Running title: Photoprotection/photoinactivation of PSII in shade leaves

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ABSTRACT

This study resolved correlations between changes in xanthophyll pigments and photosynthetic properties in attached and detached shade-grown avocado leaves upon sun exposure. Lutein epoxide (Lx) was de-epoxidized to lutein (ΔL) over 5 h, whereas violaxanthin (V) conversion to antheraxanthin (A) and zeaxanthin (Z) ceased after 1 h. During subsequent dark or shade recovery, de novo synthesis of L and Z continued, followed by epoxidation of A and Z, but not of L. Light saturated non-photochemical quenching (NPQ) was strongly and linearly correlated with decreasing [Lx] and increasing [ΔL] but showed a biphasic correlation with declining [V] and increasing [A+Z] separated when V de-epoxidation ceased. When considering [ΔL+ΔZ], the monophasic linear correlation was restored. Photochemical efficiency of PSII (F_v/F_m) and PSII/PSI (deduced from delivery of electrons to PSI in saturating single turnover flashes) showed a strong correlation in their continuous decline in sunlight and the increase in NPQ capacity. This decrease was also reflected in the initial reduction of the slope of photosynthetic electron transport (ETR) vs. PFD. Generally longer, stronger sun exposures enhanced declines in both slope and maximum ETR rates as well as F_v/F_m and PSII/PSI more severely and prevented full recovery. Interestingly, increased NPQ capacity was accompanied by slower relaxation. This was more prominent in detached leaves with closed stomata, indicating photorespiratory recycling of CO_2 provided little photoprotection to avocado shade leaves. Sun exposure of these shade leaves initiates a continuum of photoprotection, beyond full engagement of Lx-and V-cycle in the antenna, but ultimately photoinactivated PSII reaction centers.

apparatus in sun and shade, is beyond the scope of this paper. However, Matsubara et al (2005) found that Lx and L co-located with V in trimeric LHCII and LHCI from thylakoids isolated from *Inga sapindoides*. After de-epoxidation of Lx and V, followed by epoxidation of A+Z, ΔL had replaced Lx in peripheral L1 and internal L2 sites of both monomeric and trimeric Lhcs, i.e., ΔL evidently replaced A+Z in Lhcs (Matsubara et al 2007) to "lock-in" a capacity for elevated NPQ in the dark. These shade leaves seemed ideally suited for examination of the question as to whether the decline in $Ø_{PSII}$ following sunlight exposure occurred because of, or in spite of, two kinetically distinct xanthophyll cycles in these plants.

The terminology used by various authors to describe component processes in photoprotection, photoinhibition, and photoinactivation (van Kooten and Snel 1991; Horton et al 1996; Maxwell and Johnson 2000) remains a semantic minefield. Because we focus on the relationships between xanthophyll pigment composition and photoprotection in this paper, we will use the subscript designation of different components of NPQ introduced independently from several laboratories (Nilkens et al 2010; Förster et al 2011; Nichol et al 2012; Jahns and Holzwarth 2012).

In brief, the widely used nomenclature of Horton et al (1996) has been refined by designating transthylakoid ΔpH-dependent, xanthophyll-independent qE as $NPQ_{\Delta pH}$ and State transition-dependent qT as NPQ_{ST}. Preliminary experiments showed the slow state transitions in avocado shade leaves were quantitatively similar to those reported by Havaux and Lannoye (1987) but also were an early casualty of sunlight exposure. The contribution of NPQ_{ST} to NPQ overall was likely to be small (Cleland et al 1990; Tikkanen et al 2006) and was not considered further.

The different xanthophyll-dependent components of qN can be distinguished on the basis of chlorophyll fluorescence kinetics associated with differences in pigment composition. De-epoxidation in the Lx and V-cycles of avocado generates $NPQ_{\Delta LAZ}$ rather than NPQ_{AZ} in species with the V-cycle alone (presumably equivalent to qZ of Nilkens et al 2010). Conveniently, $NPQ_{\Delta LAZ}$ in avocado also accommodates the component of qI attributed to persistently high A+Z (Adams et al 1994) and ΔL following incomplete epoxidation in the dark. The distinctive component $NPQ_{\Delta L,}$ associated with low light treatments that led to persistent enhanced capacity for NPQ in the dark after epoxidation of A+Z but not L (Förster et al 2011), could not be identified in the sunlight treatments applied here. We do not wish to be drawn on mechanistic issues; indeed common mechanisms of pH sensing and xanthophyll pigment interconversions may be involved in $NPQ_{\Delta pH}$ and all xanthophyll stabilized forms of NPQ (Ruban et al 2012).

The component of qI associated with photoinactivated PSII centers is designated as NPQ_{PI} and must be distinguished using other biochemical and/or biophysical criteria. Photosystem II is well known to be susceptible to radiation damage and β-carotene has a key role in quenching of singlet O_2 in the reaction center (Telfer 2002). However, it is now clear that photoinactivation of PSII occurs under many

to minimize heating and to serve as dark controls.

Leaf discs were removed at intervals for pigment analysis during 200 (expt#1) and 300 min (expt#2, #3) and 90 min (expt#4) sun exposure, for measurements of changes in the reduction state of Q_A (1-qP), photosynthetic electron transport (ETR) and NPQ using rapid light response curves of chlorophyll fluorescence, and independent measurements of F_v/F_m and the functional fraction of PSII. Preliminary experiments with attached leaves showed no response to the "Vaseline™ patch" test predawn (Supplementary Fig. 1), confirming that stomata were closed. When the test was applied following induction in the shade and during sun exposure, marked inhibition of ETR and increase in NPQ confirmed that stomata were open (had been occluded by Vaseline™). In contrast, detached leaves showed no response of ETR or NPQ to the test during sun exposure, confirming that stomata were closed.

