Measurement of leaf day respiration using a new isotopic disequilibrium method compared with the Laisk method

Xiao Ying Gong [1], Guillaume Tcherkez [2], Johannes Wenig [1], Rudi Schäufele [1], Hans Schnyder [1]

[1] Lehrstuhl für Grünlandlehre, Technische Universität München, Alte Akademie 12, 85354 Freising, Germany.

[2] Research School of Biology, ANU College of Medicine, Biology and Environment, Australian National University, Canberra ACT 0200, Australia.

Corresponding authors: Xiao Ying Gong, Tel. +49 8161713722, Email: xgong@wzw.tum.de;
Rudi Schäufele, Tel. +49 8161715265, Email: schaeufele@wzw.tum.de

Summary

- Quantification of leaf respiration is of great importance for the understanding of plant physiology and ecosystem biogeochemical processes. Leaf respiration continues in light (R_L) but supposedly at a lower rate compared to the dark (R_D). Yet, there is no method for direct measurement of R_L and most available methods require unphysiological measurement conditions.
- A method based on isotopic disequilibrium quantified R_L ($R_{L\ 13C}$) and mesophyll conductance of young and old fully-expanded leaves of six species compared $R_{L\ 13C}$ to R_L values determined by the Laisk method ($R_{L\ Laisk}$).
- $R_{L\ 13C}$ and $R_{L\ Laisk}$ were consistently lower than R_D. Leaf ageing negatively affected photosynthetic performance, but had no significant effect on R_L or R_L/R_D as determined by both methods. $R_{L\ Laisk}$ and $R_{L\ 13C}$ were measured successively on the same leaves and correlated positively (r^2=0.38), but average $R_{L\ Laisk}$ was 28% lower than R_{L13C}. Using A/C_c curves instead of A/C_i curves, a higher photocompensation point Γ^* (by 5 μmol mol^{-1}) was found but the correction had no influence on $R_{L\ Laisk}$ estimates.
- The results suggest that the Laisk method underestimated R_L. The isotopic disequilibrium method is useful for assessing responses of R_L to irradiance and CO_2, improving our mechanistic understanding of R_L.

Key words: mitochondrial respiration, photosynthesis, carbon isotope discrimination, mesophyll conductance, photocompensation point, leaf age

INTRODUCTION

Foliar respiration is a major component of the global carbon cycle, releasing more than three times the amount of CO_2 liberated by anthropogenic emission each year (Le Quere *et al.*, 2009; Beer *et al.*, 2010), if it is assumed that foliar, i.e. plant leaf respiration constitutes 50-80% of plant respiration globally (Atkin *et al.*, 2007; Lehmeier *et al.*, 2010). Thus knowledge of the drivers and controls of leaf respiration is essential for understanding plant physiology and the global carbon budget, and that knowledge is required for improving the representation of leaf respiration in climate-vegetation models (Atkin *et al.*, 2007; Heskel *et al.*, 2013). The fact that leaf respiration rate is lower in light (R_L, also termed day respiration) compared to the dark (R_D) – when normalized to the same temperature – has long been recognized and demonstrated in leaf- (Brooks & Farquhar, 1985; Atkin *et al.*, 2000; Gong *et al.*, 2015), stand- (Schnyder *et al.*, 2003; Gong *et al.*, 2017a), and ecosystem-scale (Wehr *et al.*, 2016) studies. The inhibition of respiration by light is underpinned by the light-induced down-regulation of the activity of several enzymes of respiratory metabolism (Tcherkez *et al.*, 2005; Tcherkez *et al.*, 2012a). Yet, the quantification of R_L is technically challenging and the mechanism controlling its variation is uncertain.

In practice, R_L cannot be directly measured using conventional gas exchange measurements because R_L is masked by other concurrent, major fluxes: photosynthetic CO_2 uptake and photorespiratory CO_2 release. Net CO_2 assimilation rate can be expressed as: $A = V_c - 0.5V_o - R_L$, where V_c is the rate of carboxylation and V_o is that of oxygenation, and $0.5V_o$ is the rate of photorespiration (F). A can be further expressed as:

$$A = V_c\,(1 - \Gamma^*/C_c) - R_L \qquad (1)$$

where Γ^* is the CO_2 compensation point in the absence of day respiration and C_c is the chloroplastic CO_2 mole fraction. At a C_c that equals to Γ^*, A is equal to $-R_L$. Based on Eqn 1, R_L can be estimated from the common intersection of curves of net CO_2 assimilation rate (A) vs. intercellular CO_2 concentration (C_i) measured under low CO_2 and sub-saturating levels of irradiances (defined as $R_{L\,Laisk}$ here), as described in Laisk (1977) and further extended by Brooks & Farquhar (1985). This is based on the notion that at R_L is insensitive to light intensity. The Laisk method uses A/C_i curves instead of A/C_c curves to determine the common intersection, and thus gives the apparent, C_i-based CO_2 compensation point (C_i^*) and R_L at $C_i = C_i^*$. Although it has been widely used as a standard method for determining R_L and Γ^* (in assuming $\Gamma^*=C_i^*$)

(Farquhar *et al.*, 1980; von Caemmerer, 2000; Walker & Ort, 2015), uncertainties and limitations of the Laisk method have been intensively discussed. First, ignoring the influence of mesophyll conductance (g_m) might lead to errors in estimates of R_L and Γ^*, as $\Gamma^* = C_i^* + R_L/g_m$ (Brooks & Farquhar, 1985; von Caemmerer *et al.*, 1994; Walker & Ort, 2015). Second, the measurement must be performed at very low CO_2 that generally contrast with growth conditions (Villar *et al.*, 1994; Yin *et al.*, 2011). Experimental evidence has indicated a CO_2 effect on respiration rate in light (Gong *et al.*, 2017a) and on the abundance of transcripts encoding enzymes of the respiratory pathway in both long-term (Leakey *et al.*, 2009) and short-term (Li *et al.*, 2013) treatments. These observations raise the concern that R_L measured by the Laisk method might differ from actual R_L under growth conditions. Similarly, other methods, such as the Kok method (Kok, 1948) and a method based on chlorophyll fluorescence (Yin *et al.*, 2011), generally must be performed at low CO_2 levels or low irradiance levels and require manipulation of CO_2 assimilation rate (for a review see Yin *et al.*, 2011). Furthermore, during both Kok and Laisk measurements, variations in C_c are critical but have not been accounted for, potentially leading to errors in R_L estimates (Farquhar & Busch, 2017; Tcherkez *et al.*, 2017a b).

Techniques that allow measuring R_L without requiring modifications of environmental conditions such as CO_2 mole fraction or irradiance typically use carbon isotopes. The principle of deconvoluting CO_2 flux components by artificially created isotopic disequilibrium (i.e. labelling) has been widely explored for i.e. photorespiration (Ludwig & Canvin, 1971) or stand- (Schnyder *et al.*, 2003; Gong *et al.*, 2017a) or ecosystem-scale (Ostler *et al.*, 2016) autotrophic respiration. This type of labelling method exploits the fact that CO_2 flux components have distinct dynamics of tracer incorporation during the labelling. Abrupt changes to a $^{13}CO_2$ atmosphere were used to monitor the liberation of $^{12}CO_2$ by respiration in the first minutes following the isotopic changeover (Loreto *et al.*, 2001; Pinelli & Loreto, 2003). However, when using pure $^{13}CO_2$ this technique is relatively costly and requires a ^{13}C-sensitive infrared gas analyzer. Gong *et al.* (2015) described a leaf-level isotopic disequilibrium method to quantify R_L using CO_2 sources of natural ^{13}C abundance, which is based on concurrent measurements of photosynthetic gas exchange and $^{13}C/^{12}C$ isotope composition (denoted as δ, definition see methods) of CO_2 fluxes, i.e. online ^{13}C discrimination by net photosynthesis (online Δ). In other words, the δ-value of gross fixed CO_2 (associated with the flux V_c) responds instantaneously at the onset of labelling (i.e. abrupt change of δ of CO_2 fed to leaf), with the δ-value of the

photorespired CO_2 (flux $0.5V_o$) following with only a short delay (half-life in the order of a few minutes (Ludwig & Canvin, 1971)). By contrast, the δ-value of respired CO_2 responds rather slowly (half-life in the order of one to a few days (Schnyder *et al.*, 2003; Lehmeier *et al.*, 2008; Tcherkez *et al.*, 2012b; Gong *et al.*, 2017a). This approach requires two sets of online Δ measurements on similar leaves (or the very same leaves, as in this study), so as to examine the isotopic mass balance at the photosynthetic steady-state (Gong *et al.*, 2015). This method has the following advantages: (i) R_L measurements can be done at any setting of environmental parameters, e.g. identical to growth conditions; (ii) it measures R_L at the photosynthetic steady-state without manipulation of the photosynthesis rate; (iii) it simultaneously provides a reliable measurement on mesophyll conductance (g_m), another important parameter. As it relies on the measurements of δ-values and CO_2 exchange rates, diffusive leaks across the gasket of leaf cuvette must be minimized (Gong *et al.*, 2017b) or accounted for (Gong *et al.*, 2015).

