topic are scarce and the understanding and identification of the isotopic effects during dark-respiration defined by Tcherkez *et al.* (2003) are far from being resolved (Tcherkez & Farquhar, 2005). Taking advantage of the rapid in-tube incubation method (Werner *et al.*, 2007b), this paper aims to investigate the different metabolic processes influencing the isotope composition of respired CO_2 by analysing diurnal variation in dark-respired $\delta^{13}CO_2$ in a wide range of ecotypes and species.

Most studies on fractionation during dark respiration have been performed on fast-growing herbaceous species under laboratory conditions. Werner *et al.* (2007b) were the first to show pronounced differences in two different functional plant types under standardized conditions: no significant diurnal variation in $\delta^{13}C_{res}$ occurred in a fast-growing herb, while a pronounced $\delta^{13}C_{Light–Dark}$ amplitude of 8‰ occurred in a Mediterranean oak. Here, we explore the hypothesis that the extent of diurnal increase in $\delta^{13}C_{res}$ varies between plant functional types. We present the first species survey to characterize different functional groups in relation to structural and metabolic features such as leaf thickness, C : N ratios and photosynthesis. Further, we use pyruvate positional labelling experiments, which provide the first direct evidence of the importance of apparent fractionation processes in respiratory pathways for the observed functional differences in diurnal variation of plant $\delta^{13}C_{res}$.

Materials and Methods

Plant material – growth and experimental conditions

Controlled conditions Woody species including trees (4-yr-old *Quercus ilex* L. seedlings (height approx. 40 cm), 2-yr-old *Pinus pinea* L.) and shrubs (2-yr-old *Arbutus unedo* L., *Ceratonia siliqua* L., *Citrus hystrix* DC., *Ficus benjamina* L. and *Halimium halimifolium* L.) as well as herbaceous plants (*Tolpis barbata* Gaertn., *Oxalis triangularis* A. St-Hil.) and aromatic species (*Mentha piperita* L. and *Rosmarinus officinalis* L.) were grown under stable controlled climate conditions. Artificial light in a growth chamber was provided from 08 : 00 h to 20 : 00 h (or 09 : 00 h to 21 : 00 h) with 200 μmol m^{-2} s^{-1} for all species, up to 350 μmol m^{-2} s^{-1} for oak leaves. The air temperature was 25°C and 15°C during the light and dark periods, respectively. The relative air humidity was 60%. Plants received 150 ml of water twice a week and were fertilized once a week with 1/8th strength of Hoagland's Fertilizer Solution.

Field conditions Two herbaceous species (*Trifolium pratense* L., *Bellis perennis* L.) and three deciduous trees (*Quercus petraea* L., *Carpinus betulus* L. and *Sorbus cashmiriana* Hedl.) were sampled near the University campus of Bielefeld, Germany. Fully developed leaves from south-facing canopy were collected in June 2008 during two periods of the day: at the beginning of the light period (between 06 : 15 h and 07 : 00 h) and at the end of the light period (from 21 : 15 h to 22 : 00 h).

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At the time of collection, the mean temperature was 11°C in the morning and 25°C with a maximum light level of 1145 μmol m^{-2} s^{-1} in the afternoon.

Leaf structure parameters

Specific leaf area (SLA) was calculated as the ratio of leaf area, measured with a leaf area meter (Delta-T Scan, Cambridge, UK), to leaf dry weight, measured after drying samples for 48 h at 60°C. The relative leaf water content ((FW – DW)/FW), with FW and DW representing fresh and dry weights, respectively, was determined.

Gas exchange measurements

Net photosynthesis was measured on *H. halimifolium* and *Q. ilex* mature leaves five times during the day (after 1, 4, 7, 10 and 11 h 45 min of light) using a WALZ CMS-400 minicuvette system (WALZ, Effeltrich, Germany) equipped with an IRGA (BINOS 100, CO_2 and H_2O channels, Rosemount, Chanhassen, USA). Consecutive measurements on attached leaves were performed at 25°C under growth light intensity and 380 ppm CO_2 with controlled leaf vapour pressure deficit and waiting at least 30 min for acclimatization. Carbon dioxide accumulation along the photoperiod was expressed in mol CO_2 m^{-2} s^{-1} and calculated by multiplying the averaged net C assimilation by the considered duration of the light period (1, 4, 7, 10 and 11 h 45 min).

Changing incident light conditions

To evaluate the dependence of the diurnal variation in $\delta^{13}C_{res}$ on the daily C gain, growth light intensity was reduced to 50% by increasing the distance between the plants and the light source on *H. halimifolium* plants, grown from seeds under full light or 80 μmol m^{-2} s^{-1}, as well as in acclimatizing half of the slow-growing *Quercus ilex* trees to the low-light conditions (180 μmol m^{-2} s^{-1}) for 3 wk. Alternatively, *H. halimifolium* and *Q. ilex* were subjected to a 3-h dark period in the middle of the diurnal course.

Isotope measurements

$\delta^{13}C$ of respired CO_2 Twelve sampling times were chosen to reflect the diurnal cycle of the respired CO_2 signature: 6 : 00 h, 7 : 45 h (before the light period), 9 : 00 h, 12 : 00 h, 15 : 00 h, 18 : 00 h, 19 : 45 h (during the light period), 20 : 15 h, 20 : 30 h, 21 : 00 h, 22 : 00 h and 23 : 00 h (during dark). Sampling and analysis were performed by the rapid in-tube incubation method as described in Werner *et al.* (2007b, see below). The diurnal increase in $\delta^{13}C_{res}$ ($\delta^{13}C_{Light–Dark}$, expressed in ‰) was calculated when not otherwise specified as the difference between $\delta^{13}C_{res}$ measured at the end of the light and dark periods.

In-tube incubation measurements To measure the isotopic composition of respired CO_2, collected leaf segments or entire fully developed leaves were inserted into a 12 ml glass vial (Exetainer; Labco, High Wycombe, UK). The vials were flushed in the dark for 1 min with CO_2-free air, provided by a 10 l min^{-1} membrane pump pushing atmospheric air through two Plexiglas columns (height, 29 cm; diameter, 4 cm) of soda lime (Carbosorb Sodalime granules; BDH Laboratory Supplies, Poole, UK), as described in Werner *et al.* (2007b). Leaves were left to respire in the dark for precisely 3 min to gain sufficient CO_2 (> 350 ppm) for analysis in the mass spectrometer and minimize the incubation time. A precise incubation time is required as large isotope effects can occur within minutes upon darkening (Barbour *et al.*, 2007; Werner *et al.*, 2007b), in all functional groups (data not shown). After 3 min incubation the isotope samples were immediately measured with an IRMS (Isotope Ratio Mass Spectrometer, IsoPrime; GV, Manchester, UK) interfaced to an autosampler (Microgas; GV).

Positional ^{13}C-labelling experiments Based on Tcherkez *et al.* (2005), who use ^{13}C-labelled pyruvate molecules to quantify the relative respiratory fluxes in illuminated and darkened leaves, mature leaves from *H. halimifolium* and *O. triangularis* were fed through the transpiration stream with ^{13}C-labelled pyruvate solutions (5 mM pyruvate labelled either at the C1 or both at the C2 and C3 carbon positions: 99% ^{13}C; Cambridge Isotope Laboratories, Andover, MA, USA). Leaves were cut at the petiole, immediately recut under water and incubated in the labelled pyruvate solution in the climate chamber. After a 15-min incubation the $\delta^{13}C_{res}$ of leaves or leaf discs was determined by the in-tube incubation method as described earlier.

