

1 **Does circadian regulation lead to optimal gas exchange**  
2 **regulation?**

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29 **Running headline:** Optimal stomata and the circadian clock

30

## 31 **Summary**

32 Optimal stomatal theory is an evolutionary model proposing that leaves trade-off  
33 Carbon (C) for water to maximise C assimilation ( $A$ ) and minimise transpiration ( $E$ ),  
34 thereby generating a marginal water cost of carbon gain ( $\lambda$ ) that remains constant over  
35 short temporal scales. The circadian clock is a molecular timer of metabolism that  
36 controls  $A$  and stomatal conductance ( $g_s$ ), amongst other processes, in a broad array of  
37 plant species. Here, we test whether circadian regulation contributes towards  
38 achieving optimal stomatal behaviour. We subjected bean (*Phaseolus vulgaris*) and  
39 cotton (*Gossypium hirsutum*) canopies to fixed, continuous environmental conditions  
40 of photosynthetically active radiation, temperature and vapour pressure deficit over 48  
41 hours. We observed a significant and self-sustained circadian oscillation in  $A$  and in  
42 stomatal conductance ( $g_s$ ) which also led to a circadian oscillation in  $\lambda$ . The lack of  
43 constant marginal water cost indicates that circadian regulation does not directly lead  
44 to optimal stomatal behaviour. However, the temporal pattern in gas exchange,  
45 indicative of either maximizing  $A$  or of minimizing  $E$ , depending upon time of day,  
46 indicates that circadian regulation could contribute towards optimizing stomatal  
47 responses. More broadly, our results add to the emerging field of plant circadian  
48 ecology and show that molecular controls may partially explain leaf-level patterns  
49 observed in the field.

50

51 **Key-words:** bean, cotton, ecological strategies, gas exchange, leaf, molecular  
52 regulation, photosynthesis.

53

## 54 **Introduction**

55 Early trade-offs in ecology recognized the need to balance growth and survival  
56 (Grubb 2015). Intense resource acquisition to sustain elevated growth rates, for  
57 instance, could lead to quick resource depletion and ultimately death. As a partial  
58 explanation for plant response to this constraint, the hypothesis of optimization in  
59 stomatal conductance was developed (Cowan 1977; Cowan & Farquhar 1977). In  
60 short, the optimal stomatal conductance hypothesis proposes that stomata balance the  
61 trade-off between  $A$  ( $C$  assimilation) and  $E$  (water losses) by maintaining a constant  
62 marginal water cost ( $\lambda = \delta E / \delta A$ ; in mol  $\text{CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ ), at least over short time  
63 scales, at the point where  $A$  is maximized and  $E$  minimized (Cowan & Farquhar  
64 1977).

65 This optimal strategy was originally postulated as a conservative strategy for  
66 plants facing variation in a physical environment that, to a degree, is unpredictable or  
67 stochastic. As a result, most tests of the prediction of constant  $\lambda$  have been performed  
68 under changing vapour pressure deficit, soil water, temperature or  $[\text{CO}_2]$  (Manzoni *et al.*  
69 *et al.* 2011; Duursma *et al.* 2013; Buckley *et al.* 2014). However, there is also a degree  
70 of predictability in the variation observed in the natural environment. Chief amongst  
71 these is photoperiod, which varies deterministically as a function of day of year and of  
72 geographic location. A nearly universal adaptation to the photoperiod, and other  
73 predictable environmental cues, is the endogenous circadian clock (McClung 2006;  
74 Resco, Hartwell & Hall 2009).

75 Circadian rhythms regulate the transcription of ~30% of the plant's genome  
76 (Covington *et al.* 2008) and, amongst others, diurnal patterns of stomatal conductance  
77 and photosynthesis are partially products of circadian regulation (Hennessey, Freeden  
78 & Field 1993; Mencuccini, Mambelli & Comstock 2000). It has been shown that

79 resonance between circadian rhythms in gas exchange and environmental cues  
80 increases plant growth (Graf *et al.* 2010; de Montaigu *et al.* 2015; Kolling *et al.* 2015;  
81 Resco de Dios *et al.* 2016), and that circadian timing is related to photosynthesis rates  
82 and stomatal conductance (Edwards *et al.* 2011). Regarding the hypothesis of optimal  
83 stomatal regulation, Cowan (1982) states that “if diurnal variation in natural physical  
84 environment were regular and predictable, then optimization would require only that  
85 there be an appropriate circadian rhythm in stomatal aperture”. Given that variation in  
86 the physical environment is not entirely regular and predictable, here we seek to  
87 understand the potential role of circadian rhythms towards optimizing the trade-off in  
88  $A$  vs  $E$ .

89         Circadian biologists often mention circadian regulation as an important  
90 component of achieving optimal stomatal conductance (Hubbard & Webb 2015).  
91 However, we are unaware of any direct tests for optimality resulting from circadian  
92 regulation, and perhaps the word optimal in those studies is used in general terms, and  
93 not in relation to the specific hypothesis of time-invariant  $\lambda$ . In fact, circadian  
94 regulation in  $A$  has been documented to be uncoupled and independent from circadian  
95 regulation in  $g_s$  (Dodd, Parkinson & Webb 2004), but linkages between these two  
96 processes is a pre-requisite for optimal water use. Therefore, if circadian rhythms  
97 regulate  $A$  and  $g_s$  independently from each other, one would hypothesize that  
98 circadian regulation alone, would not lead to optimal stomatal regulation.

99         Nonetheless, there is some evidence from theoretical modeling that circadian  
100 rhythms could aid in reaching optimality. Circadian regulation serves to “anticipate”  
101 predictable environmental cues, in such a way that stomata can adjust in advance  
102 (“stomatal priming”, Resco de Dios *et al.* 2016). As such, the clock has been  
103 hypothesized to aid in attaining optimality through stomatal priming because direct

104 responses to regular diurnal fluctuations alone would inevitably lead to a lagged  
105 response (Dietze 2014). In other words, stomata show a lagged response to the  
106 environment (Vico *et al.* 2011) and, although it is not expected that optimality  
107 operates at every instant, circadian regulation could help in achieving optimality by  
108 diminishing the lags through stomatal priming (Dietze 2014).

