Original Article

Coupled response of stomatal and mesophyll conductance to light enhances photosynthesis of shade leaves under sunflecks

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ABSTRACT

Light gradients within tree canopies play a major role in the distribution of plant resources that define the photosynthetic capacity of sun and shade leaves. However, the biochemical and diffusional constraints on gas exchange in sun and shade leaves in response to light remain poorly quantified, but critical for predicting canopy carbon and water exchange. To investigate the CO_2 diffusion pathway of sun and shade leaves, leaf gas exchange was coupled with concurrent measurements of carbon isotope discrimination to measure net leaf photosynthesis (A_n), stomatal conductance (g_s) and mesophyll conductance (g_m) in *Eucalyptus tereticornis* trees grown in climate controlled whole-tree chambers. Compared to sun leaves, shade leaves had lower A_n, g_m, leaf nitrogen and photosynthetic capacity (A_{max}) but g_s was similar. When light intensity was temporarily increased for shade leaves to match that of sun leaves, both g_s and g_m increased, and A_n increased to values greater than sun leaves. We show that dynamic physiological responses of shade leaves to altered light environments have implications for up-scaling leaf level measurements and predicting whole canopy carbon gain. Despite exhibiting reduced photosynthetic capacity, the rapid up-regulation of g_m with increased light enables shade leaves to respond quickly to sunflecks.

Key-words: *Eucalyptus tereticornis*; canopy light gradients; climate warming; leaf nitrogen; shade acclimation.

INTRODUCTION

Light availability is one of the most important environmental drivers of leaf carbon (C) uptake in trees. Predicting C uptake of forests usually involves up-scaling leaf level measurements to assess whole canopy function. Because of the costs and limitations of efficient light harvesting within plant canopies, not all leaves are exposed to full sun (Niinemets 2010), making simple up-scaling based on solar irradiance problematic. Incident photosynthetic photon flux density (PPFD) declines exponentially with cumulative leaf area index (ratio of leaf area to ground area), creating a steep light gradient from the canopy top to bottom (Monsi & Saeki 2005). Consequently, structural and functional properties of leaves within canopies are

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modified to efficiently use light (Vogelman *et al.* 1996; Niinemets & Valladares 2004), as changing irradiance with canopy depth strongly affects rates of leaf net photosynthesis (A_n) (Evans 1995). To calculate whole canopy C gain, it is thus necessary to account for the non-linear response of A_n to light by distinguishing between shaded and sunlit leaves (De Pury & Farquhar 1997; Linderson *et al.* 2012).

The distribution of resources required for A_n, including leaf nitrogen (N) and supply of water, is also partially defined by canopy light gradients. As A_n has a saturating response with light and maximum rates depend, in part, on N-rich photosynthetic machinery, it has been argued that leaf N should be proportional to PPFD along the canopy light gradient to maximize canopy C gain at a given total canopy N (Field 1983; Field & Mooney 1986; Peltoniemi *et al.* 2012; Buckley *et al.* 2013). Changes in chlorophyll per unit N, chlorophyll a:b ratios, electron transport capacity per unit chlorophyll and ratios of electron transport capacity to Rubisco activity can also occur in response to changes in irradiance of the growth environment (Evans & Poorter 2001). Sun leaves frequently experience greater water limitations in the upper canopy, despite effective vascular systems that accommodate high radiation loads and transpiration (Sellin *et al.* 2008; Niinemets 2012). Optimal photosynthetic N investment in the upper canopy will be ineffective in enhancing A_n if water supply is insufficient (Niinemets 2012; Peltoniemi *et al.* 2012); thus, leaf-specific hydraulic conductance (K_L) should also be higher in the upper canopy to supply sunlit leaves with sufficient water (Hubbard *et al.* 2001; Burgess *et al.* 2006; Sellin & Kupper 2007; Sellin *et al.* 2008).

The photosynthetic rate in C_3 plants is limited by the $[CO_2]$ available for fixation by Rubisco within the chloroplast and this $[CO_2]$ is a function of the drawdown of CO_2 from the atmosphere to the site of carboxylation (Warren 2008). There are two major resistance pathways to CO_2 diffusion: the CO_2 diffusion from the atmosphere through stomata into intercellular air spaces (stomatal conductance, g_s) and the pathway from the intercellular air spaces into the chloroplast of the mesophyll cells (mesophyll conductance, g_m). Based on optimality theory, regulation of g_s within a tree canopy should act to efficiently utilize available supplies of light, N and water to maximize A_n (Peltoniemi *et al.* 2012). This is because stomata are hypothesized to exhibit an optimal behaviour to maximize C gain while simultaneously minimizing water loss through transpiration (Cowan & Farquhar 1977). Mesophyll conductance

can also impose limitations on A_n that can be as large as g_s (Warren 2008; Ubierna & Marshall 2011), reducing the efficiency of leaf N use in A_n (Niinemets 2007). Differences in photosynthetic capacity between sun and shade leaves may arise, in part, from differences in g_m (Piel *et al.* 2002; Warren *et al.* 2007), yet the trade-offs that constrain this diffusion pathway are yet to be explicitly quantified. It is likely that leaf anatomical costs associated with minimizing the length and tortuosity of the g_m diffusion pathway are necessary to maintain the benefits of a high g_m (Hassiotou *et al.* 2009). Nonetheless, like g_s, g_m may also vary dynamically in response to environmental drivers (Flexas *et al.* 2008). Thus, stomatal and mesophyll conductance should not be considered independent of each other (Griffiths & Helliker 2013), but a lack of empirical data currently hinders our ability to interpreting their coupled responses to A_n across sun and shade leaves.

How shade leaves utilize sunflecks for short-term carbon gain depends on the combined response time of g_s and g_m and the underlying photosynthetic machinery acclimated to a low light environment (Pearcy 1990; Tausz *et al.* 2005). The utilization of sunflecks in shade leaves is initially limited by delayed responses of stomata opening, which may take minutes, limiting the assimilation rate that can be achieved (Pearcy 1990; Vico *et al.* 2011; Way & Pearcy 2012). Mesophyll conductance has been shown to respond to environmental factors (e.g. CO_2, temperature or vapour pressure deficit) at timescales of minutes, possibly faster than g_s (Flexas *et al.* 2008 and references therein), yet the short-term response to light availability is unclear. For example, g_m was found to be independent of incident light intensity in wheat leaves (Tazoe *et al.* 2009) but was responsive to light in tobacco (Flexas *et al.* 2007) and *Eucalyptus* seedlings (Douthe *et al.* 2011, 2012). Anatomical parameters which regulate g_m with changing growth irradiance such as chloroplast surface area (Terashima *et al.* 2006) and mesophyll thickness (Boardman 1977; Terashima *et al.* 2001; Hanba *et al.* 2002) are also unlikely to adjust during transient fluctuations in light. The physiological behaviour of shade leaves to maximize C gain must be assessed as both a degree of acclimation to local irradiance and as a potential response to transitory light availability.