Here, we use the isotopic disequilibrium method (presented by Gong *et al.* 2015) to measure R_L ($R_{L\ 13C}$) on single leaves, and compare the results with the Laisk method ($R_{L\ Laisk}$) applied to the very same leaves. Thus, our objectives were to answer the following questions. (i) Does the isotopic disequilibrium method also show an inhibition of leaf respiration by light? (ii) Do R_L estimates from isotopic disequilibrium agree with those from the Laisk method for different species and leaves of different age effects? Or (iii) is there any consistent offset in R_L estimates obtained with the two methods, and if yes, is the offset correlated to leaf age or simply due to assumptions on internal/mesophyll conductance? To this end, $^{13}CO_2/^{12}CO_2$ exchange of leaves from plants grown with ambient CO_2 with a $\delta^{13}C$ of CO_2 ($\delta^{13}C_{CO2}$) near –10‰ was measured sequentially in the presence of CO_2 with a $\delta^{13}C_{CO2}$ of –31.2‰ and –6.3‰, and R_L of leaves was solved using isotopic mass balance equations. These measurements were immediately followed by determinations of $R_{L\ Laisk}$. The comparison of $R_{L\ 13C}$ and $R_{L\ Laisk}$ was performed on both young and old mature leaves of two grass and four legume species. Villar *et al.* (1995) have reported that ageing of leaves of an evergreen shrub led to a reduction of $R_{L\ Laisk}/R_D$ from 0.5 to 0.2. This is the reason why we included young and old leaves, since it might increase the variation range of R_L and thus enhance the method comparison. In addition, we estimated g_m of every leaf, so that A/C_i curves could be converted to A/C_c curves to estimate Γ^* and $R_{L\ Laisk}$ based on the common intersection of A/C_c curves.

MATERIALS AND METHODS

Plant material and growth conditions

Six herbaceous plant species were used, namely barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), castor bean (*Ricinus communis*), French bean (*Phaseolus vulgaris*), soybean (*Glycine max*) and broad bean (*Vicia faba*). Plants were grown from seed in plastic pots filled with quartz sand, placed in a growth chamber (PGR15, Conviron, Winnipeg, Canada) and supplied with a modified Hoagland nutrient solution with 7.5 mM nitrate (cf. Gong *et al.*, 2017b) every two to three days. Environmental conditions during plant growth were: a photosynthetic photon flux density (PPFD) of 700 µmol m^{-2} s^{-1} during the 12 h-long photoperiod per day, ambient CO_2 concentration ($[CO_2]$) of about 400 µmol mol^{-1}, air temperature of 22 °C during photo- and dark-periods, relative humidity of 50% during photoperiod and 60% during dark period. The density of plants in the growth chamber was rather low, thus leaves were not shaded. Young leaves, defined as the youngest fully expanded leaves, were measured when plants reached a stage of having 3-4 mature leaves per branch/tiller. Old leaves, defined as two age categories older than the measured young leaves, were measured about 10 days later. At that time plants had 5-7 mature leaves per branch/tiller. For dicots, the fully expanded terminal leaflets were measured. Young leaves were measured for all species, while old leaves of *G. max* and *V. faba* were not measured.

$^{13}CO_2/^{12}CO_2$ gas exchange facilities

$^{13}CO_2/^{12}CO_2$ gas exchange and labelling were performed using the protocols and facilities described in Gong *et al.* (2015) with modifications and advancements as follows. The approaches in Gong *et al.* (2015) provided a mean leak coefficient and a R_L/A for a group of similar leaves (same species and age, treated as replicates). In this study, leak coefficients were measured for each leaf and used for the correction of its gas exchange data, using the equations in Gong *et al*., (2015). To quantify R_L, the two components of A must be separated, as $A = P - R_L$, where P is the apparent photosynthesis rate ($P = V_c - F$). Briefly, we switch the CO_2 source supplied to leaf photosynthesis to create isotopic disequilibrium between P and R_L, namely, P will be immediately labelled while R_L is fed by substrate formed during plant growth (old carbon) (Gong *et al.*, 2015), thus R_L can be solved by isotopic mass balance (see below).

The leaf-level $^{13}CO_2/^{12}CO_2$ gas exchange and labelling system included a portable CO_2 exchange system (LI-6400, LI-COR Inc., Lincoln, USA) housed in a gas exchange mesocosm (chamber 1, cf. Gong *et al.*, 2015; Gong *et al.*, 2017b), and another gas exchange mesocosm (chamber 2) for the purpose of providing labelling CO_2. The air supply to both mesocosms and the LI-6400 was mixed from CO_2-free, dry air (with 21% O_2) and CO_2 of known $\delta^{13}C_{CO2}$ (cf. (Schnyder *et al.*, 2003), with $\delta^{13}C$ denoting the ^{13}C composition of a sample defined as the relative deviation of its $^{13}C/^{12}C$ ratio (R_{sample}) to that of the international VPDB standard (R_{VPDB}): $\delta^{13}C = R_{sample} / R_{VPDB} - 1$. $[CO_2]$ inside chamber 1 was monitored with an infrared gas analyzer (LI-6262, LI-COR Inc., Lincoln, USA). During leaf gas exchange measurements, the plants to be measured and the sensor head of the LI-6400 were placed inside the chamber 1. Using this setup, we separately controlled the CO_2 concentration and $\delta^{13}C_{CO2}$ in the leaf cuvette and growth chambers. The growth chamber and leaf cuvette systems were coupled to a continuous-flow stable isotope ratio mass spectrometer (IRMS; Delta^{plus} Advantage equipped with GasBench II, ThermoFinnigan, Bremen, Germany) for ^{13}C analysis of the sample air. The whole-system precision of repeated measurements on $\delta^{13}C$ was 0.09‰ (SD, n=50). For further details of the method see Gong *et al.* (2015) and Gong *et al.* (2017b).

Determinations of K_{CO2}, R_D

Measurements of each leaf started with the determination of the cuvette leak coefficient for CO_2 (K_{CO2}) with the leaf present in the cuvette during these measurements (Gong *et al.*, 2015). Each leaf was held in the leaf cuvette of the LI-6400 for more than 20 min in the dark, at a constant $[CO_2]$ of 488 ± 9 (SD) µmol mol^{-1} in the leaf cuvette (C_{out}) and 400 µmol mol^{-1} in the chamber 1 (C_M) that housed the LI-6400 measurement head (detailed measurement conditions are shown in Table S1). When gas exchange had reached a constant rate, gas exchange parameters, including $[CO_2]$ and the $\delta^{13}C$ of the incoming (C_{in} and δ_{in}) and outgoing cuvette air (C_{out} and δ_{out}) were measured with the LI-6400 and the online IRMS. Thereafter, C_M was reduced to about 200 µmol mol^{-1} and the same gas exchange parameters were measured at steady-state. Since manipulating C_M should only affect the diffusive leak between the chamber 1 housing the leaf gas exchange equipment and the internal space of the leaf cuvette but not R_D, K_{CO2} was determined as the slope of the observed net CO_2 exchange rate in the dark (N_D) and $(C_M - C_{out})/s$ relationship as:

$$N_D = R_D + K_{CO2} (C_M - C_{out})/s, \quad (2)$$

where s is the leaf area (Gong et al. 2015). Knowing the K_{CO2} of each intact leaf, CO_2 exchange data were corrected as shown in Gong *et al.* (2015) and R_D determined. Since leak coefficients for $^{12}CO_2$ and $^{13}CO_2$ were virtually the same (Gong *et al.*, 2015), K_{CO2} was used to correct both $^{12}CO_2$ and $^{13}CO_2$ flux data. Before all calculations, data of δ and rates of CO_2 fluxes were corrected for leak artefact using K_{CO2} of individual leaves and equations in Gong *et al.* (2015).

$\delta^{13}C$ of R_D was calculated as:

$$\delta_{RD} = (\delta_{in} C_{in} - \delta_{out} C_{out})/(C_{in} - C_{out}), \quad (3)$$

with δ_{in} and δ_{out} are δ measured at inlet and outlet air stream, respectively.

