Research paper

Circadian rhythms regulate the environmental responses of net CO$_2$ exchange in bean and cotton canopies

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ABSTRACT

Studies on the dependence of the rates of ecosystem gas exchange on environmental parameters often rely on the up-scaling of leaf-level response curves ('bottom-up' approach), and/or the down-scaling of ecosystem fluxes ('top-down' approach), where one takes advantage of the natural diurnal covariation between the parameter of interest and photosynthesis rates. Partly independent from environmental variation, molecular circadian clocks drive ∼24 h oscillations in leaf-level photosynthesis, stomatal conductance and other physiological processes in plants under controlled laboratory conditions. If present and of sufficient magnitude at ecosystem scales, circadian regulation could lead to different results when using the bottom-up approach (where circadian regulation exerts a negligible influence over fluxes because the environment is modified rapidly) relative to the top-down approach (where circadian regulation could affect fluxes as it requires the passage of a few hours). Here we dissected the drivers of diurnal net CO$_2$ exchange in canopies of an annual herb (bean) and of a perennial shrub (cotton) through a set of experimental manipulations to test for the importance of circadian regulation of net canopy CO$_2$ exchange, relative to that of temperature and vapor pressure deficit, and to understand whether circadian regulation could affect the derivation of environmental flux dependencies. Contrary to conventional wisdom, we observed how circadian regulation exerted controls over net CO$_2$ exchange that were of similar magnitude to the controls exerted by direct physiological responses to temperature and vapor pressure deficit. Diurnal patterns of net CO$_2$ exchange could only be explained by considering effects of environmental responses combined with circadian effects. Consequently, we observed significantly different results when inferring the dependence of photosynthesis over temperature and vapor pressure deficit when using the top-down and the bottom up approaches.

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1. Introduction

Models of land-atmosphere interactions and projections of carbon cycling under climate change rely on accurate representations of the underlying biophysical processes. Diurnal patterns are a key feature of observed ecosystem fluxes and arise largely from plant ecophysiological responses to external drivers of solar irradiance,

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temperature and relative humidity, amongst others (Hollinger et al., 1994; Jones, 2014; Richardson et al., 2007; Schwalm et al., 2010; Sellers et al., 1997). Consequently, models estimating daytime variation in ecosystem fluxes often rely upon the temporal extrapolation of short-term response functions of the flux to a given driver ("bottom-up" hereafter), or upon the temporal co-variation between fluxes and drivers ("top-down" hereafter). Concomitantly, we have known for long that the circadian clock is an important regulator of plant metabolism, including photosynthetic gas exchange in leaves (Edwards et al., 2011; Harmer et al., 2000; Lüttge, 2003). However, the role of circadian regulation of canopy-scale CO_2 exchange remains largely untested. Circadian regulation of canopy photosynthesis, if present and of sufficient magnitude, could have implications for measurement and modeling of ecosystem fluxes.

Circadian regulation leads to daily rhythms of metabolism with a period of ∼24 h that persist even in the absence of variation in environmental parameters. Circadian rhythms provide an evolutionary advantage by allowing the plant to anticipate predictable environmental patterns, such as the dawn/dusk transitions (Resco de Dios et al., 2016b), by temporally synchronizing, sequencing or separating processes that are biologically-associated or incompatible (Harmer et al., 2000) and by mediating in the response to the change in seasons thorough photoperiodic responses (Ibanez et al., 2010).

Circadian rhythms in vascular plants arise from the "circadian oscillator", a complex gene autoregulatory network that comprises interlocked transcription-translation feedback loops. The phase of the oscillator is adjusted based upon the light and temperature regimes of the previous days, a process termed "entrainment", and that allows correct tracking of dawn and dusk (Takahashi et al., 2015).

