Genetic variation in circadian regulation of nocturnal stomatal conductance enhances carbon assimilation and growth

Running head: On the function of nocturnal stomatal conductance

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ABSTRACT

Circadian resonance, whereby a plant's endogenous rhythms are tuned to match environmental cues, has been repeatedly shown to be adaptive, although the underlying mechanisms remain elusive. Concomitantly, the adaptive value of nocturnal transpiration in C_3 plants remains unknown because it occurs without carbon assimilation. These seemingly unrelated processes are interconnected because circadian regulation drives temporal patterns in nocturnal stomatal conductance, with maximum values occurring immediately before dawn for many species. We grew individuals of six *Eucalyptus camaldulensis* genotypes in naturally-lit glasshouses and measured sunset, predawn, and midday leaf gas exchange, and whole-plant biomass production. We tested whether sunrise anticipation by the circadian clock and subsequent increases in genotype predawn stomatal conductance, led to rapid stomatal opening upon illumination, ultimately affecting genotype differences in carbon assimilation and growth. We observed faster stomatal responses to light inputs at sunrise in genotypes with higher predawn stomatal conductance. Moreover, early morning and midday stomatal conductance and carbon assimilation, leaf area and total plant biomass were all positively correlated with predawn stomatal conductance across genotypes. Our results lead to the novel hypothesis that genotypic variation in the circadian-regulated capacity to anticipate sunrise could be an important factor underlying intraspecific variation in tree growth.

Keywords: adaptation; anticipation hypothesis; circadian clock; CO_2; biomass enhancement; gas exchange; genotype; memory; nocturnal transpiration; stomata.

INTRODUCTION

The circadian clock is an endogenous timer of metabolic processes that regulates transcriptional activity over time in the cells of plants and other organisms. There is ample evidence indicating that such circadian regulation is adaptive (Dodd *et al.*, 2005, Johnson & Kyriacou, 2006, Resco *et al.*, 2009b, Vaze & Sharma, 2013, Yerushalmi & Green, 2009). Circadian resonance, whereby a plant's endogenous rhythmicity is finely tuned to match environmental cues, leads to increased fitness and, conversely, fitness suffers from increasing circadian dissonance (Green *et al.*, 2002, Went, 1960). However, understanding the processes by which circadian regulation confers a fitness advantage has proven more challenging. Earlier hypotheses discussed the role of circadian regulation in shifting photophobic processes, such as DNA replication or cell division, to the nighttime period ("escape from light" hypothesis; Pittendrigh, 1993); or the separation of mutually incompatible metabolic processes, such as photosynthesis and nitrogen fixation in cyanobacteria (Mitsui *et al.*, 1986). Alternatively, the "anticipation" hypothesis, whereby circadian regulation anticipates highly predictable environmental cues and prepares cellular metabolism accordingly, remains the most common explanation for the growth and fitness advantage provided by circadian clocks. However, direct tests of this hypothesis are rare (Goodspeed *et al.*, 2012) and, as previously noted by Johnson & Kyriacou (2006), "like many other evolutionary ideas that are eminently reasonable – remains just a good idea".

Circadian regulation, in conjunction with direct physiological responses to vapor pressure deficit and other environmental drivers, is involved in the exchange of water vapor and carbon dioxide between leaves and the atmosphere, through the regulation of stomatal conductance and photosynthesis (Hennessey *et al.*, 1993, Resco

67 de Dios *et al.*, 2012). An unresolved question is whether circadian regulation, which
68 enhances water loss throughout the night, could be adaptive because there is no
69 carbon assimilation in the dark (Bucci *et al.*, 2004, Dawson *et al.*, 2007, Resco de
70 Dios *et al.*, 2013b). Indeed, the temporal pattern of nocturnal stomatal conductance,
71 largely driven by circadian rhythms, is often characterized by decreased stomatal
72 conductance in the first hours of darkness, followed by significant increases later in
73 the night, and reaching a peak immediately before dawn (Hennessey *et al.*, 1993,
74 Resco de Dios *et al.*, 2013a, Resco de Dios *et al.*, 2015).
75 One of the mechanisms hypothesized to be involved in the endogenous rise of
76 nocturnal conductance is the availability of starch because starch deficient
77 *Arabidopsis* mutants did not show enhanced predawn stomatal conductance (Lasceve
78 *et al.*, 1997). Indeed, nocturnal stomatal conductance has been reported to increase in
79 response to elevated CO_2 (Zeppel *et al.*, 2012), which is contrary to the daytime trend,
80 and increases in starch availability under elevated CO_2 could be the mechanism
81 underlying such positive response of nocturnal stomatal conductance to CO_2 (Easlon
82 & Richards, 2009). However, he generality of this observation is still under
83 examination (Resco de Dios *et al.*, 2013b), and recent studies question the relevance
84 of carbohydrates for regulating the endogenous rise in stomatal conductance (Resco
85 de Dios *et al.*, 2015).
86 There is ample evidence of genetic variation in nocturnal stomatal
87 conductance and transpiration within species (Christman *et al.*, 2008, Phillips *et al.*,
88 2010, Schoppach *et al.*, 2014). The cause and fitness implications of intra-specific
89 variation in nocturnal water use remain uncertain, but associations between circadian
90 regulated nighttime water use, daytime gas exchange rates, and plant fitness proxies
91 across genotypes may provide insight into the mechanisms and