Early cessation of de-epoxidation distinguishes V-cycle from Lx-cycle in attached avocado shade leaves exposed to sunlight

Changes in xanthophyll and carotenoid pigments in attached, fully expanded leaves of a shade-grown avocado plant were assayed predawn and after 3 and 4 h induction in the shade prior to transfer to sunlight (expt#1). Control leaves sampled predawn, as well as those in which photosynthesis was induced in the shade enclosure and discs taken from the shaded areas of exposed leaves, showed no change in F_v/F_m or change in de-epoxidation status of xanthophyll pigment pools (DES_{Lx}= [ΔL]/([Lx]+[ΔL]); DES_V= ([A+Z])/([V+A+Z])). Note that the chemical conventions of [V] to specify the concentration of a xanthophyll pigment in this case V, [ΔL] to indicate a change in [L], and [L*] to indicate [L-100] in mmol mol^{-1} Chl to facilitate stoichiometric comparison in graphical data, are used throughout.

As expected, de-epoxidation of both Lx and V commenced following exposure to sunlight and time courses of changes in xanthophyll pigments are given in Figs. 1 and 2. The slowly reversible Lx-cycle in avocado was characterized by a decline in [Lx], an increase [L] (Fig. 1 A) and in DES_{Lx} (Fig. 2 A) that showed little change in the dark overnight and in the shade enclosure next day. An unusual feature of avocado shade leaves in this and other experiments (Förster et al 2009, Matsubara et al 2012) was an initial small decline (not significant here) of [L] in the first 10 min in sunlight, despite some de-epoxidation of Lx. These dynamics were reflected in the estimate of DES_{Lx}, which was initially negative (data not shown) due to loss of L, but then increased throughout the experiment (Fig. 2 A). The increase in [L] eventually exceeded the decline in [Lx] by a factor of two, suggesting about half of the increase in [L] was due to de-novo synthesis rather than de-epoxidation. However [α-C] remained virtually unchanged (Fig. 1 C) and there was little change of [Lx], [L] or [α-C] after the plant was returned to the shade enclosure.

193 (Fig. 3 C) was clearly associated with de novo synthesis of L and a two-fold greater decline in [V] due to
194 de-epoxidation (Fig. 1 A, B). There was little further depression in 1-qP (Fig. 3 A) or change in the
195 light-limited or the maximum rate of the ETR profile (Fig. 3 B).

196 De-epoxidation of V had ceased by 60 min and period *(ii)*, between 60 and 160 min, was
197 characterized by relatively stable DES_V, continued increase in DES_{Lx} (Fig. 2 A), but continued decline in
198 F_v/F_m and PSII/PSI (Fig. 2 B). The profile of 1-qP became more complex at lower PFD and continued
199 to decline at high PFD, indicating that Q_A became more readily oxidized with increasing time in
200 sunlight (Fig. 3 D). There was little further induction of ETR (cf. Figs. 3 B and E) but $NPQ_{\Delta LAZ}$
201 evidently increased markedly (Fig. 3 F) as de-novo syntheses of L and A+Z continued (Fig. 1 A, B).
202 Measurements after 90 min exposure were intermediate between those at 60 and 120 min. Because
203 there was little change in DES_V during this time it seems plausible that that most of the increase in
204 $NPQ_{\Delta LAZ}$ was associated with the doubling in DES_{Lx}, driven by quantitatively similar increase in L
205 from de-epoxidation and de novo synthesis.

206 Period *(iii)* begins with data from the last sun exposed sample collected after 200 min (open
207 triangles) and compares recovery of photosynthetic parameters in exposed areas of the leaves after 24h in
208 the dark overnight and in the shade the next day (grey circles; 28 h after commencement of expt#1) with
209 an equivalent unexposed but induced leaf on another plant in the shade enclosure (grey squares).
210 Aluminum covered, dark control areas of leaves measured after 250 min had photosynthetic properties
211 similar to the original dark control measured predawn (data not shown). The 200 min sunlight exposure
212 showed a continuation of the trends observed in Fig. 3 D towards more oxidized Q_A, lower initial slope
213 of ETR, and strongly increased NPQ (cf. Fig. 3 D-F and G-I). After 24 h of recovery, the sunlight
214 exposed areas showed features of both controls and sunlight exposed leaf areas. The PFD response of
215 the Q_A reduction reverted from its more oxidized condition after 200 min in sunlight to levels of Q_A^-
216 similar to shade induced leaves (cf. Fig. 3 A, G). On the other hand, maximum ETR rates did not differ
217 in leaves sun exposed for 200 min, in exposed leaves after 24 h recovery or in unexposed but shade
218 induced leaves (Fig. 3 H). However, exposed tissues had a clearly lower initial slope of ETR vs. PFD
219 which did not recover.