$\delta^{13}C$ of total leaf organic material Leaves were collected 1 h before sunrise and 1 h before sunset and immediately oven-dried at 60°C for 48 h. After placing samples in desiccators overnight at room temperature, individual leaves were weighed and milled to fine powder, and 2 mg was used for mass spectrometer analysis.

Sample preparation was performed in an elemental analyser (EuroVector, Hekateck, Germany) interfaces to the IRMS. Samples are automatically combusted and analysed in a continuous-flow isotope ratio mass spectrometer (IsoPrime, GV Instruments, Manchester, UK). Samples were standardized to IAEA-CH-4 and IAEA-CH-6 (International Atomic Energy Agency, Vienna, Austria). A cross-calibrated laboratory gas standard was measured every nine samples to quantify any drift. Values are reported relative to vPDBee, and repeated measurements precision was 0.05‰.

Statistical analyses

If not indicated otherwise, all experiments were repeated independently at least three times and the standard error is

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given. Analyses of variance and LSD *post hoc* tests were performed using STATISTICA software (Statsoft Inc., Tulsa, USA) at $P < 0.05$.

Results

Diurnal variation in $\delta^{13}C$ of respired CO_2

Taking advantage of the in-tube incubation technique (Werner *et al.*, 2007b), diurnal variations of dark-respired $\delta^{13}CO_2$ of mature leaves ($\delta^{13}C_{res}$) were analysed in 16 different species grown under natural or controlled conditions (Fig. 1, Table 1). Overall, $\delta^{13}C_{res}$ ranged widely from −16 to −32‰ 15 min before sunset ($\delta^{13}C_{Light}$) and from −20 to −32‰ 15 min before sunrise ($\delta^{13}C_{Dark}$). There was a marked diurnal dynamic in $\delta^{13}C_{res}$ allowing the identification of different functional groups regarding the diurnal increase in $\delta^{13}C_{res}$ ($\delta^{13}C_{Light–Dark}$, i.e. the difference between $\delta^{13}C_{res}$ measured at the end of the light and dark periods). Generally, the functional groups followed two distinct diurnal patterns: (1) a significant increase of $\delta^{13}C_{res}$ during the light period ($\delta^{13}C_{Light–Dark}$) ranging from 1.4 to 7.9‰, Table 1) followed by a continuous decrease in $\delta^{13}C_{res}$ during the dark period; and (2) no significant changes in $\delta^{13}C_{res}$ throughout the light and dark periods (Fig. 1, Table 1). Examples of the characteristic diurnal pattern of $\delta^{13}C_{res}$ are shown in Fig. 1a. The three-slow growing evergreen species (*H. halimifolium*, *Q. ilex* and *A. unedo*) exhibited the largest $\delta^{13}C_{res}$ amplitude with $\delta^{13}C_{Light–Dark}$ values of 7.9, 7.3 and 6.9‰, respectively, while the fast-growing herbs *Tolpis barbata* and *Oxalis triangularis* showed no pronounced diurnal changes (Fig. 1a). A significant diurnal $\delta^{13}C_{res}$ increase (2.7–6.5‰) was also found in the sclerophyllous *P. pinea*, *F. benjamina*, the Mediterranean evergreen *C. siliqua* and in the aromatic species *R. officinalis*, *M. piperita* and *C. hystrix* (Table 1). In the diurnal $\delta^{13}C_{res}$ cycle the most enriched signatures were found at the end of the daylight period, whereas the most depleted values occurred during the night (Table 1). These patterns were also observed under natural conditions, with a $\delta^{13}C_{Light–Dark}$ of 4.2‰ in *C. betulus* and 2.9‰ in *Q. petraea* but no significant variation in the deciduous tree *S. cashmiriana* or in the herbs *T. pratense*, *B. perennis* (Fig. 1b, Table 1).

$\delta^{13}C$ of total leaf organic matter and of soluble sugars

No significant diurnal changes in the C isotopic signature of total leaf organic matter ($\delta^{13}C_{OM}$, measured 1 h before sunrise and sunset) was identified in any of the species investigated (Fig. 2a,b). The highest $^{13}C_{OM}$ values were obtained in the Mediterranean species *A. unedo*, and in *S. cashmiriana* (−26 to −28‰), and the most negative $^{13}C_{OM}$ values in herbs (−31 to −33‰; see Fig. 2). The $\delta^{13}C_{OM}$ was ^{13}C-depleted compared with $\delta^{13}C_{res}$ except for the two herbaceous species *B. perennis* and *T. pratense*, which exhibited almost similar $\delta^{13}C_{OM}$ and

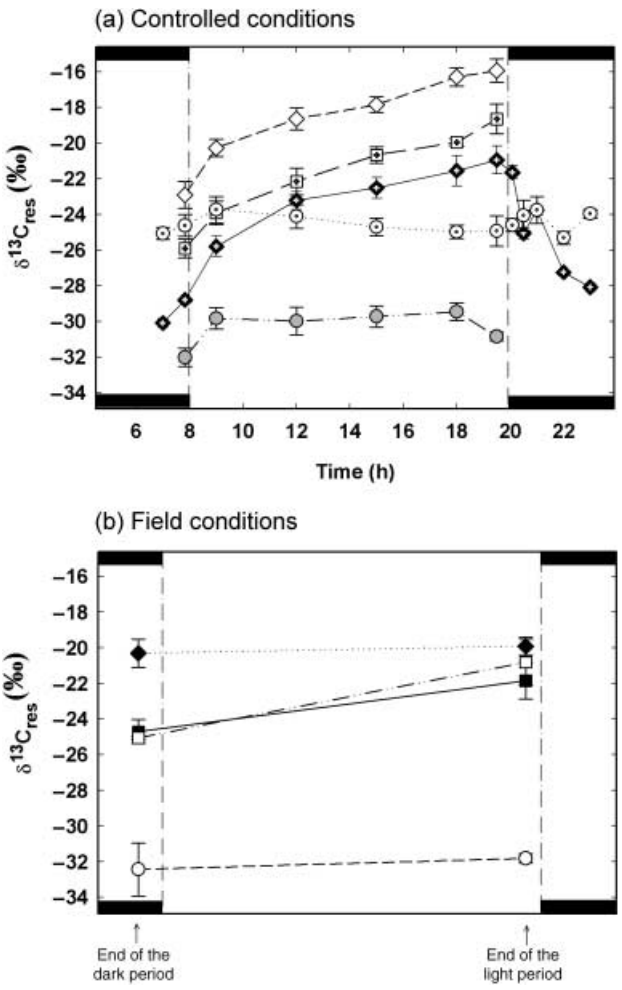

Fig. 1 Diurnal variation in the leaf dark-respired $\delta^{13}CO_2$ ($\delta^{13}C_{res}$) from nine different species. The dark period is indicated by the black bars. (a) *Halimium halimifolium* (closed diamonds), *Arbutus unedo* (open diamonds), *Quercus ilex* (squares), *Tolpis barbata* (open circles, central point) and *Oxalis triangularis* (grey circles) grown under controlled conditions (light period from 08 : 00 h to 20 : 00 h; growth light intensity 200–350 μmol m^{-2} s^{-1}). (b) *Quercus petraea* (closed squares), *Bellis perennis* (circles), *Sorbus cashmiriana* (diamonds) and *Carpinus betulus* (open squares). Leaf samples were collected in June 2008 (daylight from 07 : 00 h to 22 : 00 h. Three to eleven independent replicates (± SE).