109         Here, we propose that circadian regulation, *per se*, does not lead to optimal  
110 behavior over diurnal cycles, but that it might help in achieving optimality within  
111 field settings. More explicitly, we hypothesize that: 1) because the circadian clock  
112 regulates  $A$  and  $g_s$  independently, circadian action will lead to a time-changing  $\lambda$ ,  
113 consistent with non-optimal behavior; 2) the temporal pattern of circadian driven gas  
114 exchange will be consistent with a stomatal priming that prepares for regular  
115 environmental variation.

116         Assessing the effects of circadian regulation on daytime  $A$  and  $g_s$  under natural  
117 conditions is difficult because the influence of environmental drivers generally mask  
118 circadian regulation. Circadian regulation is most strongly expressed under a  
119 “constant environment”: when temperature, radiation, vapour pressure deficit and  
120 other environmental drivers are held experimentally constant over 24h or longer.  
121 Therefore, we addressed our questions by examining temporal variation in  $\lambda$  in an  
122 herb (bean, *Phaseolus vulgaris*) and in a shrub (cotton, *Gossypium hirsutum*) under  
123 48h of constant environmental conditions.

124         It has been noted that the optimal stomatal hypothesis cannot be tested directly  
125 with experimental manipulations such as 48h of constant environmental conditions,  
126 because optimal stomatal theory represents an evolutionary process and therefore can  
127 only be assessed under environmental conditions observed during their evolution  
128 (Cowan 2002). However, tests of the optimal stomatal hypothesis have been

129 successfully conducted in elevated CO<sub>2</sub> enrichment experiments (Barton *et al.* 2012;  
130 Medlyn *et al.* 2013), although it is clear that plants have not evolved experiencing  
131 step-function, large sudden increases in CO<sub>2</sub> concentration (Woodward 2007). We  
132 suggest that our experimental approach is somewhat similar to other approaches,  
133 except that to avoid the potential for experimental artefacts, we do not test whether the  
134 optimal stomatal theory is observed under constant environmental conditions. Instead,  
135 our goal is to assess the potential for circadian regulation to contribute to optimal  
136 stomatal behaviour in natural, varying environments.

137       Throughout the manuscript, we will present data on both the marginal water  
138 cost of carbon gain ( $\lambda = \delta E / \delta A$ ) and on  $A_{\text{net}}/g_s$  (intrinsic water use efficiency). This is  
139 for the sake of clarity as  $A_{\text{net}}/g_s$  is more often used than the marginal water cost of  
140 carbon gain, and also because those two variables tend to be inversely correlated  
141 (using the convention of this manuscript, see methods for calculation of  $\lambda$ ). However,  
142 it is important to note that  $\lambda$  is not simply the inverse of water use efficiency ( $A/E$ )  
143 because  $\lambda$  is a partial derivative, i.e. an expression of the co-variation between the two  
144 variables for a given level of  $g_s$  (see for instance (Thomas, Eamus & Bell 1999) for  
145 different methods of calculation).

146

## 147 **Materials and methods**

### 148 *Experimental set-up*

149 The experiment was performed at the Macrocosms platform of the Montpellier  
150 European Ecotron, Centre National de la Recherche Scientifique (CNRS, France). We  
151 used 6 controlled-environment units of the macrocosms platform (3 planted with bean  
152 and 3 with cotton), where the main abiotic (air temperature, humidity and CO<sub>2</sub>  
153 concentration) drivers were automatically controlled. The soil was extracted using

154 large lysimeters (2 m<sup>2</sup>, circular with a diameter of 1.6 m, weighing 7 to 8 tonnes) from  
155 the flood plain of the Saale River near Jena, Germany, and used in a previous Ecotron  
156 experiment on biodiversity (Milcu *et al.* 2014). After that experiment, the soil was  
157 ploughed down to 40 cm and fertilized with 25/25/35 NPK (MgO, SO<sub>3</sub> and other  
158 oligoelements were associated in this fertilizer: Engrais bleu universel, BINOR,  
159 Fleury-les-Aubrais, FR).

160 The soil was regularly watered to *ca.* field capacity by drip irrigation, although  
161 irrigation was stopped during each measurement campaign (few days) to avoid  
162 interference with water flux measurements. However, no significant differences (at  $P$   
163  $< 0.05$ , paired t-test,  $n=3$ ) in leaf water potential occurred between the beginning and  
164 end of these measurement campaigns, indicating no apparent effect of a potentially  
165 declining soil moisture on leaf hydration.

166 Environmental conditions within the macrocosms (excluding the experimental  
167 periods) were set to mimic outdoor conditions, but did include a 10% light reduction  
168 by the macrocosm dome cover (sheet of Fluorinated Ethylene Propylene). During  
169 experimental periods, light was controlled by placing a completely opaque fitted  
170 cover on each dome to block external light inputs (PVC coated polyester sheet Ferrari  
171 502, assembled by IASO, Lleida, Spain), and by using a set of 5 dimmable plasma  
172 lamps (GAN 300 LEP with the Luxim STA 41.02 bulb, with a sun-like light  
173 spectrum); these lamps were hung 30 cm above the plant canopy and provided a PAR  
174 of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . We measured PAR at canopy level with a quantum sensor (Li-  
175 190, LI-COR Biosciences, Lincoln, NE, USA) in each macrocosm.