92 evolutionary/ecological significance of variable nocturnal leaf gas exchange within
93 species.
94 Here, we tested whether circadian increases in predawn stomatal conductance
95 promote increased carbon assimilation and enhanced growth across six different
96 *Eucalyptus camaldulensis* genotypes. We first tested whether the different genotypes
97 varied in predawn stomatal conductance. We then hypothesized that genotype
98 predawn conductance would be positively correlated with daytime stomatal
99 conductance (Christman *et al.*, 2008, Drake *et al.*, 2013), and that the time to respond
100 to early morning radiation inputs would be shorter when stomata are "primed" prior to
101 sunrise. Finally, we tested whether genotypic variation in stomatal priming was
102 related to genotype fitness by examining the relationship between genotype predawn
103 conductance and three fitness proxies: carbon assimilation, leaf area and total
104 biomass. Experiments were conducted under two CO_2 concentrations (ambient and
105 elevated), to test whether higher predawn conductance under elevated CO_2 is
106 correlated with changes in the availability of carbohydrates (Easlon & Richards, 2009,
107 Zeppel *et al.*, 2012). Although environmental conditions of CO_2 or other
108 environmental drivers may alter the magnitude of nocturnal conductance (Barbour &
109 Buckley, 2007), its temporal pattern and predawn values are driven by circadian
110 regulation (Hennessey *et al.*, 1993, Resco de Dios *et al.*, 2013a, Resco de Dios *et al.*,
111 2015).
112
113 **MATERIALS AND METHODS**
114 **Plants and growing conditions**
115 Seedlings from six different genotypes of *E. camaldulensis* subsp. *camaldulensis* were
116 prepared from clonal hedges by the Commonwealth Scientific and Industrial Research

Organization (See Supporting Information Table S1 for full details on provenances). The hedges were half-sib seedlings originating from provenances representing different geographic and climatic origins. After reaching an average height of 24.6 cm (±0.97; SE) and a basal diameter of 1.86 mm (±0.07), genotypes were transplanted into 6.9 L cylindrical pots and grown at the naturally-lit (with 20% reduction of incident radiation) glasshouse facilities of the University of Western Sydney in Richmond, New South Wales, in southeastern Australia. Each pot contained 7.5 Kg of coarse textured soil (supplied by Australian Native Landscape, Richmond NSW, Australia), with a pH of 6.5. To ensure that no nutrient limitations occurred, the plants were fertilized every fortnight with a commercial liquid fertilizer (500 ml Aquasol, at 1.6 g l^{-1}; 23% N, 4% P, 18% K, 0.05% Zn, 0.06% Cu, 0.013% Mo, 0.15% Mn, 0.06% Fe, 0.011% B; Yates Australia, Padstow, NSW, Australia). Plants were grown in ambient (400 ppm) or elevated (640 ppm) CO_2 concentrations, and were randomly assigned to one of two glasshouse bays per CO_2 treatment, within a randomized block design. Pots were rotated between (monthly) and within (weekly) glasshouse bays to further reduce potential glasshouse bay effects on plant performance. Air temperatures (25:17 °C, average day:night) and relative humidity (45:60%) were representative of average summer values in Richmond. The pots were daily watered to field capacity (until water drained from the bottom of the pot). Further details on glasshouse design and set-up are given by Ghannoum et al. (2010). No differences in height and diameter occurred between genotypes at experiment initiation (with $P > 0.05$, ANOVA).

Measurements and statistical analyses

Leaf gas exchange was measured with 4 cross-calibrated portable photosynthesis systems (LI-6400XT, Li-Cor Inc, Lincoln, USA) two months after treatments were started in the glasshouse. Nocturnal gas exchange was measured during periods of 1 h centered at 22:30h (early night) and 03:45h (predawn). Natural dawn and dusk occurred at *ca.* 06:00h and 18:00h, respectively. Conditions inside the LI-6400XT cuvettes were set to match the growth conditions previously described. Importantly, there were no significant differences in relative humidity (ANOVA, df = 1,46, F = 1.91, P = 0.17), temperature (ANOVA, df = 1,46, F = 1.91, P = 0.17) or vapor pressure deficit (ANOVA, df = 1,46, F = 1.25, P = 0.27) in the glasshouses during periods when "early night" and "predawn" measurements were conducted. Genetic variation in nocturnal conductance was assessed by linear mixed models that included sampling time, CO_2 concentrations, genotype (with n=3-6 in each combination) and their interactions as fixed variables, and with glasshouse bay nested within CO_2 concentration as random variables. These analyses were performed after examining whether the data conformed to assumptions of homoscedasticity and normality.

To test relationships between nocturnal and early morning stomatal conductance, we monitored gas exchange briefly after dawn by logging, every 15 seconds, the responses to 300 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) during 5 minutes, and the subsequent responses at 800 μmol m^{-2} s^{-1} PAR for 5 more minutes. While 5 minutes is generally not sufficient for steady-state stomatal responses, it is sufficient to test hypotheses regarding the time to react, and values should be correlated with those at steady–state. We established whether stomatal priming led to higher early morning stomatal conductance by correlating predawn values with the maximum values at each PAR level, using mixed models that included CO_2 concentration as a random factor.

