

Canopy conundrums: building on the Biosphere 2 experience to scale measurements of inner and outer canopy photoprotection from the leaf to the landscape

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Abstract. Recognising that plant leaves are the fundamental productive units of terrestrial vegetation and the complexity of different environments in which they must function, this review considers a few of the ways in which these functions may be measured and potentially scaled to the canopy. Although canopy photosynthetic productivity is clearly the sum of all leaves in the canopy, we focus on the quest for ‘economical insights’ from measurements that might facilitate integration of leaf photosynthetic activities into canopy performance, to better inform modelling based on the ‘insights of economics’. It is focussed on the reversible downregulation of photosynthetic efficiency in response to light environment and stress and summarises various xanthophyll-independent and dependent forms of photoprotection within the inner and outer canopy of woody plants. Two main themes are developed. First, we review experiments showing the retention of leaves that grow old in the shade may involve more than the ‘payback times’ required to recover the costs of their construction and maintenance. In some cases at least, retention of these leaves may reflect selection for distinctive properties that contribute to canopy photosynthesis through utilisation of sun flecks or provide ‘back up’ capacity following damage to the outer canopy. Second, we report experiments offering hope that remote sensing of photosynthetic properties in the outer canopy (using chlorophyll fluorescence and spectral reflectance technologies) may overcome problems of access and provide integrated measurements of these properties in the canopy as a whole. Finding appropriate tools to scale photosynthesis from the leaf to the landscape still presents a challenge but this synthesis identifies some measurements and criteria in the laboratory and the field that improve our understanding of inner and outer canopy processes.

Additional keywords: avocado, chlorophyll fluorescence, LIFT, lutein-epoxide cycle, NPQ, PAM photoacclimation, photoinhibition, PRI, violaxanthin cycle.

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Introduction

‘Niggle was a painter. Not a very successful one, partly because he had many other things to do. He had a number of pictures on hand; most of them were too large and ambitious for his skill. He was the sort of painter who can paint leaves better than trees. Yet he wanted to paint a whole tree, with all of the leaves in the same style, and all of them different.’ Tolkien (2001)

(niggle *verb intrans*: to spend too much effort on minor details)

It is an honour to contribute to the Evans Review series and to celebrate one of Lloyd Evans’ many contributions to the strength of plant sciences in Australia over more than half a century. Lloyd stimulated the last author’s career in plant biology when he invited graduate students to attend an international congress convened at the opening of the Canberra Phytotron in 1962. Presentations from so many distinguished plant biologists at

the meeting were remarkable introductions to the opportunities for integrating plant research between the field and the laboratory using controlled environment facilities. Forty years on, that student's peripatetic dependence on such facilities seemed to approach its high point when the unique 'apparatus' of the Biosphere 2 Laboratory (B2 L) presented itself as a simulator for scaling experimental approaches from the laboratory to model ecosystems. Perhaps it is no surprise then that this review is illustrated with some data from B2 L and original data arising from subsequent collaborations among colleagues, several of whom shared the excitement of research in B2 L 2001–03.

Collectively the present authors have been challenged, one way or another, to understand the structural biology of reversible downregulation of photosynthetic efficiency throughout a wide range of scales of enquiry from the field to the laboratory. For some, research has ranged from pioneering airborne measurements of the transition from winter photoprotection to spring rejuvenation of light use efficiency (LUE) in the boreal forest of Siberia using remote sensing of spectral reflectance and Eddy covariance flux tower data (Nichol *et al.* 2002), to lead roles in current European projects that provide simultaneous airborne measurements of solar induced fluorescence and CO₂ fluxes (Rascher *et al.* 2009), to monitoring of stress in Antarctic moss beds using unmanned aerial vehicles (Lucieer *et al.* 2010). Others invented new techniques for remote sensing of chlorophyll fluorescence and the sensitivity of photosynthesis to stress in the canopy of the model rainforest of B2 L (Ananyev *et al.* 2005), in *Arabidopsis* mutants (Kolber *et al.* 2005) and in the field (Pieruschka *et al.* 2010). Successful combination of infrared thermal imaging and chlorophyll fluorescence imaging to assess consequences of stomatal closure at the leaf level (Omata and Takayama 2003) is now driving the design of robotic systems to monitor greenhouse crops (K. Takayama, unpubl. data). Laboratory studies have ranged from the generation and evaluation of targeted carotenoid and xanthophyll cycle mutants of *Arabidopsis* (Pogson *et al.* 1998) to natural selection of very high light resistant mutants of the model alga *Chlamydomonas* (Förster *et al.* 2001, 2005) and to current research on these pigment inter-conversions in avocado canopies (Förster *et al.* 2009, 2011).

Acknowledging that contributions to the Evans Review series are to be 'critical state-of-the-art reviews that aim to advance our understanding' our overall objective is to link these and other forms of photoprotection in leaves to processes in canopies. Our focus is the canopy conundrum, enunciated by Grace *et al.* (2007) as the 'parameterisation of vegetation structure so that leaf-scale properties may be related to the properties of the ecosystem as a whole, taking into account the structure of the canopy'. This is but one of several constraints on achieving the 'holy grail'; meaningful observation of terrestrial photosynthesis from space. The state of this art is important to most aspects of modern plant environmental biology because, as the Earth systems research community has come to accept, photosynthesis 'is the key process mediating 90% of carbon and water fluxes in the coupled biosphere–atmosphere system' (Joiner *et al.* 2011) that underpins present concerns for food security, the feasibility of bio-fuel production and sustainability of ecosystems.

This review begins with an outline of some scaling concepts and some approaches to and the tools needed for, their validation through experiments. Images of photoprotection at leaf, canopy and landscape levels are then used to illustrate the end members for this exercise. Experiments with avocado leaves that identify key properties of different forms of photoprotection within the inner and outer canopy are briefly reviewed. Examples of the expression of these processes in canopy leaves in stochastic light environments during experiments in B2 L, in the Eden Project and in the field are presented. The review concludes with a discussion of the limitations to these observations and experiments and the need to strengthen linkages between modelling approaches and measurements in scaling from the leaf to the landscape.

Scaling concepts: addressing Niggel's conundrum with measurements and models in Biosphere 2 and in FLUXNET

Barry Osmond's interest in photosynthesis in leaves of wild plants from different environments was provoked by a challenging overview of primary photosynthetic processes that identified events with relaxation times beyond picoseconds as 'eras of ignorance' and relegated events with time scales of greater than 10 s to 'ecology' (Kamen 1963). This interest found focus in collaborations with colleagues in the Department of Plant Biology, Carnegie Institution that sought to scale photosynthetic biophysics and biochemistry *in vitro* to the intact leaf and to plants in the field (Osmond *et al.* 1980). On the suggestion of Joe Berry, the scaling concepts of these authors were transformed into a two dimensional framework in which the size of the photosynthetic apparatus (in microns) was locked to relaxation times (in seconds) of relevant processes (Osmond 1989). An underlying premise was that 'one must hope to discover economical insights as opposed to the insights of economics'. Ironically, experimental plant ecophysiology now has re-focussed much basic photosynthetic research back to Kamen's domains of biophysics, biochemistry and structural biology in the quest to explain molecular mechanisms of reversible downregulation of efficiency and photoprotection in response light stress.

It was hoped that for practical purposes, these 'economical insights' would facilitate reductionist scaling; enabling the increasing complexity of whole systems to be evaluated as rather less than the sum of their parts. It was believed that such 'economical insights' would emerge from bold application of measurement techniques well grounded in photosynthetic mechanisms that would advance understanding of these processes in more complex entities at leaf, canopy and landscape scales. The urge to make measurements and do experiments at these scales was thought to complement broad integrations based on the 'insights of economics' (Chiariello *et al.* 1989) that continue to emerge from huge datasets (Wright *et al.* 2004) and demand more sophisticated techniques of meta analysis (Poorter *et al.* 2010).

Without question, our confidence in the need for integration of these complementary approaches had been strengthened by successful leaf to landscape scale expansions already achieved. For example, embedding 'economical insights' from empirically-based responses of stomata to environment in models of global

circulation models greatly facilitated coupling of transpiration and energy balance in the terrestrial biosphere (Sellers *et al.* 1992; Berry *et al.* 2010). In part, the success of this particular approach depended on the canopy integration factor (designated π and itself derived from the ‘insights of economics’) to scale whole-canopy performance to the performance of leaves at the top of the canopy. It seemed likely that the huge enclosed and climate controlled biomes in B2 L could serve as simulators for testing the reductionist scaling from leaves to canopies and to ecosystems. In short, the mission of B2 L at the turn of the 20th Century was to bring the laboratory philosophy of ‘enclose it, control it and poke it’ (attributed to K. Lachner) with replication in time rather than in space, to scaling issues in experimental ecosystem and climate change science. Indeed it seemed that B2 L presented an opportunity similar to that of Frits Went’s original phytotron (Kingsland 2009). In the history of science, few disciplines have successfully eschewed controlled experiments and such experiments were deemed necessary to bridge the Newtonian-Darwinian divide in Earth systems science (Harte 2002).

It was expected that ‘economical insights’ into ecosystem level responses to stress would emerge from the simulator of B2 L and would complement (and at times by-pass) leaf-to-canopy simulations imbued with the ‘insights of economics’. Indeed, this hope was quickly realised by experimental validation of the ‘big-leaf’ model by Lloyd *et al.* (1995) that had been developed to interpret Amazon rainforest ecosystem carbon fluxes in terms of leaf photosynthetic properties. Whole-canopy CO₂ assimilation studies at controlled [CO₂] in the huge ‘leaf chambers’ of the forest biomes of B2 L (Lin *et al.* 1998) showed how the facility could serve ‘as a new experimental system for examining the time dependent changes in ecosystem response to global change’. For example, the outer canopy/whole-canopy scaling factors used in models by Sellers *et al.* (1992) and Lloyd *et al.* (1995) were addressed by de Pury and Farquhar (1997) and shading and canopy management experiments had been designed to further validate these insights.

Supported by tools such as chlorophyll fluorescence, a small-scale indicator of photosynthetic regulation embedded in the light reactions of chloroplasts, leaf spectral reflectance embedded in chlorophyll and carotenoid pigments and stable isotope discrimination an integrator of the dark reactions embedded in CO₂ assimilation and leaf gas-exchange physiology, the time was ripe for scaling from experiments with leaves to canopies. Each of these tools found creative application in the large-scale controlled environment experiments in B2 L. For example, canopy access with hand-held measurements of chlorophyll fluorescence helped identify three photosynthetic response types in the major canopy elements during controlled drought experiments in the model tropical forest (Rascher *et al.* 2004). Guang-Hui Lin’s measurements of $\delta^{13}\text{C}$ values of whole-ecosystem CO₂ exchange in the tropical forest pointed to shifting respiratory substrates during recovery from drought (Osmond *et al.* 2004). It was shown that initial control of ecosystem level CO₂ flux response to elevated [CO₂] was by leaf area development, leading to canopy closure and light limitation in a well irrigated model poplar plantation forest, but control switched back to the leaf level as stomata closed in response to drought (Murthy *et al.* 2005). Moreover,

in the poplar forest leaf, root and system level responses relevant to potential carbon sequestration at different [CO₂] (Barron-Gafford *et al.* 2005) were differentiated using stable isotopes (Trueman and Gonzalez-Meler 2005). New methods for remote sensing of chlorophyll fluorescence were invented (Ananyev *et al.* 2005; Kolber *et al.* 2005) that, with spectral reflectance (Nichol *et al.* 2006; Rascher *et al.* 2007), revealed several unexpected photosynthetic responses in the forest canopies, as discussed below.

Today, in the absence of substantial commitments to other enclosed, controlled environment simulators on the scale of B2 L, scaling experiments are largely dependent on advances in measurements of gas exchange of whole canopies by improvements in eddy covariance and related techniques. Response functions must then be extracted from stochastic and covariant environmental data. The extraordinary flowering of flux towers, forming a network known as FLUXNET, now involves several hundred stations covering almost all the climate-space of the globe and all the continents, also presents opportunities for simultaneous and continuous probing of the photosynthetic machinery at leaf level using the above tools. FLUXNET observations demonstrate the impact of the ever-changing environment on the leaves acting *en masse*, alerting us to large scale patterns of photosynthetic behaviour arising for example from synoptic weather systems, extreme events and diffuse versus direct radiation.

Importantly for this review, these sites now provide platforms for testing of measurement systems for leaf-to-canopy scaling (Nichol *et al.* 2002; Louis *et al.* 2005) and for testing modelling protocols. Moreover, they present well calibrated targets for airborne and satellite-based remote sensing of photosynthesis in the outer canopy (Damm *et al.* 2010). The full potential of FLUXNET is only now being realised, as state-of-the-art sensors are being brought from the laboratory, modified for field use and installed within the canopy to measure fundamental processes in photosynthesis (Balzarolo *et al.* 2011). Meanwhile, phenologists are using flux towers as platforms for cameras and spectro-radiometers, to better understand the interaction between extremes of climate, photosynthesis and the seasonal development and duration of leaves (Ahrends *et al.* 2009). This trend towards enhancement of eddy-covariance by the monitoring of within canopy physiology is soon likely to extend to tropical forest ecosystems, which are considered to be especially under threat and which contain a large fraction of the world’s species and biomass-carbon.