$\delta^{13}C_{res}$ (compare Table 1 and Fig. 2). The enrichment of $\delta^{13}C_{res}$ relative to $\delta^{13}C_{OM}$ reached up to 11‰ at the end of the light period for *H. halimifolium* (Fig. 2). The isotopic composition of soluble sugars, including glucose, fructose and sucrose, and their respective concentrations were determined in four selected species: *Q. petraea*, *Q. ilex*, *H. halimifolium* and *T. barbata* at two periods of the day, just before sunset and sunrise (data not shown). Those results revealed no significant change in $\delta^{13}C$ of the three sugars between the two periods investigated and for all species. In addition, the sucrose concentration slightly decreased during the night while glucose and fructose contents remained constant throughout the day (not shown).

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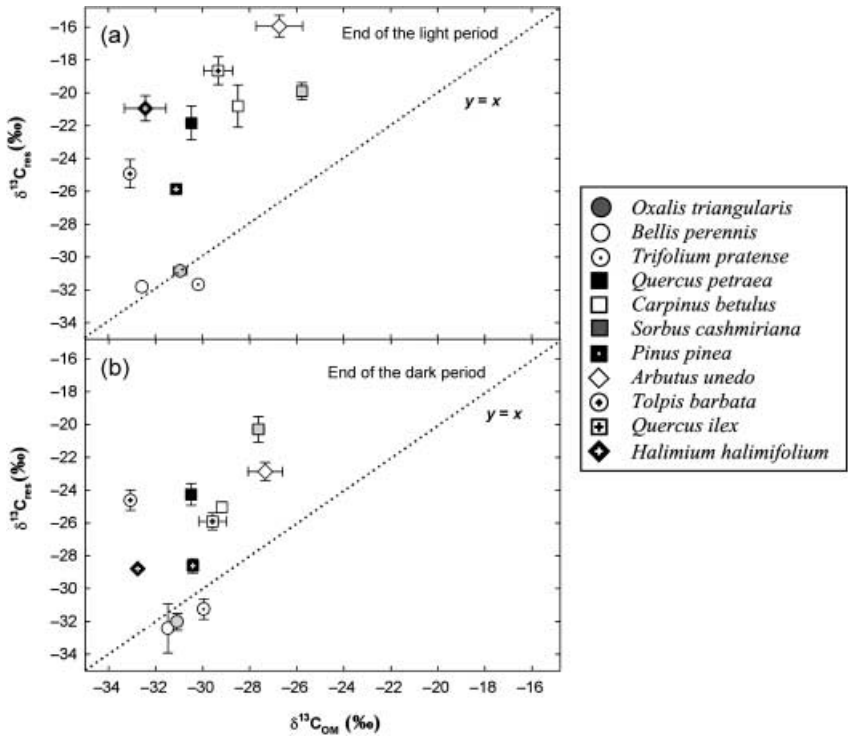

Fig. 2 Relationship between carbon isotopic signatures of leaf respired CO_2 ($\delta^{13}C_{res}$) and total leaf organic matter ($\delta^{13}C_{OM}$) both measured (a) at the end of the light period and (b) the end of the dark period of species grown either in controlled conditions in the glasshouse or under natural conditions (see the Materials and Methods section). Means (± SE) of at least three replicates from independent leaves are represented.

Table 1 Carbon isotopic composition of the dark-respired CO_2 ($\delta^{13}C_{res}$) from fully mature leaves grown either under controlled or natural (*) conditions at the end of the dark and light periods ($\delta^{13}C_{Dark}$ and $\delta^{13}C_{Light}$, respectively)

		$\delta^{13}C_{res}$ (‰)		
Functional group	Species	$\delta^{13}C_{Dark}$	$\delta^{13}C_{Light}$	$\delta^{13}C_{Light-Dark}$ (‰)
Woody				
Trees	*Carpinus betulus**	−25.0 (± 0.1)	−20.8 (± 1.3)	+4.2
	Pinus pinea	−28.6 (± 0.4)	−25.9 (± 0.2)	+2.7
	Quercus ilex.	−25.9 (± 0.5)	−18.6 (± 0.8)	+7.3
	*Quercus petraea**	−24.7 (± 0.7)	−21.8 (± 1.0)	+2.9
	*Sorbus cashmiriana**	−20.3 (± 0.8)	−19.9 (± 0.5)	ns
Shrubs	*Arbutus unedo*	−22.9 (± 0.6)	−15.9 (± 0.6)	+6.9
	Ceratonia siliqua	−25.1 (± 0.4)	−23.7 (± 0.1)	+1.4
	Citrus hystrix	−30.5 (± 1.1)	−26.4 (± 0.7)	+4.1
	Ficus benjamina	−24.4 (± 0.6)	−21.1 (± 0.4)	+3.4
	Halimium halimifolium	−28.8 (± 0.2)	−20.9 (± 0.8)	+7.9
	Rosmarinus officinalis	−27.4 (± 0.4)	−20.9 (± 0.6)	+6.5
Herbaceous	*Bellis perennis**	−32.4 (± 1.5)	−31.8 (± 0.3)	ns
	Mentha piperita	−30.2 (± 0.5)	−24.0 (± 0.8)	+6.3
	Oxalis triangularis	−32.0 (± 0.5)	−30.9 (± 0.3)	ns
	Tolpis barbata	−24.6 (± 0.6)	−24.9 (± 0.8)	ns
	*Trifolium pratense**	−31.3 (± 0.6)	−31.6 (± 0.6)	ns

*Those samples were collected in June 2008 between 06 : 15 h and 07 : 00 h ($\delta^{13}C_{Dark}$) and from 21 : 15 h to 22 : 00 h ($\delta^{13}C_{Light}$). The other species were sampled at 07 : 45 h and 19 : 45 h, that is 15 min before the end of the dark and the light period, respectively.
Diurnal changes of $\delta^{13}C_{res}$ were calculated as the difference between light and dark values ($\delta^{13}C_{Light-Dark}$, ‰ ± SE).
Data are expressed in ‰ ($n \geq 4$; ± SE). ns, No significant differences (ANOVA and LSD test, $P < 0.05$).

