Response of mesophyll conductance to light availability allows shade leaves to lie in wait

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# Summary

* overview
* method
* big result
* implication

# Introduction

The need to understand and predict carbon (C) uptake in forest ecosystems is crucially important for assessing the impacts of environmental change. Specifically, this involves accurate upscaling of leaf level measurements to predict whole canopy function. Due to the costs and limitations of efficient light harvesting plants cannot expose all leaves to full sun (Niinemets, 2010), making simple upscaling problematic. Incident PPFD declines exponentially with increasing leaf area index, creating a steep light gradient from the canopy top to bottom (Monsi & Saeki, 2005), and photosynthesis (A) increases with irradiance from shade to sun until biochemical limitation occurs (Evans, 1995). Consequently, leaves modify structural and functional properties to efficiently intercept variable light and enhance photosynthetic capacity (Vogelman *et al.*, 1996; Niinemets & Valladares, 2004). To estimate whole canopy C gain it is thus necessary to account for the non-linear response of A to light by distinguishing between shaded and sunlit leaves (De Pury & Farquhar, 1997; Linderson *et al.*, 2012).

The distribution of resources required for A, including nitrogen (N) and water, are also partially defined by canopy light gradients. As A has a saturating response with light and a dependence on N, leaf N should be distributed in the upper canopy to maximize canopy C gain (Field, 1983; Field & Mooney, 1986; Peltoniemi *et al.*, 2012). This can lead to 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 across leaf types (Evans & Poorter, 2001). Sun leaves also 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). Higher rates of A and stomatal conductance (gs) can only be sustained if the hydraulic conductance (K) is also large enough to avoid low leaf water potentials (Hubbard *et al.*, 2001). Optimal photosynthetic N investment in the upper canopy will be wasted if photosynthetic capacity exceeds hydraulic supply (Niinemets, 2012), thus K should also be higher in the upper canopy to supply sunlit leaves with sufficient water (Burgess *et al.*, 2006; Sellin & Kupper, 2007 ; Sellin *et al.*, 2008).

These theoretical distributions of N and water regulate leaf physiological traits that constrain A differently for sun and shade leaves. The balance between the costs and energetic benefits of these leaf traits affecting photosynthetic capacity create trade-offs impacting canopy C gain (Givnish, 1988). During A, CO2 diffuses from the atmosphere through stomata (gs), intercellular air space and into the chloroplast for fixation by Rubisco (mesophyll conductance, gm) (Tazoe *et al.*, 2011) and trade-offs exists that constrain each of these diffusion pathways. For optimal leaf C gain these pathways should act to minimize the resistance to CO2 diffusion while also limiting the intrinsic energy, water and construction costs which diminish the C return from A. As these processes should not be considered independent of each other and must be integrated with hydraulic constraints (Griffiths *et al.*, 2013) predicting their behaviour within canopies remains difficult.

First, gs should be efficiently distributed within a canopy to utilize supplies of light, N and water to maximize A (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). Second, variation in photosynthetic capacity between sun and shade leaves has also been hypothesized to be due to differences in gm (Duursma & Marshall, 2006). Mesophyll conductance can impose limitations on A as large as those associated with gs (Warren, 2008; Ubierna & Marshall, 2011) and these limitations can reduce the efficiency of N use in A (Niinemets, 2007). Interpreting the coupled responses of both gs and gm to A across sun and shade leaves has major implications for predicting canopy C gain, but empirical measurements across tree canopies are still lacking.

Additionally, assessing shade leaf behaviour is made difficult with accounting of short term light fluctuations within a canopy, via sunflecks. How shade leaves utilize sunflecks for short term C gain depends on the combined response time of gs and gm and the underlying photosynthetic biochemistry acclimated to a low light environment (Pearcy, 1990). For example, at short timescales the utilization of sunflecks is limited by the slow response of stomatal opening, effectively limiting the maximum assimilation rate that can be achieved (Way & Pearcy, 2012). Mesophyll conductance has been shown to respond to environmental factors across both long and rapid time scales, possibly faster than gs (Flexas *et al.*, 2008). However, anatomical parameters which regulate gm with changing irradiance such as chloroplast surface area (Terashima *et al.*, 2006) and mesophyll thickness (Boardman, 1977) are unlikely to adapt during short light fluctuations. 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 potentially affect the physiological behaviour of leaves within a canopy. This is because leaves can be exposed to different heat, water and high light stresses as temperature and vapour pressure deficit (VPD) scale positively with canopy light availability (Baldocchi *et al.*, 2002; Niinemets & Valladares, 2004; Niinemets, 2007). How these stresses affect the diffusion of CO2, through either gs or gm will have implications for upscaling A for sun and shade leaves. Additionally, light saturated rates of A are limited by the maximum rate of Rubisco carboxylation (Vcmax) or the maximum rate of photosynthetic electron transport (Jmax) 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 obviously vast, 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* Sm. trees, planted in climate controlled whole tree chambers with ambient and elevated temperature (+3°C) treatments, to empirically evaluate the distribution of resources and leaf physiological behaviour of sun and shade leaves. Our hypotheses are as follows:

1. If whole tree canopies are optimized for C gain, then leaf N, K and photosynthetic capacity were predicted to be higher in full sun leaves.

2. Under comparable leaf VPD, gs and A should be proportional across both sun and shade leaves, while gm was predicted to scale positively with photosynthetic capacity.

3. As shade leaves are constrained by their underlying biochemistry and slow physiological responses, increases in A following sunfleck simulations were not expected to reach levels of full sun leaves.

4. The effects of climate warming was predicted to be greater in sun than shade leaves, seen as increases in stomatal closure and reductions in leaf C gain during summer months.

# Materials and Methods

## Whole tree chamber experimental design

Twelve *Eucalyptus tereticornis* 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, NSW, Australia. Each chamber has a maximum height of 10 m and seedlings were grown for 15 months. A detailed description of the WTC operation and design is available in (Barton *et al.*, 2010). Six chambers were set to match outside ambient air temperatures (AT) while the remaining 6 experienced a constant elevated air temperature treatment of +3°C (ET, Figure 1). Due to the temperature treatment air humidity was not explicitly controlled. The CO2 concentration inside the chamber was set to match outside air, tracking diurnal changes. Trees were watered weekly with 70 L from March 2013 to November 2013. From December 2013 to final harvest trees were watered fortnightly with the mean monthly (100 yr) rainfall amount. In February 2013 half of the chambers (3 each of AT and ET) were subjected to a drought treatment by withholding watering. Due to a limited range of data for the drought treatment only well-watered trees are reported here.

Before seedlings were planted into each chamber they were maintained under well watered conditions in 35 L pots and kept inside each chamber. This allowed for seedlings to gain sufficient biomass before planting while also allowing them to acclimate to chamber temperature treatments. Seedlings were planted into each chamber after mean seedling height reached 100 cm. The top soils at this site, used in both pots and chambers, are an alluvial formation of low-fertility sandy loam soils with low organic matter and low water holding capacity. After 2 months, floors were installed 45 cm from the soil surface. This enabled chamber fluxes of CO2 and H2O from the whole tree canopy to be monitored. 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 diameter of 28.2±1.1 mm, height of 348±15.1 cm and an estimated 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 estimated leaf area of 6.2±0.2 m2.

## Leaf gas exchange, online carbon isotope discrimination and mesophyll conductance

Leaf gas exchange measurements were performed monthly through to the final harvest. Measurements were taken on a representative sun and shade leaf for each tree during each measurement campaign. The newest fully expanded leaf from the stem apex was chosen for gas exchange measurements and sun leaves were measured in the upper third of the canopy. In order to minimize any confounding effects of leaf age, shade leaves were measured first in the lower canopy then gradually higher during each campaign. As shade leaves most likely developed slower this assured that older leaves in the lower canopy were avoided. The nearest leaf on each branch was sampled for measurement of predawn leaf water potential.  
Prior to gas exchange measurements photosynthetic photon flux density (PPFD) was recorded at the individual leaf level and 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 full sun or full shade. A ceptometer (AccuPAR LP-80, Decagon Devices, Pullman, USA) was then used to measure 1 m integrated PPFD at the canopy height of each chosen leaf type. Five ceptometer readings were recorded within the canopy at random locations of the height of each selected leaf. The mean of these readings was assumed to represent the local light environment of full sun and shade leaves for each tree. All measurements of PPFD and gas exchange were performed on full sun days between 10:00-14:30 h.

Leaf level gas exchange was measured with a standard 6 cm2 leaf chamber using a portable gas exchange system (LI-6400, 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 online C isotope discrimination. The CO2 in the leaf chamber was set at ambient atmospheric [CO2] (400 ppm) with a flow rate of 200 mols s-1. Two identical gas exchanges 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 chamber air temperature. PPFD in the chamber was set to match the individual light environment of each leaf type (explained above). Sun flecks were then simulated for shade leaves by increasing the chamber PPFD to the light environment of the full sun leaf in the same tree. The maximum sunfleck response was then recorded once shade leaves re-stabilized in the leaf cuvette (ca. 25 min).