The circadian oscillator exerts a prevalent influence upon plant cells. About one-third of all transcripts in the model species *Arabidopsis thaliana* are regulated by the circadian clock (Covington et al., 2008). Among other processes, leaf CO_2 exchange is regulated by the circadian oscillator in a wide variety of species (Dodd et al., 2014; Edwards et al., 2011; Hennessey et al., 1993; Yakir et al., 2007). Moreover, recent studies show that photosynthesis is a key component of the circadian oscillator itself, and sugar signals mediate the output of the circadian oscillator (Haydon et al., 2013). Consequently, a diurnal pattern in leaf level assimilation can be observed even when radiation, vapor pressure deficit, temperature and other environmental drivers are held constant over a few days (Resco et al., 2009).

Although the relevance of circadian controls over cell- and leaf-level processes is well documented (Hsu and Harmer, 2014; Mas and Yanovsky, 2009; Smith and Stitt, 2007), studies on its effects at the scale of ecosystem-level gas exchange are still scarce. Some studies, which apply statistical filtering to the physical drivers to emulate constant conditions, provide circumstantial support for the importance of circadian entrainment in net ecosystem CO_2 exchange (Doughty et al., 2006; Resco de Dios et al., 2012). Moreover, circadian regulation of nocturnal stomatal conductance has been linked to whole-tree sap-flux (Resco de Dios et al., 2013) and canopy-level transpiration (Resco de Dios et al., 2015). Direct evidence for a circadian effect on photosynthesis at high organizational scales (i.e. canopy and ecosystem) has only been recently observed (Resco de Dios et al., 2016a), where 20–28% of the variation in net canopy CO_2 assimilation (A_c) was due to circadian regulation under experimental conditions where there was no temporal variation in radiation, temperature, humidity and other environmental cues during 48 h.

However, although circadian regulation is expressed at canopy scales, its potential implications for inference and interpretation of ecosystem flux dynamics are still poorly understood (Dietze, 2014;

Dodd et al., 2014; Resco et al., 2009). Here we test whether circadian regulation affects current methods of deriving environmental flux dependencies. That is, we test whether similar associations between A_c and air temperature (T_{air}), and between A_c and vapor pressure deficit (VPD), are obtained by either using the natural diurnal co-variation between A_c and drivers (where circadian action could interfere as the method requires environmental variation during a few hours), or using a short-term ramped response function (where circadian action has a negligible effect, as response curves are often conducted within minutes). We propose that there will be differences between both methods due to circadian regulation. That is, we hypothesize that canopy-level carbon assimilation is jointly regulated by endogenous circadian processes and the direct impacts of the physical environment on plant physiology (Resco de Dios et al., 2012). We address this hypothesis by examining canopy assimilation patterns in large macrocosms within an Ecotron (Milcu et al., 2014; Roy et al., 2016) planted with monocultures of *Phaseolus vulgaris* (bean, an herb) and *Gossypium hirsutum* (cotton, a shrub). These two species were chosen as the belong to contrasting functional types (annual herb vs perennial shrub) and show high agronomic and economic value.

2. Material and methods

2.1. Ecotron and general experimental set-up

The experiment was performed at the Macrocosms platform of the Montpellier European Ecotron, Centre National de la Recherche Scientifique (CNRS, France). Full details of experimental set-up have been provided elsewhere (Resco de Dios et al., 2016a; Resco de Dios et al., 2015). In short, we used 6 outdoor macrocosms (3 planted with bean and 3 with cotton) where the main abiotic (radiation, air temperature, humidity and CO_2 concentration) drivers were automatically controlled. In each macrocosm, plants were grown on a soil (area of 2 m², depth of 2 m) 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 *ca.* field capacity by drip irrigation, although irrigation was stopped during each measurement campaign (few days) to avoid interference with water flux measurements. 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 by the macrocosm 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 hung 30 cm above the plant canopy and provided a PAR of 500 μmol m⁻² s⁻¹ (the maximum possible by those lamps). We ensured target radiation was achieved by measuring PAR at canopy level with a quantum sensor (Li-190, LI-COR Biosciences, Lincoln, NE, USA) in each macrocosm (Fig. 1). CO_2 concentrations were held constant at 400 ppm.

