Light potentials of photosynthetic energy storage in the field: what limits the ability to use or dissipate rapidly increased light energy?

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The responses of plant photosynthesis to rapid fluctuations in environmental conditions are critical for efficient conversion of light energy. These responses are not well-seen laboratory conditions and are difficult to probe in field environments. We demonstrate an open science approach to this problem that combines multifaceted measurements of photosynthesis and environmental conditions, and an unsupervised statistical clustering approach. In a selected set of data on mint (Mentha sp.), we show that ‘light potentials’ for linear electron flow and non-photochemical quenching (NPQ) upon rapid light increases are strongly suppressed in leaves previously exposed to low ambient photosynthetically active radiation (PAR) or low leaf temperatures, factors that can act both independently and cooperatively. Further analyses allowed us to test specific mechanisms. With decreasing leaf temperature or PAR, limitations to photosynthesis during high light fluctuations shifted from rapidly induced NPQ to photosynthetic control of electron flow at the cytochrome b6f complex. At low temperatures, high light induced lumen acidification, but did not induce NPQ, leading to accumulation of reduced electron transfer intermediates, probably inducing photodamage, revealing a potential target for improving the efficiency and robustness of...
photosynthesis. We discuss the implications of the approach for open science efforts to understand and improve crop productivity.

1. Introduction

While oxygenic photosynthesis supplies energy to drive essentially all biology in our ecosystem, it involves highly energetic intermediates that can generate highly toxic reactive oxygen species (ROS) that can damage the organisms it powers [1]. Thus, the energy input into photosynthesis must be tightly regulated by photoprotective mechanisms that act at several key steps in the light reactions. The balance and kinetics of this regulation is an active target for crop improvement.

One class of photoprotective processes, known as non-photochemical quenching (NPQ), dissipates absorbed light energy as heat, thus diverting energy away from photosystem II (PSII) [2], decreasing the accumulation of reactive intermediates. This photoprotective capacity comes at the cost of decreased photochemical efficiency, and thus the organisms must regulate NPQ to balance the avoidance of photodamage with efficient energy conversion [3,4]. There are several forms of NPQ that differ in their mechanisms and rates of activation and deactivation. The most rapid NPQ form is qE, which is activated by acidification of the thylakoid lumen by the proton gradient ($\Delta pH$) component of the thylakoid proton motive force ($pmf$) [2]. Lumen acidification activates the violaxanthin de-epoxidase or VDE [5–8] resulting in the conversion of violaxanthin (Vx) to antheraxanthin (Ax) and zeaxanthin (Zx); and protonation of PsbS, an antenna-associated protein required for qE [2], which appear to act cooperatively in setting the extent of qE. The conversion of Vx to Ax and to Zx is typically much slower than the rapidly reversible protonation of PsbS [2], and during prolonged illumination, the responses of qE will probably be limited by the rate of acidification and de-acidification of the thylakoid lumen, which are, in turn, governed by ion movements in the chloroplasts [9–11]. Slower forms of NPQ have also been demonstrated [12], including qI, which is related to the photodamage and repair of photosystem II (PSII) or qZ, which related to the accumulation of Zx (independently from qE) [13], qH, related to cold and high light stress [13], and qT, related to antenna state transitions [14].

The acidification of the thylakoid lumen also controls electron transfer at the cytochrome $b_{6}f$ complex, a process called photosynthetic control (PCON) [15–20], which prevents the build-up of electrons on the acceptor side of photosystem I (PSI) that can lead to photodamage [15,21–23]. Interestingly, PCON and qE (both responses to lumen acidification) are expected to have opposing effects on QA redox state. High levels of PCON in the absence of qE would lead to accumulation of plastoquinol (PQH$_2$) and the reduced form of the PSII electron acceptor, QA$^-$, which can potentiate photodamage. Thus, these two processes must be tightly coordinated, with qE being activated at lumen pH somewhat less acidic than PCON [15].