Introducing *Inga* spp. and *Persea americana*

In what follows, we emphasise that although model systems remain indispensable for elucidation of the mechanisms underlying the regulation of photosynthetic efficiency in leaves and canopies, natural selection, with time on its hands, has done many interesting experiments in wild plants from which we can draw useful generalisations at all of these scales. Many of our illustrations are from experiments with leaves in canopies from two woody species of tropical origin; one ancient (*Persea americana*; Lauraceae, avocado) and the other modern (*Inga* spp.; Fabaceae). The former had been modified by ~10 000 years’ domestication in Central America before its introduction

throughout the world in the last century (Whiley *et al.* 2002). Species of the latter are fast growing trees of tropical lowland forests, tolerant of acid soils. Some species of *Inga* are prized for their fruit, others are used as shade trees in coffee plantations (Pennington 1997) and others have found application in 'alley' agro-forestry for remediation of slashed and burned landscapes of Central America (Pennington and Fernandes 1998).

Both were planted in the tropical forest mesocosm of B2 L (32°34'N; 110°51'W; elevation 1176 m) and *Inga sapindoides* was one of several species targeted for remote sensing of canopy photosynthesis. Moreover, several *Inga* spp. had been planted as rapidly growing shade trees to establish a canopy for the south-facing humid tropics biome of the Eden Project, Bodelva, Cornwall UK (50°21'N; 4°44'W; elevation 78 m) and were accessible for studies of pigment composition and measurements of photosynthesis in the inner and outer canopy using hand-held instruments. Both species retain dense, deeply shaded canopies of old leaves with very low photosynthetic activity but endowed with two xanthophyll pigment cycles that can be engaged to stabilise mechanisms of reversible downregulation of photosynthetic efficiency; i.e. mechanisms of photoprotection. Much of our recent research has been focussed on these aspects of the canopy conundrum.

A picture is worth a thousand words

In a letter to his publisher Tolkien noted that the Niggle allegory 'was the only thing I have ever done which cost me absolutely no pains at all'; he evidently dashed off the essay in a matter of hours (www.tolkienlibrary.com/reviews/leafbyniggle.htm, accessed 7 December 2011). Would that preparation of this review had been likewise! Tolkien solved the leaf-to-canopy conundrum for Niggle by having him tack each canvas of a new leaf to the architecture of a tree on the back wall of his shed; an expedient that seems to describe the approach of much research in photosynthesis today.

Understandably perhaps, we tend to become preoccupied with working at scales best served by the technologies available to advance understanding within our comfort zones, commonly extending over ~3 orders of magnitude in space and time (De Wit 1970). Nevertheless, leaf-level ecophysiological photosynthetic research has greatly expanded our mechanistic understanding of photoprotection, the reversible downregulation of photosynthetic efficiency that allows plants to engage photoprotection and achieve a modicum of 'photostasis' (Öquist and Huner 2003) in the face of some three orders of magnitude variation in light environment on a daily basis.

For the purposes of this review the three images in Fig. 1 effectively establish the end members for our perspectives on photoprotection and presage thousands of words that follow in our effort to scale these processes from the leaf to the canopy and to the landscape. Access to images of photosynthetic processes at the leaf level depended on the invention of light emitting diodes (LED) and digital photography. These inventions enabled established Pulse Amplitude Modulated (PAM) techniques for chlorophyll fluorescence analysis to be extended to imaging contexts. Working at close range (~1 to 20 cm), PAM systems use a weak measuring beam to excite intrinsic chlorophyll fluorescence (F_o) from PSII and a saturating multiple turnover

flash (~7000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for ~1 s) to close all photosystem 'traps' so as to estimate the maximum yield (F_m) of PSII fluorescence in dark adapted leaves (Ögren and Baker 1985; Schreiber *et al.* 1986; Krause and Weis 1991). The difference ($F_m - F_o$) measures variable fluorescence F_v and the ratio F_v/F_m provides an estimate of maximum efficiency of PSII (Φ_{PSII}). Subsequent flashes applied in actinic light measure F_m' so that $(F_m' - F_o)$ yields ΔF and the ratio $\Delta F/F_m'$ gives a measure of Φ_{PSII} under the prevailing light conditions. These parameters are then used to estimate the redox state of Q_A , the primary electron acceptor of PSII, photosynthetic electron transport (ETR; Genty *et al.* 1989) and non-photochemical chlorophyll fluorescence quenching (NPQ), a widely accepted index of reversible downregulation of Φ_{PSII} and of photoprotection. These methods have become all pervasive and it is difficult to overstate the revolution in photosynthesis research in the laboratory and the field over the last 25 years arising from this approach. Recognising this, the International Society of Photosynthesis Research selected Uli Schreiber as recipient of its inaugural Innovation Award in 2004.

Chlorophyll fluorescence imaging methods have rapidly expanded our understanding of photosynthetic heterogeneity arising from patchy responses of stomata and distributed metabolic regulation (Siebke and Weis 1995; Bro *et al.* 1996). An NPQ image of chlorophyll fluorescence in an avocado leaf (Fig. 1a) displays natural heterogeneity in this index of photoprotection due to patchy responses of stomata and variable access to CO_2 dictated by the heterobaric anatomy (Terashima 1992). Kotaro Takayama generated 'economical insights' into the coupling between light and dark reactions of photosynthesis in one sweep, by applying a 'Vaseline patch' to occlude stomata, prevent CO_2 access, produce sustained high NPQ under the treated area and confirm the extent of lateral diffusion of CO_2 in these leaves (Morison *et al.* 2005; Pieruschka *et al.* 2008).

In research, one never knows from whence the next needed tool might arise and NPQ in the canopies of *Arabidopsis* mutants (Fig. 1b) was measured by remote sensing with a recently developed laser-induced chlorophyll fluorescence transient (LIFT) device. This instrument was created by using the fast repetition rate fluorescence (FRRF) sensor protocols for continuous monitoring of marine phytoplankton (Kolber *et al.* 1998) to drive eye-safe lasers to target leaves in air at up to 40 m and a telescope to collect the chlorophyll fluorescence emitted (Chappelle *et al.* 1984). In one step, LIFT overcomes the impracticability of delivering a saturating flash at a distance and removes the 'black out' period of observation following saturating flashes. This pump probe method achieves estimates of $\Delta F/F_m'$ with less than 10% of the excitation delivered in saturating flashes and is thus much less intrusive, especially with shade leaves. Depending on the design of the duty cycle, other important parameters, such as photosynthetic electron transport, redox state of electron carriers and antenna size also may be obtained in real time (Kolber *et al.* 1998, 2005).

The measurements of NPQ in *Arabidopsis* mutants (Fig. 1b) were made in real time (2 s resolution) using LIFT at 14 m (Kolber *et al.* 2005) and link the PRI images to lower capacity for NPQ in *npq4-1* and higher capacity for *L5* as was previously established using PAM (Li *et al.* 2002). They were

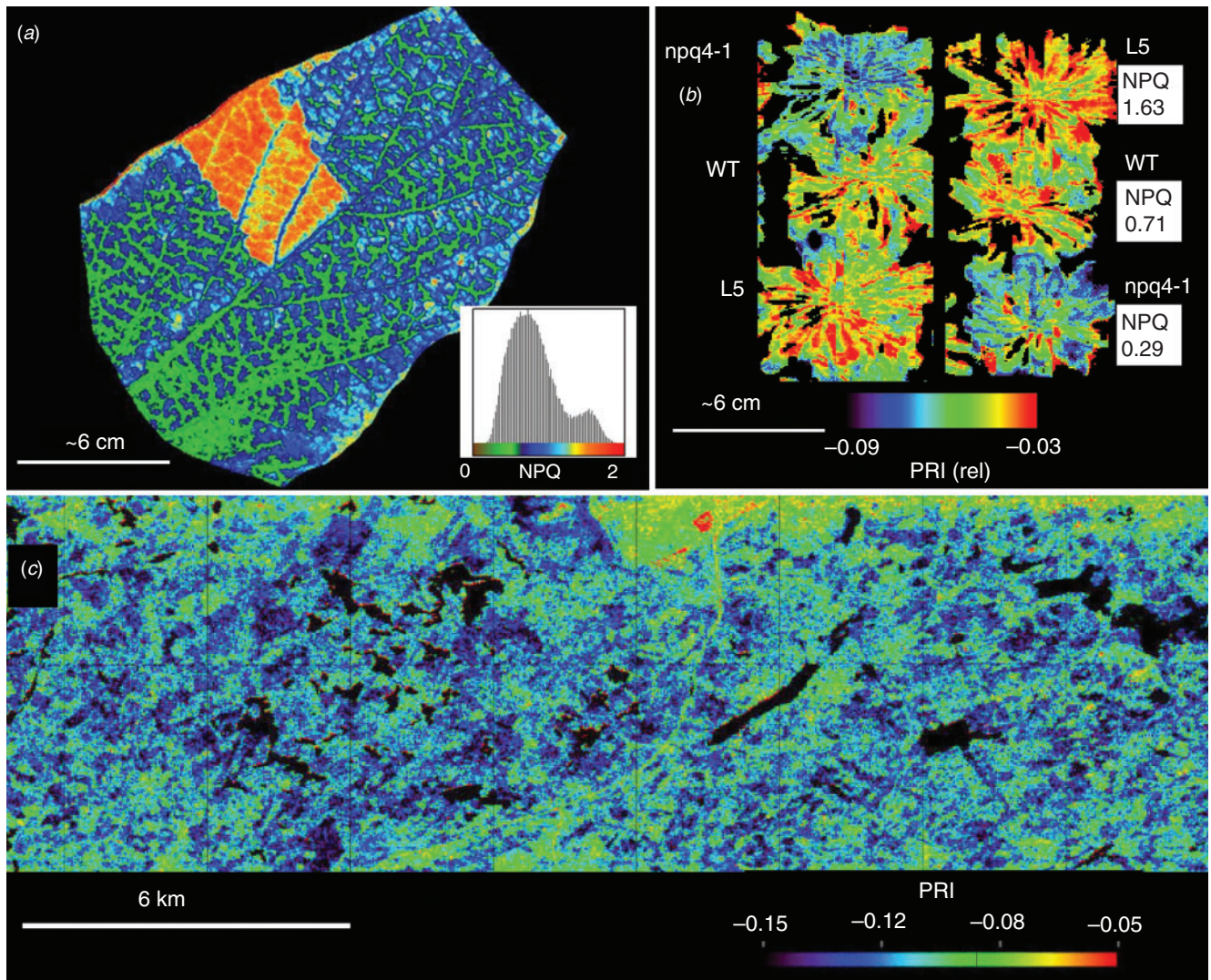


Fig. 1. Images of heterogeneous photoprotection of photosynthesis at different scales. (a) Non-photochemical quenching (NPQ) image (Technologica CF Imager; Fluorimager Two software; Technologica Ltd, Colchester, Essex, UK; Barbagallo *et al.* 2003) of a leaf of shade-grown avocado (*Persea americana*) after 3 min exposure to $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ orange light during a photosynthetic induction experiment. (b) Photochemical reflectance index (PRI) images (SOC-700; Surface Optics Corporation, San Diego, CA, USA; wavebands 531 nm and 572 nm) within a few minutes of sunlight exposure of dark adapted ‘canopies’ in wild-type *Arabidopsis* (WT) and in mutants *npq4-1* and *L5* (Li *et al.* 2002) with lower and higher capacity respectively, for NPQ through controlled expression the ΔpH sensing PsbS protein (rearranged from Rascher *et al.* 2007). Whole-canopy NPQ values were remotely sensed on the same population of mutants using LIFT (Mk 2) after 10 min exposure to $1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (recalculated from Kolber *et al.* 2005). (c) PRI image of forest canopies near the Hyytiälä Field Station, Finland, during the 76 year record heat wave in summer 2010, reconstructed from geometrically and atmospherically corrected Hyperion satellite data using wavebands 528 nm and 569 nm by G. Drolet and C. J. Nichol (unpubl. data).

used to validate independent measurements of the capacity for NPQ in these mutants obtained by spectral reflectance imaging. Spectral reflectance from leaves can indicate changes in de-epoxidation status of xanthophyll pigments that, with high thylakoid membrane ΔpH , stabilise some forms of NPQ (Gamon *et al.* 1990). The photochemical reflectance index (PRI) calculated from wavebands close to 531 and 570 nm; $\text{PRI} = (R_{531} - R_{570}) / (R_{531} + R_{570})$ was used to image NPQ in ‘canopies’ of *Arabidopsis* mutants and from a forest ecosystem (Fig. 1b, c). Compared with wild type (WT), PRI (rel) in mutant *npq4-1* (antisense to PsbS protein) decreased with time, whereas

PRI (rel) in mutant *L5* (overexpression of PsbS protein) increased (Rascher *et al.* 2007, 2009). Some may scorn application of LIFT to NPQ in *Arabidopsis* mutants as ‘overkill’. However in an era in which much of progress in plant biology begins and ends with this ‘undistinguished weed’, this evidence that absence of, or overexpression of, the PsbS protein impairs or enhances NPQ, respectively, is ‘remotely sensible’ presumably adds some weight in our efforts to scale from leaf to landscape.