Bean and cotton were planted in 5 different rows within the domes on 10th July 2013, one month before the start of the measurements, and thinned to densities of 10.5 and 9 individuals
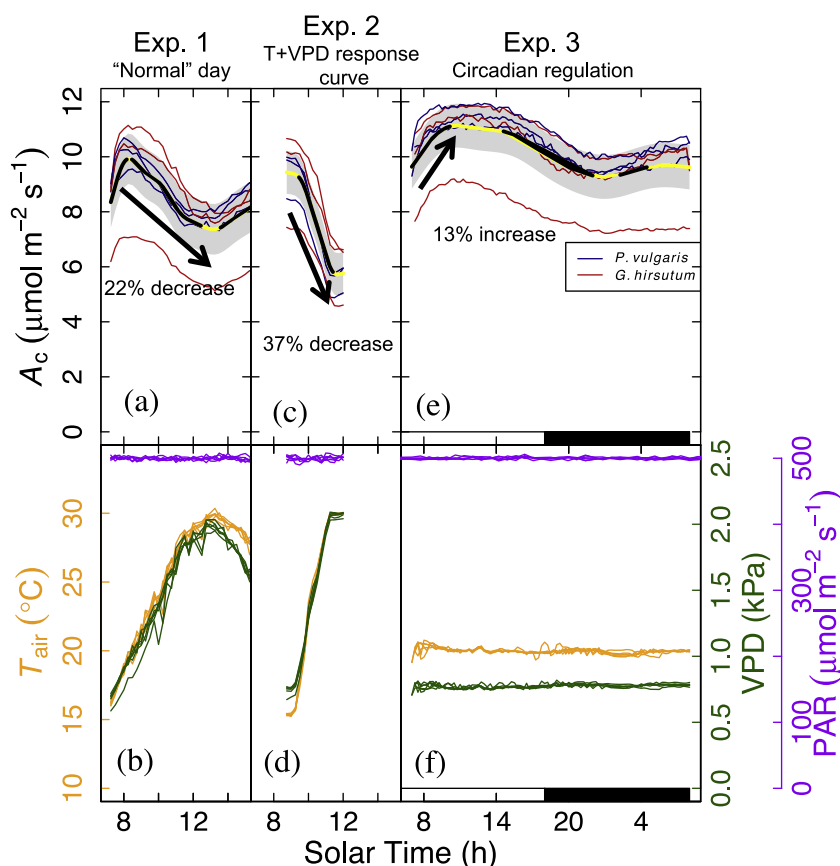
Fig. 1. Circadian regulation affects canopy-scale CO_2 fluxes in combination with direct physiological responses to temperature and vapor pressure deficit. We compared the variation in canopy assimilation (A_c) under constant photosynthetically active radiation (PAR) during: a morning and early afternoon (0600/1400 h) with the same patterns in temperature (T_{air}, 15–30 °C) and vapor pressure deficit (VPD, 0.6 kPa-2.1 kPa) as during entrainment (Exp. 1; a–b); a 2-step combined T_{air} and VPD ramped test (Exp. 2; c–d); a 24 h period with constant T_{air} and VPD (Exp. 3; e–f). Thin red and blue lines represent values measured in each of three macrocosms of each species. Thick lines (and shaded error intervals) indicate the prediction (and SE) of a single Generalized Additive Mixed Model (GAMM) across species, with yellow line portions indicating non-significant variation. White (and black) rectangles at e–f indicate the subjective day (and night) of the photoperiod during the prior entrainment period, whereas the light level was held constant. The arrow and numbers in the text indicate the percent change after linear normalization and are presented for aid in explanation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

m^{-2}, respectively. Cotton (STAM-A16 variety by INRAB/CIRAD) is a perennial shrub with an indeterminate growth habit. This cotton variety 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 species. RIL-115 is a fast growing, indeterminate dwarf variety, 0.3-0.5 m tall; it was inoculated with *Rhizobium tropici* CIAT 899 also provided by INRA. 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 Ecotron measurements have been provided elsewhere (Milcu et al., 2014).

