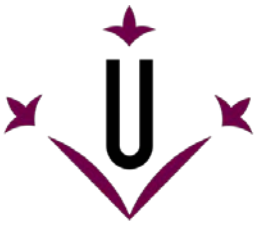

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Night and day - Circadian regulation of night-time dark respiration and light-enhanced dark respiration in plant leaves and canopies

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Abstract

- The potential of the vegetation to sequester C is determined by the balance between C-assimilation and respiration. Respiration is under environmental and substrate-driven control, but the circadian clock might also contribute to its regulation.
- To assess circadian control on night-time dark respiration (R_D) and on leaf respiration after light-to-dark transitions as an indicator for light enhanced dark respiration (LEDR) – the latter providing information on the metabolic reorganization in the leaf during light-dark transitions – we performed two experiments in macrocosms hosting canopies of bean and cotton. Under constant darkness, we tested whether circadian regulation of R_D scaled from leaf to whole canopy respiration. Under constant light, we assessed the potential for leaf-level circadian regulation of LEDR.
- There was a clear circadian oscillation of leaf-level R_D in both species and circadian patterns scaled to the canopy. Respiration in leaves transferred from light to darkness was under circadian control in cotton, but not in bean indicating species-specific controls overLEDR.
- The circadian rhythm of LEDR in cotton might indicate variable suppression of the normal cyclic function of the tricarboxylic acid cycle in the light. Since circadian regulation is assumed to act as an adaptive memory to adjust plant metabolism based on environmental conditions from previous days, circadian control of R_D may help to explain temporal variability of ecosystem respiration.

Keywords

Scaling, non-structural carbon compounds (NSC), constant light, constant darkness, adaptive memory

Introduction

Terrestrial ecosystems provide important stores for carbon (C) vulnerable to global change agents, including altered precipitation and increased temperature and CO_2 concentrations (Ciais *et al.*, 2005; Reichstein *et al.*, 2013; Schimel *et al.,* 2015). The potential of the vegetation to sequester C from the atmosphere is mainly determined by the balance between C assimilation – well studied and central in many studies (Farquhar *et al.*, 1980; Ainsworth & Long, 2005; Chaves *et al.*, 2009) – and the much less well understood complex set of processes, collectively referred to as ecosystem respiration, that return CO_2 to the atmosphere on a range of timescales (Hogberg & Read, 2006; Trumbore, 2006). There are particular conditions and systems where emissions of volatile organic compounds (VOC, e.g. Kesselmeier *et al.,* 2002, Brüggemann & Schnitzler, 2002) or wildfires (Bond Lamberty *et al.,* 2007) might be of importance, but respiration has been postulated to be the main determinant of the C balance in terrestrial ecosystems (Valentini *et al.,* 2000). Various processes are important to this balance and they are interlinked on many different spatial and temporal scales.

Plant respiration is known to be directly controlled by environmental factors among which temperature is the most important one, with plants experiencing long- and short-term acclimation (e.g. Atkin & Tjoelker, 2003). Increasing air temperatures in the future might cause substantial increases in respiratory carbon fluxes at leaf and canopy scales, which would impact the carbon balance of terrestrial vegetation (Slot & Kitajima, 2014). Plant respiration also depends on the amount and availability of respiratory substrate, which is in turn related to light availability and photosynthesis (Hogberg & Read, 2006). Moreover, the demand of sink tissues strongly affects respiration (e.g. Hagedorn *et al.*, 2016). Substrate supply depends on plant physiological processes that regulate yield and composition of C assimilates, as well as their distribution among maintenance, defense, growth, storage, and export of organic compounds to the rhizosphere (Trumbore, 2006). These processes act on timescales of hours to months depending on the plant species.

In addition to direct environmental and substrate-driven control, respiration might also be under circadian regulation, but there are conflicting reports in the literature, with circadian

rhythms observed in some species (Hillman, 1970; Hansen, 1977) but not in others (e.g. Hennessey *et al.*, 1993). The circadian clock is an endogenous timer that regulates the transcription of up to 90% of the genome in the model species *Arabidopsis thaliana* (Michael *et al.*, 2008). The interactive regulation between different clock genes with transcriptional-translational negative feedback loops is central for the function of the circadian oscillator (Alabadí *et al*., 2001) and substantial increases in photosynthesis, growth and survival is conferred by correct matching of the circadian clock period with that of the external light-dark cycle (Dodd *et al*., 2005). The daily protein expression rhythms observed for enzymes central to glycolysis (e.g. pyruvate kinase) or to the tricarboxylic acid (TCA) cycle (e.g. isocitrate dehydrogenase and succinate dehydrogenase) suggest that these respiratory pathways may also be under circadian control (Wijnen & Young, 2006). Even though the molecular mechanisms of the circadian control are well described (Harmer, 2009), the results become more ambiguous at higher organizational scales, such as the organ level, and we still lack information whether circadian rhythms scale to plant canopies or whole ecosystems. Using statistical filtering techniques, there is indirect evidence that net ecosystem CO_2 exchange (NEE) is affected by circadian regulation (Doughty *et al.*, 2006; de Dios *et al.,* 2012). Moreover, Resco de Dios et al. (2015) showed that circadian control of stomatal conductance affected night-time canopy transpiration. However, it is unknown whether these scaling effects also matter for night-time respiration. Since the temperature dependency of night-time respiration is often used to infer day-time ecosystem respiration in approaches aiming to derive photosynthetic fluxes from NEE measurements (Reichstein et al., 2005), not accounting for circadian rhythms of respiration could introduce errors to ecosystem flux separation approaches.

Dark respiration of autotrophic tissues is strongly suppressed in the light (Atkin e*t al.*, 2000; Tcherkez *et al.*, 2005) with the reorganization of the TCA cycle under illumination considered an important underlying mechanism (Tcherkez *et al.,* 2009). When light exposed leaves are transferred into darkness, an intensification of the respiratory flux is observed in the short-term that is referred to as light enhanced dark respiration (LEDR). LEDR has been defined as the enhancement of the flux of respiratory CO_2 directly after darkening of a light acclimated leaf in a photosynthesis-dependent manner (Azcon-Bieto & Osmond, 1983; Atkin *et al*., 2000). It has been observed that the CO_2 released directly after darkening is also ^{13}C

enriched (Barbour *et al.,* 2007) and that the extent of ^{13}C enrichment is related to the cumulative amount of photosynthetically fixed CO_2 during the day (Hymus *et al.,* 2005). LEDR is not simply a measurement artifact that occurs when light-acclimated leaves are darkened under experimental conditions, as it also occurs in the field in day-night transitions (Barbour *et al.*, 2011). These authors observed that an increase in $\delta^{13}C$ of leaf- and ecosystem- respired CO_2 occurs after sunset and they estimated that significant amounts of carbon could be released by LEDR, depending on the amount of cumulatively fixed carbon in the preceding light period.

