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Remote monitoring of dynamic canopy photosynthesis with high time resolution light-induced fluorescence transients

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Abstract

Understanding the net photosynthesis of plant canopies requires quantifying photosynthesis in challenging environments, principally due to the variable light intensities and qualities generated by sunlight interactions with clouds and surrounding foliage. The dynamics of sunflecks and rates of change in light intensity at the beginning and end of sustained light (SL) events makes photosynthetic measurements difficult, especially when dealing with less accessible parts of plant foliage. High time resolved photosynthetic monitoring from pulse amplitude modulated (PAM) fluorometers has limited applicability due to the invasive nature of frequently applied saturating flashes. An alternative approach used here provides remote (m), high time resolution (10 s), PAM equivalent but minimally invasive measurements of photosynthetic parameters. We assessed the efficacy of the QA flash protocol from the Light-Induced Fluorescence Transient (LIFT) technique for monitoring photosynthesis in mature outer canopy leaves of potted Persea americana Mill. cv. Haas (Avocado) trees in a semi-controlled environment and outdoors. Initially we established that LIFT measurements were leaf angle independent between ±40° from perpendicular and moreover, that estimates of 685 nm reflectance (R685) from leaves of similar chlorophyll content provide a species dependent, but reasonable proxy for incident light intensity. Photosynthetic responses during brief light events (≤10 min), and the initial stages of SL events, showed similar declines in the quantum yield of photosystem II (ΦII) with large transient increases in 'constitutive loss processes' (ΦNO) prior to dissipation of excitation by non-photochemical quenching (ΦNPQ). Our results demonstrate the capacity of LIFT to monitor photosynthesis at a distance during highly dynamic light conditions that potentially may improve models of canopy photosynthesis and estimates of plant productivity. For example, generalized additive modelling performed on the 85 dynamic light events monitored identified negative relationships between light event length and ΔΦII and Δelectron transport rate using either Δphotosynthetically active radiation or ΔR685 as indicators of leaf irradiance.

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Remote monitoring of dynamic canopy photosynthesis with high time resolution light-induced fluorescence transients

Running Head: high resolution monitoring of photosynthesis with LIFT

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ABSTRACT

Understanding the net photosynthesis of plant canopies requires quantifying photosynthesis in challenging environments, principally due to the variable light intensities and qualities generated by sunlight interactions with clouds and surrounding foliage. The dynamics of sunflecks and rates of change in light intensity at the beginning and end of sustained light (SL) events makes photosynthetic measurements difficult, especially when dealing with less accessible parts of plant foliage. High time resolved photosynthetic monitoring from pulse amplitude modulated (PAM) fluorometers has limited applicability due to the invasive nature of frequently-applied saturating flashes. An alternative approach used here provides remote (< 5 m), high time resolution (10 s), PAM equivalent but minimally-invasive measurements of photosynthetic parameters. We assessed the efficacy of the Q_A flash protocol from the Light-Induced Fluorescence Transient (LIFT) technique for monitoring photosynthesis in mature outer canopy leaves of potted avocado trees in a semi-controlled environment and outdoors. Initially we established that LIFT measurements were leaf angle independent between ± 40˚ from perpendicular and moreover, that estimates of 685 nm reflectance (R_{685}) from leaves of similar chlorophyll content provide a species dependent, but reasonable proxy for incident light intensity. Photosynthetic responses during brief light events (≤ 10 min), and the initial stages of SL events (Fig. 6), showed similar declines in the quantum yield of PSII (Φ_{II}) with large transient increases in "constitutive loss processes" (Φ_{NO}) prior to dissipation of excitation by non-photochemical quenching (Φ_{NPQ}). Our results demonstrate the capacity of LIFT to monitor photosynthesis at a distance during highly dynamic light changes that potentially may improve models of canopy photosynthesis and estimates of plant productivity. For example, generalized additive modeling performed on the 85 dynamic light events monitored here identified negative relationships between light event length and $\Delta\Phi_{II}$ and ΔETR using either ΔPAR or ΔR_{685} as indicators of leaf irradiance.

INTRODUCTION

The ability to model the total productivity of higher plants or even large-scale ecosystems requires accounting for photosynthesis occurring in dynamic light conditions in both direct light-exposed outer canopy leaves and in the shaded inner canopy foliage (Porcar-Castell et al. 2006; Niinemets 2007). These dynamic light conditions occur as light interacts with passing clouds and foliage elements causing a dynamic patchwork of light intensities of varying length. Variously, these effects can be referred to as sunflecks, sunpatches, shadeflecks or cloudflecks, depending on the cause of light fluctuation and light quality, either numbra or penumbra (Smith et al. 2013). These dynamic light events have been shown to provide a significant portion of photosynthetically active radiation (PAR) for carbon fixation to understory plants (Pearcy 1990). However, accounting for the contribution of light fluctuations to net photosynthesis has proven problematic due to: i) difficulty of accessing canopy environments, ii) difficulties in measurement of leaf-level PAR and iii) insufficient temporal resolution of photosynthesis measuring instruments. (Nichol et al. 2012; Way et al. 2012; Osmond 2014).

Laser PAM instruments have mitigated canopy access to some extent (Flexas et al. 2000; Ounis et al. 2001; Flexas et al. 2002; Louis et al. 2005). However, this method is still limited by the invasive nature of the saturating flash, and although sub-saturating PAM protocols have recently been developed (Loriaux et al. 2013), no PAM instrument delivering the non-intrusive sub-saturation flashes at a longer range (at least 1 m) is currently available. Current PAM methods for long-term monitoring, such as MONI-PAM, (Porcar-Castell et al. 2008) require fixing leaves into clips on heavy measuring heads, making it difficult to maintain the natural orientation of the examined leaf and potentially causing leaf damage. Additionally, although MONI-PAM provides reliable measures of incident PAR for estimation of photosynthetic electron transport rates (ETR), they are limited to measurement

resolutions of >30 s to avoid intrusive effects of the saturating flash (Shen et al. 1996; Apostol et al. 2001; Osmond et al. 2017).

LIFT instruments operated with the fast repetition rate (FRR) fluorescence excitation and analysis protocols were originally developed and used for measurements of marine phytoplankton (Kolber et al. 1993). In its terrestrial implementation, LIFT utilizes either LED or laser excitation sources for remote measurements of active chlorophyll fluorescence. The first application of LIFT technology at the Biosphere 2 Laboratory was based on red laser excitation and telescope optics, which induced and captured fluorescence at distances of up to 50 m (Ananyev et al. 2005). Corrected measurements of ETR from this LIFT prototype were shown to be highly comparable to those produced by PAM (Pieruschka et al. 2010). Since its first application, the LIFT approach has been used to perform daily and seasonal monitoring of various canopies, showing, for instance, photosynthetic changes with both light and temperature (Pieruschka et al. 2010) and generating maps of canopy photosynthetic heterogeneity (Pieruschka et al. 2009; Nichol et al. 2012). Importantly, long-term monitoring with time resolutions as high as 3 s has been demonstrated to be much less invasive than PAM, causing no detectable change in photosynthetic parameters during monitoring of leaves in the dark (Osmond et al. 2017).

The FRR model, upon which LIFT measurements are based, provides not only PAM comparable conventional photosynthetic parameters, but also provides measurements of broad-band radiance, reflected from an interrogated leaf at 685 nm (R_{685}), which potentially may be used as a proxy for leaf PAR. Leaf reflectance between 670 and 750 nm has been previously utilized during canopy laser PAM measurements for calculation of electron transport rates (ETR) and provided seasonal estimates similar to those calculated from MONI-PAM leaf PAR measurements (Ounis et al. 2001).

The original laser-based LIFT instrument operated at the Biosphere 2 Laboratory was not field portable (Ananyev et al. 2005). However, the current generation of LIFT instruments, which rely on blue LED excitation, are field portable (15 kg) and utilize an eye-safe blue LED excitation for measuring photosynthesis at distances of up to 5 m (Osmond et al. 2017; Wyber et al. 2017). When combined with advances in PAR sensor miniaturisation and the potential to use broadband leaf reflectance as an indicator of leaf PAR, the current generation of LIFT instruments may provide an ideal solution for measuring in vivo leaf photosynthesis under dynamic light conditions at more informative temporal resolutions. However, for successful application of LIFT technology to canopy measurements, the effects of varying leaf orientation with respect to the excitation beam needs to be understood and quantified in order to correct for leaf angular changes during growth, and to produce comparable measurements between differently oriented foliage. Moreover, the influence of leaf type, plant species, and chlorophyll content need to be known for the use of R_{685} in robust remote determination of leaf PAR and calculation of ETR.

To our best knowledge, LIFT studies involving canopy measurements have so far neglected the influences of leaf angular orientation and shadow propagation, and have sometimes relied on top-of-canopy PAR measurements. Therefore, in this paper we aimed to understand: i) the importance of leaf orientation on LIFT photosynthetic measurements, ii) determine the potential of hemispherical–conical leaf reflectance (R_{685}) sensed by LIFT to approximate leaf PAR and iii) determine what changes in LIFT-measured photosynthetic parameters can be observed (and generalised) under dynamic light conditions. We then examined the physiological and biochemical implications of photosynthetic changes under dynamic light (cause by clouds and intermittent shadows cast by nearby foliage or building architecture) and used generalised additive modelling to identify generalised predictors which

may be applied to modelling photosynthesis under dynamic light conditions and in future extended to sub-canopy environments.

MATERIALS AND METHODS

Plant material and environment

Measurements reported in this study were collected from three different avocado plants (*Persea americana* Mill. cv. Haas) grown at the University of Wollongong (UOW), Australia (34° 24' 17.5"S, 150° 52' 17.8"E). A 1.5 m plant, grown from seed in sunlight in a temperature-controlled (30˚C/18ºC day/night) greenhouse of the Research School of Biology, Australian National University, was re-potted into a 50 L pot using a commercially available fruit and citrus soil mix (Osmocote Fruit & Citrus; Bella Vista, NSW Australia) and grown for 18 months prior to measurements in a glass atrium in the School of Biological Sciences, UOW. The atrium provided a maximum glass filtered sunlight intensity of ~700 μmol photons·m^{-2}·s^{-1} with direct sunlight period limited to ~4 hours as a consequence of building architecture. Atrium temperatures ranged between 15˚C at night to 25˚C during the day, with natural direct and diffuse irradiance supplemented by ~60 μmol photons·m^{-2}·s^{-1} of light from fluorescent tubes for 8 hours as a consequence of building lighting.

Two additional plants were purchased from a commercial nursery and re-potted into 20 L pots using the same soil mix as for the atrium plant. Following re-potting these plants were transferred to the UOW Ecology Research Centre (ERC) and grown outdoors underneath a 50% black shade cloth enclosure for three months prior to measurements. The shade-enclosure was open to the NW to provide protection against strong sunlight on cool mornings but allowed for direct sunlight exposure ~4 hours after sunrise. Plants grown at the ERC experienced a maximum light intensity of ~1200 μmol photons·m^{-2}·s^{-1} with a direct light period limited to ~10 h in summer (as a consequence of local geography and enclosure

architecture) and temperatures ranging from 15˚C at night to 35˚C during the day. All plants were watered every other day with 4 litres of tap water.