^{13}C labelling

After measurements of K_{CO2}, R_D and δ_{RD}, the light source of the LI-6400 was switched on (PPFD 700 µmol m^{-2} s^{-1}) to measure the online Δ using CO_2 sources with different $\delta^{13}C$ of CO_2 (−6.3‰ and −31.2‰). The $^{12}C/^{13}C$ discrimination associated with net photosynthesis, Δ_A, was calculated according to Evans *et al.* (1986): $\Delta_A = \xi (\delta_{out} - \delta_{in})/(1+ \delta_{out} - \xi (\delta_{out} - \delta_{in}))$, where $\xi = C_{in}/(C_{in} - C_{out})$. Here, ξ was below 15 during Δ_A measurements. Measurements of Δ_A were done in the photosynthetic steady-state: after about 30 min of stabilization in the conditions similar to that of plant growth average C_{out} of 394 ± 34 (SD) µmol mol^{-1}, average relative humidity of 76 ± 10 %, block temperature of 22 °C (mean leaf temperature was 23.3 ±0.2 °C, Table S1). Online Δ was firstly measured using the depleted CO_2 source (−31.2‰), then measured with the enriched CO_2 source (−6.3‰) on each leaf. Chamber 2 was used to mix the labelling air containing the enriched CO_2 with the targeted [CO_2]. When labelling start, well mixed air in Chamber 2 was supplied to the inlet of LI-6400 with a peristaltic pump. Using this setup, the labelling air can completely flush out the air in the LI-6400 system within 8 min. The second online Δ was measured within 15 min after the start of labelling (i.e. switching of CO_2 sources), and all photosynthetic gas exchange rates are not influenced by labelling, as only $\delta^{13}C$ of CO_2 fed to leaf was changed (Gong *et al.*, 2015).

Calculations of R_L

Substituting the relationship giving the photosynthetic assimilation in the absence of day respiration P (= $V_c - F$) into equation (1) gives: $A = P - R_L$ (4)

Applying isotopic mass-balance to equation (4) gives:

$$\delta_P = (\delta_A A + \delta_{RL} R_L)/(A + R_L) \quad (5)$$

where δ_P, δ_A, δ_{RL} are the $\delta^{13}C$ of P, A and R_L, respectively. With the two sets of online Δ measurements we have:

$$\delta_{P\,d} = (\delta_{A\,d} A + \delta_{RL\,d} R_L)/(A + R_L) \quad (6)$$

$$\delta_{P\,e} = (\delta_{A\,e} A + \delta_{RL\,e} R_L)/(A + R_L) \quad (7)$$

where subscripts "d" and "e" indicates parameters measured with the ^{13}C-depleted and ^{13}C-enriched CO_2 sources, respectively. Since ^{13}C discrimination in P (Δ_P), is independent of the $\delta^{13}C$ of the CO_2 source (Farquhar *et al.*, 1989):

$$\Delta_P = (\delta_{out\,d} - \delta_{P\,d})/(1+ \delta_{P\,d}) = (\delta_{out\,e} - \delta_{P\,e})/(1+ \delta_{P\,e}). \quad (8)$$

Combining the rearranged Eqn 6-8 we have:

$$R_{L13C} = A \cdot \frac{\frac{(\delta_{Ae} - \delta_{Ad})(1+\Delta_P)}{\delta_{oute} - \delta_{outd}} - 1}{1 - \frac{(\delta_{RLe} - \delta_{RLd})(1+\Delta_P)}{\delta_{oute} - \delta_{outd}}} \quad (9a)$$

Equation (9a) includes the isotope composition of day-respired CO_2 (both under a ^{13}C-enriched and ^{13}C-depleted atmosphere) in the denominator. Under the assumption that day respiration reacts very slowly to photosynthetic input (see *Introduction*), $\delta_{RL\,d} = \delta_{RL\,e}$ and equation (9a) rearranges to:

$$R_{L13C} = A \cdot \left(\frac{(\delta_{Ae} - \delta_{Ad})(1+\Delta_P)}{\delta_{oute} - \delta_{outd}} - 1 \right) \quad (9b)$$

In practice, the approximation $\delta_{RL\,d} = \delta_{RL\,e}$ is not critical: if some C atoms photosynthetically fixed under the ^{13}C-depleted atmosphere were channelled to respiratory metabolism and liberated as CO_2 under the ^{13}C-enriched atmosphere, this would lead to a change of a few per mils only in the denominator and the change in $R_{L\ 13C}$ would be very small. In fact, during the first measurement phase (≈ 20 min) under the ^{13}C-depleted atmosphere, we expect at most 10% turn-over in leaf respiratory pools (measured by Nogues *et al.* (2004) for dark respiration) meaning a maximal putative change in δ_{RL} of about 0.6‰ (Table S2, the denominator in equation 9a would thus be equal to 0.975 instead of 1).

In Gong *et al.* (2015), the approximation that $1+\Delta_P = 1$ was used. Here, we applied a different approximation that $\Delta_P = \Delta_{A\,e}$, which was shown to be an acceptable approximation

when the enriched CO_2 source (−6.3‰) was close to that of the growth environment (−10‰) (Gong *et al.*, 2015).

Thus, R_L was calculated as follows:

$$R_{L\,13C} = A \cdot \left(\frac{\left(\delta_{Ae} - \delta_{Ad}\right)\left(1+\Delta_{Ae}\right)}{\delta_{oute} - \delta_{outd}} - 1 \right) \tag{10}$$

The δ-value of net assimilated CO_2 was calculated as: $\delta_A = (\delta_{in}\, C_{in} - \delta_{out}\, C_{out})/(C_{in} - C_{out})$. $R_{L\,13C}/R_D$ was calculated with a (small) correction accounting for the temperature difference between light and dark, using a Q_{10} of 2 (see Gong *et al.*, 2015).

Calculation of mesophyll conductance

Mesophyll conductance (g_m) is defined as $g_m = A/(C_i - C_c)$ (cf. Evans *et al.*, 1986), where C_c is the CO_2 mole fraction at the site of carboxylation in the chloroplast. Estimation of C_c was based on the photosynthetic $^{12}C/^{13}C$ discrimination model of Farquhar *et al.* (1989) (cf. Gong *et al.*, 2015). In fact, a modified equation of $^{13}C/^{12}C$ discrimination that includes both mesophyll resistance and ternary effects (Farquhar & Cernusak, 2012) is:

$$\Delta_P = \frac{1}{1-t}\left[a\frac{C_a - C_i}{C_a}\right] + \frac{1+t}{1-t}\left[a_m\frac{C_i - C_c}{C_a} + b\frac{C_c}{C_a} - f\frac{\Gamma^*}{C_a}\right] \tag{11a}$$

while the simplified equation that excludes mesophyll resistance (or assumes infinite g_m) can be written as:

$$\Delta_{Pi} = \frac{1}{1-t}\left[a\frac{C_a - C_i}{C_a}\right] + \frac{1+t}{1-t}\left[b\frac{C_i}{C_a} - f\frac{\Gamma^*}{C_a}\right] \tag{11b}$$

Therefore, the subtraction (11a) – (11b) gives:

$$\Delta_{Pi} - \Delta_P = \frac{1+t}{1-t}\cdot\frac{C_i - C_c}{C_a}\left(b - a_m\right) \tag{12}$$

where a = 4.4‰, b = 28.9‰, a_m combines dissolution and diffusion in the liquid phase so that a_m = 1.8‰ (Evans *et al.*, 1986) and f = 11‰ (Ghashghaie *et al.*, 2003; Lanigan *et al.*, 2008). Γ^*, was approximated to be equal to C_i^* measured by the Laisk method (see below). t represents the ternary correction factor (Farquhar & Cernusak, 2012):

$$t = (1+a)E/(2g_{sc}), \tag{13}$$

where E is the transpiration rate and g_{sc} is the stomatal conductance to CO_2. Here, we ignored the boundary layer resistance because air was well mixed in the leaf cuvette of the LI-6400

(Kromdijk *et al.*, 2010). Δ_P can be calculated using Eqn 6-8, assuming $\delta_{RL} = \delta_{RD}$ (Gong *et al.* 2015). Each leaf had two measurements of Δ_P using the two CO_2 sources ($\Delta_{P\,e}$ and $\Delta_{P\,d}$, and theoretically they should be very similar and can be treated as technical replicates, see also Fig. 3), thus the mean of $\Delta_{P\,e}$ and $\Delta_{P\,d}$ was used to calculate C_c using Eqn 12.