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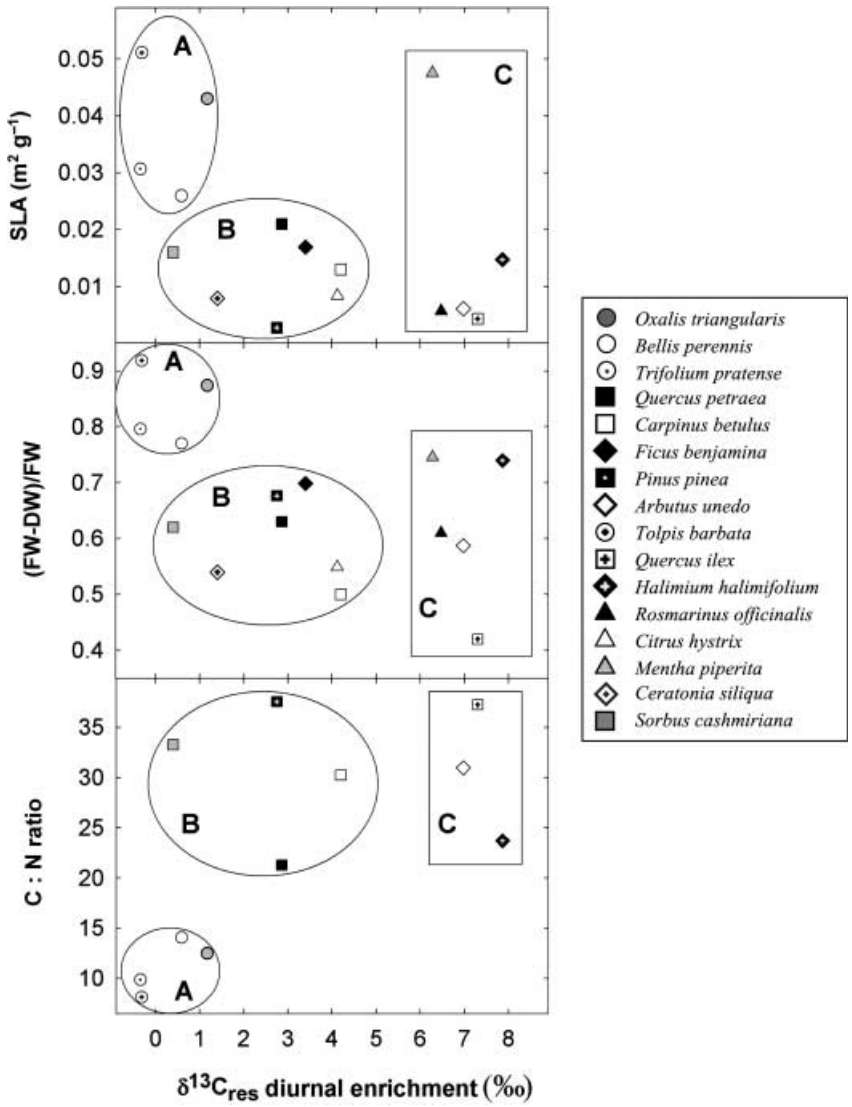

Fig. 3 Relationships between the $\delta^{13}C_{res}$ diurnal increase ($\delta^{13}C_{Light-Dark}$, corresponding to the difference between $\delta^{13}C_{res}$ measured at the end of the light period and the end of the dark period) and structural parameters. SLA (specific leaf area), leaf water content (estimated as (FW – DW)/FW, FW and DW representing the fresh and dry weights, respectively) and the total leaf carbon to nitrogen ratio (C : N ratio) of different functional types: herbaceous species (A, circles: *Bellis perennis*, *Oxalis triangularis*, *Tolpis barbata* and *Trifolium pratense*), trees (B–C, squares) including deciduous temperate trees (*Quercus petraea*, *Carpinus betulus* and *Sorbus cashmiriana*), a conifer species (*Pinus pinea*) and the Mediterranean oak (*Quercus ilex*), evergreens or semi-deciduous species (B–C, diamonds; *Arbutus unedo*, *Halimium halimifolium*, *Ficus benjamina* and *Ceratonia siliqua*) and aromatic species (B–C, triangles; *Citrus hystrix, Mentha piperita* and *Rosmarinus officinalis*).

Relationship of $\delta^{13}C_{res}$ diurnal course with basic structural parameters

The relationship between leaf characteristics (specific leaf area, SLA), water content and the C : N ratio) with the diurnal $\delta^{13}C$ enrichment in leaf respired CO_2 allowed the identification of three major functional groups (Fig. 3). Sorted by increasing $\delta^{13}C_{Light-Dark}$ amplitude, these are: herbaceous species which had the highest relative water content (> 0.75), SLA (> 0.028 $m^2\ g^{-1}$) and the lowest C : N ratio (< 15); deciduous trees, the conifer species and several shrubs (*C. hystrix*, *F. benjamina* and *C. siliqua*) with intermediate values of all leaf parameters; and Mediterranean evergreen trees and shrubs with thick leaves and hence, very low SLA (< 0.015 $m^2\ g^{-1}$), high C : N ratio (> 23) and a wide range of water contents as well as aromatic plants exhibiting different leaf structures such as needles, mesophyllic or xerophytic leaves (see Fig. 3).

Effect of diurnal CO_2 assimilation on $\delta^{13}C_{res}$

The effects of cumulative C gain on the diurnal course of $\delta^{13}C_{res}$ were investigated in plants grown under different light conditions by first, acclimatizing *Q. ilex* saplings to a 50% reduction in incident light intensity (from 350 to 180 µmol $m^{-2}\ s^{-1}$, Fig. 4a) and second, by decreasing the growth light level of *H. halimifolium* from 200 to 80 µmol $m^{-2}\ s^{-1}$ (Fig. 4b). In both cases, the diurnal increase in $\delta^{13}C_{res}$ decreased almost proportionally (from 7.3 to 3.7‰ in *Q. ilex* and from 7.9 to 4.5‰ in *H. halimifolium*, Fig. 4c,d). To assess the effect of growth light intensity or, more specifically, the cumulative incident light per day on the observed diurnal variations in $\delta^{13}C_{res}$, both *H. halimifolium* and *Q. ilex* were subjected to a 3-h dark period in the middle of the diurnal course. The observed decrease in diurnal $\delta^{13}C_{light-dark}$ amplitude was proportional to the decrease in the daily photoperiod length with a 2.1‰ and

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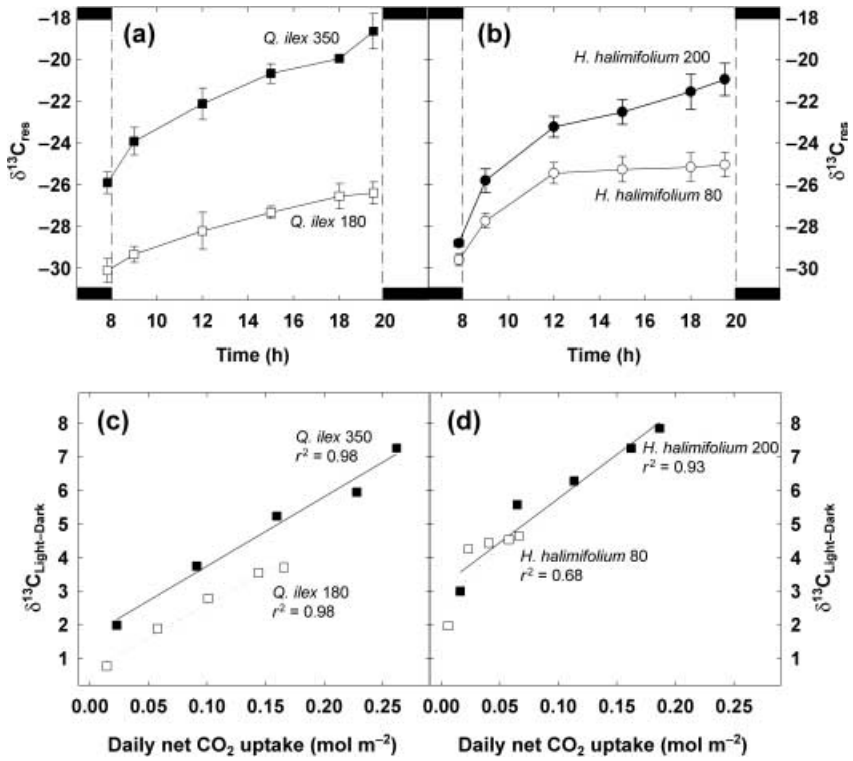

Fig. 4 Diurnal course of (mature) leaf dark-respired $\delta^{13}CO_2$ (a,b) and difference between the diurnal $\delta^{13}C_{res}$ increase obtained at five periods of the day (after 1 h, 4 h, 7 h, 10 h and 11 h and 45 min of light) relative to $\delta^{13}C_{res}$ measured at the end of the dark period ($\delta^{13}C_{Light–Dark}$, expressed in ‰) plotted against corresponding cumulative net CO_2 uptake (c,d): (a,c) *Quercus ilex* grown either under 350 μmol m^{-2} s^{-1} (*Q. ilex* 350, closed squares) or after 3 wk of acclimatization under 180 μmol m^{-2} s^{-1} (*Q. ilex* 180, open squares); (b,d) *Halimium halimifolium* plants grown under 200 or 80 μmol m^{-2} s^{-1} (*H. halimifolium* 200, closed squares; and *H. halimifolium* 80, open squares, respectively). Closed bars indicate the dark period. Symbols represent means of at least three independent replicates (± SE); r^2 of the linear relationship are given.