Once the chamber environment for leaves was 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. These gases were dried by passing through napion gas dryers in the respective gas lines, and then 12CO2 and 13CO2 concentrations were measured for each gas by the TDL. Reference, sample and 2 calibration gases were run on alternating 80 s loops (20 s each), one for each paired WTC leaf, for a total of 12 min. This allowed for 4-5 measurements per leaf and data were averaged over the last 10 s of reference and samples gases 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 TDLAS on each measurement cycle. Photosynthesis, gs, transpiration, vapor pressure deficit and intercellular [CO2] were auto logged every 15 s for each gas exchange system over the same 12 min interval.

Using online C isotope discrimination measurements, the difference between the observed discrimination and what is predicted for light saturated gas exchange is proportional to gm (Griffiths & Helliker, 2013). First, leaf discrimination was calculated by comparing the isotopic composition of the reference gas entering the chamber (13Ce) with the sample gas (13Co) such that:

(1)

where Rs is the isotopic ratio of the sample and Rstnd is the isotopic ratio of the standard Vienna Pee Dee Belemnite. Next, the observed discrimination (obs) is calculated from Evans et al. (1986):

(2)

where:

(3)

and is the ratio of the CO2 entering the well mixed leaf cuvette to the CO2 draw down by the leaf.

Second, C isotope discrimination during C3 photosynthesis () is the resultant discrimination from CO2 diffusion from the atmosphere to the site of carboxylation, consisting or a series of fractionation steps described in (Evans *et al.*, 1986). In this experiment, a modified form of this equation presented in (Evans & Von Caemmerer, 2013) with ternary effect corrections by (Farquhar & Cernusak, 2012) was used such that:

(4)

where o is the observed discrimination and i, gm , e and f are the contributions to fractionation if Ci = Cc, gm, respiration and photorespiration, respectively. The equations for each are as follows:

(5)

(6)

(7)

(8)

where the different fractionation factors include; diffusion through water (ai, 1.8‰), Rubisco carboxylation (b, 29‰), the photorespiratory fractionation (f, 16.2‰) and the combined fractionation through the boundary layer and the stomata (a'). a' is defined by:

(9)

where Cs is the CO2 partial pressure at the leaf surface, ab (2.9‰) is the fractionation from boundary layer diffusion and a is the fractionation due to diffusion in air (4.4‰) (Evans *et al.*, 1986). Ca and Ci are the atmospheric and intercellular partial pressures and is the compensation point in the absence of mitochondrial respiration in the light (Rd). In this experiment both and Rd were derived using a standard Arrhenius function with parameters for *Eucalyptus globulis* from (Crous *et al.*, 2012). The ternary effect corrections (t) are described by:

(10)

where E denotes the transpiration rate and is the total conductance to CO2 diffusion to both the boundary layer and stomatal conductance.

The CO2 diffusion from the intercellular airspace to the chloroplast, gm, is given by its relationship to the CO2 assimilation rate (A) by:

(11)

where Cc is the chloroplast CO2 partial pressure. Once gm was calculated Cc and the drawdown of CO2 from the intercellular air spaces to the site of carboxylation were then estimated using Equation 11. Examples of this approach to measure gas exchange and C isotope discrimination are presented in (Evans & Von Caemmerer, 2013). The variation in o between sun and shade leaves and the simulated sunfleck where then compared as a function of Ci:Ca.

Photosynthetic CO2 response (ACi) curves were measured at 25 °C for one sun and shade leaf for each chamber prior to the initiation of the drought treatment. Each ACi curve began at the reference [CO2] of 400 l l-1 and then consisted of 12 additional steps from [CO2] of 50 to 1800 l l-1 at 25 °C at saturating light (1800 mols m-1 s-1). From these curves the photosynthetic parameters, Jmax and Vcmax, were quantified using the biochemical model of (Farquhar *et al.*, 1980) and fit with the 'plantecophys' package (Duursma, 2015) in R (R Development Core Team, 2011).

## Leaf chemistry and hydraulic parameters

Following gas exchange measurements each leaf was collected, measured for leaf water potential (explained below), scanned for leaf area, dried to constant mass and then 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. Isotopic signatures are reported relative to the VPDP scale.

Predawn () and midday () leaf water potentials (MPa) were measured for sun and shade leaves during each gas exchange campaign using a PMS 1505D pressure chamber (PMS Instruments, Albany, OR, USA). Predawn leaf water potential was measured before sunrise on the same day as gas exchange measurements. Leaves used for gas exchange were immediately sampled for once measurements were completed. All leaves were detached and immediately stored inside foil covered bags before water potential measurements were performed. Leaf water potential and transpiration (E, mmol-2 s-1) from gas exchange were then used to calculate leaf specific hydraulic conductance (K) through the equation:

(12)

where the term Hpwg represents the gravitational potential assumed to be minimal in this experiment due to small tree size (Whitehead, 1998). Leaf level instantaneous transpiration efficiency (ITE) was calculated as leaf photosynthesis divided by transpiration. The g1 parameter was estimated from ITE to VPD response curves by fitting a rearranged optimal stomatal conductance model for ITE (Medlyn *et al.*, 2012) using non-linear regression, where K=0.5 (see Duursma *et al.*, 2013).

## Data analysis

Differences in experimental parameters to either the warming treatment or leaf type were analysed by mixed-effects models in R with WTC as a random effect. Explained variance (R2) of mixed models were computed as in (Nakagawa & Schielzeth, 2013). Confidence intervals (95 %) of mixed effect linear models of A as a function of different physiological parameters were generated using bootstrapping methods with 999 simulations in the 'lme4' package (Bates *et al.*, 2013) in R. For non-linear mixed models models confidence intervals were estimated by fitting a generalized additive model to the data with the 'mgcv' package in R. Results were considered significant at P ≤ 0.05.

# Results

## Leaf resource distribution

Across all measurement campaigns 1 m canopy integrated PPFD was significantly different between sun and shade leaves (P<0.001) and PPFD was reduced by 78% in the shade (Figure 2). The distribution of H2O, via K, was not different across sun and shade leaves (Table 1). This was the result of neither , (Table 1) nor E (Table 2) differing between leaf types. Leaf N, on an area basis, was approximately 20% higher in sun leaves than in shade leaves (Table1). Leaf mass per area was not different between leaf types. No effect of the warming treatment was detected with PPFD, , , K, E, Narea or LMA either within or across leaf types.

## Leaf photosynthesis rates and photosynthetic capacity

Mean leaf photosynthesis rates were significantly higher in sun compared to shade leaves (+23%), under their local light environment (Table 2). Following an increase in available light, A of shade leaves was significantly greater than both shade leaves at low light and sun leaves (P < 0.001). No effect of the warming treatment was detected on rates of A of sun leaves or shade leaves at low or high light. Photosynthesis within leaf types and treatments was similar through time and across the range of leaf temperatures measured (Figure S1a).

The photosynthetic parameters Jmax and Vcmax were both significantly higher in sun than shade leaves (Table 1), as evident from ACi curves from each chamber (Figure 3a). Within leaf types, no effect of the warming treatment was detected on either parameter. Within individual chambers Vcmax was positively related to leaf Narea across leaf types and temperature treatments (P = 0.01, Figure 3b). Additionally, leaf Narea was positively related to A across gas exchange campaigns and leaf types (P < 0.001, Figure 3c). As Vcmax is considered implicit with gm (results below) with conventional ACi curves, a ACc curves were also simulated to determine if treatment differences in ACi parameters where instead the result of differences in gm. When accounting for the effect of gm, photosynthetic capacity was still greater in sun leaves than shade leaves (Figure S3)

## Leaf water use efficiency

Leaf ITE was significantly greater in sun leaves than in shade leaves at low light (P = 0.001). Following an increase in available light, ITE of shade leaves did not differ from shade leaves at low light and was still significantly lower than sun leaves (P < 0.001). ITE in sun leaves was reduced in the warming treatment but no effect was noted in shade leaves with low or high light (Table 2). For all leaf types and light treatments there was a strong response of ITE to VPD, and individual data points broadly corresponded to response curves from the optimal ITE model (Figure 4a). The mean estimated g1 for sun leaves was 1.51±0.11 and for shade leaves with low and high light was 2.59±0.12 and 2.74±0.04. Within leaf types and light treatments the response of VPD to leaf temperature was similar across all measurement campaigns (Figure S2a)

Leaf 13C, as an index of water use efficiency (Marshall *et al.*, 2007), significantly decreased from sun leaves to shade leaves by ca. 2‰ (Table 1). No effects of the warming treatment on leaf 13C were detected. Leaf 13C and Narea were positively correlated for all leaves (P<0.001, Figure 4b), with less negative 13C and higher N investment in sun leaves.

## Leaf physiology

Mean gs was significantly higher in shade compared to sun leaves (+18%) under their local light environment (P = 0.004). Increases in gs led to increases in A in sun leaves, however, gs and A were not correlated in shade leaves under low light (Figure 6a). Following an increase in available light, gs of shade leaves was significantly greater than both shade leaves at low light and sun leaves ( Figure 5a). With increased light availability, both increases in A and gs in shade leaves exhibited similar behavior as observed in sun leaves.

