Field study of endogenous and exogenous contributions to diurnal stomatal conductance rhythms in *Eucalyptus pauciflora* saplings

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Abstract

Internal circadian rhythms and exogenous factors such as light intensity, vapour pressure deficit (VPD) and wind speed affect diurnal cycles of photosynthetic capacity and gas exchange in plants. Stomatal conductance of *Eucalyptus pauciflora* Sieber ex Spreng saplings was monitored in leaves both *in situ* and excised in a darkroom for 24–48 hours. We tested for evidence of endogenous rhythms by examining the change in stomatal conductance with the microclimate variables photosynthetic photon flux density (PPFD), VPD and wind speed. *In situ*, saplings showed clear diurnal cycles of stomatal conductance and increased stomatal conductance pre-dawn on only one of the two measurement mornings. Leaves placed in continuous darkness took over four hours to decrease stomatal conductance to night-time levels and failed to increase stomatal conductance for the next natural photoperiod. Variation in stomatal conductance was found to have a high correlation with light intensity variation ($R^2 = 0.6736$), but low correlation with VPD and wind speed ($R^2 = 0.0116$ & $R^2 = 0.1694$). Our results conflict on whether stomatal conductance is regulated by circadian rhythms in *E. pauciflora*, however it is possible that with a longer study and more replicates that circadian regulation would be confirmed.

Key Words

Circadian clocks, light intensity, microclimates, vapour pressure deficit, wind speed

Introduction

Plant activity, with few exceptions, exhibits diurnal cycles which usually respond to both exogenous and endogenous signals. Circadian rhythms describe the portions of these cycles, such as photosynthesis and water use, that are endogenously regulated. Plant properties such as flowering time and the range of temperatures plants can survive are also influenced by circadian rhythms (McClung 2006; Resco de Dios *et al.* 2009). As such, circadian rhythms offer proven fitness advantages to plants if rhythms match natural light intensity trends (McClung 2006). By definition, circadian cycles need to be driven by endogenous factors and therefore continue in free-running conditions (continuous light or darkness). They must also have a cyclic period of about 24 hours. Models that ignore endogenous factors are generally inadequate for describing the responses of plants to changing climates (Resco de Dios *et al.* 2012) and therefore circadian rhythms could be important for understanding plant functionality and meeting conservation goals.

Eucalyptus pauciflora is the only tree in mainland Australian to persist over 1,600 m elevation and up to the tree line. It is therefore a key species in the subalpine environment. This environment contains many cold and snow-cover specialised species and yet makes up less than 0.15 per cent of the country's land area (Costin *et al.* 2000). Plants here are adapted to life close to the limits of survival. They often rely upon snow cover for protection from the extreme cold during winter and experience mean maximum temperatures as low as 10°C in the warmest month (Costin *et al.* 2000). They have a short summer growing season and trees in particular face large challenges in collecting and maintaining biomass at high altitudes (Costin *et al.* 2000). Furthermore, climate change models project reduced snowfall and increasing temperatures in the Australian Alps (Worboys and Good 2011), which will leave trees exposed to a wider range of extreme temperatures.

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Several Australian studies into circadian rhythms have focused on *Eucalyptus* species (Resco de Dios *et al.* 2013a; Resco de Dios *et al.* 2013b) but not *E. pauciflora,* and few studies worldwide seem to focus on circadian rhythms in alpine or subalpine plant species. There is no indication in the available literature that circadian rhythms will be any different in these scenarios (Resco de Dios *et al.* 2012), but confirming this might provide insight into plant functionality at climate extremes and possible climate change responses in these plants.

Another gap in the literature exists in testing the importance of circadian rhythms in the field. Most studies are conducted with model species in laboratories with a completely controlled climate and strict 12-hour photoperiods (Resco de Dios *et al.* 2012). We have aimed to combine examination of diurnal rhythms in the field with a simple free-running test. In such a test, light incidence, wind speed and other climate factors are controlled compared to field conditions to eliminate possible exogenous drivers of a chosen plant property. Like many studies before, we use stomatal conductance to indicate diurnal rhythms.

