Rapid report

Day-length effects on carbon stores for respiration of perennial ryegrass

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Summary

• The mechanism controlling the use of stored carbon in respiration is poorly understood. Here, we explore if the reliance on stores as respiratory substrate depends on day length.

• *Lolium perenne* (perennial ryegrass) was grown in continuous light (275 μmol photons m^{-2} s^{-1}) or in a 16 : 8 h day : night regime (425 μmol m^{-2} s^{-1} during the photoperiod), with the same daily photosynthetic photon flux density (PPFD). Plants in stands were labelled with $^{13}CO_2$: $^{12}CO_2$ for various time intervals. The rates and isotopic signatures of shoot- and root-respired CO_2 were measured after labelling, and water-soluble carbohydrates were determined in biomass. The tracer kinetics in respired CO_2 was analysed with compartmental models to infer the sizes, half-lives and contributions of respiratory substrate pools.

• Stores were the main source for respiration in both treatments (*c.* 60% of all respired carbon). But, continuous light slowed the turnover (+270%) and increased the size (+160%) of the store relative to the 16 : 8 h day : night regime. This effect corresponded with a greatly elevated fructan content. Yet, day length had no effect on sizes and half-lives of other pools serving respiration.

• We suggest that the residence time of respiratory carbon was strongly influenced by partitioning of carbon to fructan stores.

Introduction

Dark respiration consumes between 30 and 80% of the gross primary production of plants (Amthor, 2000; Gifford, 2003; Van Iersel, 2003). Partly, this is met by carbon derived directly from recent photosynthesis, which therefore resides only briefly in the plant (Pärnik *et al.*, 2007; Lehmeier *et al.*, 2008; Priault *et al.*, 2009). However, a large part of respiratory carbon first cycles through storage pools (Prosser & Farrar, 1981; Carbone & Trumbore, 2007; Lehmeier *et al.*, 2008; Mortazavi *et al.*, 2009). Temporary stores effectively buffer the continuous carbon demand of respiration from the discontinuous supply from photosynthesis (see Smith & Stitt, 2007). But to date, what controls carbon partitioning between immediate consumption and storage for later use in respiration is not well understood. This lack of knowledge translates into uncertainties about the residence time of respiratory carbon in plants and, hence, ecosystems.

Carbohydrates are considered as the main storage form of respiratory substrate (ap Rees, 1980; Tcherkez *et al.*, 2003). Most plants store carbohydrates as transitory starch in chloroplasts or as sucrose and fructans (fructose-based oligo- and polysaccharides) in vacuoles. Experimental alterations

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of day length demonstrated a striking ability of starch-storing species to adjust the fluxes of daytime deposition and night-time mobilization so that the starch pool was nearly exhausted at the end of the night (Chatterton & Silvius, 1981; Fondy *et al.*, 1989; Lu *et al.*, 2005; Gibon *et al.*, 2009). This suggested an efficient use of transitory stores as supplies of terminal sinks and minimal 'excess' storage.

Similar knowledge does not exist for sucrose- and fructan-storing species (including the C_3 cereals and forage grasses). Previous investigations using carbon isotopes to partition storage- and current assimilation-derived substrate supply of respiration showed that the contribution of stores depends on environmental conditions like temperature (Prosser & Farrar, 1981), nitrogen supply (Lehmeier *et al.*, 2010) and the growing season (Carbone & Trumbore, 2007; Gamnitzer, 2010). Recently, we have shown that about half of all carbon respired by perennial ryegrass (*Lolium perenne*, a sucrose/fructan-storing grass) first cycled through stores when plants were grown in continuous light (Lehmeier *et al.*, 2008). One should expect that plants growing in day : night cycles exhibit a greater reliance on stores, since these are needed to fuel respiration in the dark period when current assimilate supply is nil.

In the present study, we test the hypothesis that the quantitative significance of temporary stores for respiration is higher in day : night cycles than in continuous light. To this end, we grew perennial ryegrass in a 16 : 8 h day : night cycle with otherwise identical environmental conditions to those in continuous light. That is, plants in these two treatments received the same total daily photosynthetic photon flux density (PPFD), not to confuse day-length effects with total irradiance effects. Plants were labelled with $^{13}CO_2$: $^{12}CO_2$ for 1 h up to several weeks, and the ^{13}C : ^{12}C ratios of respired CO_2 of shoots and roots were measured in darkness immediately after labelling. Then, the time course of tracer in respired CO_2 was analysed with compartmental models.

