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

Courtney E. Campany1, Mark G. Tjoelker1, Susanne von Caemmerer2, Remko A. Duursma1.

1 Hawkesbury Institute for the Environment, Western Sydney University, Locked Bag 1797, Penrith 2751 NSW, Australia  
2 ARC Centre of Excellence for Translational Photosynthesis, Plant Science Division, Research School of Biology, The Australian National University, Canberra 2601 ACT, Australia

Author for Correspondence:  
*Courtney Campany*  
*Tel: +61 2 4570 1421*  
*Email:* [*courtneycampany@gmail.com*](mailto:courtneycampany@gmail.com)  
  
 **Running Title**  
Mesophyll conductance response to light

# 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, 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 CO2 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 (*V*cmax and *J*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. Here 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.

## Keyword index

*Eucalyptus tereticornis*, mesophyll conductance, photosynthesis, stomatal conductance, sunflecks

# 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. Due to 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 modified to efficiently use light (Vogelman *et al.* 1996; Niinemets & Valladares 2004), as changing irradiance with canopy depth strongly affects rates of leaf photosynthesis (*A*n) (Evans 1995). To estimate 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, are 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 developed for 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 C3 plants is limited by the [CO2] available for fixation by Rubisco within the chloroplast and this [CO2] is a function of the drawdown of CO2 from the atmosphere to the site of carboxylation (Warren 2008). There are two major resistance pathways to CO2 diffusion: the CO2 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). Part of the variation in photosynthetic capacity between sun and shade leaves may arise 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. CO2, 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 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 of the growth environment 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 CO2, through either *g*s or *g*m, will have implications for up-scaling *A*n for sun and shade leaves. Additionally, light-saturated rates of *A*n are limited by the maximum rate of Rubisco carboxylation (*V*cmax) or the maximum rate of photosynthetic electron transport (*J*max) across a range of temperatures, yet their temperature dependencies are not the same (Farquhar *et al.* 1980; Medlyn *et al.* 2002). How these parameters 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 CO2 diffusion pathway via stomatal and mesophyll conductance. As leaves which developed in the shade 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 *Eucalyptus 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 are 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 6 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 (3 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 CO2 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 65cm 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±0.1 m2. For ET treatments, trees had a mean diameter of 34.1±2.1 mm, height of 418.3±23.1 cm and an leaf area of 6.2±0.2 m2. Leaf area was calculated based on complete leaf counts and mean leaf size from a subsample (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 photosynthetic photon flux density (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 10:00-14:30 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 x 3 cm) leaf cuvette 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 13C isotope discrimination. The CO2 in the leaf cuvette was set at ambient atmospheric [CO2] (400 ppm) with a flow rate of 200 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 temperature and vapour pressure deficit (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 CO2 and water vapour fluxes re-stabilized in the leaf cuvette (ca. 25 min).

Once CO2 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 12CO2 and 13CO2 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 4 or 5 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 CO2) 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:

(1)

where o is the observed discrimination and i, gm , e and f are the contributions to fractionation if *C*i = *C*c, *g*m, respiration and photorespiration, respectively. For this study, the CO2 compensation point () and respiration during the day (Rd) 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 o between sun and shade leaves and the simulated sunfleck were compared as a function of *C*i/Ca. Once *g*m was calculated, the chloroplast CO2 partial pressure (*C*c) and the drawdown of CO2 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:

(2)

## Photosynthetic capacity

Photosynthetic capacity (*A*max) of sun and shade leaves were measured at saturating light and [CO2] 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 mol m-1 s-1 and [CO2] of 1800 l l-1, with leaf temperature (*T*leaf) 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 analyzed for leaf N content and 13C. 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 CO2 and N2 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 () 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 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 () 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) with the equation:

(3)

As light intensity was increased for shade leaves before they were detached, the *E*L measured at high light was used to 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 vs. 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 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 (Figure 1). Leaf *N*a was approximately 20% higher in sun leaves compared 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 (+58%) compared to sun leaves (Table 1). This was due to differences in transpiration rates (*E*L , Table 2) between leaf types at high light, as neither leaf water potentials ( and , Table 1) differed between leaf types. No effect of the warming treatment was detected on PPFD, , , *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, Figure 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. Photosynthesis within leaf types and warming treatments was similar through time and across the range of leaf temperatures measured (Figure 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 (Figure 3a). In high light, *g*s of shade leaves was significantly greater than both shade leaves at low light and sun leaves, pooled across all measurement dates (Figure 4a). No effect of the warming treatment was detected on *g*s within or across leaf types. Stomatal conductance within leaf types and treatments was similar through time and across the range of leaf temperatures measured (Figure 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 and for shade leaves measured at low and high light was 2.59±0.12 and 2.74±0.04. 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 (Figure 5a). Within leaf types and light treatments the response of VPD to leaf temperature was similar across all measurement campaigns (Figure S4a).