Plants in natural environments are exposed to rapidly changing environmental conditions, especially light, which can change by orders of magnitude in less than a second. It has become clear that rapid and unpredictable fluctuations in light intensity can be more damaging than more gradual changes [22,24–32]. This sensitivity can partly be related to the build-up of reactive redox intermediates and thylakoid $pmf$, which can occur following low-to-high light transitions much more rapidly than the activation of photoprotective NPQ and PCON, leaving the photosynthetic apparatus prone to photodamage. Also, the slow recovery of NPQ following a decrease in light intensity can lead to substantial losses of photosynthetic efficiency [33].

Recently, it has been reported that engineering plants with increased expression levels of VDE and zeaxanthin epoxidase (ZE), resulted in accelerated formation and reversal of qE accompanied by increased plant productivity [3], suggesting that it may be possible to increase yield in crops by modifying photosynthetic regulatory responses.

On the other hand, we lack comprehensive surveys of the range of natural response of photosynthesis to real environmental fluctuations, in part because of a lack of deployable scientific equipment and methods to probe these processes in the field. Consequently, it has not been possible to assess the mechanistic bases of extant natural variations in these processes, their possible benefits or trade-offs, or which of these may be most useful for crop improvement.

Here, we introduce a method and proof-of-concept field data results to address the following questions: Can we assess the extent of natural variations in rapid responses to fluctuations in photosynthetically active radiation (PAR) intensity for both electron flow and photoprotection? How
do these limitations depend on environmental conditions? What are the mechanisms that underlie these variations in responses to rapidly fluctuating light in the field?

We define the term ‘light potential’ (LP) as the plant to respond to sudden increases in PAR, either by using it for productive photochemistry or up-regulating photoprotective mechanisms. In effect, LP is the response of photosynthesis to removal of light limitations. Here, we introduce an approach to both measure and analyse these variations in LP, focusing on one species, *Mentha* sp., under a limited set of conditions, and applied these to testing among a set of mechanisms for modulating that can be distinguished based on a range of optical measurements available using the MultispeQ 2.0 device, including: (i) PSI acceptor-side limitations to electron transfer; (ii) increased NPQ, which limits the input of light energy into photosystem II (PSII); and (iii) PCON, in which acidification of the lumen slows electron transfer at the level of plastoquinol (PQH2) oxidation by the cytochrome *b*6 complex.

The results show that the approach can effectively be used to assess the range of variations in LP under field conditions, as well as to test specific hypothetical models, setting up a broad-scale, multiple-participant, open science approach to exploring the responses across multiple species, genotypes and environments. The results also reveal, at least in *Mentha*, unexpected leaf temperature-dependent limitations in the rapid formation of NPQ that result in the accumulation of reduced PSII electron acceptor, QA, and a high thylakoid pmf, conditions likely to promote the formation of ROS.

2. Material and methods

2.1. Plants and leaf sampling

Measurements were made in a population of *Mentha spicata* (Spearmint) plants that have been maintained at the MSU Horticulture Gardens (East Lansing, MI, USA) for at least 10 years. The GPS locations of all measurements are included in the online dataset (https://photosynq.org/projects/rapid-ps-responses-pam-ecst-npqt-mint-dmk). Although it was not practical to exhaustively capture the lifecycle of the plants, the experimental strategy sampled a sufficiently wide range of conditions to allow clear patterns to emerge in the relationships between response behaviours and environmental parameters, as described below. The experiment took place over a 9-day experimental window between 21 July and 2 August 2019 (electronic supplementary material, figure S1A), sampling a range of times of day (between 5.30 and 18.40 local time), temperatures, etc. (electronic supplementary material, figure S1B). Measurements were made at multiple, alternating canopy levels and positions (subjectively at high, middle and low canopy levels) from early morning to later afternoon (electronic supplementary material, figure S1B), and at multiple locations across the plots on each day. Plants were up to 1 m in height. The leaf area index was not measured in this experiment. For the weather data, see the MSU Enviroweather website (https://mawn.geo.msu.edu/station.asp?id=msu, MSU Horticulture Teaching & Research Center, latitude: 42.6734, longitude: −84.4870, elevation: 264 m).