166 To test whether stomatal priming would also lead to faster stomatal responses,
167 we calculated the time constant (τ, the time to reach 63% [1-e^{-1}] of the maximum
168 response at each PAR level (Woodward, 1987), and determined its relationship with
169 predawn stomatal values using mixed models that included CO_2 concentration as a
170 random factor. PAR for gas exchange measurements was provided by the red-blue
171 LEDs of the LI-6400XT (with a 7-13% blue fraction, according to the manufacturer).
172 To examine the relationships between genotype stomatal conductance and
173 growth, we developed mixed model regressions of genotype predawn conductance as
174 a function of total plant carbon assimilation (also measured in the early morning), and
175 total plant leaf area and biomass. These analyses included CO_2 concentration as a
176 random factor.
177 Total plant leaf area was determined during harvest using a leaf area meter
178 (LI-3100C, Li-Cor Inc, Lincoln, Nebraska) and whole plant gas exchange was
179 determined by multiplying leaf carbon assimilation by total plant leaf area, assuming
180 a "big leaf" model structure. It is well known that big leaf scaling approaches are
181 fairly limited, especially within complex canopies, given that leaf gas exchange is
182 heterogeneous within canopies and varies as a function of factors such as leaf position
183 or age (De Pury & Farquhar, 1997), and this variation could also be under genetic
184 control. These problems would have been minimized within our design, because the
185 plants were less than three-months old, and little time had elapsed for complexity in
186 the canopy structure to be developed.
187 Total biomass (including above- and below-ground) was collected two weeks
188 after gas exchange measurements, and determined after oven drying until constant
189 mass (48 h at 70^{o}C). Immediately after removing leaves for measurements of leaf
190 area, the stem was cut and dried prior to assessing its biomass. The soil was then

191 washed and a 3 mm sieve was used to preserve the fine roots. Roots were
192 subsequently dried and weighed. Although extreme care was taken to minimize root
193 losses, we acknowledge some root biomass may have been lost using this technique.
194 However all plants were treated the same way, and any mass losses originating from
195 this technique would have affected equally all plants, regardless of their genotype or
196 CO_2 growth concentration.

197 Based on the results from this correlative approach, and as a further
198 complement to those results, we employed a path model to directly test for the
199 hypothesized mechanisms underlying relationships between predawn stomatal
200 conductance and biomass (Wright, 1934). While correlative approaches allow us to
201 test for the significance of relationships between two variables, with path models we
202 were able to directly assess the significance of the hypothesized mechanisms across
203 more complex routes. The potential routes to be tested included direct effects of
204 predawn stomatal conductance on the values of early morning stomatal conductance
205 and on the time for stomata to respond which, in turn, would affect early morning
206 carbon assimilation which would affect whole plant biomass.

207 We additionally measured the concentration of soluble carbohydrates and
208 starch to understand their relationship with potential CO_2 effects on stomatal
209 conductance. A $1cm^2$ round piece of leaf was collected at dusk and flash frozen in
210 liquid nitrogen, and analyzed following previously described methods (Mitchell *et al.*,
211 2013).

212 All analyses were performed in the R software environment using base
213 packages, *lme4* (Bates *et al.*, 2014), *car* (Fox & Weisberg, 2011), *influence.ME*
214 (Nieuwenhuis *et al.*, 2012) and *lavaan* (Rosseel, 2012). R^2 in mixed models was
215 computed following Nakagawa & Schielzeth (2014).

RESULTS

Nighttime stomatal conductance was higher at predawn than at early night (Fig. 1, Table 1), and there was significant variation in nocturnal stomatal conductance across genotypes. While there was no CO_2 effect, and no CO_2 × genotype interaction, there was a significant time × CO_2 interaction, such that conductance was higher under elevated CO_2 but only at predawn (Fig. 1a, Table 1). The effect of CO_2 on nocturnal stomatal conductance was not driven by carbohydrate availability, as we observed no CO_2 effect on starch, soluble sugars or total non-structural concentrations (only TNC shown, Table 1).

Genotypic variation in predawn (but not in early night) stomatal conductance was associated with subsequent daytime processes. For instance, genotype predawn stomatal conductance was strongly associated with early morning stomatal conductance and assimilation at both PAR levels and across CO_2 treatments (linear mixed model, $P < 0.01$, $0.35 \leq R^2 \geq 0.37$, Fig. 2). However, there was no relationship between genotype stomatal conductance in the early night, and stomatal conductance and assimilation in the early morning (linear mixed model, $P \geq 0.13$, Supporting Information Fig. S1).

τ showed a negative correlation with predawn stomatal conductance (linear mixed model, $P = 0.04$, $R^2 = 0.3$, Fig. 3a), indicating that the response time to morning light decreased as predawn conductance increased. Moreover, genotypes with more rapidly opening stomata (smaller τ) had higher leaf carbon assimilation (linear mixed model, $P < 0.0001$, $R^2 = 0.60$, Fig. 3b).