The observed carbon isotope discrimination was positively correlated with Ci:Ca for all leaf types (P < 0.001) with larger o detected for sun leaves and shade leaves at high light (Figure 7). Here, the carbon isotope discrimination associated with gm accounted for the majority of o (69.7±0.4 %) and varied little across measurement temperatures, leaf types, or warming treatments.

Mean gm was higher in sun compared to shade leaves (+27%) under their local light environment (P < 0.001). Following an increase in available light, gs of shade leaves was significantly greater than both shade leaves at low light and sun leaves (Table 2). Relationships between gm and A were nearly proportional within leaf types and light treatments (Figure 5b,c). Photosynthesis scaled positively with increases in gm for all leaves and the large increases in gm in shade leaves under high light resulted in the highest rates of A (Figure 6b). No differences in gs nor gm were detected with the warming treatment within leaf types. Additionally, neither gs nor gm varied significantly across measurements campaigns (Figures S1b and S2b, respectively) and a slightly negative relationship with gm and increasing leaf temperature was detected.

The combination of a non-limiting supply of leaf K and more open stomata lead to a significant increase in Ci in shade compared to sun leaves (Figure 8a). As increases in A in shade leaves were associated with higher rates of gs the Ci was similar than under low light but still greater than sun leaves. As the drop in CO2 for intecellular spaces to the cholorplast, Ci-Cc, measures the coordination between gm and A (Caemmerer & Evans, 2014) an increase in this drawdown was detected in shade leaves at high light (Figure 8b,c). This was a direct result of the increase in gm and its subsequent direct effect on A, when compared to both sun and shade leaves that are acclimated to their local light environment. CO2 drawdown from Ca to Ci and Ci to Cc were both relatively stable across the range of temperatures measured and gas exchange campaigns (Figure S2c and S1c, respectively).

# Discussion

Here we show that dynamic physiological responses in shade leaves to altered light environments has important implications for upscaling leaf level measurements to the canopy. Tree canopies may maintain the ability to upregulate A beyond the constraints of their local light environment, even at the cost of valuable resources. Empirical data accounting for the total conductance of CO2 reveal that shifts in gs and gm in shade leaves can lead to leaf C gain equivalent to sun leaves and may happen quicker than previously thought. Thus, failure to integrate the effects of gm into process based tree growth models may underestimate the ability of canopies to alter their photosynthetic capacity when environmental conditions change. Additionally, with measurements recorded across a large natural range of temperatures we were unable to detect any effects of a +3 °C warming treatment on leaf physiology.

## Resource distribution and photosynthetic capacity

The allocation of N constrains photosynthesis rates 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 into photosynthetic enzyme within a canopy (Mooney and Gulmon 1979). As a result, acclimation of photosynthetic capacity to irradiance is typically reflected in the key photosynthetic biochemical parameters Vcmax and Jmax (Farquhar *et al.*, 1980). Our data agree with these conventional conclusions as the distribution of N, both biochemical parameters and A were all reduced in shade leaves.

Photosynthesis in trees is also limited by the ability to supply water to the upper canopy. Using a two-leaf model Peltoniemi et al. (2012) theorizes that optimal N distribution will be proportional to light distribution only if leaf K is also optimally distributed. In this study, variation in leaf N distribution and A rates were not associated with subsequent changes in leaf K between sun and shade leaves. This is especially meaningful in trees as foliage generally operates at greater water limitations in the upper canopy. As both sun and shade leaves were acclimated to their local light environment this sub-optimal distribution of water supply has the potential to diminish the C return for the whole canopy.

The inefficient distribution of resources led to decreased instantaneous WUE in shade leaves throughout the experiment. Additionally, consistently higher leaf 13C in shade leaves suggests that this pattern was constant across leaf lifespans. From a canopy perspective this pattern in WUE appears to be detrimental to C gain as A in sun leaves was characterized by low rates of gs and low Ci compared to similar Eucalyptus species (see Drake *et al.*, 2014). As whole canopy C gain integrates the efficiency of all leaves, this begs the question of why shade leaves maintained a lower WUE while the photosynthetic efficiency of sun leaves appears at least partially constrained by water supply.

## Leaf physiology of sun and shade leaves

The pattern of wasteful water use in shade leaves is important as we hypothesized that gs and A would be proportional across sun and shade leaves. In sun leaves variation in A and gs were strongly correlated, exhibiting behaviour agreeing with optimal stomatal theory. However, lower rates of A in shade leaves were not coupled with decreases in gs, explaining the observed decreases in WUE. This is significant as optimal stomatal behaviour has been reported across a wide range of ecosystems and plant functional types; however, empirical data is often collected only on sun leaves (see 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 (Wright *et al.*, 2003) may break down when considering all leaves within a tree canopy.

This behaviour may be explained by the response of shade leaves to increases in light availability. Contrary to hypothesis, shade leaves were able to increase A to rates equivalent or even outperforming sun leaves under identical light intensity. It is possible that sustaining higher gs, at the cost of larger transpiration losses, is a strategy to increase the utilization efficiency of sunflecks. These results should not be entirely unexpected as shade leaves experience interchanging periods of sun and shade, despite canopy light extinction (Pearcy, 1990). Unfortunately, empirical studies that assess photosynthetic responses to sunflecks generally focus on seedlings (Küppers & Schneider, 1993; Leakey *et al.*, 2002) and understory plants (Chazdon & Pearcy, 1991; Brantley & Young, 2009). 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.

Increasing the drawdown of CO2 into the intercellular air spaces via gs, however, does not necessarily infer concomitant increases in A. This is because CO2 supply to the site of carboxylation, via gm, may be anatomically constrained. In this study A and gm scaled positively across leaf types and increased proportionately with increased light intensity in shade leaves. Research has suggested that aquaporins can facilitate increases in the CO2 permeability of the cell membranes resulting in rapid modulation of mesophyll conductance (Hanba *et al.*, 2004; Heinen *et al.*, 2009; Li *et al.*, 2014). This provides a potential explanation for the observed rapid increases in gm but is largely untested in leaves of tree species. Our findings support a growing wealth of evidence that gm is highly variable and can respond to environmental variables (Flexas *et al.*, 2008). Here we provide empirical data showing gm not only varies within a canopy but the up-regulation of gm plays a critical role in the photosynthetic response of shade leaves to sunflecks.

## Conclusions

Similar to Laisk (2005) we provide evidence that optimal acclimation, in this case physiological adjustment of the drawdown of CO2 in shade leaves, is possibly directed towards occasional maximums of light availability over adaptations towards a low light environment. 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. 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. For example, the potential C gain in leaves which experience high amounts of sunflecks may be large enough to tolerate excess losses from transpiration. 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 flawed unless inclusion of canopy light extinction and the integration of sunflecks on shade leaves are included (De Pury & Farquhar, 1997).

Furthermore, our findings have important implications for process based models that predict canopy C gain from rates of leaf photosynthesis. Although the distributions of resources, such as N and water, define leaf photosynthetic capacity it is the physiological behaviour of individual leaves which actually determine C gain. The observed physiological behaviour leaves in the shade, at both low and high irradiance, suggests that current theories of leaf optimal behaviour are incomplete. Furthermore, gm must now be considered as a dynamic process in tree growth models that cannot be simply parameterized. Additional empirical data are needed to determine both the mechanisms and the capacity of gm to rapidly increase CO2 drawdown. To improve our ability to predict whole canopy C gain future research should prioritize the incorporation of both sun and shade leaf physiology, which may be optimized differently.

# List of Tables

**Table 1**. *Eucalyptus tereticornis* leaf traits and the distribution of resources between full sun and shade leaves under ambient and elevated temperature treatments. Leaf mass per area, leaf Nitrogenarea, 13C, pd, l and K values represent treatment mean (± 1 standard error) across measurement campaigns (n=6). Values of Vcmax and Jmax are treatment mean(standard error) from ACi curves measured in each chamber at saturating light. Different letters represent significant differences between treatments. The P value represents the overall difference between leaf types and warming treatments.

**Table 2**. Responses of leaf level gas exchange parameters of *Eucalyptus tereticornis* trees between full sun and shade leaves under ambient and elevated temperature treatments. Each value reflects the mean (± 1 standard error) for each treatment across all gas exchange campaigns (n=6). Units for A and E are mol m-2 s-1, for gs and gm are mol m-2 s-1 and for VPDleaf are kPa. Different letters represent significant differences between treatments. The P value represents the overall difference between leaf types and both light and warming treatments.

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**Figure 1**. Daily maximum and minimum temperature (a) and total daily PPFD (b) for each chamber across the experiment duration.

**Figure 2**. Local light environment for sun and shade leaves for each gas exchange campaign. Means with standard errors represent 1 m integrated PPFD (n=5), measured with a ceptometer, at the canopy height of each selected leaf.

