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Woody clockworks: circadian regulation of night-time water use in *Eucalyptus globulus*

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Summary

- The role of the circadian clock in controlling the metabolism of entire trees has seldom been considered. We tested whether the clock influences nocturnal whole-tree water use.
- Whole-tree chambers allowed the control of environmental variables (temperature, relative humidity). Night-time stomatal conductance (g_s) and sap flow (Q) were monitored in 6- to 8-m-tall *Eucalyptus globulus* trees during nights when environmental variables were kept constant, and also when conditions varied with time. Artificial neural networks were used to quantify the relative importance of circadian regulation of g_s and Q.
- Under a constant environment, g_s and Q declined from 0 to 6 h after dusk, but increased from 6 to 12 h after dusk. While the initial decline could be attributed to multiple processes, the subsequent increase is most consistent with circadian regulation of g_s and Q.
- We conclude that endogenous regulation of g_s is an important driver of night-time Q under natural environmental variability. The proportion of nocturnal Q variation associated with circadian regulation (23–56%) was comparable to that attributed to vapor pressure deficit variation (25–58%). This study contributes to our understanding of the linkages between molecular and cellular processes related to circadian regulation, and whole-tree processes related to ecosystem gas exchange in the field.

Introduction

It is widely established that molecular regulation of the circadian clock over the transcriptome drives diel fluctuations in photosynthesis, stomatal conductance and emissions of volatile organic compounds at the leaf level under controlled growth environments (Darwin, 1898; Hennessey *et al.*, 1993; Dodd *et al.*, 2004; Wilkinson *et al.*, 2006). However, whether circadian regulation of gas exchange in whole trees is present and significant has been scantly tested (Resco *et al.*, 2009). The circadian clock is entrained by the photoperiod, which is a deterministic function of geographic location and time of year. Therefore, gas exchange modeling could be simplified, at least potentially, if the circadian clock was a significant regulator of ecosystem gas exchange. More importantly, because the temporal pattern of circadian-driven gas exchange is correlated with that of exogenous environmental cues, current modeling approaches based upon the immediate physiological response of leaves to physical drivers could be returning a 'correct' answer for a partially 'erroneous' reason. That is, they would be attributing the full magnitude of the predicted variation in gas exchange to the immediate environment,

whereas in reality that response is partly the result of a circadian-driven pattern (Resco de Dios *et al.*, 2012).

For a rhythm to be considered circadian, it needs to express a *c.* 24 h period under constant environmental conditions. Enclosing whole trees or ecosystems within controlled environments to detect circadian gas exchange regulation remains challenging. Nonetheless, a handful of studies have provided circumstantial evidence of circadian regulation on net ecosystem exchange of CO_2 (Doughty *et al.*, 2006; Resco de Dios *et al.*, 2012) and isoprene emissions (Hewitt *et al.*, 2011) by combining filtering and modeling techniques to minimize environmental variation in the datasets. However, we still lack an unequivocal and direct test of circadian regulation of gas exchange in trees beyond the leaf level and in the field, or field-like, conditions.

The temporal pattern of nocturnal stomatal conductance is largely regulated by the circadian clock (Hennessey *et al.*, 1993; Lasceve *et al.*, 1997; Caird *et al.*, 2007; Easlon & Richards, 2009; Supporting Information, Fig. S1), in concert with atmospheric water demand, which is the gradient in vapor pressure from inside-to-outside of the leaf (Barbour & Buckley, 2007; Caird *et al.*, 2007). However, it remains untested whether this circadian

stomatal control is manifested in whole-tree water flux. The temporal pattern of nocturnal sap flux, in turn, is mainly driven by stomatal conductance, stem water recharge and atmospheric factors such as vapor pressure deficit (D) and wind speed (Dawson *et al.*, 2007; Phillips *et al.*, 2010). Overcast nights are often characterized by low and consistent D, while night-time atmospheric turbulence is often reduced relative to the daytime. Thus, if circadian control of night-time stomatal conductance (g_s) affects whole-plant water use (Q), increases in g_s and Q should be comparatively easy to detect on overcast and calm nights. More specifically, where variations in D and wind speed are negligible, nocturnal g_s should exhibit a temporal pattern similar to those reported over 24 h dark cycles (Fig. S1), and Q should follow the temporal pattern of g_s after a small time lag. It is also unknown whether circadian regulation of nocturnal g_s and Q occurs under variable environmental conditions. Recently, Resco de Dios *et al.* (2012) hypothesized that endogenous controls set a predictable, time-varying 'baseline' physiological capacity that could be modified by existing environmental conditions.

Understanding the mechanisms underlying variation in nocturnal water flux is important because, on average, 5–15% of transpiration occurs in the dark (Caird *et al.*, 2007; Phillips *et al.*, 2010), with much higher observations in desert plants (Ogle *et al.*, 2012). It is difficult to estimate nocturnal water loss at the ecosystem scale using common micrometerological techniques, given the typical lack of turbulence overnight (Fisher *et al.*, 2007). Large-scale estimates of the water balance thus need to rely upon models that are often parameterized and based upon diurnal controls. A differential daytime vs night-time control over the drivers of the water flux, such as nocturnal circadian regulation, would therefore compromise our current ability to predict water fluxes.

In this study, we tested the following predictions: (1) endogenous regulation of night-time g_s (1a) affects Q (1b); (2) inclusion of endogenous regulation improves model predictions of Q; and (3) endogenous regulation is observed during nights with low or high environmental variation in D. To further simplify the potentially confounding effect of two environmental drivers (D and wind speed) affecting Q, the experiment was performed in whole-tree chambers (Barton *et al.*, 2010) where wind speed was constant throughout the night. The whole-tree chambers allowed us to test the effect of circadian regulation of Q in 6- to 8-m-tall *Eucalyptus globulus* trees grown for 12 months under field-like environmental conditions (photoperiod, temperature, D), and thus subjected to a natural entrainment of the clock central oscillator.