Instrument description and calibration

Active chlorophyll fluorescence was measured using a commercially available Light-Induced Fluorescence Transient instrument (LIFT; Soliense Inc, Shoreham, NY, USA; http://www.soliense.com/LIFT_Terrestrial.php). The LIFT instrument utilises low intensity high frequency flashes (flashlets) of blue light (470 nm) to induce fluorescence changes in leaves at distances of < 5 m. The number of flashlets delivered to leaves can be modulated to provide two different measurement protocols, designed to reduce Q_A and to observe the kinetics of electron transport (Q_A flash), or to fully reduce the PQ pool and provide PAM-analogous measurements (PQ flash) (Osmond et al 2017). Both of these protocols modulate the frequency of flashlets in two main phases, a variable length saturation phase (flashlets applied at 50% duty cycle; termed SQ_A for Q_A flashes or SPQ for PQ flashes), and a relaxation phase with an exponentially-decreasing duty cycle (termed RQ_A for Q_A flashes or RPQ for PQ flashes)(Osmond et al. 2017). The whole fluorescence transient is then fitted using the fast repetition rate (FRR) fluorescence model, which determines F_mQ_A, F'_mQ_A, F_oQ_A and $F'Q_A$ for Q_A flashes and F_mPQ, F'_mPQ, F_oPQ and $F'PQ$ for PQ flashes (Kolber 2014; Osmond et al. 2017). The Q_A flash protocol of the LIFT instrument consisted of an SQ_A saturating sequence of 300 flashlets (1.6 µs pulses) applied at 2.5 µs interval and an RQ_A phase consisting of 90 flashlets (1.6 µs pulses) with an exponential increase in the 20 µs interval described by an exponential term of 1.04. The PQ flash protocol consisted of an SQ_A phase consisting of 6000 flashlets (1.6 µs pulses) with a 20 µs interval and an RQ_A phase identical to the Q_A flash protocol.

LIFT/FRR Q_A measurements provide a non-invasive method to probe photosynthesis at informative time resolutions for monitoring photosynthesis during fluctuating light

(Osmond et al. 2017). However, as Q_A flashes are designed to only reduce the first electron acceptor Q_A they underestimate PAM F_m and F'_m by ~10% (Osmond et al. 2017). To correct for this underestimation, the PQ flash is utilized to provide a PAM-analogous reference F_m and F'_m values for the correction of LIFT F_mQ_A and F'_mQ_A measurements (Osmond et al. 2017). To correct LIFT F_mQ_A and F'_mQ_A measurements to match those from PAM a white light response curve (0 to 1000 μmol photons·m^{-2}·s^{-1} in 50 μmol increments) was performed on six avocado leaves as described in Wyber et al. 2017. At each light intensity a LIFT Q_A and PQ flash measurement were performed in quick succession (double flash; Osmond et al. 2017) and the linear regression equation between F_mQ_A or F'_mQ_A and the F_mPQ or F'_mPQ measurements used to correct LIFT F_mQ_A or F'_mQ_A during leaf monitoring (supplementary material Fig. S1).

Effect of leaf angular orientation on LIFT/FRR measurements

Leaves of avocado (n = 6) were used to assess the effect of leaf orientation on LIFT/FRR measurements. Avocado plants growing at the ERC and the School of Biological Sciences atrium (n = 3; previously exposed to ~200 μmol photons·m^{-2}·s^{-1} of diffuse morning irradiance) were transferred to the laboratory and detached leaves (two from each plant) were prepared immediately prior to measurements (~10 min). Leaves were prepared as described in Takayama et al (2013). The leaf petiole was cut underwater and the detached leaf was sealed in a water filled 1.5 mL microcentrifuge tube sealed using paraffin film. Gas exchange and chlorophyll fluorescence imaging analyses revealed little change in photosynthesis in these leaves (Takayama et al. 2013), and in the present study there was no change in F_v/F_m (measured by PAM) during 6 hours in the dark. Prepared leaves were then affixed to a vertical panel positioned on a motorized tripod (Celestron Advanced VX; Celestron, Australia) at a distance of 1 m from the LIFT fore optics. Using the motorized tripod, the leaf orientation was rotated from 0° (adaxial) to 180° (abaxial) in 10° increments, with six

196 replicate LIFT/FRR Q_A measurements performed for each leaf at each rotated angle. All
197 measurements were performed under a low level of ambient light from a combination of
198 sunlight and fluorescent tubes (~65 μmol·m^{-2}·s^{-1}) (Fig. 1).

199 **Leaf PAR approximation using reflectance at 685 nm**

200 LIFT-detected R_{685}, acquired between Q_A flashes, was assessed as a potential proxy
201 for actual leaf PAR by investigating leaves of the following species: *Alectryon subcinereus*,
202 *Eucalyptus globoidea*, *Lomandra longifolia*, *Acmena smithii*, *Asplenium nidus*, *Polyscias*
203 *elegans*, *Ficus macrophylla*, *Mangifera indica* and two groups of avocado leaves varying in
204 chlorophyll content. High (lower canopy) and low chlorophyll (upper canopy) avocado leaves
205 were collected from different locations in the canopies of avocado plants growing at the ERC
206 (n = 4) and in the UOW atrium (n = 2). Leaves of all other plants (n = 3 per plant) were
207 sourced from plants growing under natural sunlight in minimally disturbed gardens on the
208 UOW campus. Leaves from these plants were randomly sampled from leaves within reach,
209 from plants growing in different light environments. *Ficus macrophylla* and *M. indica* plants
210 were growing in shaded positions, *A. smithii*, *A. nidus* and *P. elegans* plants were growing
211 under mottled shade from surrounding foliage and *E. globoidea* and *A. subcinereus* plants
212 were found growing in full sun locations. White-light response curves were performed using
213 a quartz iodide lamp from a Rollei P355 automatic slide projector, with leaf PAR measured at
214 the leaf surface using a LS-C micro quantum light sensor (Walz, Effeltrich, Germany). Light
215 response curves were performed for the following 14 mean light intensities ± SD from 0 to
216 ~1000 μmol photons·m^{-2}·s^{-1}: 0.00 ± 0.00, 1.98 ± 0.27, 3.80 ± 0.60, 24.23 ± 3.42, 40.17 ±
217 8.72, 51.47 ± 7.84, 52.84 ± 19.08, 78.12 ± 20.29, 85.88 ± 11.23, 103.84 ± 12.55, 200.59 ±
218 25.30, 287.03 ± 38.59, 598.42 ± 46.46 and 1065.18 ± 40.43. Light intensities were modulated
219 by varying the distance and focus of the quartz iodide lamp from leaves, with the error in
220 light steps due to the manual adjustment of the light source focus and distance. During light

response curves each light step was maintained for 5 min with three replicate measurements of R_{685} at each light intensity. For each species separate light response curves were performed on three replicate detached leaves prepared as described above. All measurements were performed at a distance of 1 m, with the LIFT instrument positioned perpendicular to the leaf surface.

Total chlorophyll content of leaf replicates was assessed with a Soil-Plant Analysis Development 502 chlorophyll meter (SPAD, Spectrum Technologies Inc, USA). For the conversion of avocado SPAD measurements to chlorophyll content, a calibration curve was generated from avocado leaves varying in chlorophyll content using high-performance liquid chromatography (HPLC), as described by Pogson et al. (1996) (see supplementary material Fig. S2).

In vivo LIFT/FRR photosynthetic measurements under dynamic light

All in vivo leaf measurements were performed on the adaxial surface of fully expanded avocado leaves attached to plants and maintained in their natural orientation. LIFT measurements were restricted to leaves ≤ 1 m from the LIFT fore optic (middle to lower canopy leaves) to maintain a high temporal measurement resolution. While measurements at longer distances are possible, these require greater averaging of fluorescence transients decreasing the temporal measurement resolution. Additionally, of leaves within ≤ 1 m from the LIFT fore optic, only those where an angle between ± 40˚ relative to the LIFT beam could be achieved were selected for measurements. Measurements were made around the Southern Hemisphere summer equinox (October, November and December 2014) and (March then October and December 2015) and involved monitoring of leaves over full diurnal cycles, starting at 18:00 h the day prior and finishing at 06:00 after the following night (i.e. two nights and one day; n = 10 days). For all measurements the LIFT instrument was operated with a 10 ± 1 s time resolution, where each data point was the fitted average of six successive

Q_A fluorescence transients. Following sunset each night, reference PQ flash measurements were performed every hour until sunrise, with the maximum F_mPQ serving as a dark-adapted, PAM equivalent reference. Leaf PAR was recorded at the surface of all leaves every 10 s using either one LS-C micro quantum light sensor (cosine corrected; ± 30˚) placed in the centre of the LIFT measuring beam, or two sensors placed on either side of the measuring beam and connected to a universal light meter (ULM-500; Walz, Effeltrich, Germany). For leaf PAR measurements using two micro quantum light sensors, leaf PAR was taken as the average of both sensors.

Data analysis

Calculation of LIFT/FRR photosynthetic parameters

All photosynthetic parameters were calculated using the conventional approaches for fluorescence data collected using the PAM methodology. Data are marked by a postfix Q_A or PQ to denote the source of the fluorescence data from either the Q_A or PQ flash respectively, and with F_m and F'_m measurements with no postfix denoting the source of fluorescence data from Q_A flashes corrected to match those from PAM/PQ flash measurements. The maximum quantum yield of photosystem II was calculated as:

$$F_V/F_m = \frac{(F_mPQ - F_oPQ)}{F_mPQ}$$

for a leaf in the dark and the quantum yield of photosystem II as:

$$\phi_{II} = \frac{(F'_m - F'Q_A)}{F'_m}$$

for a leaf in the light. Electron transport rate (ETR) was calculated using the formula of Genty et al. (1989);

$$\mathrm{ETR} = \phi_{II} \times \mathrm{PAR} \times E \times \alpha$$

where PAR was the incident light intensity at the leaf surface measured by either one or two micro quantum light sensors. The energy partitioning between PSI and PSII (E) was taken as

0.5 (Maxwell et al. 2000), and the leaf absorbance (α) was measured as 0.856 ± 0.05 based upon mean ± SD absorbance of six middle to lower canopy avocado leaves, representative of those measured by LIFT (n = 2 ERC plant 1, n = 1 ERC plant 2 and n = 3 atrium), measured in an integrating sphere as described by Björkman and Demmig (1987). Partitioning of the fraction of absorbed excitation dissipated in non-photochemical quenching (Φ_{NPQ}) and constitutive heat dissipation (Φ_{NO}) were calculated by adapting the formulae of Hendrickson et al. (2004) and Klughammer et al. (2008):

$$\phi_{NPQ} = \frac{F'Q_A}{F'_m} - \frac{F'Q_A}{F_m PQ}, \text{ and}$$

$$\phi_{NO} = \left(\frac{F'Q_A}{F_m PQ}\right)$$

$$\text{Note that } \phi_{II} + \phi_{NPQ} + \phi_{NO} = 1$$

Data preparation and light fluctuation analysis

In vivo monitoring of leaves produced two different datasets with equal time resolutions (10 s: LIFT and leaf PAR), which were aligned in the software R (R Core Team 2013) by matching timestamps. Light fluctuations were manually identified; with the start of each light fluctuation defined as a rapid increase in light greater than the slow diurnal changes in the background illumination. The end of each light fluctuation was defined as the point at which leaf PAR returned to within 5% of levels measured immediately before the start of the light event. The light fluctuation length and time since the last light fluctuation were retrieved for each light event and their distribution was normalized by $\log_e$ transformation. Additionally, the initial, middle, maximum, difference (Δ), and the area under curve (AUC) were retrieved for each light event, where Δ was calculated as the middle value – the initial value (Fig. 2). Time of day was not examined due to differences in the light exposure between the two plant measurement sites; in total, 85 light fluctuations were monitored.