**Figure 3**. (a) Photosynthetic CO2 response (ACi) curves for sun and shade leaves at elevated (ET) and ambient (AT) temperature treatments. ACi curves were developed once for all trees, before the initiation of the drought treatment, at 25°C and at saturating light (1800 mols m-1 s-1). (b) The relationship between Vcmax and mean leaf nitrogenarea for each chamber, including sun leaves and shade leaves at low light. (c) the relationship between For (b,c) the dashed line represents the significant linear model fit for all leaves with a marginal and conditional r2 of 0.28 and 0.35 for (b), and 0.24 and 0.33 for (c).

**Figure 4**. (a) Response of instantaneous water use efficiency (ITE) to leaf vapor pressure deficit (VPD) for sun leaves and shade leaves at both low and high light with elevated and ambient temperature treatments. (b) The relationship between leaf 13C and leaf nitrogenarea for sun leaves and shade leaves at low light. For (a) VPD is the leaf to air pressure difference inside the gas exchange cuvette and lines represent predictions from the optimal ITE model with a g1 value for each leaf type and treatment. For (b) the dashed line represents the significant linear model fit for all leaves with a marginal and conditional r2 of 0.41 and 0.45, respectively.

**Figure 5**. The mean ± 1 standard error of stomatal conductance (a), mesophyll conductance (b) and photosynthesis rate (c) of sun leaves and shade leaves at both low and high light with standard errors.

**Figure 6**. The response of leaf photosynthesis rates to stomatal conductance (a) and mesophyll conductance (b) for sun leaves and shade leaves at both low and high light with elevated and ambient temperature treatments. The response of shade leaf physiology to high light was recorded once both photosynthesis and stomatal conductance were stable in the gas exchange leaf cuvette (ca. 25min). Lines represent either smoothed regressions from a generalized additive model fit (a) or linear model fits (b). Grey areas are approximately 95% confidence intervals from the mean.

**Figure 7**. Relationship between the observed discrimination of 13CO2 and measured Ci/Ca for sun leaves and shade leaves at both low and high light. The solid line represents the the theoretical line for C3 plants from (Evans *et al.*, 1986).

**Figure 8**. The mean ± 1 standard error of intercellular CO2 concentration (a), CO2 concentration in the chloroplasts (b) and CO2 drawdown from substomatal cavities to sites of carboxylation of sun leaves and shade leaves at both low and high light with standard error

**Figure S1**. Response of photosynthesis (a), mesophyll conductance (b) and Ci-Cc to leaf temperature for sun leaves and shade leaves at low and high light. Shaded symbols represents each monthly measurement campaign.

**Figure S2**. Response of vapor pressure deficit (a), stomatal conductance (b) and Ca-Ci to leaf temperature for sun leaves and shade leaves at low and high light. Shaded symbols represents each monthly measurement campaign.

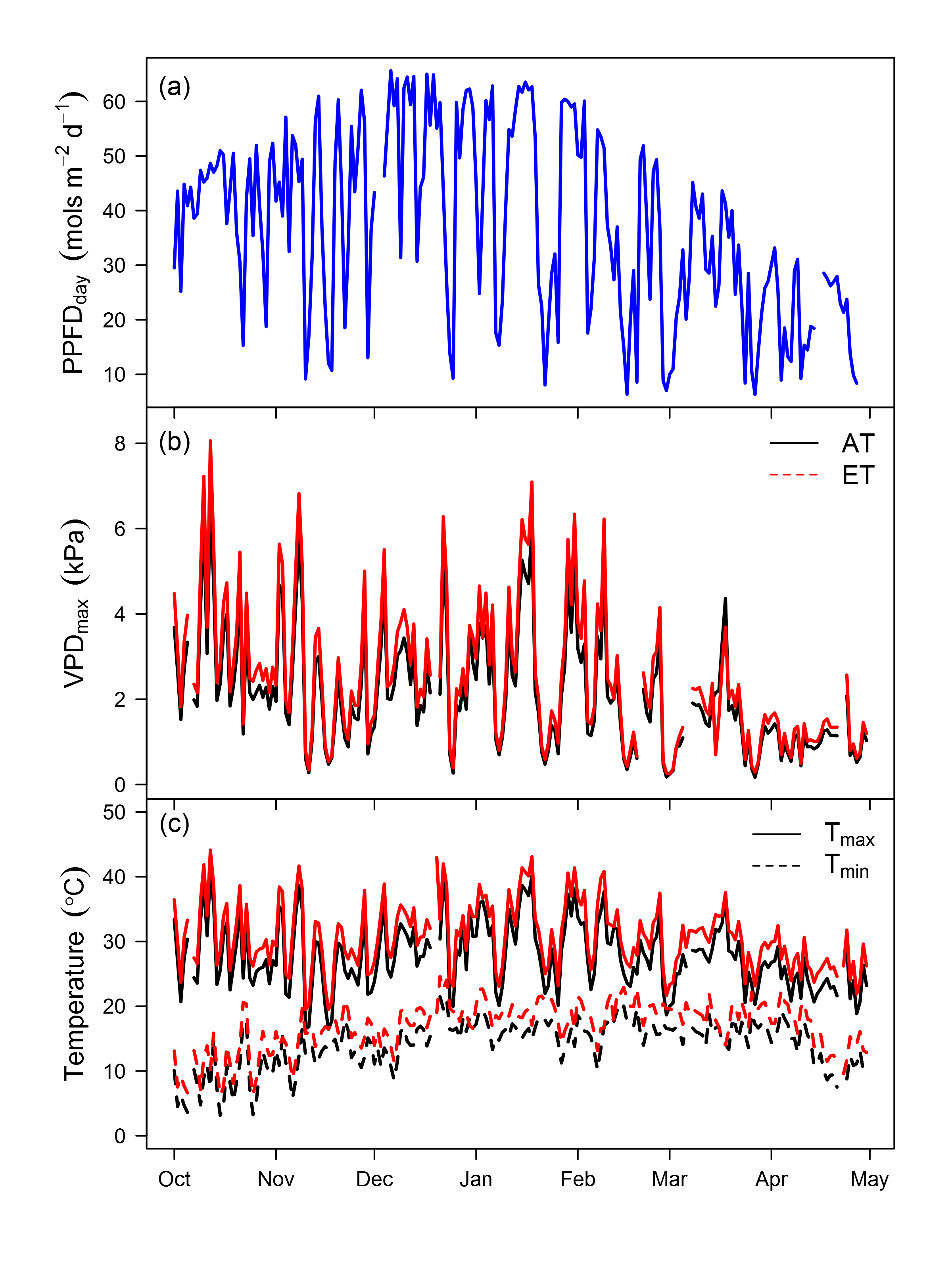
**Figure S3**. Photosynthetic CO2 response (ACc) curves for sun and shade leaves at elevated and ambient temperature treatments. Cc values were predicted with mesophyll conductance, thus curves represent chloroplastic photosynthetic parameters at 25°C and at saturating light (1800 mols m-1 s-1).

# Tables

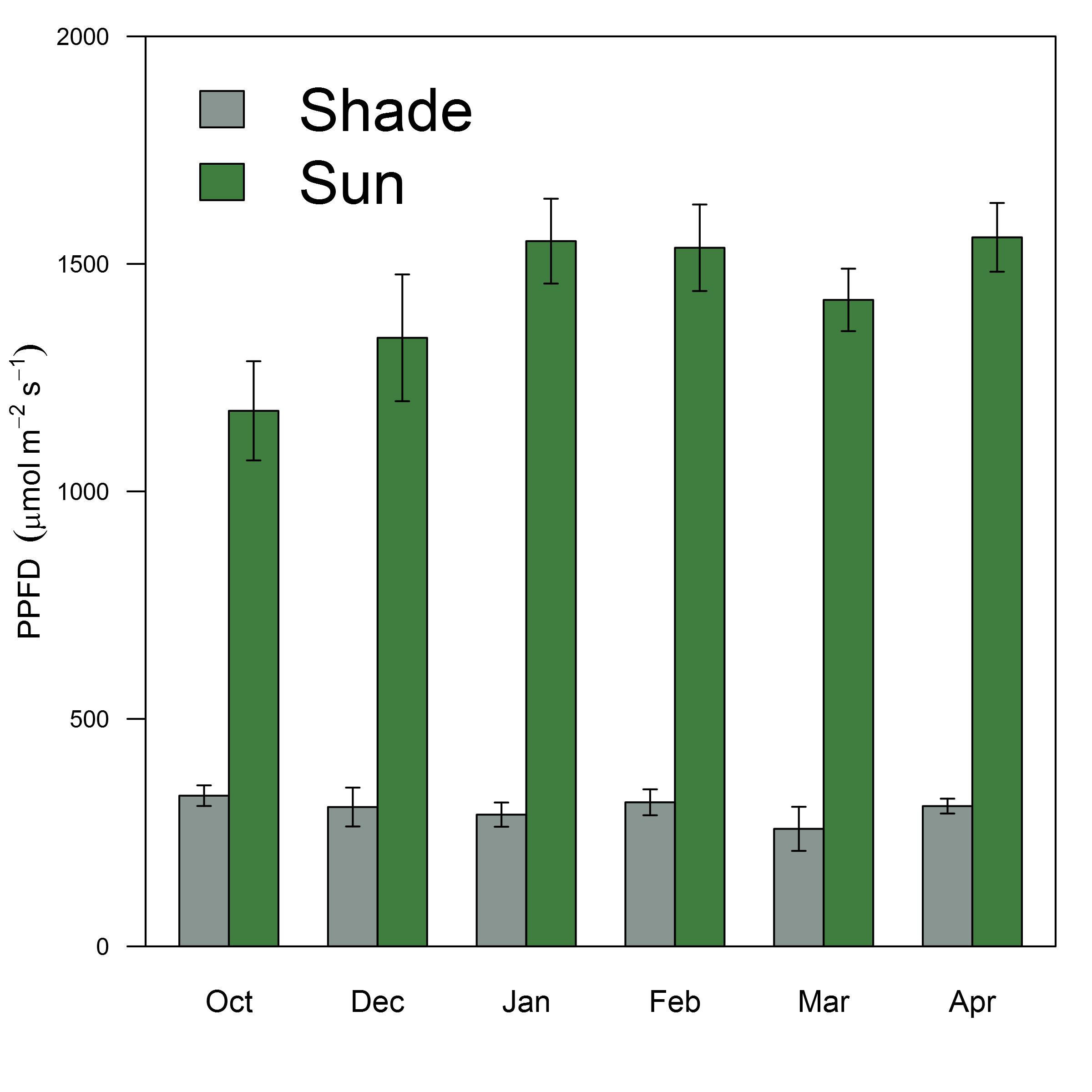
**Table 1**.