2.2. Measurements of photosynthetic and related parameters using MultispeQ 2.0

Optical measurements were made using MultispeQ 2.0 hand-held instruments (https://photosynq.com), based on that presented by Kuhlgert *et al.* [34] and calibrated using the CaliQ calibration system (https://photosynq.com/caliq). The LP protocol used in the experiments can be found in the online project information (rapid-ps-responses-with-ecs-fast-ecs-dirk-and-npqt-dmk) as illustrated in figure 1. The protocol was designed to strike a balance among the needs for sampling large numbers of leaves, the desire for detailed spectroscopic measurements and the length of time the plant could be exposed to increased or decreased PAR. The full protocol, with measurements at ambient, after 10 s full sunlight and 10 s dark required about 35–40 s, at the limit of the time scale over which most researchers could steadily clamp a leaf in the instrument. The implications of the 10 s illumination and recovery time are discussed in the Results and Discussion sections.

In the first stage of the protocol (figure 1a), the MultispeQ was programmed to continuously (at about 5 Hz) measure PAR and reproduced these levels using a red actinic LED (655 nm emission peak) illuminating the adaxial surface of the leaf. When the MultispeQ detected that a leaf was clamped in the chamber, a series of measurement sequences was initiated. After a few seconds of illumination at ambient PAR (PARamb) to allow for settling and setting of gains, the first set of measurements was made, estimating at PARamb linear electron flow (LEF) (LEFamb), NPQt (NPQamb) and other photosynthetic parameters (figure 1b).
The actinic light was then increased to approximately full sunlight (2000 µmol m\(^{-2}\) s\(^{-1}\) red light) for 10 s (figure 1c), after which the photosynthetic measurements were repeated (figure 1d), yielding measurements of LEF\(_{\text{high}}\), NPQ\(_{\text{high}}\), etc. We chose full sunlight, rather than an artificially intense super-saturation light, to estimate LPs that could occur in the field, and not the absolute maximum, and to avoid non-physiological or photoinhibitory effects. Thus, the LPs of various processes will be limited as PAR\(_{\text{amb}}\) approaches full sunlight.

The plant was then illuminated with 1000 µmol m\(^{-2}\) s\(^{-1}\) red light, indicating that the air flow in the device was sufficient to prevent substantial CO\(_2\) restriction to the leaves.

Two features of the instrument’s leaf clamp provide free air flow to the outside, preventing the depletion of CO\(_2\) during the measurements. First, there is an approximately 3 mm space (or gap) between the leaf surface and the light guides, allowing lateral air flow. Second, this gap is connected to the external environment via a pair of rectangular (2 x 3 mm) air flow guides on the sides of the light guide, leading to the instrument case, the rear of which is open to the air. We thus do not expect any significant restriction of CO\(_2\) diffusion to the leaf surface. To check this, we compared measurements of 10 separate mint leaves made with an unmodified instrument with 10 made with an additional air pump that provided approximately 300 ml min\(^{-1}\) air exchange in the leaf clamp. We observed no significant differences (p > 0.5) in LEF values measured as above, under ambient (LEF\(_{\text{amb}}\)) or high (LEF\(_{\text{high}}\), 2000 µmol m\(^{-2}\) s\(^{-1}\)) red light, indicating that the air flow in the device was sufficient to prevent substantial CO\(_2\) restriction to the leaves.
2.3. Environmental conditions during light potential measurements in the field