Materials and Methods

Study site

The whole-tree chambers are located at the Hawkesbury Forest Experiment near Sydney, Australia (33°36′40′S, 150°44′26.5′E). These chambers can control air temperature (T) and D within 0.5°C and 0.3 kPa of the set-points (Barton *et al.*, 2010). The experiment is part of a broader research program that studies the effects of increasing CO_2 and temperature on *Eucalyptus globulus* (Labill.) production. Each whole-tree chamber enclosed one tree and was assigned a combined CO_2 concentration (ambient or +240 ppm) and an air temperature (ambient or +3°C) treatment. We measured only two of the broader experiment's three replicates of each of the four CO_2 and temperature treatments. The impact of these climate change factors on endogenous regulation of nocturnal transpiration was not an important aspect of this study. Indeed, differing growth conditions were largely ignored within our design by normalizing the response of each tree, relative to its mean response, before comparing values across individuals. This design was appropriate because there is no evidence to indicate that CO_2 concentration alters circadian regulation, and the temperature compensation of endogenous regulation that might have occurred under elevated growth temperatures was not germane to our study.

Seedlings of *E. globulus* were planted in September 2010 and grown for 12 months before experiment inception. By then, tree height ranged from 6 to 8 m and base diameter ranged from 5.6 to 8.4 cm. The area is characterized by a subhumid temperate climate, with mean annual temperature, T, and precipitation of 17°C and 801 mm, respectively. Soils inside the chambers were regularly watered to field capacity. Full details on site characteristics and chamber design are given in Barton *et al.* (2010).

Leaf-level measurements

Stomatal conductance (g_s) was measured with four portable photosynthesis systems (LI-6400; Li-Cor Inc., Lincoln, NE, USA). Endogenous regulation of g_s was tested by monitoring g_s under 'constant' environmental conditions. We maintained variation of T and D inside the cuvette below 0.1°C and 0.2 kPa, respectively (Fig. 1), by setting the LI-6400 block temperature to 15°C and the desiccant to full bypass. In addition, we set T and the relative humidity in whole-tree chambers to 15°C and 80%, respectively. One leaf per tree was measured. To diminish the safety constraints associated with working at heights during the night, the leaves were haphazardly chosen between those present at ground level. Measurements were performed on two separate nights between late September and mid-October 2011. Leaves were left within the LI-6400 cuvette for the entire night (*c.* 18:00–06:00 h), defined as photosynthetically active radiation (PAR) below 1 μmol m^{-2} s^{-1}. Measurements were logged every 2 min. To compare values across trees growing under different environmental conditions, we normalized g_s values by dividing the value of g_s for individual i at time t by the mean g_s for individual i. Leaf water potential was measured on a leaf per tree every 3 h using a pressure bomb (PMS-1000; Plant Moisture Stress Instrument Company, Albany, NY, USA) on the same nights as g_s.

To compare the magnitude of the effect of endogenous regulation over g_s with D-driven variation over g_s, we measured the response of g_s to different D conditions in six individuals by either setting the block temperature in the leaf cuvette to 15°C and then changing the desiccant from full bypass to full scrub, or by setting the desiccant to full bypass and varying the temperature from 10 to 20°C. Measurements were logged 1 h after
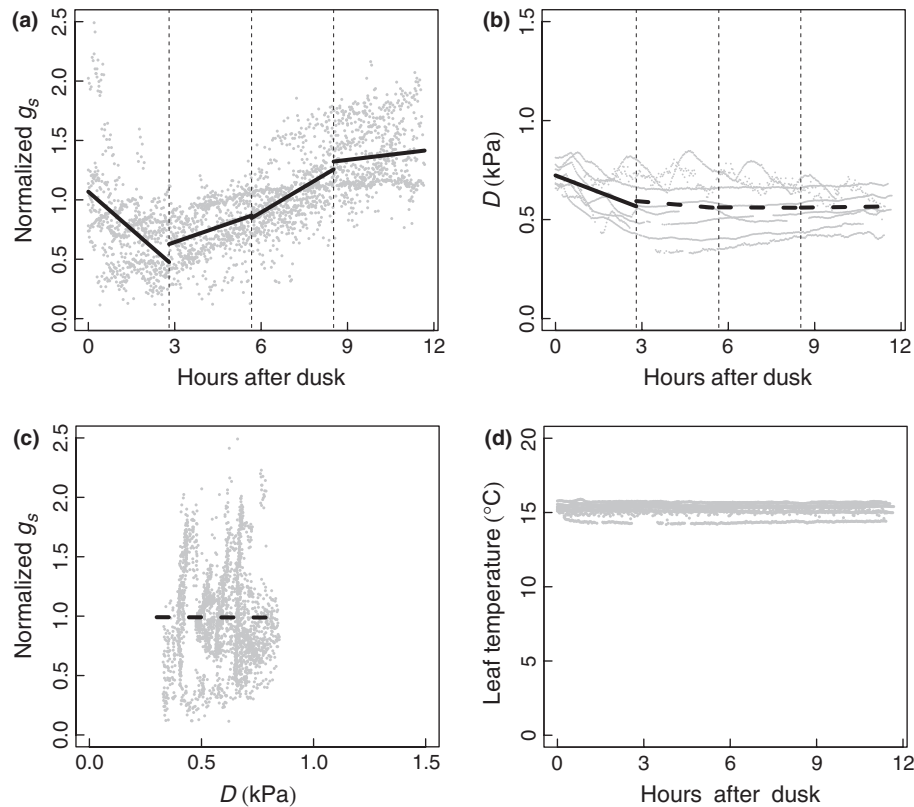
Fig. 1 Leaf-level stomatal conductance under a constant environment. We monitored the temporal pattern of stomatal conductance (g_s) in *Eucalyptus globulus* overnight (a) under 'constant' environmental conditions, that is, limited variability in the leaf cuvette block vapor pressure deficit, D (b), and temperature, T (d). Temporal trends in (a) and (b) were analyzed with the Self-Organizing Linear Output map (SOLO), which provides a flexible fitting routine by assuming no functional relationships between explanatory and dependent variables. SOLO will create different groups of data for the explanatory variable (h after dusk) depending on the number of nodes defined, and will then fit an independent line through each of the different groups. Because we were only interested in examining potential statistical differences across every c. 3 h, only four nodes were used (as indicated by the dotted lines). A simple linear regression was used to define the relationship between normalized g_s and D (c). Regardless of the fitting procedure, slopes significantly different from 0 (at $P < 0.05$) are marked by solid lines and those not significantly different from 0 by dashed lines. The temporal pattern in g_s variation under this constant environment is consistent with reports of endogenous g_s regulation driven by the circadian clock (Fig. S1). Because the trees had been growing under different environmental conditions, g_s was normalized before comparisons across individuals by dividing the g_s value for individual i at time t by the mean average g_s of individual i overnight. Units for normalized g_s are thus relative ($n = 8$ trees).