220 The PFD profiles of NPQ in period *(iii)* were particularly informative (Fig. 3 G-I). Tissue
221 exposed to 200 min sunlight showed the most rapid increase and highest level of $NPQ_{\Delta LAZ}$ (open
222 triangles) achieved during continued accumulation of L and Z. Shade induced controls were unchanged
223 compared with the starting point of the experiment (cf. grey squares in Fig. 3 C, I) consistent with the
224 absence of de-epoxidation of Lx or V (Fig. 1 A-C). After 24 h recovery, NPQ of 200 min sun exposed
225 leaves (grey circles) declined by about half (Fig. 3 I) to closely resemble the profile of $NPQ_{\Delta LAZ}$ after 60
226 min sun exposure (Fig. 3 F). However, the xanthophyll pigment compositions of the leaves at these
227 times were markedly different with $DES_{Lx} = 0.66$ after recovery (vs. 0.42 at 60 min) and $DES_V = 0.32$

detached leaves maintained higher DES_V than attached leaves (cf. Figs. 2 A, 4 C). However, there was less de novo synthesis of xanthophylls. Again, F_v/F_m and PSII/PSI continued to decline after de-epoxidation ceased (Fig. 4 D) and neither showed much recovery in the dark. Compared to attached leaves there was a larger decline, and less recovery, in PSII/PSI and F_v/F_m in detached leaves (Figs. 2 B and 4 D).

Photosynthetic parameters in sun exposed detached leaves (Fig. 5) showed interesting differences compared to attached leaves (Fig. 3). The PFD profile of Q_A reduction did not recover in the weak laboratory light (Fig. 5 A). The profiles in sun exposed and 24 h shade recovered leaves were the same and showed a distinctive transient of more reduced Q_A at low PFD prior to the expected more oxidized Q_A at higher PFD (cf. Figs. 5 A, 3 C). The ETR profiles were markedly different. Although dark control detached leaves had the same capacity for photosynthetic electron transport as attached leaves, once exposed to sunlight, detached leaves showed more extensive decline in both light-limited and maximum rate of ETR and neither was restored during 24 h shade recovery in weak laboratory light (cf. Figs. 5 B, 3 E, H). The profiles of $NPQ_{\Delta pH}$ in dark control detached and attached leaves were similar (data not shown), but 60 and 300 min sun exposure produced more rapid increases at lower PFD to similarly high $NPQ_{\Delta LAZ}$ at ~430 μmol photons m^{-2} s^{-1} followed by ~50% recovery overnight (Figs. 5 C and Fig. 1 F, I). Relaxation of $NPQ_{\Delta LAZ}$ in the dark after assay was much slower after sun exposure ($t_{1/2}$ = 890 s), became slightly faster during recovery ($t_{1/2}$ =580 s) but remained well above $NPQ_{\Delta pH}$ of induced shade leaves ($t_{1/2}$ =160 s).

These responses in pigments and photosynthetic parameters were confirmed after 60 and 90 min sun exposure in another detached leaf experiment (expt#4) at 1,200 μmol photons m^{-2} s^{-1} (Table II). De-epoxidation of both V and Lx were similar in and ceased within 60 min under these extreme light stress conditions under low [CO_2] and some de novo synthesis of both L and Z had commenced. As before, whereas there was some epoxidation of A and Z during recovery in weak lab light, Lx and L pools remained unchanged. Measurements of 1-qP indicated sustained highly reduced Q_A at low PFD throughout this experiment. There was a striking depression and even less recovery of both the light-limited and maximum rate of ETR. The sustained depression and limited recovery of F_v/F_m in this experiment was greater than previously recorded, as was the depression of PSII/PSI, and neither recovered. Overall, it seemed plausible that NPQ_{PI} was a large contributor to higher, slowly reversible NPQ and sustained lower F_v/F_m and PSII/PSI in this experiment. Despite sustained high [ΔL] and [A+Z] from de-epoxidation and de novo synthesis, photorespiratory recycling of CO_2 behind closed stomata was evidently inadequate for photoprotection in these detached shade leaves in sunlight.

Although Lx-cycle pigments correlated linearly [A+Z] with NPQ, correlation with V-cycle pigments

DISCUSSION

This paper builds on earlier observations of large, slowly reversible changes in $Ø_{PSII}$ of avocado shade leaves that showed complex relationships with de-epoxidation of Lx to ΔL and V to A+Z during short- and long-term acclimation to sunlight (Förster et al 2009). Here we examined the short-term responses of shade leaves to sun light exposure in more detail to disentangle some of the complex pigment – photosynthesis relationships, which led to some essential new insights. Interestingly, de-epoxidation of V ceased after about 60 min in sunlight and that subsequent increase in both [L] and [A+Z] from de-novo synthesis produced a two to five - fold increase in the capacity for $NPQ_{\Delta LAZ}$ over that due to $NPQ_{\Delta pH}$. The extent of recovery overnight and in the shade depended on the duration and intensity of sun exposure. Recovery was least in detached leaves with closed stomata in which excitation pressure was presumably increased when photosynthetic metabolism was limited to recycling of photorespiratory CO_2. Independent measures of the concurrent decline in light saturated ETR and the capacity of PSII to deliver electrons to PSII in saturating single turnover flashes during sun exposure, neither of which recovered in the shade, point to photoinactivation (NPQ_{PI}), in spite of the complex pigment interactions presumed to be associated with stabilization of photoprotection.

De-novo syntheses of L and Z sustain increased capacity for NPQ after cessation of V-cycle de-epoxidation in sunlight.