Concomitantly, genotypes with higher predawn stomatal conductance showed significantly higher plant carbon assimilation (linear mixed model, $P < 0.0001$, $R^2 =$

241 0.67), as well as significantly higher leaf area (linear mixed model, $P = 0.033$, $R^2 =$
242 0.29) and marginally significant higher plant biomass (linear mixed model, $P = 0.098$,
243 $R^2 = 0.19$; Fig. 4). However, there was no significant relationship between genotype
244 early night stomatal conductance and morning carbon assimilation, or leaf area (linear
245 mixed model, $P > 0.4$).
246 Finally, the path model (Fig. 5) indicated that the mechanism explaining the
247 higher biomass for genotypes with higher predawn stomatal conductance was
248 associated with the effect of predawn stomatal conductance on early morning
249 conductance (at $P < 0.10$). Additionally, we also observed that the relationship
250 between predawn stomatal conductance and early morning assimilation was mediated
251 by τ ($P < 0.05$). In turn, this early morning increase in carbon assimilation led to
252 higher biomass ($P < 0.05$).
253
254 **DISCUSSION**
255 We observed genetic correlations between nocturnal stomatal conductance and
256 genotype fitness, quantified as morning carbon assimilation, leaf area and biomass, in
257 *Eucalyptus camaldulensis* (Figs. 4, 5). These relationships between nocturnal stomatal
258 responses and carbon metabolism were only apparent for conductance at predawn
259 (Fig. 2, 3), but not at early night (Supporting Information Fig. S1). The stronger
260 relationships between predawn and daytime processes (rather than early night and
261 daytime processes) indicate that it is not nocturnal conductance *per se* that affects
262 daytime responses, but rather the magnitude of nocturnal conductance immediately
263 prior to dawn. The temporal pattern of nocturnal stomatal conductance is
264 predominantly driven by circadian regulation, which leads to gradual increases in
265 stomatal conductance through the night period (Hennessey *et al.*, 1993, Resco de Dios

266 *et al.*, 2013a, Resco de Dios *et al.*, 2015). It thus follows that strong circadian
267 regulation, with increasing stomatal conductance until predawn, fostered higher
268 daytime carbon assimilation and plant growth in *E. camaldulensis*, consistent with the
269 anticipation hypothesis.
270
271 **Circadian regulation of nocturnal conductance and plant fitness**
272 The circadian clock is known to 'orchestrate' the temporal pattern of transcription
273 (Michael *et al.*, 2008). Indeed, important differences in the transcriptome of wild-type
274 *Arabidopsis*, relative to those in arrhythmic mutants where circadian regulation has
275 been impaired, were observed one hour before dawn, and those included differences
276 in the activation state of genes related to ABA and stomatal behavior (Legnaioli *et al.*,
277 2009). Additionally, circadian rhythms in root hydraulic conductivity could also
278 provide the mechanistic basis for the endogenous rise in predawn stomatal opening
279 (Caldeira *et al.*, 2014). Here, we show that the capacity of the stomata to anticipate
280 sunrise then fostered stomatal responses to morning light, decreased diffusion
281 limitations to photosynthesis and, ultimately, was genetically correlated with
282 enhanced growth.
283 Many previous studies have documented that circadian resonance increases
284 fitness (Dodd *et al.*, 2005, Yerushalmi & Green, 2009, Yerushalmi *et al.*, 2011), and
285 these studies had attributed, but not directly tested, that such increases in fitness were
286 dominated by the clock-driven capacity for anticipating environmental cues. As far as
287 we are aware, our genetic correlations may provide some of the first direct evidence
288 linking this circadian-driven stomatal "anticipation" (that is, opening before dawn)
289 with fitness (as indicated by C assimilation, leaf area and biomass). Circadian
290 regulation is demonstrated by self-sustained oscillations with a 24 h period, a step we

291 did not take here because our previous work demonstrated that circadian regulation
292 was the main driver of predawn values of stomatal conductance (Resco de Dios *et al.*,
293 2013a, Resco de Dios *et al.*, 2015, Resco de Dios *et al.*, 2013b)).

294 We move beyond previous studies that showed that the level of predawn
295 conductance was related to early morning conductance (Drake *et al.*, 2013), by
296 additionally showing that the time for stomata to respond to light inputs is also related
297 to predawn conductance, and that this process increases carbon assimilation across
298 genotypes. This observation could provide a mechanistic explanation for previous
299 studies where relationships between nocturnal water loss and traits that confer fast
300 growth had been observed across multiple species (Daley & Phillips, 2006, Marks &
301 Lechowicz, 2007, Rohula *et al.*, 2014), or across multiple genotypes within a given
302 species (Christman *et al.*, 2008).

303 More detailed studies will be needed to describe the mechanisms underlying
304 the observed genetic correlations between pre-dawn stomatal conductance, early
305 morning photosynthesis, and total plant leaf area and biomass. In particular, the link
306 between early morning photosynthesis and biomass is based on correlations, and
307 should be considered as a hypothesis requiring further testing (Fig. 5). For example,
308 early morning photosynthesis may have a limited impact on whole day C gain, and
309 early morning assimilation may be correlated with assimilation at other times. In
310 addition to "stomatal priming" mechanistically leading to more responsive (after
311 sunset) stomata and to an enhancement of C gain, stomatal priming could also be part
312 of a trait syndrome that confers enhanced growth, and indirectly increases plant
313 fitness. At any rate, we have shown that variation in stomatal priming is an important
314 factor underlying intra-specific variation in growth.