Summary statistics for each light fluctuation were analysed using generalised additive models (GAM). Generalised additive model analyses were performed in R using the 'gam' package (Hastie et al. 1990), with separate GAM analyses run with initial, maximum, AUC and Δ values of Φ_{II}, Φ_{NPQ}, Φ_{NO} and ETR as response variables. For each response variable, all combinations of light fluctuation length, time since last light fluctuation and location, initial, maximum, AUC and Δ values for leaf PAR, R_{685}, and the initial values for Φ_{II} and Φ_{NPQ} were analysed as predictors. Initial values of Φ_{NO} and ETR were excluded as predictors from GAMs due to co-dependency with Φ_{NPQ} and Φ_{II} and leaf PAR, respectively. Additionally, raw fluorescence measurements (F_m, F'_m, F_o and F') were excluded from analyses due to dependency on distance from leaf to LIFT. For continuous predictor variables, a spline fit with two knots was used to fit the data. Model selection for each response variable was based upon the greatest deviance explained. The best models for each response variable were for the Δ values for each response variable and the predictors; light event length, time since last light event, location and either ΔR_{685} or ΔPAR. Given the strong co-dependency between ΔPAR and ΔETR, both models are presented.

RESULTS

Effect of leaf angular orientation on LIFT/FRR measurements

Changes in leaf angle away from perpendicular to the LIFT measurement beam resulted in sharp decreases in raw fluorescence parameters (F', F_v and F'_m) (Fig. 3A), with the same trend observed for both adaxial and abaxial leaf surfaces. In contrast, photosynthetic parameters based on ratios, such as Φ_{II}, were found to be relatively insensitive to changes in leaf angle (Fig. 3B). Φ_{II} measurements were found to be maintained at angles less than 40° for adaxial leaf surfaces. For abaxial leaf surfaces, Φ_{II} slowly increases by ~20% at leaf angles from 90° to 180°.

Leaf PAR approximation using reflectance at 685 nm

The possibility of using R_{685} as a proxy for leaf PAR was assessed using a series of light response curves (0 to 1000 µmol photons·m^{-2}·s^{-1}) on leaves varying in total chlorophyll content within and between species (Table 1).

LIFT R_{685} measurements were linearly related to leaf PAR measured at the leaf surface in all species ($R^2 > 0.9$). However, the determined relationships were found to be both species and chlorophyll content dependent (Fig. 4A, B and C). High chlorophyll (181.2 ± 1.5 µg·cm^{-1}) and low chlorophyll groups (36.5 ± 1.7 µg·cm^{-1}) of equal sized avocado leaves provided two distinct linear relationships ($R^2 > 0.9$) (Fig. 4C), with the low chlorophyll group exhibiting a mean increase in R_{685} of 40 ± 11% relative to the high chlorophyll group. Overall, the plants formed three general linear trends: high reflectance (*A. subcinereus, E. globoidea* and *L. longifolia*), medium reflectance (*A. smithii, A. nidus*, *P. americana* [low chlorophyll] and *P. elegans*) and low reflectance (*F. macrophylla*, *M. indica* and *P. americana* [high chlorophyll]) (Fig. 4D). Mean R_{685} measurements for the medium and high reflectance groups correspond with increasing SPAD measurements (36.2 ± 10.7 and 48.4 ± 3.7, respectively). This is, however, not the case of the low reflectance group which possessed the highest mean SPAD measurement (59.8 ± 1.8). We attempted to use R_{685} as an indicator of leaf PAR for in vivo monitoring of light fluctuations, but the relationship between R_{685} and leaf PAR was found to vary throughout the day and also just before and after light fluctuations (Fig. 5).

Changes in photosynthetic parameters during dynamic light fluctuations

The dynamic responses of photosynthetic parameters in outer canopy leaves of avocado were dependent on the frequency, duration, light intensity and time of day. Time of day was not examined in GAMs due to differences in light exposure between ERC and atrium light environments. However, differences with time of day were evident in ERC

342 measurements, which will be examined here. Initially it was convenient to characterize these
343 responses in the highly reproducible sunlight environment of the atrium in the School of
344 Biological Sciences, UOW. Two sustained light events (SL; ~45 min) and four successive
345 brief light events (BL; ~10 min) all of ~500 μmol photons·m^{-2}·s^{-1} were superimposed on the
346 background of a diffuse shade light (~50 μmol·photons·m^{-2}·s^{-1}) growth environment (Fig. 6).

347 In the shade, little energy was directed to Φ_{NO}, with ~70:30% partitioned between Φ_{II}
348 and Φ_{NO} (Fig. 6B). A ~10-fold increase in PAR over ~2 min (Fig. 6A) produced a transient
349 overshoot in ETR accompanied by redistribution in energy partitioning as ~50 % of Φ_{II} was
350 dissipated by a two phase increase in Φ_{NPQ}. The latter was accompanied by a transient near
351 doubling in Φ_{NO}. Photosynthetic ETR settled to a more noisy steady state (~65 μmol
352 electrons·m^{-2}·s^{-1}) that responded to small perturbations in PAR (Fig. 6A). After the ~5 min
353 shade event (Fig. 6A) that saw rapid redistribution of energy from Φ_{NPQ} back to Φ_{II}, the
354 second prolonged SL event resulted in a larger initial transient overshoot in ETR.
355 Interestingly, Φ_{NPQ} was immediately re-engaged to a similar steady state, with a smaller
356 transient increase in Φ_{NO}. Partitioning to Φ_{II} increased slowly as Φ_{NPQ} declined (Fig. 6B),
357 with both events tracking a small decline in PAR (Fig. 6A).

358 Initial responses in the four subsequent BLs, all at approximately the same PAR as the
359 above prolonged events, were qualitatively and quantitatively similar in terms of transients in
360 the rate of ETR and return to steady state (Fig. 6A). Moreover, they were also similar with
361 respect to the small transient in Φ_{NO} as large changes in energy partitioning took place
362 between Φ_{II} and Φ_{NPQ} (Fig. 6B). Interestingly, ETR increased by ~13% after three successive
363 BLs as Φ_{NPQ} declined. The passage of the last BL event saw ETR and energy partitioning
364 between Φ_{II}, Φ_{NPQ} and Φ_{NO} return to initial levels within a few minutes.

Monitoring of photosynthetic parameters outdoors with LIFT/FRR further expanded the above observations and it was possible to identify differing dynamic responses to fluctuating light throughout the diurnal cycle (Fig. 7A). As in the atrium, shading from structural elements of the plant enclosure generated a reproducible early morning pattern of seven oscillations in sunlight, but this time at low PAR (from ~50 to ~150 μmol photons·m^{-2}·s^{-1} over ~70 min). The sudden increase in PAR from ~50 to 1200 μmol photons·m^{-2}·s^{-1}, due to full sun exposure of previously shaded leaves, was accompanied by a brief initial transient in ETR, settling to a steady state that was similar to the maximum levels attained in the early low light oscillations. The transition to strong sunlight was also accompanied by a precipitous decline in energy partitioned to Φ_{II} from about 75% to 10%. After an initial transient increase in Φ_{NO} more than half of the dissipation was due to Φ_{NPQ} (Fig. 7B). Dynamic decreases in PAR, due to passing clouds, were reflected in these parameters that drifted slowly towards the initial morning shade conditions as ETR increased with the afternoon decline in PAR.

After ~7 h of full sunlight (~1200 to 600 μmol photons·m^{-2}·s^{-1}), late afternoon natural canopy shade provided ~40 min of highly stochastic BL events. The stronger late afternoon natural shade BL events produced an approximately 5-fold increase in ETR which peaked at about twice the ETR in full sunlight (Fig. 7A). Data from the early morning and late afternoon periods of dynamic PAR are expanded in Fig. 7C, 7D and 7E, 7F, respectively (note that the ETR and PAR scaling on Fig. 7E and 7F is 3-fold greater than that on Fig. 7C and 7D). The plants monitored outdoors showed a similar pattern of energy distribution from 06:00 to 07:00 h to that observed from the tree in the atrium at about the same PAR prior to the first SL event (c.f., Fig. 6A and 6B). In contrast to the strong BL events in the atrium, low PAR early morning oscillations produced relative small declines in Φ_{II} that scarcely perturbed Φ_{NPQ}. Clearly, under these conditions ETR proceeds with maximum efficiency with minimal engagement of photoprotective energy dissipation. Stronger stochastic BL

events occurring in the late afternoon were of similar PAR to those monitored in the atrium. Although, under similar conditions of energy partitioning, there was a striking absence of the reciprocal relationship between Φ_{II} and Φ_{NPQ} observed in the atrium (c.f., Fig. 7F and 6B).

Differentiating photosynthetic responses to sustained and brief light events of differing PAR intensities

Monitoring of photosynthetic parameters with LIFT/FRR revealed a plethora of reproducible and reversible patterns in response to abrupt changes in sunlight that invited closer attention. Before de-convolution of statistical relationships, it is helpful to examine differences in photosynthetic changes in response to light event length, either sustained light (SL; > 10 min) or brief light (BL; ≤ 10 min), and light event intensity, either strong (max PAR ≥ 500 μmol photons·m^{-2}·s^{-1}) or weak (max PAR < 500 μmol photons·m^{-2}·s^{-1}). Although, it should be noted that these groups do not define the exclusive conditions under which the described photosynthetic behaviours occur, but they describe rather generalised reactions that hold for most leaves examined within each group.