Sunlight exposure of attached avocado shade leaves led to cessation of V-cycle, but not of Lx-cycle, de-epoxidation, within 60 min (Fig 1 A, B; Supplementary Fig. 1). Incomplete de-epoxidation of the V pool in strong light is not unprecedented (Jahns and Holzwarth 2012) and seems common in shade plants (Watling et al 1997; Thiele et al 1998) as well as in Arabidopsis exposed to 2,000 µmol photons m^{-2} s^{-1} (Russell et al 1995) but the timing had not been resolved in those studies. At this stage we have no explanation for the early cessation of V de-epoxidation in sunlight but two lines of argument seem relevant. First, feedback on the capacity to maintain high lumen pH needed to promote further de-epoxidation (Pfündel et al 1994) in the face of increasing NPQ and/or declining PSII/PSI seems plausible. Consistent with such a possibility, mutations of the D1 protein in *Chlamydomonas reinhardtii* that slowed PSII electron transfer from Q_A to Q_B produced phenotypes that accumulated levels of Z in strong light similar to those in wild type but were unable to generate similarly high NPQ, possibly because impaired PSII was unable to maintain an effective ΔpH (Förster et al 2001). Second, compartmentalization of xanthophyll pools could explain the incomplete de-epoxidation. Compartmentalization of Z was invoked by Hurry et al (1997) to explain the lack of effective photoprotection with increasing [Z] in Arabidopsis *aba* mutants. These could include certain LHC-bound pigments in non-functional PSII, dissociated antennae or located elsewhere. Jahns et al

associated with A and also with ΔL enhancement of ΔpH-dependent $NPQ_{\Delta LAZ}$ (Matsubara et al 2011). But it is clear that [A+Z] also slows NPQ relaxation in the dark in avocado shade leaves (Förster et al 2011); a threshold of DES_V = ~0.1 was found for the signature effect of [A+Z] in slowing relaxation of NPQ (Matsubara et al 2012). Much more research is needed to evaluate these pigment exchanges in Lhcs during sunlight exposure in leaves with two xanthophyll cycles. The differing kinetics of the Lx cycle in avocado, *Ocotea foetans* (Esteban et al 2010) and *Inga* spp. may be especially useful in this context.

Relationships between Lx- and V-cycle pigments and enhanced NPQ in sunlight may arise from the same pigment replacement interactions detected in Lx-rich shade leaves exposed to modest light intensities (Matsubara et al 2008; Förster et al 2011). In avocado [ΔL] from de-epoxidation of Lx was retained for 48 h or more in the dark after epoxidation of A and Z to V; i.e., $NPQ_{\Delta pH} < NPQ_{\Delta LAZ} \approx NPQ_{\Delta L}$. Matsubara et al (2007) examined thylakoids of *Inga sapindoides* with both Lx- and V-cycles and demonstrated that ΔL functionally replaced Lx in the V1 and L2 binding sites of antenna Lhcs in much the same way as Z replaces V in these two xanthophyll binding sites to stabilize and enhance the capacity for NPQ. That a similar augmentation of might have occurred during sunlight exposure was suggested by the transformation of the biphasic correlation between NPQ and [ΔZ] to a monophasic correlation between NPQ as a function of the sum [ΔL+ΔZ], with a slope similar to that found for [Z] alone during de-epoxidation (Fig. 6 D). Further insight into mechanisms may be gained from Arabidopsis mutants that over express L in the absence of Z, which reportedly confers higher NPQ, and is associated with the generation of a cation radical attributed to actively quenching L similar to the signals obtained from Z (Li et al 2009a). Similar studies with avocado thylakoids may be revealing.

On the other hand, the biphasic correlation of $NPQ_{\Delta LAZ}$ with DES_V, [A+Z] or [Z] after de-epoxidation of V ceased may not be related to Z or ΔL at all, but to other factors that continued to accelerate NPQ in general. For example, the continued decline in F_v/F_m and in PSII/PSI may indicate an increase in NPQ_{PI} after cessation of de-epoxidation that adds to $NPQ_{\Delta LAZ}$ giving the impression of acceleration of NPQ expressed on the basis of the concentration of any of the de-epoxidised xanthophylls. We are unable to further partition these processes on the basis of data from expt#1, but note that both F_v/F_m and PSII/PSI recovered to a large extent overnight and in the shade next day.

De-epoxidation of Lx and V does not fully mitigate photoinactivation of PSII electron transfer during sunlight exposure

Evidently, the photosynthetic apparatus of avocado shade leaves responds to sunlight exposure in a highly coordinated and holistic manner, and seems to dynamically balance xanthophyll-dependent NPQ photoprotection against photoinactivation. The above complex interactions between NPQ and xanthophyll pigment composition in expt#1 are indicative of well-coordinated dissipation of excess

declined by ~60% and showed little recovery (Fig. 4 D).

Most importantly, the light response curves for ETR in expt#3 (Fig. 5 B) showed responses reminiscent of many early "photoinhibition" experiments (Osmond and Chow 1988; Osmond 1994; Murchie and Niyogi 2011), characterized first by inhibition of the initial slope without effect on maximum rate at saturating PFD (diagnostic of photoprotection, but not ruling out photoinactivation of PSII to an extent short of limiting whole-chain electron transport), but followed by further depression of initial slope and maximum rate with incomplete recovery in the dark and shade (diagnostic of photoinactivation). A second detached leaf experiment at 50% higher PFD for only 90 min (expt#4) led to much more drastic decline in both properties of ETR, in F_v/F_m and in PSII/PSI, despite record levels of $NPQ_{\Delta LAZ}$, DES_{Lx} and DES_V, none of which showed much recovery (Table II). It is difficult to escape the conclusion that, in these detached leaf experiments, photoinactivation was rampant.