Several studies indicated that malate accumulation over the day and decarboxylation after darkening could be a reason for the observed ^{13}C enrichment (Gessler *et al.*, 2009; Tcherkez, 2010; Werner, 2010). Werner *et al*. (2011) provided a mechanistic concept for the observed ^{13}C enrichment pattern and the linked increase of respiration during LEDR. Both can be explained by the closure of the TCA cycle, which is non-cyclic in the light (Tcherkez *et al.*, 2009), occurring immediately after the light-to-dark transition, in connection with the interplay of the malate catabolizing enzymes that facilitate the degradation of the ^{13}C enriched malate pool accumulated under illumination. The intensity of the respiration pulse and its ^{13}C isotopic enrichment seem to be directly indicative of the extent of malate accumulation in the light and the ability to degrade this malate upon darkening (Lehmann, 2014). Still, in different species different organic acids besides malate might be involved in fueling LEDR (Lehmann et al., 2016). Assessment of LEDR provides insights into the re-organization of central metabolic pathways in leaves during light-dark transitions (Werner *et al.,* 2011). The two processes (malate accumulation and degradation) seem to be directly related to the cumulative carbon assimilation before darkening, as this parameter is also correlated with LEDR. However, we do not know yet whether the processes involved are also under circadian control. Gessler *et al.* (2009) did not observe a dependence of LEDR ^{13}C enrichment on cumulative photosynthesis in *Ricinus communis*, and this observation suggests that other factors might additionally affect the metabolic pathways responsible for malate accumulation and degradation.

In order to assess circadian control on night-time dark respiration (R_D) and LEDR, we performed two experiments in experimental macrocosms (Milcu *et al.,* 2014) hosting

canopies of *Phaseolus vulgaris* (bean, a herb) and *Gossypium hirsutum* (cotton, a shrub) exposed to constant darkness and constant light, respectively. In the first experiment (constant darkness), we tested whether circadian regulation of night-time leaf R_D scaled to whole canopy respiration. In this experiment, the plant canopies were exposed, after an entrainment phase with typical diel light-dark rhythms, to constant dark conditions for 30 h with no temporal variation in air temperature [T_{air}], vapor pressure deficit [VPD], and other environmental drivers. We hypothesized that circadian control of R_D occurred on leaf-level and whole canopy scales. If true, circadian memory might need to be considered in flux separation approaches that use extrapolation of night-time respiration to the day period, solely based on its direct dependency on temperature. In the second experiment (constant light), we assessed whether respiration of light acclimated leaves transferred into darkness was affected by circadian regulation. We assume that the absolute flux measured in such darkened leaves is representative for the LEDR, which is more precisely the enhancement of dark respiration rate (following the post-illumination photorespiratory burst) of light–acclimated leaves above the rate at 'steady state' (Atkin et al. 1998). Circadian rhythms of respiration of darkened light acclimated leaves (and thus LEDR) would indicate an internal control of the underlying metabolic processes. Here, after an entrainment phase, we exposed plants to constant environmental conditions for 48h with a constant PAR. We hypothesized that LEDR as measured in light-acclimated darkened leaves is not under direct circadian control, but mainly dependent on antecedent assimilation and, thus, accumulation of respiratory substrates. In both, the constant darkness and the constant light experiments, we also evaluated the availability of non-structural carbohydrate (NSC) as most important respiratory substrate.

Material and Methods

Ecotron and general experimental set-up

The experiment was performed at the Macrocosms platform of the Montpellier European Ecotron (www.ecotron.cnrs.fr), an advanced controlled environment facility for ecosystem research of the Centre National de la Recherche Scientifique (CNRS, France). We used 12

experimental domes/macrocosms (6 planted with bean and 6 with cotton) where air temperature, humidity, and CO_2 concentration were automatically controlled. In each macrocosm, plants were grown on a soil (area of 2 m^2, depth of 2 m) contained in a lysimeter, resting on a weighing platform and aboveground enclosed in a transparent dome-shaped cover. The soil was collected from the flood plain of the Saale River near Jena, Germany, and used in a previous Ecotron experiment on biodiversity (Milcu *et al.,* 2014). After that experiment, the soil was ploughed down to 40 cm and fertilized with 25/25/35 NPK (MgO, SO_3 and other oligoelements were associated in this fertilizer: Engrais bleu universel, BINOR, Fleury-les-Aubrais, FR). The soil was regularly watered to or close to field capacity by drip irrigation, although irrigation was stopped during each measurement campaign (few days) to avoid interference with water flux measurements that were additionally performed (c.f. Resco de Dios *et al.,* 2015). However, no significant differences (at $P < 0.05$, paired t-test, n=3) in leaf water potential occurred between the beginning and end of these measurement campaigns, indicating no effect of a potentially declining soil moisture on leaf hydration.

Environmental conditions within the macrocosms (excluding the experimental periods) were set to mimic outdoor conditions, but did include a 10% light reduction of solar radiation by the dome cover. During experimental periods, light was controlled by placing a completely opaque fitted cover on each dome to block external light inputs (PVC coated polyester sheet Ferrari 502, assembled by IASO, Lleida, Spain), and by using a set of 5 dimmable plasma lamps (GAN 300 LEP with the Luxim STA 41.02 bulb, with a sun-like light spectrum); these lamps were installed 30 cm above the plant canopy and provided a PAR of 500 μmol m^{-2} s^{-1} at the top of the canopy.

The wind speed in the domes was between 0.9-1 m s^{-1} leading to the canopy being well coupled to the dome atmosphere (Resco de Dios *et al.,* 2015). The concrete surface in the domes around the lysimeters were covered with epoxy-resin to prevent CO_2 absorption.

Bean and cotton were planted in 5 different rows within the lysimeters on 10th July 2013, one month before the start of the measurements, and thinned to densities of 10.5 and 9 individuals per m^2, respectively. Cotton (STAM-A16 variety by INRAB/CIRAD) is a perennial shrub with an indeterminate growth habit. STAM-A16 grows to 1.5-2 m tall and has a pyramidal shape and short branches. Bean (recombinant inbred line RIL-115 bred by INRA

Eco&Sol) is an annual herbaceous legume. RIL-115 is a fast growing, indeterminate dwarf variety, 0.3-0.5 m tall; it was inoculated with *Rhizobium tropici* CIAT 899 provided by INRA.