315 In response to a decrease in predawn relative humidity, Auchincloss *et al.*
316 (2014) observed a decline in predawn stomatal conductance, but no change in
317 subsequent daytime carbon assimilation in sunflower. The authors interpreted this
318 result as proof that predawn stomatal regulation does not affect early morning
319 photosynthesis; however, our results provide an alternative explanation to this finding.
320 The circadian clock regulates the temporal pattern of transcription based on the
321 conditions experienced in the previous days (Graf *et al.*, 2010). Therefore, the
322 application of a "pulse" of low relative humidity, which leads to immediate reductions
323 in predawn stomatal conductance, would not have altered circadian regulation. The
324 stomata exposed to low humidity would have been pre-conditioned to respond to early
325 morning radiation inputs, and would have done so at the same rate as stomata exposed
326 to high humidity, following the removal of the environmental constraint of high vapor
327 pressure deficit.
328
329 **CO_2 effects on nocturnal stomatal conductance**
330 The positive effect of CO_2 growth concentration on stomatal conductance was only
331 significant at predawn (Table 1, Fig. 1). Earlier work hypothesized that the positive
332 effect of CO_2 on stomatal conductance, when occurring, could be attributed to
333 increases in carbohydrate concentrations, as carbohydrates provide some of the
334 osmoticant and C skeletons for ATP production necessary to regulate stomatal
335 opening (Easlon & Richards, 2009). However, we are not aware of previous studies
336 directly testing this hypothesis by measuring carbohydrate concentrations and
337 nocturnal conductance under elevated CO_2. Our data did not support this hypothesis
338 because we did not observe a change in carbohydrate concentrations under elevated

339 CO_2. Alternative mechanisms could be driving this response, including changing
340 stomatal sensitivities to ABA under elevated CO_2 (Levine *et al.*, 2009).
341
342 **Implications and outlook**
343 *Eucalyptus camaldulensis* is a riverine species, with relatively low values of nocturnal
344 conductance, compared to species from drier environments. We did not have enough
345 data to meaningfully test for associations between water availability at the genotype's
346 origin and nocturnal conductance. However, it is tempting to hypothesize that our
347 findings provide an explanation for a major conundrum in nocturnal water research,
348 which is why the rates of nocturnal conductance and water loss are typically highest
349 (up to 25% of daytime water loss or more) in species growing in deserts (Ogle *et al.*,
350 2012), savannas (Rosado *et al.*, 2012) and Mediterranean ecosystems (Barbeta *et al.*,
351 2012), which are some of the driest environments on Earth. The period for carbon
352 assimilation in C_3 or C_4 plants in water-limited ecosystems is very narrow, typically
353 only a few hours in the early morning, before the daily onset of strongly desiccating
354 atmospheric conditions (Huxman *et al.*, 2004, Resco *et al.*, 2009a). Therefore, it
355 would be adaptive to have high predawn stomatal conductance in water-limited
356 ecosystems, which would lead to rapid stomatal response to early morning light
357 coincident with lower vapour pressure deficit; this strategy would maximise carbon
358 assimilation before environmental conditions cause stomatal closure. Although *E.*
359 *camaldulensis* grows in riparian environments, seedlings lacking groundwater access
360 will still be exposed to intermittency of water availability.
361 Perhaps the next major challenge will be to understand why predawn stomatal
362 conductance varies among genotypes and species, instead of being always high, and
363 whether this is due to differences in the strength of clock regulation, the degree of

clock resonance, or local site factors. Differences in circadian period and intensity have often been related to differences in photoperiod. Genotypes of *E. camaldulensis* came from different provenances (Supporting Information Table S1) and the distance between provenances may have been large enough to drive major clinal variation in photoperiod (Hut *et al.*, 2013). Moreover, variation in the perception of blue light across genotypes may have contributed to the pre-dawn stomatal response as well (Taiz & Zeiger, 2006).

It is important to note that high nocturnal conductance may conflict with other demands for water during the night. Decreasing nocturnal conductance and water loss is necessary to reduce xylem tension and thus favor leaf expansion and growth (Müller *et al.*, 2014), stem refilling and cavitation repair (Daley & Phillips, 2006), and hydraulic lift (Neumann *et al.*, 2014). Additionally, increasing nocturnal water use may decrease daytime water availability. Future work could address how these multiple trade-offs for water allocation overnight interact across genotypes and species, ultimately affecting fitness across a wide range of environmental stress intensities and types.

More broadly, the finding that genotypes with higher predawn conductance show higher daytime gas exchange and productivity may represent an important mechanism by which intraspecific variation in plant growth occurs, where genetic variation in the capacity for anticipating sunrise would be an important trait underlying differences in C uptake and growth. This novel hypothesis on the mechanisms driving intraspecific variation deserves further testing. Our results thus contribute to the up and coming field of ecological memory (Ogle *et al.*, 2015), by demonstrating the selective advantage of plants using information from the recent past to prepare plant metabolism to respond to predictable changes in the environment.