It should be noted that equation (11a) simply represents the model of photosynthetic fractionation where the term associated with day respiration has been omitted. That is, the full model following Farquhar *et al*. (1989) notations is:

$$\Delta_A = \Delta_P - \frac{1+t}{1-t} \cdot \frac{eR_L}{kC_a} \qquad (14)$$

where e is the isotope fractionation by day respiration, with respect to net fixed photosynthates which are assumed to represent the respiratory substrates. However, considering that respiratory substrate pool turn-over is slow and mostly disconnected from photosynthesis at time scales less than the duration of the measurements (30-45min), day respiration is fed by a distinct carbon source and thus equation (14) has to be changed to (Tcherkez *et al.*, 2011):

$$\Delta_A = \Delta_P - \frac{1+t}{1-t} \cdot \frac{eR_L}{A} \qquad (15)$$

In Eqn 15, e is still expressed relative to net fixed CO_2 (i.e. $e = (\delta_A - \delta_{RL})/(\delta_{RL} + 1)$). Under our conditions, t is very small (< 0.1‰) , thus Eqn 15 can be rearranged as

$$e = (\Delta_P - \Delta_A) \cdot A/R_L. \qquad (16)$$

Measurement of R_L and C_i^* using the Laisk method

After online Δ measurements, each single leaf was measured for R_L using the Laisk method (Laisk, 1977; Brooks & Farquhar, 1985) with the LI-6400 open system. Briefly, A/C_i curves were obtained at three levels of PPFD, 50-70, 100-150, and 250 µmol m^{-2} s^{-1}, and C_{out} was decreased from 110 to 50 µmol mol^{-1} step-wise at each PPFD. Average relative humidity was 77±9% and block temperature 22 °C (meaning that leaf temperature was 22.4±0.2 °C, Table S1). Again, the observed A and C_i values were firstly corrected for leak artefacts. The coordinates of the common intersection of A/C_i curves provided the estimates of $R_{L\,Laisk}$ and C_i^* (Fig. S4). We also tested the slope-intersection regression approach suggested by Walker & Ort (2015), a modified Laisk method, but it yielded very similar results (data not shown) as the common intersection approach in the original Laisk method.

Using Laisk measurements, we estimated $R_{L\ Laisk\ CC}$ and Γ^* from the A/C_c curves (cf. Fig. S4). For this purpose, we established the relationship between g_{sc} and g_m across the measured leaves, and g_{sc}/g_m was plotted against A or C_{out} to check whether the g_{sc} - to - g_m ratio was independent of photosynthesis rate or CO_2 mole fraction. Using the g_{sc}/g_m relationship, g_m along A/C_i curves was estimated from measured g_{sc}, and thus A/C_i curves could be converted into A/C_c curves.

RESULTS

R_L across species and leaf age

As expected, both $R_{L\ Laisk}$ and $R_{L\ 13C}$ were consistently lower than R_D, demonstrating that the labeling technique generally also shows an inhibition of leaf respiration in the light compared to the dark (Table 1). Further, leaf age had no effect on $R_{L\ Laisk}$ or $R_{L\ 13C}$ (Table 1). Also, both methods showed similar species effects: *H. vulgare* and *P. vulgaris* had higher $R_{L\ Laisk}$ and $R_{L\ 13C}$ than the other species; *T. aestivum* had the lowest $R_{L\ Laisk}$ and $R_{L\ 13C}$ of young leaves and *R. communis* the lowest $R_{L\ Laisk}$ and $R_{L\ 13C}$ of old leaves. Pooling over all $R_{L\ 13C}$ and $R_{L\ Laisk}$ paired data, a significant positive correlation was found (r^2=0.38, p<0.001, Fig. 1). Importantly, however, $R_{L\ Laisk}$ was systematically smaller than $R_{L\ 13C}$ by 28% (averaged over all leaves), and this effect was similar for the different species and age classes (Fig. 1). As a result, the ratio of respiration in light to that in darkness at the same temperature (R_L/R_D) was higher for the isotopic disequilibrium method than the Laisk method: $R_{L\ 13C}/R_D$ ranged between 0.6 and 1.3 with a mean of 0.9, and $R_{L\ Laisk}/R_D$ ranged between 0.4 and 0.9 with a mean of 0.7 (Fig. 2). Both measurements showed a tendency of increasing R_L/R_D with leaf ageing; however, a significant age effect on $R_{L\ 13C}/R_D$ was detected in *P. vulgaris* while a clear age effect on $R_{L\ Laisk}/R_D$ was found in *P. vulgaris*, *T. aestivum* and *R. communis* (Fig. 2). R_D was not significantly different between age classes, but differed between species, with *T. aestivum* having the smallest R_D value of all species.

Photosynthetic parameters

Leaf ageing had clear effects on many gas exchange parameters (Table 1) when averaged across species. Old leaves had an approx. 30% lower net CO_2 assimilation rate (A), 58% lower stomatal conductance to water vapour (g_{sw}), 47% lower mesophyll conductance (g_m), 11% lower ratio of

internal-to-atmospheric CO_2 mole fraction (C_i/C_a), and a 19% lower ratio of chloroplastic-to-atmospheric CO_2 mole fraction (C_c/C_a), as compared to young leaves. On the other hand, old leaves had a 13% higher C_i^* and 46% higher intrinsic water-use efficiency ($WUE_i = A/g_{sw}$) compared with young leaves, averaged across species (Table 1). Nevertheless, A and g_m in *P. vulgaris* did not differ significantly between the two age classes. Across individual leaves of all species and age classes, R_L was not significantly correlated to A, g_{sc} or g_m ($r^2<0.1$, $p>0.05$), but was significantly correlated to R_D, with both methods ($r^2_{13C}=0.34$, $r^2_{Laisk}=0.45$, $p<0.001$).

Isotope fractionation and mesophyll conductance

Carbon isotope discrimination during net CO_2 assimilation (Δ_A) showed clear differences during the two sets of online Δ measurements (Fig. 3), that is, the observed discrimination was influenced by the isotope composition of inlet CO_2. This was due to the isotopic disequilibrium between respiratory (R_L) and photosynthetic (P) CO_2 fluxes. By contrast, Δ_P was not influenced by CO_2 sources in any species (Fig. 3) supporting the accuracy of flux partitioning of P and R_L. Furthermore, the calculation using Eqn 16 yielded estimates of e of −16.5‰ with ^{13}C- depleted inlet CO_2 and +11.2‰ with ^{13}C-enriched inlet CO_2 (averaged across species). Those estimates were close to values that could be simply computed from the $\delta^{13}C$ difference between growth CO_2 source and outlet CO_2 (that is, $e = \delta_{out} - \delta_{growth\ CO2}$ where $\delta_{growth\ CO2} = -10‰$ and δ_{out} denotes the isotopic composition of CO_2 in the leaf cuvette during measurements in light), assuming there was no fractionation between photosynthates and respired CO_2 (Wingate *et al.*, 2007): e obtained in this way was −18.3‰ and + 6.1‰ with ^{13}C-depleted and ^{13}C-enriched inlet CO_2, respectively. The agreement between the two calculations of e again indicates that our flux partitioning of P and R_L was performed properly.

g_m was calculated from carbon isotope discrimination during apparent photosynthesis (Δ_P) using equation (12). As measured under conditions similar to growth conditions using our isotopic disequilibrium method, g_m and g_{sc} showed a strong linear correlation across young and old leaves of all species ($g_{sc}=0.67g_m+0.01$, $r^2=0.82$, $p<0.001$, Fig. S1). Meanwhile, g_{sc}/g_m showed no significant correlation with A ($p>0.05$, $r^2<0.1$) or CO_2 mole fraction in the leaf cuvette (C_{out}, $p>0.05$, $r^2<0.1$). The g_m-g_{sc} relationship was used to calculate g_m of each leaf during Laisk measurements (A/C_i curves) and thus to calculate Γ^* and $R_{L\ Laisk\ cc}$ using A/C_c curves (cf. Fig. S4). This established that C_i^* was generally lower than Γ^* with a mean absolute difference of 5 μmol

mol^{-1} for both young and old leaves (Fig. 4a), while $R_{L\ Laisk\ cc}$ (obtained from A/C_c courves) was not different from $R_{L\ Laisk}$ (obtained from A/C_i curves; Fig. 4b). An example of the offset in the common intersection point is given in Fig. S4.

DISCUSSION

In this work, R_L was measured using both an isotopic disequilibrium method and the classical Laisk method on single leaves of different species, and values obtained therefrom were compared.

Reliability of R_L values derived from isotopic disequilibrium

The present results showed a positive correlation between the two sets of R_L measurements across all species and age classes, while on average $R_{L\ Laisk}$ estimates were 28% smaller than $R_{L\ 13C}$. To our knowledge, this is the first comparison of R_L estimated from the Laisk method and an isotopic disequilibrium method that does not require manipulation of photosynthetic gas exchange rates using non-physiological environmental conditions. It is not totally unexpected that the two methods provided consistently different R_L estimates, given that the measurements were performed with contrasting environmental conditions and different theoretical bases. The isotopic disequilibrium method measures CO_2 efflux that is not labelled (i.e. respiration fuelled by old carbon) during leaf photosynthesis. An important assumption involved is that after a short period of labelling, no tracer (new carbon) has been incorporated into respiration. Any contribution of new carbon to the respiratory CO_2 efflux will lead to an underestimation of R_L. The potential error seems to be negligible, since our calculations using Eqn 8 (Table S2) showed that this assumption might have led to a 2.5% underestimation of R_L only, thus cannot explain the offset between R_L estimates measured by the two methods. Also, in perennial ryegrass, no new carbon was observed in shoot dark respiration for about 2 h following a 1 h-long labelling period (Lehmeier *et al.*, 2008), again suggesting insignificant underestimation of R_L by short-term labelling (30-45min). The labelling dynamics in shoot respiration should be similar to that of single leaves considering that leaf respiration contributes to about half of total plant respiration (Atkin *et al.*, 2007). However, information on labelling dynamics of single leaves is currently very limited, thus the kinetics of label appearance in day respired CO_2 and its putative environmental dependence should be studied in a greater number of species.