Measuring techniques

Each macrocosm was designed as an open gas exchange system to continuously measure CO_2 net ecosystem exchange by measuring the air flow at the inlet of each dome (thermal mass flowmeter Sensyflow iG, ABB, Zurich, CH) and by sequentially (every 12 min) measuring the CO_2 concentration at each inlet and outlet, using a multiplexer system coupled with two LI-7000 CO_2/H_2O analyzers (LI-COR Biosciences, Lincoln, NE, USA). Soil fluxes were prevented from mixing with canopy air by covering the soil with a plastic sheet during the entire experimental period and by applying a slight overpressure in the dome (+ 5 Pa) compared to the soil compartment (see Resco de Dios *et al.,* 2015) (possible soil CO_2 contamination on aboveground fluxes was tested and its absence was confirmed at experiment initiation).

For each crop, three macrocosms were dedicated to leaf-level measurements (researchers entered periodically) and the remaining three macrocosms were 'undisturbed' (i.e. no entry) and dedicated to canopy gas exchange measurements. During the experiment, bean and cotton generally remained at the inflorescence emergence developmental growth stage (codes 51-59 in BBCH scale, the standard phenological scale within the crop industry) (Feller *et al.*, 1995; Munger *et al.*, 1998). Further details on the Ecotron equipment and methodology used to measure canopy-level CO_2 and water fluxes have been provided elsewhere (Milcu et al., 2014; Roy et al., 2016).

We measured leaf gas exchange using a portable photosynthesis system (LI-6400XT, Li-Cor, Lincoln, Nebraska, USA), after setting the leaf cuvette to the same temperature and humidity as the air in the macrocosms. We conducted spot gas exchange measurements every 4 hours in three leaves within each macrocosm, and average values for each of the 3 macrocosms per species were used in subsequent analyses. Different leaves from different individuals were measured during each measurement round. Leaf temperature was independently measured at the time of gas exchange measurements with an infra-red thermometer (MS LT, Optris GmbH, Berlin, Germany) and no significant difference with air

temperature recorded by the T_{air} probe (PC33, Mitchell Instrument SAS, Lyon, France) was observed.

The following constant dark and constant light experiments were performed between 8th August and 8th September 2013.

Constant dark experiment

In order to assess whether the hypothesized leaf circadian regulation of R_D scaled up to affect whole canopy respiration, we conducted a constant dark experiment. For that purpose, canopies were originally entrained ("changing" conditions) by mimicking the temporal patterns in T_{air} (28/19 °C, max/min) and VPD (0.5/1.7 kPa) of an average sunny day in August in Montpellier. Photoperiod was set to 12 h of darkness and 12 h of light during entrainment, and a maximum PAR of 500 μmol m^{-2} s^{-1} (at canopy height) was provided by the plasma lamps (see above). After a 5-day entrainment period, we maintained PAR, T_{air}, and VPD constant at night values, for 30 hours starting (free running period) at solar midnight ("constant" conditions). We determined net ecosystem CO_2 exchange (canopy respiration) in the domes as explained above and assessed leaf level R_D with a portable photosynthesis system (LI-6400XT, LI-Cor Biosciences, Lincoln, NE).

Constant light experiment

We tested whether the respiration of light acclimated leaves transferred into darkness and (light enhanced dark respiration, LEDR) was subject to circadian regulation. The entrainment period was similar to the constant dark experiment. After 5 days of entrainment (see CO_2 fluxes air temperature and relative air humidity in Figs S1 and S2, we maintained PAR, T_{air}, and VPD at constant levels (see above) for 48 h starting at solar noon. To assess LEDR, we determined leaf dark respiration in light-acclimated leaves by transferring them into the LI-6400XT gas exchange cuvettes with no light. During the transfer into the cuvette, shading of the leaf was avoided. Prior to LEDR measurements, we determined leaf net photosynthesis (A_{net}) by setting cuvettes to the same environmental conditions as the macrocosm dome and cumulative A_{net} was calculated by assuming the point measurement being representative for

half of the time period to the preceding and half of the period to the subsequent measurement.

Non-structural carbohydrate analyses

In both the constant dark and the constant light experiments, every 4 hours (in time with the leaf-level gas exchange measurements) leaves from 3 individual plants were collected in each macrocosm and bulked to one sample for NSC analysis and directly quenched in liquid nitrogen to stop metabolic activity. Thus for a given time point, one leaf sample from each of the 3 macrocosms per species were collected and oven-dried at 60°C. NSCs are defined here as free, low molecular weight sugars (glucose, fructose and sucrose) plus starch. They were analyzed following the protocol of Hoch *et al.* (2003) with slight modifications as described in Plavcova *et al.* (2016). The NSC concentrations are expressed on a percentage dry matter basis.

Statistical Analyses

We examined statistical significance of temporal patterns of leaf and canopy level respiration with Generalized Additive Mixed Model (GAMM) fitted with automated smoothness selection (Wood, 2006) in the R software environment (*mgcv* library in R 3.1.2, The R Foundation for Statistical Computing, Vienna, Austria), including macrocosms as a random factor. This approach was chosen because it makes no *a priori* assumption about the functional relationship between variables. We accounted for temporal autocorrelation in the residuals by adding a first-order autoregressive process structure (*nlme* library; (Pinheiro & Bates, 2000)). Significant temporal variation in the GAMM best-fit line was analyzed after computation of the first derivative (the slope, or rate of change) with the finite differences method. We also computed standard errors (SE) and a 95% point-wise confidence interval for the first derivative. The trend was subsequently deemed significant when the derivative confidence interval was bounded away from zero at the 95% level; for full details on this method, see Curtis & Simpson (2014). Non-significant periods, reflecting lack of local statistically significant trending, are illustrated on the figures by the dotted line portions, and

1 significant differences occur elsewhere. The relationship between leaf respiration, canopy
2 respiration, environmental parameters, and NSC were determined by calculating Pearson
3 product-moment correlation coefficient in OriginPro 2016 (OriginLabs; Northampton, MA,
4 USA).