**Table 2**.

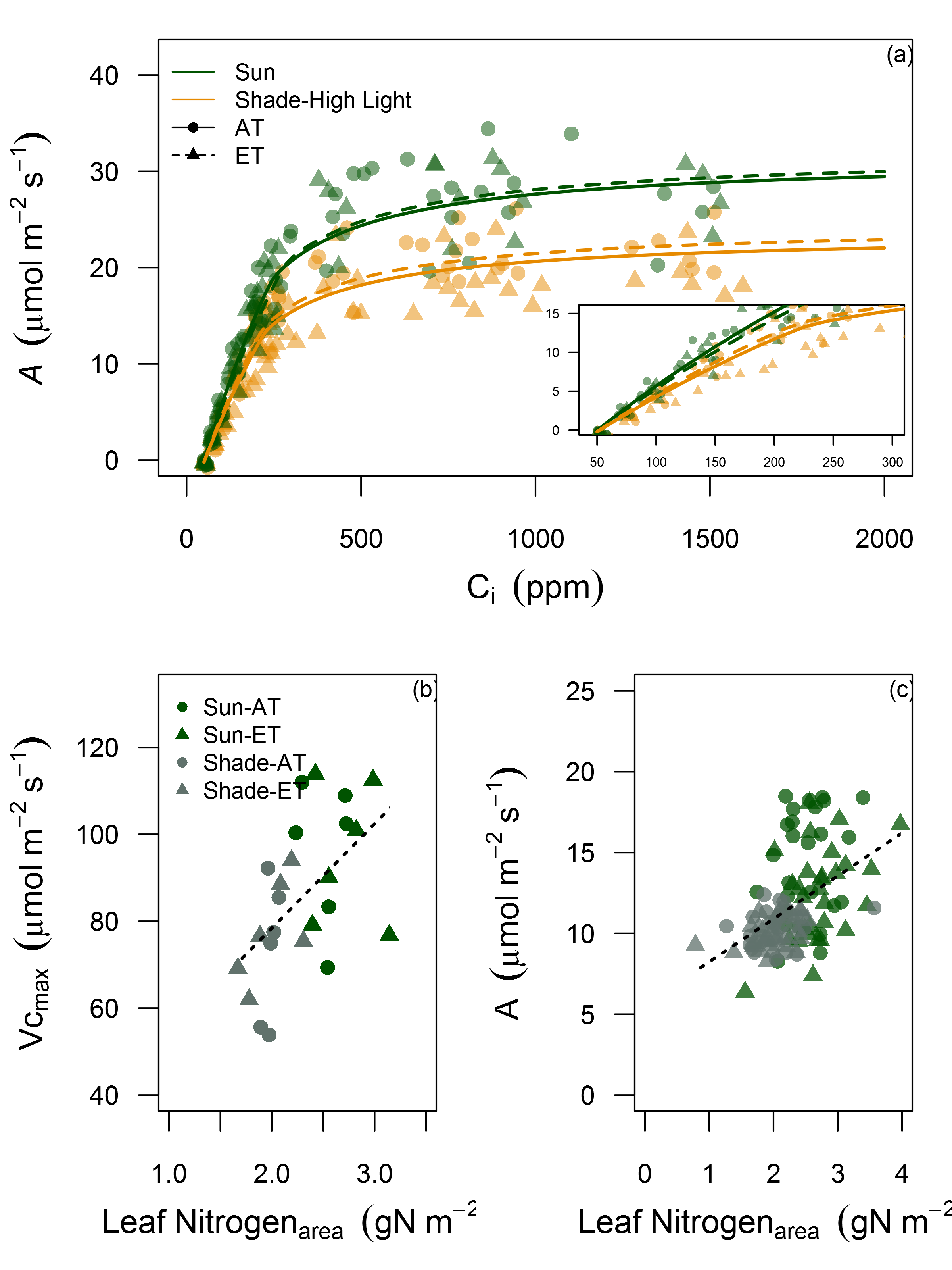
# Figures



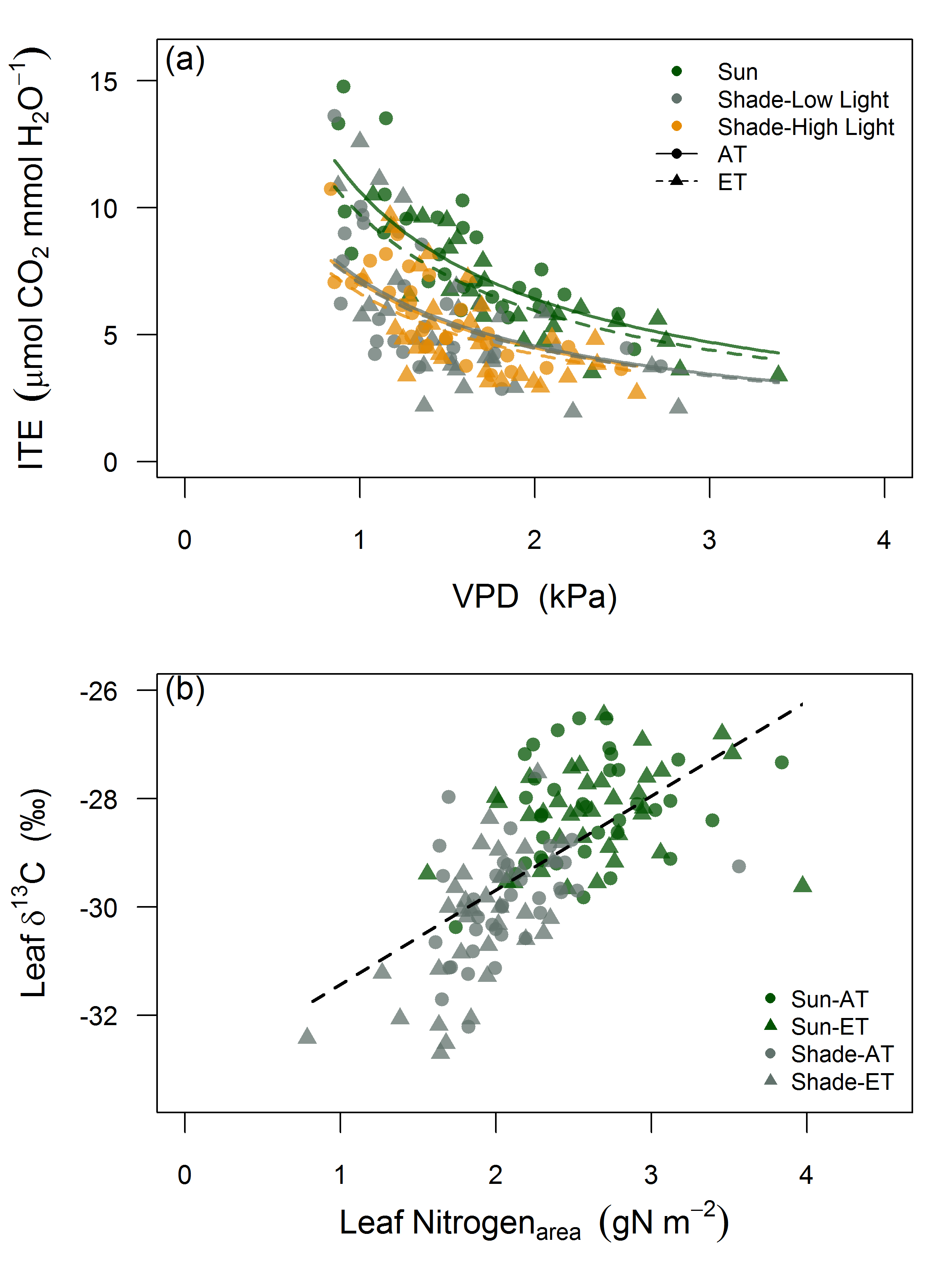
**Figure 1**



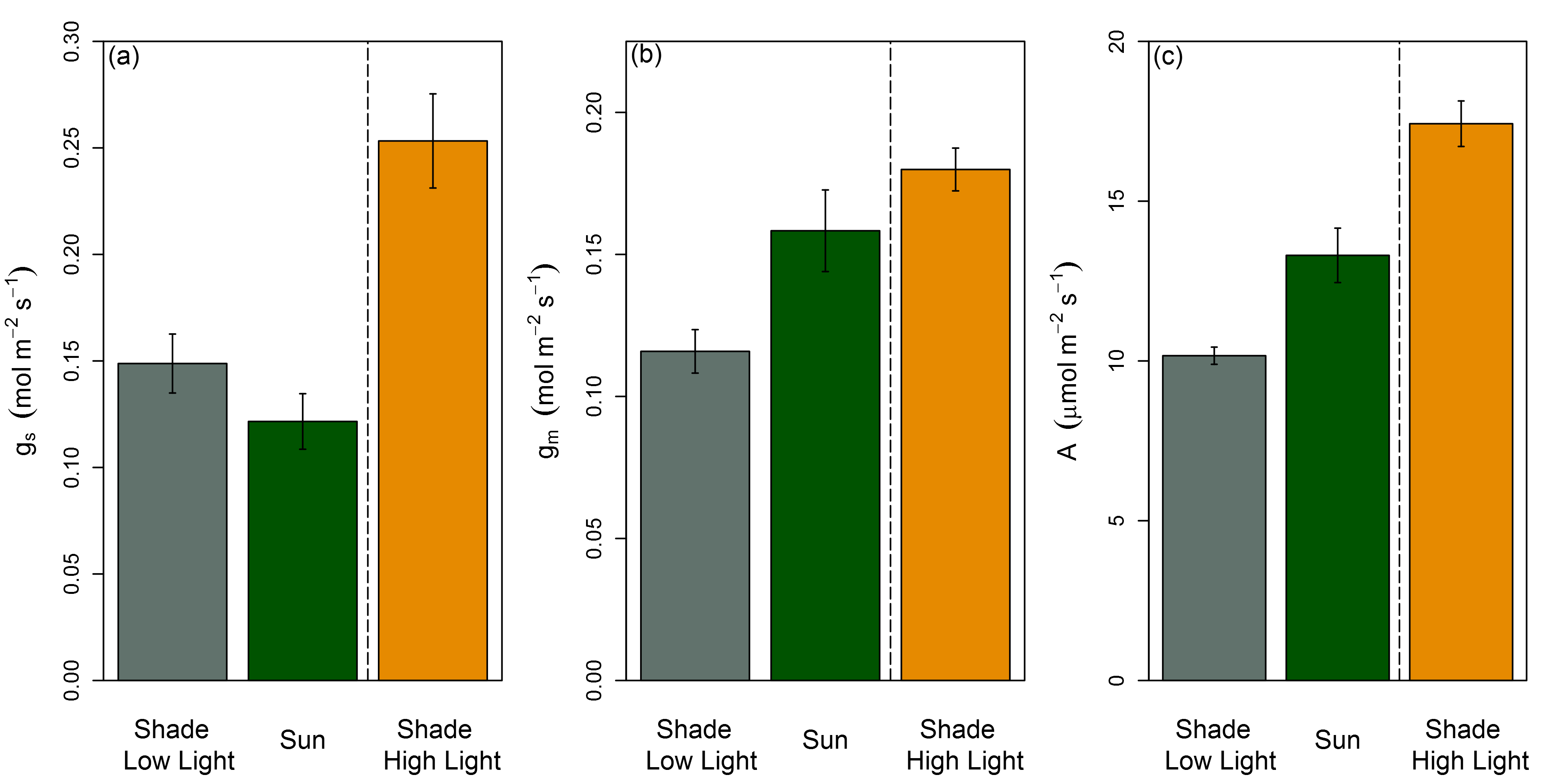