176 Bean and cotton were planted in 5 different rows within the macrocosms on  
177 10<sup>th</sup> July 2013, one month before the start of the measurements, and thinned to  
178 densities of 10.5 and 9 individuals m<sup>-2</sup>, respectively. Cotton (STAM-A16 variety by



179 INRAB/CIRAD) is a perennial shrub with an indeterminate growth habit. This cotton  
180 variety grows to 1.5-2 m tall and has a pyramidal shape and short branches. Bean  
181 (recombinant inbred line RIL-115 bred by INRA Eco&Sol) is an annual herbaceous  
182 species. RIL-115 is a fast growing, indeterminate dwarf variety, 0.3-0.5 m tall; it was  
183 inoculated with *Rhizobium tropici* CIAT 899 also provided by INRA. During the  
184 experiment, bean and cotton generally remained at the inflorescence emergence  
185 developmental growth stage (codes 51-59 in BBCH scale, the standard phenological  
186 scale within the crop industry; Feller *et al.* 1995; Munger *et al.* 1998). Further details  
187 on Ecotron measurements have been provided elsewhere (Resco de Dios *et al.* 2015).

188         During each experimental period, plants were entrained for five days under  
189 environmental conditions that mimicked the pattern observed in an average August  
190 sunny day in Montpellier in terms of  $T_{\text{air}}$  (28/19° C, diurnal max/min) and VPD.  
191 However, we kept radiation levels much lower (at 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at canopy level)  
192 because previous research proposed that stomatal behavior should follow optimal  
193 theory when photosynthesis is light- (and not CO<sub>2</sub>) limited (Medlyn *et al.* 2011), and  
194 our PAR values ensured this was the case. After 5 days of entrainment, we maintained  
195 environmental conditions constant starting at solar noon and for the next 48 h.

196

### 197 *Measurements*

198 We characterized the general pattern of gas exchange during the last day of  
199 entrainment and during constant environmental conditions by monitoring, every 4  
200 hours, gas exchange (LI-6400XT, Li-Cor, Lincoln, Nebraska, USA) in three different  
201 leaves, within each of the three domes, per species that were available. To diminish  
202 redundancy in the presentation, only  $A_{\text{net}}/g_s$  will be shown to characterize this general  
203 pattern.

204 To obtain enough resolution to test for temporal changes in the marginal water  
205 cost of carbon gain, we additionally measured gas exchange every 2 minutes by using  
206 2-3 additional portable photosynthesis systems per species and day. Each instrument  
207 was continuously deployed on a leaf for 24 h, and the Auto-Log function was used.  
208 Measurements were conducted over 48 h with an effective  $n = 3$  per species (1-2  
209 leaves were measured per macrocosm, in a total of 3 macrocosms). To diminish  
210 redundancy in the presentation, only  $A_{\text{net}}$  and  $g_s$  (along with  $g_1$ ) will be shown from  
211 these high-resolution measurements (but not  $A_{\text{net}}/g_s$ ).

212

### 213 *Analyses*

214 The marginal water cost used was estimated from parameter  $g_1$  in the stomatal model  
215 of Medlyn *et al.* (2011). This model was derived from optimal stomatal theory, such  
216 that the  $g_1$  is inversely proportional to the root square of  $\lambda$ . We calculated the  
217 marginal water cost from the Medlyn *et al.* (2011), and assuming minimal  
218 conductance ( $g_0$ ) was 0, so that we could compare the variability observed in our  
219 experiment with that observed in a recent synthesis reporting  $g_1$  values for 314 species  
220 from 10 different functional types (Lin *et al.* 2015). We calculated values of  $g_1$   
221 separately for each hour.

222 We examined statistical significance of temporal patterns in  $g_1$  with a  
223 Generalized Additive Mixed Model (GAMM) fitted with automated smoothness  
224 selection (Wood 2006) in the R software environment (*mgcv* library in R 3.1.2, The R  
225 Foundation for Statistical Computing, Vienna, Austria), including macrocosms as a  
226 random factor. This approach was chosen because there are no *a priori* assumptions  
227 about the functional relationship between variables. We accounted for temporal  
228 autocorrelation in the residuals by adding a first-order autoregressive process structure

229 (nlme library, Pinheiro & Bates 2000). Significant temporal variation in the GAMM  
230 best-fit line was analysed after computation of the first derivative (the slope, or rate of  
231 change) with the finite differences method. We also computed standard errors and a  
232 95% point-wise confidence interval for the first derivative. The trend was  
233 subsequently deemed significant when the derivative confidence interval was bounded  
234 away from zero at the 95% level (for full details on this method see Curtis & Simpson  
235 2014). Periods with no significant variation are illustrated on the figures by the yellow  
236 line portions, and significant differences occur elsewhere. The magnitude of the range  
237 in variation driven by the circadian clock was calculated using GAMM maximum and  
238 minimum predicted values.

239

## 240 **Results**

241 We observed a self-sustained oscillation in  $A_{\text{net}}$ ,  $g_s$  and  $A_{\text{net}}/g_s$  that showed a ~24 h  
242 periodicity (Figs. 1, 2, Table 1). That is, there was a significant variation in  $A_{\text{net}}$  and  $g_s$   
243 in the absence of variation in environmental drivers, and this variation showed a  
244 diurnal cycle. Although  $A_{\text{net}}$  and  $g_s$  generally followed the same pattern in that they  
245 both concurrently showed either a positive or a negative trend, the magnitude of the  
246 oscillation was larger in  $g_s$  (54-84% change, Table 1), than in  $A_{\text{net}}$  (28-42% change,  
247 Table 1) over a 24 h cycle in constant environmental conditions. In turn, this led to a  
248 significant variation in instantaneous water use efficiency ( $A_{\text{net}}/g_s$ ) that was 46-74% of  
249 that during entrainment (Table 1). If we only consider the oscillation during the  
250 subjective day (the time under constant conditions when it would have normally been  
251 daytime during entrainment) we still observe a significant and time-dependent  
252 variation in  $A_{\text{net}}$ ,  $g_s$  and  $A_{\text{net}}/g_s$ , although of smaller magnitude than during the whole  
253 24 h cycle (Figs. 1, 2).