Climate warming may also affect the physiological behaviour of leaves within a canopy. Leaves are exposed to different heat, water and high light stresses as temperature and vapour pressure deficit (VPD) vary with canopy light availability (Baldocchi *et al.* 2002; Niinemets & Valladares 2004; Niinemets 2007). How these stresses affect the diffusion of CO_2, through either g_s or g_m, will have implications for up-scaling A_n for sun and shade leaves. How these parameters, in combination with photosynthetic capacity, are differentially affected by warming may impact constraints of N distribution and leaf photosynthetic capacity across light gradients. The impacts of warming on plant physiological processes are diverse, yet differentiating their impacts on leaf physiology within a canopy will be essential to evaluate whole tree responses to a changing climate.

In this study, we use *Eucalyptus tereticornis* trees, planted in naturally sunlit climate controlled whole-tree chambers with ambient and elevated temperature (ambient +3 °C) treatments, to test whether the distribution of N, water supply

capacity and leaf physiological traits result in higher photosynthetic capacity in sun leaves compared to shade leaves. We further aimed to quantify the constraints on A_n in sun and shade leaves arising from photosynthetic capacity and the CO_2 diffusion pathway via stomatal and mesophyll conductance. As leaves which were acclimated to shade conditions were expected to have lower photosynthetic capacity and correspondingly lower diffusive conductance (both g_s and g_m) than sun leaves, we predicted that sunfleck simulations would increase A_n and g_s in shade leaves, but reach values lower than that attained by sun leaves in similar conditions. We further predicted that climate warming would decrease g_s and leaf C gain in sun leaves more so than shade leaves during summer months, as increased evaporative demand from higher temperatures and irradiance lead to stomatal closure.

MATERIALS AND METHODS

Whole-tree chamber experimental design

Twelve *E. tereticornis* Sm. seedlings, chosen from a single local Cumberland plain cohort, were planted in March 2013 into 12 whole-tree chambers (WTC) at the Hawkesbury Forest Experiment site near Richmond, New South Wales, Australia. The top soils at this site, used in the chambers, are an alluvial formation of low-fertility sandy loam soils (380 and 108 mg kg^{-1} total N and phosphorus respectively) with low organic matter (0.7%) and low water holding capacity. A root exclusion barrier extended from chamber walls to the hard layer (ca. 1 m) and roots were allowed to grow freely below the barrier. Each chamber was 9 m tall, which accommodated growth of trees for 15 months. A detailed description of the WTC operation and design is available in Barton *et al.* (2010) and methods for this experiment in Drake *et al.* (2016). Coppiced *E. saligna* trees were present in the area between the whole-tree chambers, and these coppiced trees grew at rates similar to the trees in the chambers, thus providing ample shade. Six chambers were set to match outside ambient air temperatures (AT) while the remaining six experienced an elevated air temperature treatment of ambient +3 °C (ET, Figure S1). The trees grew quickly and developed large canopies, with height growth reaching the top of the WTCs at the end of the experiment. Trees were watered weekly with 70 L from March 2013 to September 2013. From October 2013 to the end of the experiment trees were watered every 15 days with the mean monthly rainfall amount for Richmond, NSW. In February 2014 half of the chambers (three each of AT and ET) were subjected to a drought treatment by withholding watering. Because of the difficulty of measuring g_m in the droughted trees (the necessary CO_2 drawdown was not achieved), we here report only data from well-watered trees, which reduces the sample size from $n = 6$ to $n = 3$ for the final 3 months of the experiment.

Leaf gas exchange measurements were initiated in October 2013 when trees had both ample height growth and canopy development for realistic canopy light gradients to be measured. At this point, trees under AT treatment had a mean stem diameter (measured at 65 cm from the stem base) of 28.2 ± 1.1 mm (SE), height of 348 ± 15.1 cm and a total leaf area of

$3.9 \pm 0.1\,m^2$. For ET treatments, trees had a mean diameter of $34.1 \pm 2.1\,mm$, height of $418.3 \pm 23.1\,cm$ and a leaf area of $6.2 \pm 0.2\,m^2$. Leaf area was calculated based on complete leaf counts and mean leaf size from a subsample from each tree (Drake *et al.* 2016).

Leaf gas exchange, coupled with concurrent measurements of carbon isotope discrimination to estimate mesophyll conductance

Leaf gas exchange measurements were performed six times, beginning in October 2013 and monthly from December 2013 to April 2014. Measurements were taken on a representative sun and shade leaf for each tree during each measurement campaign. The newest fully expanded leaf from the branch apex was chosen for gas exchange measurements, and sun leaves were measured in the upper third of the canopy. Here, shade leaves are defined as inner-canopy leaves developing on secondary branches in a low light environment. Shade leaves were always measured in the lower canopy, but leaves were sampled on subsequent higher branches across measurement campaigns to minimize confounding effects of leaf age. As shade leaves most likely developed more slowly, this assured that older leaves in the bottom canopy were avoided.

Prior to gas exchange measurements, PPFD was recorded both as a point measurement at the individual leaf level and a spatially averaged measurement at the canopy position for each selected leaf. A hand-held quantum sensor (Li-Cor, Lincoln, NE, USA) was used to record leaf level PPFD to ensure that chosen leaves were positioned in the desired light environment, either sun or shade. A ceptometer (AccuPAR LP-80, Decagon Devices, Pullman, USA) was then used to measure a spatially averaged PPFD at the canopy height of each chosen leaf type. Each ceptometer reading integrated an array of 80 sensors over a total length of 84 cm. Five ceptometer readings were recorded at different locations within the canopy, but at the same height and close to each selected leaf. The mean of these readings was assumed to represent the local light environment of sun and shade leaves for each tree. All measurements of PPFD and gas exchange were performed on sunny days between 10:00 and 14:30 h.

Concurrent gas exchange and C isotope discrimination measurements were conducted based on methods described in Tazoe *et al.* (2011) and Evans & von Caemmerer (2013). Leaf level gas exchange was measured with a standard ($2 \times 3\,cm$) leaf cuvette and red/blue light source using a portable gas exchange system (LI-6400XT, Li-Cor, Lincoln, NE, USA). This system was coupled with a tunable diode laser (TDL; TGA100, Campbell Scientific, Inc., Logan, UT, USA) for concurrent measurements of ^{13}C isotope discrimination. The CO_2 in the leaf cuvette was set at ambient atmospheric $[CO_2]$ ($400\,\mu l\,l^{-1}$) with a flow rate of $200\,\mu mol\,s^{-1}$. Two identical gas exchange systems were run simultaneously, one in each of a randomly chosen WTC for each temperature treatment. Leaf temperatures were controlled at the current AT or ET WTC air temperature, and measurements were made at ambient air humidity. Measurements were thus made across a range of air

temperatures and vapour pressure deficits (kPa) across the six campaigns. PPFD in the cuvette was set to match the individual light environment of each leaf type (explained above). Sustained periods of high irradiance (sunflecks) were simulated for shade leaves by increasing the leaf cuvette PPFD (Li-Cor red/blue light source) to match the light environment of the sun leaf in the same tree. The maximum sunfleck response of shade leaves was then recorded once CO_2 and water vapour fluxes re-stabilized in the leaf cuvette (ca. 25 min).