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

## Leaf carbon isotope discrimination and mesophyll conductance

The observed carbon isotope discrimination () measured during photosynthesis was positively correlated with *C*i/*C*a for both leaf types (P < 0.001), with larger o detected for sun leaves and shade leaves at high light than shade leaves at low light (Figure S2). Carbon isotope discrimination associated with *g*m accounted for the majority of (69.7±0.4%) and varied little across measurement temperatures, leaf types, or warming treatments. The remainder of the variation in consists of the contributions of *g*s, respiration and photorespiration to discrimination.

Mean *g*m was higher in sun compared to shade leaves (+27%) under their local light environment (P < 0.001). Following the increase in PPFD from low to high light on the same leaf, *g*m values of shade leaves increased on average 55% after approximately 25 min (Figure 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 (Figure 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 (Figure 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 (Figure S3b), but a weak negative relationship with increasing leaf temperature was detected with sun and shade leaves under their local light environment (P = 0.001 for sun leaves, P = 0.04 for shade leaves).

## Variation in intercellular and chloroplastic CO2 concentrations

Higher *g*s in shade leaves under low and high light led to significant increases in *C*i compared to sun leaves (Figure 7a). The chloroplast CO2 partial pressure was comparable between shade leaves when measured at both low and high light conditions (Figure 7c). In sun leaves *C*c was significantly lower than shade leaves, consistent with a lower *C*i. The drawdown of CO2 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 (Figure 7c). This result demonstrates the approximately proportional relationship between *g*m and *A*n across all leaves. The CO2 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 (Figure S4c and S3c, respectively).

# Discussion

Here we show that *A*n in leaves within canopies of *Eucalyptus 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 CO2 conductance pathway should be accounted for when testing optimizations of canopy C uptake and water use in future studies. Additionally, with measurements recorded across a large natural range of air temperatures only minimal effects a +3 °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, both measures of photosynthetic capacity, 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). Similar LMA in sun and shade leaves could be the result of leaf formation under comparable light conditions or possible unmeasured differences in total non-structural carbohydrates contents between leaf types that 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 ability to supply 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) theorizes that optimal N distribution will be proportional to light distribution only if *K*L is also optimally distributed. In this study, variation in leaf N distribution and *A*n rates were not associated with subsequent changes in *K*L between sun and shade leaves. Thus, no direct relationship between water supply capacity and N distribution or *A*n within the canopy were detected.

## 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 13C 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 observation that *g*m accounted for nearly 70% of the variation in o. Higher *g*s in shade leaves relative to the differences in *A*n, appears to result in 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 maintain 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). As a result, the often used economic framework of balancing costs of using water versus N allocation to predict *A*n (Wright *et al.* 2003) may break down when considering all leaves within a tree canopy.

It is possible that higher *g*s in shade leaves is a strategy to take advantage of high light quickly in shade leaves, avoiding the delay with stomatal opening (Tausz *et al.* 2005). 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), though 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. Possible mechanisms for the rapid (~25 min) response of *g*m to light intensity include regulation of aquaporins, which can facilitate increases in the CO2 permeability of the cell membranes (Hanba *et al.* 2004; Heinen *et al.* 2009; Li *et al.* 2014) or movement of chloroplasts to facilitate CO2 diffusion (Tholen *et al.* 2008). Our findings support growing evidence that *g*m is highly variable and can respond to environmental variables (Flexas *et al.* 2007, 2008). Here we provide empirical data showing *g*m not only varies within a canopy, but that the up-regulation of *g*m plays a critical role in the photosynthetic response of shade leaves to sunflecks.**

*fixed vs variable controls of gm* 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 CO2 permeability of the cell membranes (Hanba *et al.* 2004; Heinen *et al.* 2009; Li *et al.* 2014) or movement of chloroplasts to facilitate CO2 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 supports 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 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 PFFD 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.