**Figure 2**.



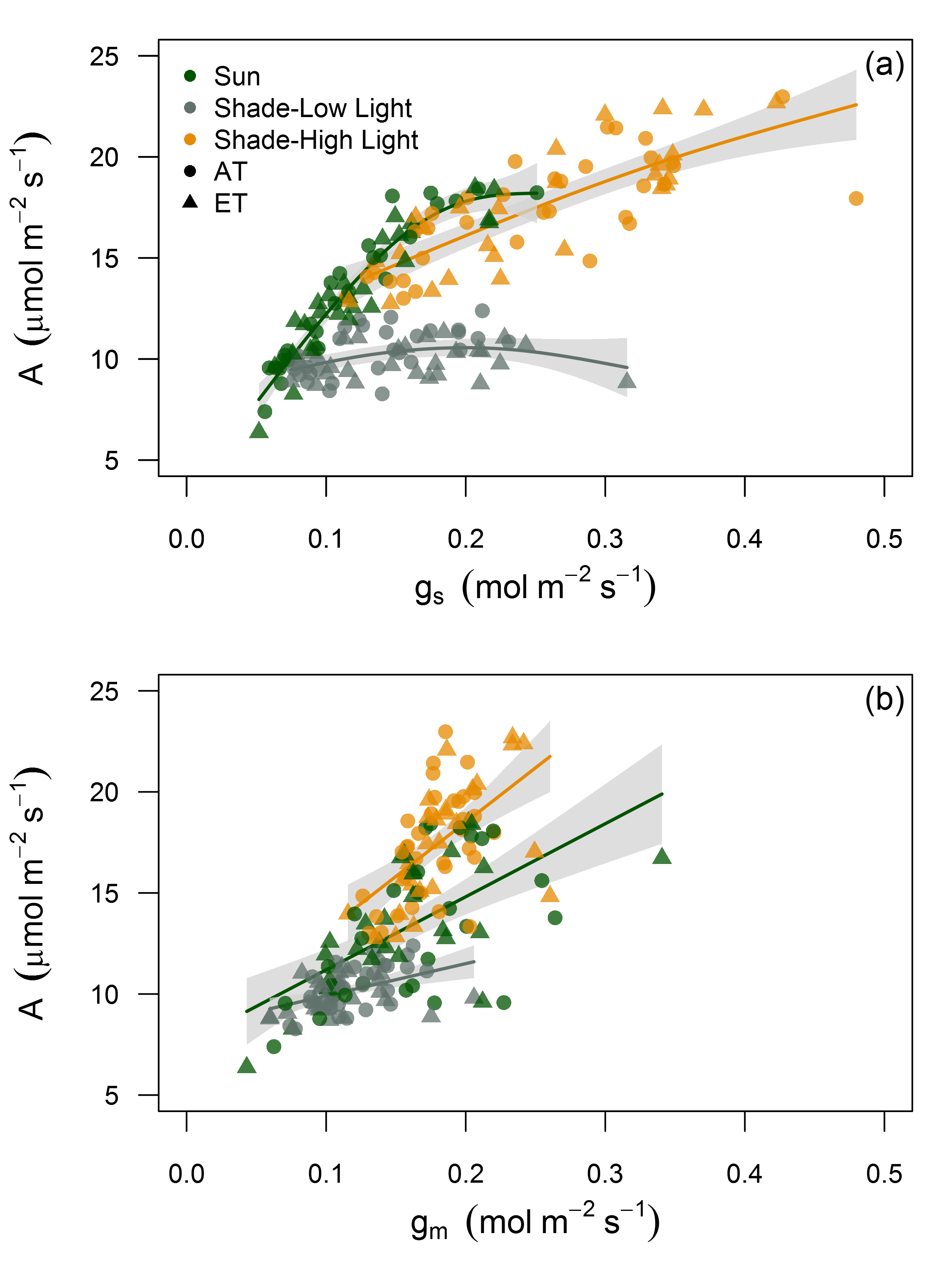
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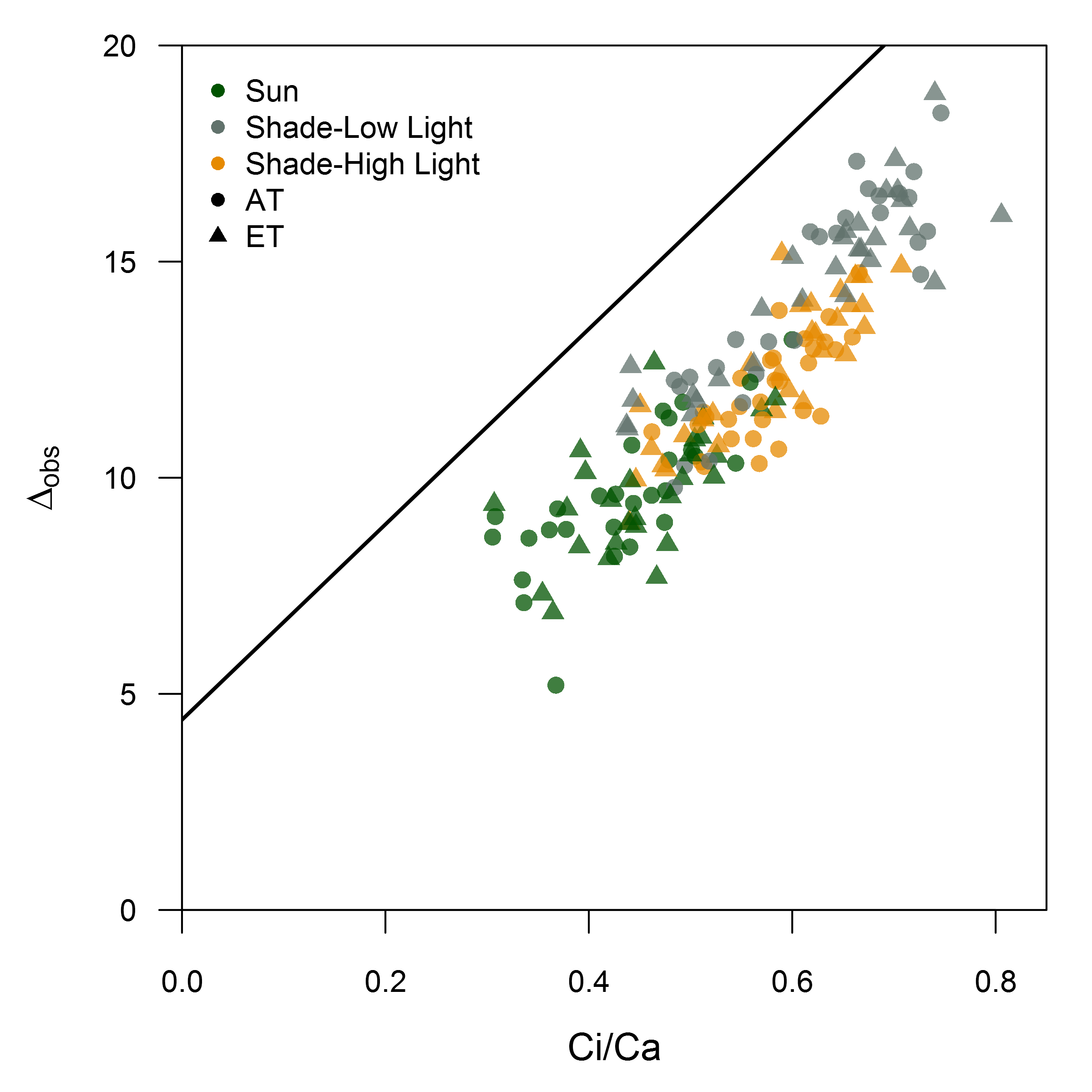
**Figure 4**.