Electronic supplementary material, figure S1A–C shows the distributions of environmental factors (light intensities, leaf temperatures) for the measurements analysed in this study. The MultispeQ sensor was positioned by the user to be parallel to the leaf surface, so that the cosine-corrected PAR sensor should effectively estimate PAR absorbed by the leaf surfaces *in situ* throughout their canopy, and thus the ambient PAR (PAR<sub>amb</sub>) values were dependent on both time of day (diurnal cycle, electronic supplementary material, figure S1B) and by leaf angle (electronic supplementary material, figure S1C). Ambient temperature and leaf temperatures (T<sub>leaf</sub>) were dependent on time of day, with obvious influences from weather-related fluctuations (electronic supplementary material, figure S1A, B). We chose to compare results with T<sub>leaf</sub>, rather than ambient temperature, to better reflect the effects on leaf photosynthetic processes. We note that previous results, e.g. Kuhlgert *et al.* [34], indicate that there may also be significant interactions between canopy position and photosynthetic parameters, though the current experiment did not explicitly record these positions, but rather sampled them as described in Material and methods.

2.4. Data calculations and cleaning

Data from the PhotosynQ platform were reprocessed and cleaned to improve the estimation of decay constants for ECS and near-infrared absorbance changes. As with any field experiments, some results were found to have obvious errors or be out of acceptable ranges, and were removed from the analysis. However, all original data were maintained in the online platform, allowing the reader to explore and reanalyse the effects of our data cleaning procedures. The rules and code for data flagging are defined in the Jupyter Notebook (see electronic supplementary material ‘Data Cleaning Notebook’). A total of 292 points were flagged from a total of 1346 original measurements. The majority of the flagged measurements (179) were due to a defective device. The remaining 113 flagged points can be attributed to user error (e.g. leaf movements during measurements) or poor signal-to-noise that resulted in parameter values outside the theoretical ranges.

3. Results

3.1. Field measurements of photosynthetic parameters under ambient and rapidly elevated PAR

Figure 2a shows LEF measured at PAR<sub>amb</sub> (LEF<sub>amb</sub>) plotted against ambient PAR<sub>amb</sub> and leaf temperature (T<sub>leaf</sub>, see coloration of points). The plots use the square root of PAR to better resolve the results at lower PAR<sub>amb</sub> and to partially linearize the responses. LEF<sub>amb</sub> increased with increasing PAR<sub>amb</sub> with a roughly hyperbolic dependence and an apparent half-saturation point of about 350 µmol photons m<sup>−2</sup> s<sup>−1</sup>, reaching maximum values of about 250 µmol electrons m<sup>−2</sup> s<sup>−1</sup> at 1700 µmol photons m<sup>−2</sup> s<sup>−1</sup>. Upon 10 s of exposure to 2000 µmol photons m<sup>−2</sup> s<sup>−1</sup> increased LEF to generally higher values (LEF<sub>high</sub> figure 2b), indicating that LEF<sub>amb</sub> was at least partly light-limited under all of the conditions. Note that each LEF<sub>amb</sub> point was taken on different leaves at different times (Material and methods) and has corresponding LEF<sub>high</sub> and LEF<sub>high-amb</sub> measurements. The relationship between measurements is illustrated in electronic supplementary material, figure S2, which shows selected pairs of LEF<sub>amb</sub> and LEF<sub>high</sub> connected by vertical line segments. The extent of LEF<sub>high</sub> was not uniform, but appeared to be strongly suppressed at low PAR<sub>amb</sub> and/or low T<sub>leaf</sub>. The high light-induced difference in LEF (LEF<sub>high-amb</sub>) increased with PAR<sub>amb</sub> at low light, reaching a peak at about 200 µmol photons m<sup>−2</sup> s<sup>−1</sup>, above which it declined as PAR<sub>amb</sub> approached PAR<sub>high</sub> and LEF<sub>high</sub> became light-saturated. The suppression of LEF<sub>high</sub> was due to large decreases in the quantum efficiencies of PSII (Phi2, figure 2d). Phi2 at PAR<sub>amb</sub> (Phi2<sub>amb</sub>) were highest at low PAR<sub>amb</sub>, and progressively saturated as light was increased. The opposite behaviour was seen with Phi2 measured after 10 s of high light (Phi2<sub>high</sub>, figure 2d, grey symbols) which was lowest at low PAR<sub>amb</sub> and gradually increased with PAR<sub>amb</sub>.
3.2. Gaussian mixture model clustering analysis of field data