Strong light, from both BL and SL events, produced photosynthetic changes dependent on the duration of the light event (Fig. 8). For a strong SL event outdoors (Fig. 8A, 8C), photosynthetic changes were quantitatively similar to that in Fig. 7A, 7B (and to that in the atrium; Fig. 6A, 6B) but with ~60% higher rates of ETR at ~900 μmol photons·m^{-2}·s^{-1} for ~90 min. Initial transient increase in the rate of ETR and Φ_{NO} preceded changes in Φ_{NPQ} by about 5 min (Fig. 8A, 8C), but otherwise changes in energy partitioning were also qualitatively similar those in the atrium.

In contrast, different photosynthetic responses were observed during strong BL events that were faster than the initial increases in the rates of ETR and Φ_{NO} in SL events (Fig. 8B, 8D). For example, in a leaf that had previously been exposed to weak sunlight (~100 μmol

photons·m^{-2}·s^{-1}; Fig. 8B), a strong BL event (~1,000 μmol photons·m^{-2}·s^{-1}; ~2 min.) produced a markedly different energy partitioning dynamic. The short strong BL event produced a decline in Φ_{II}, which coincided with an equal drop in Φ_{NPQ}, resulting in a much amplified Φ_{NO} transient. This photosynthetic response to a short strong BL event in a sun leaf on a dull day appears to stimulate PSII energy dissipation processes in the same manner as observed in the initial exposure to a strong SL event in the atrium (Fig. 6B). However, during the midday BL event the duration of the light event is shorter than the time required for Φ_{NPQ} engagement.

Sustained as well as brief sunlight exposures on another cloudy day are compared in Fig. 9. The lower maximum PAR in both events (~220 μmol photons·m^{-2}·s^{-1}) did not produce large initial transients in ETR (Fig. 9A) and as expected, much lower rates of ETR were achieved than in strong PAR events (~50 vs. 125 μmol electrons·m^{-2}·s^{-1} c.f., Fig. 9A, 9B vs. 8A, 8B). However, the long (~25 min) weak sunlight event exposed protracted changes in energy partitioning similar to those in the short strong BL event monitored in another leaf a month earlier (c.f., Fig. 8C and 8D). Notably, the 1 min BL event with a similar PAR at midday did not elicit a change in Φ_{NO} (cf., Fig 8D) and the small decline in Φ_{II} was mirrored in a small increase in Φ_{NPQ}.

Generalized additive model analyses

To identify generalized relationships between changes in photosynthetic parameters in response to light event properties, which might be useful for photosynthetic modelling, generalized additive models were created. Generalised additive models generated for each photosynthetic response variable consistently showed indicators of leaf irradiance (ΔR_{685} and ΔPAR) as significant predictor variables ($P \leq 0.003$**). Exceptions to this were ΔETR and Φ_{NO} for models run with ΔR_{685} ($P = 0.266$) and ΔPAR ($P = 0.065$) respectively (Table 2).

The length of light events was found to be a significant predictor of $\Delta\Phi_{II}$, $\Delta\Phi_{NPQ}$ and ΔETR when ΔPAR was included in models ($P < 0.001$). In contrast, light event length was found to be a significant predictor of only $\Delta\Phi_{II}$ ($P = 0.021$) and ΔETR ($P = 0.001$) when ΔR_{685} was included in models as an indicator of leaf irradiance. The time since last light event was a significant predictor of $\Delta\Phi_{NPQ}$ in models run using both indicator of leaf irradiance (ΔR_{685}; $P = 0.004$ and ΔPAR; $P = 0.002$) and a significant predictor of $\Delta\Phi_{II}$ ($P = 0.045$) and $\Delta\Phi_{NO}$ ($P = 0.029$) in models run with ΔR_{685} and ΔPAR respectively. Sample location (ERC or atrium) was found to be a significant predictor of both $\Delta\Phi_{NPQ}$ (ΔR_{685}; $P <0.001$ and ΔPAR; $P = 0.04$) and $\Delta\Phi_{NO}$ (ΔR_{685}; $P = 0.004$ and ΔPAR; $P = 0.028$) in models with both ΔR_{685} and ΔPAR as predictors.

Partial response graphs of each response variable plotted against either ΔPAR or ΔR_{685} showed the same trends irrespective of using ΔPAR or ΔR_{685} as an indicator of leaf irradiance, with the exception of ETR, which showed a positive relationship with increasing ΔPAR and a flat relationship with increasing ΔR_{685} (see supplementary data Fig. S3 to S10). The direction of relationships with indicators of leaf irradiance (ΔPAR or ΔR_{685}) was as expected for ΔETR, $\Delta\Phi_{II}$ and $\Delta\Phi_{NPQ}$. Positive relationships with increasing leaf irradiance (ΔPAR or ΔR_{685}) were identified for ΔETR and $\Delta\Phi_{NPQ}$, while a negative relationship was identified for $\Delta\Phi_{II}$. Positive relationships between $\Delta\Phi_{NPQ}$ and leaf irradiance showed a plateau with high levels of leaf irradiance. Interestingly, $\Delta\Phi_{NO}$, unlike all other parameters, showed a flat relationship with low levels of leaf irradiance and a positive relationship with high levels of leaf irradiance (ΔPAR > 400 μmol photons$\cdot m^{-2}\cdot s^{-1}$ and ΔR_{685} > 500 AU). Additionally, negative relationships were identified between light event length and $\Delta\Phi_{II}$ and ΔETR, and time since last light event and $\Delta\Phi_{NPQ}$ in models using either ΔPAR or ΔR_{685} as an indicator of leaf irradiance. For models incorporating ΔPAR as a predictor, a positive relationship was also identified between light event length and $\Delta\Phi_{NPQ}$. For sample location,

light fluctuations measured in the School of Biological Sciences atrium showed lower values of $\Delta\Phi_{NO}$ and higher values of $\Delta\Phi_{NPQ}$ for both indicators of leaf irradiance than measurements at the ERC.

DISCUSSION

Remote non-invasive and high temporal resolution measurements of photosynthesis are essential for quantifying photosynthesis under dynamic light conditions. Attempts to remotely monitor photosynthesis in canopies with actively induced fluorescence approaches have used either laser PAM (Flexas et al. 2000; Ounis et al. 2001; Flexas et al. 2002) or LIFT instruments (Ananyev et al. 2005; Pieruschka et al. 2009; Pieruschka et al. 2010; Pieruschka et al. 2014). Although studies have investigated the effect of leaf shape, orientation and arrangement on light interception (Cohen et al. 1987; Jordan et al. 1993), no study, to our best knowledge, has investigated the effect of leaf angularity on remote active fluorescence measurements, nor a possible use of reflectance at 685 nm as a proxy of leaf PAR. We addressed both of these issues and utilized LIFT technology for remote near-proximity measurements of avocado leaf photosynthesis during SL and BL events in vivo.

Effect of leaf angular orientation on LIFT/FRR measurements

Maintaining the natural orientation of leaves in canopies during measurements of photosynthesis is important for correctly capturing the contribution of individual leaves to net canopy photosynthesis. We found that LIFT raw fluorescence measurements (e.g. F', F'_m) are sensitive to leaf angle, while Φ_{II} is relatively insensitive, except at very steep angles. The raw fluorescence changes due to leaf angularity are probably related to elongation of the LIFT measurement beam, which consequently lowers excitation energies delivered to the leaf surface and fluorescence returned to the sensor. Although leaf fluorescence emissions are generally considered to be isotropically emitted from the leaf (Pinto et al. 2017), another

factor affecting the amplitude of the returned fluorescence signal is the possible non-uniformity of the angular distribution of the emitted fluorescence radiation. Irrespectively, in the case of Φ_{II}, the decrease in both F' and F'_m are corrected for by internal ratio of the calculations. Nevertheless, at steep leaf angles the fluorescence signal becomes very low, reducing the signal-to-noise ratio below a level required for reliable assessment of Φ_{II} by LIFT/FRR.

Monitoring of photosynthesis in avocado leaves is aided by availability of large mature leaves, which often hang perpendicularly relative to the LIFT measuring beam. However, it might be impossible to ensure that leaves are in optimal angular positions and that measurements are collected from the adaxial surface in canopies, where leaves are held in planophile (prevailingly horizontal) angular positions. In accordance with the results from PAM measurement (Schreiber et al. 1977; Schreiber et al. 1996), our LIFT measurements of the abaxial leaf surface demonstrated a slight underestimation of Φ_{II}. However, for photosynthetic monitoring of planophile leaves it is not currently known how light intensity changes at the leaf adaxial side affect photosynthetic measurements conducted on the abaxial leaf side. Moreover, rapid leaf movement driven by wind still presents a considerable challenge to modelling and measurements (Burgess et al. 2016) both in terms of the frequency needed to capture rapidly changing PAR (Roden et al. 1993) and the observational uncertainties due to large variations in leaf angle.

Leaf PAR approximation using reflectance at 685 nm

Although accurate estimates of leaf PAR are essential for deriving the actual ETR (Genty et al. 1989), acquisition of leaf PAR measurements in canopy environments with traditional PAR sensors is difficult unless the geometries of both sensor and leaf are constrained. We employed two different sensor arrangements for measurements of leaf PAR,

511 both of which presented challenging problems. The use of a single PAR sensor placed in the
512 centre of the LIFT measurement beam resulted in underestimation of ETRs during the start of
513 light fluctuations, when illumination was first recorded by a portion of the LIFT measurement
514 beam and only later by the PAR sensor. This issue was addressed by using two PAR sensors
515 placed on either side of the LIFT measurement beam. This allowed the averaging of PAR
516 from both sensors, which compensated the underestimation of ETR during the start of light
517 fluctuations. However, we observed several cases where light fluctuations travelled over only
518 a single sensor and where averaging of the two PAR sensors consequently did not match the
519 expected changes in photosynthetic parameters. In these cases, the change in R_{685} may
520 actually better represent changes in photosynthesis. This problem highlights the need for a
521 reliable method of estimating leaf PAR remotely and within an equally sized measurement
522 footprint.

523 As previously shown by Ounis et al. (2001), broad band red leaf reflectance is
524 strongly correlated with leaf PAR. However, our results show that the gradients of these
525 relationships are species dependent and strongly influenced by chlorophyll content and the
526 structure of foliar tissues. We found species dependent relationships could be generalised into
527 three different relationships (high, medium and low reflectance), which may be potentially
528 related to the plant growth environment. Leaves collected from plants naturally growing on
529 the UOW campus were found under different light environments, broadly correlating with the
530 three generalised reflectance trends. High reflectance trend plants were collected from full
531 sun exposed conditions, medium reflectance trend plants were found under partially exposed
532 conditions and low reflectance leaves were collected from the shaded canopies of a large fig
533 and mango tree. The different gradients in these three generalised trends may be partially
534 explained by the strong absorbance of 685 nm light by chlorophylls, which is evident in
535 differences between high and low chlorophyll avocado leaves and partially in leaf SPAD

measurements. Furthermore it is likely that scattering by species-specific internal leaf structures and reflection by cuticle properties also influence the gradients of these relationships.

