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Endogenous circadian rhythms in pigment composition induce changes in photochemical efficiency in plant canopies

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Abstract:	There is increasing evidence that the circadian clock is a significant driver of photosynthesis that becomes apparent when environmental cues are experimentally held constant. We studied whether the composition of photosynthetic pigments is under circadian regulation, and whether pigment oscillations lead to rhythmic changes in photochemical efficiency. To address these questions, canopies of bean and cotton were maintained, after an entrainment phase, under constant (light or darkness) conditions for 30-48h. Photosynthesis and quantum yield peaked at subjective noon and non-photochemical quenching peaked at night. These oscillations were not associated to parallel changes in carbohydrate content or xanthophyll cycle activity. We observed robust oscillations of Chla/b during constant light in both species, and also under constant darkness in bean, peaking when it would have been night during the entrainment (subjective nights).

	These oscillations could be attributed to the synthesis and/or degradation of trimeric light-harvesting complex II (reflected by the rhythmic changes in Chla/b), with the antenna size minimal at night and maximal around subjective noon. Considering together the oscillations of pigments and photochemistry, the observed pattern of changes is counterintuitive if we assume that the plant strategy is to avoid photo-damage, but consistent with a strategy where non-stressed plants maximize photosynthesis.

Endogenous circadian rhythms in pigment composition induce changes in photochemical efficiency in plant canopies

Running title: Circadian rhythms and pigments

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ABSTRACT

There is increasing evidence that the circadian clock is a significant driver of photosynthesis that becomes apparent when environmental cues are experimentally held constant. We studied whether the composition of photosynthetic pigments is under circadian regulation, and whether pigment oscillations lead to rhythmic changes in photochemical efficiency. To address these questions, canopies of bean and cotton were maintained, after an entrainment phase, under constant (light or darkness) conditions for 30-48h. Photosynthesis and quantum yield peaked at subjective noon and non-photochemical quenching peaked at night. These oscillations were not associated to parallel changes in carbohydrate content or xanthophyll cycle activity. We observed robust oscillations of Chl*a/b* during constant light in both species, and also under constant darkness in bean, ~~with peaks~~peaking when it would have been night during the entrainment (~~during~~ subjective nights). These oscillations could be attributed to the synthesis and/or degradation of trimeric light-harvesting complex II (reflected by the rhythmic changes in Chl*a/b*), with the antenna size minimal at night and maximal around subjective noon. Considering together the oscillations of pigments and photochemistry, the observed pattern of changes is counterintuitive if we assume that the plant strategy is to avoid photo-damage, but consistent with a strategy where non-stressed plants maximize photosynthesis.

INTRODUCTION

Because of Earth's rotation, light and temperature oscillate over the course of a day in a very predictable manner. As a consequence of such rhythmic oscillations, optimal and unfavourable time intervals for physiological activities can be anticipated. To be able to take advantage of these predictable oscillations, living organisms have developed a mechanism, the circadian clock, which coordinates physiological processes with environmental conditions. Circadian clocks are ubiquitous in nature and present in almost all groups of organisms examined to date (cyanobacteria, fungi, algae, plants, insects or vertebrates) (Bell-Pedersen *et al*. 2005).

In plants, the circadian clock originates from a feedback system of coordinated gene expression. In a process known as entrainment (McClung 2006; 2013), external cues such as photoperiod set the circadian clock aiming to synchronize plant performance with environmental fluctuations (Hotta *et al*. 2007). These circadian oscillations are masked by the alternating light/dark cycles, being usually revealed when plants are deprived of external cues and maintained under constant environmental conditions for protracted periods of time (>24h). Therefore, circadian regulation is more apparent, but not more important in constant conditions. Components of the clock include transcription factors that regulate the expression of other genes involved in the clock output, particularly those that regulate physiological processes and developmental events (Hanano *et al*. 2008). As a result of such coordinated regulation of gene expression, it has been estimated that at least one-third of the *Arabidopsis* transcriptome shows circadian resonance (Covington *et al*. 2008), including as much as 70% of chloroplast-encoded genes (Noordally *et al*. 2013). Through the control of gene expression, circadian clocks regulate the abundance and activity of proteins involved in physiological processes and, consequently, of metabolite pools. However, the mechanistic linkage between transcription and the final physiological output is still not well understood.

Photosynthetic responses are typically clock-controlled and, under constant environmental conditions, circadian rhythms are among the main drivers of photosynthesis (Dodd *et al*. 2014). These regulatory effects on photosynthesis are achieved through the hierarchical oscillation of the clock on each type of cell (Endo 2016) modulating the structure and dynamics of the photosynthetic apparatus (Dodd *et al*. 2014; Harmer *et al.* 2000). Furthermore, some evidence also points to an important

regulatory role under oscillating "natural" conditions, at plant and even at ecosystem levels (Doughty *et al.* 2006; Resco de Dios *et al.* 2012, Resco de Dios *et al.* 2016b). In fact, the expression of genes involved in biosynthesis of carotenoids (Covington *et al.* 2008; Pan *et al.* 2009), chlorophylls (Harmer 2009; Khan *et al.* 2010) and pigment binding proteins (Schmid 2008) have been documented to oscillate synchronically during day/night cycles. Carbohydrate levels also affect the expression of circadian-regulated genes, controlling and being controlled by photosynthetic rate (Haydon *et al.* 2013).

The internal maintenance of rhythms in photosynthesis provides an adaptive advantage, such as the capacity to anticipate predictable environmental change (Yerushalmi & Green 2009; Hotta *et al.* 2007; Salmela *et al.* 2016). Diurnal rhythmicity in photosynthesis could potentially lead to two contrasting strategies: i) a conservative strategy of maximizing photoprotection at peak light intensities, at the expense of potentially losing efficiency or, ii) a more risky strategy of maximizing light harvesting, at the expense of potentially suffering photo-damage. It has been shown that *Arabidopsis* plants with internal clocks in resonance with day-night cycles are able to fix more carbon, grow faster and survive better than mutants with impaired rhythmicity (Dodd *et al.* 2005). Studies with non-model species are scarcer in the literature, but most support an adaptive role for the circadian rhythms of photosynthesis; e.g. assimilation rates and biomass accumulation correlate positively with the length of circadian periods in *Brassica rapa* (Yarkhunova *et al.* 2016), above-ground biomass is higher with clock periods close to 24h in *Boechera stricta* (Salmela *et al.* 2016), and genotypic variation in the capacity to anticipate sunrise correlates with photosynthesis and growth in *Eucalyptus camaldulensis* (Resco de Dios *et al.* 2016a).

Overall, from molecular to organelle scale, the control over photosynthetic processes by circadian clocks is well documented. There are also hints from indirect approaches (statistical filtering, e.g. Resco de Dios *et al.* 2012; 2016b) that the circadian control of photosynthesis scales up to ecosystem-level fluxes. However, the assessment of the processes driving circadian regulation of photosynthesis has been typically performed at molecular scales by studying rhythmic regulation in the transcriptome and metabolome (Dodd *et al.* 2014). This contrasts with the "classical" approach in ecophysiology, where C assimilation is considered to be determined either by diffusional (resistance to CO_2 diffusion from the stomata to the site of carboxylation) or biochemical limitations

(Farquhar & Sharkey 1982; Flexas *et al.* 2012). While the literature is rich in molecular assessments of circadian regulation of photosynthesis, integrative studies on circadian control of photosynthesis at ecophysiological scales are, to the best of our knowledge, non-existent (*cf* review by Dodd *et al.* 2014).

Furthermore, whether the contribution of these rhythms to plant fitness differs across different species has been rarely tested. For instance, if contrasting patterns of daily rhythms in photochemical activity exist among different life forms, that could indicate the existence of trade-offs that modify the physiological output so as to adapt the photosynthetic performance to different life strategies. Therefore, in the present work we aim at, first, characterising the photosynthetic output of circadian rhythms at scales relevant for ecophysiology, that is whether photosynthesis is regulated by diffusional or biochemical constraints; second, whether such photosynthetic output differs among species with different life-history strategies; and third, whether the photosynthetic output also involves circadian changes in photosynthetic pigment composition. To accomplish our goals and with the aim to understand the clock function beyond the *Arabidopsis* model, we have characterised photosynthetic responses under constant environmental conditions in two species of high agronomic value belonging to contrasting life forms: bean (*Phaseolus vulgaris*), an annual herb, and cotton (*Gossypium hirsutum*), a perennial shrub.