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555 **SUPPORTING INFORMATION**

556 Additional supporting information may be found in the online version of this article at
557 the publisher's web-site.

558 **Fig. S1:** Early night stomatal conductance (g_s) is not positively correlated with the
559 maximum g_s (a-b) and carbon assimilation (A, c-d) measured in response to 300 (b, d)
560 and 800 μmol m^{-2} s^{-1} (a, c) of photosynthetically active radiation in the early morning.
561 Values indicate the mean (n=4, ± SE) for each genotype under each CO_2
562 concentration. p-values reflect the results of Wald tests on linear mixed model that
563 included CO_2 concentrations as a random factor.

564 **Table S1:** Origin for each of the six genotypes of *Eucalyptus camaldulensis* used in
565 this study

566 **Table 1.** Results of linear mixed models on the effects of CO_2, genotype and their
567 interactions on nocturnal stomatal conductance (g_s) and on total non-structural
568 carbohydrates (TNC), with sampling time as an additional fixed factor affecting g_s,
569 and with glasshouse bay nested within CO_2 concentration as random variables.

	g_s			TNC		
Factor	χ^2	**Df**	***P*-value**	χ^2	**Df**	***P*-value**
CO_2	1.56	1	0.211	1.68	1	0.194
Genotype	12.34	5	0.030	28.59	5	<0.001
Sampling	78.09	1	<0.001			
CO_2 × Genotype	3.63	5	0.604	2.02	5	0.846
CO_2 × Sampling	5.70	1	0.017			
Genotype × Sampling	5.39	5	0.370			
CO_2 × Genotype × Sampling	1.76	5	0.893			

570

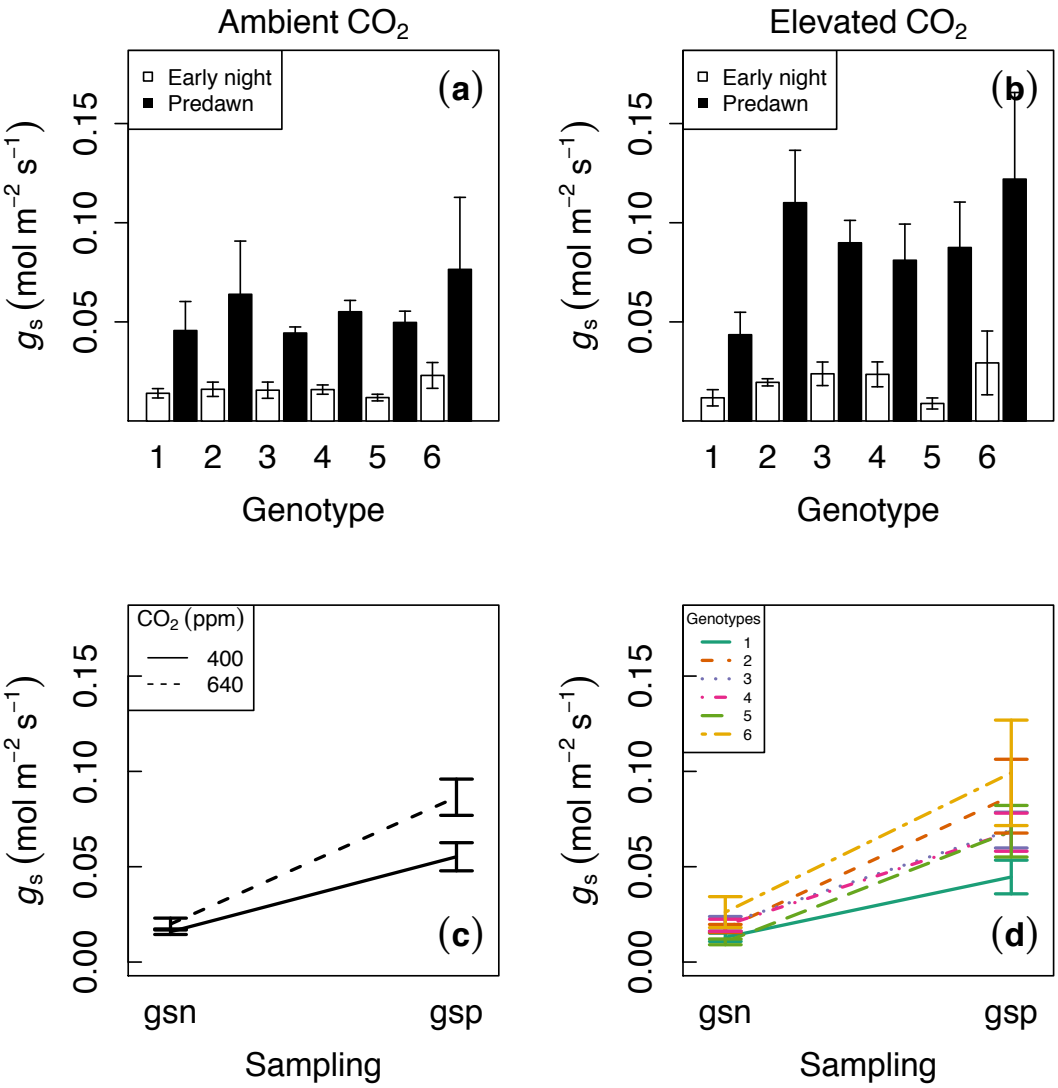
571 **Figure 1:** Mean (± SE) stomatal conductance (g_s) for each sampling time (gsn and
572 gsp indicate g_s at early night and at predawn, respectively) and genotype under
573 ambient (a) and elevated (b) CO_2, and effect of the interactions between sampling
574 time and growth CO_2 (c) or genotype (d) on g_s.
575
576 **Figure 2:** Predawn stomatal conductance (g_s) is positively correlated with the
577 maximum g_s (a-b) and carbon assimilation (A, c-d) measured in response to 300 (b, d)
578 and 800 μmol m^{-2} s^{-1} (a, c) of photosynthetically active radiation in the early morning.
579 Values indicate the mean (n=4, ± SE) for each genotype under each CO_2
580 concentration. *p*-values reflect the results of Wald tests on linear mixed model that
581 included CO_2 concentration as a random factor.
582
583 **Figure 3:** Time to reach 63% of the final stomatal conductance (g_s) in response to 5
584 minutes at 800 μmol m^{-2} s^{-1} of photosynthetically active radiation in the early morning
585 (τ) is significantly correlated with predawn g_s (a). In turn, τ is significantly correlated
586 with early morning plant carbon assimilation (A_p, b). These results indicate that
587 carbon input is related to stomatal responsiveness (τ) which, in turn, is related to
588 predawn g_s. Values indicate the mean (n=4, ± SE) for each genotype under each CO_2
589 concentration. *p*-values reflect the results of Wald tests on linear mixed models that
590 included CO_2 concentration as a random factor. Small error bars may be hidden.
591
592 **Figure 4:** Values of early morning plant carbon assimilation (A_p), leaf area and total
593 biomass are significantly correlated with predawn stomatal conductance (g_s). Values
594 indicate the mean (n=4, ± SE) for each genotype under each CO_2 concentration. Small
595 error bars may be hidden. *p*-values reflect the results of Wald tests on linear mixed