2.2. Measuring techniques

Each unit of the Macrocosms platform 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). Belowground fluxes were prevented from mixing with canopy air by covering the soil with a plastic sheet during the entire experimental period. Substantial internal air mixing within the dome

(2 vols per min) reduced the canopy boundary layer and minimized the CO_2 concentration gradients within the dome. A slight atmospheric over-pressure (5–10 Pa) applied to the plastic sheet (through the slits made for the plant stems) covering the soil minimized potential mixing of soil respiration fluxes with aboveground fluxes. Indeed, we observed negligible CO_2 flux at the onset of the experiment (immediately after seed germination, when there was no significant carbon assimilation), indicating lack of significant CO_2 efflux on the canopy above the plastic sheet. Further details on flux measurements have been provided elsewhere (Resco de Dios et al., 2016a; Resco de Dios et al., 2015; Roy et al., 2016).

2.3. Experiments

We tested whether diel flux patterns could be explained by direct physiological responses to environmental changes alone, or whether including circadian regulation would add explanatory power. In our first experiment (Exp. 1 in Fig. 1), we documented the morning and early afternoon (0600h–1400 h) patterns of net canopy CO_2 exchange (A_c) under "normal" (i.e. same as during the five entrainment days) patterns in T_{air} and VPD (15 °C and 0.6 kPa at 0600 h/30 °C and 2.1 kPa at 1400 h). This variation in T_{air} was double the long-term average observed in Montpellier in August (22–30 °C in that time, J. Roy *unpublished data*) and was chosen to maximize the influence of direct responses to T_{air} and VPD. To test if the variation in fluxes during this period was regulated by direct
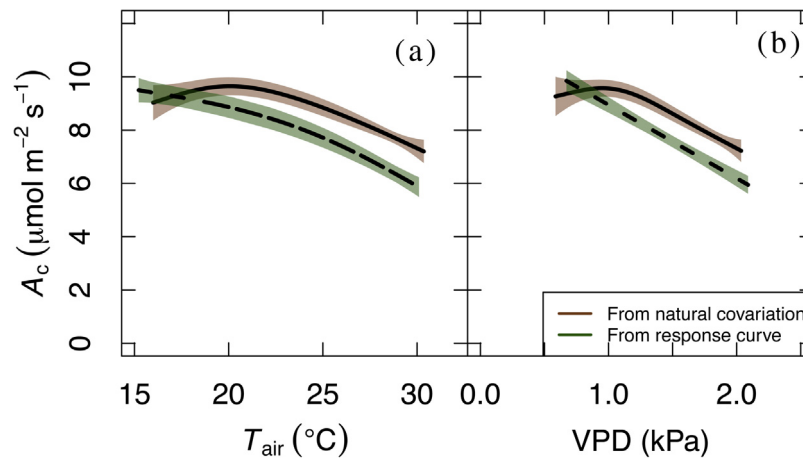
Fig. 2. The derivation of environmental flux dependencies is affected by circadian regulation in bean and cotton canopies. We modeled the relationship between A_c and T_{air} (a) and between A_c and VPD (b) using the natural co-variation that exists during a "normal" day but with constant radiation (data from Fig. 1a–b; brown line); and also during a response curve (data from Fig. 1c–d; green dashed line). Modeling was done with a single Generalized Additive Model across species (given lack of differences in Fig. 1). The shaded confidence interval indicates the 95% CI in the GAM prediction and, hence, when intervals do not overlap, significant differences on the dependence of A_c on T_{air} and VPD exist. Photosynthetic values at the starting and ending environment conditions (15 and 30 °C and 0.6 and 2.1 kPa) are at steady state (>30 min of acclimation), but values are transient elsewhere. The divergence at increasing temperatures is interpreted as the result of circadian regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

physiological responses alone, we conducted a ramped response function (Exp. 2 in Fig. 1), where T_{air} and VPD were raised (from 15° to 30 °C and from 0.6 to 2.1 kPa, respectively) quickly (2-h period) in order to prevent circadian regulation from exerting a significant impact. Alternatively, to test if the variation in fluxes between 0600 and 1400 h (Exp. 1) was regulated by circadian regulation, we maintained T_{air} and VPD constant (at ~17 °C and ~0.85 kPa, respectively) for 24 h (Exp. 3 in Fig. 1). Significant differences in the change in A_c across experiments were tested in paired t-tests.