At this stage biochemical analyses of damage and repair in the D1 reaction center protein of PSII (Russell et al 1995; Förster et al 2005) would greatly assist further attempts to resolve the relative impact of photoprotection vs photoinactivation in avocado shade leaves. Anderson et al (1998) estimated that on an average day in an average leaf, all of the D1 protein is damaged and repaired at least once, and it is likely these processes are accelerated in sun exposed shade leaves. For example, transfer of the shade plant *Monstera deliciosa* to sun led to accumulation of damaged D1 protein that was 'labeled' for repair by phosphorylation (i.e., D1*), despite retention of high [A+Z] (Ebbert et al 2001). A fraction of D1* was retained during dark recovery, when dephosphorylation of D1* protein would normally initiate the D1 repair cycle (Chow and Aro 2005). Perhaps D1* centers comprise the population of more rapidly quenching centers that correlate with the decline in functional fraction of PSII in photoinactivated in *Capsicum annuum* (Matsubara and Chow 2004)? Weak light is required for the D1 repair cycle and perhaps the much slower recovery of F_v/F_m and PSII/PSI in the dark in expt#2 reflects this requirement. The hypothesis that accumulation of these reaction center proteins following light stress "favors maximal energy dissipation over photochemistry" (Ebbert et al 2001) seems compatible with the photoprotective function proposed for this population of rapidly quenching centers (Matsubara and Chow 2004). Furthermore, as first reported by Depka et al (1998) and recently explored by Beisel et al (2010), there also may be links between PSII reaction center turnover, release of β-carotene and de novo syntheses and retention of Z following extreme light stress (Cazonelli and Pogson 2010).

None of this is unexpected, but the new data reported here are relevant to the early events in long held hypotheses of photoinhibition and acclimation (Anderson and Osmond 1987). Shade leaves in general have low rates of photosynthesis, low concentrations of Rubisco, low capacities for photosynthetic ETR, and importantly, low pools of xanthophyll pigments for photoprotection (Demmig-Adams 1998). Avocado shade leaves were no exception, with maximum capacities of light

demonstrated to also afford photoprotection (Matsubara and Chow 2004).

CONCLUSIONS

Sun exposure of avocado shade leaves revealed four major co-occurring changes in pigment composition and photosynthetic activities. (*i*) De-epoxidation in both the Lx- and V-cycles was incomplete and ceased after the ~ 60 min in sunlight, but was followed by further de-novo synthesis of L and Z. (*ii*) Whereas light saturated NPQ was linearly correlated with decline in [Lx] and increase in [ΔL] from either source, the transition to de novo synthesis was marked by a break point in a biphasic correlation of NPQ with DES_V, [A+Z] or [ΔZ]. Restoration of monophasic correlation in plots against ([ΔL]+[ΔZ]) suggested augmented stabilization of NPQ by L and Z. (*iii*) Photochemical efficiency of PSII (F_v/F_m) and the independently estimated functional fraction of PSII centers declined continuously in sunlight and were also highly correlated with NPQ, indicating a participation of photoinactivated PSII centers in photoprotection. (*iv*) Photorespiratory CO_2 recycling evidently contributed little to mitigation of light stress in detached leaves with closed stomata, presumably because these shade leaves had very low Rubisco and electron transport capacity. More research is needed to resolve the interactions between the two xanthophyll cycles as well as the relationships between various components of NPQ and the functional state of PSII centers. Avocado shade leaves transferred to sunlight present many opportunities for further detailed exploration of underlying mechanisms.

MATERIALS and METHODS

Plant material and growth conditions

Seedlings of avocado *(Persea americana* Mill, cv Edranol) were purchased from Vallance's Nursery (Mullumbimby NSW 2482 Australia) and grown in a shade enclosure in a temperature controlled glasshouse for 24 months (18 °C night/ 29 °C day) as described earlier (Förster et al 2009; 2011). Shade provided by several layers of neutral beige colored shade netting and was unrepresentative of changes in the spectral composition of natural shade due to absorption of shorter wavelength light by other canopy leaves. Mature fully expanded leaves ranged from 20-35 cm in length and were thinner and deeper green in the shade than in the sun. They have numerous small stomata that are restricted to the underside and photosynthetic parameters were routinely measured from the upper epidermis, but measurements from the lower epidermis were not significantly different. As judged by the "Vaseline patch" test (application of Vaseline halves ETR and doubles NPQ when stomata are open, but has no effect if they are closed; Supplementary Fig. 1), stomata on attached leaves in the shade enclosure were closed predawn, opened in the course of photosynthetic induction in the shade (or in response to actinic light treatments) and closed again during recovery in the dark lab overnight. Using the same criteria,

arranged and exposed to sunlight as in the above attached leaf experiments. Expt#3 was a close approximation to expt#1, but with assays 1 and 5 h after transfer to sunlight, and after recovery in the dark 24 h after commencement of the experiment. Detached leaves in expt#4 (Table II) experienced the strongest sunlight exposure (1,200 μmol photons m^{-2} s^{-1}), with leaf discs removed for pigment analysis after 15, 30, 60 and 90 min and after recovery overnight. Photosynthetic parameters were measured in discs from exposed areas after 60 and 90 min at 1,200 μmol photons m^{-2} s^{-1}, then again after 23 h recovery in the dark lab overnight. Controls were measured before exposure then after 90 min and again after 24 h.