596 models that included CO_2 concentration as a random factor.

597 **Figure 5:** Results and significance levels for the path model on hypothesized
598 relationships between relating predawn stomatal conductance ($g_{s,p}$), the time constant
599 of stomatal responses (τ, with high τ indicating slow-responsive stomata), early
600 morning stomatal conductance ($g_{s,em}$) and carbon assimilation ($A_{p,em}$) and final
601 biomass. Straight and dashed lines indicate hypothesized positive and negative
602 relationships, respectively. Significance levels are indicated by NS, * and ** which
603 indicate $p > 0.10$, $p < 0.10$ and $p < 0.001$, respectively.

604

605 Fig. 1



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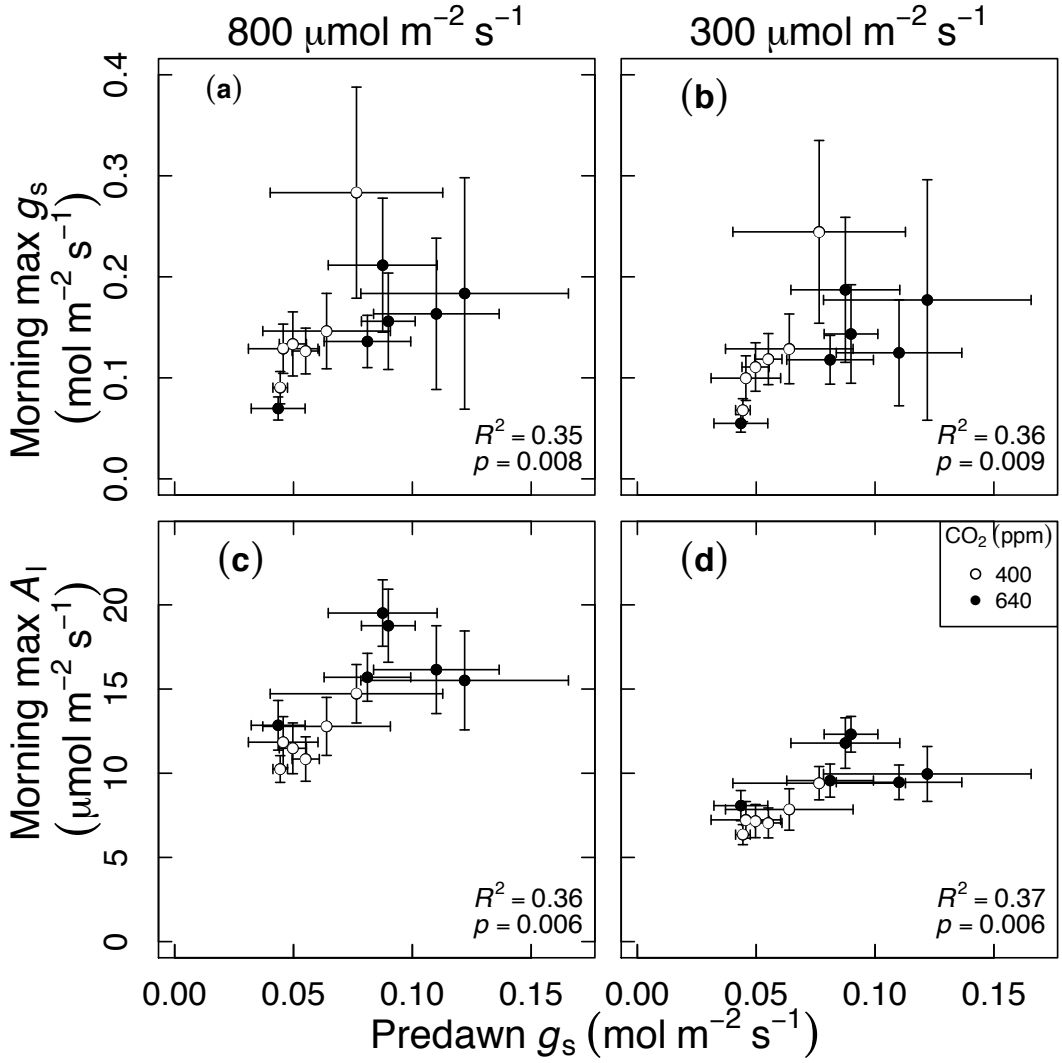
608 Fig. 2