# Acknowledgements

We thank Craig Barton and Burhan Amiji for maintaining the whole-tree chambers experiment and for their outstanding technical assistance. We thank Sune Linder and the Swedish University for Agricultural Science for providing the whole-tree chambers. The complete dataset can be downloaded from *link to figshare will be added*.

# Tables

**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 m2), leaf nitrogen per unit area (*N*a, gN m-2), 13C (‰), pre-dawn leaf water potential (pd, MPa), midday leaf water potential (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 *V*cmax and *J*max are treatment mean (± 1 standard error) from A-*C*i curves measured in each chamber at 25 °C and saturating light. 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** | **pd** | **L** | **13C** |
| Sun | AT | 114.1 (4.5) a | 28.4 (2.2) b | 2.63 (0.08) b | 1.71 (0.21) 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 | 1.78 (0.18) 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 | 2.73 (0.22) 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 | 2.79 (0.22) 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 |

**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, mol 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 CO2 concentration (*C*i, mol mol-1), chloroplastic CO2 concentration (*C*c, mol mol-1) and leaf-to-air vapour pressure deficit (VPD, kPa) are given as the mean (± 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 | 0.163 (0.005) c | 8.26 (0.48) b | 1.78 (0.07) a | 1.60 (0.04) ab | 179.8 ( 3.2) a | 92.2 ( 2.9) a |
|  |  | ET | 13.1 (0.3) b | 0.123 (0.005) a | 0.153 (0.007) bc | 6.57 (0.39) ab | 2.21 (0.09) a | 1.90 (0.05) b | 187.9 ( 2.9) a | 92.2 ( 2.8) a |
| Shade | Low | AT | 10.4 (0.1) a | 0.150 (0.005) a | 0.117 (0.004) ab | 6.24 (0.50) a | 1.93 (0.07) a | 1.40 (0.04) a | 255.4 ( 3.8) b | 160.0 ( 4.1) c |
|  |  | ET | 10.0 (0.1) a | 0.146 (0.005) a | 0.116 (0.004) a | 5.43 (0.51) a | 2.23 (0.09) a | 1.60 (0.05) a | 253.8 ( 4.1) b | 160.3 ( 3.5) bc |
|  | High | AT | 18.1 (0.3) c | 0.255 (0.007) b | 0.184 (0.003) c | 5.85 (0.33) a | 3.42 (0.12) b | 1.40 (0.04) a | 237.4 ( 2.2) b | 137.4 ( 1.9) b |
|  |  | ET | 16.7 (0.2) c | 0.246 (0.009) b | 0.177 (0.003) c | 5.02 (0.35) a | 3.81 (0.15) b | 1.70 (0.04) ab | 238.1 ( 3.2) b | 141.7 ( 2.8) bc |
| P value |  |  | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.005 | 0.001 | 0.001 |

# Figures

**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.  
  
**Figure 2**. The relationship between mean leaf N per area (*N*a) for each chamber and photosynthetic capacity (*A*~max) for sun and shade leaves grown under elevated (ET) and ambient (AT) temperature treatments. *A*~max represents the net photosynthetic rate at the highest Ci values taken from A-*C*i curves measured once on all trees, in February 2014, at 25°C and at saturating light (1800 mols m-1 s-1). 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.  
  
**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.  
  
**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.  
  
**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 13C (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 CO2 and water vapour fluxes were stable in the leaf cuvette, which took approximately 25 minutes after light intensity was increased. The dashed line is the 1:1 relationship.  
  
**Figure 7**. Boxplots of (a) intercellular CO2 concentration (*C*i), (b) CO2 concentration in the chloroplasts (*C*c) and (c) CO2 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.

# References

Allen M.T. & Pearcy R.W. (2000) Stomatal behavior and photosynthetic performance under dynamic light regimes in a seasonally dry tropical rain forest. *Oecologia* **122**, 470–478.

Aspinwall M.J., Drake J.E., Campany C., Vårhammar A., Ghannoum O., Tissue D.T., … Tjoelker M.G. (2016) Convergent acclimation of leaf photosynthesis and respiration to prevailing ambient temperatures under current and warmer climates in Eucalyptus tereticornis. *New Phytologist*.