Measurements of photosynthetic properties

Photosynthetic parameters were conveniently and reproducibly measured in air using the Photosynthesis Yield Analyzer MINI-PAM fitted with leaf clip holder 2030-B (www.walz.com). Chlorophyll fluorescence was measured randomly from a spot of about 12 mm diameter in control and exposed areas on attached or detached leaves, or on discs cut from these leaves and supported on a moist glass fiber filter during measurement. Assay protocols were optimized with respect to saturating flash intensity and actinic light treatments to minimize pigment de-epoxidation and artifacts in photosynthetic parameters during measurements on the extremely light sensitive shade leaves (Förster et al 2011). Rapid light response curves (RLRC) had a dwell time of 30 s at each of 8 steps from darkness to ~400 μmol photons m^{-2} s^{-1} (to ~600 μmol photons m^{-2} s^{-1} in expt#4) with a saturating flash at the end of each step. Dark relaxation of NPQ was usually followed for 220 s at the end of RLRC, but the short time intervals between sampling in expt#1 made this impossible so only a few representative dark relaxation profiles were collected. Light intensities during the experiments were measured with the light sensor fitted to the leaf clip of the MINI-PAM system.

Calculation of photosynthetic parameters

The MINI-PAM apparatus measured intrinsic chlorophyll fluorescence (F) from a spot on a leaf during a saturating flash (F_m) to give the quantum yield of photochemical energy conversion $Ø_{PSII}$ or $F_v/F_m = (F_m-F)/F_m$. Photosynthetic electron transport was calculated from fluorescence yield in subsequent saturating flashes under actinic light (F_m') using the quantum yield of photochemical energy conversion ($\Delta F/F_m' = F_m'-F/F_m'$), and the PFD measured at that spot (adjusted for absorptance of 0.85 and assuming equal light absorption in PSII and PSI). Sunlight exposure of shade-grown leaves led to large, slowly relaxing (hours to days) decreases in F_m, as well as marked decline (-20%) in F within 100 s after actinic light was extinguished (presumably due to the absence of background far red light in the MINI-PAM) that produced misleading results from automated calculation of NPQ and qP. These problems were addressed by recalculating all NPQ data using F_m measured on the leaf kept in the dark overnight before light-treatment and/or F_m from Aluminum foil shaded areas of the leaf during and after treatment. Likewise, 1-qP data were recalculated using the minimum value of F obtained

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910 **Figure 5.** Changes in Q_A reduction (1-qP), in ETR, and increase in NPQ in detached avocado shade
911 leaves measured by rapid light response curves (RLRC) of chlorophyll fluorescence in expt#3.
912 Photosynthetic parameters of control leaves were measured pre-dawn (dark), after 60 and 300min
913 sun exposure (exp 60; exp 300) and after recovery in weak laboratory light (5 μmol photons m^{-2} s^{-1})
914 over the next 19 h (rec 24 h) (mean ± SE, n=4; error bars appear when SE exceeds symbol size).

915 **Figure 6.** Light saturated NPQ (from Fig. 3) was linearly correlated with changes in pool sizes of
916 Lx- and V-cycle pigments during sunlight exposure (from Fig. 1) in expt#1. (**A)** The correlation of
917 NPQ with the decline in [Lx] and increase in [L*] was monophasic. (**B**) In contrast, the correlations
918 between the increase in NPQ and changes in DES_V were biphasic, separating after cessation of
919 de-epoxidation and commencement of de-novo synthesis. (**C**) Similar biphasic correlations were
920 found between the increase in NPQ and the increase in [A+Z]. (**D**) Interestingly, the correlation
921 between NPQ and [Z] was biphasic whereas with [ΔZ]+[ΔL] it was monophasic. (mean ± SE; n=3;
922 error bars appear whenever SE exceeds size of symbols).

923 **Figure 7.** Linear correlations of photosynthetic properties measured in expt#1. (**A**) The increase in
924 light saturated NPQ (from Fig. 3 C, F, I) correlated negatively with functional fraction of PSII
925 (PSII/PSI from Fig. 2 B). (**B**) The increase in F_v/F_m correlated positively with PSII/PSI during sun
926 exposure (from Fig. 2 B). (**C**) The reduction state of the Q_A pool (1-qP from Fig. 3 A, D and G)
927 showed similar positive correlation with PSII/ PSI at ~half saturating PFD (123 μmol photons m^{-2}
928 s^{-1}) and at light saturation (433 μmol photons m^{-2} s^{-1}) PSI (from Fig. 3 A, D, G); (mean ± SE; n=3;
929 error bars appear when SE exceeds symbol size).

930 **Figure 8.** A holistic, traffic light-inspired schematic summary of interactions between reversible
931 photoprotection and photoinactivation with irradiance and time, based on ancient Chinese insights
932 into the complementarity of opposite concepts. Commencing with rapidly reversible $NPQ_{\Delta pH}$,
933 increasing irradiance and/or time engage more slowly reversible dissipation of excess excitation as
934 heat (amber) through various forms of xanthophyll-dependent photoprotection in the antenna
935 (NPQ_{AZ}, $NPQ_{\Delta LAZ}$ and $NPQ_{\Delta L}$). Even more slowly reversible dissipation of excess excitation
936 (shown in red) follows from the accumulation of photoinactivated PSII centers (NPQ_{PI}) due to
937 imbalance in the rates of PSII damage and repair.

Table II. Summary of changes in xanthophyll pigment composition and associated changes in photosynthetic parameters in rapid light response curves in detached, sun exposed avocado shade leaves in expt#4 (Means ± SE; n = 4).