254           The pattern in  $A_{\text{net}}/g_s$  was such that water use efficiency increased in the first  
255 subjective afternoon under constant conditions (hours 0-6 in Fig. 2c), it remained  
256 constant in the first hours of the night, and then it increased again from the subjective  
257 midnight (hour 12 in Fig. 2c) until the following subjective noon (hour 24 in Fig. 2c).  
258 The rhythm dampens slightly in the second 24 h period under constant conditions  
259 (hours 24-48), because the clock is flexible and becomes entrained every day  
260 (Hennessey & Field 1991; Graf *et al.* 2010). However, we can still observe temporal  
261 fluctuations similar to those in the previous day (although with a smaller degree of  
262 significance). Because this study mostly focused on the implications of clock  
263 regulation within field settings, the results during the first day are most important  
264 because this reflects the period of highest influence of natural environmental  
265 variation.

266           We also observed a diurnal self-sustained cycle in  $g_1$  (Fig 1). That is, we did  
267 not observe homeostasis in the marginal water cost despite lack of variation in  
268 environmental drivers. Instead, we observed a pattern that was generally opposite to  
269 that found in water use efficiency:  $g_1$  significantly declined during the subjective  
270 afternoon in both species (although with a more pronounced decline in cotton), and a  
271 significant increase during the subjective night occurred for both species, that  
272 continued into the subjective morning for cotton.

273

## 274 **Discussion**

### 275 *The importance of circadian regulation towards achieving optimization*

276 We observed a significant and self-sustained 24 h oscillation in  $A_{\text{net}}$  and  $g_s$ , of  
277 different magnitude for each process, and that ultimately led to a diurnal oscillation in  
278 intrinsic water use efficiency and in the marginal cost of water, despite the absence of

279 variation in environmental drivers. Diurnal variation in  $g_1$  ranged from 5.5 to 0.5 over  
280 the 24 h cycle, and from 5.5 to 1.7 when we only consider variation during the  
281 subjective daytime (Fig. 1). There are many processes that could explain an afternoon  
282 decline in  $A_{\text{net}}$ , including feedback inhibition from starch accumulation,  
283 photorespiration and stomatal feedbacks, amongst others (Azcón-Bieto 1983; Jones  
284 1998; Flexas *et al.* 2006). Similarly, a multitude of processes could explain the  
285 afternoon decline in  $g_s$ , including hydraulic feedbacks and depletion of stem  
286 capacitors (Jones 1998; Zhang *et al.* 2014). However, the only process that can  
287 explain a self-sustained 24 h cycle is the circadian clock (Resco, Hartwell & Hall  
288 2009). We can therefore conclude that, in the absence of variation in the physical  
289 environment, circadian regulation of stomatal behaviour *per se* does not directly lead  
290 to an optimization, because  $g_1$  was not constant throughout the experiment. However,  
291 as we will discuss further below, the pattern of variation in  $g_1$  indicates that circadian  
292 regulation could be an important contributor to achieving optimality in the field.

293 Under the well-watered and fertilized conditions of this experiment, where  
294 radiation was probably the only limiting factor, we observed a stronger relative  
295 fluctuation in  $g_s$  than in  $A_{\text{net}}$ . This result is consistent with previous studies (Doughty  
296 *et al.* 2006; Yakir *et al.* 2007) and suggests that stronger clock regulation over  $g_s$  than  
297 over  $A_{\text{net}}$  could be widespread across  $C_3$  plants. On the one hand, these temporal  
298 patterns could be interpreted as an indication that the clock fosters a maximization of  
299  $A$  at the time of maximal potential for assimilation ( $A$  peaked at the subjective noon)  
300 which, in turn, would be aided by a maximal  $g_s$  which decreases diffusional  
301 limitations. On the other hand, the stronger decrease in  $g_s$ , relative to that in  $A_{\text{net}}$ ,  
302 during the subjective morning and afternoon, when conditions would have become  
303 less favourable for assimilation in a naturally fluctuating environment, is consistent

304 with a conservative water use strategy. Therefore, this result is consistent with the  
305 hypothesis that circadian-driven stomatal priming could contribute towards reaching  
306 optimality (Dietze 2014).

307         Nonetheless, this stomatal regulation strategy contrasts with other work that  
308 has shown that circadian regulation tends towards “wasting” water at times when  
309 there is no  $A$ . Circadian regulation is one of the main drivers of the temporal pattern of  
310 nocturnal  $g_s$ , which increases constantly from *ca.* midnight until predawn (Caird,  
311 Richards & Donovan 2007; Resco de Dios et al. 2015). There is no  $A$  overnight and,  
312 therefore nocturnal water use is not directly linked with assimilation. However,  
313 different studies have linked higher predawn  $g_s$  with higher  $A$  early in the morning  
314 (although whether or not this has a significant effect on plant growth is still under  
315 discussion, cf. Auchincloss *et al.* 2014; Resco de Dios *et al.* 2016). Therefore,  
316 compensation could occur if we observe circadian regulation over the full diurnal  
317 cycle, where an increase in  $A$  at different times (*e.g.*: subjective noon in this study or  
318 early morning in the cited work) would be accompanied by higher water losses  
319 (maximum  $g_s$  at subjective noon, and increase in  $g_s$  overnight, respectively); but a  
320 more conservative water use occurs at other times (when the relative decline in  $g_s$  is  
321 higher than in  $A$ , such as the afternoon or evening).