Once CO_2 and water vapour flux values were stable for each leaf measurement, the sample and reference gas lines were diverted to the TDL via T-junctions inserted into the reference gas tube and match valve outlet of the LI-6400XT. The gas streams were dried by passing through napion gas dryers in the respective gas lines, and then $^{12}CO_2$ and $^{13}CO_2$ concentrations were measured for each gas stream by the TDL. Reference, sample and two calibration gases were run on alternating 80 s loops (20 s each), one for each AT and ET leaf at a matched canopy position, for a total of 12 min. This allowed for four or five measurements per leaf and data were averaged over the last 10 s of reference line and sample line gas streams for calculations. The two calibration gases were drawn from compressed air tanks (330 and 740 ppm CO_2) in order to correct for gain drift of the TDL on each measurement cycle. All gas exchange variables were auto-logged every 15 s for each gas exchange system over the 12 min interval.

Mesophyll conductance was calculated from carbon isotope discrimination with equations and fractionation factors as presented in Evans & von Caemmerer (2013), including the ternary corrections proposed by Farquhar & Cernusak (2012), such that:

$$\Delta_o = \Delta_i\text{-}\Delta_{gm}\text{-}\Delta_e\text{-}\Delta_f \qquad (1)$$

where Δ_o is the observed discrimination and Δ_i, Δ_{gm}, Δ_e and Δ_f are the contributions to fractionation if $C_i = C_c$, g_m, respiration and photorespiration, respectively. C_i and C_c are the intercellular and chloroplast CO_2 partial pressures. For this study, the CO_2 compensation point (Γ_*) and respiration during the day (R_d) parameters originally derived for tobacco plants (von Caemmerer *et al.* 1994) were replaced with parameters derived for *Eucalyptus globulus* from Crous *et al.* (2012) when calculating g_m. Full descriptions of the carbon isotope discrimination equations, with ternary corrections, are presented in Supporting Information Methods S1. The variation in Δ_o between sun and shade leaves and the simulated sunfleck were compared as a function of C_i/C_a. Once g_m was calculated, C_c and the drawdown of CO_2 from the intercellular air spaces to the site of carboxylation were estimated from the relationship between g_m and leaf photosynthesis rate (A_n) by:

$$g_m = \frac{A_n}{C_i - C_c}. \qquad (2)$$

Photosynthetic capacity

Photosynthetic capacity (A_{max}) of sun and shade leaves was measured at saturating light and $[CO_2]$ for one sun and shade

leaf for each WTC. Measurements were completed in February 2014, when all trees were well-watered in each temperature treatment ($n = 6$). A_{max} measurements were made at a PPFD of $1800\,\mu mol\,m^{-1}\,s^{-1}$ and [CO_2] of $1800\,\mu l\,l^{-1}$, with leaf temperature held constant at $25\,°C$.

Leaf nitrogen and hydraulic conductance

Following gas exchange measurements, each leaf was collected, measured for leaf water potential (explained below), scanned for leaf area, oven-dried and weighed. These leaves were then milled and analysed for leaf N content and $\delta^{13}C$. Leaf samples were analysed on a Delta V Advantage coupled to a Flash HT and Conflo IV (Thermo Fisher Scientific, Bremen, Germany) in dual-reactor setup. Samples were flash combusted at $1000\,°C$ and converted to CO_2 and N_2 and then subjected to stable isotope ratio mass spectrometry. Leaf N is reported on an area basis (N_a, g m^{-2}) and isotopic signatures of dry matter are reported relative to standard Vienna Pee Dee Belemnite.

Prior to the gas exchange measurements, predawn (Ψ_{pd}) leaf water potentials (MPa) were measured for a separate set of sun and shade leaves using a PMS 1505D pressure chamber (PMS Instruments, Albany, OR, USA). The leaf closest to the leaf used for gas exchange was sampled for measurement of Ψ_{pd} before sunrise on the same day as gas exchange measurements. Following the gas exchange measurements, leaves used for gas exchange were immediately sampled for midday (Ψ_L) leaf water potentials. All leaves were detached and immediately stored inside foil covered bags before water potential measurements were performed. Leaf water potentials and transpiration (E_L, mmol m^{-2} s^{-1}) from gas exchange in the leaf cuvette were then used to calculate leaf-specific hydraulic conductance (K_L, mmol m^{-2} s^{-1} MPa^{-1}) from the equation (Whitehead 1998; Flexas *et al*. 2013):

$$K_L = \frac{E_L}{\Psi_{pd} - \Psi_L}. \tag{3}$$

As light intensity was increased for shade leaves before they were detached for measurement of Ψ_L, the E_L measured at high light was used to calculate K_L for all shade leaves.

Leaf level instantaneous transpiration efficiency (ITE) was calculated as A_n divided by E_L. The g_1 parameter, describing plant water-use strategy, was estimated from ITE versus VPD response curves by fitting a rearranged optimal g_s model for ITE (Medlyn *et al*. 2011) using non-linear regression (see Duursma *et al*. 2013). Small values of g_1 indicate that transpiration is comparatively costly in C terms and reflecting conservative water use (increased water-use efficiency), whereas large values imply a lower C cost and decreased water-use efficiency (Lin *et al*. 2015).

Data analysis

Differences in responses of dependent variables to either the warming treatment or leaf type were analysed with linear mixed-effects models with WTC as a random effect, and leaf type (sun, shade or shade in the sun) and warming treatment, and the interaction as fixed effects. The interaction was never significant, so we only report results of the tests of the main effects. Explained variance (R^2) of mixed models was computed as in Nakagawa & Schielzeth (2013), in which the marginal R^2 represents variance explained by fixed factors and the conditional R^2 by both fixed and random factors. Confidence intervals (95%) of linear mixed-effects models were generated using bootstrapping methods with 999 simulations, using the 'bootMer' function in the 'lme4' package (Bates *et al*. 2015). For non-linear relationships, confidence intervals were estimated by fitting a generalized additive model to the data with the 'mgcv' package (Wood 2006), using WTC as a random effect. All tests of statistical significance were conducted at an α of 0.05. For multiple post-hoc comparisons, we used Tukey pairwise comparison tests with the 'multcomp' package (Hothorn *et al*. 2008). In all results, we report the mean ± one standard error for the mean (SE). All analyses were performed with R 3.3.0 (R Development Core Team 2011).

RESULTS

Leaf resource distribution

Across six measurement campaigns over the 7 month period, PPFD was reduced on average by >75% in the shade (Fig. 1). Leaf N_a was approximately 20% higher in sun leaves compared
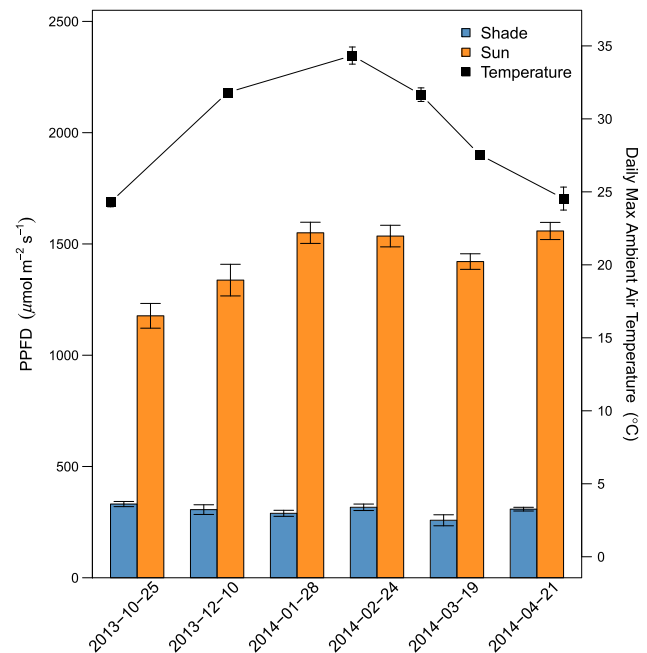

Figure 1. Environmental conditions during the six measurement campaigns. Bars represent the local light environment for sun and shade leaves during six gas exchange campaigns from October 2013 to April 2014 (as photosynthetic photon flux density, PPFD). Means ± 1 standard error represent PPFD measured with a ceptometer at the height of each selected leaf in the canopy. Points connected by a line represent the mean (±1 standard error) daily maximum air temperature during each campaign period. Each date represents the starting date for each measurement campaign; each campaign was completed in 3 days.