Maps of PRI from space indicate stress in forest canopies near the Hyytiälä Field Station in Finland (Fig. 1c), reconstructed

using narrow band spectral reflectance data from the Hyperion satellite, show less negative PRI (red pixels) in areas more remote from lakes (black pixels). These canopies may have experienced higher water stress and perhaps higher NPQ associated with closure of stomata, during the record high temperatures of summer 2010. Spectral reflectance measurements at this scale provide a versatile, but potentially more ambiguous approach to observing downregulation of photosynthetic efficiency. However, when corroborated by pigment analyses at ground level, PRI yields convincing evidence of canopy and ecosystem responses to stress (Nichol *et al.* 2002). Asner *et al.* (2004) used spaceborne imaging parameters (including PRI) to observe drought stress and estimate net primary productivity (NPP) in adjacent 1 ha Amazon rainforest sites, one of which had been fitted with 'drain-out' panels to prevent precipitation from reaching the soil during the rainy season. There seems little doubt that PRI will continue to have an important role in observing canopy photosynthesis from space (Hilker *et al.* 2011).

The actual correlation between PRI and NPQ varies with species, leaf morphology and canopy architecture and although diel variation in PRI is primarily driven by xanthophyll de-epoxidation in strong light and epoxidation in the dark, variation in PRI over longer periods is particularly sensitive to changes in the total pools of carotenoids and chlorophylls (Sims and Gamon 2002). Busch *et al.* (2009) identified particularly important limitations, suggesting it may be impracticable to detect both zeaxanthin-independent NPQ and sustained forms of zeaxanthin-dependent NPQ at low temperature using PRI. Peñuelas *et al.* (2011) found a similar R^2 (~0.6) for LUE vs PRI in studies of leaves, canopies and ecosystems. We can imagine Niggler's sigh of relief on encountering a recent meta-analysis of PRI that concluded there is a surprising degree of 'functional convergence of biochemical, physiological and structural components affecting leaf, canopy and ecosystem carbon uptake efficiencies' Garbalsky *et al.* (2011).

Differing distribution of pigments and differing kinetics of two xanthophyll cycles in canopies of woody plants

Leaf pigment composition is a major determinant of PRI, so we briefly review recent data that have expanded our understanding of these relationships. Although Yamamoto *et al.* (1962) showed violaxanthin (V) to be cyclically inter-converted to zeaxanthin (Z) via antheraxanthin (A), in spinach, lima bean leaves and lettuce, 15 years were to pass before a link between these pigment inter-conversions and reversible downregulation of photosynthetic efficiency in strong light was appreciated. Demmig *et al.* (1987) discovered the link between NPQ and much subsequent research led by Demmig-Adams and Adams III (1992, 2006) established the near universal association of the V-cycle with this process. The correlation between NPQ and de-epoxidation of V was so widespread that more than a decade elapsed before the role of another analogous xanthophyll cycle, the light-dependent inter-conversion of lutein epoxide (Lx) and lutein (L), previously described in green tomato fruit (Rabinowitch *et al.* 1975), attracted the attention of ecophysiologicalists.

A fully functional Lx-cycle in photosynthetic tissues, in which de-epoxidation of Lx to L in the light augments the already large pool of L (i.e. $L + \Delta L$), followed by epoxidation of ΔL in the dark, was re-discovered in *Cuscuta reflexa* (Bungard *et al.* 1999), then in mistletoes (Matsubara *et al.* 2001, 2003) and most recently in *Ocotea foetans* (Esteban *et al.* 2010). A truncated form of this cycle, with very slow epoxidation of ΔL to Lx in the dark overnight or in the shade, has been extensively researched in the deeply shaded inner canopy of oaks (García-Plazaola *et al.* 2003), of *Inga* spp. (Matsubara *et al.* 2005, 2007, 2008) and in *Laurus* and *Persea* (avocado) (Esteban *et al.* 2007, 2008; Förster *et al.* 2009, 2011) and other species. This interest in the truncated Lx-cycle arose because retention of ΔL after epoxidation of A+Z, allowed direct demonstration of the long suspected role for L in augmenting the capacity for NPQ, as detailed below. The biochemical relationships of the two cycles were summarised in fig. 1 of García-Plazaola *et al.* (2007).

Recent surveys of woody plants (Esteban *et al.* 2007; Matsubara *et al.* 2008, 2009) have revealed the accumulation of high [Lx] in shade leaves of many species. For example, on average the inner canopy leaves of seven *Inga* spp. in the Eden Project had ratios of [Lx]/[V] of 0.93 ± 0.11 , whereas this ratio in outer canopy leaves was 0.35 ± 0.03 (J. Grace, C.J. Nichol and B. Osmond, unpubl. data). Subsequent field measurements of other *Inga* spp. at the Smithsonian Tropical Research Institute in Panama (Matsubara *et al.* 2008) and of avocado in Eastern Australian orchards further confirmed this distinctive pigment composition of inner and outer canopy leaves in these species. However, it should be noted that some other Lx-rich species show little difference in [Lx] between inner and outer canopy leaves (Matsubara *et al.* 2009).

The distinctive accumulation of Lx in old inner canopy leaves of *Inga* spp. and avocado is presumably a product of metabolic pathways and leaf environment (Matsubara *et al.* 2003; García-Plazaola *et al.* 2007; Esteban *et al.* 2008). In *Inga*, avocado and other plants (Matsubara *et al.* 2009) a 'leaky' zeaxanthin epoxidase (ZE) with some affinity for L, evidently catalyses the slow production of Lx from L in the dark. Evidently, Lx accumulates in deeply shaded inner canopy leaves because they are infrequently exposed to light strong enough to activate violaxanthin de-epoxidase (VDE) for conversion of Lx back to L. This enzyme has affinity for both V and Lx *in vivo* and is activated each day in the outer canopy (as evidenced by conversion of V to A+Z) where it maintains low levels of Lx.

Analyses of these pigments with leaf age in both *Inga* and avocado confirm these hypotheses (Fig. 2). Lutein concentration in leaflets on a N-facing branch in a recently pruned *I. edulis* canopy in the Eden Project declined about 4-fold with age and [Lx] increased 2-fold (Fig. 2a). Light intensity before sampling at the Spring Equinox was such that only traces of A (and no Z) were detected so de-epoxidation of Lx was presumably minimal (Fig. 2b). Much the same relationships were found with increasing leaf age in a single vertical stem of an avocado seedling in sunlight in an open glasshouse in Canberra. Concentrations of xanthophylls in these leaves were usually about twice those in *Inga* leaflets, but developmental trends with age and the shading of old leaves produced similar trends in [Lx] or [L] that did not change with time of sampling

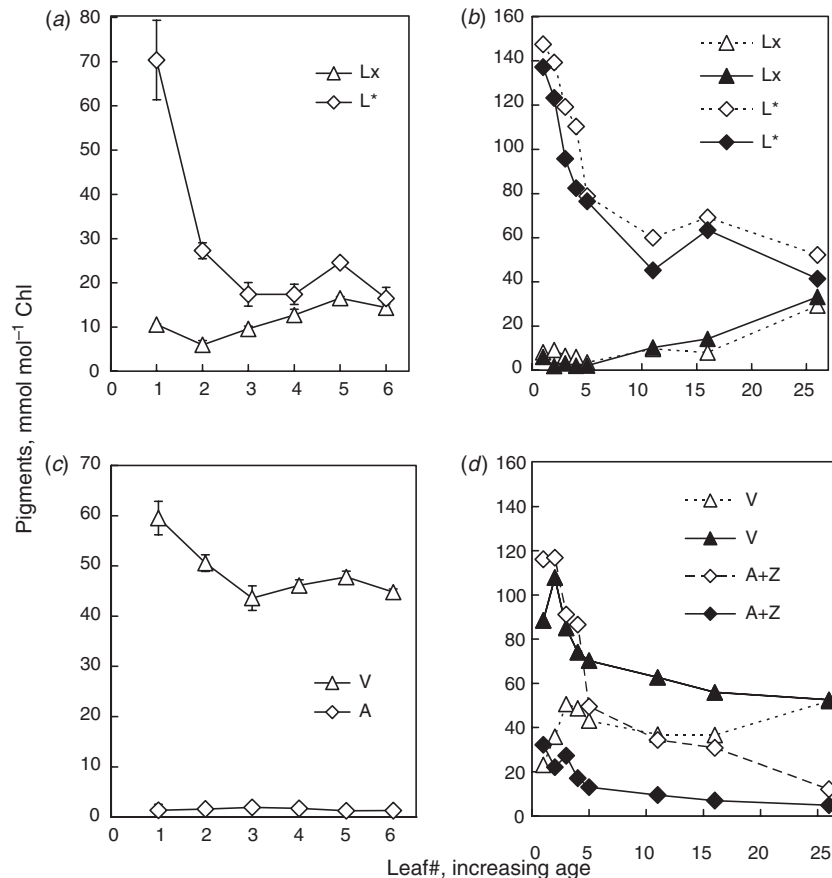


Fig. 2. (a, b) Changing concentrations of xanthophyll and carotene pigment concentrations during development in leaflets of *I. edulis* on an N-facing branch (NH) in weak sunlight in the Eden Project (3–90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at time of collection). (a) Age dependent decline in [L] and increase in [Lx] and (b) decline in [V] (with traces of [A]). (means \pm s.e.; $n=4$). (c, d) Leaves of avocado sampled before dawn (closed symbols) and again in full sunlight at noon (open symbols; 1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in an open glasshouse in Canberra. (c) Age-dependent decline in [L] and increase in [Lx] were similar to *I. edulis*, but in (d) all leaves showed extensive de-epoxidation of V to A + Z at noon. Note that the much higher [L] has been reduced by 100 mmol mol^{-1} Chl to facilitate stoichiometric comparison with other pigments so that the scale for [L*] corresponds to 100–270 mmol mol^{-1} Chl in (a) and 100–260 mmol mol^{-1} Chl in (c).

(Fig. 2c). In contrast, de-epoxidation of V at noon clearly dominated diel changes in pigment composition of fully expanded leaves 3 to 16 (Fig. 2d). Lx-rich mature leaves of shade grown avocado transferred to sunlight showed substantial de-epoxidation of Lx and V after a few hours in sunlight. Recovery of [Lx] required more than 30 days after return to the shade whereas [V] was restored overnight (Förster *et al.* 2009).

The chemical structure of L shares many similarities with A and Z and all three de-epoxidised xanthophylls are loosely bound to the L1 site of light harvesting chlorophyll-protein complexes in the antenna of the photosystems, in addition to L tightly bound in the core L2 of the complexes (Croce *et al.* 1999; Matsubara *et al.* 2007). These old shade leaves of *Inga* spp. and avocado have permitted a direct approach to the enigmatic role of L in photoprotection (García-Plazaola *et al.* 2007), providing ‘economical insights’ into the stabilisation of NPQ by ΔL ,

with distinctive functional implications for inner and outer canopy photosynthesis, as discussed below.

Xanthophyll pigments, photoprotection and photoinhibition: rapidly- and slowly-reversible regulation of photosynthetic efficiency in sun and shade leaves of avocado

After years of attention to mechanisms underlying high photosynthetic efficiency, research from plant ecophysiology has redirected attention toward the likelihood that much of terrestrial photosynthesis takes place at low photochemical efficiency in sunlight. Unable to draw the blinds, as it were, or move into the shade, natural selection has endowed the photosynthetic apparatus with a range of photoprotective mechanisms to dispose of light, in excess of that which can be used in photochemistry, as heat. Reversible regulation of

photosynthetic efficiency is now held to involve at least four interacting forms of NPQ that vary in their thresholds of light intensity and time for engagement and relaxation. These are usually assayed by PAM measurements of chlorophyll fluorescence quenching and the reliability and portability of devices such as MINI-PAM (Heinz Walz GmbH, Effeltrich, Germany) has seen a rapid expansion of research in which chlorophyll fluorescence based estimation of photosynthetic electron transport (ETR) is used as a surrogate (with qualifications) for photosynthetic CO₂ assimilation and of NPQ as an index of various components of photoprotection.

The components of NPQ were originally designated as *qE*, *qN* and *qI* (Horton *et al.* 1996) and all are mechanisms to dispose of excitation (as heat) in excess of the capacity of photosynthesis to carry out photochemistry under the conditions of measurement. This nomenclature has become less precise with time as more diverse responses have been explored and mechanisms have been advanced (Maxwell and Johnson 2000). For our purposes, we will use a subscript designation of NPQ associated with or independent from, specific xanthophyll pigment compositions (Förster *et al.* 2011), quantitative aspects of which are summarised in data from experiments with mature leaves of avocado (Fig. 3).