Stomatal conductance is a key determinant of the availability of carbon dioxide for photosynthesis. Therefore, stomatal conductance usually increases with light intensity during the day, such that plants maximise carbon availability and photosynthetic capacity at times of high light (Korner and Cochrane 1985). However, higher stomatal conductance also leads to plant water loss and therefore stomatal conductance decreases with the onset of night to conserve water. Late in the night, stomatal conductance usually increases again in ‘anticipation for dawn’ (Resco de Dios *et al.* 2013a). This pre-dawn response may be used to indicate endogenous regulation is occurring in field samples. Stomatal conductance is also highly dependent on other environmental conditions, decreasing with vapour pressure deficit (VPD) and wind speed, especially in drought conditions (Caldwell 1970; Resco de Dios *et al.* 2013a). It is thus important to monitor these variables in field studies of stomatal conductance.

We aimed to examine the contributions of exogenous and endogenous factors to regulating the diurnal cycles of stomatal conductance in *E. pauciflora*. Additionally, we hypothesised that under free-running conditions (constant darkness) these rhythms would continue, indicating the importance of the circadian clock in stomatal regulation.

Methods

Site and sapling choice, treatments and time of experiment

Five field growing saplings of *Eucalyptus pauciflora* Sieber ex Spreng were chosen behind the Southern Alps Ski Lodge, Charlotte Pass Village, NSW (1,765 m elevation, 36°26'S, 148°20'E). The saplings were chosen for apparent health and were all within 1 to 2.5 m in height. The three treatments were: saplings sampled *in situ*, dark treated excised branches and excised branches kept in natural light conditions outdoors. F_v/F_m values, as a proxy for leaf health, were ascertained to be above 0.75 (no units) for all 25 leaves selected for treatments using a Hansatech photosynthetic efficiency analyser (PEA). Branches for dark treatment were cut from trees on Day 1 at about 1pm, stems placed in water and kept in a darkroom for about 24 hours. Measurements started from 5pm the day of harvest and continued for 21 hours. The branches for the natural light treatment were harvested in the same manner on Day 2 at about 9pm. These branches were kept outdoors at the site of the other saplings and measurements were taken for about 24h.

Stomatal conductance measurements

Stomatal conductance (mmol m^{-2} s^{-1}) was measured via vapour flux at approximately 3h intervals with an SC-1 Leaf Porometer (ICT International, Australia), desiccator attached. Two leaves per sapling were measured for the *in situ* field saplings and natural light treatment and one leaf each for the dark treatment. Due to the variance of outside conditions, stomatal conductance was measured twice per leaf for outdoor samples. The exact time of each measurement was logged and then grouped into 1-hour sections. When leaves were wet the best of care was taken to dry them before measurement but due to the sensitivity of the equipment to water this may have affected some results.

Microclimate variables

Microclimate variables were also measured at the time of stomatal conductance measurements, at each sapling or treatment site. Using a Kestrel portable weather meter (Kestrel Instruments, USA), the average wind speed was measured over a 30-second period (km/hr) as well as the ambient temperature (°C) and relative humidity (%). Light intensity was measured as photosynthetic photon flux density (PPFD) with a Li-Cor quantum flux meter (µmol m^{-2} s^{-1}, Li-Cor, USA). Vapour pressure deficit was calculated from temperature and relative humidity by the formulae below.

Mathematical formulae (from Maciel *et al.* 2016)

Saturation vapour pressure (SVP) calculated from temperature (T):

$$SVP\ (kPa) = \frac{610.7 \times 10^{7.5T/(237.3+T)}}{1000} \tag{1}$$

Vapour pressure deficit (VPD) calculated from SVP and relative humidity (RH):

$$VPD\ (kPa) = \frac{100 - RH}{100} \times SVP \tag{2}$$

Statistical analysis

A Student *t*-test was used to compare the differences in stomatal conductance between pre-dawn and 11pm the same night (both nights) and pre-dawn on Day 2 and Day 3. Measured values of microclimate variables between 8pm and 5am were compared between the two nights via Student *t*-tests. Pearson correlations were used to test the relationship between each microclimate variable and stomatal conductance from the field sapling measurements.