Table 1. Leaf morphological and physiological traits of sun and shade leaves under ambient and elevated temperature treatments for *Eucalyptus tereticornis*. Leaf mass per area (LMA, g m^{-2}), leaf nitrogen per unit area (N_a, gN m^{-2}), δ^{13}C (‰), pre-dawn leaf water potential (Ψ_{pd}, MPa), midday leaf water potential (Ψ_L, MPa) and leaf-specific hydraulic conductance (K_L, mmol m^{-2} s^{-1} MPa^{-1}) are given as treatment means (±1 standard error) across six measurement campaigns. Values of A_{max} (μmol m^{-2} s^{-1}) were measured once on all trees for each leaf type at 25 °C and at both saturating light and [CO_2]. Different letters represent significant differences between leaf type and temperature treatments (based on a Tukey test). The *P* value represents the overall effect between each unique combination of leaf type and temperature treatment for each trait.

Leaf	Temperature	LMA	A_{max}	N_a	K_l	Ψ_{pd}	Ψ_L	δ^{13}C
Sun	AT	114.1 (4.5) a	28.4 (2.2) b	2.63 (0.08) b	1.69 (0.18) b	−0.32 (0.04) a	−1.64 (0.11) a	−28.1 (0.18) b
	ET	109.9 (4.8) a	27.5 (1.2) b	2.60 (0.09) b	1.74 (0.15) b	−0.32 (0.03) a	−1.74 (0.10) a	−28.3 (0.17) b
Shade	AT	118.3 (4.4) a	21.8 (0.9) a	2.13 (0.07) a	3.00 (0.21) a	−0.27 (0.02) a	−1.56 (0.09) a	−29.9 (0.17) a
	ET	113.1 (4.3) a	19.2 (0.9) a	1.88 (0.06) a	3.09 (0.21) a	−0.29 (0.02) a	−1.64 (0.11) a	−30.4 (0.22) a
P value		0.781	0.001	0.001	0.001	0.3486	0.6385	0.001

to shade leaves (Table 1). Leaf mass per area (LMA) was not different between leaf types (Table 1). Leaf-specific hydraulic conductance (K_L) was significantly higher in shade leaves (+44%) compared to sun leaves (Table 1). This was because of differences in transpiration rates (E_L, Table 2) between leaf types at high light, as neither leaf water potentials (Ψ_{pd} and Ψ_L, Table 1) differed between leaf types. No effect of the warming treatment was detected on PPFD, Ψ_{pd}, Ψ_L, K_L, E_L, N_a or LMA either within or across leaf types (*P* > 0.05).

Photosynthetic capacity and leaf photosynthesis rates

Mean photosynthetic capacity (A_{max}) of sun leaves was significantly higher (+27%) compared to shade leaves, when measured at 25 °C (Table 1). Within leaf types, no effect of the warming treatment was detected on A_{max}. A_{max} was positively related to mean leaf N_a across leaf types and temperature treatments (*P* = 0.01, Fig. 2).

Mean A_n was significantly higher in sun compared to shade leaves (+23%), when measured at their local light environment and temperatures (Table 2). Following an increase in light intensity to match high-light conditions ('sunfleck simulations'), A_n of shade leaves increased to values significantly greater than sun leaves at high light (*P* < 0.001, Table 2). No effect of the warming treatment was detected on A_n of sun leaves measured at high light or shade leaves at either low or high light. Rates of photosynthesis within leaf types and warming treatments were similar through time and across the range of leaf temperatures measured (Fig. S3a).

Stomatal conductance and leaf water-use efficiency

On average, g_s was 18% higher in shade compared to sun leaves under their local light environment (Table 2). Photosynthesis was positively correlated with g_s in all leaves measured under high light conditions; however, g_s and A_n were not correlated in shade leaves under low light (Fig. 3a). In high light, g_s of shade leaves increased compared to low light and was significantly greater than sun leaves, pooled across all measurement dates (Fig. 4a). No effect of the warming treatment was detected on g_s within or across leaf types. Stomatal conductances

within leaf types and treatments were similar through time and across the range of leaf temperatures measured (Fig. S4b).

Measured under ambient light and temperature, leaf instantaneous transpiration efficiency (ITE) was significantly greater in sun leaves than in shade leaves at low light (+21%, *P* = 0.001). Following an increase in PPFD to high-light conditions, ITE of shade leaves did not differ from shade leaves at low light and was therefore still significantly lower than sun leaves (*P* < 0.001). ITE in sun leaves was reduced in the warming treatment compared to ambient, but no warming effect was detected in shade leaves measured at low or high light (Table 2). The mean estimated g_1 for sun leaves was 1.51 ± 0.11. Mean g_1 was 2.59 ± 0.12 for shade leaves measured at low light and 2.74 ± 0.04 when measured at high light. For all leaf types and light treatments there was a strong response of ITE to VPD and individual data points broadly corresponded to the fitted response curves from the optimal ITE model with a specified g_1 value for each leaf type and treatment (Fig. 5a). Within leaf types and light treatments, the response of VPD to leaf temperature was similar across all measurement campaigns (Fig. S4a).

Bulk-leaf δ^{13}C was significantly lower in shade leaves compared to sun leaves by ca. 2‰ (Table 1). No effects of the warming treatment on leaf δ^{13}C were detected. Leaf δ^{13}C and N_a were positively correlated for all leaves (*P* < 0.001, Fig. 5b), with less negative δ^{13}C and higher N investment in sun leaves than shade leaves.

Leaf carbon isotope discrimination and mesophyll conductance

The observed carbon isotope discrimination (Δ_o) measured during photosynthesis was positively correlated with C_i/C_a for both leaf types (*P* < 0.001), with larger Δ_o detected for sun leaves and shade leaves at high light than shade leaves at low light (Fig. S2). Carbon isotope discrimination associated with g_m accounted for the majority of Δ_o (69.7 ± 0.3%) and varied little across measurement temperatures, leaf types, or warming treatments. The remainder of the variation in Δ_o consists of the contributions of g_s, respiration and photorespiration to discrimination.

Mean g_m was higher in sun compared to shade leaves (+25%) under their local light environment (*P* < 0.001).