altering humidity or temperature to each of those two levels. One leaf per tree was measured between 23:00 and 04:00 h, which corresponded to 5–10 h after dusk, on two consecutive nights in late October.

To understand if measurement errors (arising from the low nocturnal flux rates) propagated through our dataset, we monitored g_s for 3–5 h in empty cuvettes, where g_s was not expected to be significantly different from 0, and then assessed the slope of the regression between g_s and D, which was also expected to be indistinguishable from 0. In the empty cuvettes, g_s was at least one order of magnitude lower than the values observed when a leaf was inside the chamber. Stomatal conductance was significantly < 0 in two instruments (-2.66 ± 0.57 and -3.4 ± 0.65 mmol m^{-2} s^{-1}, mean $\pm$ 95% CI), not significantly different from 0 in one instrument (-0.35 ± 1.77 mmol m^{-2} s^{-1}), and significantly higher in the last instrument (1.37 ± 0.3 mmol m^{-2} s^{-1}). The slope of the regression between g_s and D was never significantly different from 0, and the 95% CIs (calculated using the Markov Chain Monte Carlo method for linear regression) ranged from -3.54 to 12.08, -3.54 to 2.67, -0.27

to 2.69, and -6.47 to 3.85 mmol m^{-2} s^{-1} kPa^{-1} for each instrument. Given these results, we concluded that leaf observations were reliable and that a general correction was not required. Therefore, we only discarded some clearly unreliable measurements (i.e. negative g_s values, or values higher than two times the SE) from the continuous monitoring under constant conditions (as seen by the straight lines in Fig. S2) and the D-response curve (which has 15, instead of 24 (four treatments × six individuals) measurement points).

Sap flow measurements

Sap velocity in *E. globulus* was measured with the heat ratio method (HRM30; ICT International Pty Ltd, Armidale, NSW, Australia). Probes were installed on 13 September 2011; measurements began 2 d later and continued until 6 November 2011. Three 3.5-cm-long probe needles were installed at 0.6 cm distance between each other, below the lowest branch (c. 10.2 cm above the ground), and after removing the 0.5–0.8 cm thick bark. Only one set of sensors was installed per tree, given the

relatively small tree diameter at implant height. All sensors were oriented towards the west.

Sap velocity was measured every 10 min and transformed to whole-tree water use (Q in $l\,h^{-1}$) following Burgess *et al.* (2001). We corrected for wounding by assuming a 1.8 mm wound diameter, and for radial variability by dividing total sapwood area into concentric rings at the midpoints of each of the two measurement depths (1.25 and 2.75 cm from the probe hub) following Burgess *et al.* (2001). Trees were felled at experiment termination, in order to measure wood density (0.39–0.49 g cm^{-3}) and moisture content (0.61–0.68 g cm^{-3}). Wood density and moisture content were determined by measuring the fresh mass of small stem discs (2 cm thick with bark removed) near the site previously occupied by probe needles, the displacement method was used to determine volume, and dry mass was determined after oven-drying at 70°C for 2 d. Zero flow corrections were made by measuring sap flux for 1–7 d after tree felling (depending on tree), and subtracting these values to those measured during the experiment. Visual examination of the cut stem indicated that only sapwood was present within the woody tissue of these young trees.

Our objective was to test for endogenous regulation of whole-tree water use in a natural environment. Thus, instead of subjecting individuals to an arbitrary set of restricted environmental conditions, we compared the temporal pattern of Q during nights with little variation in D (ΔD), with Q during nights characterized by a marked gradient in ΔD. Therefore, nights representing our 'constant' environment were those nights with a ΔD so small that the coefficient of determination of the linear regression model between Q and D was minimal. Conversely, selected nights with a marked gradient in ΔD were those when maximal ΔD was observed.

Statistical analyses and modeling

We tested our first prediction (endogenous regulation of nighttime g_s affects Q) by examining temporal patterns under constant conditions. Temporal patterns were evaluated with the artificial neural network known as the Self-Organising Linear Output (SOLO) linear map (Hsu *et al.*, 2002). We used SOLO for our time series analyses because it was specifically designed to identify complex temporal patterns and it provides a flexible environment, where no *a priori* functional relationship between dependent and independent variables is assumed. SOLO organizes the dataset into different nodes based on similarity between the values of the explanatory variable, and fits individual regression lines through each group. Four nodes were used in this analysis, because we were interested in broad temporal patterns, that is, changes in the slope of g_s or Q vs time every 3 h (cf. Figs 1, 2).