We will not deal with ‘external photoprotection’ in which changes in leaf angle with time scales of minutes (Ludlow and Björkman 1984; Lovelock *et al.* 1994), or with slower changes (days to weeks) in leaf surface reflective properties (Robinson and Osmond 1994), both of which substantially reduce absorption of light and engagement of the photoprotective processes described below. Nor will we deal with ‘internal photoprotection’ based on chloroplast movements that achieve the same end (Brugnoli and Björkman 1992).

NPQ_{ΔPH}: energy-dependent (qE), antheraxanthin and zeaxanthin -independent chlorophyll fluorescence quenching

Rapid response methods for interrogating leaf photosynthesis by a combination of gas-exchange and optical methods have been especially successful in linking biophysics and biochemistry to photosynthetic physiology *in vivo* (Laisk and Oja 1998). In recognition of importance of this approach and of their pioneering contributions over many years, these authors were awarded the second Innovation Prize by the International Society of Photosynthesis Research in 2010. Kirschbaum and Pearcy (1988) found the rate of O₂ evolution *in vivo* peaked in ~2 s, before declining by 60% to match the rate of CO₂ fixation after 10 s during an artificial sun-fleck on a shade leaf of *Alocasia macrorrhiza*. These fast kinetics *in vivo* are difficult to follow using PAM, but LIFT has the capability of monitor $\Delta F/F_m'$ in real time (with resolution of 0.5–3 s) and provide independent confirmation of this decline in photosynthesis in the first seconds of induction (see below).

Earlier work *in vitro* using isolated chloroplasts correlated rapidly reversible chlorophyll fluorescence quenching with light induced acidification of the thylakoid lumen and lower photosynthetic efficiency (Briantais *et al.* 1979; Horton 1983). Described then as energy dependent chlorophyll fluorescence quenching (*qE*), we have designated these rapid quenching

processes as NPQ_{ΔPH}. Finazzi *et al.* (2004) demonstrated a Z-independent form of NPQ in barley and wild-type *Arabidopsis* grown in and exposed, to 100 μmol photons m⁻² s⁻¹. The response was absent in the *npq4* mutant of *Arabidopsis* lacking the pH sensing PsbS protein and was attributed to early, reversible inactivation of a proportion of PSII reaction centres (see NPQ_{PI}; below). Huner *et al.* (2005) reviewed evidenced that NPQ_{ΔPH} may involve excitation quenching in both antenna and reaction centres.

In general, the *in vivo* capacity of NPQ_{ΔPH} in leaves is most conveniently and reliably measured pre-dawn (maximum F_v/F_m) when stomata are closed (as confirmed by lack of response of ETR and NPQ to a ‘Vaseline patch’; Fig. 1a), using PAM measurements in rapid light response curves that minimise xanthophyll de-epoxidation during assay (Förster *et al.* 2011). Conditions should be chosen so as to induce photosynthesis under controlled conditions in the assay, leading to stabilisation of redox state in the electron transport chain (indicated by $1 - qP$), saturation of photosynthetic electron transport rate (ETR) and saturation of NPQ. Under these conditions, internal CO₂ supply will quickly stabilise at the CO₂ compensation point at the prevailing temperature, maximise the excitation pressure on PSII centers and minimise the potentially large variations in NPQ due to physiological factors.

Mature leaves of sun-grown avocado have about twice the capacity for zeaxanthin-independent NPQ_{ΔPH} that shade leaves have when measured under comparable conditions (Fig. 3a, b). The photosynthetic apparatus of sun leaves expresses this NPQ capacity even though it attains 3-fold higher ETR. Presumably all components of acclimation to growth in sunlight conspire to assure a higher capacity for light use in sun leaves, as well as higher capacity for NPQ_{ΔPH}. Importantly, NPQ_{ΔPH} measured in this way relaxes rapidly ($t_{1/2} \sim 40\text{--}65$ s) in both sun and shade leaves.

Various shades of qN: ΔL, A, Z (and ΔpH)-dependent NPQ in species with one or two xanthophyll cycles

De-epoxidation of Lx and V to ΔL, A and Z stabilises and augments NPQ_{ΔPH} for minutes to hours, depending on species, on light intensity and duration and access to CO₂ or other sinks for ATP and NADPH. The pigment inter-conversions associated with this form of NPQ (also known as *qN*) are catalysed by the enzyme VDE activated by low pH and sustained by high ΔpH (Pföndel *et al.* 1994). The capacity to sense or maintain high ΔpH is crucial and mutants of *Chlamydomonas* (Förster *et al.* 2001) and *Arabidopsis* (Li *et al.* 2002) are known that accumulate high levels of Z in strong light but are unable to achieve high capacity for NPQ.

NPQ_{AZ} in species that do not accumulate Lx

This is by far the most widely distributed, near universal and best understood form of A and Z-dependent NPQ in terrestrial plants. Since Thayer and Björkman (1990) there has been widespread interest in the capacity of NPQ_{AZ} in leaves in sun and shade. In general, sun leaves have higher [V + A + Z] (Fig. 3a) and higher NPQ_{AZ} than shade leaves (Demmig 1996).

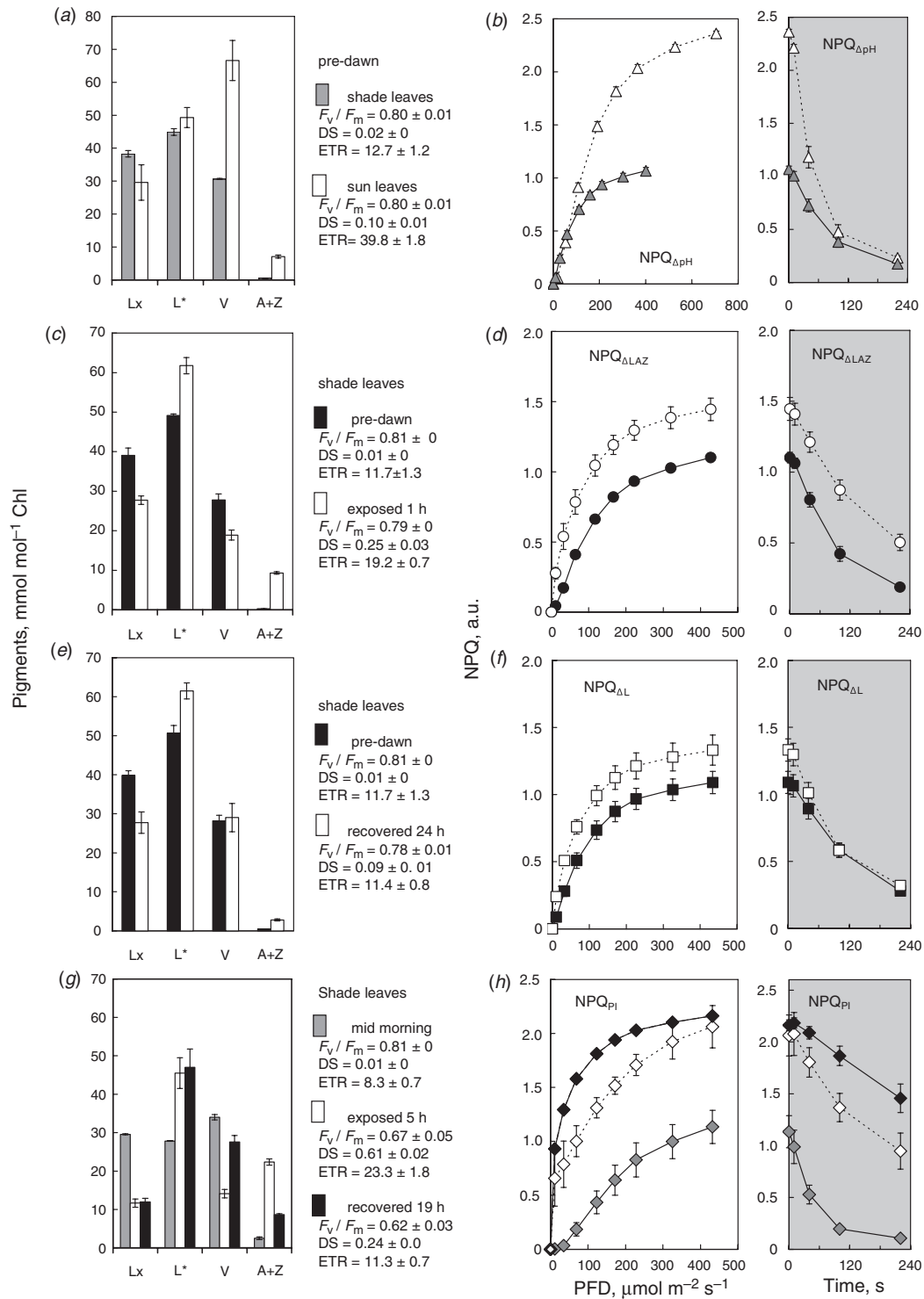


Fig. 3. Designation and properties of rapidly and slowly reversible forms of NPQ in mature leaves of avocado measured in rapid light response curves (30 s at each PFD followed 220 s in the dark) that minimised xanthophyll pigment de-epoxidation during assay (Förster *et al.* 2011). Leaf pigment compositions are shown in *a*, *c*, *e* and *g*. Note that scale for [L*] corresponds to 100–180 mmol mol⁻¹ Chl in (*a*) and 100–170 mmol mol⁻¹ Chl in (*c*, *e* and *g*). The different actinic treatments used to bring about the changes in pigment composition are outlined in the text column, along with F_v/F_m , de-epoxidation status ($DS = [A + Z]/[V + A + Z]$) and ETR (at ~400 μmol photons m⁻² s⁻¹). (*b*) NPQ_{ΔpH} in sun and shade leaves pre-dawn. (*d*) NPQ_{ΔpH} pre-dawn and NPQ_{ΔLAZ} after 1 h exposure to 200 μmol photons m⁻² s⁻¹. (*f*) NPQ_{ΔpH} pre-dawn and NPQ_{ΔL} after overnight epoxidation of A and Z in the experiment in (*d*). (*h*) NPQ_{ΔpH} pre-dawn and NPQ_{PI} after exposure to 800 μmol photons m⁻² s⁻¹ for 5 h and after 19 h recovery in the dark. (means ± s.e.; $n = 3-4$).

NPQ_{ΔLAZ} in species that accumulate Lx

In avocado shade leaves exposed to a low light intensity that led to de-epoxidation of Lx and V (Fig. 3c) the capacity for NPQ_{ΔLAZ} increased by 30–40% compared with NPQ_{ΔPH}, but the $t_{1/2}$ for relaxation in the dark doubled (Fig. 3d). Slower relaxation in the dark is a feature of A and Z stabilised NPQ. Johnson *et al.* (2008) associated slower dark relaxation with higher DS rather than [A + Z]. Avocado data are consistent with this; the [A + Z] concentration in shade leaves was similar to that in sun leaves pre-dawn, yet sun leaves showed much faster dark relaxation of NPQ_{ΔPH}. There may be no way of knowing if NPQ_{ΔLAZ} substitutes or simply augments NPQ_{ΔPH}, as a common mechanism may be involved (Johnson *et al.* 2009).

NPQ_{ΔL}: a special case in Lx-rich shade leaves

When avocado leaves were returned to shade elevated NPQ_{ΔL} persisted after epoxidation of A + Z and with lower DS, dark relaxation of NPQ_{ΔL} after assay was identical to that of NPQ_{ΔPH} in controls (Fig. 3e). This diagnostic feature shared by NPQ_{ΔPH} and NPQ_{ΔL} evidently has a threshold DS = ~0.1 in avocado. Elevated NPQ_{ΔL} persisted for up to 72 h in the dark in some experiments, as long as some ΔL from de-epoxidation of Lx was present (Förster *et al.* 2011). We will consider some implications of these properties of NPQ_{ΔL} later.

The mechanisms involved in all the above forms of ΔL, A and Z-dependent NPQ are vigorously debated. It seems likely that the xanthophylls are associated with structural changes in major light harvesting complexes (de Bianchi *et al.* 2010) that produce populations of more rapidly quenching antenna complexes (Gilmore *et al.* 1998; Li *et al.* 2002) and dissipate excitation before transfer to reaction centres. *Arabidopsis* mutants have been constructed to show that Z and L produce similar radical cations (quenching centres) when NPQ is engaged (Li *et al.* 2009) and the equivalence of A and ΔL sustained NPQ in avocado leaves has been explored using fluorescence lifetime imaging (Matsubara *et al.* 2011).

NPQ_{PI} (q1): slowly reversible xanthophyll-dependent and independent-photoprotection

Depending on growth conditions, temperature and other stress factors, the above processes of photoprotection may be inadequate to prevent delivery of excess excitation to PSII reaction centres. For example, exposure of avocado shade leaves to sunlight led to a decline in F_v/F_m that was not reversible overnight (Fig. 3g) and to sustained high NPQ_{PI} (>2.0). Independent assays based on delivery of electrons from PSII to P700+ in single turnover flashes (Losciale *et al.* 2008), showed a decline in the functional fraction of PSII (H-S. Jia, W. S. Chow and B. Osmond, unpubl. data). However, with residual high levels of ΔL, A and Z after recovery and persistent high DS (Fig. 3g), it was no surprise to discover very slow relaxation of NPQ_{PI} in the dark after assay (Fig. 3h).