Materials and Methods

Continuous light data have been presented before (Lehmeier *et al.*, 2008). In this work these data served as the reference against which the effect of day length on respiratory substrate stores was tested.

In both treatments, *Lolium perenne* L. cv Acento was grown from seeds in growth chambers which formed part of a custom-made $^{13}CO_2$: $^{12}CO_2$ gas exchange and labelling facility (Schnyder *et al.*, 2003). In the continuous-light treatment, plants were grown with constant illumination at a PPFD of 275 μmol m^{-2} s^{-1}. Day : night plants were grown in alternating 16 h light (PPFD, 425 μmol m^{-2} s^{-1}) and 8 h dark periods. Further chamber settings were the same in both treatments, including air temperature (20°C), CO_2 concentration (360 μl l^{-1}) and nutrient supply (see Supporting Information, Methods S1).

In each treatment, half of the plants were grown in a chamber with ^{13}C-enriched CO_2, and the other half in ^{13}C-depleted CO_2. When closed stands were established, the $\delta^{13}C$ of CO_2 was changed for intervals ranging from 1 h to 25 d (^{13}C-enriched $\rightarrow$ ^{13}C-depleted atmosphere or vice versa). At the end of each labelling interval, the rates of shoot and root respiration as well as the $\delta^{13}C$ of shoot- and root-respired CO_2 of individual plants were measured in the dark (Lötscher *et al.*, 2004; Klumpp *et al.*, 2005). After 5–6 h (continuous-light treatment) or 6–7 h (day : night treatment) of respiration measurements, plants were harvested. Carbon and nitrogen elemental contents and water-soluble carbohydrate fractions were determined in shoot and root biomass. Respiration of nonlabelled control plants was measured likewise. This determined the end members for a two-source mixing model which was used to calculate the fractions of unlabelled carbon in CO_2 respired by the labelled plants (Fig. 1).

The tracer time courses contain information about the respiratory carbon supply system: carbon fluxes in the system, pool sizes and half-lives, and the contribution of pools to respiration. This information was extracted with compartmental analysis. In plants of the day : night treatment metabolite pool sizes and fluxes must have fluctuated during the day as a result of the discontinuous photosynthesis (Farrar & Farrar, 1986). To account for this nonsteadiness, we restricted the compartmental analysis to the day-by-day timescale (Lattanzi *et al.*, 2005), for

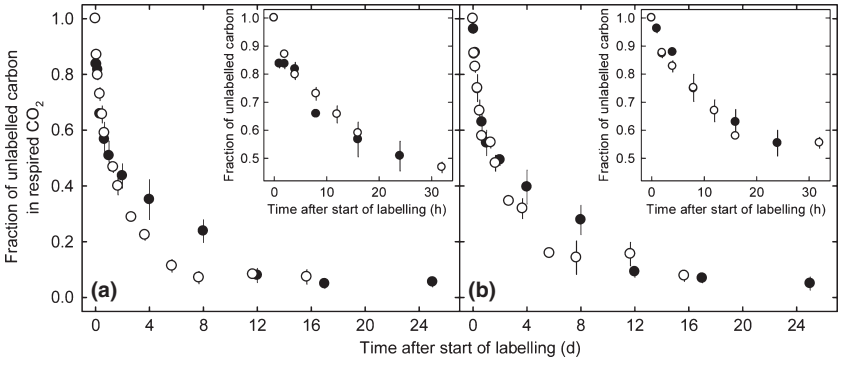

Fig. 1 The fraction of unlabelled carbon ($f_{unlabelled\text{-}C}$) in CO_2 evolved in dark respiration of shoots (a) and roots (b) of *Lolium perenne* (perennial ryegrass) plants grown in continuous light (closed symbols) or in a 16 : 8 h day : night regime (open symbols) during labelling. Each value is the mean of four to six replicate plants (± 1 SE). Data points at 2 h of labelling duration in root-respired CO_2 overlap. Insets expand the first 34 h. The continuous-light data are taken from Lehmeier *et al.* (2008).