Our laboratory light response curves showed strong correlations between R_{685} and leaf PAR, however, the relationship between PAR and R_{685} measured in the field varied before and after light fluctuations, and also over the course of a diurnal cycle. These variations might be driven by changes in the spectral composition of combined direct and indirect solar irradiation during a diurnal cycle, and multi-angular anisotropy of leaf reflectance, i.e. variations in specular and diffuse leaf reflectance depending on actual solar altitude and zenith. These effects on reflected light estimates of leaf PAR were recognized by Ounis et al. (2001). However, our measurements show that more work is needed to assess these factors in order to accurately approximate absolute PAR values from leaf R_{685} in canopy environments.

To allow for the use of R_{685} as a proxy for leaf PAR, leaf biochemical and physical properties may potentially be retrieved from spectral measurements using leaf radiative transfer models such as PROSPECT (Malenovský et al. 2006), while changes in solar spectral composition and variations in direct and diffuse irradiance can be modelled for exposed outer canopy leaves (Emde et al. 2016). However, accounting for changes in the spectral quality and intensity of light within inner canopies may prove to be too complex, making use of R_{685} as a proxy of leaf PAR in the inner canopy unfeasible.

Changes in photosynthetic parameters during dynamic light fluctuations

Our results demonstrate the applicability of the high frequency LIFT protocol for chlorophyll fluorescence based measurements of photosynthesis during BL and SL events in avocado leaves, complementing the application of this technique to the ground truthing of solar induced fluorescence (Wyber et al. 2017). The time resolution of such measurements

560 achieved here with LIFT/FRR is ~2 orders of magnitude faster than that achieved to Adams
561 et al. (1999) in studies of changes in xanthophyll cycle-dependent energy dissipation in two
562 vines growing in the understorey of an open Eucalyptus forest with PAM. Like these authors,
563 we sought to partition energy from absorbed PAR into three component processes;
564 photochemical quenching (Φ_{II}), non-photochemical quenching (Φ_{NPQ}) and still poorly
565 specified constitutive losses (Φ_{NO}), all monitored by the small fraction of excitation emitted
566 as fluorescence (Hendrickson et al. 2004; Kramer et al. 2004).

567 Our measurements with LIFT/FRR during a rapid increase in PAR confirm that induction of
568 ETR and decline in Φ_{II} is faster than increase in Φ_{NPQ}, and because $\Phi_{II} + \Phi_{NPQ} + \Phi_{NO} = 1$,
569 results in strong transients in Φ_{NO} in the first 10 min (Fig. 6). The plethora of "constitutive
570 loss processes" embraced by Φ_{NO} is rapidly reversible and is mitigated in SL (and in repeated
571 BL events) by induction of Φ_{NPQ} (Fig. 8C and Fig. 7E, F respectively). While changes in
572 electron transfer happen very rapidly over seconds, ΔpH-dependent NPQ, linked with the
573 enzymatic changes in xanthophyll and lutein pigment cycles, occurs over minutes to hours
574 (García-Plazaola et al. 2007; Demmig-Adams et al. 2012). The transient in Φ_{NO} and ETR
575 occurred over ~10 min and likely corresponds to the slow induction of ΔpH-dependent NPQ
576 (Krause et al. 1991; Adams et al. 1999; Maxwell et al. 2000; Müller et al. 2001; Demmig-
577 Adams et al. 2012; Jia et al. 2013). It is important to note that SL events at high PAR produce
578 high Φ_{NPQ}, presumably associated with de-epoxidation of violaxanthin and lutein epoxide,
579 leading to accumulation of zeaxanthin and lutein in avocado leaves (Matsubara et al. 2005;
580 García-Plazaola et al. 2007; Jia et al. 2013). Although Φ_{NPQ} declines in the afternoon, it is
581 about twice morning levels, and much stronger BL events are not associated with the
582 transients in Φ_{NO} observed in the morning (Figs. 7E, F). Clearly, ~6 h prior exposure to an
583 average of >800 μmol photons$\cdot m^{-2} \cdot s^{-1}$ sunlight had effectively damped energy partitioning
584 processes.

585 Complementary declines in Φ_{II} and increases in Φ_{NO} with little engagement of NPQ
586 were apparent during weak morning BL events (Fig. 7C, D). An unexpected decline in Φ_{NPQ}
587 associated with strong transient increases in ETR and Φ_{NO} was observed in short strong BL
588 events in leaves acclimated at > 50 μmol photons m^{-2} s^{-1} (Fig. 8D), as well as in low PAR SL
589 events on cloudy days (Fig. 9C). This decrease in Φ_{NPQ} may reflect the sensitivity of the LIFT
590 assay in which the ultra-fast probing of PSII by blue light may maintain a low level of steady
591 state NPQ. Increases in light from a weak SL or BL event may then potentially increase the
592 PSI oxidizing potential causing NPQ to drop. However, further investigation of the
593 mechanisms underpinning these photosynthetic responses is required to confirm this
594 hypothesis.

595 **Generalized additive model analyses**

596 Generalized additive models were run for each photosynthetic parameter to
597 understand the importance of various components of light fluctuations on different
598 photosynthetic processes. We found that more complex models, which also incorporated the
599 pre-light fluctuation states of photosynthetic parameters, showed no improvement over
600 simpler models. This suggests that when analysed without respect to the light fluctuation time
601 of day or sequential order, that the pre-light fluctuation states of photosynthetic parameters
602 have insignificant influence on photosynthetic changes during the light event. The priming of
603 leaves by an initial SF has already been well documented (Way et al. 2012) and although it
604 was not evident in the initial states of photosynthetic parameters, we did observe a priming
605 effect of the first SL event, each day, in atrium leaves. This priming was evident in a lower
606 initial ETR and higher Φ_{NO} than in a following SL event of equal intensity and duration (Fig.
607 6A, 6B), which occurred, presumably, because higher ETR capacity had been induced but
608 was not expressed in the first SL event. It is likely that this priming effect may be captured in
609 statistical analyses where light fluctuations are examined with respect to time of day and

sequential order. Additionally, the significance of time since last light event in GAM analyses can be seen in the decrease in Φ_{NPQ} during closely spaced BL events (Fig. 6B).

Sample location proved to be a significant predictor of $\Delta\Phi_{NPQ}$ and $\Delta\Phi_{NO}$, with both ΔPAR and ΔR_{685} included as predictors. In both cases, light fluctuations in leaves grown in the atrium had higher levels of $\Delta\Phi_{NPQ}$ and lower $\Delta\Phi_{NO}$. In general, light fluctuations in the atrium reached a maximum PAR of ~700 μmol photons$\cdot m^{-2}\cdot s^{-1}$ in contrast to 1200 μmol photons$\cdot m^{-2}\cdot s^{-1}$ reached during light events at the ERC. This indicates that for the same ΔPAR, higher $\Delta\Phi_{NPQ}$ and lower $\Delta\Phi_{NO}$ were achieved for leaves in the atrium. This is likely a result of differences in leaf age/leaf acclimation.

The direction of changes in $\Delta\Phi_{II}$, $\Delta\Phi_{NPQ}$ and ΔETR matched the expected changes in Φ_{II}, Φ_{NPQ} and ΔETR under increasing light. The strong relationship between ETR and PAR was expected, given their co-dependency, but the insignificance of the relationship between R_{685} and ΔETR suggests R_{685}, at least in the case of ΔETR prediction, may be a poor proxy for leaf irradiance compared with on-the-leaf PAR measurements under dynamic light conditions.

The results of GAM analyses identified highly significant relationships between photosynthetic measurements and light fluctuation properties that may be useful for modelling photosynthesis in dynamic outer canopy light environments. However, these trends represent those from young (~2 year old) re-potted avocado plants, which may have had some degree of pot binding. Both leaf age and pot binding have been shown to influence leaf photosynthetic responses (Poorter et al. 2012). Old deep shade leaves in established orchard trees have been shown to have lower ETRs and NPQ (Matsubara et al. 2012), while pot binding has been shown to limit leaf photosynthetic rates, through restricted root biomass in pot bound plants (Poorter et al. 2012). Moreover, while ETR is commonly calculated with the assumption of equal energy partitioning between PSII and PSI (E = 0.5), measurements of

sunflecks and other light fluctuations in inner canopies, where far-red enriched diffuse light is punctuated by specular sunlight, likely represents a situation where the assumption of equal energy partitioning does not hold. As such, the deployment of LIFT for monitoring of dynamic light fluctuations in established orchard trees, and the measurement of *E* during dynamic light fluctuations is required to determine if the generalised trends identified from GAM analysis are found in established older plants.

Conclusion

The ability to effectively monitor light fluctuations in canopies is essential for understanding photosynthetic regulation during SL and BL events in different canopy layers and for modelling the total productivity of plants (Porcar-Castell et al. 2006). This study showed that LIFT can be usefully deployed outdoors to perform high time resolved measurements of photosynthesis in outer canopy leaves in their natural orientation. LIFT was capable of providing measurements of Φ_{II} that are relatively insensitive to changes in leaf angular position and to resolve effects of SL and BL events on leaf photosynthesis. It also showed the potential of leaf reflectance at 685 nm to be used as an indicator of leaf PAR under conditions of fixed leaf chlorophyll and light quality. For modelling photosynthesis in canopies, statistically significant relationships between light event properties and photosynthetic parameter responses were identified from potted avocado plants.

The availability of programmable LED arrays for dynamic light environments in the laboratory (e.g., Alter et al. 2012) and advances in modelling interactions between plant architecture and dynamic light environments (e.g., Burgess et al. 2016) undoubtedly will accelerate our understanding of these processes in future. The time resolution of the automated remote monitoring of chlorophyll fluorescence with LIFT/FRR is approaching that achieved decades ago in dynamic light response studies in fixed gas exchange systems. With

659 the use of currently available miniature light sensors and the ability to automate leaf
660 measurements using a motorized tripod, it now is possible to monitor canopy photosynthesis
661 in mature orchards with precision. Such studies will be the subject of subsequent reports and
662 potentially will support improved models of canopy photosynthesis and estimates of plant
663 productivity at larger spatial scales.