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METHODS

Experimental design

The experiment was performed at the Macrocosms platform of the Montpellier European Ecotron, Centre National de la Recherche Scientifique (CNRS, France, www.ecotron.cnrs.fr). We used 6 controlled environment units/macrocosms (3 planted with bean and 3 with cotton) where the main abiotic drivers (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. The intact soil monoliths were extracted 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).

Bean and cotton were planted in 5 rows within each macrocosm on 10th July 2013, one month before the start of the measurements, and thinned to densities of 9 to 11 individuals m^{-2}. 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 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 (Munger *et al.* 1998; codes 51-59 in BBCH scale, the standard phenological scale within the crop industry; Feller *et al.* 1995).

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 (sheet of Fluorinated Ethylene Propylene). The soil was regularly watered to 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 (Resco de Dios *et al*. 2015).

During experimental periods, the natural light was blocked by placing a completely opaque fitted cover (PVC coated polyester sheet Ferrari 502, assembled by IASO, Lleida, Spain) on each dome, which allowed full control of the light regime using a set of 5 dimmable plasma lamps (GAN 300 LEP with the Luxim STA 41.02 bulb, delivering a sun-like light spectrum~~, Fig. S1~~) (Resco de Dios *et al*. 2016b). The lamps were hung 30 cm above the plant canopy and provided a PAR of 500 µmol m^{-2} s^{-1} at the top of the canopy, when not dimmed. We measured PAR at canopy level with a quantum sensor (Li-190, LI-COR Biosciences, Lincoln, NE, USA) in each macrocosm. The plants adapted to the new conditions during a entrainment period of five days, in which photoperiod was set to 12 h of darkness and 12 h of light, with gradual changes in light intensity. After the entrainment period, in the night-time experiments we maintained PAR, air temperature (T_{air}) and vapour pressure deficit (VPD) constant at midnight values for 30 hours starting at solar midnight. In the daytime experiments, we maintained PAR, T_{air} and VPD constant at noon values for 48 hours starting at solar noon.

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Leaf gas exchange and chlorophyll florescence

We measured leaf net assimilation rate (A_{net}), stomatal conductance (g_s), maximum assimilation rate (A_{max}) and chlorophyll fluorescence using portable photosynthesis systems (LI-6400-40XT, Li-Cor, Lincoln, Nebraska, USA), after setting the leaf cuvette to the same environmental conditions as in the macrocosms, except for A_{max} which was measured at saturating PAR (2,000 µmol m^{-2} s^{-1}) and CO_2 (2,000 ppm). We conducted measurements every 4 h in three leaves situated in the upper light-exposed part of the canopy 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 (intercept = -4.3 ± 4.5 [mean ± 95%CI]; slope = 1.15 ± 0.17; R^2 = 0.89). Chlorophyll fluorescence measurements were made immediately after gas exchange

measurements. During daytime, steady-state fluorescence (Fs) was measured, followed by a saturating pulse of *ca*. 8000 µmol m^{-2} s^{-1} to determine maximum fluorescence in the light (Fm'). Derived values of effective quantum yield (Φ_{PSII}) were estimated as (Fm'-Fs)/Fm' (Genty *et al.* 1989). During nightime, dark-acclimated minimal fluorescence (Fo) was measured, followed by a saturating flash to determine the maximum fluorescence in the dark (Fm). Fm determination allowed the calculation of non-photochemical quenching (NPQ) as (Fm-Fm')/Fm'.

Pigment determination

Following each set of gas exchange measurements, we collected leaves from two plants (one per individual) per measuring round in each macrocosm, which were immediately frozen in liquid nitrogen and stored at -80°C until biochemical analysis. Frozen samples were homogenised with a mortar in pure acetone solution buffered with $CaCO_3$. The extracts were centrifuged at 16100g for 20 min, and supernatants were filtered with 0.2 µm PTFE filters (Teknokroma, Spain). Chlorophylls (Chl) and carotenoids (Car) separation were performed by HPLC with a reverse phase C18 column (Spherisorb ODS1, 4.6 × 250 mm, Waters, Milford, MA, USA) with a photodiode array (PDA) detector, following the method by García-Plazaola & Becerril (1999, 2001). The total VAZ pool was calculated as the sum of violaxanthin, antheraxantin and zeaxantin. The de-epoxidation index (AZ/VAZ) was calculated as the sum of antheraxantin and zeaxantin) divided by VAZ.

Non-structural carbohydrates

The same leaf samples that were used for pigment analyses were also used for the determination of non-structural carbohydrates (NSC), defined here as the sum of starch and the three most abundant low molecular weight sugars: sucrose, glucose and fructose. NSC were analysed photometrically after enzymatic conversions of the target carbohydrates following a modified version of the protocol described in Hoch *et al.* (2002). The dried leaves were ground to fine powder on a ball mill (MM 400, Retsch, Germany) and stored well-sealed over silica gel until analyses. Approximately 10 mg of plant powder was extracted with 2 ml distilled water in glass vials over steam for 30 min. An aliquot of the extract was used for the determination of low molecular

carbohydrates after enzymatic conversion of fructose and sucrose to glucose (using phosphoglucose isomerase and invertase form bakers yeast). The concentration of total free glucose was then determined on a 96-well multiplate photometer (Multiscan EX, Thermo Scientific, Waltham, MA, USA) after enzymatic conversion of glucose to gluconat-6-phosphate using a glucose hexokinase (GHK) assay reagent (G3292). Following the degradation of starch to glucose with amyloglucosidase from *Aspergillus niger* at 49 °C overnight, NSC was determined in a separate analysis. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). The concentration of starch was calculated as NSC minus the free low molecular carbohydrates. Tissue concentrations were given on % dry matter basis.

Statistical analyses

We examined temporal patterns of 14 variables: (A_{net}, g_s, A_{net}/C_i, A_{max}, Fm, Fm', ΦPSII, NPQ, Chla/b, Car/Chl, VAZ/Chl, AZ/VAZ, NSC, starch) with Generalized Additive Model (GAM) fitted with automated smoothness selection (10-15 nodes, Wood, 2006) in the R software environment (*mgcv* library in R 3.1.2, The R Foundation for Statistical Computing, Vienna, Austria). We used best-fit line from model predictions to estimate the extent of the diurnal oscillation (maximum minus minimum) during entrainment and during free-running (constant condition) phases.

RESULTS

Circadian oscillations of net assimilation rate (A_{net}) were observed when plants were shifted to continuous light conditions after the 5-day entrainment period (Fig. 1a). During entrainment, A_{net} in *P. vulgaris* ranged from -3.6 µmol m^{-2} s^{-1} during the night up to a maximum of 19.9 µmol m^{-2} s^{-1} at noon (as estimated from the GAM best fit line). During the constant conditions phase, A_{net} oscillated from 7.7 to 15.8 µmol m^{-2} s^{-1}. Therefore the oscillation observed during constant conditions (15.8-7.7=8.1 µmol m^{-2} s^{-1}) was 34% of that observed during entrainment (period -24 to 0h in Figure 1) (8.1/24.3×100). Similarly, for cotton we observed that the oscillation in A_{net} was 37% of that recorded during entrainment. Circadian oscillations of stomatal conductance (g_s) were also observed, representing 72% and 63% of those in the entrainment phase in bean and cotton, respectively (Fig. 1b). Under constant light conditions, both parameters (A_{net} and g_s) peaked around subjective noon and declined during subjective nights (when it would have been noon or night, respectively, during the entrainment phase). In both species, the frequency of the oscillation was similar and close to 24 h, but the relative magnitude of the oscillation in A_{net} was 2- to 4-fold smaller than in g_s. Oscillations in the A_{net} to intercellular CO_2 concentration (~~/~~C_i) (A_{net}/C_i) (Fig. 1c) were weaker in bean (18%), but maintained closer to the entrainment phase in cotton (69%) and attenuated in both species after the first 24 h in constant conditions. Contrasting with these parameters, maximum assimilation rate (A_{max}) did not show any consistent rhythmic oscillation (Fig. 1d).

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As observed in A_{net}/C_i, effective quantum yield (Φ_{PSII}) oscillated rhythmically during the first 24 h of continuous light in both species (Fig. 2a). The rhythm showed a tendency to weaken during the second 24 h cycle. In the case of bean, this oscillation was particularly remarkable, attaining an amplitude of 0.064, which was half of that measured in the entrainment phase (0.140). In both species, non-photochemical quenching (NPQ) can also be described by an oscillatory behaviour (Fig. ~~2b~~2c). The amplitude of the oscillation was maintained during the whole illumination period in bean, but it dampened towards the end of the 48-h period of constant illumination in cotton. Oscillations in NPQ were oppositely phased with those in Φ_{PSII}, peaking during subjective nights. As measurements of NPQ require illumination, it was not possible to compare the 24-h amplitude of this rhythm with that during the entrainment phase (which contains dark periods).