322         Our study is, to the best of our knowledge, the first to report a circadian  
323 pattern in  $g_1$ . As previously mentioned, the optimal stomatal hypothesis does not  
324 present specific predictions about what should happen under environmental conditions  
325 that do not naturally occur in the field (Cowan 2002). One could argue that 24h of  
326 continuous light does occur above the Polar circle, but not as a constant light intensity  
327 as utilised in our experiment, and moreover, bean and cotton did not evolve in these  
328 constant light environments. However, all species experience cloudy days over their

329 lifespans. Under cloudy days, temporal variation in temperature, vapour pressure  
330 deficit and other environmental drivers is generally minimal. Therefore, plants do  
331 often experience environmental conditions that are roughly constant for a few hours.  
332 It is therefore notable that the largest change in  $g_1$  occurred in the first 6 hours after  
333 conditions were kept constant (1200h to 1800h solar time) and this change in  $g_1$   
334 (from 5.5 to 1.7, see above) was significant. In fact, a recent global synthesis shows  
335 that mean  $g_1$  values across different functional types (in a study encompassing 314  
336 species) ranged between 1.6 and 7.2 (Lin *et al.* 2015). Subsequently, we encourage  
337 field studies of leaf-level gas exchange conducted at high temporal resolution to  
338 assess the extent of temporal variation in  $g_1$  under cloudy days.

339

#### 340 *Implications and mechanisms*

341 It has been argued that more biological realism must be incorporated into optimality  
342 models to generate a better understanding of optimal behaviour and its constraints  
343 (Niinemets 2012). Our results indicate that circadian regulation might be one of the  
344 most important processes to be included in these models. For instance, it is well  
345 documented that hysteresis in the  $E$ -VPD relationship generally exists, with higher  $E$   
346 values in the morning than in the afternoon, at any given VPD. There are different  
347 processes that could explain this phenomenon (O'Grady, Eamus & Hutley 1999;  
348 Tuzet, Perrier & Leuning 2003; Unsworth *et al.* 2004) and, one of them, is the lag  
349 between peaks in radiation and VPD (radiation peaks at solar noon, but VPD peaks a  
350 few hours later) (Zhang *et al.* 2014). Circadian rhythms could contribute to this  
351 phenomenon. The clock is often considered to be entrained by both temperature and  
352 radiation (Millar 2004). However, the pattern of  $A$  and  $g_s$  resembles more closely that  
353 of radiation, in that both  $A$  and  $g_s$  peaked at subjective noon, which was the same time

354 for PAR during entrainment. However,  $T_{\text{air}}$  and VPD peaked at 1400h during  
355 entrainment, and circadian regulation would have already started to decrease stomatal  
356 conductance at that time. Therefore, circadian-driven stomatal closure after radiation  
357 peaks at noon (which are more pronounced than the decline in  $A$ ), in concert with  
358 radiation-VPD lags, could be a contributing factor in the documented hysteresis in E-  
359 VPD relationships; however, this is not currently accounted for in models.

360         Circadian clocks in plants have traditionally been assumed to be cell  
361 autonomous and not coordinated across cells or plant tissues (Endo *et al.* 2014).  
362 However, recent research has observed that a hierarchy exists in plants in that the  
363 clock in the leaf vascular tissue regulates the clock in the mesophyll leaf tissue (Endo  
364 *et al.* 2014). Although speculative, it is tempting to hypothesize that clock-controlled  
365 hydraulic signals over vascular tissue could also be part of the response driving  
366 hysteresis in diurnal transpiration cycles.

367         The effect of circadian regulation on stomatal physiology is still being  
368 debated. In *Arabidopsis*, it has been proposed that the central oscillator of the clock  
369 directly controls stomatal behaviour because TOC1 (a component of the central  
370 oscillator) regulates ABA signalling (Legnaioli, Cuevas & Mas 2009). However, other  
371 studies have documented that time-dependent circadian regulation of  $g_s$  is  
372 independent of ABA concentration in beans (Mencuccini, Mambelli & Comstock  
373 2000). Another line of research proposes that it is through  $[Ca_{2+}]_{\text{cyt}}$  signalling that the  
374 circadian clock regulates stomatal movements (Hubbard & Webb 2015). Circadian  
375 regulation of  $A$  is relatively better understood, and it involves the joint regulation of  
376 the light harvesting complex, the carboxylating enzyme Rubisco, and feedbacks from  
377 carbohydrates (Dodd *et al.* 2014). However, most studies have been conducted at the  
378 molecular level with *Arabidopsis*, and the mechanism of action at “phenotypic” or



379 eco-physiological scales, as well as the degree to which processes in *Arabidopsis* are  
380 generalizable to other species, remain unknown.

381

### 382 *Conclusions*

383 It has been known for long that the circadian clock could be an important an important  
384 component underlying plant fitness. Understanding the reason why the circadian clock  
385 is adaptive has proven more challenging. Here we developed the first formal test of  
386 the hypothesis that the circadian clock leads towards optimal stomatal regulation and,  
387 indeed, the strong stomatal regulation under constant environmental conditions points  
388 to the circadian clock as an important component. Although we did not observe a  
389 constant marginal water cost under constant conditions, which is necessary for  
390 stomatal regulation to be optimal, the optimal stomatal hypothesis would also not  
391 have predicted that to occur given the artificiality of the experimental treatment.  
392 Importantly, the temporal patterns observed indicate how variation in stomatal  
393 regulation was consistent with a circadian-driven stomatal priming that prepares gas  
394 exchange in advance of regular environmental fluctuation. Although our experiments  
395 were not conducted under conditions typical of field settings, the strong fluctuation in  
396  $A$  and  $g_s$  indicate that circadian regulation could be an important component  
397 underlying optimal behaviour in the field. These results add to the emerging field of  
398 plant circadian ecology and show that one of the mechanisms by which the circadian  
399 clock increases plant fitness is by contributing towards reaching optimal stomatal  
400 behaviour. Further studies will need to clarify whether the large changes observed in  
401  $g_1$  under the subjective afternoon also occur in other species and under cloudy  
402 conditions.

403

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416

## 417 **Data Accessibility**

418 Data are freely accessible upon registration from  
419 <http://www.ecotron.cnrs.fr/index.php/en/component/users/?view=login>.