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676 **CONFLICT OF INTEREST**

677 The authors declare no conflict of interest.

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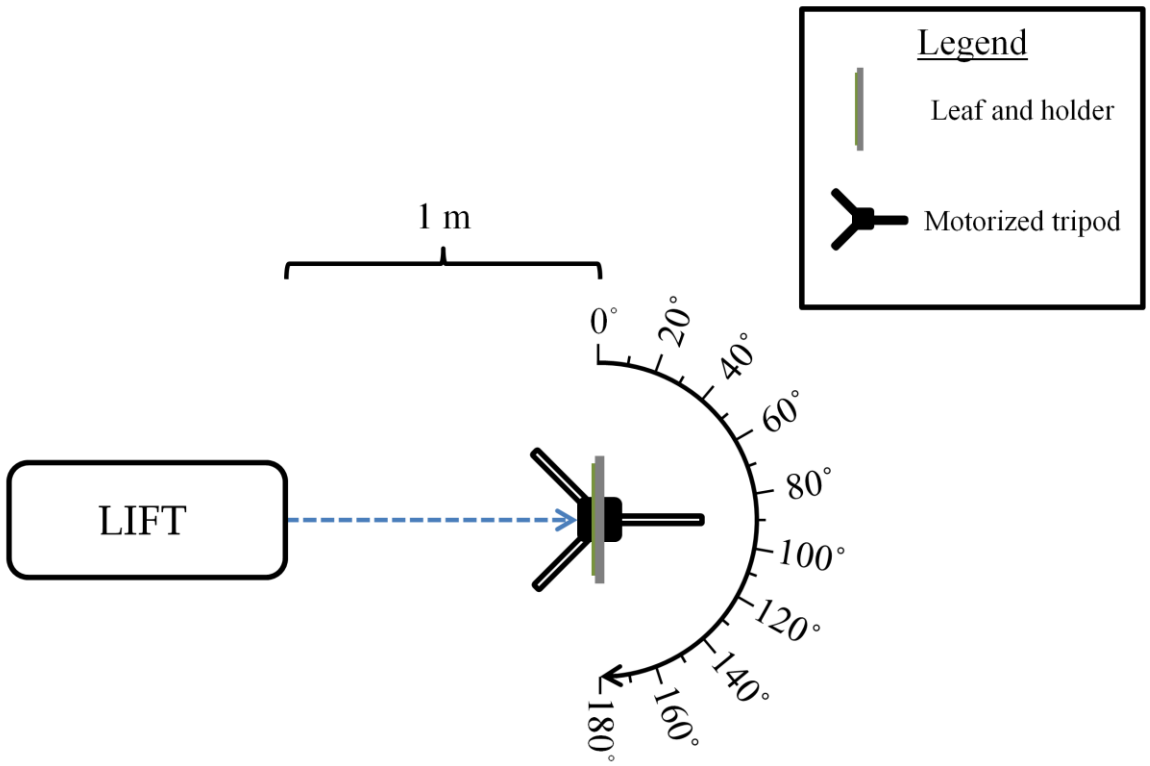

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828 Fig. 1. LIFT leaf angle measurement setup viewed from a nadir perspective. The blue broken arrow indicates the
829 measurement beam of the LIFT, perpendicular to the tripod mounted leaf and sample holder. The solid black
830 line indicates the rotation direction of the leaf and sample holder, where measurements from 0˚ to 80˚ indicate
831 measurements from the leaf adaxial surface and measurements at 100˚ to 180˚ indicate measurements from the
832 leaf abaxial surface.

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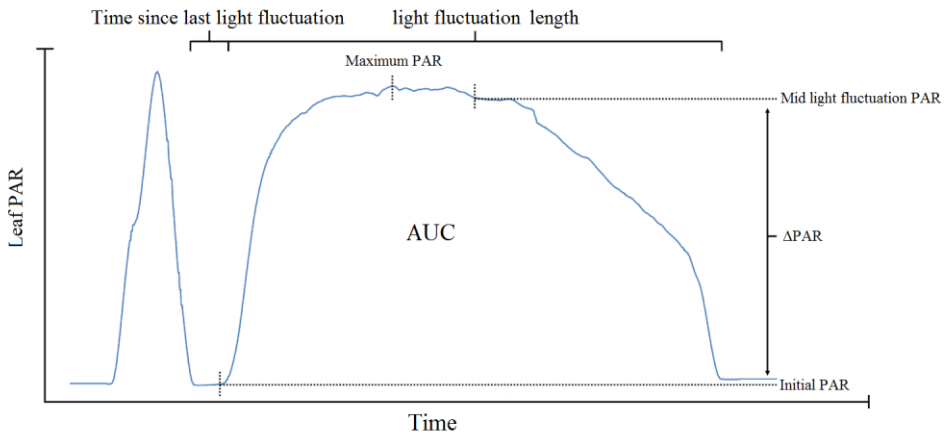

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838 Fig. 2. Leaf photosynthetically active radiation (PAR) measured during two successive light fluctuations. Figure
839 illustrates the parameters retrieved for each light fluctuation for generalized additive model analysis, where
840 AUC = the area under PAR intensity curve for a given light fluctuation and initial, maximum and mid refer to
841 the PAR immediately prior to the light fluctuation, the maximum achieved PAR during a light fluctuation and
842 the PAR half way through the light fluctuation respectively. ΔPAR refers to the PAR change in during a light
843 fluctuation as the difference between the initial and the mid light fluctuation PAR. For generalized additive
844 model analysis the same parameters were retrieved for each measured parameter during each light fluctuation.

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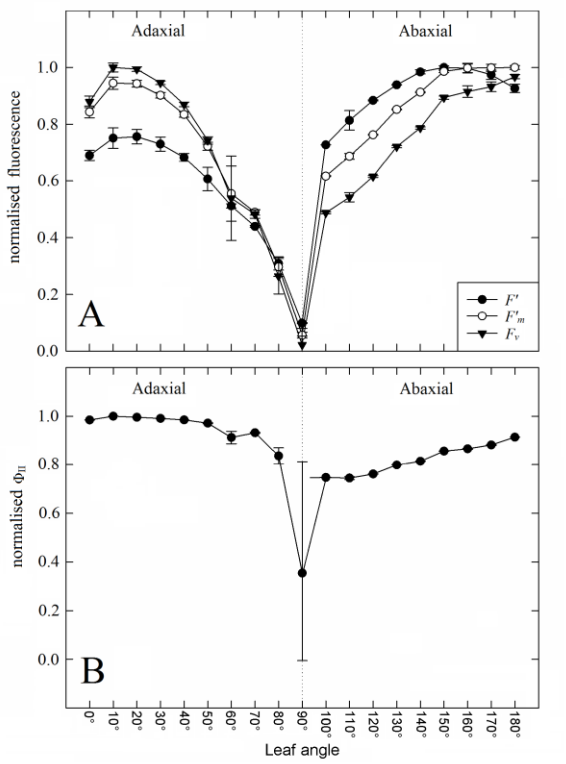

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857 Fig. 3. Relationship between avocado leaf adaxial and abaxial LIFT/FRR measurements and changes in leaf
858 angle. Measurements were performed on avocado leaves (n = 6) positioned 1.0 m from the LIFT instrument.
859 Leaves were rotated 180˚ degrees relative to the LIFT measuring beam in 10° increments using a motorized
860 tripod, where replicate LIFT measurements were taken for each angle (n = 6). The leaf angle changes in each
861 measured parameter were normalised to the maximum to allow direct comparison. Panel A shows raw
862 fluorescence parameters and panel B shows Φ_{II}. All measurements are means ± SD.

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869 Table 1. Plant species and mean SPAD values ± SD (n = 3) used to assess LIFT-detected R_{685} as a proxy for leaf
870 PAR. Leaves were collected from naturally growing plants on the University of Wollongong campus. SPAD
871 measurements were used to control for chlorophyll content between species replicates. Samples are grouped
872 based on the measured intensity of R_{685}, where underlined SPAD / chlorophyll contents (Chl) represent the
873 mean ± SD of all measurements within each group.

Species scientific name	**Common name**	**SPAD / total Chl (µg.cm^{-1})**
<u>High reflectance at 685 nm</u>		<u>48.4 ± 3.7</u>
Alectryon subcinereus	(Native Quince)	47.4 ± 3.3
Eucalyptus globoidea	(White stringy bark)	50.9 ± 4.9
Lomandra longifolia	(Spiny-head mat-rush)	46.9 ± 3.0
<u>Medium reflectance at 685 nm</u>		<u>36.2 ± 10.7</u>
Acmena smithii	(Lilli Pilly)	28.4 ± 2.0
Asplenium nidus	(Bird's-nest fern)	33.0 ± 1.1
Persea americana	(Avocado) low chlorophyll	30.3 ± 2.0 / 36.5 ± 1.7
Polyscias elegans	(Celery wood)	53.2 ± 5.2
<u>Low reflectance at 685 nm</u>		<u>59.8 ± 1.8</u>
Ficus macrophylla	(Fig tree)	61.5 ± 2.2
Mangifera indica	(Mango)	59.2 ± 1.6
Persea americana	(Avocado) high chlorophyll	58.5 ± 1.5 / 181.2 ± 1.5

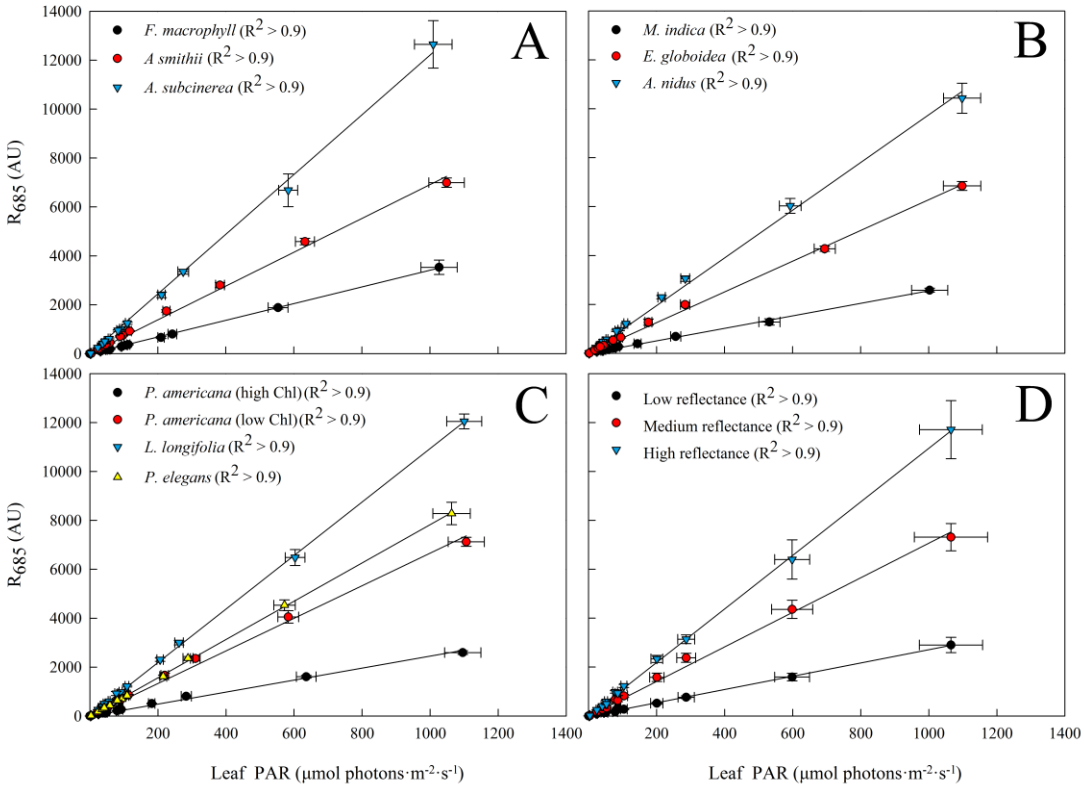
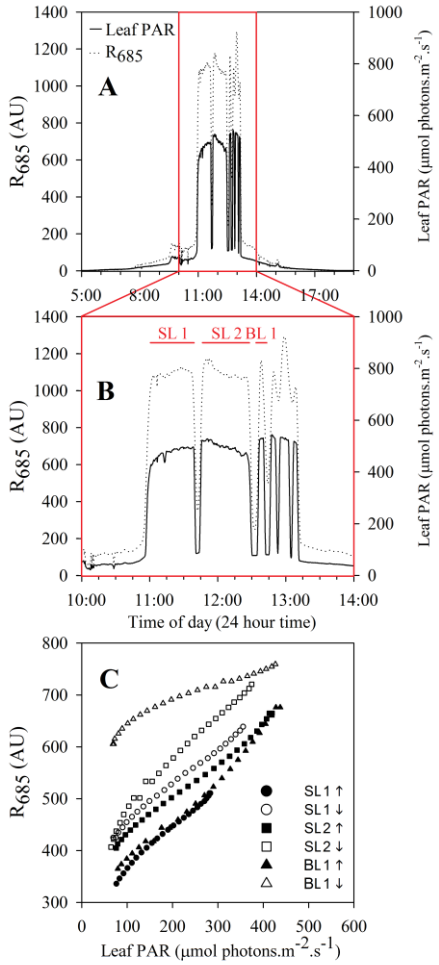