To ensure the results from this experiment were robust, we conducted a similar test but allowed only T_{air} to vary (15/30 °C, 0600 h/1400 h) while maintaining constant VPD (at ~0.85 kPa; Exp 4 in Appendix A in Supplementary material). Again, to test if temporal changes in A_c were solely regulated by physiological responses to changes in T_{air}, we conducted a ramped response function (Exp. 5 in Appendix A in Supplementary material) under constant light and VPD.

A potential artefact in this experiment may be that stomata and photosynthesis do not respond immediately to external light stimuli, although they often take less than 30 min to exhibit full response (Mott and Woodrow, 1993). However, we minimized this potential impact by exposing leaves to the target PAR, T_{air} and VPD for an entire hour before beginning the experiments (dawn occurred at 0500 h). Our experiments will not be representative of sunny conditions, as PAR would have been ~4 times larger (~2000 µmol m^{-2} s^{-1} at full sunlight in Montpellier). We are unaware of any study indicating that the effect of circadian regulation diminishes as PAR increases, but we note that our tests are representative only of circadian regulation when photosynthesis is light-limited. Importantly, PAR of ~500 µmol m^{-2} s^{-1} does occur in the field, specially under cloudy conditions.

In the ramped tests (Exps. 2 and 5), T_{air} and VPD were linearly raised over 2 h, and maintained at each target level for 1 h. Only values collected after 30 min at each T_{air} and VPD target point during the response functions were used for further calculations. Two hours was chosen to balance the conflicting requirements of controlling circadian variation and avoiding possible damage to the photosynthetic apparatus due to a sudden increase in T_{air} (Sharkey, 2005)

2.4. Statistical analyses

We examined statistical significance of temporal patterns 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 and 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 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 and Simpson, 2014). Non-significant periods, reflecting lack of local statistically significant trending, are illustrated on the figures by the yellow line portions, and significant differences occur elsewhere.

3. Results

In our first experiment (Exp. 1 in Fig. 1a–b), we documented the "natural" variation in morning and early afternoon (0600 h/1400 h) patterns of net canopy CO_2 exchange (A_c) under controlled (i.e. same as during the five entrainment days) patterns in T_{air} and VPD (15 °C and 0.6 kPa at 0600 h/30 °C and 2.1 kPa at 1400 h) but under constant levels of light (500 µmol m^{-2} s^{-1}). During this time, A_c decreased from 9.85 µmol m^{-2} s^{-1} at 0800 h to 7.3 µmol m^{-2} s^{-1} at 1400 h across species (Fig. 1). For ease of explanation, we normalized A_c values through linear rescaling (with 0 as minimum), by dividing measured values over the maximum A_c measured in all experiments (11.3 µmol m^{-2} s^{-1}, Fig. 1). After normalization, the calculated relative decline in A_c associated with a change in T_{air} of 15 °C along with a change in VPD of 0.6 kPa was 22% ((9.85 − 7.3)/11.3) × 100; Appendix B in Supplementary material).

In our second experiment (Exp. 2), we tested whether the decline in A_c (observed in Exp. 1) matched that experienced during a ramped response function, where T_{air} and VPD were quickly raised over the course of two hours (from 15 to 30 °C and from 0.6 to 2.1 kPa, respectively) also under 500 μmol m^{-2} s^{-1} of PAR (Exp. 2 in Fig. 1c–d). However, this short-term response resulted in an A_c decline in Exp. 2 (37%, Fig. 1c–d, Appendix B in Supplementary material) that was significantly larger than the decline in Exp. 1 (22%; paired t-test, n = 6, P = 0.0008). This suggests that realized direct T_{air} and VPD effects on A_c were dampened (relative to short-term responses) throughout the course of the experimental period.