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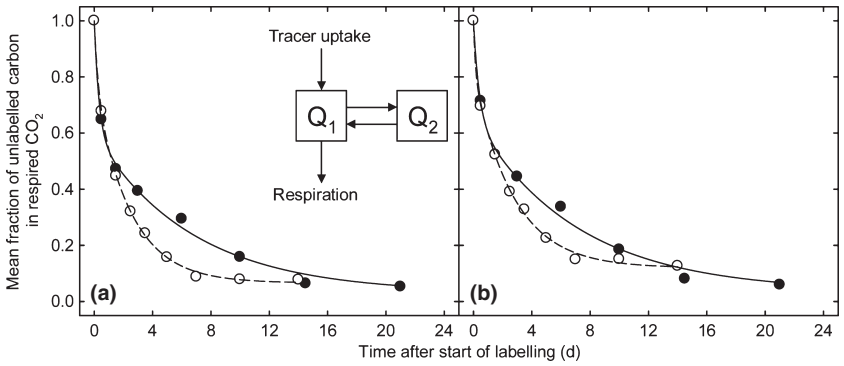

Fig. 2 The mean fraction of unlabelled carbon in CO_2 dark-respired by shoots (a) and roots (b) during a day (or multiples of that) of *Lolium perenne* (perennial ryegrass) plants grown in continuous light (closed symbols) or in a 16 : 8 h day : night regime (open symbols) during labelling. Values at day 2 in root respiration overlap. Lines denote the predictions of the two-pool model shown in (a) (which was provided with an asymptote; see the Supporting Information Methods S1) as applied to the mean fraction of unlabelled carbon in respired CO_2 of plants grown in continuous light (solid lines) or in the 16 : 8 h day : night regime (dashed lines). The mean fraction of unlabelled carbon respired by shoots of plants grown in day : night cycles was calculated under the assumption that the ratio between the respiration rate in light and in darkness was 0.7.

which relevant features were approximately steady in both treatments. For this, we calculated the mean fraction of unlabelled carbon in CO_2 respired during a 1-d-long period (or multiples thereof) as respiration-weighted averages of the fraction of unlabelled carbon of a given day (or multiples thereof, depending on the available time resolution of the data shown in Fig. 1). The mean fraction of unlabelled carbon in respired CO_2 in both treatments (Fig. 2) then served to infer differences and similarities in the respiratory carbon supply systems using compartmental analysis.

For the day : night treatment, the shoot respiration rate in light (R_{day}) may have been lower than that in darkness (R_{night}; Peisker & Apel, 2001; Pärnik *et al.*, 2007). This could modify the relative contributions of pools to respiration. To assess the possible effects, we calculated the mean fraction of unlabelled carbon in respired CO_2 in assuming that R_{day} : R_{night} was 1, 0.7 or 0.35. See Methods S1 for a comprehensive description of materials and methods.

Results

General growth and respiration parameters

All plants remained vegetative during the entire experimental period. Average specific growth rates of shoots and roots, obtained by a regression line to $\log_e$-transformed carbon mass observed over time, were similar in both light treatments, as were the plants' shoot : root ratios of *c.* 4 : 1 (Table 1). However, specific shoot respiration rate was 23% higher in plants grown in day : night cycles than in continuous light. Overall, the photosynthetic carbon-use efficiency (= growth rate/(growth rate + respiration rate)) was similar: 0.66 for plants in day : night cycles and 0.68 for plants in continuous light (Table 1).

Table 1 Experimental and growth parameters of perennial ryegrass (*Lolium perenne*) grown in continuous light or in a 16 : 8 h day : night regime

	Continuous light	Day : night
Irradiance (photosynthetic photon flux density)		
μmol m^{-2} s^{-1}	275	425
mol m^{-2} d^{-1}	24	24
Specific dark respiration rate		
Plant, mg plant-respired C g^{-1} plant C h^{-1}	1.50 ± 0.02	1.67 ± 0.03*
Shoot, mg shoot-respired C g^{-1} plant C h^{-1}	0.97 ± 0.02	1.19 ± 0.03*
Root, mg root-respired C g^{-1} plant C h^{-1}	0.53 ± 0.01	0.48 ± 0.01*
Specific growth rate		
Plant, mg C g^{-1} plant C h^{-1}	3.23 ± 0.21	3.19 ± 0.54 ns
Shoot, mg C g^{-1} shoot C h^{-1}	3.53 ± 0.23	3.42 ± 0.56 ns
Root, mg C g^{-1} root C h^{-1}	3.00 ± 0.28	2.40 ± 0.54 ns
Shoot : root ratio	3.84 ± 0.14	4.15 ± 0.08 ns
Nitrogen content (mg g^{-1} DW)		
Shoot	18.3 ± 0.5	29.4 ± 0.8*
Root	10.7 ± 0.3	12.6 ± 0.5*
Photosynthetic carbon-use efficiency	0.68 ± 0.05	0.66 ± 0.13 ns

Values are means of 60 (continuous light) and 121 (day : night cycles) replicate plants ± 1 SE. *n* for respiration rates and nitrogen content in the day : night treatment was 55. Some of the continuous-light data have been published previously (Lehmeier *et al.*, 2008, 2010).
*, $P \leq 0.05$; ns, not significant, $P > 0.05$.