Baldocchi D.D., Wilson K.B. & Gu L. (2002) How the environment, canopy structure and canopy physiological functioning influence carbon, water and energy fluxes of a temperate broad-leaved deciduous forestâan assessment with the biophysical model CANOAK. *Tree Physiology* **22**, 1065–1077.

Barton C.V.M., Ellsworth D.S., Medlyn B.E., Duursma R.A., Tissue D.T., Adams M.A., … Linder S. (2010) Whole-tree chambers for elevated atmospheric CO2 experimentation and tree scale flux measurements in south-eastern Australia: The Hawkesbury Forest Experiment. *Agricultural and Forest Meteorology* **150**, 941–951.

Bates D., Maechler M., Bolker B. & Walker S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**, 1–48.

Boardman N.K. (1977) Comparative photosynthesis of sun and shade plants. *Annual Review of Plant Physiology* **28**, 355–377.

Brantley S.T. & Young D.R. (2009) Contribution of sunflecks is minimal in expanding shrub thickets compared to temperate forest. *Ecology* **90**, 1021–1029.

Brooks J.R., Sprugel D.G. & Hinckley T.M. (1996) The effects of light acclimation during and after foliage expansion on photosynthesis of *abies amabilis* foliage within the canopy. *Oecologia* **107**, 21–32.

Buckley T.N., Cescatti A. & Farquhar G.D. (2013) What does optimization theory actually predict about crown profiles of photosynthetic capacity when models incorporate greater realism? *Plant, Cell & Environment* **36**, 1547–1563.

Burgess S.S.O., Pittermann J. & Dawson T.E. (2006) Hydraulic efficiency and safety of branch xylem increases with height in *Sequoia sempervirens* (D. Don) crowns. *Plant, Cell & Environment* **29**, 229–239.

Chazdon R.L. & Pearcy R.W. (1991) The importance of sunflecks for forest understory plants. *Bioscience* **41**, 760–766.

Cowan I.R. & Farquhar G.D. (1977) Stomatal function in relation to leaf metabolism and environment. In: *Symposia of the society for experimental biology*, pp. 471–505.

Crous K.Y., Zaragoza-Castells J., Ellsworth D.S., Duursma R.A., Loew M., Tissue D.T. & Atkin O.K. (2012) Light inhibition of leaf respiration in field-grown *Eucalyptus saligna* in whole-tree chambers under elevated atmospheric CO2 and summer drought. *Plant, Cell & Environment* **35**, 966–981.

De Pury D.G.G. & Farquhar G.D. (1997) Simple scaling of photosynthesis from leaves to canopies without the errors of big-leaf models. *Plant Cell and Environment* **20**, 537–557.

Douthe C., Dreyer E., Epron D. & Warren C.R. (2011) Mesophyll conductance to CO2, assessed from online TDL-AS records of 13CO2 discrimination, displays small but significant short-term responses to CO2 and irradiance in *Eucalyptus* seedlings. *Journal of Experimental Botany* **62**, 5335–5346.

Douthe C., Dreyer E., Brendel O. & Warren C.R. (2012) Is mesophyll conductance to CO2 in leaves of three *Eucalyptus* species sensitive to short-term changes of irradiance under ambient as well as low CO2? *Functional Plant Biology* **39**, 435–448.

Drake J.E., Tjoelker M.G., Aspinwall M.J., Reich P.B., Barton C.V.M., Medlyn B.E. & Duursma R.A. (2016) Does physiological acclimation to climate warming stabilize the ratio of canopy respiration to photosynthesis? *New Phytologist*.

Duursma R.A., Payton P., Bange M.P., Broughton K.J., Smith R.A., Medlyn B.E. & Tissue D.T. (2013) Near-optimal response of instantaneous transpiration efficiency to vapour pressure deficit, temperature and [CO2] in cotton (*Gossypium hirsutum* L.). *Agricultural and Forest Meteorology* **168**, 168–176.

Evans J.R. (1995) Carbon fixation profiles do reflect light absorption profiles in leaves. *Functional Plant Biology* **22**, 865–873.

Evans J. & Poorter H. (2001) Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area and nitrogen partitioning in maximizing carbon gain. *Plant, Cell & Environment* **24**, 755–767.