We tested our second prediction (improved model fitting under consideration of circadian regulation), by comparing the performance of SOLO based solely on D, on time, or on both D and time for all the nights of the experiment. An increased number of nodes was used (25 nodes) because we attempted to maximize the goodness of fit of the model. We wanted to ensure high accuracy and precision in the prediction of Q as a function of D,
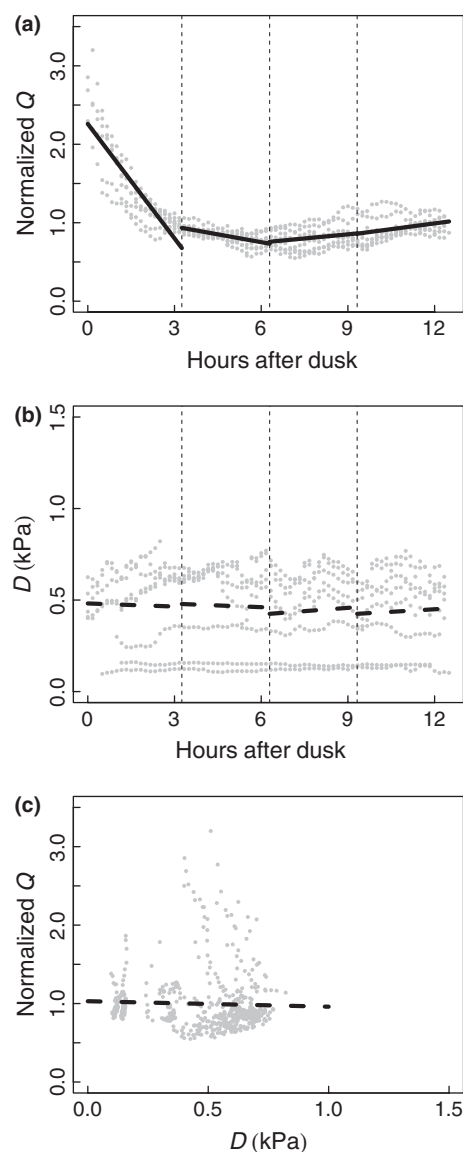

Fig. 2 Normalized whole-plant water use under a constant environment. The temporal pattern of normalized total water used (Q, a) in *Eucalyptus globulus* overnight under limited temporal variation in vapor pressure deficit (D, b) was characterized by marked decrease in the first 6 h after dusk, and an increase after midnight, resulting in no significant relationship between Q and D (c). Temporal patterns (a, b) were analyzed using SOLO as in Fig. 1, where the dotted lines indicate the different groups of data. Regardless of the fitting procedure, slopes significantly different from 0 (at $P < 0.05$) are marked by solid lines and those not significantly different from 0 are marked by dashed lines. These results are consistent with circadian regulation of g_s affecting whole-tree water use. We normalized Q values (as for stomatal conductance in Fig. 1) before statistical analyses to ensure comparability between individuals grown under different environmental conditions. Units for normalized Q are thus relative ($n = 8$ trees).

so that we could obtain a conservative estimate of the importance of circadian controls when they were later introduced into the model.

We acknowledge that describing endogenous regulation in a Q model as a function of time is generally unsatisfactory because it is not a mechanistic model. However, this has been a common

approach in studies of circadian regulation of gas exchange, given the current lack in understanding of the underlying physiological mechanisms (Williams & Gorton, 1998; Hewitt *et al.*, 2011). Moreover, we feel this approach is justified because D and wind speed are currently considered to be the two main atmospheric drivers of nocturnal Q (Phillips *et al.*, 2010), but wind speed variation was negligible within the whole-tree chambers (the circulating fans within the whole-tree chamber ensured a constant coupling of the tree with the surrounding atmosphere), variation in Q after midnight was not dependent upon stem refilling (as described in further detail in the Discussion section) and thus, other than D (which was a parameter in the model), no other factors known to affect Q covary with time.

Our third prediction, that endogenous regulation is observed during nights with low and high ΔD, was tested by examining the correlation between the residuals of the D-based Q model with time, and then assessing the variation in the R^2 of this correlation with ΔD; and by comparing the slope of the regression between Q and D (dQ/dD) at different times during a single night at approximately midnight (6.0–6.5 h after dusk) and at the end of the night near predawn (10–10.5 h after dusk), and separately for nights with low and high ΔD (< or > 0.5 kPa, respectively). The comparison of dQ/dD across time depends upon comparing Q values within a similar range of variation in D. We thus restricted the analyses of dQ/dD to nights showing comparable D-values at predawn and at midnight, which ranged between 0.03 and 0.74 kPa for nights with low ΔD and between 0.03 and 1.12 kPa for nights with low ΔD.

Results

Prediction 1a: endogenous regulation of stomatal conductance

The slope of the temporal pattern of normalized g_s under a constant environment was significantly negative ($-0.21 \pm 0.017\,h^{-1}$, mean $\pm$ SE) during the first c. 3 h after dusk, and significantly positive afterwards. The highest positive slope occurred during 6–9 h ($0.14 \pm 0.01\,h^{-1}$), and was followed by a significantly lower (yet positive) slope during c. 9–12 h after dusk ($0.04 \pm 0.01\,h^{-1}$, Fig. 1a). The initial decline in g_s coincided with a significant decline in D during c. 0–3 h ($-0.05 \pm 0.004\,kPa\,h^{-1}$, Fig. 1b), but the slope of the temporal pattern in D was not significantly different from 0 during c. 3–6, 6–9 or 9–12 h after dusk (t-test, $P > 0.05$). This mismatch in the pattern of temporal variation between g_s and D led to a lack of significant correlation between these variables (Fig. 1c, $P = 0.29$). We normalized g_s, which has relative units, to compare values across individuals that had been exposed to different growth conditions (see Fig. S2 for absolute values).

The magnitude of variation in absolute g_s under constant D (8–36 mmol m^{-2} s^{-1} across all trees, Fig. S2) was larger than that observed by artificially changing D from 0.4 to 1.6 kPa (2–25 mmol m^{-2} s^{-1}) in a response curve (Fig. S3). In turn, the range of variation in D (1.6–0.4 = 1.2 kPa; Fig. S3) in these response curves was significantly larger (t-test, $P < 0.0001$) than the average variation of D at the research site, which ranged from 0.20 ± 0.15 to 0.70 ± 0.07 kPa over the study period (Fig. 4). Leaf water potential showed no clear temporal pattern and only varied between -0.3 and -0.6 MPa (Fig. S4).