Consequently, we observed significant differences on the association of A_c with T_{air} and VPD (Fig. 2) using the natural diurnal co-variation (Exp. 1), or the short-term ramped response function (Exp. 2). We tested whether this difference could be driven by circadian regulation in our third experiment (Exp. 3). In Exp. 3 we maintained constant conditions of PAR, T_{air} and VPD (Exp. 3 in Fig. 1e–f). Under these constant conditions, we observed that A_c increased by 13% between 0600 h and 1000 h. In addition, A_c remained stable between 1000 h and 1400 h, and subsequently declined during the rest of the afternoon, rising again towards the end of the following subjective night despite being exposed to constant levels of light, T_{air} and VPD throughout the entire period.

In summary, in Exp. 1, there were 3 factors varying: time, T_{air} and VPD, which allowed for a "top-down" extrapolation of environmental dependencies based on the temporal co-variation of fluxes and their meteorological drivers (Hollinger et al., 1994). In Exp. 2, we varied only T_{air} and VPD during a short amount of time, to avoid circadian interference, and to quantify the portion of the response in Exp. 1 that depended solely on these meteorological drivers (emulating the "bottom-up" approach for extrapolation of environmental dependencies based on response curves). Combining Exp. 1 and Exp. 2 we could therefore test whether the same result is obtained by the natural co-variation and by the response function methods of inferring environmental flux dependencies. Additionally, in Exp. 3, only time varied (with T_{air} and VPD being constant), which allowed for examination of the role of circadian regulation to explain the divergence in the results between Exp. 1 and Exp. 2. The 22% A_c decline during the normal T_{air} and VPD conditions (Exp. 1) was larger than the 37% decline during the short-term T_{air} and VPD environmental response (Exp. 2). This shorter A_c decline in Exp. 1, relative to Exp. 2, suggests that temporal changes in A_c cannot be solely explained by direct physiological responses to the environment, but must also include the combined effect of clock-regulated responses. The additional experiments previously explained (Exps. 4, 5), where only T_{air} was altered for further replication, corroborated the need to account for a combined effect of clock regulation and direct environmental responses (Appendices A, B in Supplementary material).

4. Discussion

Circadian regulation is not confined to the molecular regulation of leaf-level function, but based on evidence shown it also regulates canopy-level gas exchange. When the environment was kept constant for 24 h, we observed that A_c significantly increased in the "subjective" morning (time when it would have been morning during entrainment), significantly decreased in the subjective afternoon, and then significantly increased again after the middle of the subjective night (Fig. 1e). There is a myriad of processes that could affect diurnal patterns of carbon fluxes, such as carbohydrate accumulation (Azcón-Bieto, 1983), hydraulic feedbacks (Jones, 1998) or changes in Rubisco activation state (Mott and Woodrow, 1993), to name but a few. However, hydraulic and car-

bohydrate feedbacks will generally tend towards monotonically decrease A and E over time, and Rubisco activation usually requires ∼30 min of illumination (and the experiment started after 1 h under target radiation). Instead, here we observed how diurnal variation under a constant environment showed self-sustained period of ∼24 h. The only mechanism currently known to create such a self-sustained 24 h cycle is the circadian clock (McClung, 2006; Müller et al., 2014) and can be explained from interactions between the central oscillator of the clock and photosynthesis (Haydon et al., 2013).

T_{air} and VPD, after irradiance, are often considered the major physical regulators of diurnal CO_2 flux patterns. Here, we observed that the 13% variation in A_c during the experimental period was regulated by circadian regulation (in constant environmental conditions) and that it was approximately one-third of the 37% change in A_c due to a combined change of T_{air} (of 15 °C) and VPD (of 1.5 kPa, Fig. 1), highlighting the importance of circadian regulation of canopy carbon assimilation.