Results

Dark respiration – constant dark experiment

Figure 1 shows the time courses of canopy and leaf level R_D under constant darkness. Canopy respiration in bean shows significant temporal variation with an increase from 0 to 6 hours under constant environmental conditions and a subsequent decreasing tendency indicating circadian control. Leaf level R_D also showed significant variations during the constant dark period. The increase in R_D during the initial 6 hours and the subsequent decrease as observed for the canopy was also present for leaf level R_D. We note that there was an instrument failure around 18-24 h solar time under constant conditions, where leaf level R_D could not be measured. In cotton, canopy respiration generally showed temporal patterns comparable to bean. However, the initial increase, and large parts of the decrease, in respiration over time were not significant. Leaf level R_D in contrast showed significant variation over time indicating a circadian rhythm: during the first 6 hours, R_D increased, remained constant for another 6 hours, and then declined for almost 12 hours. For both canopy and leaf respiration, the maximum rates in the free-running period were higher in bean (R_{leaf}: 4.9 μmol m^{-2} s^{-1}; R_{canopy}: 1.6 μmol m^{-2} s^{-1}) than in cotton (R_{leaf}: 3.2 μmol m^{-2} s^{-1}; R_{canopy}: 1.1 µmol m^{-2} s^{-1}).

In leaves of bean, total NSC showed a maximum value of 5.2 % at 13:00 h solar time in the light (entrainment) period and decreased after the onset of darkness (Fig. 2). After 8 hours of constant dark, total NSC dropped to 0.7% and thereafter, further decreased and reached 0.3% after 32 hours in darkness. Both, starch and sugar contributed more or less equally to this pattern with sugars generally more abundant than starch. In contrast to bean, total NSC concentrations in cotton did not show a clear day-night variation in the entrainment period. Whilst starch concentration was highest in the afternoon/evening and lowest at the beginning of the light period, soluble sugars tended to decrease over the light period. Comparable to bean, NSC concentrations in cotton decreased under constant darkness. After 8 hours under constant night conditions, NSC dropped from 3.6% to 1.9% and reached 0.4% after 32 hours. At the beginning of the constant dark period, soluble sugars contributed

between 60% and 90% of NSC, but after 12-16 hours in constant darkness, the contribution of starch and sugars was comparable.

In order to assess whether (i) circadian leaf R_D patterns scaled to the canopy level and (ii) whether other parameters were related to respiration in constant dark, we performed correlation analyses (Table 1). For bean, there was no significant correlation between canopy and leaf level respiration; most likely this occurred due to lack of data points for the leaf level experiments because of instrument failure, as previously mentioned. Moreover, leaf and canopy respiration were not related to sugars, starch or total NSC. In cotton, there was a clear and significant positive correlation between leaf and canopy level respiration, but independent of NSC. Canopy respiration rates of bean and cotton were in contrast significantly correlated, which indicates that the pattern was comparable across species and that the lack of correlation between leaf and canopy R_D for bean was due to data scarcity after instrument malfunction. The fact that we did not find any relationship between RH, air or leaf temperature and respiration patterns clearly shows that direct environmental cues were not responsible for the variations shown in Fig. 1.

Light enhanced dark respiration - Constant light experiment

Fig. 3 depicts the temporal pattern of respiration of light acclimated leaves transferred into darkness under constant light. When we assume in a first approximation constant “steady state” respiration the measured parameter also indicates temporal variation in LEDR. When light-acclimated bean leaves were darkened, there was a slight increase in measured LEDR over the period of constant light, but no clear circadian oscillations. In contrast, cotton exhibited significant increases and decreases within a period of approximately 24 hours, indicating a circadian component in LEDR. As for bean there was also a general tendency for slightly increased LEDR over time for cotton.

At the end of the dark period, NSC strongly increased in bean and reached a value of 9.5% at 2 hours after constant light (Fig. 4). Thereafter, NSC values increased only slightly, reaching a maximum of 10.5% after 36 hours of constant light. This further slight increase was mainly due to starch, whilst soluble sugars remained more or less constant in the light. In cotton,

the NSC pattern under constant light was more complex: there was a first peak (7.4%) after 12 hours of constant light, then a subsequent decrease, and then a second peak after 36 hours (9.2%) thus corresponding to a 24-hour oscillation.

LEDR of bean was strongly correlated to total NSC and its components (soluble sugars and starch), whereas in cotton, the correlation was observed for sugars and total NSC, but not for starch (Table 2). Moreover, LEDR over the constant light period showed high and significant correlation between the two species even though cotton showed significant circadian variations and bean did not. The correlation was most likely due to the increase over time that was observed in both species. LEDR was significantly related to cumulative leaf net photosynthetic rate over the constant light phase in bean, but this correlation was not found in cotton (Fig. 5).

Discussion

24h oscillations of carbon fluxes under constant conditions as observed here (e.g. Figs 1; 3b) might be affected by many different processes such as carbohydrate accumulation or depletion or hydraulic feedbacks (Jones, 1998). While we took into account the effect of changes in NSC concentrations (Figs. 2 and 4), hydraulic feedbacks were not considered but these would cause monotonic increases or decreases of fluxes rather than oscillations. The only mechanism currently known to create self–sustained 24h cycles is the circadian clock (McClung, 2006, Müller et al., 2014) with interactions between the central oscillator of the clock and the different processes involved in dark respiration and LEDR.

Scaling of circadian regulation in R_D from leaves to canopies

Whilst there is a strong consensus that circadian control is of central importance for the control of gene expression and central metabolic pathways (e.g. Harmer, 2009; De Caluwé *et al.,* 2016), evidence is less clear when moving up in scale. On the organ (leaf) level, significant effects of the circadian clock have been observed for stomatal conductance (Resco de Dios *et al.,* 2013; 2015; Williams and Gorton 1998), but on the canopy and

ecosystem level only a few studies are available (e.g. Resco de Dios *et al.*, 2015). Concerning respiration, the situation is comparably ambiguous with clear indication of circadian control in some species (Hillman, 1970; Hansen, 1977), but not in others (e.g. Hennessey *et al*., 1993).

In our experiments, there was a significant circadian oscillation of leaf level R_D in cotton under constant darkness and a comparable pattern was observed for bean. This result contradicts the previous findings of Hennessey *et al*. (1993), who reported that no rhythm in respiration occurred in bean plants/leaves transferred to constant darkness. It might be assumed that a fast depletion of the respiratory substrate was the reason for the lack of rhythmicity in Hennessey *et al.* (1993), because plants were entrained under much lower radiation (200 μmol m^{-2} s^{-1}). We also observed a reduction of NSC concentration in the leaves of both species, and although the circadian rhythm was sustained during the experiment, it would likely be dampened if the experiment had lasted longer due to lack of carbohydrate substrates for respiration. Different NSC storage capacities in different cultivars of bean might also explain differences between our study and Hennessey *et al*. (1993). The sugar depletion, and its potential dampening of diurnal rhythmicity, also suggests that the importance of circadian regulation as a driver of R_D may have been underestimated in our experiment.