Table 2. Leaf gas exchange parameters for sun and shade leaves under ambient and elevated temperature treatments for *Eucalyptus tereticornis*. Leaf net photosynthesis rate (A_n, μmol m^{-2} s^{-1}), leaf transpiration rate (E_L, mmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}), g_m (mol m^{-2} s^{-1} bar^{-1}), intercellular CO$_2$ concentration (C_i, μbar), chloroplastic CO$_2$ concentration (C_c, μbar) and leaf-to-air vapour pressure deficit (VPD, kPa) are given as the mean ($\pm$1 standard error) for each treatment across six gas exchange campaigns. Different letters represent significant differences between leaf type, light environment and temperature treatments (based on a Tukey test). The P value represents the overall effect between each unique combination of leaf type, light environment and temperature treatment for each parameter.

| Leaf	Light	Temperature	A_n	g_s	g_m	ITE	E_L	VPD	C_i	C_c
Sun	High	AT	13.5 (0.3) b	0.122 (0.005) a	0.195 (0.006) ac	8.26 (0.48) b	1.78 (0.07) a	1.60 (0.04) ab	179.8 (3.2) a	106.4 (2.8) a
		ET	13.1 (0.3) b	0.123 (0.005) a	0.182 (0.008) ac	6.57 (0.39) ab	2.21 (0.09) a	1.90 (0.05) b	187.9 (2.9) a	108.4 (2.6) a
Shade	Low	AT	10.4 (0.1) a	0.150 (0.005) a	0.162 (0.006) ab	6.24 (0.50) a	1.93 (0.07) a	1.40 (0.04) a	255.4 (3.8) b	184.3 (4.2) cd
		ET	10.0 (0.1) a	0.146 (0.005) a	0.161 (0.007) a	5.43 (0.51) a	2.23 (0.09) a	1.60 (0.05) a	253.8 (4.1) b	185.2 (3.8) d
	High	AT	18.1 (0.3) c	0.255 (0.007) b	0.212 (0.004) c	5.85 (0.33) a	3.42 (0.12) b	1.40 (0.04) a	237.4 (2.2) b	151.3 (2.0) b
		ET	16.7 (0.2) c	0.246 (0.009) b	0.208 (0.004) bc	5.02 (0.35) a	3.81 (0.15) b	1.70 (0.04) ab	238.1 (3.2) b	156.4 (2.9) bc
P value			0.001	0.001	0.001	0.001	0.001	0.005	0.001	0.001
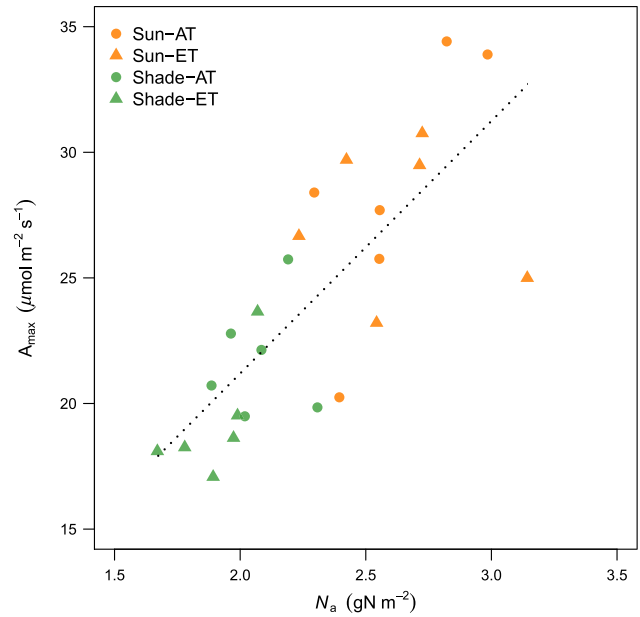
Figure 2. Photosynthetic capacity (A_{max}) was positively correlated with mean leaf N per area (N_a) for sun and shade leaves grown under elevated (ET) and ambient (AT) temperature treatments. Leaf N_a is the mean of each leaf type, in well-watered treatments, sampled across six gas exchange campaigns in each whole-tree chamber. A_{max} was measured once on all trees for each leaf type, before the initiation of the drought treatment, at 25 °C and at both saturating light and [CO$_2$]. The dashed line represents the significant linear model fit for all leaves, with a marginal and conditional R^2 of 0.59 and 0.62.

Following the increase in PPFD from low to high light on the same leaf, g_m values of shade leaves increased on average 37% after approximately 25 min (Fig. 6). These measured values of g_m for shade leaves at high light were also indistinguishable than those of sun leaves (Table 2). Proportional increases in g_m were matched by proportional increases in A_n from low to high light in shade leaves (Fig. 4b,c). Photosynthesis scaled positively with increases in g_m for all leaves, with similar intercepts but different slopes between leaf type and light treatment ($P = 0.019$). The large increases in g_m in shade leaves under high light likely resulted in the highest rates of A_n (Fig. 3b). No differences in g_m were detected with the warming treatment within leaf types. Mesophyll conductance did not vary across measurements campaigns within leaf types and light treatments (Fig. S3b), but a weak negative relationship with increasing leaf temperature was detected with sun and shade leaves under their local light environment ($P = 0.002$ for sun leaves, $P = 0.044$ for shade leaves).

Variation in intercellular and chloroplastic CO$_2$ concentrations

Higher g_s in shade leaves under low and high light led to significant increases in C_i compared to sun leaves (Fig. 7a). The chloroplast CO$_2$ partial pressure was comparable between shade leaves when measured at both low and high light conditions (Fig. 7c). In sun leaves C_c was significantly lower than shade
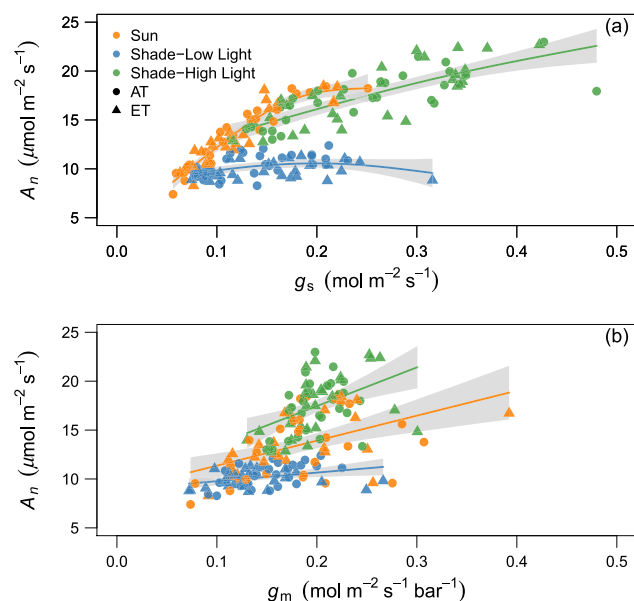
Figure 3. Relationships between leaf net photosynthesis rate (A_n), stomatal conductance (g_s, panel (a)) and mesophyll conductance (g_m, panel (b)) for sun leaves and shade leaves in low or high light. Sun leaves were measured at high light, shade leaves were measured at both low and high light. Data are combined across elevated (ET, triangles) and ambient (ET, circles) temperature treatments, and six measurement campaigns. Lines represent either smoothed regressions from a generalized additive model fit (a) or linear model fits (b), fitted across all data but accounting for random effects (see Methods). Grey areas are 95% confidence intervals for the mean.

leaves, consistent with a lower C_i. The drawdown of CO_2 from intercellular spaces to the chloroplast, $C_i - C_c$, measures the coordination between g_m and A_n (von Caemmerer & Evans 2014). This drawdown was similar between sun and shade leaves measured at their local light and temperature environment and increased marginally in shade leaves at high light (Fig. 7c). This result demonstrates the approximately proportional relationship between g_m and A_n across all leaves. The CO_2 drawdown from C_a to C_i and C_i to C_c were both relatively stable across the range of temperatures measured and gas exchange campaigns (Fig. S4c and S3c, respectively).