2.7‰ decrease in $\delta^{13}C_{Light–Dark}$ in *H. halimifolium* and *Q. ilex* subjected to the 3 h-dark period, respectively, compared with plants exposed to a full-time photoperiod of 12 h of light (see the Supporting Information, Fig. S1).

Cumulative CO_2 fixation during the light period was calculated from diurnal courses of net C assimilation rates and plotted against the observed diurnal $\delta^{13}C_{res}$ variations (Fig. 4c,d). A highly significant linear correlation was obtained for *Q. ilex* and *H. halimifolium* leaves under full ($P < 0.05$; $r^2 \geq 0.93$) and reduced light intensities ($P < 0.05$; $r^2 \geq 0.68$; Fig. 4c,d). Furthermore, the slopes of these relations were not markedly different, particularly for *Q. ilex* leaves (Fig. 4c).

Measurements on developing leaves

To evaluate whether the respiratory energy demand of a growing leaf does influence the isotopic composition of leaf-respired CO_2, growing versus fully mature leaves of *H. halimifolium* and *A. unedo* were compared (Fig. 5). The extent of $\delta^{13}C_{res}$ diurnal increase was markedly decreased in growing compared with mature leaves ($\delta^{13}C_{Light–Dark}$ of 6‰ and 7.9‰, respectively, Fig. 5a). Similar results were obtained for *A. unedo* (with $\delta^{13}C_{Light–Dark}$ of 2‰ and 7‰ in growing and mature leaves, respectively, Fig. 5b).

Positional ^{13}C-labelling experiments

The diurnal course of $\delta^{13}C_{res}$ of mature leaves from two selected species, with and without diurnal increase of leaf respired CO_2 (*H. halimifolium* and *O. triangularis* respectively), was investigated after addition of ^{13}C-enriched pyruvate into their transpiration stream (either enriched at the first, or second and third carbon atom positions, ^{13}C1 and ^{13}C2-3-enriched, respectively). When ^{13}C1-enriched pyruvate was supplied, $\delta^{13}C_{res}$ continuously increased during the light period in *H. halimifolium* leaves and decreased very rapidly after 1 h of darkness (Fig. 6c), following the diurnal pattern of unlabelled $\delta^{13}C_{res}$ (Fig. 1). In *O. triangularis*, $\delta^{13}C_{res}$ of ^{13}C1-labelled pyruvate was slightly higher in the light than in the dark but remained constant during the light (Fig. 6d). The addition of ^{13}C2-3-enriched pyruvate did not reveal any significant variations in any of the species (Fig. 6c,d).

Discussion

The primary objective of this study was to investigate species-specific differences in the magnitude of diurnal variation in $\delta^{13}C$ of leaf dark-respired CO_2 ($\delta^{13}C_{res}$). The diurnal amplitude in $\delta^{13}C_{res}$ varied from 0 to 8‰ among the species examined. Such differences could be attributed to distinct functional plant groups: all evergreen, slow-growing or aromatic species studied exhibited large variation in $\delta^{13}C_{res}$ with a common diel pattern. The $\delta^{13}C_{res}$ continuously increased during the light period compared with the morning values, followed by a decrease during the dark period. Conversely, herbaceous and fast-growing plants did not exhibit marked temporal variations. These results precisely match previous data obtained for *T. barbata* (Werner *et al.*, 2007b), for one

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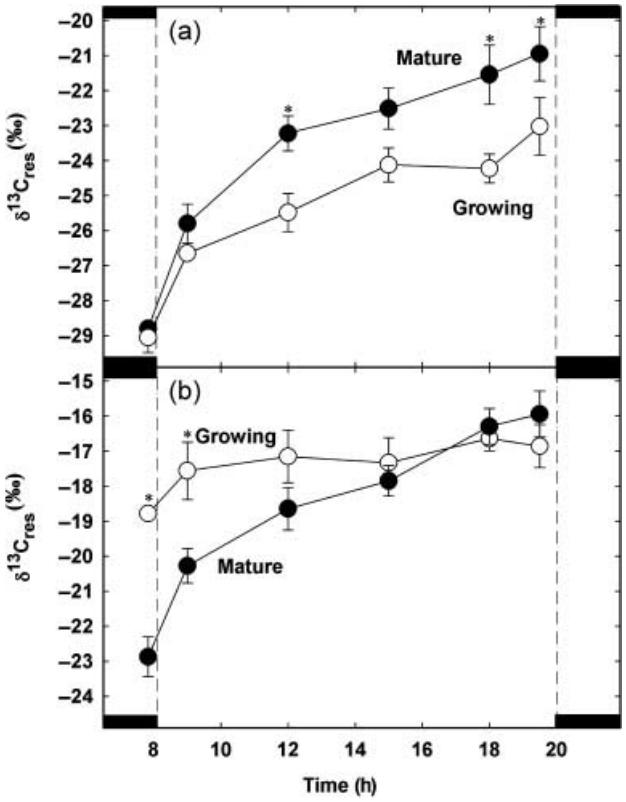

Fig. 5 Diurnal course of the dark-respired $\delta^{13}CO_2$ from fully mature (Mature, closed circles) and growing (Growing, open circles) (a) *Halimium halimifolium* and (b) *Arbutus unedo* leaves. The dark period is indicated by closed bars. Symbols represent means of three to six independent measurements (± SE) and statistically significant differences between growing and mature leaves are indicated by an asterisk ($P < 0.05$).

Pinus species (*Pinus elliottii*; Prater *et al.*, 2006) and for *Q. ilex* (Hymus *et al.*, 2005; Werner *et al.*, 2007b) in natural conditions, being a strong indication that measured $\delta^{13}C_{res}$ on glasshouse-grown species were fully representative. Nevertheless, changes in $\delta^{13}C_{res}$ diurnal patterns could occur under natural conditions with, for example, drought or temperature.