These data confirm earlier studies of the limited photoprotective capacity of NPQ_{AZ} in wild-type *Arabidopsis* after de-epoxidation ceased. Russell *et al.* (1995) used two biochemical methods to measure the loss of D1 protein and a direct measurement of functional PSII centres (O_2 yield from saturating single turnover flashes) to demonstrate

photoinactivation in these experiments. Matsubara and Chow (2004) showed that NPQ_{PI} is accompanied by the generation of a new population of short-lifetime quenching centres from photoinactivated PSII that dissipate excess excitation as heat before delivery to remaining functional PSII centres. Other experiments showed that in an average leaf, in an average light environment, all of the D1 protein in PSII is turned over at least once daily (Anderson *et al.* 1997; Chow and Aro 2005) and any stress that accelerates D1 turnover and/or impairs the repair cycle, may accelerate NPQ_{PI}.

These data are also consistent with many studies showing severe stress in strong light leads to persistently high [A + Z], especially at low temperatures in overwintering evergreen species (Adams *et al.* 1995; Demmig-Adams *et al.* 2006). Evidently, these stresses must impair epoxidation and potentiate a slowly relaxing form of NPQ_{AZ} that is also described as photoinhibition (q1). They are also associated with impairments in the D1 recovery cycle (Ebbert *et al.* 2005; Zarter *et al.* 2006). A similar high [A + Z] syndrome during high temperature stress exhibited rather more complex control of the expression slowly reversible NPQ (Barker *et al.* 2002).

Expression of different forms of NPQ in canopies in stochastic light environments

Mechanisms underlying the diversity of photoprotective processes discussed above will exercise the ingenuity of biochemists and biophysicists for some time to come. However, for practical purposes of scaling to the canopy and beyond, the whole is a good deal less than the sum of the component parts. Just as it seems that all the diversity of structure and metabolism in leaves of C_4 plants confers a common, robust leaf level advantage with respect leaf water and nutrient economy of CO_2 assimilation and light use in air (von Caemmerer and Osmond 2009), so all components of NPQ have the potential to secure stability and function of the photosynthetic apparatus in the face of a stochastic light environment, especially in conjunction with other stresses. In the following we illustrate some examples of this diversity.

Zeaxanthin-independent NPQ_{ΔPH} in the outer canopy of *Inga* spp. during morning sun flecks and in the shaded understory of the Eden Project

We were particularly interested in the extent to which NPQ_{ΔPH} might be engaged in the outer canopy of woody plants during patchy morning sunshine. Species of *Inga* grow rapidly in the warm (28°C) environment of the S-facing Eden Project, yet at 50°N, the outer canopy experiences low light environments for much of the year. Spot estimates of outer canopy $\Delta F/F_m'$ and of NPQ showed $\Delta F/F_m'$ declined by ~50% in brief sun-exposures of up to 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and settled at this value in sun flecks up to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 4a). Dynamic NPQ in these plants was zeaxanthin-independent; traces of A (but no Z) were found in only one sample of *I. sertulifera* during the decline in $\Delta F/F_m'$ and in *I. ilta* and *I. sapindoides* over the higher range of sun fleck PFD. Outer canopy leaflets of *I. edulis* and *I. spectabilis* showed a linear increase in NPQ during sun flecks up to 140 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, but without detectable A or Z (Fig. 4b). Inner canopy leaves had no detectable A.

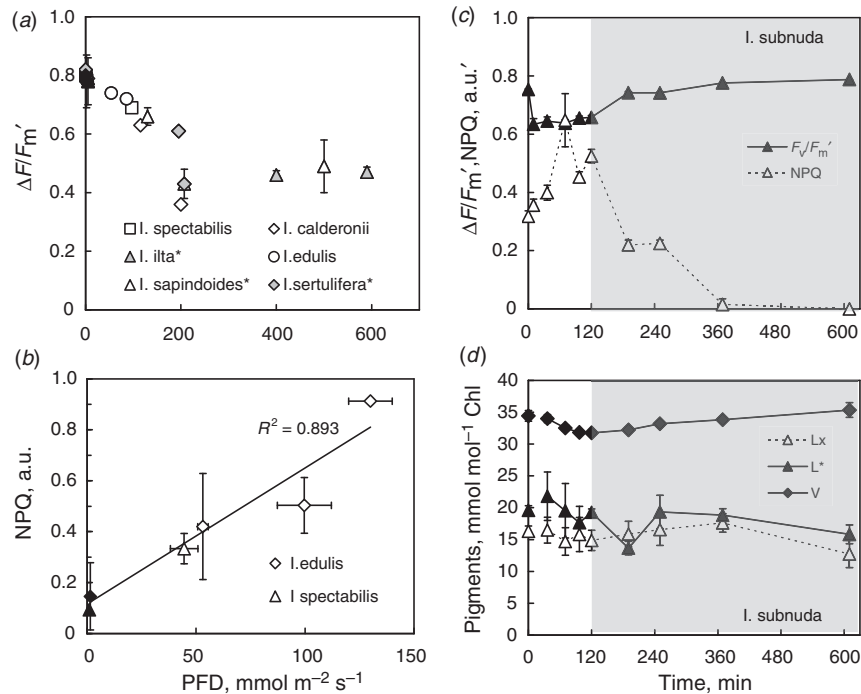


Fig. 4. Photosynthetic parameters in outer canopy leaflets of *Inga* spp. in the Eden Project measured between 0930 and 1300 hours and during artificial illumination in the understory. (a) Spot estimates of $\Delta F/F_m'$ during intermittent sunshine; leaves of species marked with asterisk sometimes contained traces of A, but Z was not detected in any samples. (b) Spot estimates of NPQ (referenced to dark samples measured 2100–2200 hours the previous night) during a cloudy morning; none of the leaves measured contained A or Z. Inner canopy leaves (black symbols) and outer canopy leaves (open and grey symbols). (c) Decline in $\Delta F/F_m'$ and increase NPQ in leaflets of understory *I. subnuda* during 2 h exposure to $120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ were accompanied by small changes in xanthophyll pigments. (d). No A or Z was detected and changes in [Lx] and [L*] were not significant. Note that the scale for [L*] corresponds to $100\text{--}180 \text{ mmol mol}^{-1} \text{ Chl}$ in (d). (mean \pm s.e., $n = 3\text{--}5$).

Further experiments with the small leaf *I. subnuda* growing in the understory produced similar results. Exposure for 2 h to $120\text{--}140 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (white light; halogen source) saturated ETR, caused a clear and sustained decline in $\Delta F/F_m'$ and a doubling of NPQ (Fig. 4c). Although [V] declined slightly no A or Z was found (Fig. 4d). There was no change in [Lx] or [L]. Together these data show that morning sun-flecks in the Eden Project at this time of year engaged zeaxanthin-independent $\text{NPQ}_{\Delta\text{PH}}$ in the outer canopy and in the understory throughout the day. These observations may be a first demonstration of $\text{NPQ}_{\Delta\text{PH}}$ in the outer canopy under simulated field conditions.

Accumulation of Lx may improve photosynthetic efficiency in shaded canopies then provide a 'perfect switch' to 'lock in' photoprotection after de-epoxidation

Concern with inner vs outer canopy photosynthesis might seem a lost cause in scaling from leaf-to-canopy and beyond. For example, although spot measurements of inner canopy avocado leaves in the field at noon show very high $\Delta F/F_m'$, rates of ETR achieved in the outer canopy are so far in excess of those achieved by inner canopy leaves at the prevailing PFD as to make the latter seem trivial. Nevertheless, two potentially important functional implications have followed from discovery

of the distinctive distribution of two xanthophyll cycles in the canopies of these plants.

First, noting the high F_v/F_m in leaves that accumulated Lx in the inner canopy of *I. sapindoides*, Shizue Matsubara and her colleagues reconstituted a recombinant model light harvesting complex (Lhcb5) with Lx and other xanthophylls. They found higher fluorescence yield when reconstituted with Lx than with other xanthophylls and suggested that accumulation of Lx in the shade may facilitate excitation energy transfer to chlorophyll in light limiting conditions (Matsubara *et al.* 2007). In *Ocotea foetans*, with a fully reversible Lx-cycle, the comparatively rapid restoration of [Lx] after short (3 min) artificial sun flecks is consistent with such a role (Esteban *et al.* 2010).

Second, a sustained increase in capacity for $\text{NPQ}_{\Delta\text{L}}$ in shade leaves is due to a small addition (ΔL ; 5–10%) to the total pool of L following de-epoxidation of Lx (Fig. 3e,f). First observed in the field (García-Plazaola *et al.* 2003; Matsubara *et al.* 2008), this phenomenon was also confirmed in *I. sapindoides* growing in the Eden Project. Modest light exposure of inner canopy leaves produced the expected de-epoxidation of Lx and V, followed by epoxidation of A + Z overnight (Fig. 5a). The separate time courses of induction of $\text{NPQ}_{\Delta\text{PH}}$, $\text{NPQ}_{\Delta\text{LAZ}}$ and $\text{NPQ}_{\Delta\text{L}}$ were measured using actinic light of 60 and $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ so as to minimise the likelihood of de-epoxidation during assay.

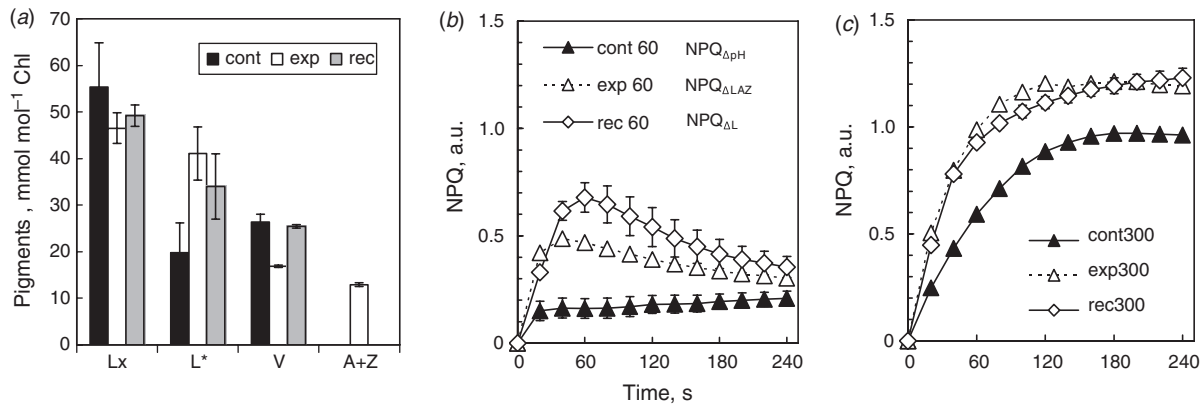


Fig. 5. Capacities for different forms of NPQ measured in inner canopy leaves of *I. sapindoides* Willd. before and after 1 h actinic exposure to $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ from halogen lamps and after epoxidation of A + Z, but not of ΔL , in the same leaves wrapped in foil overnight. (a) Depoxidation of Lx and V. Note that the scale of $[\text{L}^*]$ corresponds to $100\text{--}170 \text{ mmol mol}^{-1} \text{Chl}$. (b, c) Photosynthetic induction curves measured with MINI-PAM at low (b) $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance and high (c) $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance before (cont) and after actinic exposure (exp) and again after epoxidation of A + Z overnight (rec). In both cases residual ΔL from Lx sustained higher capacity for $\text{NPQ}_{\Delta\text{L}}$ overnight in the absence of A + Z (Mean \pm s.e., $n = 2\text{--}3$).

The capacity of $\text{NPQ}_{\Delta\text{LAZ}}$ and $\text{NPQ}_{\Delta\text{L}}$ exceeded $\text{NPQ}_{\Delta\text{pH}}$ when measured at the lower PFD (Fig. 5b), but declined slowly with time (as ETR was induced; data not shown). The transient increased capacity for xanthophyll-dependent NPQ in low light was about the same as the difference between $\text{NPQ}_{\Delta\text{pH}}$ and $\text{NPQ}_{\Delta\text{LAZ}}$ or $\text{NPQ}_{\Delta\text{L}}$ measured at higher PFD (Fig. 5c) and similar to those measured in avocado shade leaves (Fig. 3). Higher capacity for $\text{NPQ}_{\Delta\text{L}}$ in avocado shade leaves was ‘locked-in’ for days in the dark after ΔL replaced A and Z and as no increase in $[\text{Lx}]$ was detected in prolonged dark, metabolism of ΔL evidently did not involve epoxidation (Förster *et al.* 2011). Importantly, although the role of ΔL in sustained $\text{NPQ}_{\Delta\text{L}}$ mimics that of persistently high $[\text{Z}]$ in sustaining slowly reversible forms of NPQ_{AZ} during low temperature stress, it does so without the penalty of very slow relaxation in the dark (cf. Fig. 3f, h).