Fig. 4. Relationships between leaf-level PAR and LIFT measured reflected light at 685 nm (R_{685}) for leaves of 8 different plant species. Light response curves were performed on detached leaves with the LIFT instrument at a fixed distance of 1 m and measuring beam perpendicular to the leaf surface. All measurements are means (n = 3) ± SD with linear fits. Individual relationships derived from triplicate leaf measurements of each species are shown in panel A, B and C. In panel D, species relationships have been plotted as generalised trends for low reflectance leaves (*P. americana* [High chl], *F. macrophylla* and *M. indica*), medium reflectance leaves (*A. nidus*, *A. smithii*, *P. elegans* and *P. americana* [low chl]) and high reflectance leaves (*A. subcinereus*, *L. longifolia* and *E. globoidea*).



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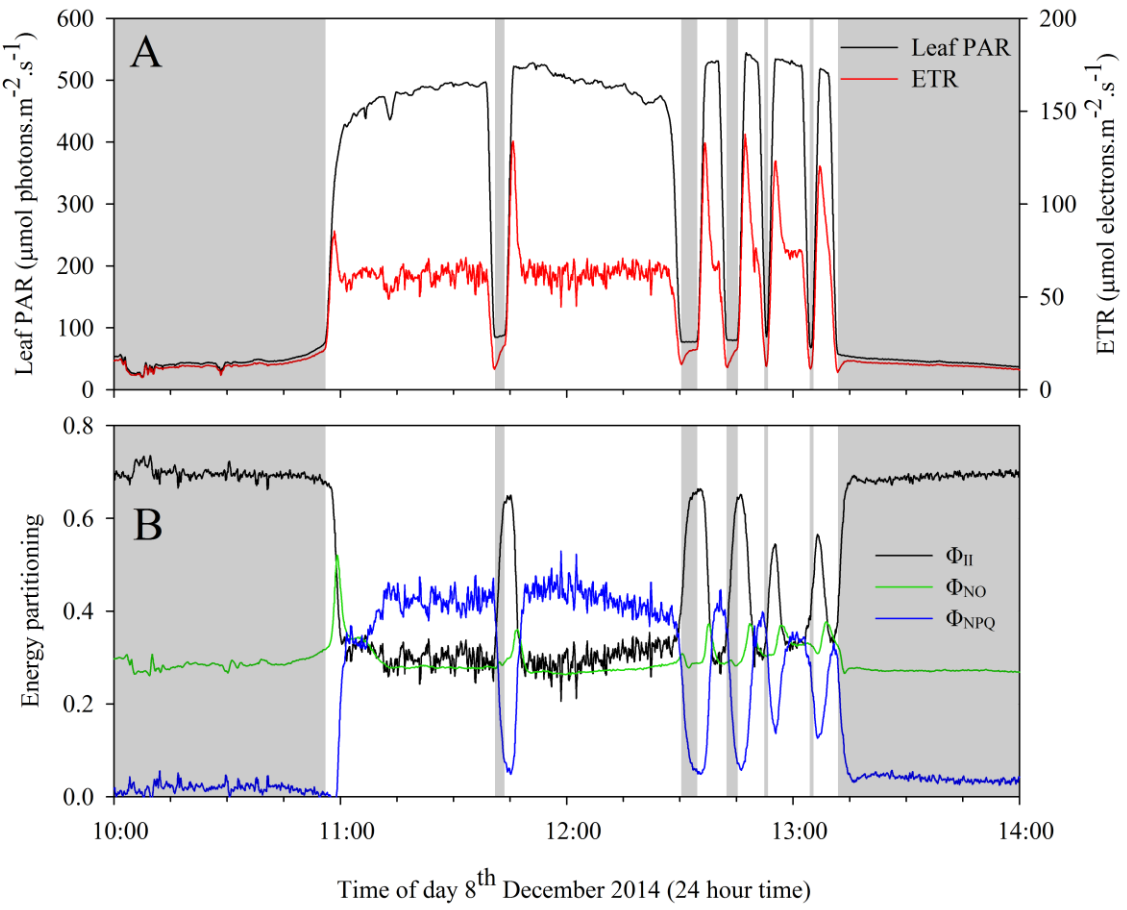
885 Fig. 5. Relationship between leaf PAR and R_{685} measured during a single day on an exposed outer canopy
886 avocado leaf from a plant grown indoors in a glass atrium. During cloud free days the structural beams in the
887 roof of the atrium cast regularly spaced shadows inducing two sustained light events (SL; ~45 min) and four
888 brief light events (~10 min). Panel A shows changes in R_{685} (dotted line) and leaf PAR (solid line) over a full
889 diurnal cycle and panel B shows changes between 10:00 and 14:00 on the same day (red box in panel A). Panel
890 C shows the relationships for two sustained light events and a brief light event (SL1, SL2, BL1; red bars in
891 panel B), where solid symbols show relationships during the initial light event PAR increase (↑) and empty
892 symbols during the subsequent light event PAR decrease (↓).



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894 Fig. 6. Photosynthetic changes in an outer canopy avocado leaf to dynamic changes in sunlight intensity in a
895 glass atrium. On cloud free days structural roof beams cast regularly spaced shadows (grey bars) creating two
896 sustained light events (~45 min) and four brief light events (~10 min) of comparable light intensity. Panel A,
897 incident PAR and ETR estimated from a micro quantum light sensor and LIFT/FRR measurements of
898 chlorophyll fluorescence monitored at 10 s intervals. Panel B, energy partitioning between three component
899 photosynthetic processes.