The potential role of circadian regulation as a regulator of net canopy CO_2 flux dynamics should be considered further in assessments of canopy carbon assimilation. If endogenous circadian effects were negligible, we would have observed a similar dependence of photosynthesis on the physical drivers of T_{air} and VPD in experiments 1 (Fig. 1a–b) and 2 (Fig. 1c–d). That is, either using the natural diurnal co-variation between A_c and drivers, or the short-term ramped response function, we would have observed comparable associations between A_c and T_{air}, and between A_c and VPD. These two methodological approaches are commonly used and often considered interchangeable when characterizing gas exchange responses in relation to environmental drivers (Evans et al., 2010). However, we observed significant differences between these two approaches (Fig. 2), which are plausibly explained as arising from circadian regulation of A_c.

The observation that circadian regulation affects A_c, and that it shows a combined effect with direct physiological responses to the environment, suggests that incorporating circadian regulation might improve model fits of ecosystem gas exchange in field conditions. A previous study that incorporated a circadian oscillator into a biochemical model of leaf photosynthesis, documented small increases in model goodness-of-fit (Williams and Gorton, 1998). However, other studies have argued that failure to account for circadian regulation could lead models to provide the "right" answer for a partially "incomplete" reason (Resco de Dios et al., 2012). It is important to note that the circadian clock has a temporal pattern, which co-varies with the temporal cues of the environmental drivers. Therefore, any model that considers variation in these environmental drivers is indirectly incorporating circadian regulation. Adding a circadian oscillator to ecosystem level models may increase the degree of biological realism, but it is unclear whether it would also enhance model accuracy when tested against the conditions for which it has been calibrated (e.g. flux gap filling or other models of "the present"). Whether or not incorporation of circadian regulation into flux modeling will be important to predict flux dynamics under novel conditions beyond calibration, such as those expected under global change, is a different issue and will also need testing in future studies.

4.1. Future research directions

Flux dependencies and patterns have, to some degree, been successfully derived from either the temporal co-variation between fluxes and drivers or from response functions. We argue we should additionally design studies to fully understand the extent of "circadian interference" on carbon fluxes, by documenting the degree of generality of circadian regulation of leaf-level gas exchange. We know circadian rhythmicity is nearly universal in plants, but the

relative impact of circadian regulation compared to environmental conditions is generally unknown (Resco et al., 2009). Additional studies at canopy or ecosystem level, under natural conditions, could address endogenous regulation when down-scaling fluxes by analyzing environmental dependencies separately for different time intervals. Furthermore, we propose that up-scaling ramped response curves will require additional measurements to test the time period that data can be extrapolated beyond the range of collection.

Few studies have attempted to assess circadian regulation of gas exchange in canopies because it is difficult to experimentally control and manipulate irradiance (which is needed for direct tests of circadian regulation) at levels commonly observed in the field. In our study, a maximum PAR level of $500 \, \mu mol \, m^{-2} \, s^{-1}$ may represent a cloudy day, but not full sun conditions which are four times higher. Subsequently, the non-saturating PAR levels might have influenced our results because photosynthesis shows smaller temperature sensitivity under limiting PAR compared to photosynthetically saturating PAR. Moreover, because photosynthesis is part of the circadian oscillator, limiting radiation could have influenced the circadian oscillation through altering sugar concentrations (Haydon et al., 2013), although that was unlikely to be the case here, as some species showed saturating carbohydrate concentrations (García-Plazaola et al., 2017). Further studies could address the role of circadian regulation under saturating radiation.

While the role of circadian rhythms in animal ecology has been recognized for long (Houadria et al., 2016), we advocate for a deeper consideration of circadian rhythms to understand plant ecology and ecosystem processes. Plants show adaptations to their environment and the photoperiod, which varies deterministically as a function of geographical location and time of year, shows much higher predictability than drought, fire occurrence, or other environmental drivers. For instance, circadian-driven increases in nocturnal stomatal conductance are often observed between midnight and dawn (Resco de Dios et al., 2013), and in rubisco activation which often starts also at predawn (Harmer et al., 2000) (explaining the increase in A_c after the subjective night in Exp. 3). The circadian clock is thus an adaptation to predict photoperiodic changes (Troein et al., 2009), amongst others, and we should thus expect it to exert important controls over plant physiology and ecology.

Acknowledgements

Appendix A. Supplementary data

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