The accumulation of Lx, its ready conversion to ΔL during a long sun fleck and the subsequent slow metabolism in the shade might well comprise a ‘perfect switch’ from high LUE in the shade to ‘locked-in’, faster relaxing photoprotection for the shade leaf. Moreover, these ecophysiological studies with leaves of woody plants in the field and controlled environments provided the first direct evidence for the long suspected possibility that L, like A and Z has a role in photoprotection. They presaged recent biophysical studies showing engagement of NPQ in a Z-free, L overexpressing *Arabidopsis* mutant generates a cation radical analogous to that arising from Z in other mutants (Li *et al.* 2009).

Persistent elevated NPQ in the shade in the outer canopy at high temperatures

Remote sensing of photosynthetic parameters in less accessible areas of the outer canopy of *I. sapindoides* and *Pterocarpus indicus* in the tropical forest biome of B2 L were made possible with prototype LIFT (Mk1). In the northern hemisphere targeted N-facing, outer mid-canopy leaves of *I. sapindoides* had low $[\text{A} + \text{Z}]$ ($\text{DS} = 0.07\text{--}0.17$) yet attained NPQ of $1.5\text{--}2.0$ during morning exposures of $1000\text{--}2000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$

(Ananyev *et al.* 2005). Similar peak NPQ values were obtained by mid-day PAM measurements in the outer canopy of *I. sapindoides* at 2 sites in B2 L but these leaflets contained higher $[\text{A} + \text{Z}]$ (Matsubara *et al.* 2005). The more shaded canopy of *P. indicus* (Fig. 6a–d) had only traces of Lx and of A ($\text{DS} = 0.01\text{--}0.03$) and attained only lower $\text{NPQ}_{\Delta\text{pH}}$ (1.0 at $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Even in the bright light of Arizona it seems that $\text{NPQ}_{\Delta\text{pH}}$ had a role in photoprotection that was comparable to $\text{NPQ}_{\Delta\text{LAZ}}$ in these species.

Both species showed unexpectedly high mid-afternoon NPQ (>2) in targeted areas of the outer canopy that became shaded as the day progressed (Fig. 6d). This phenomenon was further interrogated mid-afternoon by an intense sun fleck from a large mirror. In both species the sun fleck drove $\Delta F/F_m'$ to near zero and NPQ to extreme values ($\sim 4\text{--}5.5$) that relaxed fully overnight. It is not known whether the high NPQ involved depoxidation of xanthophylls. Interestingly, the more frequently sun exposed outer canopy of *Inga* showed no capacity for increased ETR in the sun fleck (Ananyev *et al.* 2005) whereas ETR accelerated to the maximum achieved during morning sun exposure in the more shaded outer canopy of *Pterocarpus* (Fig. 6c).

High afternoon NPQ in the shade in both species was attributed to high air temperatures in the canopy. Reversible inactivation of CO_2 fixation by Rubisco in response to high temperature is well established (Weis 1981; Weis and Berry 1988; Law and Crafts-Brandner 1999). Perhaps the different responses of ETR to the sun fleck were due to higher temperatures in the mid canopy of *Inga* canopy compared with those in lower canopy of *Pterocarpus*. $\Delta F/F_m'$, ETR and NPQ recovered fully overnight in both canopies so we eliminate $\text{NPQ}_{\Delta\text{L}}$ and NPQ_{Pi} as contributors and ascribe the response to $\text{NPQ}_{\Delta\text{pH}}$ and/or NPQ_{AZ} . As an aside, one has to concede that LIFT (Mk1) uncovered the limited success of the engineers’ best efforts to provide sufficient air movement and control temperatures in the top of the B2 L tropical forest enclosure. It remains to be seen whether LIFT will expose similar temperature

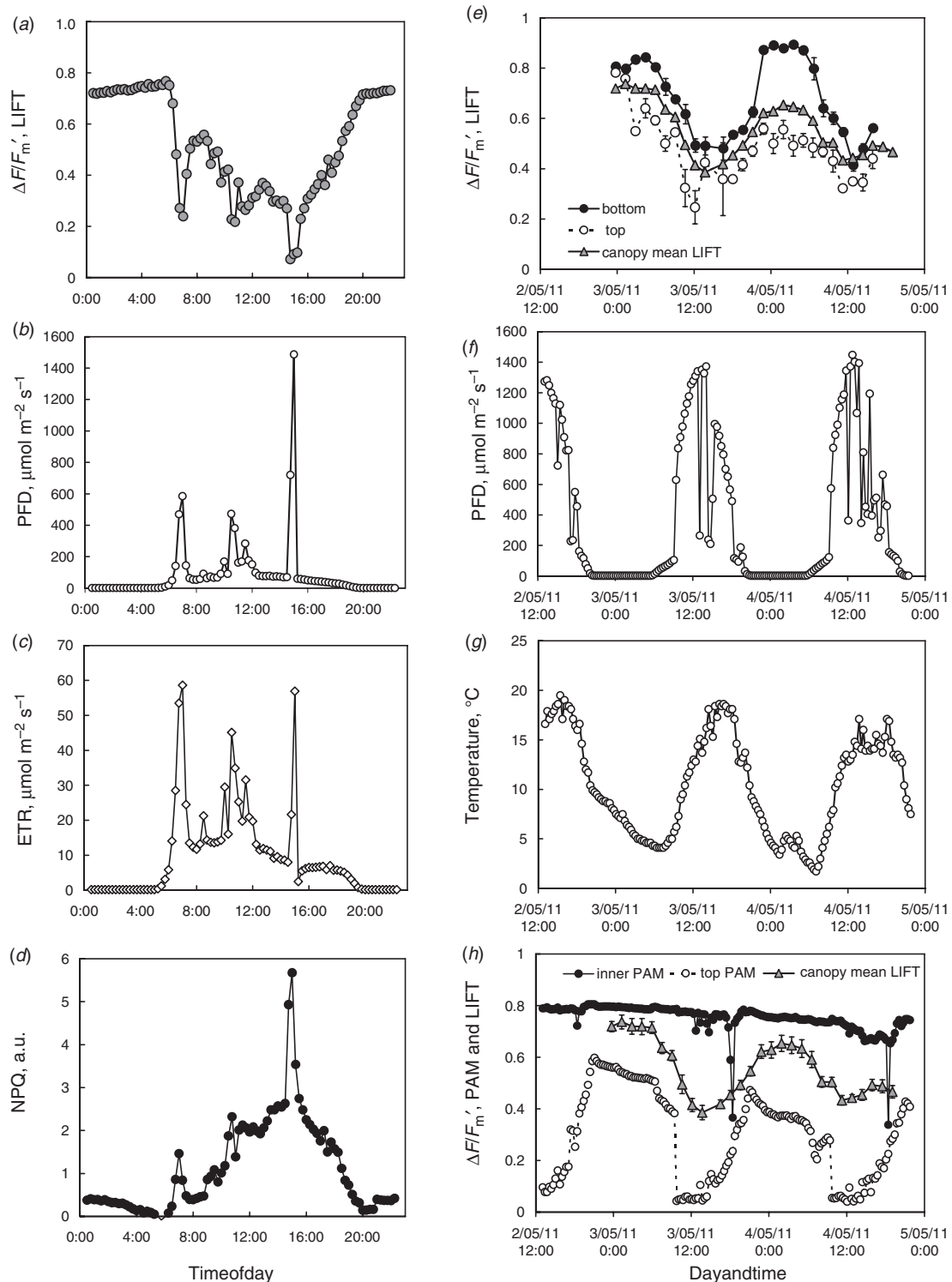


Fig. 6. (a–d) Remote sensing of photosynthetic parameters in the shaded outer canopy of *Pterocarpus indicus* at 15 m in the B2 L tropical forest biome using prototype LIFT (Mk 1). (a) $\Delta F/F_m'$ showing decline in response to natural PFD, a sustained decline in afternoon shade and strong depression during an artificial sun fleck (b). (c) ETR calculated from (a) and (b). (d) NPQ showing an increase in afternoon shade afternoons at high temperature and very high values attained during the sun fleck. (redrawn from Ananyev *et al.* 2005). (e–h) Remote sensing of photosynthetic parameters in the S-facing outer canopy of *Philadelphus coronarius* at 12 m measured with LIFT (Mk 2). (e) $\Delta F/F_m'$ in shaded lower and sun exposed top canopy targets and canopy mean of 12 targets. (f) E-facing PFD at top of canopy, (g) average canopy temperature and (h) inner canopy and top canopy $\Delta F/F_m'$ measured by MONITORING-PAM (Heinz Walz GmbH, Effeltrich, Germany), with outer canopy mean from LIFT.

dependent increases in NPQ in the shade in natural rainforest canopies.

Diel patterns of heterogeneity in outer canopy $\Delta F/F_m'$ (LIFT) following cold nights

Successful deployment of LIFT (Mk2) in B2L led to further testing of the apparatus in other laboratory and field applications. When calibrated against the 'gold standard' of PAM and gas-exchange measurements, LIFT clearly identified different photosynthetic responses to high light and low night temperatures in cold sensitive tomato and pepper plants, in avocado and in native grassland (Pieruschka *et al.* 2010). The potential of raster-LIFT for monitoring spatial heterogeneity of photosynthesis in the outer canopy was demonstrated using a potted avocado plant (Pieruschka *et al.* 2009) and our preliminary efforts to exploit this capability are shown in Fig. 6e–h.

The telescope of LIFT (Mk2) is currently limited to repeated observation of 12 targets. A small canopy of *Philadelphus coronarius* (~2.5 m high \times 1.5 m deep) growing in the phenology garden at Forschungszentrum Jülich was interrogated at 12 m over 6 days in May 2011. Twelve targets on the sun-exposed S-face (NH) of the canopy (cover photo, this issue) were visited sequentially every ~220 s and data from five visits were averaged to report mean values for each target every 18–19 min. Three MONI-PAM units (Porcar-Castell *et al.* 2008) were fixed to a bamboo tripod within the canopy, monitoring sun-exposed top canopy leaves facing east and west, with a third on a deeply shaded inner canopy leaf and set to report at 20 min intervals. Wind events required repeated leaf replacements in the PAM units and also produced unacceptably high standard errors in some peripheral LIFT targets due to leaf movements that resulted in 'miss-hits'. Photos of a representative target area (cover photo this issue) showed a cylinder of excitation ~10 cm diam. and ~10 cm deep that was intercepted by several small leaves (~3 \times 5 cm) as well as some chlorophyll rich floral buds. It was clear that movements of adjacent leaves occurred frequently and that leaf movements in the target space evidently provided a modicum of integration over the period of observation.

The canopy had experienced two weeks of warm temperatures (>25°C day / ~15°C night) and bright sunlight before the first 2 days of LIFT observation when a chilling event was experienced ('Eisheiligen'; 18°C day/2–4°C night). Compared with the upper sun exposed canopy, the shaded lower canopy showed much smaller diel change in $\Delta F/F_m'$ and full recovery during cool nights (Fig. 6e). In contrast, the top canopy showed earlier, faster and further decline in $\Delta F/F_m'$ during the day and later, slower and incomplete recovery in the cool nights. Inner canopy $\Delta F/F_m'$ (PAM) was similar to nocturnal $\Delta F/F_m'$ (LIFT) in the lower shaded canopy and interestingly, MONI-PAM caught afternoon sun flecks at the same time on successive days (c.f. Fig. 6e, h). The precipitous drop in $\Delta F/F_m'$ at the same time each day in the E-facing outer canopy was evidently due to the orientation of the selected leaf and the monitoring unit. As before PAM units in the upper canopy recorded much larger decline in $\Delta F/F_m'$ at high PFD than that measured by LIFT in the adjacent target (c.f. Fig. 6e, h). The canopy average $\Delta F/F_m'$ of 12 LIFT targets was intermediate between the upper and lower canopy values (Fig. 6e)

and between inner and outer canopy PAM values (Fig. 6h). Clearly, further development of raster-LIFT technology will provide more detailed profiles of $\Delta F/F_m'$ in the outer canopy.

Sun flecks revisited: photoprotection in highly stochastic light environments

Pearcy (1990) reported that understory sun flecks generally range in duration and intensity from a few seconds at 150–200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to >10 min at 1500–2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and Adams *et al.* (1999) found two vines growing in an open eucalypt forest experienced a similar range of sun flecks. Some representative measurements of sun flecks in avocado canopies at midday (M.V. Mickelbart unpubl. data) ranged from 20 s (~150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; background ~18 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) to 7 min (~120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; background ~4 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

Within-canopy light gradients are known to produce similar correlations of [V + A + Z] with increasing PFD and with seasonal variation in light interception (Niinemets *et al.* 1998), but there are few studies of the roles of V de-epoxidation during a sun fleck and its impact on the efficiency of assimilation. If de-epoxidation occurs during a sun fleck, efficiency of assimilation in the low light that follows the sun fleck is presumably reduced by the slow relaxation of NPQ_{AZ} described above. Adams *et al.* (1999) found with the vines *Smilax australis* and *Stephania japonica* growing in an open *Eucalyptus* forest, residual A + Z from incomplete overnight epoxidation (predawn DS ~0.25–0.35) primed subsequent rapid and sustained engagement of NPQ_{AZ} (from ~0.5 to ~2.5) after the first 'parcel' of sun flecks. Although some parallels can be drawn with the classical studies that revealed post-sun fleck CO₂ assimilation (Pearcy 1988), lower photosynthetic efficiency due to sustained NPQ_{AZ} during sun flecks in a stochastic low light environment seems counter intuitive to optimisation of CO₂ assimilation. It may be that induction of photosynthesis in the shade environment primes higher capacity for assimilation in the sun fleck (Pearcy *et al.* 1997) and compensates for the sustained higher NPQ_{AZ}.