Results

Field samples showed the expected cyclic changes in stomatal conductance, with peaks at midday and depressions during the night (Figure 1). Anticipation of the dawn, via increasing stomatal conductance pre-dawn, was apparent only on the second dawn (see 19 and 43h, Figure 1). Upon the second dawn, stomatal conductance was higher than at 11pm the previous evening ($p = 0.001$) and higher than at the first dawn ($p = 0.002$). Wind speed, VPD and VPD's constituents – ambient temperature and relative humidity – were significantly different on the two nights (all $p < 0.001$ except relative humidity which was $p = 0.044$), possibly contributing to the different pre-dawn responses. Whilst overall VPD was significantly lower on the first night, VPD measured at the first dawn was higher than on the second dawn (Figure 2). Wind speed was different between the early parts of each night but similar in the pre-dawn period (Figure 3).

For cut branches under natural light stomatal conductance rose during the day, particularly between 8am and 3pm (shown between 46 and 53h in Figure 1). High variance in these measurements may be attributed to the rain on Day 3. The mean stomatal conductance of cut branches at midday on the third day (14h after excision) was approximately half that of the field saplings. In the dark treatment, 14 hours after excision coincides with 2–3am, before the beginning of the next natural photoperiod. There was no indication of a pre-dawn increase in conductance in these cut branches.

The dark treated leaves maintained levels of stomatal conductance comparable to the field saplings for the initial 12 hours logged but failed to increase again for daylight hours on the second day. After four hours of dark treatment, leaves still showed levels of stomatal conductance similar to the field saplings outside (shown at 8 h in Figure 1). The stomatal conductance was low overnight and maintained this level for the remaining natural daylight hours. This suggests that light rather than endogenous rhythms controls stomatal conductance.

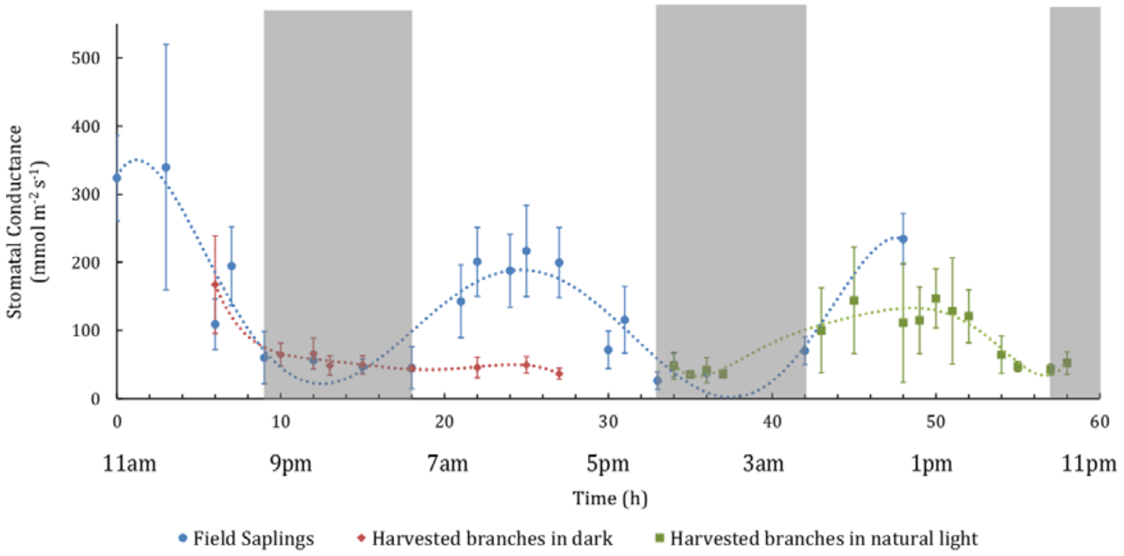

Figure 1: The stomatal conductance of leaves over experiment time for each of the three treatments. The x-axis represents the 60h period in which measurements were taken: from 11am on 12 December to 11pm on 14 December 2016. Shaded sections represent times of darkness of approx. 8pm to 5am (10–19h, 34–43h and 58–60h). Each datum was averaged with values logged within the same hour (e.g. values logged between 5 and 6pm were averaged separately to those logged between 6 and 7pm). Error bars are standard deviation for that hour.