Oscillations in Φ_{PSII} and NPQ in plants maintained under continuous illumination were due to rhythmic changes in the maximal fluorescence of illuminated leaves (Fm'), which peaked during subjective days (Fig. ~~3a~~2b). During the first 24 h the amplitude of these oscillations was 3-fold higher in bean compared to cotton. Interestingly, the maximal fluorescence of dark-adapted leaves (Fm) also oscillated in bean, but not in cotton, when plants were maintained in continuous darkness and constant environmental conditions (Fig. ~~3b~~2d).

In bean plants entrained to day/night cycles, there was a robust oscillation of the chlorophyll *a* to chlorophyll *b* ratio (Chl *a/b*) that was maintained when plants were transferred to continuous light (Fig. ~~4a~~3a). Under constant conditions this ratio peaked during subjective nights, with the amplitude of the oscillation being even higher than during the entrainment phase (115% and 182% higher in bean and cotton, respectively). Furthermore, under continuous darkness (Fig. ~~4b~~3b), the same oscillation of Chl *a/b* was maintained, with identical magnitude (0.41 vs 0.46 in continuous light or darkness, respectively) and peak time. In cotton, the oscillation was attenuated compared to bean, and disappeared during the second 24 h cycle. The total pool of xanthophyll cycle pigments expressed on a chlorophyll basis (VAZ/Chl) oscillated rhythmically, but was differently phased in bean and cotton (Fig. ~~5a~~4a). In bean, VAZ/Chl peaked around subjective noon, while in cotton it peaked around subjective dusk~~, and the oscillation period was progressively shortened~~. The same oscillatory trends were observed in the carotenoid to chlorophyll ratio (Car/Chl) (Fig. ~~5c~~54b). In contrast, the de-epoxidation index (AZ/VAZ) varied greatly among plants and sampling times, but did not show any consistent oscillatory pattern (Fig ~~5b~~54c); however, an increasing trend during continuous illumination was observed.

During the entrainment phase, total non-structural carbohydrates (NSC) accumulated during the day and were consumed during the night in both species (Fig. ~~6~~5). However, when transferred to continuous light, they followed a distinct pattern in bean and cotton. In bean, NSC and starch accumulated very fast during the first subjective day, and after the start of the first subjective night the accumulation rate slowed, but continued at a constant rate until the end of the experiment. In the case of cotton it described a rhythmic oscillation, accumulating during the subjective day, with a peak around dusk, and a subsequent decrease during the course of the subjective night.

As a consequence of the parallel oscillation under constant light of A_{net} and g_s, a tight correlation between them, with different slope for bean and cotton, was observed (Fig. ~~7a~~6a). However, when both species were considered together (red+blue dots in Figure ~~7a~~6a), the linearity of the relationship disappeared at g_s values higher than 0.2 mol m^{-2} s^{-1}. These observations may indicate that g_s was the leading process controlling A_{net} oscillations by changing C_i (Fig. ~~7b~~6b). However, contrasting with g_s, the correlation between C_i and A_{net} was only significant in bean. The observed oscillations in A_{net} also correlated linearly with changes in Φ_{PSII} in both species (Fig. ~~7e~~6c). Finally, A_{net} oscillations were negatively related to changes in Chl *a/b* in bean (Fig. ~~7d~~6d).

DISCUSSION

Carbon assimilation and stomatal opening are known to be clock-controlled processes in plants (Pallas *et al*. 1974; Hennessey *et al*. 1993). Accordingly, we observed parallel oscillations of A_{net} and g_s in bean and cotton (Fig. 1). Initially, it would appear that stomatal conductance is the process that leads the oscillation through the regulation of the availability of CO_2 for carboxylation. However, as described in *Arabidopsis* and other plants (Dodd *et al*. 2004; Wyka *et al*. 2005), both processes were not functionally related since A_{net} was not related to C_i (Fig. ~~6b~~7b), at least in ~~bean~~cotton. The effect of g_s on A_{net} is generally indirect through regulation of CO_2 supply. Hence, if C_i and A_{net} are not correlated, we can discard g_s as a significant driver of A_{net} variation under continuous light. Alternatively, circadian rhythms in A_{net} could also be driven by oscillations in mesophyll conductance, Rubisco activity, light harvesting efficiency, feedback interactions of assimilates with photosynthesis or electron transport. The first two explanations seem unlikely considering that neither A_{net}/C_i nor A_{max} showed a rhythmic pattern sustained more than 24h (Fig. 1).

~~Recently, rhythmic changes in photochemical quenching have been characterised in *Arabidopsis* and identified as controlled by a phototropin-related mechanism (Litthauer *et al*. 2015).~~ In the present study, fluorescence parameters indicative of photochemical use of energy (Φ_{PSII} ~~ΔF/Fm'~~ and NPQ) also oscillated rhythmically in bean and cotton (Fig. 2). However, both parameters showed an opposite behaviour: Φ_{PSII}~~ΔF/Fm'~~, which describes the yield of photon capture, peaked during subjective days, while NPQ, which is a proxy of the rate of energy dissipation, peaked during subjective nights. The

346 opposite behaviour of both parameters is expected, as both parameters are affected by
347 Fm' but in opposite directions. However, oscillations in Fm' (Fig. ~~3a~~2b) may be
348 generated by processes other than photochemistry, such as antenna size adjustments or
349 chloroplast movements (Cazzaniga *et al*. 2013). Surprisingly, under continuous
350 darkness, Fm also oscillated, at least in bean, and this oscillation cannot be explained by
351 any light-triggered phenomenon, such as chloroplast relocation. Thus, changes in
352 antenna size and/or photochemical efficiency are likely the factors involved in such
353 oscillation.

354 Functionally, these trends imply that plants maximize efficiency during the day and
355 dissipation at night. A study of delayed fluorescence revealed that nucleus-controlled
356 rhythms in PSII photochemistry are present in most plant species (Gould *et al*. 2009).
357 However, this rhythmic pattern is not universal; e.g. oscillations in photochemistry
358 follow an opposite pattern in the CAM plant *Kalanchoe daigremontiana*, peaking at
359 night, as a CAM plant is expected to follow the opposite patter to C3/C4 photosynthesis
360 (Wyka *et al*. 2005). These different rhythms indicate coordination between the
361 physiological output of the clock and the requirements of different photosynthetic
362 pathways and life strategies, but in-depth knowledge regarding this oscillation remains
363 limited.

364 The amplitude of NPQ is regulated by three factors (García-Plazaola *et al*. 2012): the
365 generation of a proton gradient across the thylakoid membrane, the presence of the
366 protein PsbS, and the formation of zeaxanthin (Z) through the xanthophyll cycle.
367 Among them, a differential xanthophyll cycle activity could justify these oscillations,
368 mainly considering that the expression of the two enzymes that participate in the cycle,
369 VDE (violaxanthin de-epoxidase) (Zhao *et al*. 2012; Covington *et al*. 2008) and ZE
370 (zeaxanthin epoxidase) (Audran *et al*. 1998), is also clock-controlled. However, we did
371 not find any evidence pointing to a circadian regulation of xanthophyll cycle activity
372 (Fig. ~~5c~~4c). Similarly, in an experiment with coral endosymbiotic algae, Sorek *et al*.
373 (2013) failed to detect circadian rhythm in the diadinoxanthin cycle, while Fv/Fm
374 maintained the oscillation, suggesting the involvement of factors independent of
375 xanthophyll cycle activity. Alternatively, changes in NPQ could be the consequence,
376 rather than the cause, of the oscillating pattern of carbon assimilation. Decreased energy
377 usage for photosynthesis during subjective nights implies greater proton gradient and,

consequently, higher NPQ. If this is the case, then another factor must trigger the oscillations in assimilation rate.

A straightforward explanation might be that the clock is under feedback control by the products of photosynthesis (sucrose and starch), controlling and being controlled by carbon assimilation (Müller *et al*. 2014). This could be the case in cotton, in that the starch content oscillated (Fig ~~6~~5), peaking at subjective dusk and later metabolized during subjective night. Equally, in *Arabidopsis* starch and sugar are metabolized at night in a process that is under circadian control (Graf *et al*. 2010). As observed in cotton, metabolization proceeds almost linearly, ensuring that starch availability is maintained until the following dawn (Gibon *et al*. 2004). In bean, there was also a complete consumption of starch during the night, but contrasting with cotton, the rhythm was not observed in the free-running phase under constant light. In fact, as described by Hennessey *et al*. (1993) for this species, non-structural carbohydrates accumulated steadily under continuous illumination, but apparently without inducing feedback inhibition of photosynthesis.