Does $R_{L\ Laisk}$ respond to environmental conditions imposed during measurement?

Estimates of R_L differ between methods (Villar *et al.*, 1994; Yin *et al.*, 2011), and this effect is likely related to the different measurement conditions. Importantly, the response of R_L to environmental conditions like irradiance and CO_2 concentration is not well understood to date, mainly due to methodological limitations. Light has long been recognized to inhibit R_L so that R_L is believed to be higher at very low light, a phenomenon that is possibly also at the origin of the Kok effect (Brooks & Farquhar, 1985; Villar *et al.*, 1994; Atkin *et al.*, 2000; Yin *et al.*, 2011). However, the effect of light at higher levels is not well documented. It is notable that both the Laisk and Kok method require manipulation of PAR, so the effect of PAR on R_L cannot be quantified with these methods. Also, uncertainty remains as to whether there is a short-term response of R_L to CO_2 mole fraction. Early reports of a decrease of leaf R_D with short-term increase of CO_2 (see the discussion by Amthor (2000) and Yin *et al.* (2011)), were suggested to be largely attributable to CO_2 diffusive leaks during gas exchange measurements (Amthor, 2000; Jahnke & Krewitt, 2002; Long *et al.*, 2004; Gong *et al.*, 2015). Results of the short-term CO_2 response of day respiration are scarce. However, using ^{13}C-labelling, it was shown that respiratory metabolism (TCA pathway) increased as CO_2 mole fraction decreased (Tcherkez *et al.*, 2008), while there seemed little effect on R_L assessed with the Kok method (Tcherkez *et al.*, 2012b). CO_2 mole fraction can potentially impact on R_L via changes in nitrogen assimilation caused by altered rates of photorespiration (Tcherkez *et al.*, 2012a; Abadie *et al.*, 2016). On the one hand, increased photorespiration at low CO_2 is believed to cause high mitochondrial NADH levels and thus inhibit TCA decarboxylases. On the other hand, the increased demand for carbon skeletons to assimilate nitrogen at high photorespiration should stimulate day respiratory metabolism (Abadie *et al*. 2016). However, the contribution of TCA decarboxylations to total respiratory CO_2 production in the light is rather small when compared to pyruvate dehydrogenation (Tcherkez *et al.*, 2008). Therefore, the net effect of CO_2 on R_L itself may be modest. Still, a short-term change in CO_2 mole fraction may in principle influence R_L, and thus the possibility that R_L is misestimated by the Laisk method cannot be excluded. This could contribute to explaining why Laisk estimates of R_L are smaller than ^{13}C-derived estimates, as shown here.

Further, the low CO_2 conditions used with the Laisk method may provoke a diffusive leak as the (non-controlled) CO_2 concentration outside the cuvette is higher than inside. That would increase the estimate R_L if not accounted for properly, further affecting the relationship between $R_{L\ Laisk}$ and $R_{L\ 13C}$. In the present work, however, the leak effect was accounted for. Also, the leak coefficients of intact leaves (K_{CO2}) measured here were generally very low, much lower than the producer-suggested value of 0.44 μmol s^{-1}. Nevertheless, we found a clear leak artefact on $R_{L\ Laisk}$ of *V. faba*. The diffusive leak had no significant effect on estimates of C_i^* of young leaves of *V. faba* and *R. communis* (Fig. S2). Importantly, leak artefacts on R_D are also not ignorable given that measurements of R_D of small leaves are quite close to the detecting limit of currently available infra-red gas analysers. Since K_{CO2} may vary substantially between species and leave age classes, leak effects should be minimized (cf. Gong *et al.* 2017b) or accounted for by the measurement of the leak coefficient for every single leaf, as done here.

Does g_m influence R_L estimates?

Another potential uncertainty associated with the C_i-based Laisk method is the assumption on mesophyll conductance. The compensation point in the absence of day respiration, Γ^*, is a C_c-based value and thus should be determined from A/C_c curves rather than A/C_i curves. In other words, the use of A/C_i curves to estimate C_i^* (as a proxy of C_c) involves the assumption that g_m is infinite. Consequently, assuming an infinite g_m might lead to errors in the estimated Γ^* and R_L by the Laisk method (von Caemmerer, 2013; Walker & Ort, 2015). Here, g_m of each leaf was quantified using online Δ measurements, and demonstrated that g_m of older leaves was 47% smaller than that of young leaves, in agreement with studies using both online Δ or florescence methods (reviewed in Flexas *et al.* (2008). The estimates of g_m obtained here were not very sensitive to errors in Γ^*. In fact, the difference between species and age classes was not influenced by changes in Γ^* within 20 μmol mol^{-1} (Fig. S3). Other methods like the constant J method were suggested to be sensitive to errors in Γ^* (Harley *et al.*, 1992). Furthermore, the robust relationship between g_{sw} and g_m across all species found here was similar to that reported in tree leaves (Whitehead *et al.*, 2011). Knowing the relationship between g_{sc} and g_m allowed us to estimate g_m and thus convert A/C_i curves into A/C_c curves in the Laisk method. That way, we were able to derive the parameters of interest (Γ^* and R_L) from A/C_c curves (cf. Fig. S4). These calculations assumed that the g_{sc}-g_m relationship was the same under the measurement condition

of the Laisk method and normal growth condition, which is supported by the fact that g_{sc}-to-g_m ratio showed no significant correlation with net assimilation rate or CO_2 mole fraction in the leaf cuvette. Furthermore, analyses of published data also showed a strong g_{sc} - g_m relationship across species and growth conditions (Flexas *et al.*, 2013). Importantly, however, our results show that the Laisk method based on A/C_i curves systematically underestimated Γ^* (by 5 μmol mol^{-1}) but not R_L (i.e. R_L determined from A/C_i curves and A/C_c curves were identical).

Although statistical significance was found in *T. aestivum* only, the age effect on both C_i^* and the C_c-based value of Γ^* suggested that there was some error in the Laisk method. In fact, Γ^* is given by $[O_2]/2S_{c/o}$ (where $[O_2]$ is oxygen mole fraction at carboxylation sites and $S_{c/o}$ is Rubisco specificity) and is thus not expected to change with leaf age. Assuming a single conductance term from intercellular spaces (C_i) to the site of carboxylation (C_c) is perhaps not completely realistic, as some authors suggested that there is some resistance of the chloroplast envelope to intracellular CO_2 movement (von Caemmerer, 2000), thereby leading to a lack of common intersection in Laisk curves (Tholen *et al.*, 2012). According to the model of Tholen *et al.* (2012), $\Gamma^* = C_i^* + R_L/g_{wp} - F/g_{ch}$, with total mesophyll conductance subdivided into conductance associated with cell wall and plasmalemma (g_{wp}) and chloroplast envelope and stroma (g_{ch}). Under such an assuption, the offset of C_i^* and apparent Γ^* between age classes can be explained by a small increase in photorespiration F (according to the difference of C_c) and a small decrease in g_{ch} with ageing. Improving the representation of mesophyll conductance in the Laisk method is beyond the scope of the present paper, but our results suggest that the estimates of Γ^* or R_L obtained via the Laisk method are not precise enough (Gu & Sun, 2014), and should be viewed as approximations of actual Γ^* and R_L.