Prediction 1b: endogenous regulation of total water use

Normalized Q under a constant environment showed a generally similar temporal pattern to that of normalized g_s (Figs 2a, 3a): a significant decrease during c. 0–3 h ($-0.48 \pm 0.02\,h^{-1}$), and a significant increase between c. 6–9 ($0.03 \pm 0.01\,h^{-1}$) and 9–12 h after dusk ($0.05 \pm 0.01\,h^{-1}$). The only differences from the g_s pattern were that Q showed a significant decline during c. 3–6 h ($-0.06 \pm 0.007\,h^{-1}$) and the rate of increase in Q was not significantly different between c. 6–9 and 9–12 h after dusk (t-test, $P = 0.19$). No clear temporal pattern was observed in either D or T (Figs 2b, 3c) and normalized Q was not significantly correlated with D ($P = 0.34$, Fig. 2c).

Under a 'changing' environment (i.e. nights with maximal D variation), Q, D and T continuously declined overnight
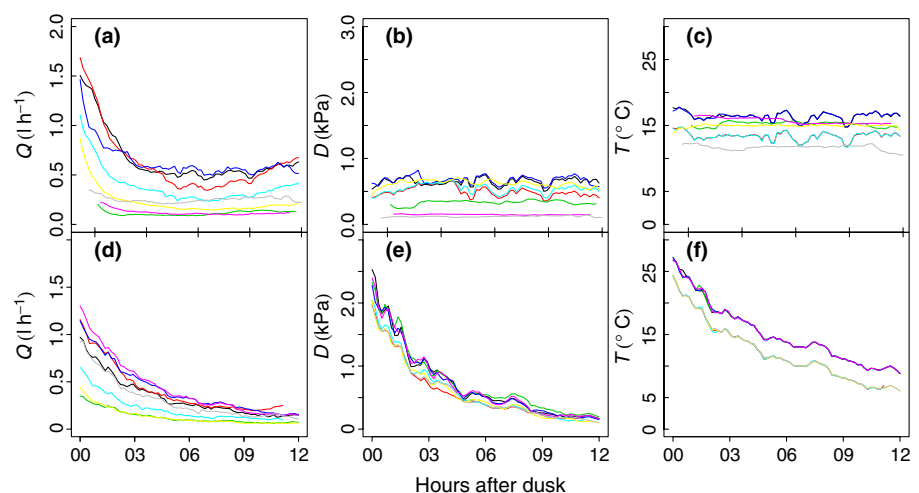

Fig. 3 Absolute whole-plant water use under a constant and a changing environment. (a–f) Temporal evolution of absolute water use (Q), vapor pressure deficit (D) and air temperature (T) under constant conditions (a–c), or under a changing environment (d–f) in *Eucalyptus globulus*. Different colors indicate different trees ($n = 8$ trees; some values may be hidden when values overlap).

(Fig. 3d–f). The average magnitude of variation in absolute Q under constant conditions after midnight ($0.32 \pm 0.06\,1\,h^{-1}$) was significantly higher (t-test, $P = 0.04$) and doubled that under a changing environment ($0.16 \pm 0.02\,1\,h^{-1}$, Fig. 3). The range in D over both conditions was significantly different (t-test, $P < 0.0001$), ranging from 0.2 (± 0.04) kPa in the constant environment to 2.06 (± 0.07) kPa under a changing environment.

Prediction 2: considering endogenous regulation improves model fitting

Adding time to the D-based neural network model of Q decreased the value of the Akaike information criterion (AIC, an index of model parsimony, with lower values indicating higher parsimony) significantly (-7488 to $-10\,213$ averaged across all trees), increased R^2 by 23% (0.58–0.81), and decreased the root-mean-square error (RMSE) of the model from 0.088 to 0.058 (Table 1). When the Q model was run with time as the only explanatory variable, we observed smaller predictive capability in that AIC, R^2 and RMSE were -7195, 0.56 and 0.094, respectively (Table 1).

Prediction 3: endogenous regulation is observed during nights with low and high ΔD

We observed an exponential decline in the importance of time to explain residual variation in the D-based Q model depending on the degree of environmental variability. Time explained between 42 and 77% of the residual variation at ΔD of $c.$ 0 kPa, and the percentage of residual variation stabilized at $\Delta D > 0.5$ kPa between 6 and 22% (depending on tree, Fig. 4).

Temporal changes in the slope of the regression between Q and D were not significantly altered by ΔD being $<$ or > 0.5 kPa (Fig. 5). The slope was always significantly higher (t-test, $P < 0.05$) at predawn than at midnight.

Discussion

We observed that endogenous regulation of stomatal conductance affected whole-tree water use during the night as expected
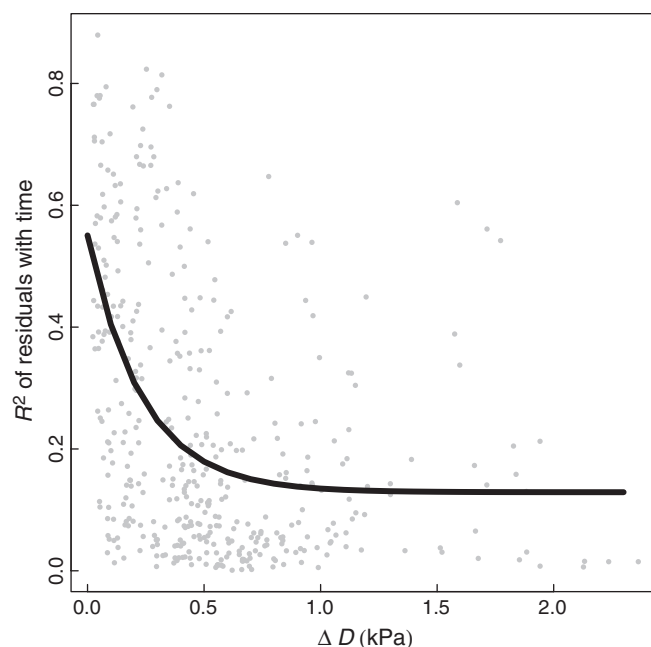

