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

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4 Víctor Resco de Dios^{* a b}, Arthur Gessler^{c d}, Juan Pedro Ferrio^b, Josu G
5 Alday^b, Michael Bahn^e, Jorge del Castillo^b, Sébastien Devidal^f, Sonia
6 García-Muñoz^g, Zachary Kayler^d, Damien Landais^f, Paula Martín-
7 Gómez^b, Alexandru Milcu^{f h}, Clément Piel^f, Karin Pirhofer-Walzl^d,
8 Olivier Ravel^f, Serajis Salekinⁱ, David T Tissue^a, Mark G Tjoelker^a,
9 Jordi Voltas^b, Jacques Roy^f

10

11 ^a Hawkesbury Institute for the Environment, Western Sydney University, Richmond,
12 Australia.

13 ^b Department of Crop and Forest Sciences-AGROTECNIO Center, Universitat de
14 Lleida, Lleida, Spain.

15 ^c Swiss Federal Institute for Forest, Snow and Landscape Research WSL Long-term
16 Forest Ecosystem Research (LWF), Birmensdorf, Switzerland.

17 ^d Institute for Landscape Biogeochemistry, Leibniz-Centre for Agricultural Landscape
18 Research (ZALF), Müncheberg, Germany.

19 ^e Institute of Ecology, University of Innsbruck, Innsbruck, Austria.

20 ^f Ecotron Européen de Montpellier, CNRS, Montferrier-sur-Lez, France.

21 ^g IMIDRA, Finca “El Encín”, Alcalá de Henares, Madrid, Spain.

22 ^h CNRS, Centre d’Ecologie Fonctionnelle et Evolutive Montpellier, France

23 ⁱ Erasmus Mundus Master on Mediterranean Forestry and Natural Resources
 24 Management, Universitat de Lleida, Lleida, Spain.
 25 * Corresponding author: Víctor Resco de Dios, Department of Crop and Forest
 26 Sciences-AGROTECNIO Center, Universitat de Lleida, E 25198 Lleida, Spain.
 27 v.rescodedios@gmail.com

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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 (λ) 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 λ . 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 CO₂ mol⁻¹ H₂O), 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 λ have been performed
68 under changing vapour pressure deficit, soil water, temperature or [CO₂] (Manzoni *et al.*
69 *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 λ . 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 λ ,
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 λ 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 CO₂ 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 CO₂ 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_{net}/g_s (intrinsic water use efficiency). This is
139 for the sake of clarity as A_{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 λ). However,
142 it is important to note that λ is not simply the inverse of water use efficiency (A/E)
143 because λ 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 CO₂
153 concentration) drivers were automatically controlled. The soil was extracted using

154 large lysimeters (2 m², 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, SO₃ 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 μmol m⁻² 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 10th July 2013, one month before the start of the measurements, and thinned to
178 densities of 10.5 and 9 individuals m⁻², respectively. Cotton (STAM-A16 variety by

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

During each experimental period, plants were entrained for five days under environmental conditions that mimicked the pattern observed in an average August sunny day in Montpellier in terms of T_{air} (28/19° C, diurnal max/min) and VPD. However, we kept radiation levels much lower (at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level) because previous research proposed that stomatal behavior should follow optimal theory when photosynthesis is light- (and not CO₂) limited (Medlyn *et al.* 2011), and our PAR values ensured this was the case. After 5 days of entrainment, we maintained environmental conditions constant starting at solar noon and for the next 48 h.

Measurements

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

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

Analyses

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

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

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

Results

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

254 The pattern in A_{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_{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

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

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

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

339

340 *Implications and mechanisms*

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

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

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

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

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

Conclusions

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

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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>.

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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_{net}) stomatal conductance (g_s)
590 and a parameter proportional to the marginal water cost of carbon gain (g_1).
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_{net}) over stomatal conductance
601 (g_s). During entrainment, environmental conditions of Temperature (T_{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_{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_{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