VPD varied between 0.4 and 2.0 kPa across the field saplings experiment with a mean of 1.1 kPa (Figure 2). VPD also varied in the dark treatment as temperature and relative humidity could not be held constant. Wind speeds were relatively low for the area (mean 4.3 and range 0–12 km h^{-1}) and highly variable (Figure 3). They were highly dependent on gusting in the 30 seconds over which measurements were made. PPFD was the only variable to explain a significant proportion of the variance in stomatal conductance in the field saplings. PPFD was 0 throughout the night, the mean daytime value was 660 µmol m^{-2} s^{-1}, and the maximum reading was 1995 µmol m^{-2} s^{-1} on Day 1. As expected, stomatal conductance increased linearly with PPFD (Figure 4). The R^2 value was 0.6736, indicating that approximately 67 per cent of the variance in stomatal conductance may be attributed to changes in PPFD. Wind speed showed some explanation of the variance in stomatal conductance in correlation tests ($R^2 = 0.1694$) but VPD showed almost none ($R^2 = 0.0116$).

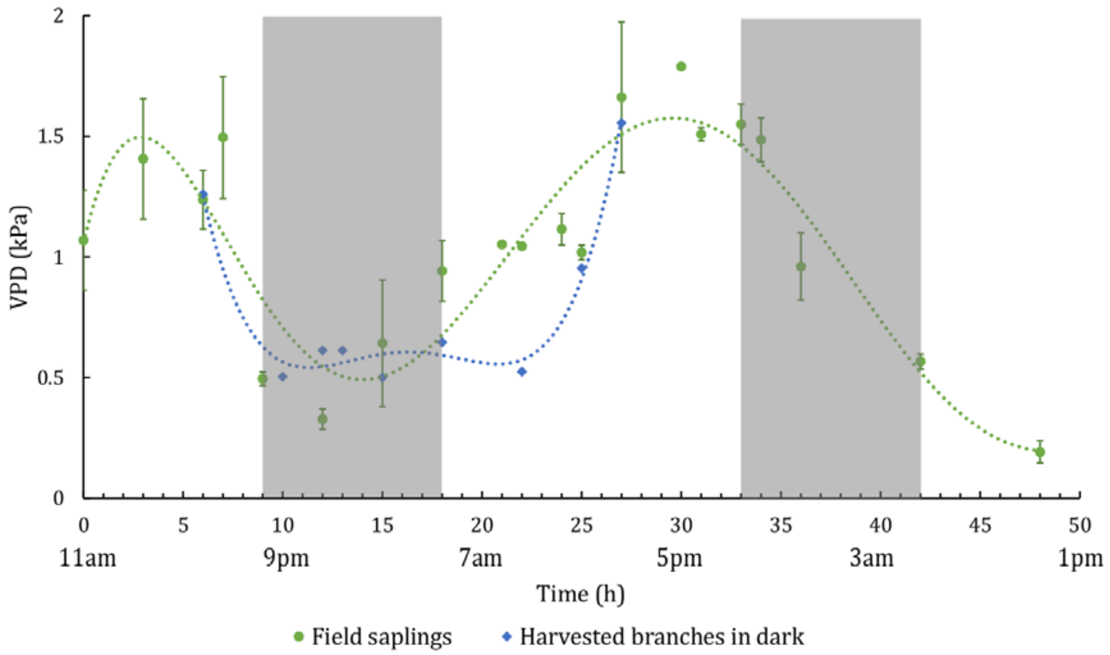

Figure 2: Vapour pressure deficit (VPD) over the field sapling and dark treatments. The time x-axis represents and shading as per Figure 1. Error bars are standard deviation and shown for field saplings only because only one measurement per time segment was taken for dark treatment. Note that the VPD in the field saplings is much lower overall for the first night than the second although the first dawn (right border of the shaded section) has a higher VPD than the second. VPD between environments was generally similar.