420

421

## 422 **References**

423 Auchincloss, L., Easlon, H.M., Levine, D., Donovan, L. & Richards, J.H. (2014) Pre-  
424 dawn stomatal opening does not substantially enhance early-morning  
425 photosynthesis in *Helianthus annuus*. *Plant Cell and Environment*, **37**, 1364-  
426 1370.  
427 Azcón-Bieto, J. (1983) Inhibition of photosynthesis by carbohydrates in wheat leaves.  
428 *Plant Physiology*, **73**, 681-686.

- 429 Barton, C.V.M., Duursma, R.A., Medlyn, B.E., Ellsworth, D.S., Eamus, D., Tissue,  
430 D.T., Adams, M.A., Conroy, J., Crous, K.Y., Liberloo, M., Löw, M., Linder,  
431 S. & McMurtrie, R.E. (2012) Effects of elevated atmospheric [CO<sub>2</sub>] on  
432 instantaneous transpiration efficiency at leaf and canopy scales in *Eucalyptus*  
433 *saligna*. *Global Change Biology*, **18**, 585-595.
- 434 Buckley, T.N., Martorell, S., Diaz-Espejo, A., Tomas, M. & Medrano, H. (2014) Is  
435 stomatal conductance optimized over both time and space in plant crowns? A  
436 field test in grapevine (*Vitis vinifera*). *Plant Cell and Environment*, **37**, 2707-  
437 2721.
- 438 Caird, M.A., Richards, J.H. & Donovan, L.A. (2007) Nighttime Stomatal  
439 Conductance and Transpiration in C<sub>3</sub> and C<sub>4</sub> Plants. *Plant Physiology*, **143**, 4-  
440 10.
- 441 Covington, M.F., Maloof, J.N., Straume, M., Kay, S.A. & Harmer, S.L. (2008) Global  
442 transcriptome analysis reveals circadian regulation of key pathways in plant  
443 growth and development. *Genome Biology*, **9**, R130.
- 444 Cowan, I.R. (1977) Stomatal behaviour and environment *Advances in Botany*  
445 *Research*, **4**, 117-228.
- 446 Cowan, I.R. (2002) Fit, Fitter, Fittest; Where Does Optimisation Fit In? *Silva*  
447 *Fennica*, **36**, 745-754.
- 448 Cowan, I.R. & Farquhar, G.D. (1977) Stomatal function in relation to leaf metabolism  
449 and environment. *Integration of Activity in the Higher Plant* (ed. D.H.  
450 Jennings). Cambridge University Press, Cambridge.
- 451 Curtis, C.J. & Simpson, G.L. (2014) Trends in bulk deposition of acidity in the UK,  
452 1988–2007, assessed using additive models. *Ecological Indicators*, **37**, 274-  
453 286.

- 454 de Montaigu, A., Giakountis, A., Rubin, M., Toth, R., Cremer, F., Sokolova, V., Porri,  
455 A., Reymond, M., Weinig, C. & Coupland, G. (2015) Natural diversity in  
456 daily rhythms of gene expression contributes to phenotypic variation.  
457 *Proceedings of the National Academy of Sciences of the USA*, **112**, 905-910.
- 458 Dietze, M.C. (2014) Gaps in knowledge and data driving uncertainty in models of  
459 photosynthesis. *Photosynth. Research*, **119**, 3-14.
- 460 Dodd, A.N., Kusakina, J., Hall, A., Gould, P.D. & Hanaoka, M. (2014) The circadian  
461 regulation of photosynthesis. *Photosynthesis Research*, **119**, 181-190.
- 462 Dodd, A.N., Parkinson, K. & Webb, A.A.R. (2004) Independent circadian regulation  
463 of assimilation and stomatal conductance in the *ztl-1* mutant of *Arabidopsis*.  
464 *New Phytologist*, **162**, 63-70.
- 465 Doughty, C., Goulden, M.L., Miller, S. & da Rocha, H. (2006) Circadian rhythms  
466 constrain leaf and canopy gas exchange in an Amazonian forest. *Geophysical*  
467 *Research Letters*, **33**, L15404.
- 468 Duursma, R.A., Payton, P., Bange, M.P., Broughton, K.J., Smith, R.A., Medlyn, B.E.  
469 & Tissue, D.T. (2013) Near-optimal response of instantaneous transpiration  
470 efficiency to vapour pressure deficit, temperature and [CO<sub>2</sub>] in cotton  
471 (*Gossypium hirsutum* L.). *Agricultural and Forest Meteorology*, **168**, 168-  
472 176.
- 473 Edwards, C.E., Ewers, B.E., Williams, D.G., Xie, Q., Lou, P., Xu, X., McClung, C.R.  
474 & Weinig, C. (2011) The genetic architecture of ecophysiological and  
475 circadian traits in *Brassica rapa*. *Genetics*, **189**, 375-390.
- 476 Endo, M., Shimizu, H., Nohales, M.A., Araki, T. & Kay, S.A. (2014) Tissue-specific  
477 clocks in *Arabidopsis* show asymmetric coupling. *Nature*, **515**, 419-422.