A simple linear effects model applied over the entire dataset (electronic supplementary material, table S1A) indicated strong correlations between LEFamb and both PARamb and Tleaf, suggesting that both environmental factors controlled LEFamb. However, such correlations may be coincidental since PAR and Tleaf are both expected to be dependent on weather or time of day, as is clear from the strong statistical correlations between PAR and Tleaf. Also, the effects are likely to be co-dependent. For example, at low PARamb, LEFamb should be light-limited, and thus have minimal dependence on Tleaf, but at higher PARamb, may be more strongly controlled by temperature-dependent processes.

One approach to disentangling these effects would be to slice the data into segments, e.g. at different ranges of PARamb, and test for correlations with Tleaf within each segment. However, arbitrarily chosen ranges for the segments can add bias, or fail to detect more complex interactions. We thus applied a Gaussian mixture model (GMM) clustering approach based on those presented earlier [41,42]. Because GMM is an unsupervised machine learning method, it can reduce bias in the selection of clusters that represent regions of distinct interactions among environmental and photosynthetic parameters. GMM assumes that the data points from the population of interest are being drawn from a combination (or mixture) of Gaussian distributions with certain parameters, and performs an optimization scheme to a sum of K Gaussian distributions, allowing for a completely unsupervised process, avoiding potential user bias. An expectation–maximization (EM) algorithm was used to fit the GMM to the dataset, generating a series of Gaussian components (clusters) with distributions characterized by specific means and covariance matrices. The optimal number of clusters was determined using the Bayesian information criterion (BIC), the value of the maximized log likelihood, with a penalty on the number of parameters in the model [41–44]. This approach also allows comparison of models with differing
parametrizations and/or differing numbers of clusters, because the volumes, shapes and orientations of the covariances can be constrained to those described by defined models [41].

Clusters obtained through GMM have both within cluster (intraclass) and between cluster (interclass) variations. In order to test for intercluster variation, we used the clustering assignment obtained for one parameter (or response) and applied it on another response. Here we want to investigate what would be the distinctive behaviour of different responses if we have used the same configuration. Using the same set of cluster assignments to different responses, one might be skeptical of the clustering behaviour as responses interact differently with PAR<sub>amb</sub> and T<sub>leaf</sub>. In that case, we might not be able to directly compare the intercluster behaviours of responses. To mitigate this issue, we use the GMM clustering as a tool to create a 'baseline' clustering configuration for one response and use that configuration over other responses. We set up our hypothesis as two responses are similar under the same configuration against they are not. If the interaction pattern of one response with PAR<sub>amb</sub> and T<sub>leaf</sub> changes over the other response, we reject our hypothesis and imply that different configurations of PAR<sub>amb</sub> and T<sub>leaf</sub> interact differently with responses. By doing this we are able to disentangle the effect of PAR<sub>amb</sub> and T<sub>leaf</sub> and infer regarding the intraclass variations as to be a key element to determine variations in the interactions between parameters and variations in environmental conditions, e.g. to assess if a relationship is modulated in different ways under different ranges of conditions. Also, as will be seen in the Discussion, interclass variations (differences in the mean and covariances between clusters) can be used to differentiate distinct patterns of behaviour, or mechanistic interactions, between conditions.