Alternatively, rhythms in photosynthesis could be explained by the synthesis/degradation of chlorophyll and its binding proteins, a process that has been observed in young leaves of wheat where LHCII content peaks at noon coinciding with the minimum Chl*a/b* ratio (Busheva *et al*. 1991). As occurred with photochemical responses, Chl*a/b* ratio oscillated in bean, and less markedly in cotton. Furthermore, the oscillations in bean occurred both under continuous illumination and under continuous darkness (Fig ~~4b~~3b). Chl*a/b* is the resultant of two factors: on one hand, Chl*a* is present in antennae and reaction centers of both PSI and PSII, while Chl*b* is exclusively bound to antenna proteins of both photosystems (Hogewoning *et al*. 2012). On the other hand, PSII is comparatively enriched in Chl*b*, with most of it bound to major light-harvesting complexes (LHCs). These complexes form trimers that are bound in variable ways to dimeric PSII core complexes (C2) forming the C2S2, the C2S2M, the C2S2M2 or the C2S2M2L2 super-complexes, with respectively 2, 3, 4 or 6 trimers (Derks *et al*. 2015). As a consequence of the different amount of LHC trimers bound to PSII, Chl*a/b* reflects the PSII/PSI stoichiometry, but also the relative antenna size (Evans 1988). In shaded leaves of higher plants, which optimize light harvesting at the expense of energy conversion and photoprotection, high LHC relative to PSII and low PSII/PSI ratio are mirrored in Chl*a/b* ratios in the range of 2 to 2.8. Conversely, in high light acclimated

leaves, Chl*a/b* values are higher (2.8 to 4) (Hogewoning *et al*. 2012; Esteban *et al.* 2015). Thus, the reported oscillations of Chl*a/b*, whose adjustments ranged between 3.3 and 3.6 for bean and between 3.4 and 3.7 for cotton, are likely due to the synthesis and/or degradation of trimeric LHCIIs~~, that bind most of Chl*b*~~. Using the model proposed by Esteban *et al*. (2015) this range of oscillation, assuming a PSII/PSI ratio of 2 (Antal *et al*. 2013), would represent a net daily variation of around 0.8 LHCII trimers per PSII dimer. Considering that Chl-binding proteins represent about 20% of leaf N (Hötensteiner 2006), this turnover rate represents a tremendous metabolic effort in terms of energy and N use.

It is considered that the abundance and binding properties of LHCII trimers regulate the acclimation capacity to long-term changes in light environment (Kouril *et al*. 2013; Ware *et al*. 2015), while the stoichiometry of minor antenna complexes and reaction centers is usually maintained stable (Ballottari *et al*. 2007). Interestingly, Chl*a/b* peaked during subjective nights, implying that the capacity for light harvesting (larger antenna) is minimal at night, being maximal around subjective noon. This interpretation is consistent with the described midday peaks in the circadian patterns of expression of genes involved in Chl biosynthesis (Fukushima *et al*. 2009; Harmer *et al*. 2000; Khan *et al*. 2010), carotenoid biosynthesis (Facella *et al*. 2008; Ragni and Ribera d'Alcalà 2007; Pan *et al.* 2009), Chl-binding proteins (in particular LHCII, whose expression is maximum at noon) (Hotta *et al.* 2007; Schmid *et al.* 2008) and carbon assimilation (Harmer *et al.* 2000). This pattern of change seems counterintuitive if we assume that the plant strategy is to avoid photo-damage, but is fully consistent with a model in which non-stressed plants maximize photosynthesis. In fact, considering that the risk of photo-oxidative damage is higher around sunrise due to the combination of high light and sub-optimal temperatures, the enhancement of photo-protective response at night could be considered as a pre-emptive response. This is also supported by the fact that before sunrise there is an enhancement in the expression of photo-protective genes such as those of flavonoids (Harmer *et al*. 2000), tocopherols and carotenoids (Covington *et al*. 2008) and cold protection (Yakir *et al*. 2007). ~~However, considering that light excess is concomitant with reactive oxygen species (ROS) generation, it is surprising that ROS responsive genes are not clock regulated (Sanchez *et al*. 2011).~~ All these processes could, at least partially, contribute to explain the marked oscillations of carbon assimilation (Fig. 1) that cannot be solely ascribed to changes in stomatal

conductance, being in agreement with the proposal that links circadian oscillations in chlorophyll content with carbon assimilation (Müller *et al*. 2014).

As indicated before, the structure of photosynthetic apparatus determines the Chl*a/b* ratio, and this parameter has shown a robust oscillatory behaviour. This fact, together with the feasibility of Chl*a/b* determination, makes this parameter an excellent reporter of the photosynthetic output of circadian oscillations. Furthermore, as Chl*a/b* can be easily estimated with reflectance indexes (Siebke & Ball 2009), it can be used as a non-invasive reporter of rhythmicity in phenotyping or remote sensing platforms using hyperspectral images (Pan *et al*. 2015), complementing other circadian reporters currently available such as delayed chlorophyll fluorescence and transgenic luciferase (Tindall *et al*. 2016).

Overall, the present results suggest that there is no single, universal response to the dilemma between maximizing light harvesting and avoiding photo-damage. We have studied two species and have found two types of clock-responses in photosynthetic pigments. Thus, in bean there was a higher circadian regulation of photochemical processes and pigment composition, while in cotton carbohydrate metabolism was apparently clock regulated. As a consequence, extrapolation of the responses from *Arabidopsis* and other model plants to other species is not always appropriate (Müller *et al*. 2014), making necessary the use of additional reporters of circadian rhythms.

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484 REFERENCES

485 Antal T.K., Kovalenko I.B., Rubin A.B. & Yyystjärvi E. (2013) Photosynthesis-related
486 quantitites for education and modeling. *Photosynthesis Research* 117, 1-30.

487 Audran C., Borel C., Frey A., Sotta B., Meyer C., Simonneau T. & Marion-Poll A.
488 (1998) Expression studies of the zeaxanthin epoxidase gene in *Nicotiana*
489 *plumbaginifolia*. *Plant Physiology* 118, 1021-1028.

490 Ballottari M., Dall'Osto L., Morosinoto T. & Bassi R. (2007) Contrasting behavior of
491 higher plant photosystem I and II antenna systems during acclimation. *The Journal*
492 *of Biological Chemistry* 282, 8947-8958.

493 Bell-Pedersen D., Cassone V.M., Earnest D.J., Golden S.S., Hardin P.E., Thomas T.L.
494 & Zoran M.J. (2005) Circadian rhythms from multiple oscillators: lessons from
495 diverse organisms. *Nature Reviews Genetics* 6, 544-556.

496 Busheva M., Garab G., Liker E., Toth Z., Szell M. & Nagy F. (1991) Diurnal
497 fluctuations in the content and functional properties of the light harvesting
498 chlorophyll a/b complex in thylakoid membranes. *Plant Physiology* 95, 997-1003.

499 Cazzaniga S., Dall'Osto L., Kong S.G., Wada M. & Bassi R. (2013) Interaction
500 between avoidance of photon absorption, excess energy dissipation and zeaxanthin
501 synthesis against photooxidative stress in Arabidopsis. *Plant Journal* 76, 568-579.

502 Covington M.F., Maloof J.N., Straume M., Kay S.A. & Harmer S.L. (2008) Global
503 transcriptome analysis reveals circadian regulation of key pathways in plant growth
504 and development. *Genome Biology* 9, R130.

505 Derks A., Schaven K. & Bruce D. (2015) Diverse mechanisms for photoprotection in
506 photosynthesis. Dynamic regulation of photosystem II excitation in response to rapid
507 environmental change. *Biochimica et Biophysica Acta* 1847, 468-485.

508 Dodd A.N., Parkinson K. & Webb A.A.R. (2004) Independent circadian regulation of
509 assimilation and stomatal conductance in the *ztl-1* mutant of *Arabidopsis*. *New*
510 *Phytologist* 162, 63-70.

511 Dodd A.N., Salathia N., Hall A., Kévei E., Tóth R., Nagy F., ..., Webb A.A.R. (2005)
512 Plant circadian clocks increase photosynthesis, growth, survival, and competitive
513 advantage. *Science* 309, 630-633.

514 Dodd A.N., Kusakina J., Hall A., Gould P.D. & Hanaoka M. (2014) The circadian
515 regulation of photosynthesis. *Photosynthesis Research* 119, 181-190.