It is important to note that in bean, maximum R_D during the constant dark period was only slightly lower than respiration during the light phase of the entrainment period. This occurred even though temperature was more than 5°C lower in the dark and LEDR in darkened light adapted leaves should have caused an additional CO_2 efflux burst in the light period. In cotton, in contrast, maximum R_D during the dark period was approximately two times lower than respiration during the light phase. Comparable to leaf level R_D, canopy respiration in both species showed an initial increase at the beginning of the constant dark period and a decrease thereafter, followed by an additional smaller peak. Whilst this circadian pattern was mostly significant in bean, it was much less clearly expressed in cotton. However, the high and significant correlation between canopy respiration of cotton and bean is a clear indication of a similar oscillation. Moreover, the circadian oscillation of leaf-level R_D was clearly affecting canopy R_D in cotton. Although the oscillation between leaf and

canopy R_D was not significant in bean, this was most likely due the lack of data coverage as a result of instrument failure. The patterns for canopy respiration were, however, similar between the two species (Table 1) and thus it is reasonable to assume a comparable regulation.

The lack of correlation between respiration, both on the leaf and canopy levels, with total NSC, sugar and starch content indicates that substrate availability/limitation was not responsible for the observed circadian patterns of respiration. Despite the fact that starch synthesis and degradation is under strict circadian control (Weise *et al.,* 2006), it has been shown that under constant darkness, carbohydrate levels drop quickly, as observed in our study, but also transcript levels of starch-degrading enzymes declined (Lu *et al*., 2005) thus resulting in a rather gradual decrease in substrate levels for respiration. Fukushima *et al.* (2009) showed that mitochondrial functions and activities are closely coupled with the circadian system in plants, and thus the activity of TCA cycle enzymes rather than substrate availability seems to be responsible for the rhythmicity of respiration.

Based upon our results, we accept our first hypothesis although more data for additional vegetation types need to be acquired in order to substantiate our finding that circadian control of respiration on the leaf level scales to canopies and thus the aboveground compartment of ecosystems. Our observation that circadian control of leaf-level respiration scales to the canopy is in agreement with observations for night-time transpiration (Resco de Dios *et al.,* 2013; 2015) and daytime carbon dioxide net exchange (Doughty *et al.*, 2006; Resco de Dios *et al.,* 2012), but in contrast to results for ecosystem respiration – including above- and belowground respiration (Resco de Dios *et al.*, 2012). There are at least two possible reasons why Resco de Dios *et al*. (2012) did not find evidence of circadian regulation on ecosystem respiration. One reason may be that ecosystem respiration is the result of above- and below-ground respiration. If circadian regulation does not occur in soil respiration or if its rhythmicity is phase shifted, this might have masked rhythms in canopy fluxes, because flux towers do not measure above- and below-ground fluxes separately. Another potential reason may be that the previous study was based on indirect evidence from eddy covariance data, which often do not provide accurate estimates of fluxes under low turbulent conditions, which are typical during night-time. Nonetheless, the present

study is the first direct test of circadian control of respiration at canopy scale and further studies will be needed to confirm the generality of this finding.

Implications of circadian R_D regulation on night-to-day extrapolations

Eddy covariance approaches are commonly used to characterize ecosystem carbon exchange and to calibrate and validate ecosystem carbon balance models (Reichstein *et al.,* 2005). In order to partition net ecosystem exchange during daytime into its component fluxes ecosystem gross primary productivity and ecosystem respiration (R_{eco}), often temperature dependency of nighttime R_{eco} is assessed to infer daytime values (c.f. Lasslop *et al.*, 2010). With this approach it is assumed that day- and nighttime R_{eco} show the same response to temperature and sophisticated algorithms that consider temporal changes in temperature sensitivity of R_{eco} are applied. Our results show that dark respiration at the leaf and canopy levels exhibits considerable fluctuation with time under constant darkness and constant temperature. In order to estimate the strength of circadian oscillations compared to temperature driven variations in a normal day and night cycle, we used a "Q_{10} approach" to describe the temperature dependency. Many models calculate, as a first approximation, temperature responses of respiratory CO_2 efflux from plants, soils, and ecosystems by using exponential functions with a Q_{10} that often ranges between 1.2-2.5 (Mahecha *et al.* 2010, Tjoelke*r et al.,* 2001). While a full calculation of Q_{10} within our experimental systems would have been beyond the scope of our study, we will assume it took a value of 2 simply for illustrative purposes. A Q_{10} of 2 will be close to the average of temperature sensitivities of different species (Tjoelker *et al.* 2001). In other words, we assumed that Q_{10} was 2 simply to compare the potential importance of temperature with that of circadian rhythms in a way that would realistically reflect the influence of the former. Thus, taking the minimum (leaf level) respiration rate during the first subjective night (at the beginning of the constant dark period; bean: 2.4 μmol m^{-2} s^{-1}, cotton: 2.1 μmol m^{-2} s^{-1}) and the constant temperature (ca. 20°C) at that time as reference values, we calculated a respiration rate for an assumed temperature of 28°C (equaling our maximum daytime temperature in the light). For bean and cotton, the calculated rates for leaf R_D were 4.1 and 3.7 μmol m^{-2} s^{-1}, respectively. These rates were comparable to the maximum respiration rates during the constant dark period at 20°C (bean: 4.6 μmol m^{-2} s^{-1}, cotton: 3.4 μmol m^{-2} s^{-1}). The leaf respiration rates measured

during daytime in the light at 28°C were higher than the Q_{10} derived values, which is reasonable since these measured rates should be affected by LEDR. For canopy respiration, 25% (cotton) to 57% (bean) of the variation expected by an 8°C temperature rise (based on a Q_{10} of 2) was observed at constant darkness and constant temperature.

Our findings indicate that at least part of the day-night variation of leaf and whole canopy respiration is not solely temperature controlled, but also triggered by the circadian clock. Such an internal control might buffer the direct temperature dependency of respiration, and thus energy demanding metabolic processes, over the diurnal time scale in general. Under strong temperature variations within a few days, metabolic imbalances might thus be avoided and over longer time spans, temperature acclimation (Atkin & Tjoelker, 2003) might be facilitated.