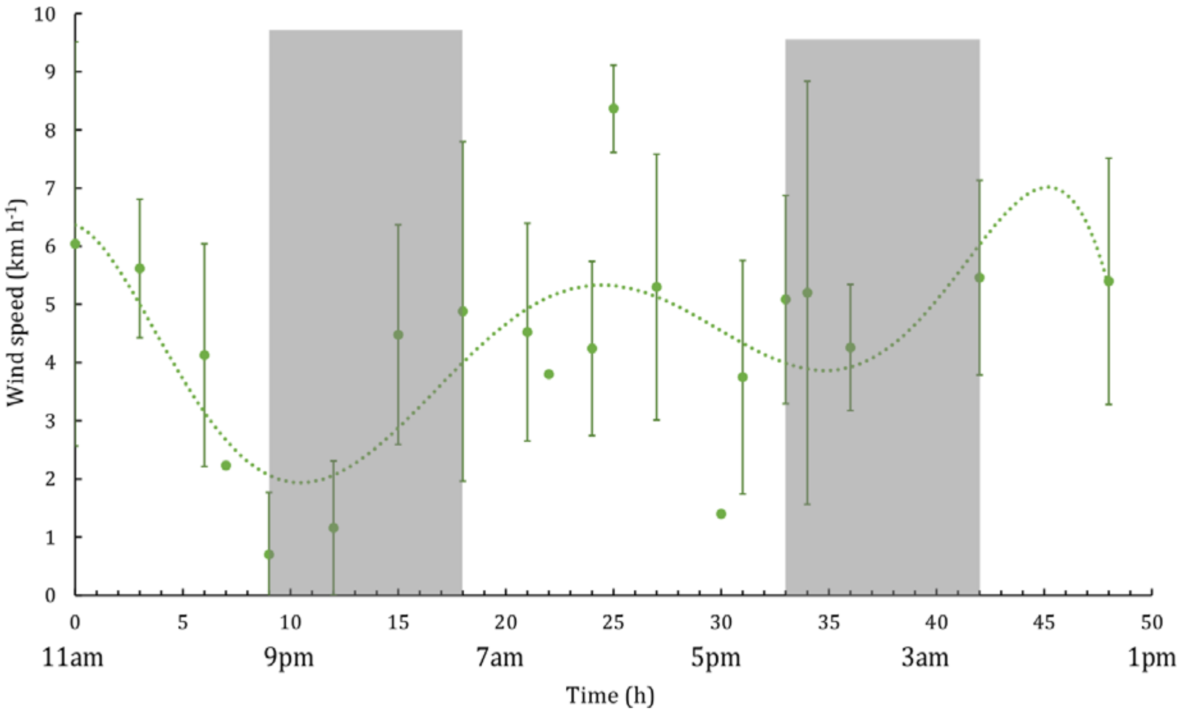

Figure 3: Wind speed as measured over the course of the field sapling experiment. The time x-axis represents and shading as per Figure 1. Error bars are standard deviation from the mean at each hour segment. Wind speed was highly variable but overall quite low.