Fig. 4 Effect of time on model residuals as a function of environmental variability. To obtain a model of sap flow (Q) based on vapor pressure deficit (D) and with high predictive capacity, we parameterized the Self-Organising Linear Output (SOLO) artificial neural network, which has been proposed to serve as the benchmark of land surface models, separately for every night and tree in this study. The residuals of this model were correlated with time (via a first-degree Fourier regression: residuals $= a + b\cos(t) + c\cos(t) + \varepsilon$) and the R^2 of this correlation is shown on the y-axis. The x-axis indicates the range in variation in D for every single night (ΔD). The values in the y-axis diminished as ΔD increased, and remained relatively low and stable after $\Delta D > 0.5$ kPa. This result would seem to indicate that endogenous regulation of water fluxes is only important during nights showing small variation in D in *Eucalyptus globulus*. However, we argue that this is an artifact arising from the fact that D has a very marked temporal pattern when there is a large ΔD and, thus, any model of total water use that is based on D is indirectly capturing endogenously driven Q variation, as shown in Fig. 5.

from circadian-driven regulation. The magnitude of the variation in g_s under nearly constant D was significantly larger than that observed when D declined strongly from dusk to dawn. This endogenous regulation of nocturnal g_s, in turn, led to
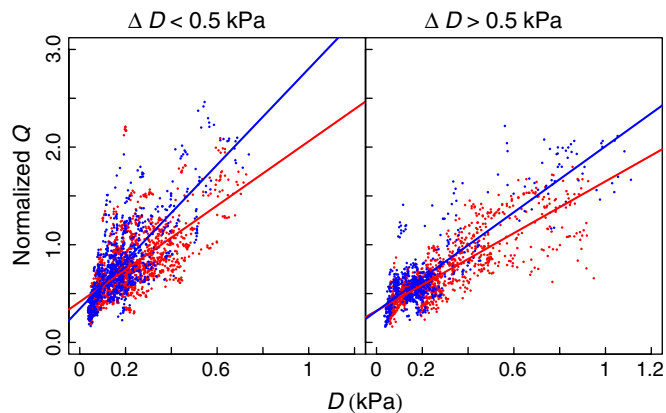
Table 1 Effects of circadian regulation on model fitting. Akaike information criterion (AIC), R^2 and root-mean-square error (RMSE) of Self-Organising Linear Output (SOLO) parameterized with 25 nodes as a function of vapor pressure deficit (D), time (t), or D and t, for each of the eight *Eucalyptus globulus* individuals studied

Tree	AIC			R^2			RMSE		
	$f(D)$	$f(t)$	$f(D, t)$	$f(D)$	$f(t)$	$f(D, t)$	$f(D)$	$f(t)$	$f(D, t)$
1	-4974.337	-6242.824	-7562.346	0.406	0.588	0.722	0.116	0.097	0.080
2	-4399.624	-3912.096	-8338.481	0.649	0.596	0.889	0.127	0.136	0.071
3	-11562.756	-13250.818	-14054.965	0.338	0.593	0.682	0.045	0.035	0.031
4	-7407.925	-4348.206	-9323.616	0.777	0.462	0.874	0.082	0.127	0.062
5	-8237.002	-8715.989	-11256.917	0.522	0.584	0.803	0.073	0.068	0.047
6	-5265.913	-5120.876	-8119.154	0.627	0.611	0.838	0.112	0.114	0.074
7	-11979.244	-11821.716	-14763.110	0.596	0.577	0.821	0.042	0.043	0.028
8	-6075.427	-4147.732	-8283.651	0.697	0.472	0.842	0.099	0.131	0.072
Average	-7487.779	-7195.032	-10212.780	0.576	0.560	0.809	0.087	0.094	0.058

Fig. 5 Effects of clock regulation on plant water use. We compared the slopes of normalized sap flow (Q) in response to vapor pressure deficit (D) for eight *Eucalyptus globulus* trees between nights with little variation in D ($\Delta D < 0.5$ kPa) and nights with large variation in D ($\Delta D > 0.5$ kPa). We observed that the slope of this regression was always significantly higher (t-test, P-value < 0.05) at predawn (●; 04:30–05:00 h) than at midnight (●; 00:00–00:30 h). This shows the effect of endogenous regulation over the whole-plant water flux, independent of the degree of environmental variation. Lines indicate the result of linear fitting.

endogenously controlled variation in Q that was significantly larger than the variation in Q during nights with a maximum range in D.

Mechanisms underlying endogenous regulation

Although the initial decline in g_s during the first 3 h after dusk coincides with the observed pattern of g_s under constant conditions and is thus consistent with reports of circadian regulation of g_s in the dark (Fig. S1), it could also be explained by the decline in D (given the positive slope of the g_s vs D relationship in this species, Fig. S2) or by the delayed kinetics of stomatal relaxation in response to darkness (Raschke, 1970). However, the only physiological mechanism known and tested to date that can explain the stomatal opening in the period c. 3–12 h after dusk is the circadian clock (Fig. S1). We therefore conclude that the circadian clock is the most plausible driver of the increase in g_s between c. 3 and 12 h after dusk.