Reichstein *et al*. (2005) stated that temperature sensitivity of R_{eco} might not be constant, but variable over the course of the growing season. In fact, they observed that the temperature sensitivity of R_{eco}, derived from long-term (annual) data sets, did not reflect the short-term (hourly) temperature sensitivity that is effectively used when extrapolating from night- to daytime respiration. They attributed the varying short-term temperature sensitivity of R_{eco}, to differences in the overall activity of leaves, roots as well as in tissue growth over time. Our results suggest that on a short time scale circadian regulation of respiration could also be involved. Circadian regulation is known to act as a "memory bank" of processes in the recent past to adjust organismal metabolism accordingly (Boikoglou et al., 2011). In such a case the measured "apparent" temperature sensitivity will not only reflect the reaction towards current conditions but also the impact of the environmental conditions of previous days (in the case of our experiment the entrainment period) and thus contains an internal "memory"-related component that is not directly temperature dependent. Any change in this component will change the "apparent" response of respiration to temperature and might thus contribute to the observed short-term variability in temperature sensitivity of R_{eco}.

Circadian control of Light Enhanced Dark Respiration

In photosynthesizing leaves, respiration is strongly repressed but when transferring these leaves into the dark, LEDR induces a burst of CO_2 release. Here we measured not exactly this increase in respiration above a baseline value of dark respiration (c.f. Atkin et al. 1998). As a consequence, diurnal variations in baseline respiration might also be involved in the observed diurnal patterns. Especially diurnal variations in respiratory sink demand as related to hormonal control of growth might be a factor affecting baseline respiration (Nozue and Maloof, 2006; Caldeira et al. 2014). To test if this could be the case we compared R_D values of cotton from the first approx. 12 h after start of the constant dark conditions, when sugar depletion and its potential effect on R_D was not strong, yet, with respiration values of light acclimated darkened leaves at the same subjective time (Fig. 6). R_D is assumed to be the steady state baseline value of respiration and the difference between the two parameters would be "real LEDR". Fig. 6 shows that though variations in R_D affected the calculated difference both, "real LEDR" and respiration of darkened light acclimated leaves show the same general pattern with a maximum around subjective noon. We thus consider that the analysis of respiration darkened of light acclimated leaves over time provides an adequate measure of LEDR and its circadian variation. Under constant light conditions, there was a marked difference in LEDR patterns between the two species. While CO_2 efflux from darkened light-acclimated leaves did not show a clear circadian oscillation in bean, cotton leaves in contrast did show a circadian pattern of LEDR. A commonality between the two species was the slight overall tendency in respiratory CO_2 efflux to increase over the free-running period.

The NSC patterns were comparable to the two different LEDR patterns. In bean, we observed (after a steep rise at the beginning of the constant light period) a slight increase in NSC mainly due to starch accumulation over the free-running period, whereas in cotton, NSC showed a ~24 hours oscillation. Variations in starch concentrations under continuous light, comparable to cotton, have been observed in *A. thaliana* and associated with the regulation of starch breakdown via maltose (Espinoza *et al.*, 2010). In our case, however, not only starch but also soluble sugars show a circadian rhythm, and thus the patterns observed are not a result of a circadian shift of assimilate allocation to starch as e.g. shown by Kölling *et al.* (2015). Thus, the circadian pattern is either source (photosynthesis) or sink (export of sugars out of the leaf) controlled. Peuke *et al.* (2001) observed sugar transport in the phloem

to be constant over the day-night cycle and thus strong circadian variations of phloem loading seem to be unlikely. Resco de Dios *et al*. (2016) showed, however, circadian oscillations in CO_2 assimilation rates in cotton under constant light that were phase shifted to the NSC patterns observed here, i.e. showing maxima at subjective midday and minima at midnight. Sugars are known to be important signaling compounds involved in modulating the circadian oscillator (Dodd e*t al*., 2015) and sugar leaf carbohydrate accumulation exerts negative feedback on photosynthesis (Goldschmidt & Huber, 1992; van Gestel *et al.,* 2005). Photosynthetic minima occurred when NSC showed maxima and vice versa, indicating a feedback mechanism. However, while photosynthesis in bean showed circadian oscillations comparable to cotton (Resco De Dios *et al*. 2016), this did not occur for NSC, suggesting a lack of regulatory feedbacks between sugars and photosynthetic carbon assimilation in the legume species.

The inverse relationship between photosynthesis and sugars for one species and the lack of a relationship in the other species also explains the differences among these species regarding the relationship between LEDR and cumulative A_{net} over the constant light period. It is well known that the extent of the LEDR burst is related to the accumulated net CO_2 assimilation in the preceding light period (Azcon-Bieto & Osmond, 1983). This observation gave rise to the assumption that LEDR reflects the level of photosynthetic metabolites available to the mitochondria following a period of illumination (Atkin *et al.*, 2000); LEDR was shown to be directly related to the malate pool consumed after darkening (Gessler *et al.*, 2009). For bean, a relationship between LEDR and cumulative photosynthesis as well as the NSC pools was observed, suggesting that NSC accumulation is related to the size of the malate pool which is the specific substrate for LEDR (Lehmann *et al.*, 2015). For cotton, the intensity of LEDR was also positively related to sugar concentration, but due to the lack of continuous accumulation of photoassimilates not to cumulative photosynthesis rate. Given the specific substrate for LEDR, we have to assume that not only the sugar, but also the malate pool oscillated diurnally. According to Gessler *et al*. (2009), the malate net production flux is given by the fixation of HCO_3^- to phosphoenolpyruvate by PEPc to produce oxaloacetate minus the oxaloacetate consumption by the TCA. Until present, it was assumed that the repression of the TCA cycle enzymes (plus the non-cyclic nature of the TCA cycle) (Tcherkez *et al.,* 2009), as well as the increase in PEPc activity during daytime (Gousset-

Dupont *et al.,* 2005), were directly light-driven and thus resulting in a continuous accumulation of malate in the light. Our results for cotton, however, indicate that either the malate producing or the malate consuming pathways or both are under circadian control. This effect might also be indirect and related to the circadian oscillation of photosynthesis since TCA enzymes (and thus malate consumption) are known to be inhibited by large NAD(P)H/NAD(P) ratios (Igamberdiev & Gardeström, 2003). The decrease in photosynthesis occurring in the subjective night (Resco De Dios *et al*. 2016) might have consequently led to a partial release of the repression of the TCA cycle via its effect on the redox ratio. This leads us to reformulate our second hypothesis, insofar that in one species (bean) LEDR and the underlying mechanisms do not seem to be under circadian control, whereas in the other species (cotton), both LEDR intensity and the linked metabolite levels show circadian oscillation.