There has long been evidence for enriched $\delta^{13}C$ signals in leaf-respired CO_2 relative to leaf organic matter or respiratory substrates (Park & Epstein, 1961; Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Tcherkez *et al.*, 2003; Klumpp *et al.*, 2005), which has been explained by apparent fractionation processes in the respiratory pathways (Ghashghaie *et al.*, 2001, 2003; Tcherkez *et al.*, 2003). Our positional labelling experiments (Fig. 6) provide first evidence, that these processes are also involved in the diurnal changes in $\delta^{13}C_{res}$. In short, enrichment in dark leaf-respired $\delta^{13}CO_2$ is mainly attributed to the heterogeneous ^{13}C-distribution within hexose molecules (Fig. 6), where C3 and C4 are ^{13}C-enriched compared with other positions because of fractionation in the aldolase reaction (Rossmann *et al.*, 1991; Gleixner & Schmidt, 1997). During glycolysis, C1 of pyruvate derived from enriched C3 and C4 of glucose molecules is decarboxylated by pyruvate dehydrogenase (PDH), releasing ^{13}C-enriched CO_2, while the lighter carbon atoms are incorporated in acetyl-CoA and decarboxylated in the Krebs cycle. Acetyl-CoA molecules are partially deviated to the biosynthesis of metabolites, for example, fatty acids and secondary compounds, well known to be ^{13}C-depleted compared with carbohydrates (Park & Epstein, 1961). Accordingly, Ghashghaie *et al.* (2001) proposed that if the carbohydrate molecule is fully consumed during dark respiration no apparent fractionation will be observed (i.e. the overall CO_2 released by dark respiration carries the isotopic signature of the substrate). By contrast, in the case of a deviation of light carbon (acetyl-CoA) into biosynthetic pathways, the overall respired CO_2 is ^{13}C-enriched.

The pyruvate positional ^{13}C-labelling provides direct evidence that changes in the relative activity of the PDH-reaction (decarboxylation of $^{13}C1$-labelled pyruvate) and Krebs cycle (decarboxylation of $^{13}C2$-3-labelled pyruvate) do occur (Fig. 6). Moreover, it indicates the importance of changes in relative C flux rates through both respiratory pathways: the diurnal increase in $\delta^{13}C_{res}$ was caused by a marked increase in the C flux through PDH into secondary metabolism relative to the Krebs cycle activity, which remained constant throughout the day (for *H. halimifolium*, Fig. 6c). Hence, diurnal variations in $\delta^{13}C_{res}$ are related to an increased metabolic activity of the PDH, which exceeded the C flow into Krebs cycle by several times. By contrast, a stable low activity of both pathways was observed in the herb *O. triangularis* (Fig. 6d), which is consistent with the lack of diurnal variation in $\delta^{13}C_{res}$ in herbaceous, fast-growing species. Nevertheless, the amount of CO_2 released from C1 by PDH exceeded the CO_2 released in the Krebs cycle ($^{13}C2$-3-labelled pyruvate, Fig. 6d) even in this species. This indicates that not all the glucose molecules are fully respired even in fast-growing species, but that some acetyl-CoA molecules and/or intermediates of the Krebs cycle, which are precursors for multiple anabolic and catabolic reactions, may always be allocated into other pathways.

Effect on the balance between carbon supply and demand on $\delta^{13}C_{res}$

Differences between functional plant groups may be attributed to marked differences in magnitude of C supply through photosynthesis ('supply function') relative to the respiratory demand for growth and maintenance respiration ('demand function').

The demand function is sustained by distinct respiratory energy demand of the functional groups: slow-growing, evergreen woody species, with high diel variation in $\delta^{13}C_{res}$, generally exhibit a low demand in respiratory substrates in contrast to actively growing species with a high respiratory energy demand (e.g. herbs). The respiratory demand of a species can be broken up into growth and maintenance respiration. The latter contributes to a larger proportion of total plant

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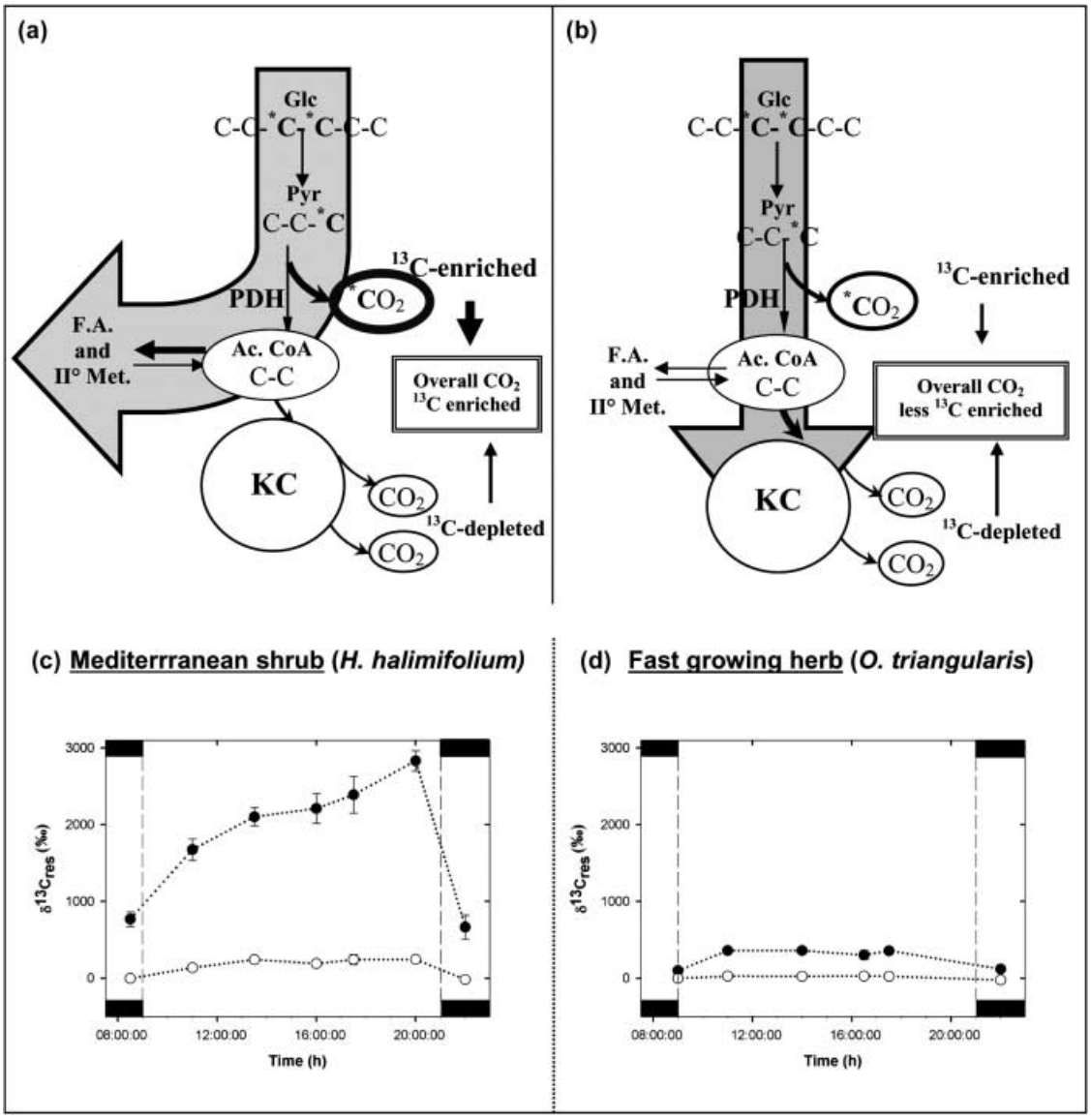