Evans J.R. & von Caemmerer S. (2013) Temperature response of carbon isotope discrimination and mesophyll conductance in tobacco. *Plant, Cell & Environment* **36**, 745–756.

Farquhar G.D. & Cernusak L.A. (2012) Ternary effects on the gas exchange of isotopologues of carbon dioxide. *Plant, Cell & Environment* **35**, 1221–1231.

Farquhar G.D., Caemmerer S. von & Berry J.A. (1980) A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species. *Planta* **149**, 78–90.

Field C. (1983) Allocating leaf nitrogen for the maximization of carbon gain: leaf age as a control on the allocation program. *Oecologia* **56**, 341–347.

Field C.H. & Mooney H.A. (1986) Photosynthesis–nitrogen relationship in wild plants. In: *On the economy of plant form and function: Proceedings of the sixth maria moors cabot symposium*, pp. 25–55. Cambridge University Press.

Flexas J., Diaz-Espejo A., Galmes J., Kaldenhoff R., Medrano H. & Ribas-Carbó M. (2007) Rapid variations of mesophyll conductance in response to changes in CO2 concentration around leaves. *Plant, Cell & Environment* **30**, 1284–1298.

Flexas J., Ribas-Carbó M., Diaz-Espejo A., Galmes J. & Medrano H. (2008) Mesophyll conductance to CO2: current knowledge and future prospects. *Plant, Cell & Environment* **31**, 602–621.

Flexas J., Scoffoni C., Gago J. & Sack L. (2013) Leaf mesophyll conductance and leaf hydraulic conductance: an introduction to their measurement and coordination. *Journal of Experimental Botany* **64**, 3965–3981.

Franks P.J. (2006) Higher rates of leaf gas exchange are associated with higher leaf hydrodynamic pressure gradients. *Plant, Cell & Environment* **29**, 584–592.

Griffiths H. & Helliker B.R. (2013) Mesophyll conductance: internal insights of leaf carbon exchange. *Plant, Cell & Environment* **36**, 733–735.

Hanba Y.T., Kogami H. & Terashima I. (2002) The effect of growth irradiance on leaf anatomy and photosynthesis in *Acer* species differing in light demand. *Plant, Cell & Environment* **25**, 1021–1030.

Hanba Y.T., Shibasaka M., Hayashi Y., Hayakawa T., Kasamo K., Terashima I. & Katsuhara M. (2004) Overexpression of the barley aquaporin HvPIP2; 1 increases internal CO2 conductance and CO2 assimilation in the leaves of transgenic rice plants. *Plant and Cell Physiology* **45**, 521–529.

Hassiotou F., Ludwig M., Renton M., Veneklaas E.J. & Evans J.R. (2009) Influence of leaf dry mass per area, CO2, and irradiance on mesophyll conductance in sclerophylls. *Journal of Experimental Botany* **60**, 2303–2314.

Heinen R.B., Ye Q. & Chaumont F. (2009) Role of aquaporins in leaf physiology. *Journal of Experimental Botany* **60**, 2971–2985.

Hothorn T., Bretz F. & Westfall P. (2008) Simultaneous inference in general parametric models. *Biometrical Journal* **50**, 346–363.

Hubbard R.M., Ryan M.G., Stiller V. & Sperry J.S. (2001) Stomatal conductance and photosynthesis vary linearly with plant hydraulic conductance in ponderosa pine. *Plant, Cell & Environment* **24**, 113–121.

Küppers M. & Schneider H. (1993) Leaf gas exchange of beech (*Fagus sylvatica* L.) seedlings in lightflecks: effects of fleck length and leaf temperature in leaves grown in deep and partial shade. *Trees* **7**, 160–168.

Leakey A.D.B., Press M.C., Scholes J.D. & Watling J.R. (2002) Relative enhancement of photosynthesis and growth at elevated CO2 is greater under sunflecks than uniform irradiance in a tropical rain forest tree seedling. *Plant, Cell & Environment* **25**, 1701–1714.

Leakey A.D.B., Press M.C. & Scholes J.D. (2003) High-temperature inhibition of photosynthesis is greater under sunflecks than uniform irradiance in a tropical rain forest tree seedling. *Plant, Cell & Environment* **26**, 1681–1690.