Contents of water-soluble carbohydrates and nitrogen in shoot and root biomass

Total water-soluble carbohydrates accounted for 183 mg C g^{-1} total plant C in day : night cycles. This was almost 40% less than in plants grown in continuous light

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Table 2 Contents of water-soluble carbohydrate (WSC; mg C g^{-1} plant C) fractions in shoots and roots of perennial ryegrass (*Lolium perenne*) grown in continuous light or in a 16 : 8 h day : night regime

	Continuous light		Day : night	
	Shoot	Root	Shoot	Root
Fructan	235 ± 20a	6.3 ± 0.7b	102 ± 18c	3.6 ± 0.5b
Sucrose	21 ± 1a	2.3 ± 0.2b	30 ± 2c	2.2 ± 0.2b
Glucose	10 ± 1a	1.5 ± 0.2b	21 ± 2c	1.3 ± 0.2b
Fructose	10 ± 1a	4.0 ± 0.5b	20 ± 2c	3.1 ± 0.5b
Total WSC	276 ± 20	14 ± 0.9	173 ± 18	10 ± 0.8

Values are means of six (continuous light) or eight (day : night) replicate plants ± 1 SE. Different letters within a row denote statistically significant differences based on multiple *t*-tests ($P \leq 0.05$).

(290 mg C g^{-1} C; Table 2). In both treatments, 95% of total water-soluble carbohydrates were located in shoot biomass. Fructans accounted for 58% of total water-soluble carbohydrates in plants in day : night cycles, but 83% in continuous light. This resulted in fructan : sucrose mass ratios of 3 in day : night cycles and 10 in continuous light.

The nitrogen content of shoots in day : night cycles was *c.* 30 mg g^{-1} DW, *c.* 60% higher than that in continuous light (Table 1). The nitrogen content of roots in day : night cycles was 18% higher than in continuous light.

Tracer time course in respired CO_2

The tracer kinetics of shoot-respired CO_2 in both treatments can be divided into two major phases (Fig. 1a). In the first phase, $f_{unlabelled\text{-}C}$ in respired CO_2 decreased very rapidly. This was observed in shoots and roots of both treatments. The second phase commenced at *c.* 1 d of labelling, that is, when plants of both treatments had received the same dose of PPFD. Thereafter, $f_{unlabelled\text{-}C}$ decreased at slower rates in both treatments, but the rate of decrease was more rapid in day : night cycles where $f_{unlabelled\text{-}C}$ decreased to 0.06 until day 8 of labelling. Conversely, in continuous light it took *c.* 12 d until $f_{unlabelled\text{-}C}$ had decreased to the same level. In both treatments, there was a small residual respiratory (asymptotic) activity which was not exchanged by tracer within the timeframe of labelling. In essence, a very similar pattern was observed in both treatments for root-respired CO_2 (Fig. 1b).

Compartmental modelling of respiratory carbon pools

The transformation of the tracer kinetics in Fig. 1 to the mean fraction of unlabelled carbon in respired CO_2 per interval of 24 h (or multiples thereof; Fig. 2), conserved the two-phase pattern seen in Fig. 1 and the differences between treatments. Assumptions about the degree of light inhibition of shoot respiration in day : night cycles had little effect on the results of compartmental modelling (Table S1) and did not affect any conclusions of the present work.

Table 3 Optimized parameters of a two-pool compartmental model of respiratory substrates, applied to the mean fraction of unlabelled carbon respired by shoots and roots of perennial ryegrass (*Lolium perenne*) plants grown in continuous light or in a 16 : 8 h day : night regime (Fig. 2)

	Continuous light		Day : night	
	Shoot	Root	Shoot	Root
Size (mg C g^{-1} plant C)				
Q_1	17 ± 4	12 ± 3	22 ± 4	6 ± 2
Q_2	75 ± 39	45 ± 22	29 ± 17	17 ± 9
Half-life (h)				
Q_1	5.8 ± 1.3	7.3 ± 1.7	5.7 ± 1.7	2.7 ± 0.8
Q_2	50 ± 14	45 ± 22	13 ± 3	12 ± 1
Fractional contribution (%)				
Current assimilates	46 ± 10	41 ± 10	39 ± 12	26 ± 9
Temporary stores	51 ± 10	55 ± 10	54 ± 12	62 ± 9
Asymptote	3 ± 3	4 ± 1	7 ± 1	12 ± 1

The model included an asymptote which represented a small source of respired carbon that released no tracer during the entire labelling period. The equations describing the model in terms of pool sizes, fluxes and rate constants are given in the Supporting Information section. For the day : night treatment, it was assumed that the shoot dark respiration rate during light was 0.7 times the shoot respiration rate measured in darkness. Values ± 95% CI were calculated from model-optimized rate constants (and the measured respiration rates for the size estimation).