516 Doughty C., Goulden M.L., Miller S. & da Rocha H. (2006) Circadian rhythms
517 constrain leaf and canopy gas exchange in an Amazonian forest. *Geophysical*
518 *Research Letters* 33, L15404.

519 Endo M. (2016) Tissue-specific circadian clocks in plants. *Current Opinion in Plant*
520 *Biology* 29, 44-49.

521 Esteban R., Barrutia O., Artetxe U., Fernández-Marín B., Hernández A. & García-
522 Plazaola J.I. (2015). Internal and external factors affecting photosynthetic pigment
523 composition in plants: a meta-analytic approach. *New Phytologist* 206, 268-280.

524 Evans J.R. (1988) Acclimation by the thylakoid membranes to growth irradiance and
525 the partitioning of nitrogen between soluble and thylakoid proteins. *Australian*
526 *Journal of Plant Physiology* 15, 93-106.

527 Facella P., Lopez L., Carbone F., Galbraith D.W., Giuliano G. & Perrotta G. (2008)
528 Diurnal and circadian rhythms in the tomato transcriptome and their modulation by
529 cryptochrome photoreceptors. *PLoS ONE* 3, e2798.

530 Farquhar G.D. & Sharkey T.D. (1982) Stomatal conductance and photosynthesis.
531 *Annual Review of Plant Physiology and Plant Molecular Biology* 33, 317-345.

532 Flexas J., Barbour M.M., Brendel O., Cabrera H.M., Carriqui M., Diaz-Espejo A., …,
533 Warren C.R. (2012) Mesophyll diffusion conductance to CO_2: an unappreciated
534 central player in photosynthesis. *Plant Science* 193-194, 70-84.

535 Fukushima A., Kusano M., Nakamichi N., Kobayasho M., Hayashi N., Sakakibara H.,
536 Mizuno T. & Saito K. (2009). Impact of clock-associated Arabidopsis pseudo-
537 response regulators in metabolic coordination. *Proceedings of the National Academy*
538 *of Sciences USA* 106, 7251-7256.

539 García-Plazaola J.I. & Becerril J.M. (1999) A rapid HPLC method to measure lipophilic
540 antioxidants in stressed plants: simultaneous determination of carotenoids and
541 tocopherols. *Phytochemical Analysis* 10, 307-313.

542 García-Plazaola J.I. & Becerril J.M. (2001) Seasonal changes in photosynthetic
543 pigments and antioxidants in beech (*Fagus sylvatica*) in a Mediterranean climate:

implications for tree decline diagnosis. *Australian Journal of Plant Physiology* 28, 225-232.

García-Plazaola J.I., Esteban R., Fernández-Marín B., Kranner I. & Porcar-Castell A. (2012) Thermal energy dissipation and xanthophyll cycles beyond the Arabidopsis model. *Photosynthesis Research* 113, 89-103.

Genty B., Briantais J.M. & Baker N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990, 87-92.

Gibon Y., Bläsing O.E., Palacios-Rojas N., Pankovic D., Hendriks J.H.M., Fisahn J., …, Stitt M. (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. *Plant Journal* 39, 847-862.

Gould P.D., Diaz P., Hogben C., Kusakina J., Salem R., Hartwell J. & Hall A. (2009) Delayed fluorescence as a universal tool for the measurement of circadian rhythms in higher plants. *Plant Journal* 58: 893-901.

Graf A., Schlereth A., Stitt M., & Smith A.M. (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. *Proceedings of the National Academy of Sciences USA* 107, 9458-9463.

Hanano S., Stracke R., Jakoby M., Merkle T., Domagalska M.A., Weisshaar B. & Davis S.J. (2008) A systematic survey in *Arabidopsis thaliana* of transcription factors that modulate circadian parameters. *BMC Genomics* 9, 182.

Harmer S.L., Hogenesch J.B., Straume M., Chang H.S., Han B., Zhu T., …, Kay S.A. (2000) Orchestated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290, 2110-2113.

Harmer S.L. (2009) The circadian system in higher plants. *Annual Review of Plant Biology* 60, 357-377.

Haydon M.J., Mielczarek O., Robertson F.C., Hubbard K., & Webb A.A.R. (2013) Photosynthetic entrainment of the *Arabidopsis thaliana* circadian clock. *Nature* 502, 689-692.

574 Hennessey T., Freeden A. & Field C. (1993) Environmental effects on circadian
575 rhythms in photosynthesis and stomatal opening. *Planta* 189, 369-376.

576 Hoch G., Popp M. & Körner C. (2002) Altitudinal increase of mobile carbon pools in
577 *Pinus cembrasuggests* sink limitation of growth at the Swiss treeline. *Oikos* 98, 361-
578 374.

579 Hogewoning S.W., Wientjes E., Douwstra P., Trouwborst G., van Leperen W., Croce R.
580 & Harbison J. (2012) Photosynthetic quantum yield dynamics: from photosystems to
581 leaves. *The Plant Cell* 24, 1921-1935.

582 Hörtensteiner S. (2006) Chlorophyll degradation during senescence. *Annual Review of*
583 *Plant Biology* 57, 55-77.

584 Hotta C.T., Gardner M.J., Hubbard K.E., Baek S.J., Dalchau N., Suhita D., Dodd A.N.
585 & Webb A.A.R. (2007) Modulation of environmental responses of plants by
586 circadian clocks. *Plant Cell and Environment* 30, 333-349.

587 Khan S., Rowe S.C. & Harmon F.G. (2010) Coordination of the maize transcriptome by
588 a conserved circadian clock. *BMC Plant Biology* 10, 126.

589 Kouril R., Wientjes E., Bultema J.B., Croce R. & Boekema E.J. (2013) High-light vs.
590 low-light: effect of light acclimation on photosystem II composition and organization
591 in *Arabidopsis thaliana*. *Biochimica et Biophysica Acta* 1827, 411-419.

592 ~~Litthauer S., Battle M.W., Lawson T. & Jones M.A. (2015) Phototropins maintain~~
593 ~~robust circadian oscillation of PSII operating efficiency under blue light. *The Plant*~~
594 ~~*Journal* 83, 1034-1045.~~

595 McClung C.R. (2006) Plant circadian rhythms. *The Plant Cell* 18, 792-803.

596 McClung C.R. (2013) Beyond Arabidopsis: the circadian clock in non-model plant
597 species. *Seminars in Cell & Developmental Biology* 24, 430-436.

598 Milcu A., Roscher C., Gessler A., Bachmann D., Gockele A., Guderle M., …, Roy J.
599 (2014) Functional diversity of leaf nitrogen concentrations drives grassland carbon
600 fluxes. *Ecology Letters* 17: 435-444.

601 Müller L.M., von Korff M. & Davis S.J. (2014) Connections between circadian clocks
602 and carbon metabolism reveal species-specific effects on growth control. *Journal of*
603 *Experimental Botany* 65: 2915-2923.

Munger P., Bleiholder H., Hack H., Heß M., Strauss R., van den Boom T. & Weber E. (1998) Phenological growth stages of the peanut plant (*Arachis hypogaea* L.) Codification and description according to the BBCH Scale. *Journal of Agronomy and Crop Science* 180, 101-107.

Noordally Z.B., Ishii K., Atkins K.A., Wetherill S.J., Kusakina J., Walton A.J., …, Dodd A.N. (2013) Circadian control of chloroplast transcription by a nuclear-encoded timing signal. *Science* 339, 1316-1319.

Pallas J.E., Samish Y.B. & Willmer C.M. (1974) Endogenous rhythmic activity of photosynthesis, transpiration, dark respiration, and carbon dioxide compensation point of peanut leaves. *Plant Physiology* 53, 907-911.

Pan Y., Michael T.P., Hudson M.E., Kay S.A., Chory J. & Schuler M.A. (2009) Cytochrome P450 monooxygenase as reporters for circadian-regulated pathways. *Plant Physiology* 150, 858-878.

Pan W.J., Wang X., Deng Y.R., Li J.H., Chen W., Chiang J.Y., Yang J.B. & Zheng L. (2015) Nondestructive and intuitive determination of circadian chlorophyll rhythms in soybean leaves using multispectral imaging. *Sientific Reports* 5, 11108.

Ragni M. & Ribera d'Alcalà M. (2007) Circadian variability in the photobiology of *Phaeodactylum tricornutum*: pigment content. *Journal of Plancton Research* 29, 141-156.

Resco de Dios V., Goulden M.L., Ogle K., Richardson A.D., Hollinger D.Y., Davidson E.A., …, Moreno J.M. (2012) Endogenous circadian regulation of carbon dioxide exchange in terrestrial ecosystems. *Global Change Biology* 18, 1956-1970.