- 478 Feller, C., Bleiholder, H., Buhr, L., Hack, H., Hess, M., Klose, R., Meier, U., Stauss,  
479 R., Boom, T.v.d. & Weber, E. (1995) Phänologische Entwicklungsstadien von  
480 Gemüsepflanzen: II. Fruchtgemüse und Hülsenfrüchte. Nachrichtenbl. Deut.  
481 *Pflanzenschutzd*, **47**, 217–232.
- 482 Flexas, J., Bota, J., Galmes, J., Medrano, H. & Ribas-Carbo, M. (2006) Keeping a  
483 positive carbon balance under adverse conditions: responses of photosynthesis  
484 and respiration to water stress. *Physiologia Plantarum*, **127**, 343-352.
- 485 Graf, A., Schlereth, A., Stitt, M. & Smith, A.M. (2010) Circadian control of  
486 carbohydrate availability for growth in Arabidopsis plants at night.  
487 *Proceedings of the National Academy of Sciences of the USA*, **107**, 9458-9463.
- 488 Grubb, P.J. (2015) Trade-offs in interspecific comparisons in plant ecology and how  
489 plants overcome proposed constraints. *Plant Ecol Divers*,  
490 **doi:10.1080/17550874.2015.1048761**.
- 491 Hennessey, T., Freeden, A. & Field, C. (1993) Environmental effects on circadian  
492 rhythms in photosynthesis and stomatal opening. *Planta*, **189**, 369-376.
- 493 Hennessey, T.L. & Field, C.B. (1991) Circadian rhythms in photosynthesis:  
494 oscillations in carbon assimilation and stomatal conductance under constant  
495 conditions. *Plant Physiology*, **96**, 831-836.
- 496 Hubbard, K.E. & Webb, A.A.R. (2015) Circadian rhythms in stomata: Physiological  
497 and molecular aspects. *Rhythms in Plants* (eds S. Mancuso & S. Shabala), pp.  
498 231-255. Springer International Publishing Switzerland.
- 499 Jones, H. (1998) Stomatal control of photosynthesis and transpiration. *Journal of*  
500 *Experimental Botany*, **49**, 387-398.
- 501 Kolling, K., Thalmann, M., Muller, A., Jenny, C. & Zeeman, S.C. (2015) Carbon  
502 partitioning in Arabidopsis thaliana is a dynamic process controlled by the

- 503 plants metabolic status and its circadian clock. *Plant Cell and Environment*,  
504 **38**, 1965-1979.
- 505 Legnaioli, T., Cuevas, J. & Mas, P. (2009) TOC1 functions as a molecular switch  
506 connecting the circadian clock with plant responses to drought. *EMBO*  
507 *Journal*, **28**, 3745-3757.
- 508 Lin, Y.-S., Medlyn, B.E., Duursma, R.A., Prentice, I.C., Wang, H., Baig, S., Eamus,  
509 D., Resco de Dios, V., Mitchell, P., Ellsworth, D.S., Op de Beeck, M., Wallin,  
510 G., Uddling, J., Tarvainen, L., Linderson, M.-J., Cernusak, L.A., Nippert, J.B.,  
511 Ocheltree, T.W., Tissue, D.T., Martin-StPaul, N.K., Rogers, A., Warren, J.M.,  
512 De Angelis, P., Hikosaka, K., Han, Q., Onoda, Y., Gimeno, T.E., Barton,  
513 C.V.M., Bennie, J., Bonal, D., Bosc, A., Löw, M., Macinins-Ng, C., Rey, A.,  
514 Rowland, L., Setterfield, S.A., Tausz-Posch, S., Zaragoza-Castells, J.,  
515 Broadmeadow, M.S.J., Drake, J.E., Freeman, M., Ghannoum, O., Hutley,  
516 L.B., Kelly, J.W., Kikuzawa, K., Kolari, P., Koyama, K., Limousin, J.-M.,  
517 Meir, P., Lola da Costa, A.C., Mikkelsen, T.N., Salinas, N., Sun, W. &  
518 Wingate, L. (2015) Optimal stomatal behaviour around the world. *Nature*  
519 *Climate Change*, **5**, 459-464.
- 520 Manzoni, S., Vico, G., Katul, G., Fay, P.A., Polley, W., Palmroth, S. & Porporato, A.  
521 (2011) Optimizing stomatal conductance for maximum carbon gain under  
522 water stress: a meta-analysis across plant functional types and climates.  
523 *Functional Ecology*, **25**, 456-467.
- 524 McClung, C.R. (2006) Plant Circadian Rhythms. *Plant Cell*, **18**, 792-803.
- 525 Medlyn, B.E., Duursma, R.A., De Kauwe, M.G. & Prentice, I.C. (2013) The optimal  
526 stomatal response to atmospheric CO<sub>2</sub> concentration: Alternative solutions,

527 alternative interpretations. *Agricultural and Forest Meteorology*, **182-183**,  
528 200-203.

529 Medlyn, B.E., Duursma, R.A., Eamus, D., Ellsworth, D.S., Prentice, I.C., Barton,  
530 C.V.M., Crous, K.Y., De Angelis, P., Freeman, M. & Wingate, L. (2011)  
531 Reconciling the optimal and empirical approaches to modelling stomatal  
532 conductance. *Global Change Biology*, **17**, 2134-2144.

533 Mencuccini, M., Mambelli, S. & Comstock, J. (2000) Stomatal responsiveness to leaf  
534 water status in common bean (*Phaseolus vulgaris* L.) is a function of time of  
535 day. *Plant Cell and Environment*, **23**, 1109-1118.

536 Milcu, A., Roscher, C., Gessler, A., Bachmann, D., Gockele, A., Guderle, M.,  
537 Landais, D., Piel, C., Escape, C., Devidal, S., Ravel, O., Buchmann, N.,  
538 Gleixner, G., Hildebrandt, A. & Roy, J. (2014) Functional diversity of leaf  
539 nitrogen concentrations drives grassland carbon fluxes. *Ecology Letters*, **17**,  
540 435-444.

541 Millar, A. (2004) Input signals to the plant circadian clock. *Journal of Experimental*  
542 *Botany*, **55**, 277-283.

543 Munger, L., Bleiholder, H., Hack, H., Hess, M., Stauss, R., Boom, T.v.d. & Weber, E.  
544 (1998) Phenological growth stages of the peanut plant (*Arachis hypogaea* L.)  
545 Codification and description according to the BBCH Scale – with figures.  
546 *Journal of Agronomy and Crop Science*, **180**, 101-107.

547 Niinemets, U. (2012) Optimization of foliage photosynthetic capacity in tree  
548 canopies: towards identifying missing constraints. *Tree Physiology*, **32**, 505-  
549 509.