The two-pool model depicted in Fig. 2(a) was a simple model with biological significance able to fit the tracer time courses of shoots and roots of both light treatments (Fig. 2). One-pool models were statistically inferior, and more complex variations of the two-pool model were overparameterized. The two-pool model consisted of a pool Q_1 which received tracer from current photosynthesis, conveyed it to respiration and exchanged carbon with a temporary storage pool (Q_2). The model included an asymptote, which represented a small flux of carbon from a source that remained unlabelled during the experimental period (see Methods S1; Figs 1, 2). Therefore, this source could not be characterized as a mixing pool in terms of its size and half-life.

Substrate pool sizes, half-lives and contributions to respiration

The light regime had a large effect on the size of the total respiratory supply system (i.e. the sum of Q_1 and Q_2 of shoot and root; Table 3). In day : night plants, it accounted for 74 mg C g^{-1} C, only about half that of plants in continuous light. This was because of a smaller size of the temporary store Q_2 in day : night plants.

The kinetic properties of Q_1 were similar in both treatments, exhibiting a half-life between 3 and 7 h (Table 3). By contrast, the half-life of Q_2 was *c.* 0.5 d in day : night

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and *c.* 2 d in continuous light. Within a treatment, half-lives of pools supplying shoot and root respiration were almost the same.

More than 50% of all respired carbon first cycled through the store Q_2. This was true for shoots and roots in both treatments (Table 3). Current assimilation supplied *c.* 40% of all respired carbon, except for root respiration of plants in day : night cycles (26%; Table 3).

Discussion

The fractional contribution of stores as a substrate for respiration was independent of day length

The hypothesis of this investigation was that stores become a greater source of carbon for respiration when day length gets shorter. The work falsified this hypothesis: the relative contributions of stores to respiration were virtually identical in continuous-light and day : night cycles.

We believe that any responses observed here were true day-length responses, since environmental conditions were the same in the two treatments, including the daily dose of PPFD. Plant respiration rates in day : night cycles were slightly higher, but relative growth rates were the same in both treatments (Table 1). This suggests that daily rates of photosynthesis were not very different between the treatments, as irradiance was well below light saturation levels for photosynthesis (Gay & Thomas, 1995; Spiering *et al.*, 2006). Nevertheless, day length affected respiratory pool sizes and fluxes, and, possibly, also the metabolic identity of respiratory stores.

Perhaps the most striking result of the present work was that such a large fraction (50–60%) of respired carbon first cycled through a store, and that this occurred in both treatments (Table 3). The size of the store was much larger than was required to sustain respiratory activity even in the day : night cycle. Thus, the size of Q_2 in day : night cycles of 46 mg g^{-1} C would have allowed sustaining current dark respiration rates of those plants theoretically for 28 h (Tables 1, 3), much longer than the 'normal' dark period of 8 h. The surplus was even higher in continuous light (Tables 1, 3), where it was sufficient to supply respiration for 80 h. Even larger contributions were found for ryegrass grown in nitrogen-limited conditions (Lehmeier *et al.*, 2010).

Clearly, the sucrose/fructan-storing grass stored more carbon in its respiratory storage pool (Q_2) than was 'needed' to buffer day : night fluctuations in respiratory substrate. This differs from reports of plants that store transient starch: these plants adjusted storage fluxes to environmental changes such as the length of the dark period to deposit only as much starch during the daytime as they 'anticipated' using during the following night (Chatterton & Silvius, 1981; Fondy *et al.*, 1989; Lu *et al.*, 2005; Smith & Stitt, 2007; Gibon *et al.*, 2009). Our findings may not be directly comparable, as these studies did not characterize the respiratory substrate supply system. But such an apparently fundamental difference makes it worth pursuing labelling studies to characterize sink supply systems in plants with other forms of carbon storage.