DISCUSSION

Here we show that A_n in leaves within canopies of *E. tereticornis* is limited by the local light environment; however, shade leaves increased rates of leaf C gain exceeding sun leaves when light availability increased. Although shade leaves in lower light environments exhibited relatively high g_s and C_i, the increases in g_m under periods of high light availability enabled the up-regulation of A_n. Although it is well-known that shade leaves experience transient periods of sun and shade (Pearcy 1990), a lack of empirical data within tree canopies currently impedes our ability to predict whole canopy C gain. These findings offer new insights into how aspects of leaf gas exchange physiology may be optimized differently in sun and shade leaves and reveal how the total leaf CO_2 conductance pathway should be accounted for when testing optimizations of canopy C uptake and water
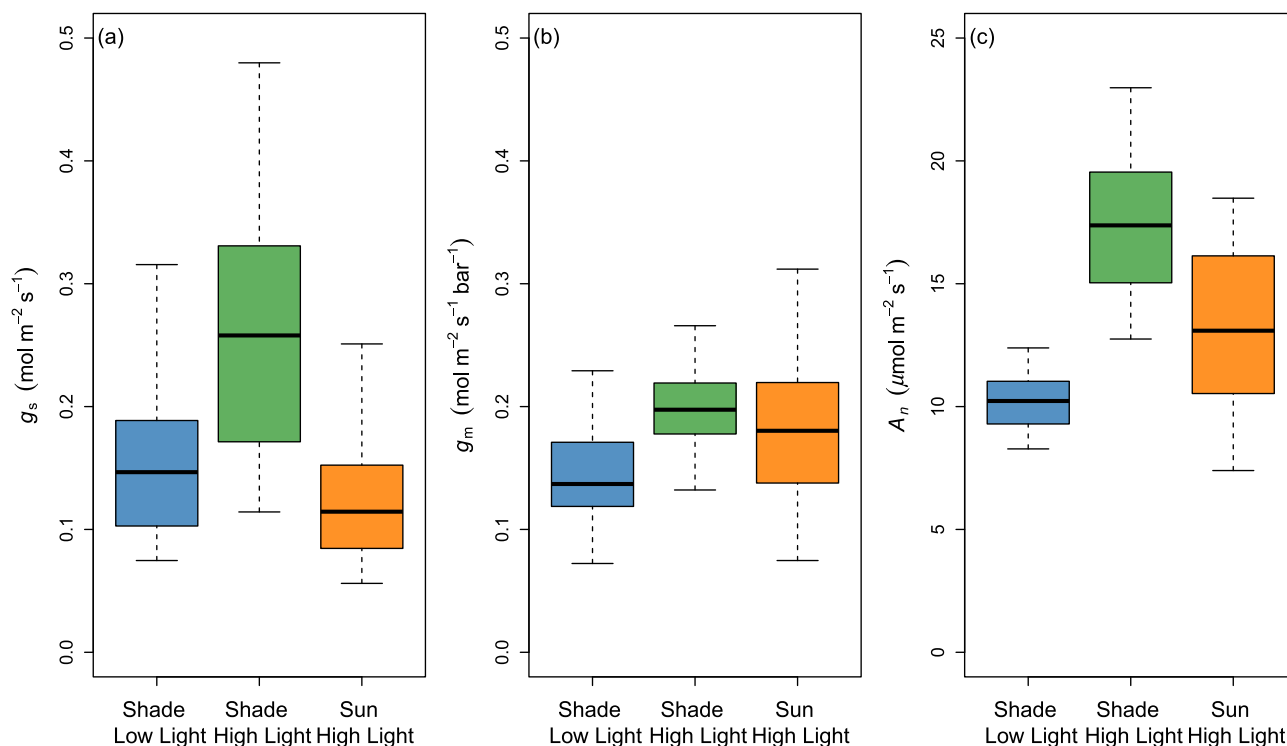

Figure 4. Box plots of stomatal conductance (g_s) (a), mesophyll conductance (g_m) (b) and leaf net photosynthesis rate (A_n) (c) of sun leaves and shade leaves at low and high light pooled across six measurement dates and both temperature treatments. For statistical comparisons, see Table 2.
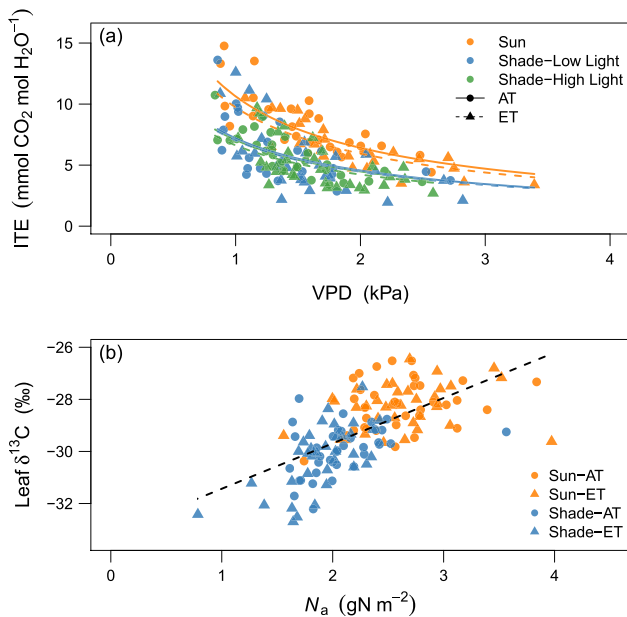
Figure 5. Two measures of water-use efficiency related to vapour pressure deficit and leaf nitrogen per area (N_a). (a) Instantaneous transpiration efficiency (ITE) declines with VPD, and is lower for shade leaves (either when measured in high or low light). VPD is the leaf to air pressure difference inside the gas exchange cuvette and lines represent predictions from the optimal ITE model with a g_1 value for each leaf type and temperature treatment (AT, ambient temperature; ET, elevated temperature). (b) The relationship between leaf $\delta^{13}C$ (higher values indicate higher water-use efficiency) and leaf N_a for sun leaves at high light and shade leaves at low light. The dashed line represents the significant linear model fit across all leaves with a marginal and conditional R^2 of 0.41 and 0.45, respectively.

Figure 6. Mesophyll conductance (g_m) measured on shade leaves in low light, and in high light (on the same leaves). After measurement in the shade, the photosynthetic photon flux density (PPFD) was increased to match the light environment of full sun leaves in the same tree. All six measurement dates are included, and symbols reflect the temperature treatment (AT, ambient temperature; ET, elevated temperature). Measurements of g_m were recorded after CO_2 and water vapour fluxes were stable in the leaf cuvette, which took approximately 25 min after light intensity was increased. The dashed line is the 1:1 relationship.

use. Additionally, with measurements recorded across a large natural range of air temperatures only minimal effects of a $+3\,^{\circ}C$ warming treatment were detected on leaf physiology, consistent with temperature acclimation demonstrated in this experiment (Aspinwall *et al.* 2016).