Resco de Dios V., Roy J., Ferrio J.P., Alday J.G., Landais D., Milcu A., & Gessler A. (2015) Processes driving nocturnal transpiration and implications for estimating land evapotranspiration. *Scientific Reports* 5, 10975.

Resco de Dios V., Loik M.E., Smith R.A., Aspinwall M.J. & Tissue D.T. (2016a) Genetic variation in circadian regulation of nocturnal stomatal conductance enhances plant fitness. *Plant Cell and Environment* 39, 3-11.

Resco de Dios V., Gessler A., Ferrio J.P., Alday J.G., Bahn M., del Castillo J., …, Roy J. (2016b) Circadian rhythms have significant effects on leaf-to-canopy gas exchange under field conditions. *Giga Science* ~~doi: 10.1101/054593~~5, 43.

635 Salmela M.J., Greenham K., Lou P., McClung C.R., Ewers B.E. & Weinig C. (2016)
636 Variation in circadian rhythms is maintained among and within populations in
637 *Boechera stricta*. *Plant Cell and Environment* 39, 1293-1303.

638 ~~Sanchez A, Shin J. & Davis S.J. (2011) Abiotic stress and the plant circadian clock.~~
639 ~~*Plant Signalling and Behaviour* 6, 223-231.~~

640 Schmid V.H.R. (2008) Light-harvesting complexes of vascular plants. *Cellular and*
641 *Molecular Life Sciences* 65, 3619-3639.

642 Siebke K. & Ball M.C. (2009) Non-destructive measurement of chlorophyll *b*:*a* ratios
643 and identification of photosynthetic pathways in grasses by reflectance spectroscopy.
644 *Functional Plant Biology* 36, 857-866.

645 Sorek M., Yacobi Y.Z., Roopin M., Berman-Frank I. & Levy O. (2013) Photosynthetic
646 circadian rhythmicity patterns of *Symbiodium*, the coral endosymbiotic algae.
647 *Proceedings of the Royal Society B* 280, 20122942.

648 Tindall A.J., Waller J., Greenwood M., Gould P.D., Hartwell J. & Hall A. (2016) A
649 comparison of high-throughput techniques for assaying circadian rhythms in plants.
650 *Plant Methods* 11, p. 32.

651 Ware M.A., Belgio E. & Ruban A.V. (2015) Photoprotective capacity of non-
652 photochemical quenching in plants acclimated to different light intensities.
653 *Photosynthesis Research* 126, 261-274.

654 Wood S.N. (2006) *Generalized Additive Models: An Introduction. R*. (Chapman &
655 Hall/CRC: Boca Raton, Florida).

656 Wyka TP, Duarte HM & Lüttge UE (2005) Redundancy of stomatal control for the
657 circadian photosynthetic rhythm in *Kalanchoë daigremontiana* Hamet et Perrier.
658 *Plant Biology* 7: 176-181.

659 Yakir E., Hilman D., Harir Y. & Green R.M. (2007) Regulation of output from the plant
660 circadian clock. *FEBS Journal* 274, 335-345.

661 Yarkhunova Y., Edwards C.E., Ewers B.E., Baker R.L., Aston T.L., McClung C.R.,
662 Lou P. & Weinig C. (2016) Selection during crop diversification involves correlated
663 evolution of the circadian clock and ecophysiological traits in *Brassica rapa*. *New*
664 *Phytol*ogist 210, 133-144.

665 Yerushalmi S. & Green R.M. (2009) Evidence for the adaptive significance of circadian
666 rhythms. *Ecology Letters* 12, 970-981.

667 Zhao W., Wang S., Li X., Huang H., Sui X. & Zhang Z. (2012) Molecular cloning and
668 characterization of the light-regulation and circadian-rhythm of the *VDE* gene
669 promoter from *Zingiber officinale*. *Plant Cell Reports* 31, 1381-1392.

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FIGURES

Figure 1. Net assimilation (A_{net}) (a), stomatal conductance (g_s) (b), A_{net}/C_i ratio (c) and photosynthetic capacity (A_{max}) (d) in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves during the last cycle of the entrainment phase and under continuous illumination and constant environmental conditions. The grey section corresponds to the last dark period of an entrainment phase of five days. Black and white segments on the X-axis represent subjective nights (i.e. when the plants would have naturally experienced night-time conditions) and days (i.e. when the plants would have naturally experienced day-time conditions), respectively. Time zero represents the first subjective noon after transfer to constant conditions. Temporal patterns were examined with Generalized Additive Model (GAM) fitted with automated smoothness selection (Wood 2006). Shaded areas indicate SE of GAM fitting.

Figure 2. Actual photochemical efficiency of PSII (Φ_{PSII}) (a), maximal fluorescence of illuminated leaves at steady-state (Fm') under continuous illumination and constant environmental conditions (b), non-photochemical quenching (NPQ) (~~b~~c) and maximal fluorescence of dark-adapted leaves (Fm) during the last cycle of the entrainment phase and under continuous darkness and constant environmental conditions (d) in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves during the last cycle of the entrainment phase and under continuous illumination and constant environmental conditions. The grey section corresponds to the last dark period of an entrainment phase of five days. Black and white segments on the X-axis represent subjective nights and days, respectively. Statistical analysis and data presentation as in Fig. 1.

~~**Figure 3**. Maximal fluorescence of illuminated leaves at steady state (Fm') under continuous illumination and constant environmental conditions (a) and maximal fluorescence of dark-adapted leaves (Fm) during the last cycle of the entrainment phase and under continuous darkness and constant environmental conditions (b), in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves. The grey section corresponds to the last dark period of an entrainment phase of five days. Black and white segments on the X-axis represent subjective nights and days, respectively. Statistical analysis and data presentation as in Fig. 1.~~

Figure 43. Ratio of chlorophyll *a* to chlorophyll *b* (Chl*a*/*b*) in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves under continuous illumination and constant environmental conditions (a) and in bean leaves during the last cycle of the entrainment phase and under continuous darkness and constant environmental conditions (b). The grey section corresponds to the last dark period of an entrainment phase of five days. Black and white segments on the X-axis represent subjective nights and days, respectively. Statistical analysis and data presentation as in Fig. 1.

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Figure 54. Ratio of total pool of xanthophyll cycle pigments (violaxanthin + antheraxanthin + zeaxanthin) to chlorophyll (VAZ/Chl) (a), deepoxidation state of the xanthophyll cycle (AZ/VAZ) (b) and total carotenoid to chlorophyll (c) in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves during the last cycle of the entrainment phase and under continuous illumination and constant environmental conditions. The grey section corresponds to the last dark period of an entrainment phase of five days. Black and white segments on the X-axis represent subjective nights and days, respectively. Statistical analysis and data presentation as in Fig. 1.

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Figure 65. Total pool of non-structural carbohydrates (NSC) (a) and starch (b) in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves during the last cycle of the entrainment phase and under continuous illumination and constant environmental conditions. The grey section corresponds to the last dark period of an entrainment phase of five days. Black and white segments on the X-axis represent subjective nights and days, respectively. Statistical analysis and data presentation as in Fig. 1.