Conclusions

Our results clearly indicate that night-time dark respiration (R_D) on the leaf-level is under circadian control and that the circadian patterns scale to the canopy level. Since circadian regulation is assumed to act as an adaptive memory to adjust plant metabolism, based on the environmental conditions experienced in previous days, it might be worth to explore further if this internal regulation mechanism affects measured temperature sensitivity of canopy or even whole ecosystem respiration. That is, since temperature and circadian regulation both co-vary with time, temperature effects on ecosystem respiration may be, at least potentially, confounded by circadian regulation of respiration. If so it might contribute to the observation that temperature sensitivity of ecosystem respiration is not constant but variable over time. Assessments of LEDR can provide deeper insights into the metabolic re-organization in the leaf during light-dark transitions. The circadian rhythm of LEDR in cotton might indicate variable suppression of the “normal cyclic” function of the TCA cycle in the light. Although it needs to be clarified why such rhythmicity is not present in bean, our

results point to differences in the regulatory feedbacks between the clock, sugars, and photosynthetic carbon assimilation.

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Figure Legends

Fig. 1: Assessments of the circadian regulation of canopy (a, c) and leaf level respiration (b, d) fluxes during constant darkness. Environmental conditions of air temperature (T) and relative air humidity (RH) simulated an average August day in Montpellier, with 500 μmol m^{-2} s^{-1} PAR during daytime conditions before the onset of the constant dark period. RH and T were set constant to night conditions for 36 hours starting at midnight (dashed vertical line). In a) and c) daytime CO_2 fluxes at the time before constant darkness are not shown and only the respiration rates in the dark (grey shaded area) are displayed. The white and black rectangles at the base indicate the subjective day (when it would have been daytime during entrainment) and subjective night, respectively, under constant conditions. The thin lines in a) and c) represent ± standard deviation of the means of three replicate macrocosms. In b) and d) squares represent mean respiration values from measurements of three plants per dome (all 3 domes were measured within a 60 min interval). Bold lines in a) to d) (and shaded SE intervals) indicate the prediction of the GAMM. Significant variations in ecosystem respiration are indicated by solid portions of the GAMM best-fit lines, non-significant variations by the dotted portions. For the measurements of leaf respiration in bean (b) no data could be acquired between 17 hours and 15 hours after the onset of constant darkness due to instrument failure.

Fig. 2: Temporal course of NSC concentrations in the leaves of bean (a) and cotton (b) during constant darkness. Environmental conditions of air temperature (T) and relative air humidity (RH) simulated an average August day in Montpellier, with 500 μmol m^{-2} s^{-1} PAR during daytime conditions (white area) before the onset of the constant dark period (grey shaded area). RH and T were set constant to night conditions for 36 hours starting at midnight (dashed vertical line). The white and black rectangles at the base indicate the subjective day (when it would have been daytime during entrainment) and subjective night, respectively, under constant conditions. Data shown are mean values (± SD) from 3 domes.

Fig. 3: Assessments of circadian regulation of light enhanced dark respiration (LEDR) during constant light for bean (a) and cotton (b). Environmental conditions of dome air temperature and vapor pressure deficit simulated an average August day in Montpellier, with 500 μmol m^{-2} s^{-1} PAR during the light period, and remained constant for the following 48 hours starting at solar noon (dashed vertical line). The grey shaded area indicates the dark period during entrainment. Respiration fluxes have been measured on the leaf level in the in gas exchange cuvettes with no light, irrespective of the light conditions in the domes. Data shown are mean values from measurements of three plants per macrocosm/dome (all 3 domes were measured within a 60 min interval). The bold lines (and shaded SE intervals) indicate the prediction of the GAMM. Significant variations in leaf dark respiration are indicated by solid portions of the GAMM best-fit lines, non-significant variations by the dotted portions.

Fig. 4: Temporal course of NSC concentrations in the leaves of bean (a) and cotton (b) during constant light. Environmental conditions of dome air temperature and vapor pressure deficit simulated an average August day in Montpellier, with 500 μmol m^{-2} s^{-1} PAR during the light period, and remained constant for the following 48 hours starting at solar noon (dashed vertical line). The grey shaded area indicates the dark period during entrainment. The white and black rectangles at the base indicate the subjective day (when it

would have been daytime during entrainment) and subjective night, respectively, under constant conditions. Data shown are mean values (± SD) from 3 domes.

Fig. 5: Relationship between cumulative net photosynthesis (A_{net}) over the constant light period and dark respiration of darkened light acclimated leaves (LEDR) in bean and cotton. Cumulative A_{net} and LEDR were both determined at the same time in the constant light experiment and only values from the constant free running phase have been included. Here, negative values are given for respiration and positive values for (cumulative) photosynthesis. Note that both photosynthesis and respiration fluxes are given with positive signs.

Fig. 6: Comparison between dark respiration and respiration of darkened light acclimated leaves to estimate "real" LEDR in cotton. In the constant light experiment as shown in Fig 3, we measured not exactly the increase in respiration above a baseline value of dark respiration, which is referred to as LEDR. To test if our measurements are a still a proxy for LEDR, we compared leaf level respiration (R_D) values from the first approx. 12 h after start of the constant dark conditions (c.f. Fig 1) with respiration values of light acclimated darkened leaves at the same subjective time (Fig. 3). Respiration under constant darkness is assumed to be the steady state baseline value of respiration and the difference between the two parameters would equal "real LEDR".