These are complex relationships but two recent studies are beginning to unravel the connections. Artificial sun fleck experiments by Esteban *et al.* (2010) suggest the reversible Lx-cycle in *Ocotea foetans* may facilitate photoprotection due to NPQ_{AL} and relax rapidly enough to restore high efficiency in low light. Using a novel computer regime to deal with the highly variable within canopy light environment in a tropical rainforest Rascher *et al.* (2011) showed photosynthesis of 16 epiphytes was well adapted to an integrated 3 h window of variable irradiance. Interestingly, some species that were chronically photoinhibited pre-dawn (NPQ_{PI}) showed further acute, but recoverable (xanthophyll-dependent?) photoinhibition, during sun flecks throughout the day.

Our PAM assays of photosynthetic parameters in dark adapted leaves using induction curves or rapid light response curves (Förster *et al.* 2011) are tantamount to simulation of some forms of sun fleck. The 20 min induction protocols used in Fig. 1a resulted in de-epoxidation of V whereas the light response curves of Fig. 3 did not. Opening of stomata and induction of photosynthesis in avocado leaves in the shade lowered NPQ_{APH} compared with pre-dawn (B. Osmond

unpubl. data) and may have preserved the high photosynthetic efficiency possibly associated with high [Lx] (Matsubara *et al.* 2007). The faster relaxation of $\text{NPQ}_{\Delta\text{PH}}$ and $\text{NPQ}_{\Delta\text{L}}$ (compared with $\text{NPQ}_{\Delta\text{LAZ}}$) could sustain this advantage during subsequent sun flecks, but more work is needed.

Improved less-intrusive measurement and long-term monitoring techniques now becoming available will facilitate more detailed understanding of these phenomena. Small distributed PFD sensors (Leuchner *et al.* 2005) and smaller rapidly responding light meters (ULM-500, Heinz Walz GmbH) will help monitor the duration and intensity of sun flecks in different canopies. Although there is no doubt that MONI-PAM will be useful for long-term studies, mechanistic understanding will require techniques free of the saturating flash of PAM. Smaller LIFT based devices are emerging to meet the needs of close quarter remote sensing within canopies.

Real time monitoring of $\Delta F/F_m'$ (LIFT) in a shade grown *Alocasia* leaf during a light curve, analogous to those Fig. 3, but measured at 50 cm using LIFT (Mk3), is shown in Fig. 7. This flexible bench-top device uses blue LED excitation, smaller optics and delivered fluorescence components in real time with resolution of 0.5–3 s (Fig. 7a). It is much less intrusive than MINI-PAM but estimates of $\Delta F/F_m'$ from the two devices were outstandingly correlated (Fig. 7b) so estimates of ETR also calibrated well at low PFD (Fig. 7c). Empirically determined corrections against the ‘gold-standard’ of PAM can be used to accommodate the lower $\Delta F/F_m'$ of LIFT at high PFD (Pieruschka *et al.* 2010). Much further laboratory evaluation of this instrument is needed to establish its suitability for evaluation of sun flecks within the inner canopy.

Prolonged exposure of shade leaves to sunlight leads to prolonged low F_v/F_m (Förster *et al.* 2009) with sustained slowly relaxing $\text{NPQ}_{\Delta\text{LAZ}}$ and NPQ_{PI} (Fig. 3f, h). These forms of photoprotection persisted for days to weeks as large and equivalent de-novo synthesis of both L and A + Z built higher capacity for photoprotection as avocado shade leaves acclimated to become sun leaves. Although [A + Z] oscillated on a diel basis, [L] did not, suggesting that de-novo synthesis of L may serve the primary role in photoprotection during acclimation, fine tuned by the V-cycle. Evidence that ΔL from Lx can replace A + Z and sustain higher $\text{NPQ}_{\Delta\text{L}}$ in shade leaves, suggests that the diurnal increase in [L] and the more rapidly reversible Lx-cycle in sun exposed photosynthetic tissues of species such as *Cuscuta reflexa* (Bungard *et al.* 1999) and *Ocotea foetans* (Esteban *et al.* 2010) augment photoprotection associated with [A + Z].

Other assays of chlorophyll fluorescence at canopy and ecosystem scales

Measurements of steady-state fluorescence (F_s) using a PAM system modified with a laser excitation source (Ounis *et al.* 2001) have proved valuable in relating photosynthetic efficiency to stress, particularly water stress (Flexas *et al.* 2002). The scale of these observations has been expanded by exploiting the weak, solar-induced F_s , observed in the Fraunhofer lines of the solar spectrum (Meroni *et al.* 2009). Perhaps the most exciting application for the passive quantification of sun-induced fluorescence is its potential for airborne and satellite observation (Rascher *et al.* 2009).

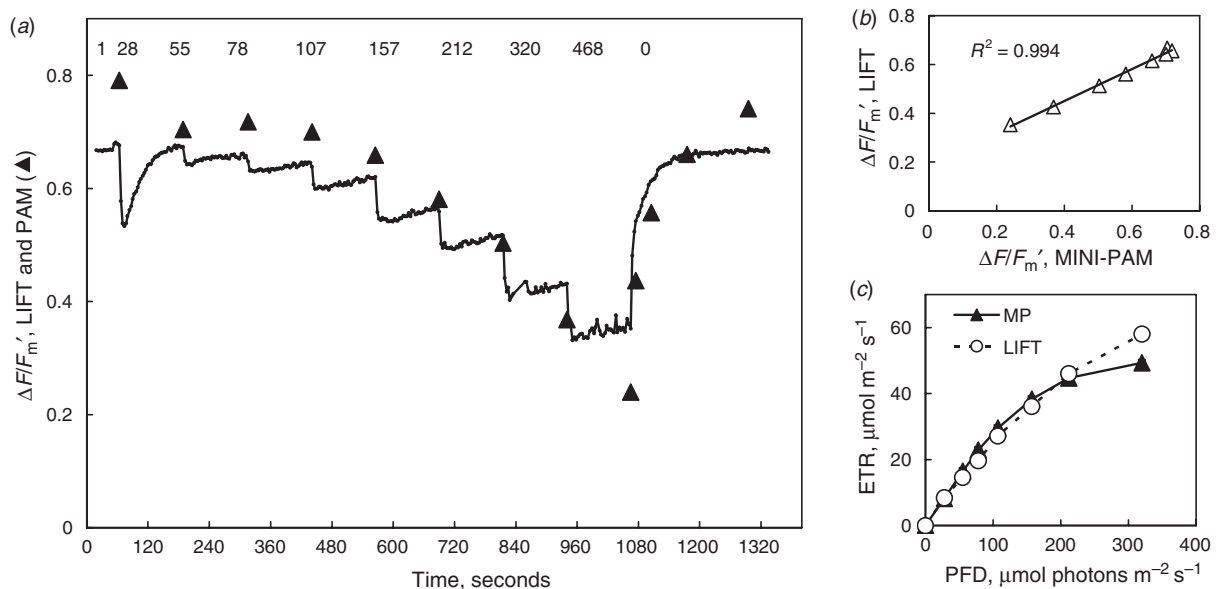


Fig. 7. Remote sensing of photosynthetic parameters in leaves of shade grown *Alocasia macrorrhiza* at 50 cm using the bench top blue LED LIFT (Mk3). (a) Kinetics of $\Delta F/F_m'$ LIFT and MINI-PAM (closed triangles) on adjacent areas of leaf measured in a light response curve (2 min at each PFD shown in the upper band) using the leaf clip of MINI-PAM as a light source in both instances. The total excitation load from LIFT during the experiment was $\sim 4500 \mu\text{mol photons m}^{-2}$ or $\sim 2.6\%$ of the total actinic + detector PFD. In contrast, the saturating flashes from MINI-PAM contributed a total of $\sim 91000 \mu\text{mol photons m}^{-2}$ or an additional $\sim 37.4\%$ to the total excitation load perhaps explaining the progressively larger decline in $\Delta F/F_m'$ with increasing PFD. (b) Correlation between $\Delta F/F_m'$ LIFT and $\Delta F/F_m'$ MINI-PAM measured on adjacent spots on the leaf using the same actinic light source. (c) Photosynthetic ETR calculated from $\Delta F/F_m'$ measured on adjacent spots on a leaf by LIFT and MINI-PAM (MP).

As explained by Joiner *et al.* (2011) 'space-based missions that were specifically designed to measure CO₂ concentrations can also be used to measure an important value-added carbon product: regional-scale chlorophyll fluorescence ... that could provide important information on photosynthetic efficiency of terrestrial ecosystems at a global scale'. Clearly observations at this scale are in their infancy and some limitations are already evident. Three of nine sites worldwide were located over Australia but unfortunately and to the surprise of none, these revealed that 'active fluorescence occurs over Australia primarily along the continental edges in the austral spring/early summer' (Joiner *et al.* 2011). This approach is at the core the European Space Agency program to evaluate the potential of a fluorescence satellite (FLEX). If successful, passive remote sensing of sun-induced fluorescence may provide an innovative global remote sensing tool fundamentally complementing and extending the range of local systems that may be installed on towers and mobilised on rail tracks to survey field plots (Daumard *et al.* 2010).

The potential for and some limitations to, scaling up using PRI, LIFT and PAM

Clearly, the emerging and sometimes conflicting priorities for food security, bio-fuels and sustainable ecosystems demand more than the proofs of concept implicit in all of the above leaf to landscape capabilities for measurement of LUE and its expression in stochastic light environments. It seems inescapable now that we have to find ways to respond to the large question of whether and how, the plant biosphere can sustain humankind in the face of global change. So we will conclude this review with some thoughts on the potential for and some limitations to, expansion of our insights in photosynthesis from leaf-to-canopy to ecosystem processes.

The need to strengthen linkage between 'insights of economics' and 'economical insights' from experiments

Our focus on retention of inner canopy leaves as they grow old in the shade, especially the retention of leaves with unusual accessory pigment compositions, has presented 'economical insights' and novel, but testable hypotheses relevant to responses to the stochastic light environment, at least in some species. Our discussion of improved methods for measuring photosynthetic efficiency in the inner and outer canopy will complement bottom-up integration of leaf-to-canopy processes based on the 'insights of economics'. This emphasis on measurements rather than models may be a reaction to many treatments of inner canopy leaf photosynthesis that in the past seem to have focussed on modelling 'pay back' times for construction and maintenance. Two recent studies encourage our growing sense that sometimes the retention of leaves in deep shade may involve more than repayment of the mortgage. First, measurements and modelling in European forests by Poorter *et al.* (2006) showed that in spite of 5- to 20-fold larger payback times than outer canopy leaves, investment in inner canopy leaves exposed to as little as 5% of seasonal irradiance paid a dividend. Second, Reich *et al.* (2009) reported that, in the dry sclerophyll woodland of eastern

Australia, leaves of most species retained positive carbon balance (remained productive) throughout their typical life-span.

Looking ahead: improving measurements of inner canopy photosynthesis

Until now we have been dependent on continuing heroic efforts to measure and integrate inner canopy attributes with outer canopy measurements. After a career devoted to measurements of leaf responses to sun flecks and acclimation to shade Pearcy *et al.* (2005) assessed the trade-offs between biomechanical costs of canopy architecture, light use efficiency, photoinhibition and whole-plant carbon gain. These simulations showed that without these structural investments photoinhibition was likely to be much greater and carbon gain much reduced. Perhaps the most comprehensive review yet of scaling leaf physiology to canopy performance (Niinemets 2007) highlighted other complex constraints on the potential for photosynthetic acclimation within the canopy, especially from interactions of light with foliage age and species specific leaf and shoot structural properties that determine responses in dynamic light environments.

Our fascination with the Lx-cycle illustrates but one, potentially significant insight arising from experiments with wild plants and further progress will depend on dogged experiments to resolve some of the uncertainties identified above. The excellent 'practical guide' to the use of PAM devices (Maxwell and Johnson 2000) and the review of 'common pitfalls' (Logan *et al.* 2007) notwithstanding, shade leaves are especially sensitive to photoinhibition arising from repeated saturating flashes (Shen *et al.* 1996; Apostol *et al.* 2001). Further development of LIFT and a range of other sensor-needs have to be moved to the front line of innovative technology and not remain dependent on the vision and ingenuity of a few dedicated enthusiasts. The whole arsenal of science and engineering needs to be mobilised if we are to substantially refine our understanding of the canopy conundrum and provide the tools needed for scaling the contributions of shade leaves to the whole canopy.

Our experience that repeated saturating flashes may deliver as much light as the actinic exposure (Förster *et al.* 2011) and thereby cause de-epoxidation of xanthophyll pigments during induction or light response curve assays has implications beyond inner canopy research. This possibility often seems to be overlooked in high PFD (800–2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) induction experiments with wild-type *Arabidopsis* when used as reference for interpreting mechanisms of pigment associated photoprotection in *Arabidopsis* mutants grown in low light laboratory conditions. Clearly the more targeted, much less intrusive pump probe technology of LIFT, with its richer diagnosis of photosynthetic parameters, also has potential for many high throughput phenotyping applications.