Resource distribution and photosynthetic capacity

The allocation of N_a along canopy gradients constrains A_n and is thus a key trait in determining the relative contribution of individual leaves to canopy C gain. Decreasing light availability should decrease the investment of N into photosynthetic enzymes within a canopy (Mooney & Gulmon 1979). As a result, acclimation of photosynthetic capacity to irradiance is typically reflected in the key photosynthetic biochemical parameters V_{cmax} and J_{max} (Farquhar *et al.* 1980). Our data agree with these conventional conclusions as the distribution of N_a, A_{max} and A_n were all reduced in shade leaves. Leaf mass per area (LMA), however, was not different between sun and shade leaves, consistent with small differences reported in a clonal *Eucalyptus* plantation (Nouvellon *et al.* 2010), but in contrast to data for many other species (Niinemets *et al.* 2015). Similar LMA in sun and shade leaves could be the result of initial leaf formation and expansion under comparable light conditions
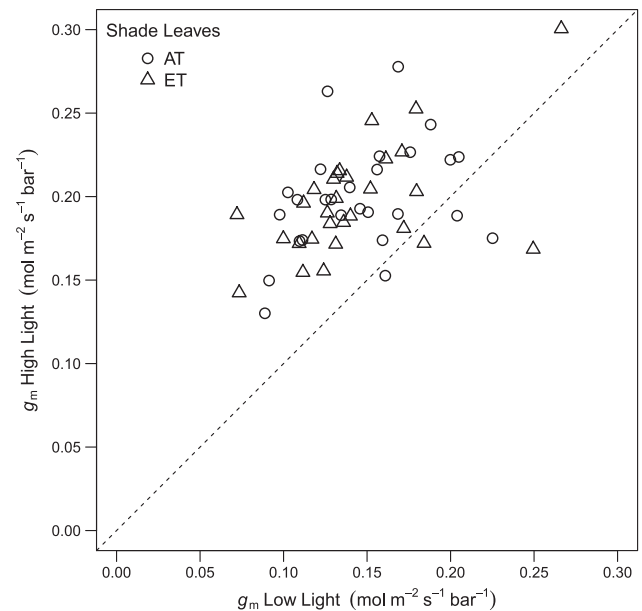
and subsequent shade arising during canopy development in this evergreen species. Unmeasured differences in total non-structural carbohydrate contents between leaf types may also have masked differences in leaf thickness or density. For example, sun leaves of *Abies amabilis* foliage that became shaded exhibited physiology similar to shaded leaves, while retaining the morphology of sun leaves (Brooks *et al.* 1996).

Photosynthesis is also limited by the supply of water to the upper canopy. Ultimately, the ability of tree hydraulic architecture to supply water to foliage across increasing pathlengths affects productivity and survival (Sellin *et al.* 2008). Using a two-leaf model, Peltoniemi *et al.* (2012) theorized that optimal N distribution will be proportional to light distribution only if K_L is also optimally distributed. We found higher K_L in shade leaves compared to sun leaves, contrary to the prediction by Peltoniemi *et al.* (2012), but consistent with higher g_s attained by shade leaves in high light conditions. This finding is consistent with the idea that g_s is regulated, at least in part, by K_L (Hubbard *et al.* 2001).

Physiological behaviour of sun and shade leaves

Unexpected higher g_s in shade leaves compared to sun leaves led to decreased ITE in shade leaves throughout the experiment. Consistently lower leaf $\delta^{13}C$ in shade leaves also suggests that observed higher C_i and C_c in shade leaves was prevalent long term (Figure S2) (Marshall *et al.* 2007), despite the
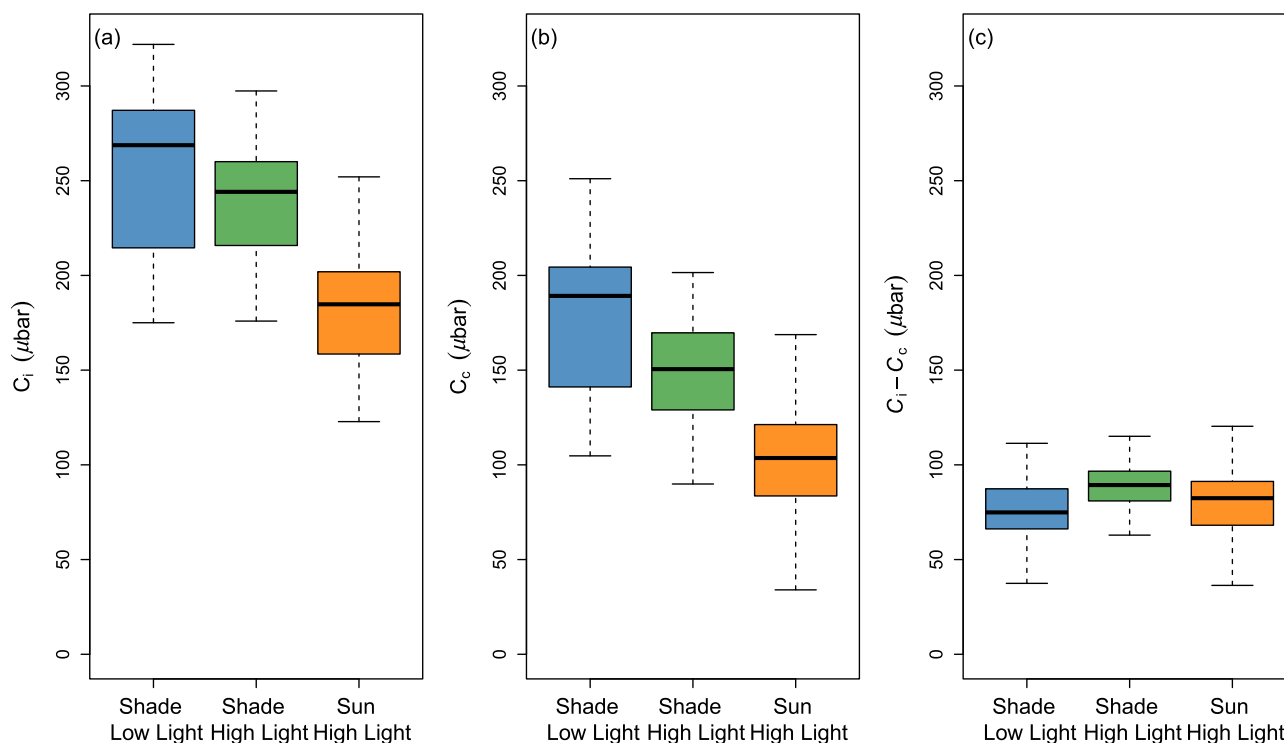
Figure 7. Boxplots of (a) intercellular CO_2 concentration (C_i), (b) CO_2 concentration in the chloroplasts (C_c) and (c) CO_2 drawdown from substomatal cavities to sites of carboxylation of sun leaves and shade leaves at both low and high light ($C_i - C_c$). All data across six measurement dates and both temperature treatments are included. For statistical comparisons, see Table 2.

observation that g_m accounted for nearly 70% of the variation in $\varDelta_o$. Higher g_s in shade leaves relative to the differences in A_n, appears to result in comparatively inefficient water use as more water is used per unit photosynthesis. As whole canopy C gain integrates the efficiency of all leaves, this raises the question of why shade leaves exhibit a lower water-use efficiency compared to sun leaves.