Li G., Santoni V. & Maurel C. (2014) Plant aquaporins: roles in plant physiology. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1840**, 1574–1582.

Lin Y.-S., Medlyn B.E., Duursma R.A., Prentice I.C., Wang H., Baig S., … Others (2015) Optimal stomatal behaviour around the world. *Nature Climate Change* **5**, 459–464.

Linderson M.-L., Mikkelsen T.N., Ibrom A., Lindroth A., Ro-Poulsen H. & Pilegaard K. (2012) Up-scaling of water use efficiency from leaf to canopy as based on leaf gas exchange relationships and the modeled in-canopy light distribution. *Agricultural and Forest Meteorology* **152**, 201–211.

Marshall J.D., Brooks J.R. & Lajtha K. (2007) Sources of variation in the stable isotopic composition of plants. In: *Stable isotopes in ecology and environmental science* (eds Michener R, Lajtha K), pp. 22–60. Blackwell Publishing Ltd, Oxford, UK.

Medlyn B.E., Dreyer E., Ellsworth D., Forstreuter M., Harley P.C., Kirschbaum M.U.F., … Others (2002) Temperature response of parameters of a biochemically based model of photosynthesis. II. A review of experimental data. *Plant, Cell & Environment* **25**, 1167–1179.

Medlyn B.E., Duursma R.A., Eamus D., Ellsworth D.S., Prentice I.C., Barton C.V.M., … Wingate L. (2011) Reconciling the optimal and empirical approaches to modelling stomatal conductance. *Global Change Biology* **17**, 2134–2144.

Milla-Moreno E.A., McKown A.D., Guy R.D. & Soolanayakanahally R.Y. (2016) Leaf mass area predicts palisade structural properties linked to mesophyll conductance in balsam poplar (*populus balsamifera* l.). *Botany* **94**, 1–15.

Monsi M. & Saeki T. (2005) On the factor light in plant communities and its importance for matter production. *Annals of Botany* **95**, 549–567.

Mooney H.A. & Gulmon S.L. (1979) Environmental and evolutionary constraints on the photosynthetic characteristics of higher plants. In: *Topics in plant population biology*, Vols. 316-337 (eds Solbrig OT, Jain S, Johnson GB, Raven PH). Springer, New York.

Nakagawa S. & Schielzeth H. (2013) A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution* **4**, 133–142.

Niinemets Ü. (2007) Photosynthesis and resource distribution through plant canopies. *Plant, Cell & Environment* **30**, 1052–1071.

Niinemets Ü. (2010) A review of light interception in plant stands from leaf to canopy in different plant functional types and in species with varying shade tolerance. *Ecological Research* **25**, 693–714.

Niinemets Ü. (2012) Optimization of foliage photosynthetic capacity in tree canopies: towards identifying missing constraints. *Tree Physiology* **32**, 505–509.

Niinemets Ü. & Valladares F. (2004) Photosynthetic acclimation to simultaneous and interacting environmental stresses along natural light gradients: optimality and constraints. *Plant Biology* **6**, 254–268.

Nouvellon Y., Laclau J.-P., Epron D., Kinana A., Mabiala A., Roupsard O., … Saint-André L. (2010) Within-stand and seasonal variations of specific leaf area in a clonal Eucalyptus plantation in the Republic of Congo. *Forest Ecology and Management* **259**, 1796–1807.

Pearcy R.W. (1990) Sunflecks and photosynthesis in plant canopies. *Annual Review of Plant Biology* **41**, 421–453.

Peltoniemi M.S., Duursma R.A. & Medlyn B.E. (2012) Co-optimal distribution of leaf nitrogen and hydraulic conductance in plant canopies. *Tree Physiology* **32**, 510–519.

Pepin S. & Livingston N.J. (1997) Rates of stomatal opening in conifer seedlings in relation to air temperature and daily carbon gain. *Plant, Cell & Environment* **20**, 1462–1472.

Piel C., Frak E., Le Roux X. & Genty B. (2002) Effect of local irradiance on CO2 transfer conductance of mesophyll in walnut. *Journal of Experimental Botany* **53**, 2423–2430.

Prentice I.C., Dong N., Gleason S.M., Maire V. & Wright I.J. (2014) Balancing the costs of carbon gain and water transport: testing a new theoretical framework for plant functional ecology. *Ecology Letters* **17**, 82–91.