Looking ahead: some needed observations and experiments in the outer canopy

Comprehensive evaluation of outer canopy photosynthesis from ground or tower based systems using PRI, LIFT and solar-induced chlorophyll fluorescence is clearly possible.

Hand-held measurements of zeaxanthin-independent NPQ_{ΔpH} in the outer canopy of *Inga* spp. and the understory (Fig. 4) suggest that the short-term responses should be detectable using LIFT, but not with PRI, suggesting comparative measurements might be revealing. Although solar-induced fluorescence does not contain all the information of LIFT, this fluorescence signal also originates from the same core photosynthetic processes and also should allow detection of changes in photosynthetic efficiency independent of the pigment changes detected by PRI

(Damm *et al.* 2010). However, we should not forget that PRI imaging identified *Arabidopsis* mutants differing in the ability to sense ΔpH and engage NPQ (Fig. 1b), despite similar DS after exposure to high PFD (Li *et al.* 2002), so detailed comparative evaluation of these different remote sensing systems is clearly a high priority.

It is unknown whether the capability of PRI to detect zeaxanthin-dependent NPQ is modified or otherwise compromised by the presence of the Lx-cycle, but Rascher

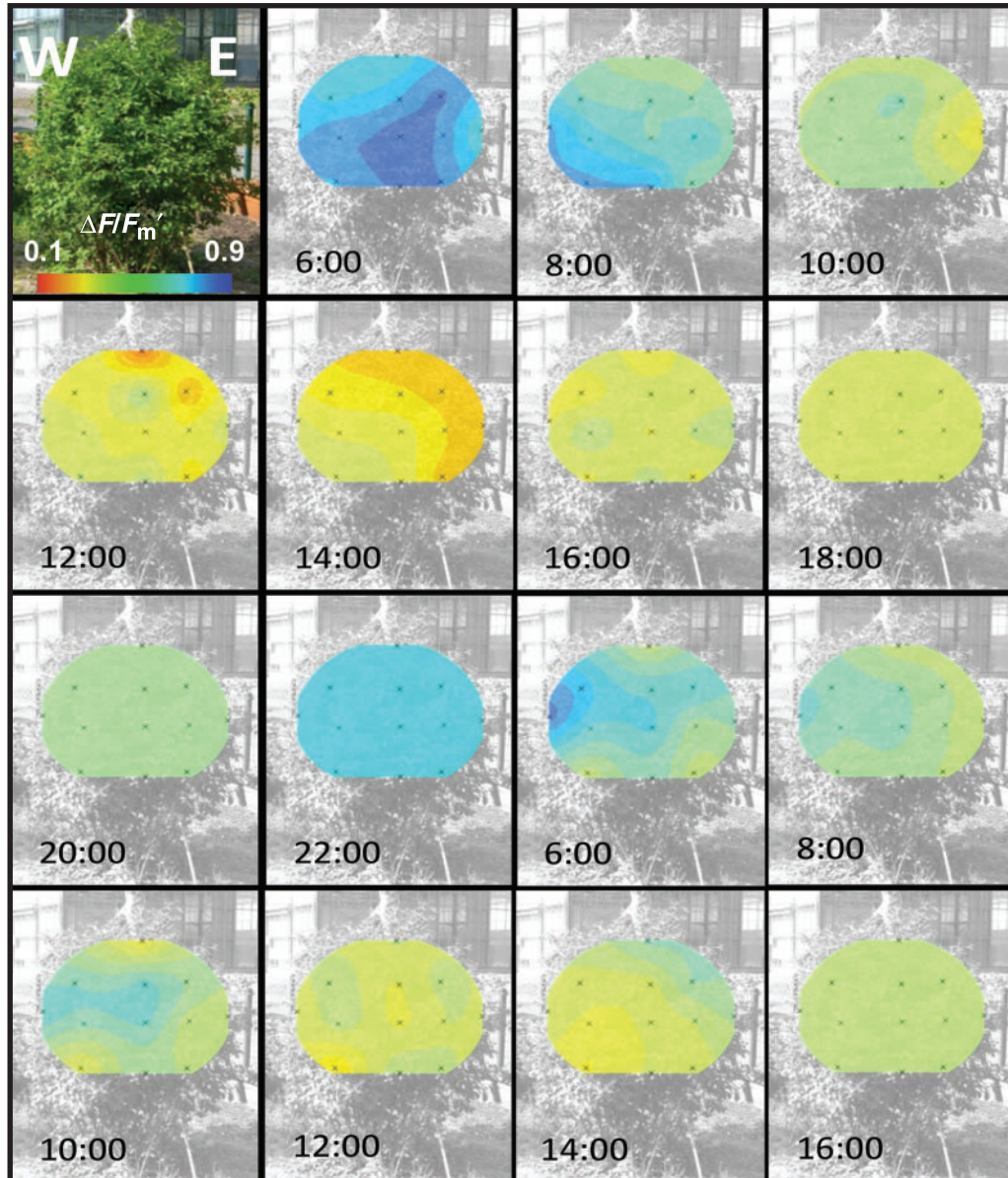


Fig. 8. Pictorial reconstruction of $\Delta F/F_m'$ LIFT on the S-facing (NH) outer canopy of *Philadelphus coronarius* at 2 h intervals following the first cool night of 2–3 May 2011. Images from 0600–2200 hours on 3 May 2011 are followed by those from 0600 to 1600 hours on 4 May 2011. High $\Delta F/F_m'$ throughout the canopy before dawn soon gave way to lower values in the upper E-canopy that persisted until recovery commenced mid afternoon. The gradients from top to bottom (cf. Fig. 8e) and E to W are clear on both days and some acclimation (higher $\Delta F/F_m'$) is evident on 4 May compared with 3 May. Interpolation between the twelve targets (marked x) was performed with a kriging algorithm using the software ArcGIS (ASRI, Redland, CA, USA).

et al. (2007) noted weaker correlation between PRI and both NPQ and F_v/F_m in Lx-rich *I. sapindoides*, compared with *P. indicus*, during leaf dehydration experiments. It is unlikely that spectral properties of Lx itself will be important because relatively few species have high outer canopy Lx (Matsubara *et al.* 2009). Inner canopy [Lx] will be exposed to PRI only after major disturbance following storms or pruning and then de-epoxidation, reversible or otherwise, may be complete in the first diel. Comparisons of high capacity for NPQ in the shaded outer canopy at high ambient temperatures (Fig. 6d) using PRI and LIFT might be especially valuable if Lx-accumulating and non-accumulating species were targeted.

Nevertheless, reconsideration of the importance of reflectance signals attributable to L is needed. Outer canopy 'flushing' of new leaves in tropical species such as avocado and *Inga*, presents an opportunity for examination of relationships between seasonality and productivity. These leaves are rich in L and V (Fig. 2) but remain as sink leaves for 2–4 weeks. Although cloudiness may present problems for PRI investigation of these species in their forest habitats, there would seem to be remarkable opportunities for evaluation of these factors at all scales in avocado orchards in different regions. For example, clear sky conditions are common in expansive Australian and Chilean orchards in which hugely contrasting planting and pruning practices are deployed (Whiley *et al.* 2002).

The limitations to potential applications of LIFT are not very different from those confronted already by PRI (Garbulsky *et al.* 2011). For example, dealing with the highly variable and dynamic geometry of chlorophyll fluorescence excitation and emission in the target space (cover picture this issue) presumably resembles similar problems encountered in the reflectance domain. Recent experience with long-term sequential LIFT measurements in tree canopies with differing leaf morphologies has revealed quantitatively different responses that require creative interpretation (R. Pieruschka, unpubl. data). So, again, one wonders if 'fuzzy' interrogation of a large number of outer canopy targets by raster-LIFT, or more sophisticated spectral reflectance imaging, might in the end provide useful alternatives to simple spot measurements on individual leaves using MINI- or MONI-PAM; by letting structural elements in the canopy speak as it were.

By way of example, the 12 data points targeted on the *Philadelphus* canopy (Fig. 6e–h) were interpolated in maps of $\Delta F/F_m'$ (LIFT) and although this interpolation was a rather limited (Fig. 8), it provided valuable information on variation in photosynthetic efficiency over the canopy. Uniformly high $\Delta F/F_m'$ at dawn (0600 hours) on day 1 soon gave way to declining values in the upper canopy and on the E-side. The W-side showed smaller decline in $\Delta F/F_m'$ and recovered in the cool night whereas recovery of $\Delta F/F_m'$ on the E-side at 0600 hours on day 2 was incomplete. Evidently, the combination of light stress in the previous day and the low night temperature reduced the capacity of the E-facing canopy to recover. Interestingly, E-side $\Delta F/F_m'$ values on the second day were higher than on the first under similar PFD and the W-side now showed the larger decline. Perhaps some acclimation of photosynthesis was already underway in the E-facing canopy on the second cold day? This possibility cannot be resolved in the preliminary experiment but the potential for more detailed, higher resolution $\Delta F/F_m'$ maps is

already obvious. One cannot escape the feeling that Niggle might be smiling as he looks down as these efforts to 'to paint a whole tree, with all of the leaves in the same style and all of them different' approach realisation.

At this stage, further evaluation of LIFT is mainly constrained by the availability of instruments and operators in a position to explore applications to the extent that, for example, PAM has been explored. Technical issues, such as low signal-to-noise at high PFD, just when sensitivity is most needed, presumably can be addressed. Likewise, precision raster requirements and data handling capabilities should now be within reach and can be coupled with small distributed light sensors (Leuchner *et al.* 2005) or with new optical techniques for remote estimation of PAR at target. Co-alignment of stereo imaging or LIDAR for surface structure (Biskup *et al.* 2009) may be important, perhaps essential, for future development of LIFT for interrogation of complex canopies. Active chlorophyll fluorescence is potentially a much richer signal than reflectance, but excitation energy requirements and emission capture limitations, may constrain measurements to a range of a few hundred meters using unmanned aerial vehicles (blimps, helicopters or drones; Lucieer *et al.* 2010). The intrinsically interesting outer canopy responses to a stochastic light environment under cloudy conditions are unlikely to be susceptible to spaceborne remote sensing.

A clear and persistent need

Effectively we are confronted once again with a need similar to that met by Lloyd Evans and the Canberra Phytotron in 1962, but on a much larger scale and seemingly with much greater urgency. Now, half a century after the opening of the facility, much of the Canberra Phytotron has been renovated as a High Resolution Plant Phenomics Centre. Now, as then, Australian plant biologists are taking a lead in responding to the grand challenges of the time through this and other infrastructure initiatives such as the Plant Accelerator at the University of Adelaide and at ANU and CSIRO Canberra (Finkel 2009; Furbank 2009). Unfortunately, these infrastructure investments have yet to be secured by recruitment of cohorts of creative users; the potential of such facilities cannot be realised otherwise. Experience shows that in the plant sciences research community here as elsewhere, the expectation that 'the market alone' (including short-term competitive grant cycles) can sustain the most effective use of such investments is fraught.

Six years ago the XVII International Botanical Congress in Vienna resolved (in Resolution 2, in part):

'We cannot simply continue to observe the uncontrolled global environmental change. As a matter of urgency, facilities for controlled, ecosystem-scale experiments are required now, supported by commitments that match those presently devoted to space and planetary sciences. Without such facilities, experiments and international research activities, we have little prospect of understanding, anticipating and taking advantage of the mechanisms that underlie functional biodiversity in plants of major forest biomes such as the boreal,

temperate, humid and dry tropical forests. These ecosystem processes mediate most of the exchanges between the terrestrial biosphere and the atmosphere and are vital to sustainable human habitation of Earth.'

Berry (2012) has related the history of a remarkable NASA-funded experiment that challenged self-assembled teams of scientists from several disciplines to find paths for the integration of terrestrial plant ecophysiology into Earth systems science. In the precarious economic environment of today, it may be politic to point out that this model and its achievements were more dependent on trust in the creativity of individuals than on huge investments in resources managed from the top. It is indeed heartening that the European Research Council will introduce (on an experimental basis) new 'ERC Synergy' grants in 2012 'bringing together in a bottom-up mode two to four individual PIs whose project crucially depends on their mutual (and typically complementary) knowledge, expertise and excellence, combined in a way that includes novel ways of working together' (Nowotny 2011). If implemented to facilitate creatively coordinated individual research campaigns, perhaps focussed on key FLUXNET sites and phenotyping centres, such actions could do much to resolve our canopy conundrums.

The distinctive insights from B2 L show that contributions from large scale controlled environment facilities should not be underestimated. It is heartening that B2 L, now in the hands of researchers from many disciplines at the University of Arizona Tucson, may again prove an indispensable asset for Earth systems and experimental climate change science. But as noted in the IBC Vienna resolution, a sea change will be required in the level of resources available if we are to predict outcomes of humankind's uncontrolled experiment as it tests the limits to sustainability of planet Earth.

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