Conclusions and perspectives

This study showed a high variation in R_L of similar leaves measured by both methods, and R_L was positively correlated to R_D, but not to net CO_2 assimilation rate or other parameters. These observations do not support the assumption that leaf R_L is a fixed proportion of photosynthesis or maximum V_c as used in many models (cf. De Kauwe *et al.* (2016)), but suggest that scaling R_L to R_D is a more reliable approach for the modelling purpose. We found a tendency for R_L/R_D to increase during leaf aging, and this finding is not in agreement with that reported by Villar *et al.* (1995). The average age difference between young and old mature leaves was about 16-20 days

in our study, much shorter than that of tree leaves (about 2 years) in the study of Villar *et al.* (1995). Taken as a whole, our results show that R_L estimates obtained using the isotopic disequilibrium method and the Laisk method were positively correlated, but R_L estimated by the isotopic disequilibrium method was generally higher than that measured by the Laisk method. Both methods captured the difference in R_L between species but found no effect of leaf ageing. Although R_L estimates differed between measurement techniques, most leaf-level studies (including the present study) support the notion that R_L is lower than R_D (Villar *et al.*, 1994; Yin *et al.*, 2011; Gong *et al.*, 2015; Tcherkez *et al.*, 2017a). Mesocosm-scale ^{13}C labelling study also showed that stand R_L is inhibited by light (Schnyder *et al.*, 2003; Gong *et al.*, 2017a). Previous comparisons between Laisk and Kok methods showed a systematic difference between R_L estimates, with R_L estimated by the Kok method being generally lower than that measured by the Laisk method (Villar *et al.*, 1994; Yin *et al.*, 2011). Also in the case of the Kok method, it has been recently suggested that the apparent inhibition of respiration by light is at least partially explained by considerable changes in C_c during the manipulation of irradiance (Farquhar & Busch, 2017), in addition to other changes such as that in photochemical yield (for a review, see Tcherkez *et al*. 2017ab). Thus, our study suggests that common methods (Laisk or Kok) likely provide underestimated R_L values and thus overestimated inhibition of day respiration by light. For the mechanistic understanding of day respiratory metabolism, the response of R_L to light and CO_2 mole fraction should be assessed in further studies, and the isotopic disequilibrium method is suitable for such a purpose since it does not require irradiance and CO_2 alterations.

ACKNOWLEDGMENTS

We thank The New Phytologist Trust for supporting the 18th New Phytologist Workshop 'The Kok effect: beyond the artefact, emerging leaf mechanisms (KOALA)' Angers, France, July 2016. We also thank all participants of this workshop for enlightening discussion and comments. This research was supported by the Deutsche Forschungsgemeinschaft (DFG SCHN 557/7-1).

AUTHOR CONTRIBUTIONS

R.S. and X.Y.G. designed and planned the research; J.W. and R.S. performed the gas exchange measurements and isotope analyses; J.W., R.S. and X.Y.G. analyzed the data; G.T., R.S., H.S., and X.Y.G. discussed the results and implications; X.Y.G. wrote the first draft, and all authors contributed to the revision.

REFERENCES

Abadie C, Boex-Fontvieille ERA, Carroll AJ, Tcherkez G. 2016. In vivo stoichiometry of photorespiratory metabolism. *Nature Plants* **2**: 15220.

Amthor JS. 2000. The McCree-de Wit-Penning de Vries-Thornley respiration paradigms: 30 years later. *Annals of Botany* **86**(1): 1-20.

Atkin OK, Evans JR, Ball MC, Lambers H, Pons TL. 2000. Leaf respiration of snow gum in the light and dark. interactions between temperature and irradiance. *Plant Physiology* **122**(3): 915-923.

Atkin OK, Scheurwater I, Pons TL. 2007. Respiration as a percentage of daily photosynthesis in whole plants is homeostatic at moderate, but not high, growth temperatures. *New Phytologist* **174**(2): 367-380.

Beer C, Reichstein M, Tomelleri E, Ciais P, Jung M, Carvalhais N, Rodenbeck C, Arain MA, Baldocchi D, Bonan GB, et al. 2010. Terrestrial gross carbon dioxide uptake: global distribution and covariation with climate. *Science* **329**(5993): 834-838.

Brooks A, Farquhar GD. 1985. Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase / oxygenase and the rate of respiration in the light - estimates from gas-exchange measurements on spinach. *Planta* **165**(3): 397-406.

De Kauwe MG, Lin YS, Wright IJ, Medlyn BE, Crous KY, Ellsworth DS, Maire V, Prentice IC, Atkin OK, Rogers A, et al. 2016. A test of the 'one-point method' for estimating maximum carboxylation capacity from field-measured, light-saturated photosynthesis. *New Phytologist* **210**(3): 1130-1144.

Evans JR, Sharkey TD, Berry JA, Farquhar GD. 1986. Carbon isotope discrimination measured concurrently with gas-exchange to investigate CO_2 diffusion in leaves of higher plants. *Australian Journal of Plant Physiology* **13**(2): 281-292.

Farquhar GD, Busch FA. 2017. Changes in the chloroplastic CO_2 concentration explain much of the observed Kok effect: a model. *New Phytologist* **214**(2): 570-584.

Farquhar GD, Cernusak LA. 2012. Ternary effects on the gas exchange of isotopologues of carbon dioxide. *Plant, Cell and Environment* **35**(7): 1221-1231.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503-537.

Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical-model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149**(1): 78-90.

Flexas J, Niinemets U, Galle A, Barbour MM, Centritto M, Diaz-Espejo A, Douthe C, Galmes J, Ribas-Carbo M, Rodriguez P, et al. 2013. Diffusional conductances to CO_2 as a target for

increasing photosynthesis and photosynthetic water-use efficiency. *Photosynthesis Research* **117**(1-3): 45-59.

Flexas J, Ribas-Carbo M, Diaz-Espejo A, Galmes J, Medrano H. 2008. Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell and Environment* **31**(5): 602-621.

Ghashghaie J, Badeck F-W, Lanigan G, Nogués S, Tcherkez G, Deléens E, Cornic G, Griffiths H. 2003. Carbon isotope fractionation during dark respiration and photorespiration in C_3 plants. *Phytochemistry Reviews* **2**(1): 145-161.

Gong XY, Schäufele R, Feneis W, Schnyder H. 2015. $^{13}CO_2/^{12}CO_2$ exchange fluxes in a clamp-on leaf cuvette: disentangling artefacts and flux components. *Plant, Cell and Environment* **38**(11): 2417-2432.

Gong XY, Schäufele R, Lehmeier CA, Tcherkez G, Schnyder H. 2017a. Atmospheric CO_2 mole fraction affects stand-scale carbon use efficiency of sunflower by stimulating respiration in light. *Plant, Cell and Environment* **40**(3): 401-412.

Gong XY, Schäufele R, Schnyder H. 2017b. Bundle-sheath leakiness and intrinsic water use efficiency of a perennial C_4 grass are increased at high vapour pressure deficit during growth. *Journal of Experimental Botany* **68**(2): 321-333.

Gu LH, Sun Y. 2014. Artefactual responses of mesophyll conductance to CO_2 and irradiance estimated with the variable *J* and online isotope discrimination methods. *Plant, Cell and Environment* **37**(5): 1231-1249.

Harley PC, Loreto F, Dimarco G, Sharkey TD. 1992. Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98**(4): 1429-1436.

Heskel MA, Atkin OK, Turnbull MH, Griffin KL. 2013. Bringing the Kok effect to light: A review on the integration of daytime respiration and net ecosystem exchange. *Ecosphere* **4**(8).

Jahnke S, Krewitt M. 2002. Atmospheric CO2 concentration may directly affect leaf respiration measurement in tobacco, but not respiration itself. *Plant, Cell and Environment* **25**(5): 641-651.

Kok B. 1948. A critical consideration of the quantum yield of *Chlorella* photosynthesis. *Enzymologia* **13**: 1-56.

Kromdijk J, Griffiths H, Schepers HE. 2010. Can the progressive increase of C4 bundle sheath leakiness at low PFD be explained by incomplete suppression of photorespiration? *Plant, Cell & Environment* **33**: 1935-1948.

Laisk AK. 1977. *Kinetics of Photosynthesis and Photorespiration in C_3 Plants*. Moscow: Nauka.

Lanigan GJ, Betson N, Griffiths H, Seibt U. 2008. Carbon isotope fractionation during photorespiration and carboxylation in *Senecio*. *Plant Physiology* **148**(4): 2013-2020.

Le Quere C, Raupach MR, Canadell JG, Marland G, Bopp L, Ciais P, Conway TJ, Doney SC, Feely RA, Foster P, et al. 2009. Trends in the sources and sinks of carbon dioxide. *Nature Geoscience* **2**(12): 831-836.

Leakey ADB, Xu F, Gillespie KM, McGrath JM, Ainsworth EA, Ort DR. 2009. Genomic basis for stimulated respiration by plants growing under elevated carbon dioxide. *Proceedings of the National Academy of Sciences of the United States of America* **106**(9): 3597-3602.

Lehmeier CA, Lattanzi FA, Gamnitzer U, Schaufele R, Schnyder H. 2010. Day-length effects on carbon stores for respiration of perennial ryegrass. *New Phytologist* **188**(3): 719-725.

Lehmeier CA, Lattanzi FA, Schäufele R, Wild M, Schnyder H. 2008. Root and shoot respiration of perennial ryegrass are supplied by the same substrate pools: assessment by dynamic ^{13}C labeling and compartmental analysis of tracer kinetics. *Plant Physiology* **148**(2): 1148-1158.

Li X, Zhang GQ, Sun B, Zhang S, Zhang YQ, Liao YWK, Zhou YH, Xia XJ, Shi K, Yu JQ. 2013. Stimulated leaf dark respiration in tomato in an elevated carbon dioxide atmosphere. *Scientific Reports* **3**: ARTN 3433.