R Development Core Team R. (2011) R: A language and environment for statistical computing. **1**, 409.

Schymanski S.J., Or D. & Zwieniecki M.A. (2013) Stomatal control and leaf thermal and hydraulic capacitances under rapid environmental fluctuations. *PloS ONE* **8**, e54231.

Sellin A. & Kupper P. (2007) Effects of enhanced hydraulic supply for foliage on stomatal responses in little-leaf linden (*Tilia cordata* Mill.). *European Journal of Forest Research* **126**, 241–251.

Sellin A., Õunapuu E. & Kupper P. (2008) Effects of light intensity and duration on leaf hydraulic conductance and distribution of resistance in shoots of silver birch (*Betula pendula*). *Physiologia Plantarum* **134**, 412–420.

Tausz M., Warren C.R. & Adams M.A. (2005) Dynamic light use and protection from excess light in upper canopy and coppice leaves of *Nothofagus cunninghamii* in an old growth, cool temperate rainforest in Victoria, Australia. *New Phytologist* **165**, 143–156.

Tazoe Y., von Caemmerer S., Badger M.R. & Evans J.R. (2009) Light and CO2 do not affect the mesophyll conductance to CO2 diffusion in wheat leaves. *Journal of Experimental Botany* **60**, 2291–2301.

Tazoe Y., von Caemmerer S., Estavillo G.M. & Evans J.R. (2011) Using tunable diode laser spectroscopy to measure carbon isotope discrimination and mesophyll conductance to CO2 diffusion dynamically at different CO2 concentrations. *Plant, Cell & Environment* **34**, 580–591.

Terashima I., Miyazawa S.-I. & Hanba Y.T. (2001) Why are sun leaves thicker than shade leaves? Consideration based on analyses of CO2 diffusion in the leaf. *Journal of Plant Research* **114**, 93–105.

Terashima I., Hanba Y.T., Tazoe Y., Vyas P. & Yano S. (2006) Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO2 diffusion. *Journal of Experimental Botany* **57**, 343–354.

Tholen D., Boom C., Noguchi K., Ueda S., Katase T. & Terashima I. (2008) The chloroplast avoidance response decreases internal conductance to CO2 diffusion in Arabidopsis thaliana leaves. *Plant, Cell & Environment* **31**, 1688–1700.

Tjoelker M.G., Volin J.C., Oleksyn J. & Reich P.B. (1995) Interaction of ozone pollution and light effects on photosynthesis in a forest canopy experiment. *Plant, Cell & Environment* **18**, 895–905.

Ubierna N. & Marshall J.D. (2011) Estimation of canopy average mesophyll conductance using C of phloem contents. *Plant, Cell & Environment* **34**, 1521–1535.

Vico G., Manzoni S., Palmroth S. & Katul G. (2011) Effects of stomatal delays on the economics of leaf gas exchange under intermittent light regimes. *New Phytologist* **192**, 640–652.

Vogelman T.C., Nishio J.N. & Smith W.K. (1996) Leaves and light capture: light propagation and gradients of carbon fixation within leaves. *Trends in Plant Science* **1**, 65–70.

von Caemmerer S. & Evans J.R. (2014) Temperature responses of mesophyll conductance differ greatly between species. *Plant, Cell & Environment* **38**, 629–637.

von Caemmerer S., Evans J.R., Hudson G.S. & Andrews T.J. (1994) The kinetics of ribulose-1, 5isphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. *Planta* **195**, 88–97.

Warren C.R. (2008) Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO2 transfer. *Journal of Experimental Botany* **59**, 1475–1487.

Warren C.R., Löw M., Matyssek R. & Tausz M. (2007) Internal conductance to CO2 transfer of adult *Fagus sylvatica*: variation between sun and shade leaves and due to free-air ozone fumigation. *Environmental and Experimental Botany* **59**, 130–138.

Way D.A. & Pearcy R.W. (2012) Sunflecks in trees and forests: from photosynthetic physiology to global change biology. *Tree Physiology* **32**, 1066–1081.

Wood S.N. (2006) Generalized additive models : An introduction with R. CRC Press, 410 pp.

Wright I.J., Reich P.B. & Westoby M. (2003) Least-cost input mixtures of water and nitrogen for photosynthesis. *The American Naturalist* **161**, 98–111.