Supplementary Material

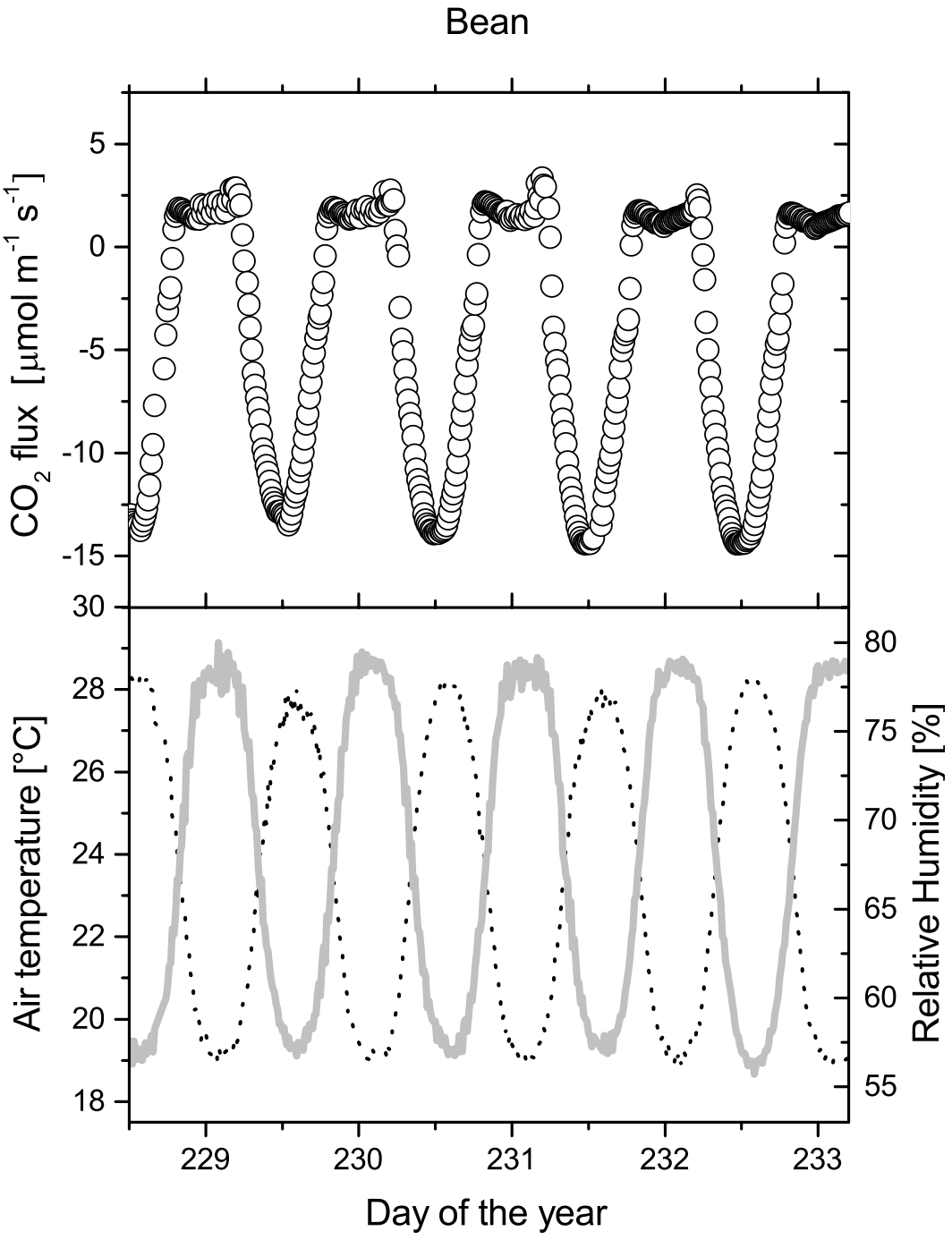

Fig S1: Diel variations of dome CO_2 flux, air temperature and relative air humidity in the entrainment period for bean for the constant darkness experiment. Note that photosynthetic fluxes are negative while net respiration is positive. Values given are means of three domes.

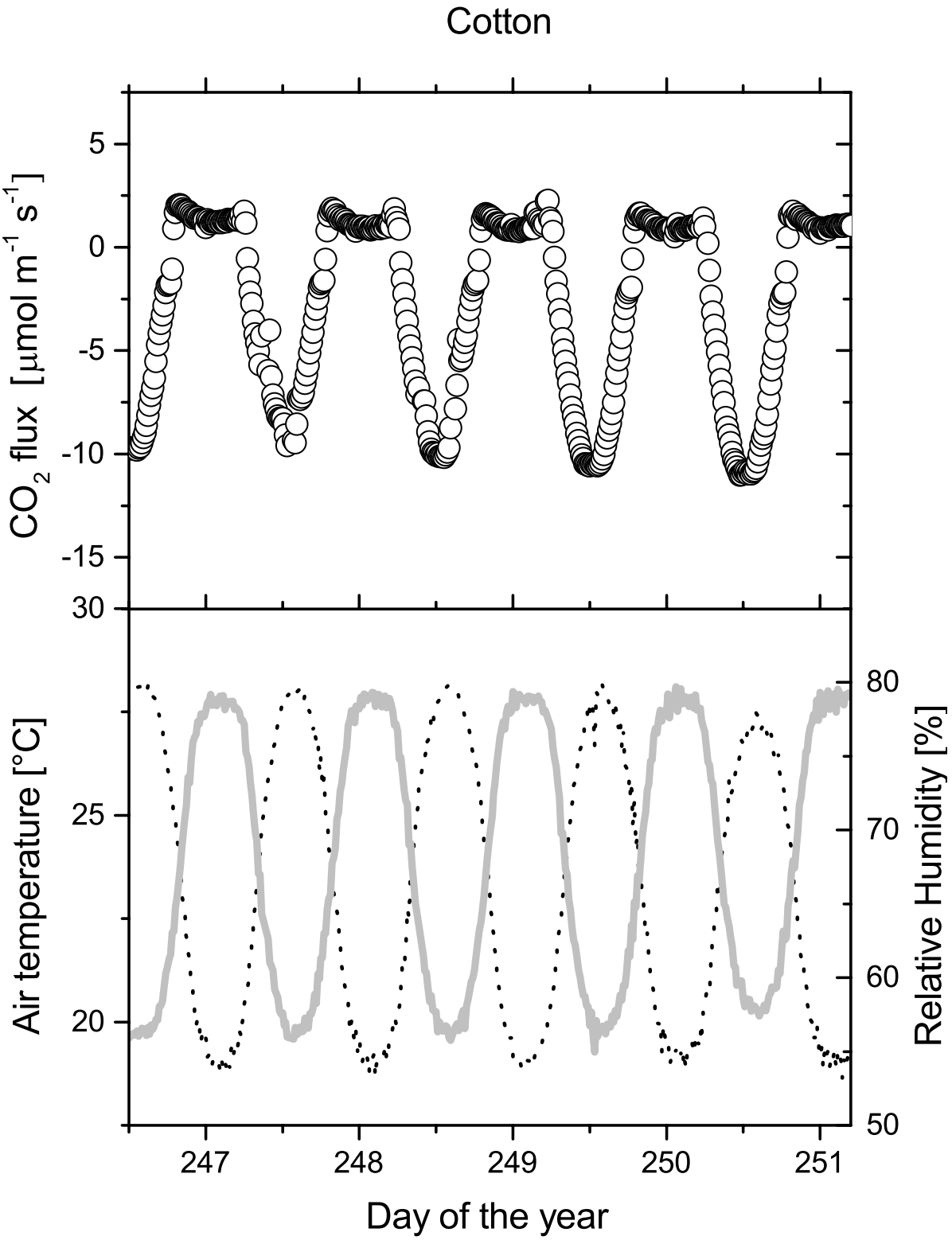

Fig S2: Diel variations of dome CO_2 flux, air temperature and relative air humidity in the entrainment period for cotton for the constant darkness experiment. Note that net photosynthetic fluxes are negative while net respiration is positive. Values given are means of three domes.