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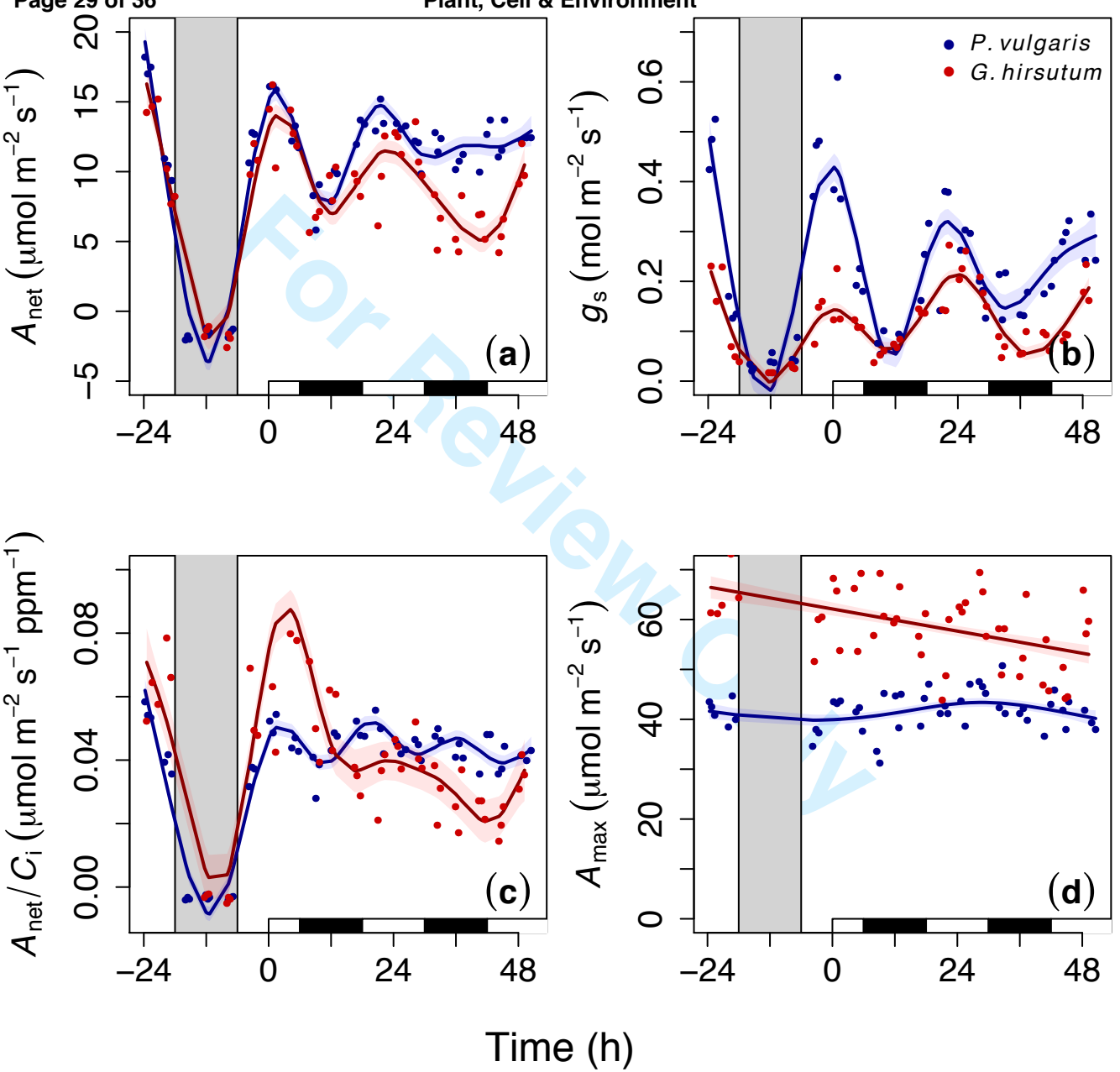
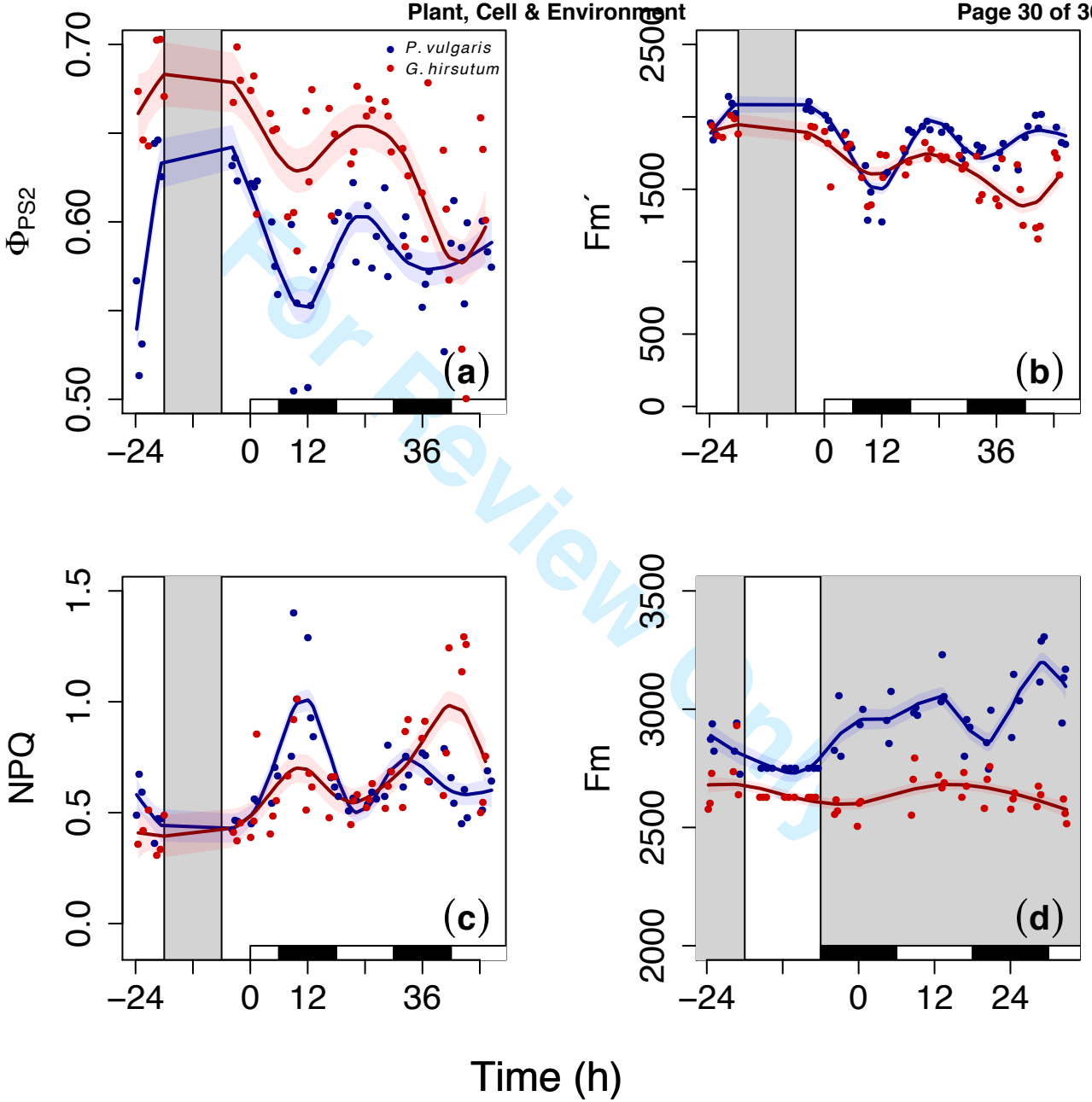
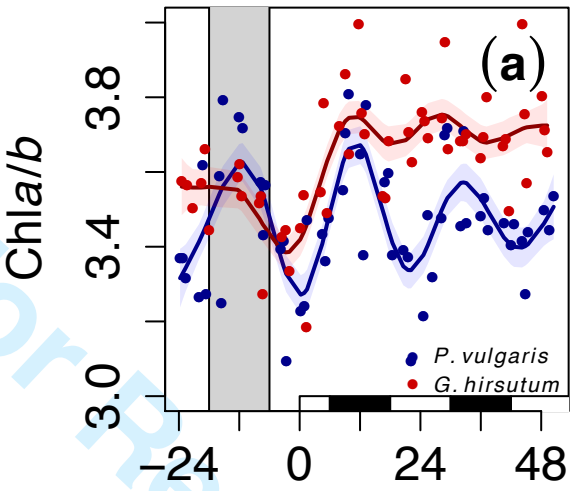
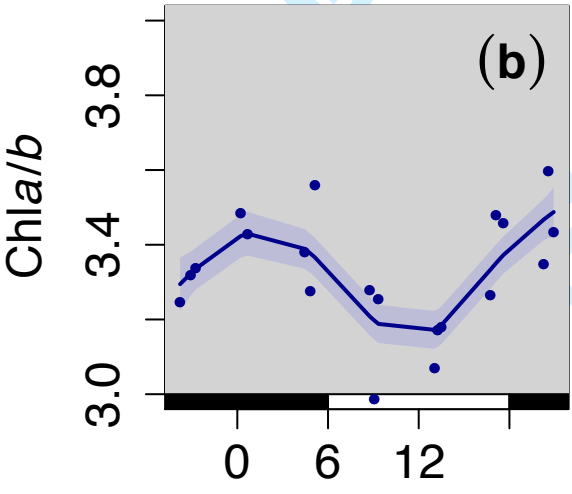
Figure 76. Relationship between net assimilation (A_{net}) and potential drivers: stomatal conductance (g_s) (a), internal CO_2 concentration (C_i) (b), actual photochemical efficiency of PSII (Φ_{PSII}) (c) and ratio of chlorophyll *a* to chlorophyll *b* (Chl*a*/*b*) (d) in bean (*P. vulgaris*) and cotton (*G. hirsutum*) leaves during the last cycle of the entrainment phase and under continuous illumination and constant environmental

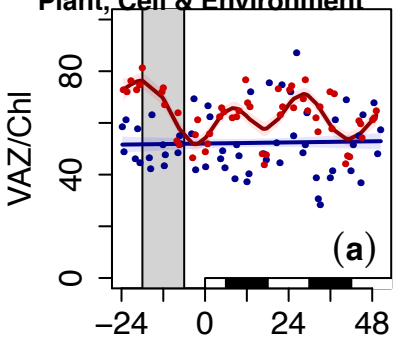
conditions. Linear regressions are shown when significant at $P<0.05$. Dotted lines represent non-significant regressions.