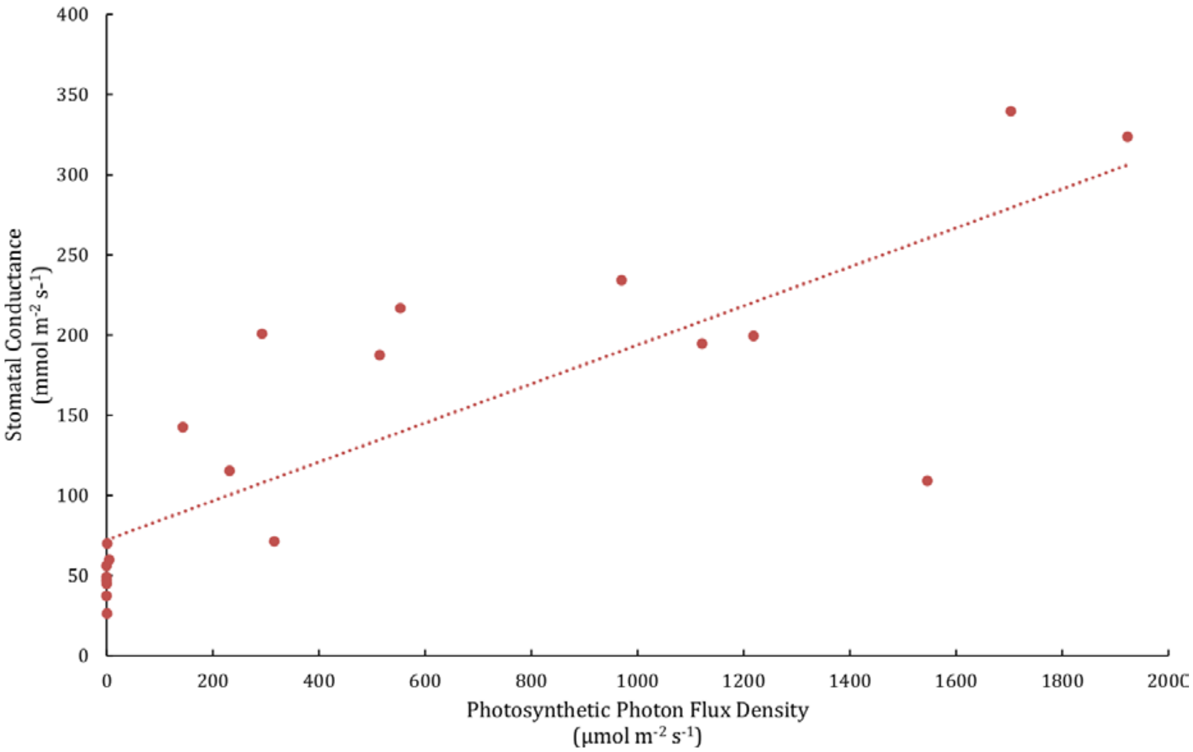

Figure 4: Stomatal conductance of field saplings increased with PPFD (Photosynthetic Photon Flux Density). R^2 = 0.6736

Discussion

Circadian rhythms are generally thought to regulate stomatal conductance in conjunction with exogenous factors such as light intensity, wind speed and VPD. For *E. pauciflora* in natural conditions, we found that light intensity was the only variable of these to have a strong predictive power on stomatal conductance. Regulation via endogenous factors was not clear under constant dark free-running conditions, possibly due to artefacts of the excision method. A pre-dawn increase in stomatal conductance, suggestive of endogenous regulation, was observed in natural conditions on uncut branches, but only on one of the two mornings.

Effect of microclimate variables on stomatal conductance

It has previously been established that stomatal conductance decreases with wind speed (Caldwell 1970). Though we measured wind speed the measurements were highly variable due to gusts and we did not find that they were associated with changes in stomatal conductance. As the field site was fairly sheltered it is reasonable to expect that stomata would not respond to these wind speeds (Caldwell 1970); however, that does not rule out wind speed as a factor for *E. pauciflora* at windier sites such as ridgelines or under more constant high wind conditions. Stomatal conductance is determined by VPD in water-limited circumstances (Resco de Dios *et al.* 2009). Natural run-off water was continuously moving through the study area and so saplings were unlikely to have been water stressed. Thus, the lack of correlation between VPD and stomatal conductance is understandable. We did, however, find a strong correlation between stomatal conductance and light intensity, which was expected due to the close link between stomatal conductance and photosynthesis.

Circadian rhythms of stomatal conductance

Following this study, the question of whether circadian cycles of stomatal conductance occur in *E. pauciflora* remains unresolved. Results conflict on whether diurnal rhythms of stomatal conductance are circadian (i.e. endogenous) or are responding only to light intensity.