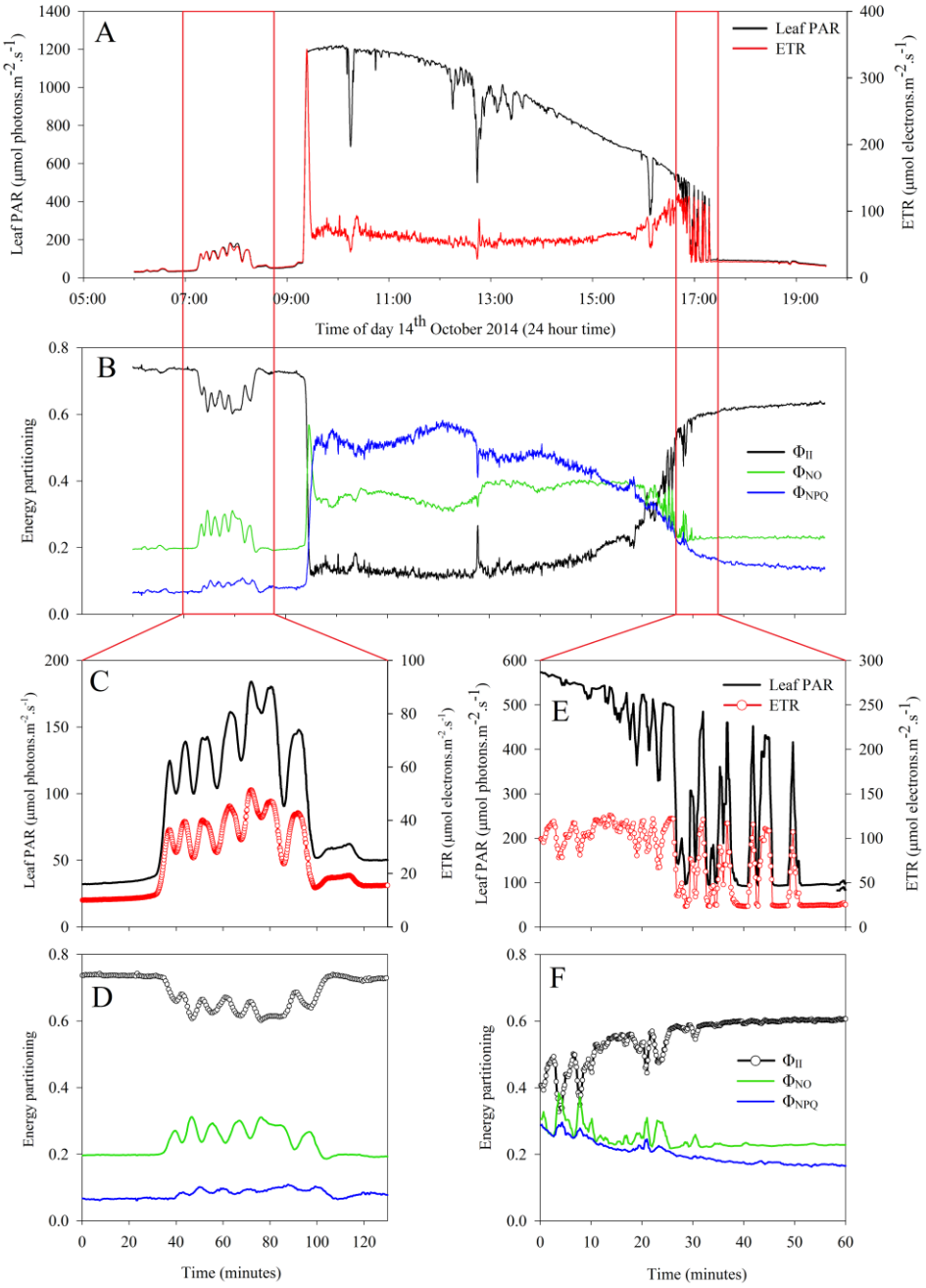
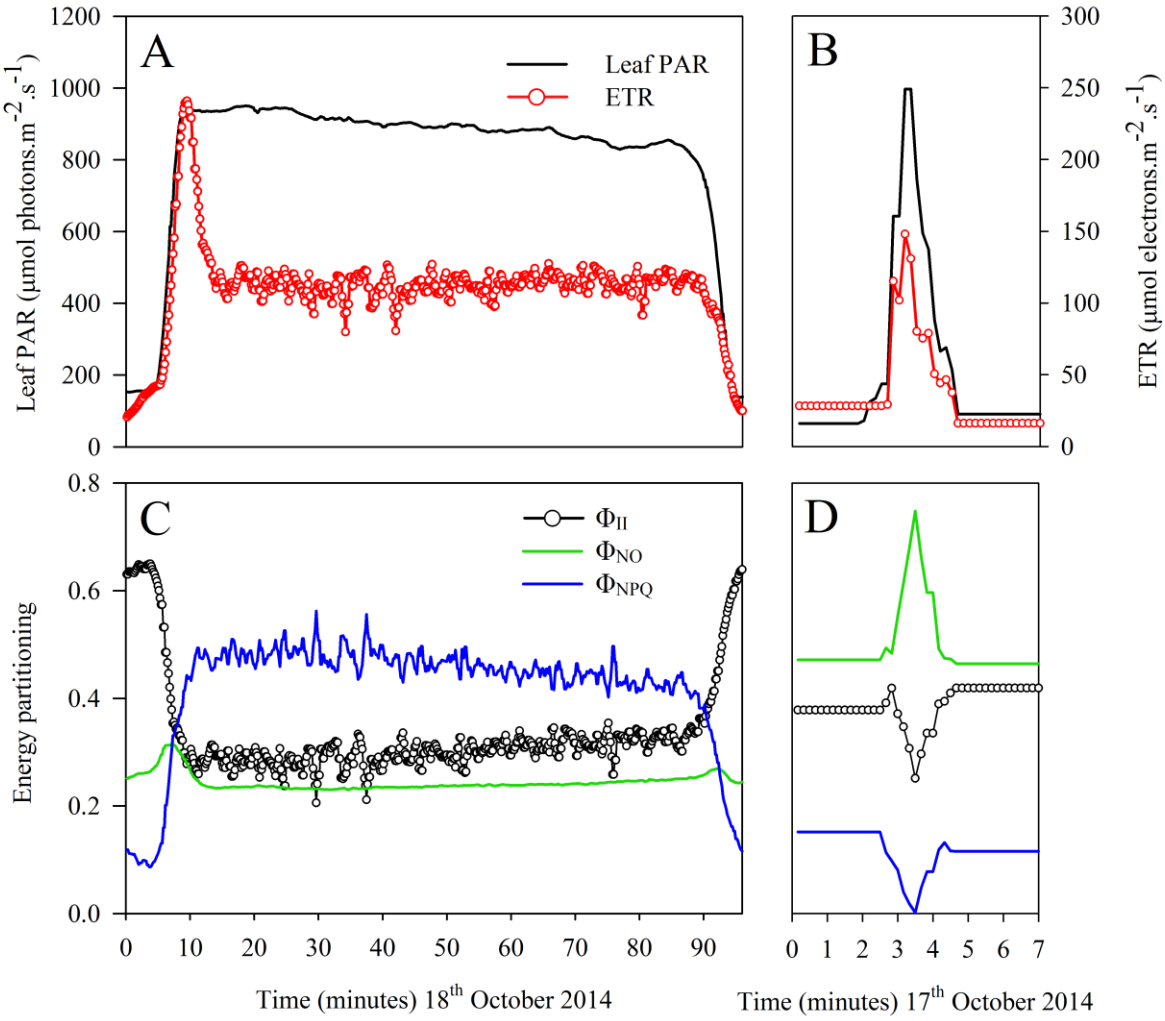


Fig. 7. Photosynthetic changes in response to dynamic sunlight fluctuations in an outer canopy leaf of an avocado plant outdoors at the ERC at different times of the day. Morning light fluctuations are due to shadows from the shade house framework before sudden exposure to direct sunlight, while evening light fluctuations are due to natural shade from adjacent vegetation. Panel A, incident PAR and ETR at measured at 10 s intervals, panel B, energy partitioning between three component photosynthetic processes. Data from early morning and late afternoon brief light events are shown at expanded scales in panels C, D and E, F respectively (red boxes of panels A and B; N. B. the scale of the latter is three times larger than the former).



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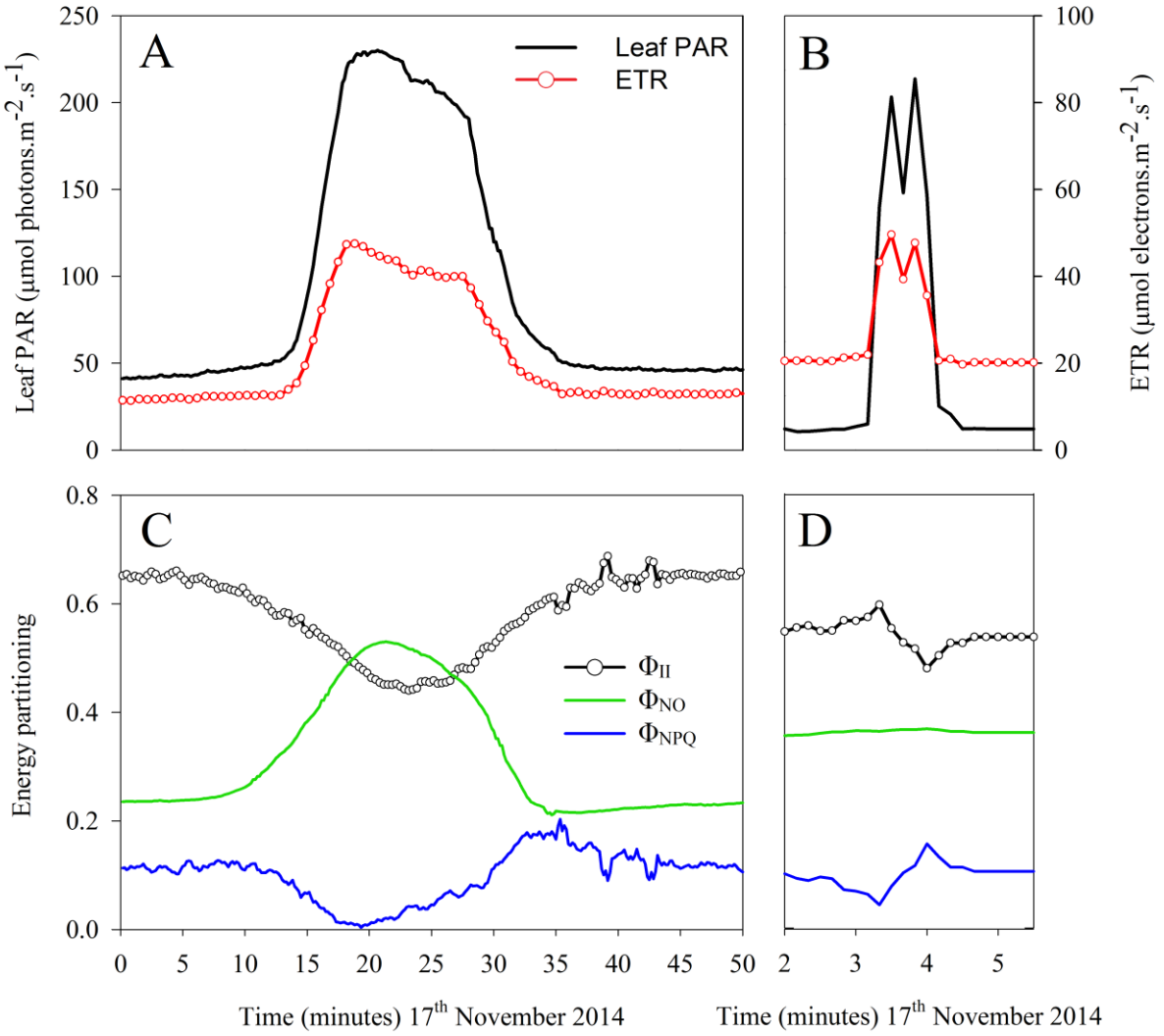
909 Fig. 8. Photosynthetic parameters during a midday strong sustained light event (A and C) and a midday brief
910 light event (B and D) in two different leaves on an avocado plant grown in a shade house at the ERC and
911 monitored by LIFT/FRR with PAR collected at 10 s intervals.



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913 Fig. 9. Photosynthetic parameters during a morning weak sustained light event (A and C) and a midday brief
914 light event (B and D) in a leaf of a sun grown avocado plant at the ERC monitored by LIFT/FRR with PAR
915 collected at 10 s intervals.

916 Table 2. Results of general additive models created for the Δ values of photosynthetic parameters measured
917 during 85 dynamic light fluctuations on middle to lower avocado leaves using the LIFT instrument. Models
918 have been run for the Δ value of each measured response variable and the predictor variables: sustained light or
919 brief light event length (SL/BL length), time since last sustained light or brief light event (time since last
920 SL/BL), sample location and either ΔR_{685} (top) or ΔPAR (bottom). For each model the deviance explained is
921 given in brackets (dev explained). P values are given for each predictor variable, where significant vectors are
922 marked by *** = P < 0.001, ** = P ≥ 0.001 & P < 0.01 and * = P ≥ 0.01 & ≤ 0.05.

	Predictor			
	ΔR_{685}	Ln(SL/BL length)	Ln (time since last SL/BL)	Sample location
Response (dev explained)				
$\Delta\phi_{II}$ (0.703)	<0.001***	0.021*	0.045*	0.109
$\Delta\phi_{NPQ}$ (0.576)	<0.001***	0.215	0.004**	<0.001***
$\Delta\phi_{NO}$ (0.353)	0.003**	0.668	0.092	0.004**
ΔETR (0.375)	0.266	<0.001***	0.144	0.229
	ΔPAR	Ln(SL/BL length)	Ln (time since last SL/BL)	Sample location
$\Delta\phi_{II}$ (0.503)	<0.001***	<0.001***	0.077	0.546
$\Delta\phi_{NPQ}$ (0.524)	<0.001***	<0.001***	0.002**	0.04*
$\Delta\phi_{NO}$ (0.461)	0.065	0.094	0.029*	0.028*
ΔETR (0.726)	<0.001***	<0.001***	0.376	0.331

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