Did day length affect the metabolic identity of temporary stores supplying respiration?

While the fractional contribution of stores to respiration was similar in both light regimes, their half-life was considerably shorter in day : night (0.5 d) than in continuous-light (2 d) treatments. Both sucrose and fructan may form part of vacuolar stores in grasses (Wagner *et al.*, 1983; Borland & Farrar, 1988). Indeed, these were the only fractions whose size was sufficient to accommodate the size of Q_2 in both treatments (Tables 2, 3). The much lower fructan : sucrose ratio in plants grown in day : night cycles than in continuous light (Table 2) suggests that sucrose was a quantitatively more important storage carbohydrate for respiration in day : night cycles.

This interpretation agrees with the half-life of Q_2 in day : night plants being close to values reported for vacuolar sucrose (Bell & Incoll, 1982; Farrar, 1989), and suggests that the longer half-life of Q_2 in continuous-light plants reflects a slower turnover of vacuolar fructans as compared with vacuolar sucrose. If this is true, the residence time of respiratory carbon was mainly influenced by the ratio of carbon partitioning between sucrose and fructan in storage deposition fluxes.

Why did day length elicit differences in shoot fructan content and in the size of stores (despite, in principle, equal resource supply in both treatments)? We suggest these are linked responses. Fructan and sucrose concentrations are thought to be associated, fructan synthesis kicking in before sucrose concentration reaches values which could cause osmotic imbalances or a feedback inhibition of photosynthesis (Pollock & Cairns, 1991; Cairns *et al.*, 2000). In day : night cycles, sucrose concentration probably remained below critical values for part of the day (Sicher *et al.*, 1984; Borland & Farrar, 1985). But in continuous light, a continuous provision of assimilates would have led to the larger fructan : sucrose ratio. Since fructans turn over more slowly than sucrose, the similar proportion of carbon diverted to storage (Table 3) would have led to a bigger storage pool size. This strategy of temporary storage use appears to have been effective, as growth rates and photosynthetic carbon-use efficiency were similar in both treatments (Table 1).

Day length affected shoot respiration rates and nitrogen content but not plants' growth performance

Plants in day : night cycles exhibited a 23% higher shoot respiration rate than those in continuous light. One reason for this was likely posed by the 61% higher nitrogen

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content in shoot biomass (Table 1), since requirements of respiration for *de novo* synthesis of amino-compounds and turnover of proteins are closely associated with nitrogen content (Amthor, 2000). The probable cause of the difference in shoot nitrogen content between treatments is less clear. Part of it may have been a dilution effect: the carbohydrate content was lower in day : night plants (Table 2), so that shoot nitrogen content per unit carbohydrate-free dry matter was only 33% higher.

Possibly, continuous illumination lowered the capacity of plants to assimilate nitrate, as compared with plants in day : night cycles. The tricarboxylic acid (TCA) cycle, an important carbon source for nitrate assimilation, is known to have reduced activity in illuminated leaves (Atkin *et al.*, 2000; Nunes-Nesi *et al.*, 2007). With ample nitrogen supply, as used here, nitrate assimilation occurs mainly in the shoots of temperate grasses (Andrews *et al.*, 1992). Recent findings suggest that the alternation of light and dark periods may be important for a balanced functioning of nitrogen metabolism (Gauthier *et al.*, 2010). However, despite the lower shoot nitrogen content, it is hard to argue that plants in continuous illumination experienced a nitrogen limitation compared with plants in day : night cycles, since growth rates and photosynthetic carbon-use efficiency were similar (Table 1).

In conclusion, this work highlights a remarkable flexibility of the respiratory substrate supply system of perennial ryegrass in response to day length. The adjustment occurred not in the proportion of current assimilates that were stored, but in the rate with which the carbon store was turned over. This, in turn, was probably linked to a change in the biochemical nature of the stored substrate, specifically the fructan : sucrose ratio. Such an adjustment would serve plants to sustain high growth rates in a changing environment. While this may be of general validity for plants (cf. Smith & Stitt, 2007), there seems to be a substantial difference regarding how this is achieved between sucrose/fructan- and starch-storing species.

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Supporting Information

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

Table S1 Optimized parameters of the two-pool compartmental model shown in Fig. 2(a), applied to the mean fraction of unlabelled carbon in CO_2 respired by shoots of plants grown in day : night cycles with either 0 or 65% reduced shoot respiration rate in light as compared with the shoot respiration rate in darkness

Methods S1 A comprehensive description of all materials and methods used for the present study.

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