Even if g_s and Q were both measured independently of each other, both consistently indicated the effect of circadian regulation across different scales. The temporal pattern of variation in Q under constant conditions was only slightly different from that of g_s. Q had a much stronger decline over the first 3 h of the night than g_s, the decrease continued until 6 h after dusk, and this initial decline in Q was not accompanied by a decline in D. The initial decrease in Q could be related to a sluggish stomatal response and/or stem water recharge occurring early in the night. In a previous experiment in these whole-tree chambers, Zeppel *et al.* (2011) observed stem refilling in *Eucalyptus saligna* up to 5 h after dusk. Under constant conditions, Q started to rise 6 h after dusk, which was slightly later than g_s (which started to rise 3 h after dusk). This delay in the

increase of Q may be the result of the combination of: a decrease in stem refilling during the first hours of the night; the commonly observed lag in sap flow behind leaf- or canopy-level conductance (Granier *et al.*, 1996); and the fact that g_s and D are both relatively small overnight (and time to develop a relatively large change in g_s would thus be necessary before an effect on Q might be detectable). However, the most plausible driver of the increase in Q c. 6–12 h after dusk is the effect of clock-driven controls over g_s.

We do not expect that varying patterns of rehydration or gradients in water potential from roots to leaves have a significant effect on the circadian pattern observed in our experiment (c. 6–12 h after dusk; Figs 2, 3). This is because transpiration increasingly relies on stored water reserves with increasing soil water limitation. As stated in the Materials and Methods section, our plants were regularly well watered. Hence, the volumetric contribution of capacitance to transpiration during the day, and posterior refilling of emptied capacitors during the night, should have been minimal to 0. Secondly, refilling of capacitors decreases from late afternoon into the night, which would explain, at least partly, the continuous decline in Q observed in the first 6 h after dusk (Fig. 2). However, it can be excluded that the increase of Q later in the night is related to refilling, as this process, if existent, would have led to the opposite pattern (a continuous decrease in Q overnight). However, we can envisage that rehydration kinetics have the potential to overwrite circadian dynamics under certain circumstances – for instance, in trees with very large capacitors, or once severe drought stress is affecting plant hydraulic functioning and refilling cannot be completed during the night-time (Pfautsch & Adams, 2013).

Lack of increased predawn stomatal opening has been found in *Arabidopsis* mutants with disrupted rhythmicity (Dodd *et al.*, 2004). Similarly, *Arabidopsis* mutants with starch deficiency do not show enhanced g_s later in the night (Lasceve *et al.*, 1997). These results suggest that circadian regulation of nocturnal g_s may be mediated by starch metabolism, presumably through the formation of osmolites for guard cell regulation (Caird *et al.*, 2007).

Circadian rhythms are often considered adaptive, as they anticipate predictable environmental changes, such as the onset of dawn or dusk (Yerushalmi & Green, 2009). For example, g_s often shows a delayed response to radiation, and increasing predawn g_s may reduce this time lag, potentially increasing the efficiency of carbon assimilation shortly after dawn (Mansfield & Heath, 1961). Alternatively, increased night-time transpiration later in the night may enhance oxygen delivery for parenchyma respiration at a period when oxygen concentrations would be critically low otherwise (Gansert, 2003; Daley & Phillips, 2006), or could facilitate nutrient uptake by mass-flow (Gessler *et al.*, 2002).

Quantifying the importance of endogenous controls

Circadian regulation of Q significantly decreased uncertainty in our D-based Q model. The proportion of the variance in the

model explained by D and time was 23% higher than that explained by D alone. However, this does not imply that endogenous regulation 'only' explains an additional 23% of the variability in Q. As a tentative upper bound on the relative importance of time, we ran the Q model with time as the sole independent variable and obtained $R^2 = 0.56$, which indicates that circadian regulation could potentially explain between 23% and up to 56% of the variation in Q, which is comparable to the 25–58% of the variation in Q explained by D. Disentangling the effect of the clock from that of D in affecting Q is necessarily confounded by the very marked temporal pattern of D, which implies that any D-based model of Q is already indirectly capturing at least part of the endogenous, time-varying, regulation of Q.

There would seem to be only a limited role for endogenous controls over Q under variable environmental conditions, as the proportion of residual variation of the D-based model explained by time is relatively small when ΔD exceeds 0.5 kPa (Fig. 4). We argue that this decrease in the proportion of residual variation explained by time with ΔD occurs because variation in D has a marked temporal pattern and our D-based model is indirectly capturing this endogenous control. Indeed, the slope of the linear regression between Q and D was always significantly larger at predawn (10 h after dusk) than at midnight (6 h after dusk), regardless of whether ΔD was larger or smaller than 0.5 kPa (Fig. 5), indicating an endogenous control of Q independent of the degree of environmental variation. As previously mentioned, refilling for these *Eucalypt* trees has been reported to finish by 23:00 h, which is 5 h after dusk (Zeppel *et al.*, 2011). Thus, stem refilling could not have contributed to differences in this slope.

The marked temporal pattern of D, and its strong correlation with Q, may thus be masking the possibility that Q is controlled by a combination of endogenous and exogenous factors. An important research priority is thus to disentangle clock- from D-driven regulation of stomatal conductance and sap flux, as the temporal pattern of D could lead current models, based on the direct effects of the physical environment on leaf physiology, to produce a 'correct' result for a partially 'erroneous' reason. Overall, this could potentially compromise the ability to predict fluxes under future nonanalog, novel conditions.

Interspecific variation on the degree to which the clock regulates leaf-to-whole-tree gas exchange and water use is likely to occur (Doughty *et al.*, 2006). However, with the possible exception of trees with fairly large capacitances or requiring a long time for stem water recharge (Pfautsch & Adams, 2013), current theoretical and empirical evidence suggests widespread circadian regulation of g_s, affecting Q and its modeling on trees. First, daily rhythmicity is nearly universal, with light and day–night transition signaling occurring through the phytochromes. Indeed, the transcriptional feedbacks and genetics underlying circadian regulation in higher plants have been relatively conserved across evolution (Harmer, 2009). In the case of circadian regulation of g_s, reciprocal regulation between the key clock component TOC1 and the putative abscisic acid receptor

ABAR might be the mechanistic basis for a fine-tuned switch to modulate the plant sensitivity to ABA and thus stomatal control during the diel course (Legnaioli *et al.*, 2009). Secondly, an increase in g_s late in the night has been widely reported across multiple taxa in the literature (reviewed by Caird *et al.*, 2007; Ogle *et al.*, 2012).