Long SP, Ainsworth EA, Rogers A, Ort DR. 2004. Rising atmospheric carbon dioxide: plants face the future. *Annual Review of Plant Biology* **55**: 591-628.

Ludwig LJ, Canvin DT. 1971. The rate of photorespiration during photosynthesis and the relationship of the substrate of light respiration to the products of photosynthesis in sunflower leaves. *Plant Physiology* **48**: 712-719.

Nogues S, Tcherkez G, Cornic G, Ghashghaie J. 2004. Respiratory carbon metabolism following illumination in intact french bean leaves using $^{13}C/^{12}C$ isotope labeling. *Plant Physiology* **136**(2): 3245-3254.

Ostler U, Schleip I, Lattanzi FA, Schnyder H. 2016. Carbon dynamics in aboveground biomass of co-dominant plant species in a temperate grassland ecosystem: same or different? *New Phytologist* **210**(2): 471-484.

Schnyder H, Schäufele R, Lötscher M, Gebbing T. 2003. Disentangling CO_2 fluxes: direct measurements of mesocosm-scale natural abundance $^{13}CO_2/^{12}CO_2$ gas exchange, ^{13}C discrimination, and labelling of CO_2 exchange flux components in controlled environments. *Plant, Cell and Environment* **26**(11): 1863-1874.

Tcherkez G, Bligny R, Gout E, Mahe A, Hodges M, Cornic G. 2008. Respiratory metabolism of illuminated leaves depends on CO_2 and O_2 conditions. *Proceedings of the National Academy of Sciences of the United States of America* **105**(2): 797-802.

Tcherkez G, Boex-Fontvieille E, Mahe A, Hodges M. 2012a. Respiratory carbon fluxes in leaves. *Current Opinion in Plant Biology* **15**(3): 308-314.

Tcherkez G, Cornic G, Bligny R, Gout E, Ghashghaie J. 2005. In vivo respiratory metabolism of illuminated leaves. *Plant Physiology* **138**(3): 1596-1606.

Tcherkez G, Gauthier P, Buckley TN, Busch FA, Barbour MM, Bruhn D, Heskel MA, Gong XY, Crous K, Griffin KL, et al. 2017a. Tracking the origins of the Kok effect, 70 years after its discovery. *New Phytologist* **214**(2): 506-510.

Tcherkez G, Gauthier P, Buckley TN, Busch FA, Barbour MM, Bruhn D, Heskel MA, Gong XY, Crous K, Griffin KL, et al. 2017b. Leaf day respiration: low CO_2 flux but high significance for metabolism and carbon balance. *New Phytologist*,in press DOI: 10.1111/nph.14816.

Tcherkez G, MahÉ A, GuÉRard F, Boex-Fontvieille ERA, Gout E, Lamothe M, Barbour MM, Bligny R. 2012b. Short-term effects of CO_2 and O_2 on citrate metabolism in illuminated leaves. *Plant, Cell and Environment* **35**(12): 2208-2220.

Tcherkez G, Mauve C, Lamothe M, Le Bras C, Grapin A. 2011. The $^{13}C/^{12}C$ isotopic signal of day-respired CO_2 in variegated leaves of *Pelargonium x hortorum*. *Plant, Cell and Environment* **34**(2): 270-283.

Tholen D, Ethier G, Genty B, Pepin S, Zhu XG. 2012. Variable mesophyll conductance revisited: theoretical background and experimental implications. *Plant, Cell and Environment* **35**(12): 2087-2103.

Villar R, Held AA, Merino J. 1994. Comparison of methods to estimate dark respiration in the light in leaves of two woody species. *Plant Physiology* **105**(1): 167-172.

Villar R, Held AA, Merino J. 1995. Dark leaf respiration in light and darkness of an evergreen and a deciduous plant-species. *Plant Physiology* **107**(2): 421-427.

von Caemmerer S. 2000. *Biochemical models of leaf photosynthesis*. Collingwood: CSIRO.

von Caemmerer S. 2013. Steady-state models of photosynthesis. *Plant, Cell and Environment* **36**(9): 1617-1630.

von Caemmerer S, Evans JR, Hudson GS, Andrews TJ. 1994. The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. *Planta* **195**(1): 88-97.

Walker BJ, Ort DR. 2015. Improved method for measuring the apparent CO_2 photocompensation point resolves the impact of multiple internal conductances to CO_2 to net gas exchange. *Plant, Cell and Environment* **38**(11): 2462-2474.

Wehr R, Munger JW, McManus JB, Nelson DD, Zahniser MS, Davidson EA, Wofsy SC, Saleska SR. 2016. Seasonality of temperate forest photosynthesis and daytime respiration. *Nature* **534**(7609): 680-683.

Whitehead D, Barbour MM, Griffin KL, Turnbull MH, Tissue DT. 2011. Effects of leaf age and tree size on stomatal and mesophyll limitations to photosynthesis in mountain beech (*Nothofagus solandrii* var. *cliffortiodes*). *Tree Physiology* **31**(9): 985-996.

Wingate L, Seibt U, Moncrieff JB, Jarvis PG, Lloyd J. 2007. Variations in ^{13}C discrimination during CO_2 exchange by *Picea sitchensis* branches in the field. *Plant, Cell and Environment* **30**(5): 600-616.

Yin XY, Sun ZP, Struik PC, Gu JF. 2011. Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements. *Journal of Experimental Botany* **62**(10): 3489-3499.

TABLE AND FIGURES

Table 1. Gas exchange parameters of young and old leaves measured under the same environmental conditions as during growth. Gas exchange parameters include: respiration rate in light measured using the isotopic disequilibrium method ($R_{L\ 13C}$, μmol m^{-2} s^{-1}) or the Laisk method ($R_{L\ Laisk}$, μmol m^{-2} s^{-1}), respiration rate in the dark (R_D, μmol m^{-2} s^{-1}), leak coefficient (K_{CO2}, μmol s^{-1}), net assimilation rate (A, μmol m^{-2} s^{-1}), transpiration rate (E, mmol m^{-2} s^{-1}), stomatal conductance to water vapour (g_{sw}, mol m^{-2} s^{-1}), mesophyll conductance (g_m, mol m^{-2} s^{-1}), apparent chloroplastic CO_2 photocompensation point (C_i^*, μmol mol^{-1}), intrinsic water use efficiency ($WUE_i=A/g_{sw}$, μmol mol^{-1}), ratio of internal to atmospheric CO_2 concentration (C_i/C_a), ratio of chloroplastic to atmospheric CO_2 concentration (C_c/C_a). Data are shown as mean±SE (n=4), significant treatment effects (age, species and their interaction, $a\times s$) were marked with * when $0.01<p<0.05$ or with ** when $p<0.01$.

	H. vulgare		*T. aestivum*		*P. vulgaris*		*R. communis*		*Significance*		
	Young	Old	Young	Old	Young	Old	Young	Old	*age*	*species*	*a×s*
$R_{L\ 13C}$	1.8±0.1	1.7±0.3	1.0±0.1	1.4±0.2	1.5±0.1	2.2±0.2	1.5±0.1	1.0±0.1	*0.45*	**	*
$R_{L\ Laisk}$	1.1±0.1	1.4±0.2	0.7±0.1	0.9±0.1	1.3±0.1	1.7±0.3	0.8±0.2	0.7±0.1	*0.25*	**	*0.31*
R_D	1.9±0.2	1.8±0.1	1.0±0.2	1.0±0.1	2.3±0.1	2.2±0.2	2.0±0.4	1.1±0.1	*0.07*	**	*0.08*
K_{CO2}	0.19±0.06	0.27±0.07	0.11±0.02	0.15±0.01	0.24±0.07	0.21±0.05	0.12±0.04	0.18±0.07	*0.32*	*0.14*	*0.75*
A	14.1±0.5	7.5±1.7	15.4± 0.6	8.9±1.0	7.3±0.9	8.3±0.6	9.0±1.9	7.2±1.8	**	**	**
E	1.5±0.1	1.1±0.1	1.4±0.1	1.1±0.2	1.0±0.1	1.0±0.1	0.9±0.2	0.7±0.2	*0.06*	**	*0.71*
g_{sw}	0.26±0.03	0.11±0.02	0.37±0.06	0.11±0.03	0.21±0.08	0.12±0.02	0.16±0.09	0.08±0.03	**	*0.21*	*0.36*
g_m	0.25±0.02	0.08±0.03	0.32±0.04	0.12±0.02	0.09±0.02	0.10±0.01	0.12±0.03	0.08±0.03	**	**	**
C_i^*	41.8±1.1	45.9±3.8	40.6±0.9	53.6±2.1	37.3±2.1	42.0±3.9	44.9±2.2	48.9±7.4	**	*0.10*	*0.41*
WUE_i	57±6	70±9	45±5	91±12	46±9	73±5	87±22	109±23	**	*	*0.58*
C_i/C_a	0.72±0.03	0.72±0.04	0.77±0.02	0.64±0.04	0.80±0.03	0.69±0.02	0.63±0.07	0.55±0.07	*	*	*0.48*
C_c/C_a	0.56±0.03	0.49±0.02	0.62±0.02	0.46±0.02	0.56±0.06	0.48±0.01	0.43±0.08	0.32±0.08	**	**	*0.79*

Fig. 1 Correlation between respiration rate in light measured using the isotopic disequilibrium method ($R_{L\ 13C}$) and the Laisk method ($R_{L\ Laisk}$). Open symbols represent data of young leaves and filled symbols represent old leaves; *V. faba* and *G. max* were measured only on young leaves. Lines are the regression line (black solid line), upper and lower 95% confidence limits (dotted lines) and the 1:1 line (dashed line). Each symbol represents both parameter measured on the same leaf.