Fig. 6 Major expected fluxes of respiratory substrates (grey arrows) explaining $\delta^{13}C$ of dark-respired CO_2 depending on the respiratory energy demand (a,b) and diurnal course of $\delta^{13}C_{res}$ of two selected species with and without diurnal increase in $\delta^{13}C_{res}$ (Mediterranean shrub *Halimium halimifolium* (c) and a fast-growing herb *Oxalis triangularis* (d), respectively) fed with $^{13}C1$ or $^{13}C2$-3 labelled pyruvate. (a,b) Carbon atoms C3 and C4 of glucose (Glc) and thus C1 of pyruvate (Pyr) which is decarboxylated during pyruvate dehydrogenase (PDH) reaction are ^{13}C-enriched (*C in bold type) while depleted carbons which form acetyl-CoA (Ac. CoA) enter the Krebs cycle (KC). Tinted arrows indicate the major carbon flow. (F.A.) represents fatty acids and (II° Met.) secondary metabolites. Adapted from Werner *et al*. (2007a) based on Tcherkez *et al*. (2003). Lower panels show the diurnal course of the amount of CO_2 decarboxylated by the PDH ($^{13}C1$ pyruvate, closed circles) and in the Krebs cycle ($^{13}C2$-3 pyruvate, open circles), $n = 4–8 \pm$ SE.

respired CO_2 in slow-growing as opposed to fast-growing plants (Amthor, 1984). Given the differences in leaf structure such as higher lignin content in woody compared with herbaceous plants, Bowling *et al*. (2008) estimated that growth respiration would produce twice as enriched $\delta^{13}C_{res}$ in woody than in herbaceous plants.

The demand function is further supported by the reduced diurnal $\delta^{13}C_{res}$ variation in growing leaves, with a higher respiratory energy demand, compared with mature leaves from the same evergreen plants (Fig. 5). Most evergreen species exhibit a flush-like growth during a short period (Werner *et al*., 1999) and woody species can sustain new growth from C reserves of the previous year (Damesin & Lelarge, 2003). By contrast, in fast-growing species, the growing shoots may provide a strong C sink for the whole plant. This is further supported by Ocheltree & Marshall (2004) who found that the enrichment in $\delta^{13}C_{res}$ of *Helianthus annuus* relative to soluble sugars was negatively correlated to its relative growth rate. It supports the importance of maintenance vs growth respiration to explain isotopic increase in $\delta^{13}C_{res}$.

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However, differences in respiratory energy demand between functional groups are not expected to change on a diurnal time-scale. Hence, what seems to be of greater importance is the balance between the respiratory energy demand and the C supply rate, which can indeed exhibit marked diurnal changes. The supply function is reinforced by the fact that the increase in $\delta^{13}C_{res}$ is linearly related to the cumulative CO_2 fixation during the light period (Fig. 4), as already reported for *Q. ilex* leaves (Hymus *et al.*, 2005). Moreover, by impairing the potential CO_2 accumulation through a decreased light intensity or by interrupting the light period, the diurnal increase in $\delta^{13}C_{res}$ declined almost proportionally. The positive linear relationship between the increase in $\delta^{13}C_{res}$ and the cumulative CO_2 uptake may indicate that with the diurnal accumulation of metabolites in excess of the respiratory demand a larger proportion can be diverted into secondary metabolism, as shown through the pyruvate-labelling experiments (Fig. 6c). This is supported by findings of Prater *et al.* (2006) who induced less enriched $\delta^{13}C_{res}$ on *P. elliottii* needles by artificial shading of leaves. Thus, the diurnal increase in $\delta^{13}C_{res}$ can be attributed to the increasing flux into secondary metabolism with increasing C supply during the day when the sugar pools are filled and the respiratory demand is met.

Influence of fractionation of enzymes and respiratory substrates on $\delta^{13}C_{res}$

The observed diurnal variation in $\delta^{13}C_{res}$ of up to 8‰ exceeds the variation that can be expected from the heterogeneous intramolecular ^{13}C distribution of a glucose molecule (6‰, Rossmann *et al.*, 1991; Hobbie & Werner, 2004). However, isotope effects of the respiratory decarboxylating enzymes could increase the difference between CO_2 evolved via PDH reaction and the Krebs cycle. Tcherkez & Farquhar (2005) assumed that PDH fractionates (Melzer & Schmidt, 1987) but they also suggested that this fractionation would not be evident in case of full decarboxylation of pyruvate molecules (Tcherkez & Farquhar, 2005). Nevertheless, they have shown with quantum chemical calculations, that the enzyme citrate synthase, which catalyses the first step of the Krebs cycle, has an isotope effect of 23‰ (Tcherkez & Farquhar, 2005). Calculations of the overall isotope effects revealed that the Krebs cycle is a source of ^{13}C depletion, both in organic acids that are intermediates in the cycle and in the respired CO_2 (Tcherkez & Farquhar, 2005). Hence, this process could increase the difference in $\delta^{13}C_{res}$ above the expected difference originating from heterogeneous distribution within the glucose molecule.

The extent to which respiratory processes fractionate is further dependent on the pool sizes of the substrates (i.e. with increasing pool sizes during the day there is a higher probability for fractionation to occur). Further, the observed increase in $\delta^{13}C_{res}$ could be caused by an increase in $\delta^{13}C$ of the respiratory substrates through either (i) a diurnal decrease in photosynthetic discrimination via temporal variation of stomatal and internal conductance and Rubisco activity; or (ii) a shift to respiratory sources with more enriched isotopic signatures or (iii) a change in the relative flux rates from respiratory substrates with different $\delta^{13}C$.

Leaf respiration uses several C sources, including soluble sugars, starch, lipids or amino acids with a rapid turnover and different isotopic characteristics and residence times (Schnyder *et al.*, 2003; Nogués *et al.*, 2004). Further, a change in isotopic signature of stored vs fresh assimilates, that can contribute to up to 50% of respiration, can account for variation in $\delta^{13}C_{res}$ (Schnyder *et al.*, 2003; Nogués *et al.*, 2004). The $\delta^{13}C_{res}$ has been shown to vary over the course of various days in darkness under constant environmental conditions. Those changes were associated with the depletion of different substrate pools and/or shifts in the relative contributions of dark-respiratory pathways (Tcherkez *et al.*, 2003). Could these processes also occur in the light?

We did not observe diurnal changes in either the pool sizes or $\delta^{13}C$ of different sugars (data not shown) or leaf organic matter (Fig. 2), in agreement with other recent works that reported little diurnal variation in $\delta^{13}C$ of different respiratory substrates, despite marked diurnal variations in $\delta^{13}C_{res}$ (Hymus *et al.*, 2005; Göttlicher *et al.*, 2006). Furthermore, marked diurnal changes in photosynthetic discrimination and thus, in $\delta^{13}C$ of fresh assimilates are unlikely under the controlled conditions in the climate chamber. Vapour pressure deficit and temperature were kept constant in the glasshouses, thus, changes in those parameters can also be ruled out as a potential source of variations. Further, as these plants were grown for a prolonged period under constant conditions, there was presumably no substantial difference in the isotopic signature of old and new C reserves. However, there are further potential fractionation processes, such as transitory starch accumulation and remobilization, which have been found to govern the diel rhythm of $\delta^{13}C_{res}$ in short-term turnover pools of soluble sugars in leaves and phloem-transported organic matter (Tcherkez *et al.*, 2004; Gessler *et al.*, 2007).