Implications

The observation of a circadian control over g_s that scales up to affect whole-tree water use is important in several ways. First, it implies that the control of the central clock oscillator over the transcriptome ultimately affects the regulation of water use in whole trees. This finding is in strong contrast with our current understanding of biosphere–atmosphere interactions, which relies largely on the direct physiological responses to environmental drivers. That is, it indicates an additional physiological regulation that has been poorly appreciated and recognized but that helps to integrate large trees.

Secondly, previous studies on the amplitude and period of circadian regulation of leaf gas exchange were conducted in artificial and highly controlled conditions inside a growth chamber. Here, we selected to track sap flux during cloudy nights that naturally undergo little variation in environmental conditions, to show that the stability in environmental variation required for circadian regulation also occurs in nature. In other words, circadian regulation of gas exchange is a naturally occurring phenomenon affecting whole trees in the field, or under field-like environmental variation.

Future research directions

Evidence suggests that the circadian clock may be a key regulator of diurnal fluxes of water, carbon and volatile organic compound (VOC) emissions over entire ecosystems. Price & Black (1989) noted that time of day was the parameter that better explained the residuals of diel fluctuations in a g_s model based on Penman–Monteith (Monteith & Unsworth, 2008) and parameterized for a conifer forest. By focusing on night-time water flux, we demonstrated that the circadian clock partially regulates whole-tree water use, and recent studies present indirect evidence that the clock may also regulate net ecosystem exchange of CO_2 (Doughty *et al.*, 2006; Resco de Dios *et al.*, 2012) and isoprene emissions (Hewitt *et al.*, 2011) between terrestrial ecosystems and the atmosphere. Considering the impact of circadian clock regulation on model output, we propose a more thorough examination of how the clock regulates ecosystem fluxes. The challenge is that we can only ensure circadian regulation after environmental conditions have remained constant over a 24 h cycle, and enclosing whole trees or ecosystems within chambers that maintain constant values of light, temperature and other physical drivers is logistically complex.

We propose further research (Fig. 6) to effectively bridge the gap between our relatively robust knowledge of circadian regulation at the transcriptome level and our limited understanding at the ecosystem level:
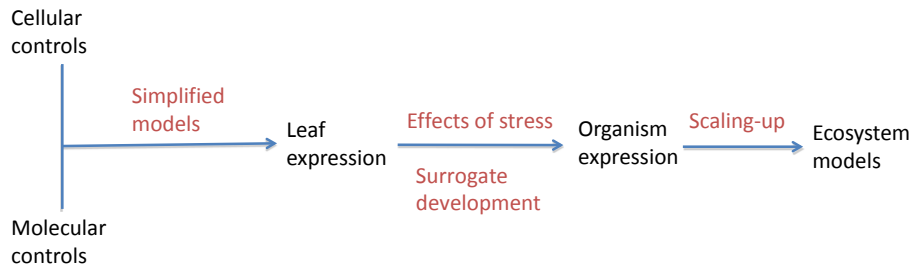
Fig. 6 Future research directions. Conceptual figure on the future research priorities to understand the effect of circadian regulation over the exchanges of water, carbon and volatile organic compounds (VOCs) between terrestrial ecosystems and the atmosphere. We propose the joint development by molecular biologists and physiologists, along with ecosystem scientists and land surface modelers, of mechanistic but simplified models of circadian regulation over leaf-level fluxes. There is a paucity of studies on how circadian regulation of leaf gas exchange is affected by biotic and abiotic stresses, which also constitutes an important research priority. To scale up from leaves to organisms, we advocate an exploration of whether nocturnal water flux may serve as a surrogate for circadian regulation in daytime water, carbon and VOC fluxes. We will then be able to effectively scale and model circadian regulation of gas exchange from the transcriptome to the ecosystem, which would avoid current pitfalls and has the potential to simplify biosphere–atmosphere interactions

(1) We suggest the simplification of current mechanistic models of circadian action from the molecular to the leaf scale (Dalchau, 2012), so that they can be readily incorporated into large-scale models without computational limitations or excessive data demand for parameterization.

(2) We propose examination of the impact of drought, shade, insect attacks or other abiotic and biotic stresses on the expression of the circadian clock at high levels of organization.

(3) We suggest the development of whole-tree chambers where ambient light can be controlled to effectively mimic outdoor conditions. Alternatively, but more speculatively, to diminish logistic and financial constrains, we propose exploring the use of nocturnal water fluxes as a surrogate or model system to facilitate research on circadian effects at the organismal level. That is, we propose further studies to examine whether the expression of circadian regulation over whole-tree water use during the night will indicate, and be correlated with, the expression of circadian regulation of whole-tree diurnal water use, carbon assimilation and partitioning and isoprene emissions. Subsequently, we can predict the occurrence and magnitude of the circadian oscillation in these three processes based on circadian regulation of nocturnal water flux. Addressing circadian regulation of biological processes using night-time sap flux is advantageous because it has considerably smaller constraints than maintenance of daytime environmental conditions required to unravel circadian control over water, carbon and VOC fluxes. Considering that all circadian rhythms are driven by a single central oscillator (McClung, 2011), it seems feasible that studying one process driven by circadian regulation could serve as a surrogate for other processes.

(4) We propose integrating simplified models of circadian regulation at the leaf level, with empirical tests on the effect of stresses on circadian controls, to inform scaling to ecosystem fluxes and models.

Given that the circadian clock is driven by the photoperiod, which deterministically varies with place and time, gas exchange modeling could be simplified in the long term, at least potentially, if the circadian clock was a widespread and significant regulator of ecosystem gas exchange.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Thirty-six-hour cycle of stomatal conductance under constant dark conditions.

Fig. S2 Temporal pattern of stomatal conductance under constant conditions without normalization.

Fig. S3 Response of normalized stomatal conductance to changing vapor pressure deficit.

Fig. S4 Temporal pattern of predawn water potential.

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