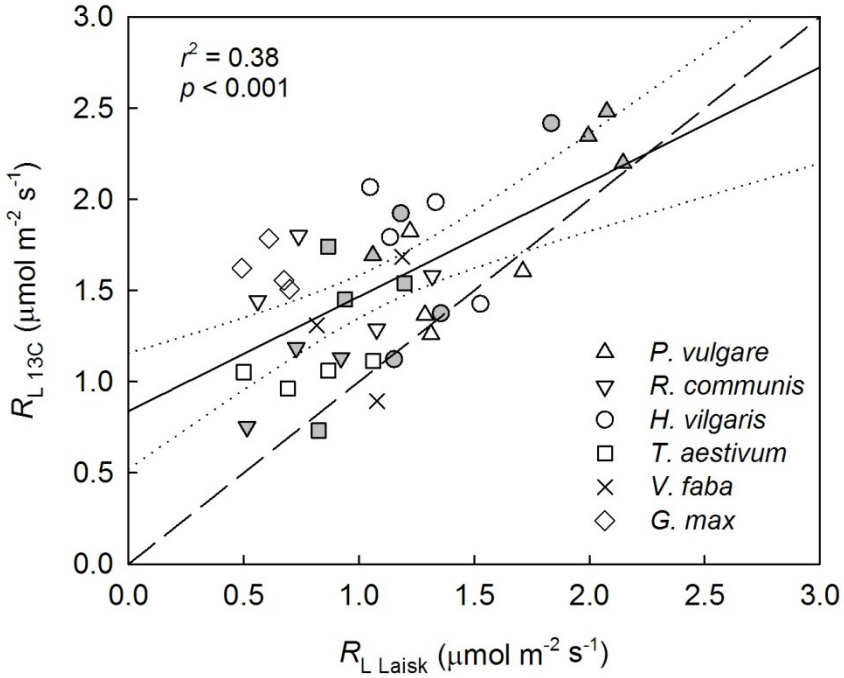

Fig. 2 The ratio of respiration in light to that in darkness measured by the isotopic disequilibrium method ($R_{L\ 13C}/R_D$, blue bars) or the Laisk method ($R_{L\ Laisk}/R_D$, black bars) of young (Y, open bars) and old (O, filled bars) leaves. Different letters indicate significant difference between means within each species measured by the same method ($p<0.05$, $n=4$).

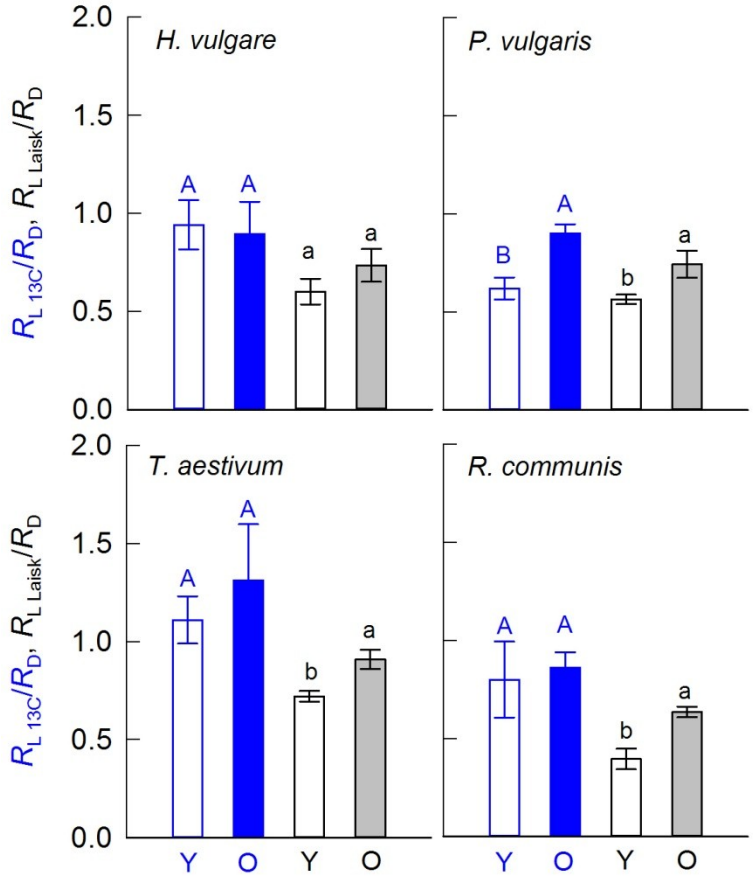

Fig. 3 Carbon isotope discrimination during net CO_2 assimilation (Δ_A, triangles) and during apparent photosynthesis (Δ_P, circles) measured using an ^{13}C enriched CO_2 source (red symbols) or a ^{13}C depleted CO_2 source (blue symbols). Panels a - d show data of individual species, and error bars are standard errors (n=4).

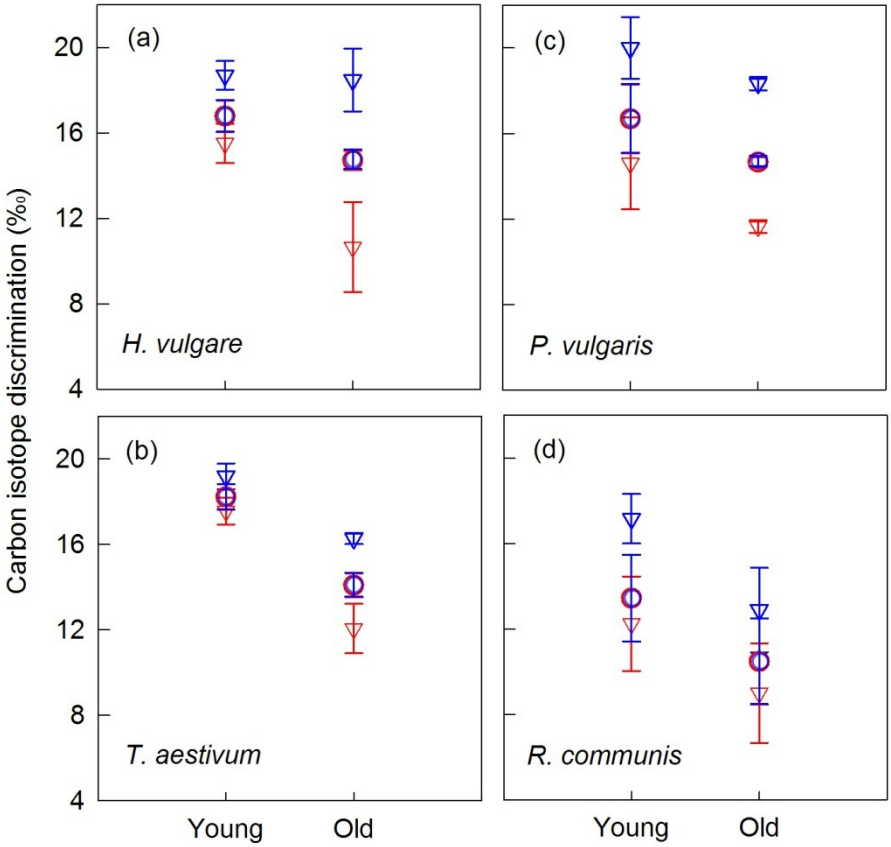

Fig. 4 Relationship between (a) chloroplastic CO_2 photocompensation point (Γ^*) and apparent chloroplastic CO_2 photocompensation point (C_i^*), and between (b) respiration in light measured using modified Laisk method based on C_c ($R_{L\ Laisk\ cc}$) and that measured using the Laisk method ($R_{L\ Laisk}$). Species are separately marked with different symbols (see Fig. 1), blue symbols represent data of young leaves and red symbols represent old leaves. Black solid line is the regression line, and dashed line is the 1:1 line. Each symbol represents the mean and standard error of a species (n=4).