Light-enhanced dark respiration

Upon darkening of a leaf an immediate increase followed by a subsequent decrease in $\delta^{13}C_{res}$ occurs (Werner *et al.*, 2007b), and hence the time of dark-incubation is important. It has been argued that this transient peak is related to light-enhanced dark respiration (LEDR; Barbour *et al.*, 2007) that can be observed as a post-illuminatory respiration pulse (Atkin *et al.*, 1998). Although the metabolic origin of such an effect is not well known (Atkin *et al.*, 1998), organic acids might be the respiratory substrates during this peak (Cornic, 1973), which might have a different $\delta^{13}C$ signature from glucose. Barbour *et al.* (2007) have suggested that malate could be a substrate for respiration by the NAD^+ malic enzyme during the LEDR peak, which would produce enriched CO_2. Our on-going work does not provide a strong support for this hypothesis. First, the transient decrease in $\delta^{13}C_{res}$ typically lasts for 30–60 min (up to 120 min, Werner *et al.*, 2007b) which is longer

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than the time frame of LEDR and second, we observed this transient peak during the decarboxylation of ^{13}C1-labelled pyruvate, which may not be expected if the major source of LEDR is the decarboxylation of (unlabelled) organic acid via the NAD^+ malic enzyme. An alternative explanation could be a rapid increase in PDH activity, which is downregulated during the light period (Tcherkez *et al.*, 2005), and subsequent rapid decarboxylation of the available sugar pools, reflecting the pool size and C flow rates. However, more research is needed to clarify the underlying processes. Nevertheless, the diurnal increase in $\delta^{13}C_{res}$ has similar amplitude when measured after 30 min darkening (*Q. ilex*, data not shown) and the reported values are consistent with data from Hymus *et al.* (2005) who incubated leaves for approx. 15–30 min. This indicates, that even if the LEDR has an influence on $\delta^{13}C_{res}$ immediately upon darkening, the diurnal pattern observed in this study will be maintained.

Differences in $\delta^{13}C_{res}$ between functional plant groups

Our data cover a broad spectrum of leaf-respired CO_2 isotopic signatures, ranging from −16 to −32‰, which may be partially attributed to differences in photosynthetic discrimination between functional groups, reflected in differences in $\delta^{13}C$ of leaf organic matter (Fig. 2). Differences in leaf structure, such as SLA, leaf water content and C : N ratios (Fig. 3) may also play a role through its effect on stomatal and mesophyll CO_2 conductance. Evergreen species have a lower internal CO_2 conductance compared with deciduous trees and herbaceous plants (e.g. 0.1 and 0.24 for *Q. ilex* and *Q. petraea,* respectively; Roupsard *et al.*, 1996; for references on mesophyll conductance see Ethier & Livingston, 2004). Mesophyll conductance can vary rapidly with incident light, temperature, CO_2 and humidity (Piel *et al.*, 2002; Warren & Dreyer, 2006; Flexas *et al.*, 2007), however, to our knowledge there are no reports on diurnal changes in mesophyll conductance. Stomatal conductance remained constant over the day in species with marked increase in $\delta^{13}C_{res}$ (such as *Q. ilex*, *H. halimifolium* and *A. unedo*; data not shown). Further, a diurnal $\delta^{13}C_{res}$ increase was observed for all leaf types investigated (including needles, sclerophyllous and mesophyllous leaves; see Figs 1 and 3) and in *M. piperita*, a herbaceous aromatic plant with larger SLA and (most probably) high mesophyll conductance. Thus, while species might be clustered into functional groups by leaf morphology, this is not likely to account for the observed diurnal patterns.

One common function of species with a high diurnal increase in $\delta^{13}C_{res}$ seems to be an increased biosynthesis of secondary metabolites for defence, stress avoidance or aromatic compounds. For 10 species of this group, including the aromatic herb *M. piperita* (Nogués *et al.*, 2006), we found a literature reference for volatile compound emission, particularly isoprene (no data were available for the remaining species).

In *Q. cilex* and *P. pinea,* for example, monoterpene emissions were found to be highly variable at the diurnal timescale, and markedly dependent on the incident light level (Staudt *et al.*, 1997; Sabillon & Cremades, 2001). Isoprene emission is closely linked to photosynthesis and can exhibit a marked diurnal increase (Rapparini *et al.*, 2004). Although several pathways can be involved in isoprene synthesis, Affek & Yakir (2003) showed that 72–91% of emitted isoprene was derived from recently fixed C. An inverse relationship between dark respiration rate and isoprene emission was found in several studies and it has been hypothesized that these two processes compete for the same substrate (Rosenstiel *et al.*, 2003, 2004), which would be in agreement with our results. Loreto *et al.* (2007) confirmed this relationship for young leaves only, showing that the decrease in respiratory demand when leaves mature is accompanied by a progressive and rapid increase in isoprene emission during leaf development. This is in agreement with the reduced diurnal increase in $\delta^{13}C_{res}$ in young leaves (Fig. 5). Further, several species with marked diurnal variation in $\delta^{13}C_{res}$ are aromatic species (e.g. *R. officinalis* and *M. piperita*), which do allocate C into secondary compound synthesis of aromatic volatile molecules.

Hence, the diurnal increase in $\delta^{13}C_{res}$ may be attributed to the increasing flux into secondary metabolism with increasing C supply during the day when the sugar pools are filled and the respiratory demand is met.

It will be important to evaluate the impact of these dynamics in $\delta^{13}C_{res}$ at other spatial scales, from organs (e.g. shoots, stems and roots) to the plant level and for other ecosystem compartments (e.g. soil) to identify, how variations in $\delta^{13}C_{res}$ of different ecosystem compartments will influence the integrated signal of ecosystem respiration ($\delta^{13}C_R$). There is now increasing evidence that significant diurnal cycles in $\delta^{13}C_{res}$ also occur in many other respiratory sources in the ecosystem, such as trunk (Maunoury *et al.*, 2007; Kodama *et al.*, 2008), soil (Kodama *et al.*, 2008), roots (S. Unger *et al.*, unpublished) and ecosystem (Bowling *et al.*, 2003; Knohl *et al.*, 2005; Werner *et al.*, 2006; Kodama *et al.*, 2008). Such knowledge is of major importance as it may affect the reliability of our estimates of ecosystem respiration, which is used in many modelling approaches to partition ecosystem C fluxes (Yakir & Wang, 1996; Bowling *et al.*, 2001; Knohl & Buchmann, 2005) and may thus affect our predictions on ecosystem response to environmental changes.

Concluding remarks

This is the first large species survey on short-term $\delta^{13}C_{res}$ variations that could be attributed to marked apparent fractionation processes in the respiratory pathways. Our results support the hypothesis that the diurnal increase in $\delta^{13}C_{res}$ is enhanced during the light period in species with a high investment in secondary metabolism, whereas fast-growing herbs and grasses with a high respiratory energy demand do not show this diurnal pattern. Pyruvate positional ^{13}C-labelling provided the first direct evidence that diurnal variations in $\delta^{13}C_{res}$ are related to increased metabolic activity of PDH at

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low constant Krebs cycle activity, and point out the importance of changes in relative flux rates between both pathways. Differences between functional groups may be attributed to marked differences in the balance between the C supply through the amount of fresh assimilates during photosynthesis versus the respiratory demand for growth and maintenance respiration. Hence, C isotope composition of plant-respired CO_2 contains information on the fate of respiratory substrates, and may, therefore, provide a nonintrusive way to identify changes in C allocation patterns. These short-term variations in $\delta^{13}C_{res}$ have marked implications at larger scales, particularly for isotope partitioning studies at the ecosystem level.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Characteristic diurnal course of $\delta^{13}C_{res}$ from (a) *Quercus ilex* and (b) *Halimium halimifolium* mature leaves for which the 12-h photoperiod was decreased by including a 3-h dark period in the middle of the light period.

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