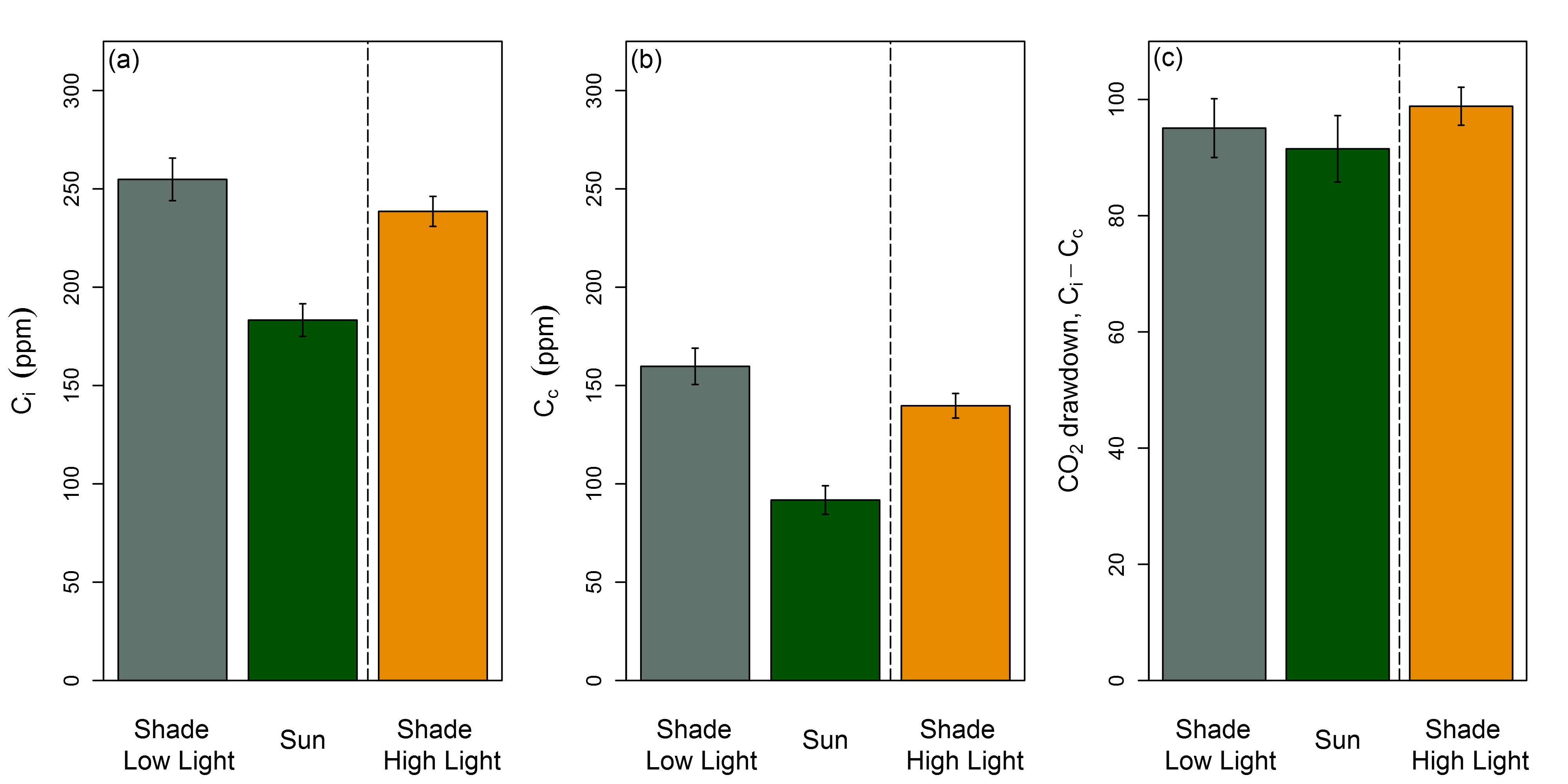
**Figure 5**.