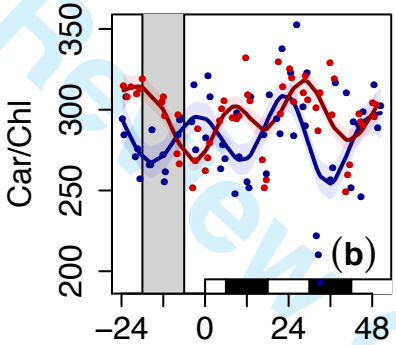


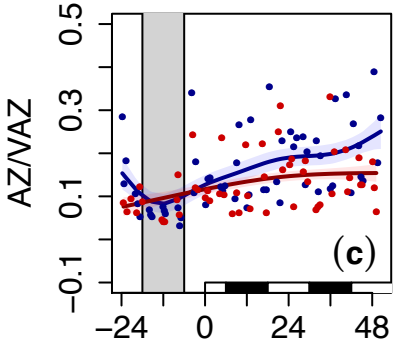


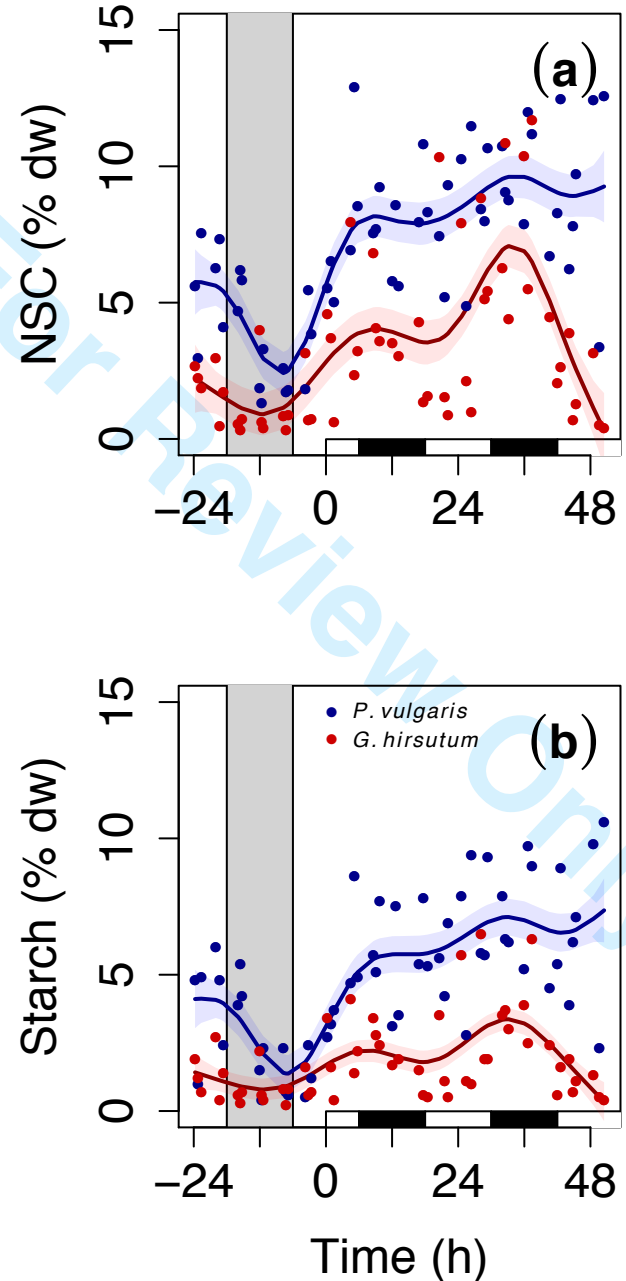


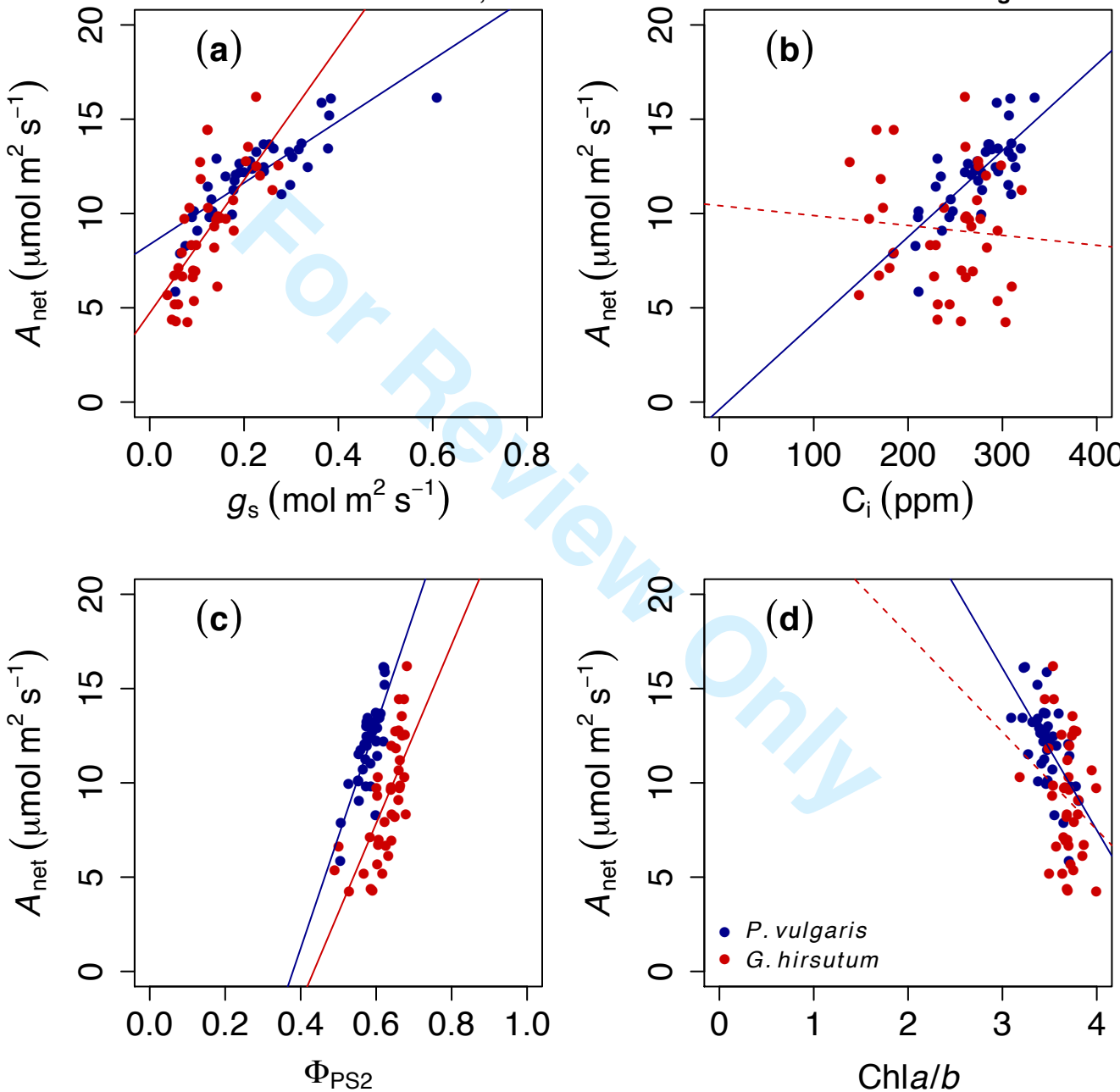












Macrocosms platform of the Montpellier European Ecotron (Courtesy of J. Roy)

623x467mm (180 x 180 DPI)

Macrocosms platform of the Montpellier European Ecotron (Courtesy of J. Roy).

623x467mm (180 x 180 DPI)

Macrocosms platform of the Montpellier European Ecotron (Courtesy of J. I. García-Plazaola)

192x144mm (300 x 300 DPI)