In sun leaves, A_n and g_s were strongly correlated, exhibiting behaviour broadly consistent with optimal stomatal theory (Fig. 5). However, lower rates of A_n in shade leaves were not coupled with decreases in g_s, leading to the observed decreases in ITE. This is significant as optimal stomatal regulation to balance C gain with water loss has been reported across a wide range of ecosystems and plant functional types; however, empirical data is often collected only on sun leaves (e.g. Prentice *et al.* 2014; Lin *et al.* 2015). For the entire canopy to exhibit optimal regulation of stomatal conductance, that is, to maximize photosynthesis minus transpiration cost (Cowan & Farquhar 1977), sun and shade leaves should have equal ITE – provided environmental drivers are similar (Buckley *et al.* 2014). In our study, higher g_s in shade leaves may in part be explained by higher K_L, which effectively reduces the cost of water transport, allowing more profligate water use in shade leaves. Consistent with our results, Buckley *et al.* (2014) found that shade leaves in a grapevine canopy exhibited higher g_s than expected based on their environment and comparison to sun leaves. Further work is needed to confirm the generality of this observation.

It is also possible that higher g_s in shade leaves is a strategy to take advantage transient periods of high light quickly in shade leaves, avoiding the delay associated with stomatal opening (Tausz *et al.* 2005; Vico *et al.* 2011). Our results support this hypothesis, as shade leaves increased A_n to values equal or even outperforming sun leaves when subjected to a brief period of identical high-light intensity. Transpiration-induced cooling in shade leaves, by keeping stomata open, has also been suggested as an effective strategy to reduce sunfleck-induced rapid increase in leaf temperature (Schymanski *et al.* 2013), which has been shown to inhibit C gain (Leakey *et al.* 2003). However, this response likely occurs at very high air temperatures and may not explain the observed g_s in shade leaves across the large natural seasonal range of temperatures included in our study. How prevalent each of these strategies are within tree canopies is still unknown, as empirical studies assessing photosynthetic responses to sunflecks generally focus on seedlings (Küppers & Schneider 1993; Pepin & Livingston 1997; Leakey *et al.* 2002) and understory plants, often in deep shade (Chazdon & Pearcy 1991; Allen & Pearcy 2000; Brantley & Young 2009), although leaves within a forest canopy exhibited a similar uncoupling of A_n and g_s across a light gradient (Tjoelker *et al.* 1995). Thus, our findings highlight a critical need for empirical measurements of shade leaves under dynamic light environments in order to accurately scale C gain from leaf to canopy (see De Pury & Farquhar 1997).

We found that A_n and g_m scaled positively across leaf types (Fig. 3b) and, unexpectedly, increased in shade leaves when light intensity was increased to values similar in sun leaves. Comparable LMA within the canopy likely allowed for this up-regulation of g_m, which played a critical role in the

photosynthetic response of shade leaves to sunflecks. Components of LMA, such as palisade thickness and cell surface exposed to intercellular air space, set an upper limit for g_m, but considerable variation in g_m can exist for a given LMA (Flexas *et al.* 2008; Milla-Moreno *et al.* 2016). Possible mechanisms for the rapid (~25 min) up-regulation of g_m to light intensity include regulation of aquaporins, which can facilitate increases in the CO_2 permeability of the cell membranes (Hanba *et al.* 2004; Heinen *et al.* 2009; Li *et al.* 2014) or movement of chloroplasts to facilitate CO_2 diffusion (Tholen *et al.* 2008). It has also been proposed that g_m and K_L should be coordinated, as optimizing hydraulic supply allows both g_s and g_m to enhance A_n (Flexas *et al.* 2013). Here, higher K_L in shade leaves under high light lends support to the potential coordination K_L and g_m and also to the association of higher A_n with higher K_L when light becomes saturating, as reported by Franks (2006).

If shade leaves 'lie in wait' for sunflecks, then perhaps we should consider an alternate leaf economic strategy to maximize C gain, beyond conventional trade-offs associated with canopy resource distribution. This is because the role of g_s in regulating photosynthetic induction impacts the capacity of a leaf to utilize sunflecks (Way & Pearcy 2012). If the valuation of sunflecks as a C resource is large enough, then costs of sub-optimal stomatal behaviour could be offset over the leaf lifespan or across the entire canopy when considering both sun and shade leaf types (Vico *et al.* 2011). For example, the potential C gain in leaves where sunflecks constitute a large proportion of total daily PPFD may be large enough to accommodate the cost of increased water use in the shade. However, accounting for the heterogeneous nature of light within a canopy remains a current challenge for empirical and modelling studies. Thus, models which predict leaf photosynthesis from N distribution within a canopy will be incomplete unless inclusion of canopy light extinction and the integration of sunflecks on shade leaves are included (De Pury & Farquhar 1997).

CONCLUSIONS

Here we show that dynamic physiological responses of shade leaves to altered light environments have important implications for up-scaling leaf level measurements to the canopy. Although resource allocation constrains leaf photosynthetic capacity, it is the physiological behaviour of individual leaves that determines C gain. These findings suggest that current theories of leaf optimal behaviour should be extended to include dynamic light environments, which will have implications for process-based models that predict canopy C gain from rates of leaf photosynthesis. Furthermore, the dynamic nature of g_m cannot be simply parameterized in vegetation models, and further experimental evidence is needed so that it can be represented properly. Additional empirical data, across multiple tree species, are needed to determine both the mechanisms and the capacity of g_m to respond to environmental drivers.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Daily maximum and minimum temperature (a), daily maximum VPD (b) and total daily PPFD (c) for each chamber across the experiment duration.

Figure S2. Relationship between the observed discrimination of $^{13}CO_2$ measured during photosynthesis (Δ_o) and measured C_i/C_a for sun leaves measured at high light and shade leaves measured at both low and high light. The solid line represents the theoretical line for C3 plants from Evans *et al.* (1986).

Figure S3. Response of A_n (a), g_m (b) and $C_i - C_c$ to leaf temperature for sun leaves and shade leaves at low and high light. Shaded symbols represent each monthly measurement campaign. Solid lines, coloured by leaf and light type, are fitted line for the relationship with each parameter and leaf temperature across all measurement campaigns. All parameters with no relationship are fitted with zero slope and the overall mean value for each treatment combination. Weak negative relationships with g_m and increasing leaf temperature were detected with sun and shade leaves under their local light environment ($R^2 = 0.16$ and 0.08, respectively).

Figure S4. Response of VPD (a), g_s (b) and $C_a - C_i$ to leaf temperature for sun leaves and shade leaves at low and high light. Shaded symbols represent each monthly measurement campaign. Solid lines, coloured by leaf and light type, are fitted line for the relationship with each parameter and leaf temperature across all measurement campaigns. All parameters with no relationship are fitted with zero slope and the overall mean value for each treatment combination. Leaf VPD inside the gas exchange cuvette was positively correlated with increasing leaf temperature for sun leaves and shade leaves at low and high light ($R^2 = 0.73$, 0.58 and 0.72, respectively).

Methods S1. Description of the calculation of g_m from carbon isotope discrimination during C3 photosynthesis