**Figure 6**.

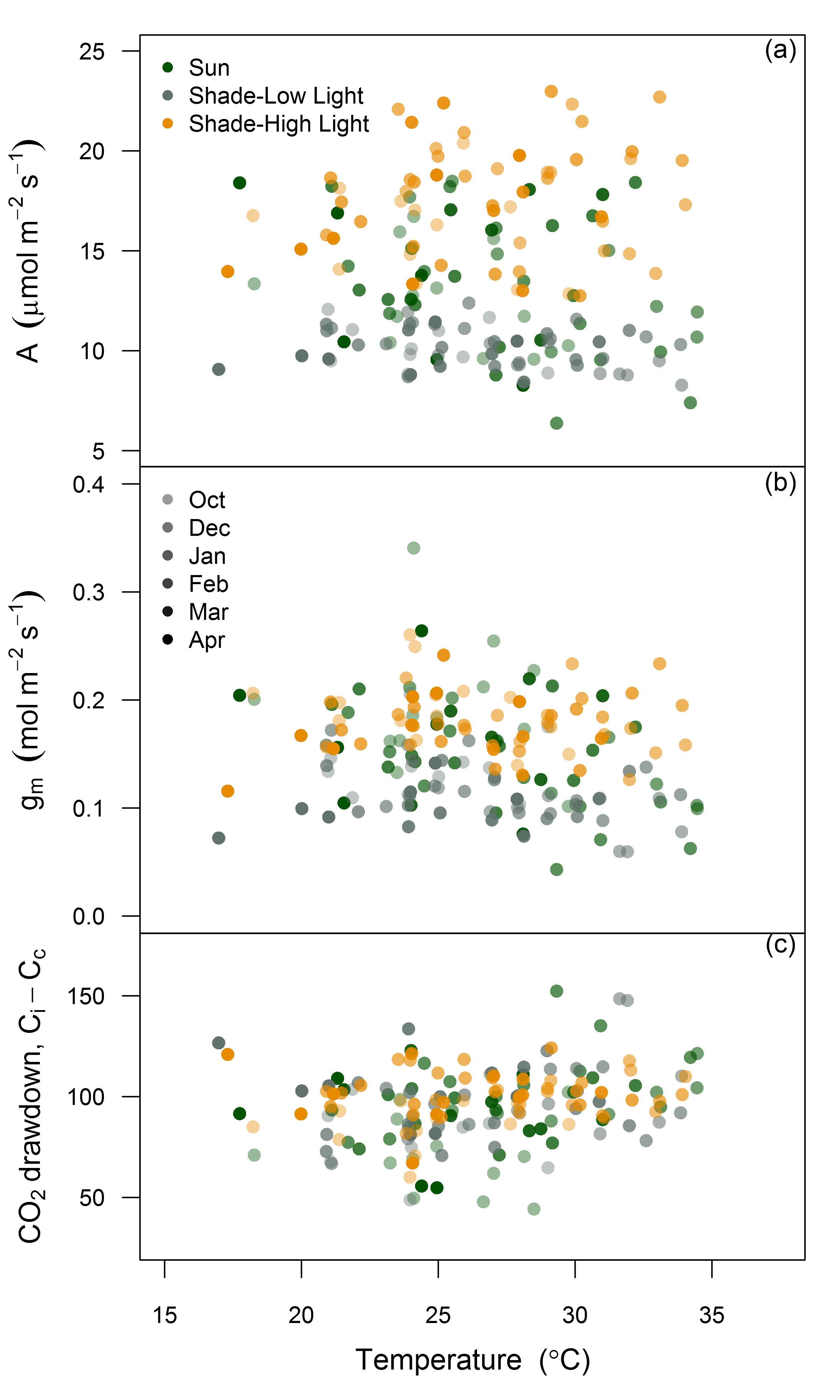


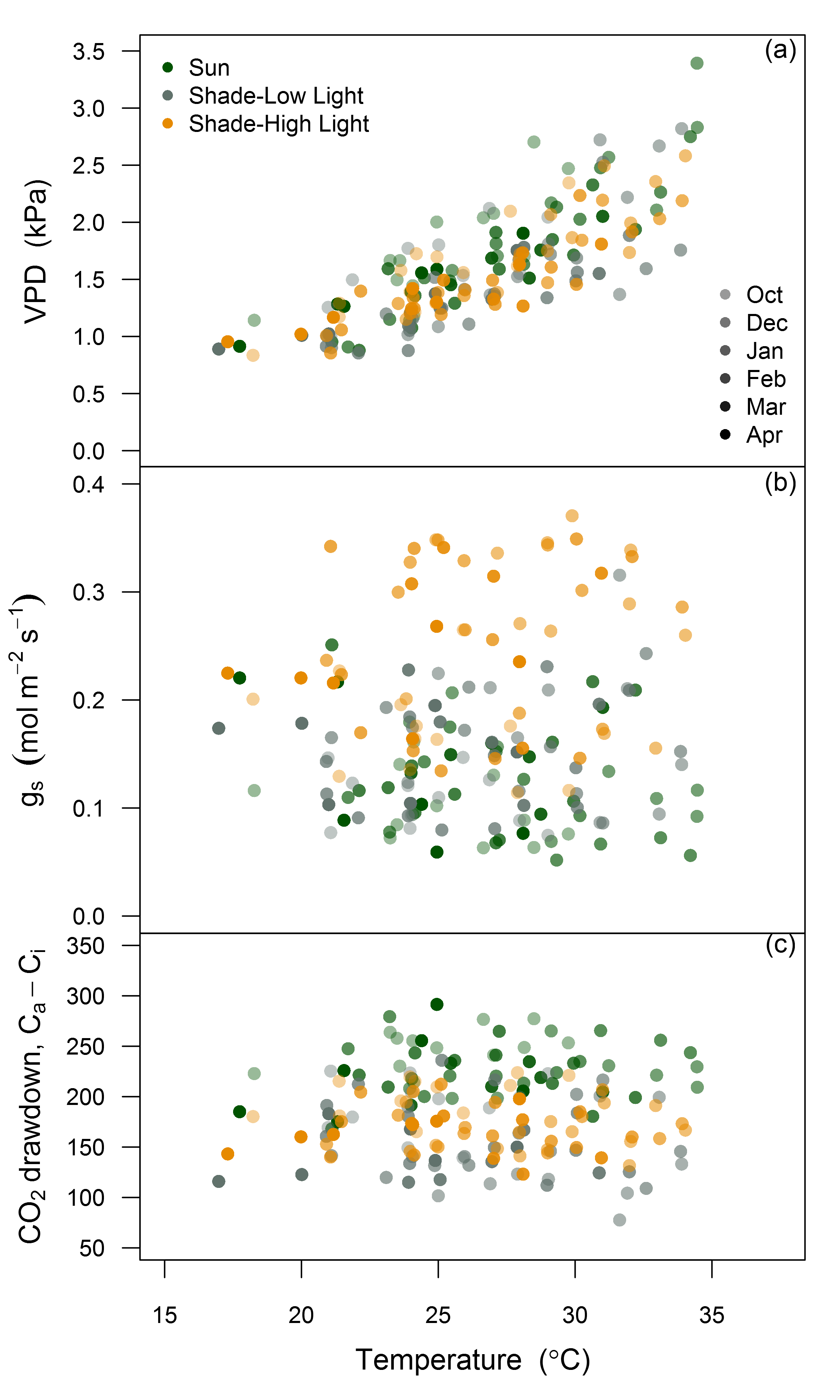
**Figure 7**.

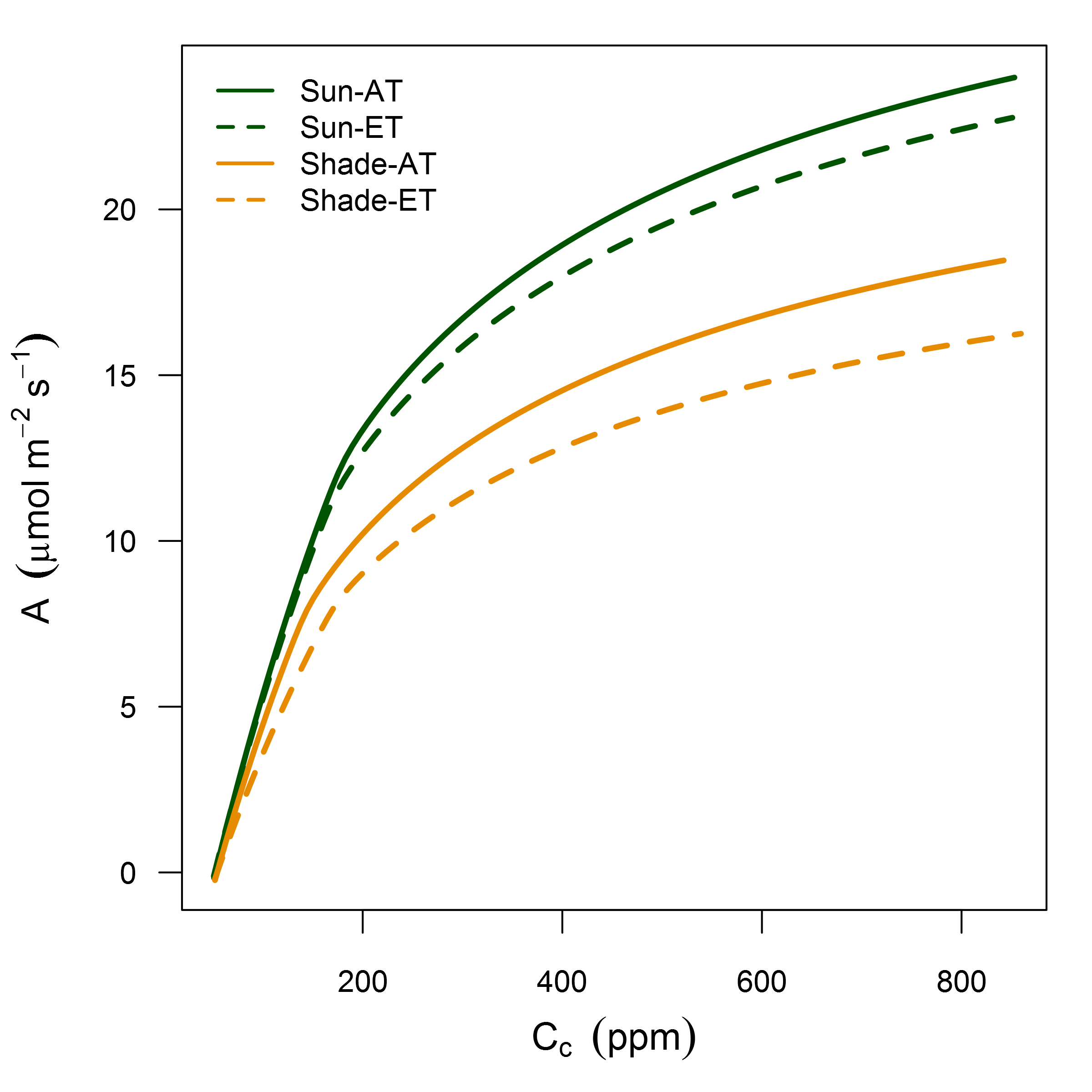


**Figure 8**.

# Supporting Information

 **Figure S1**.

 **Figure S2**.



**Figure S3**.

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