Detached leaves; expt#4 (1,200 μmol photons m^{-2} s^{-1})	Shade leaf pre-dawn	60 min sunlight	90 min sunlight	Recovered 22 h shade
Pigments (mmol mol^{-1} Chl) and de-epoxidation states (DES)				
[Lx]	30.4 ± 2.3	12.4 ± 0.6	10.5 ± 0.9	11.6 ± 0.6
[L]	104.7 ± 1.1	133.9 ± 0.8	130.9 ± 2.3	130.1 ± 1.5
DES_{Lx}	0	0.68	0.71	0.69
[V]	31.8 ± 0.2	10.4 ± 0.2	9.8 ± 0.2	16.3 ± 1.5
[A]	0.2 ± 0.2	2.5 ± 0.3	3 .0 ± 0.1	7.9 ± 0.9
[Z]	2.4 ± 0.4	29.1 ± 0.5	28.4 ± 1.6	17.5 ± 1.5
DES_V	0.08 ± 0	0.76 ± 0	0.76 ± 0.01	0.61 ± 0.04
Photosynthetic parameters from initial slope and saturation at PFD (μmol photons m^{-2} s^{-1})				
1-qP (94)	0.78 ± 0.01	0.91 ± 0.01	0.95 ± 0.02	0.88 ± 0.01
(467)	0.92 ± 0.01	0.94 ± 0.01	0.98 ± 0.03	0.85 ± 0.03
ETR (94)	5.3 ± 0.2	0.9 ± 0.2	0.3 ± 0.2	1.0 ± 0.1
(467)	9.4 ± 1.2	1.7 ± 0.5	0.3 ± 0.3	3.0 ± 0.3
NPQ (94)	0.74 ± 0.02	2.59 ± 0.08	3.83 ± 0.23	3.45 ± 0.47
(467)	1.38 ± 0.03	3.13 ± 0.08	4.48 ± 0.26	4.22 ± 0.47
Photosynthetic parameters from independent measurements				
F_v/F_m	0.79 ± 0	0.18 ± 0	0.14 ± 0.03	0.18 ± 0.06
PSII/PSI (arbitrary ratio)	0.56 ± 0.01	0.22 ± 0	0.28 ± 0.03	0.32 ± 0.06

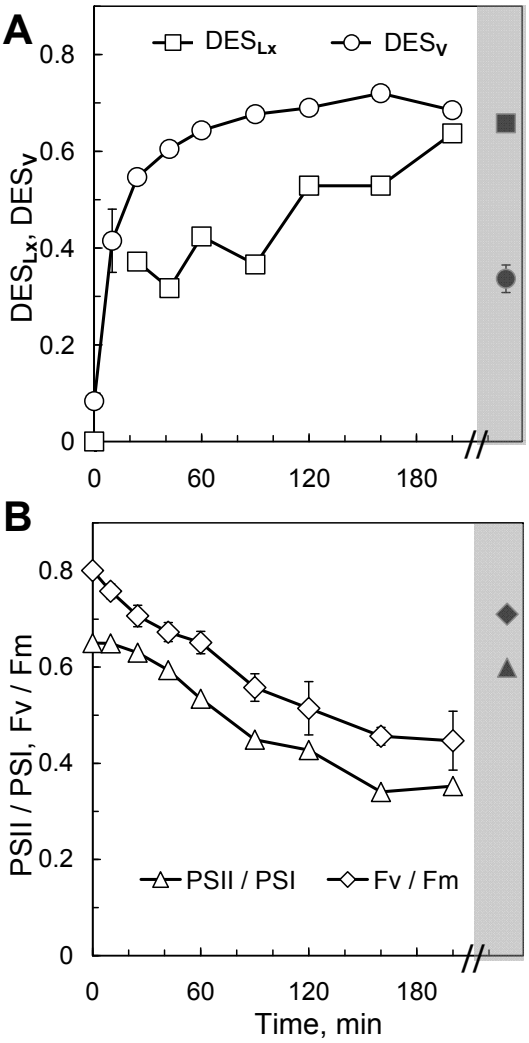


Figure 2. Photochemical efficiency of PSII continues to decline after de-epoxidation of V ceases. **(A)** Times courses of change in de-epoxidation status of the V cycle (DSV = [A+Z]/[V+A+Z]) and the Lx cycle (DSLx = [ΔL]/[Lx+ΔL]) in expt#1. **(B)** Decline in the photochemical efficiency of PSII (F_v/F_m) in sunlight in expt#1 and in the functional fraction of PSII, indicated by the arbitrary ratio PSII/PSI measured as the capacity of a single turnover saturating flash to deliver electrons to PSI (mean ±SE; n=3; error bars appear when SE exceeds symbol size).