The inconsistent response of stomatal conductance pre-dawn is the first piece of evidence against circadian regulation. Studies on *Eucalyptus* species and many other plants agree that stomatal conductance increases pre-dawn in response to circadian rhythms (Resco de Dios *et al.* 2009; Resco de Dios *et al.* 2013a; Resco de Dios *et al.* 2013b) which we cannot verify in this experiment. The same studies concede, however, that there is little information on the significance of circadian regulation in the field. Therefore the exogenous factors such as changing VPD and extended summer photoperiod (3h longer than usual laboratory conditions) may help explain these results. Wind speed was relatively similar pre-dawn and so in this case probably did not contribute to the difference. VPD was higher pre-dawn on the first day, perhaps causing the belated response; however, as we have already mentioned VPD is usually only correlated with stomatal conductance in low water scenarios (Resco de Dios *et al.* 2009). Further examination of the different pre-dawn responses observed here is necessary to clarify this question.

The second factor opposing the circadian hypothesis is the cessation of the diurnal stomatal conductance cycle in continuous darkness. Total dark free-running experiments often experience severe dampening or only a single cycle indicating circadian regulation (Martin and Meidner 1971). Additionally, the performance of our excised natural light control indicated the harvesting procedure dampened leaf responses. Before the beginning of the next natural photoperiod in the dark treatment, stomatal conductance might already have been reduced by the excision procedure. Combined dampening due to both the dark treatment and the excision procedure is possible and may have masked any stomatal conductance changes due to endogenous rhythms. Regardless the cycle cannot be called circadian without evidence of its continuation in free-running conditions.

The two factors that hinted at circadian regulation are the existence of at least one pre-dawn response and the slow midday response to darkness. Without the stimulus of light, any increase in stomatal conductance before dawn has no explanation as yet, except that it must be driven by an internal clock, hence a circadian rhythm (Resco de Dios *et al.* 2013a). Studies claim that circadian rhythms are responsible for slower responses to darkness at midday than at dusk (Martin and Meidner 1971). Even so, the response of *E. pauciflora* was slower than might be expected, taking more than 4 hours to reduce stomatal conductance to night-time levels. To confirm this result, response time to darkness (or light) at different times of day should be included and compared.

The smallest modification we would recommend to the dark treatment used here would be harvesting and initiating treatment at night. This would ensure that branches are kept in unnatural conditions for the least possible time before the expected circadian increase in stomatal conductance at dawn. It would be even more beneficial to include a continuous light treatment as studies on circadian rhythms commonly use light rather than dark because multiple cycles can be observed with less significant dampening (Martin and Meidner 1971).

Wider significance

If, upon further study, stomatal conductance turns out to be not under circadian control in *E. pauciflora* then the evolutionary and functional consequences are vast. Why should a plant with a short growing season and limited by cold temperatures not attain the fitness advantage inherent in circadian anticipation of photoperiod? If *E. pauciflora* does regulate stomatal conductance via circadian rhythms this might explain how it can survive the restrictions of its environment. If, however, as our results suggest, use of circadian rhythms to regulate stomatal conductance is conditional, this calls into question the usefulness of laboratory studies on these properties. Modelling for future climates would need to be adjusted so that circadian rhythms are expressed only some of the time.

Conclusions

In summary, our ability to draw conclusions from these findings is limited by the low number of replicates and short space of time for measurement. Under natural conditions, *E. pauciflora* did show a diurnal cycle of stomatal conductance, correlated strongly with light intensity but not with other exogenous factors. Evidence of a slow midday response to darkness hints at circadian regulation, but the inability to produce a continuation of the cycle in free-running conditions contradicts this hypothesis. Given a new experiment, it is likely that circadian regulation of stomatal conductance in *E. pauciflora* will be confirmed. However, if stomatal conductance is not in this case regulated by circadian rhythms, other processes may be. One question raised from the field results is why were the pre-dawn responses different between the two days? Could circadian regulation pre-dawn be conditional on exogenous factors?

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