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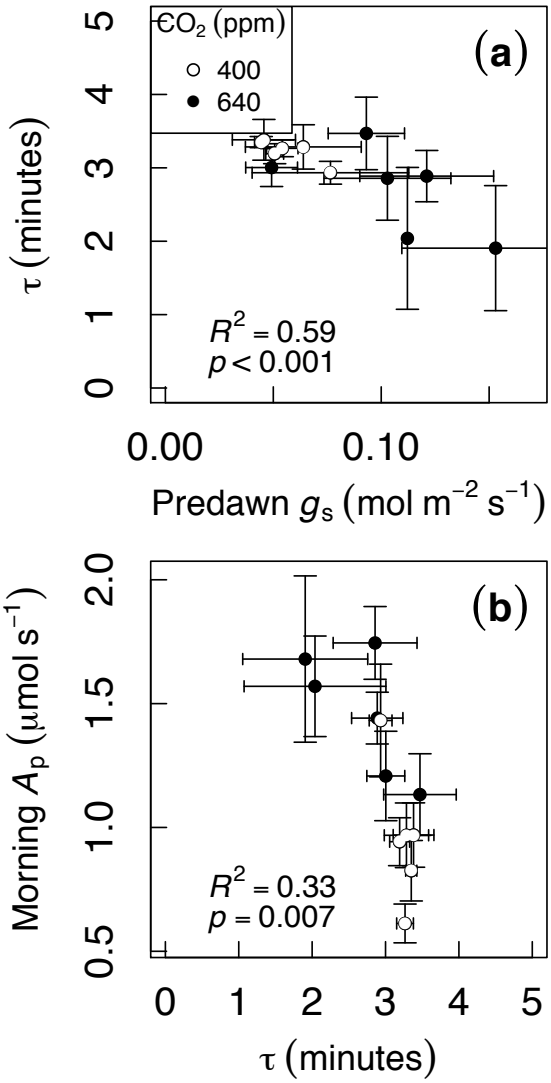
611 Fig. 3



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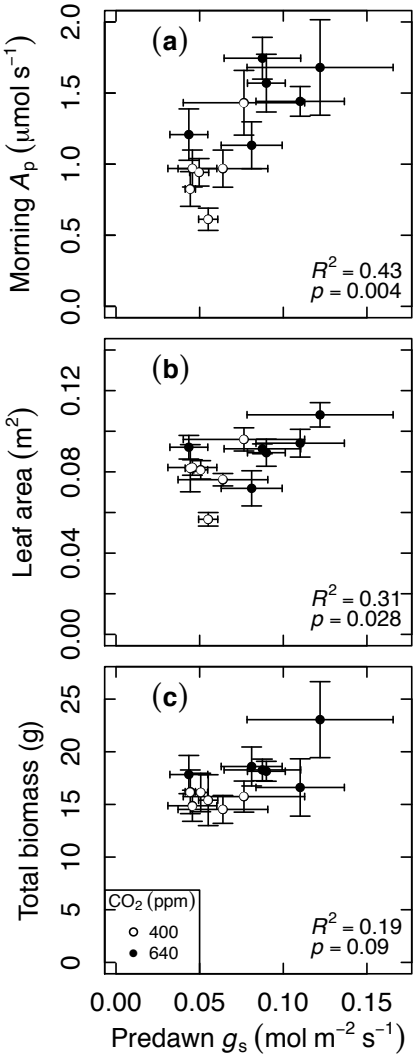
614 Fig. 4



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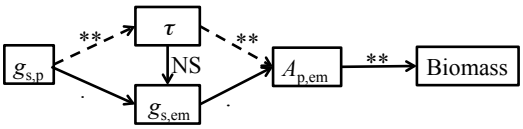

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