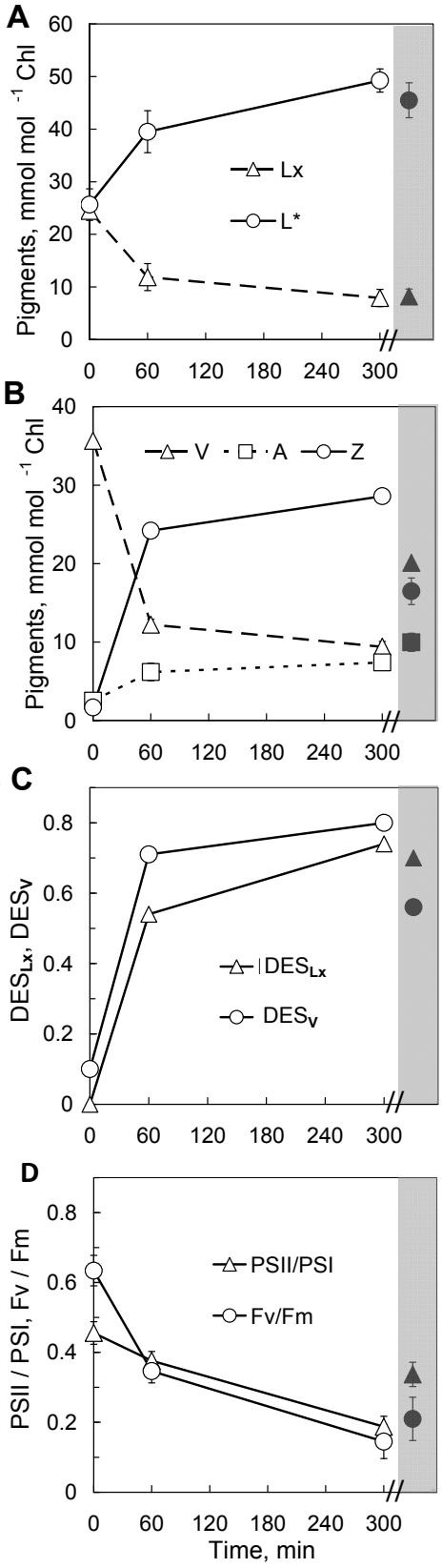
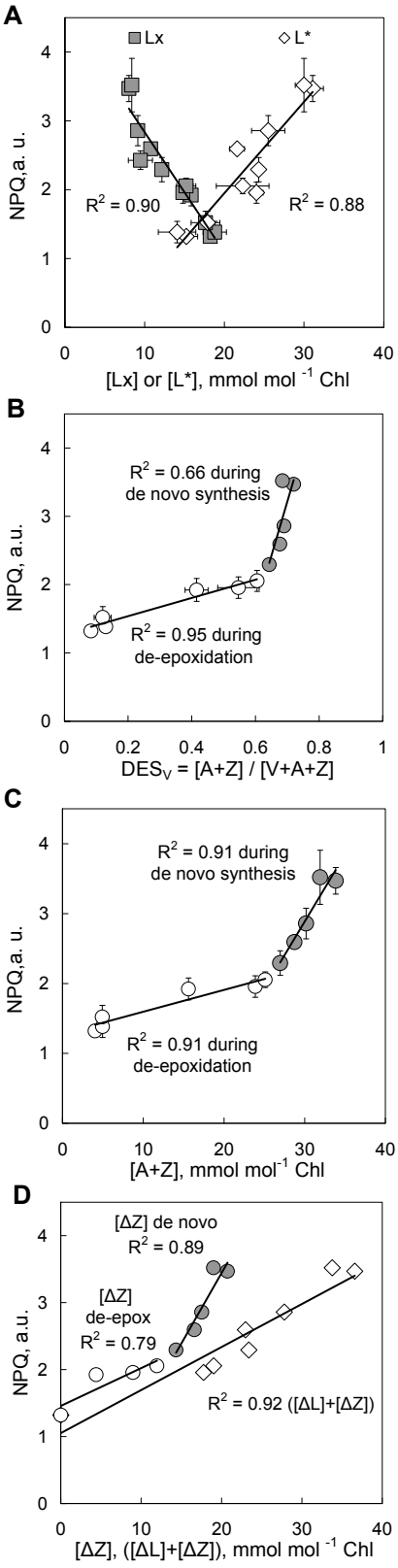


Figure 4. Changes in pigments and photosynthetic parameters in detached avocado shade leaves exposed on water during exposure to sunlight (750 µmol photons m^{-2} s^{-1}) and after recovery for 23 h in the shade enclosure (black symbols in shaded panel of graphs) in expt#3. (**A**) Changes in [Lx] and [L*] (Note the scale for [L*] is 100-160 mmol mol^{-1} Chl), (**B**) in V-cycle pigments and de-epoxidation status and (**C**) in de-epoxidation status of Lx and V cycles (DSLx and DSV respectively). (**D**) Independent measurements of PSII/PSI and F_v/F_m (mean ± SE, n=4; error bars appear when SE exceeds symbol size).



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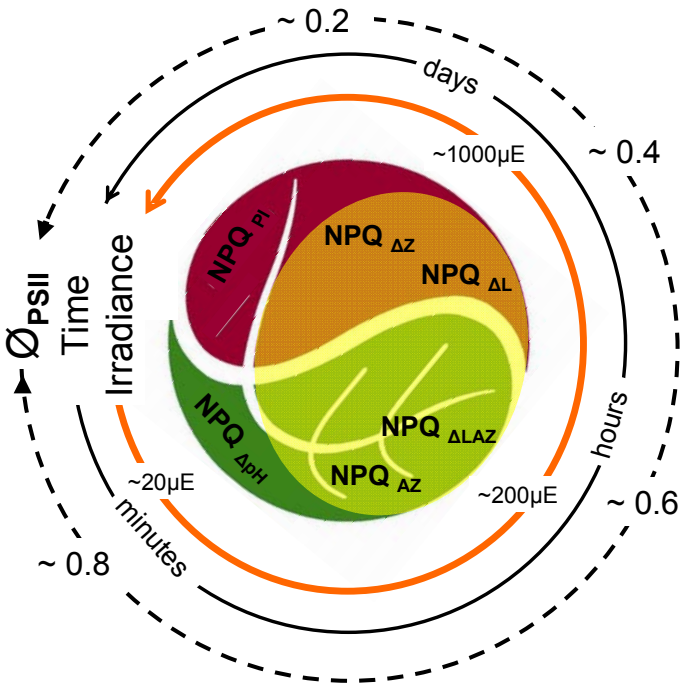

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