- 550 O'Grady, A.P., Eamus, D. & Hutley, L.B. (1999) Transpiration increases during the  
551 dry season: Patterns of tree water use in eucalypt open-forests of northern  
552 Australia. *Tree Physiology*, **19**, 591-597.
- 553 Pinheiro, J.C. & Bates, D.M. (2000) *Mixed-Effects Models in S and S-PLUS*.  
554 Springer, Secaucus, NJ, USA
- 555 Resco de Dios, V., Loik, M.E., Smith, R.A., Aspinwall, M.J. & Tissue, D.T. (2016)  
556 Genetic variation in circadian regulation of nocturnal stomatal conductance  
557 enhances plant fitness. *Plant Cell and Environment*, **39**, 3-11.
- 558 Resco de Dios, V., Roy, J., Ferrio, J.P., Alday, J.G., Landais, D., Milcu, A. & Gessler,  
559 A. (2015) Processes driving nocturnal transpiration and implications for  
560 estimating land evapotranspiration *Scientific Reports*, **5**, 10975.
- 561 Resco, V., Hartwell, J. & Hall, A. (2009) Ecological implications of plants' ability to  
562 tell the time. *Ecology Letters*, **12**, 583-592.
- 563 Thomas, D., Eamus, D. & Bell, D. (1999) Optimization theory of stomatal behaviour.  
564 I. A critical evaluation of five methods of calculation. *Journal of Experimental*  
565 *Botany*, **50**, 385-392.
- 566 Tuzet, A., Perrier, A. & Leuning, R. (2003) A coupled model of stomatal  
567 conductance, photosynthesis and transpiration. *Plant, Cell and Environment*,  
568 **26**, 1097-1116.
- 569 Unsworth, M.H., Phillips, N., Link, T., Bond, B.J., Falk, M., Harmon, M.E.,  
570 Hinckley, T.M., Marks, D. & U, K.T.P. (2004) Components and controls of  
571 water flux in an old-growth Douglas-fir-western hemlock ecosystem.  
572 *Ecosystems*, **7**, 468-481.



- 573 Vico, G., Manzoni, S., Palmroth, S. & Katul, G. (2011) Effects of stomatal delays on  
574 the economics of leaf gas exchange under intermittent light regimes. *New*  
575 *Phytologist*, **192**, 640-652.
- 576 Wood, S.N. (2006) *Generalized Additive Models: An Introduction*. R. Chapman &  
577 Hall/CRC, Boca Raton, Florida.
- 578 Woodward, F.I. (2007) An inconvenient truth. *New Phytologist*, **174**, 469-470.
- 579 Yakir, E., Hilman, D., Harir, Y. & Green, R.M. (2007) Regulation of output from the  
580 plant circadian clock. *FEBS Journal*, **274**, 335-345.
- 581 Zhang, Q., Manzoni, S., Katul, G., Porporato, A. & Yang, D. (2014) The hysteretic  
582 evapotranspiration—Vapor pressure deficit relation. *Journal of Geophysical*  
583 *Research: Biogeosciences*, **119**, 2013JG002484.
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## 587 **Figures**

588 Figure 1: Circadian oscillation in the marginal water cost. The dots (with small SE  
589 bars hidden) indicate hourly values of assimilation ( $A_{\text{net}}$ ) stomatal conductance ( $g_s$ )  
590 and a parameter proportional to the marginal water cost of carbon gain ( $g_l$ ).  
591 Measurements were taken concomitantly to those under constant conditions reported  
592 in Fig. 2, although data from both days were pooled together to increase sample size.  
593 The white and black rectangles at the base indicate the subjective day (when it would  
594 have been daytime during entrainment) and subjective night, respectively, under  
595 constant conditions. Lines (and shaded error intervals) indicate the prediction (and  
596 SE) of Generalized Additive Model (GAM) fitting separately for each species (some  
597 lines may overlap), and portions which are not yellow indicate significant temporal  
598 variation.

599

600 Figure 2. Circadian regulation of leaf assimilation ( $A_{\text{net}}$ ) over stomatal conductance  
601 ( $g_s$ ). During entrainment, environmental conditions of Temperature ( $T_{\text{air}}$ ) and Vapor  
602 Pressure Deficit (VPD) mimicked those outdoors, with  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR (first 24  
603 h shown), and remained constant for 48 h starting at solar noon. The grey and white  
604 backgrounds indicates when PAR was at  $0 (\mu\text{mol m}^{-2} \text{s}^{-1})$  or higher, respectively.  
605 White and black rectangles at the base indicate the subjective day (when it would  
606 have been daytime during entrainment) and subjective night, respectively, under  
607 constant conditions. Dots represent measured values at each of three replicate  
608 macrocosms, and thick lines (and shaded error intervals) indicate the prediction (and  
609 SE) of Generalized Additive Mixed Model (GAMM) fitting separately for each  
610 species (some lines may overlap). GAMM best-fit line portions which are not yellow  
611 indicate significant temporal variation. Values in (b-d) were measured by the different

612 macrocosms every 15 minutes and values in (a) were measured every 4 hours with a  
613 portable photosynthesis system (the cuvette was set to match macrocosm conditions).  
614

**Table 1:** Quantification of the circadian-driven range in variation of diurnal gas exchange. The variation in fluxes under constant environmental conditions was derived from Generalized Additive Mixed Model predictions in Fig. 2.

Process	Species	Variation under constant conditions		
		Maximum	Minimum	% Change
$A_{\text{net}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	<i>P. vulgaris</i>	15.42	11.02	28.48
	<i>G. hirsutum</i>	16.97	9.73	42.65
$g_s$ ( $\text{mol m}^{-2} \text{s}^{-1}$ )	<i>P. vulgaris</i>	0.31	0.14	54.03
	<i>G. hirsutum</i>	0.41	0.063	84.85
$A_{\text{net}}/g_s$ ( $\mu\text{mol CO}_2 / \text{mol H}_2\text{O}$ )	<i>P. vulgaris</i>	95.37	51.17	46.35
	<i>G. hirsutum</i>	156.23	40.52	74.06