As shown in electronic supplementary material, figure S3, GMM analysis of LEF<sub>amb</sub>, PAR<sub>amb</sub> and T<sub>leaf</sub>, found six distinct, compact clusters that differed in the mode of interaction among the photosynthetic and environmental parameters. Encompassing points with lower PAR<sub>amb</sub> showed strong (clusters 1, 2, 4 and 5) dependence of LEF<sub>amb</sub> on PAR<sub>amb</sub> with little contributions from T<sub>leaf</sub>. By contrast, two clusters (3 and 6), which included points at higher PAR<sub>amb</sub>, showed substantial dependencies on both PAR<sub>amb</sub> and T<sub>leaf</sub>. These results are consistent with LEF being predominantly light-limited at low ambient PAR, but increasingly limited by temperature-dependent processes at higher PAR. The presence of these two classes of clusters indicates that PAR<sub>amb</sub> and T<sub>leaf</sub> are likely to affect LEF<sub>amb</sub> in independent ways. The fact that the shapes of the clusters were not determined with individual slicing under the individual parameters for PAR<sub>amb</sub> and T<sub>leaf</sub> but with a co-dependence on both PAR<sub>amb</sub> and T<sub>leaf</sub> suggests that, under some conditions, these effects interact, e.g. T<sub>leaf</sub> may affect the dependence of LEF<sub>amb</sub> on PAR<sub>amb</sub>. GMM identified five distinct clusters for interactions among LEF<sub>high</sub>, PAR<sub>amb</sub> and T<sub>leaf</sub> (electronic supplementary material, figure S4). In contrast to the results on LEF<sub>amb</sub> clusters at lower PAR<sub>amb</sub> (1, 2 and 4) showed LEF<sub>high</sub> dependencies on both T<sub>leaf</sub> and PAR<sub>amb</sub>, while cluster 3 showed correlations with T<sub>leaf</sub>, but not with PAR<sub>amb</sub>. The stronger dependence on T<sub>leaf</sub> of LEF<sub>high</sub> compared with LEF<sub>amb</sub> implies that the exposure to high light revealed additional rate limitations in LEF<sub>high</sub> that were more strongly controlled by both T<sub>leaf</sub> and PAR<sub>amb</sub> and that, at least under some conditions, these effects were independent of each other.

3.3. Analysis of NPQ

NPQ, measured under PAR<sub>amb</sub> (NPQ<sub>amb</sub>, figure 3a) showed a positive correlation to PAR<sub>amb</sub> with an apparent tendency for smaller values at lower T<sub>leaf</sub>. NPQ<sub>amb</sub> showed considerable variations, compared with LEF<sub>amb</sub> even at low PAR<sub>amb</sub> consistent with the idea that NPQ is governed not only by PAR but by metabolic, developmental or other environmental parameters.

Figure 3b shows NPQ<sub> </sub>values measured at 10 s full sunlight (NPQ<sub>high</sub>). The NPQ LP, or light-induced differences in NPQ (NPQ<sub>high-amb</sub>) are shown in figure 3c. While NPQ<sub>high-amb</sub> was always positive, both NPQ<sub>high-amb</sub> and NPQ<sub>high</sub> were suppressed at low PAR<sub>amb</sub> or T<sub>leaf</sub>. NPQ measured after the 10 s dark recovery period (NPQ<sub>rec</sub>, figure 3f) was consistently lower than NPQ<sub>amb</sub> and NPQ<sub>high</sub>. The difference between NPQ<sub>amb</sub> and NPQ<sub>rec</sub> (NPQ<sub>amb-rec</sub>, figure 3d) ranged from slightly negative at low PAR<sub>amb</sub>, where the majority of NPQ<sub>amb</sub> was rapidly reversible, to about one at the higher PAR<sub>amb</sub>, where about half of NPQ<sub>amb</sub> was rapidly reversed.

Overall, these results indicate that a large fraction (in many cases the majority) of NPQ<sub>amb</sub> as well as NPQ<sub>high</sub> recovered within 10 s of darkness and can probably be attributed to qE, and thus, under our conditions, qE is likely to be the most important form of NPQ for rapid adjustments to photoprotection. The residual, more slowly reversible, components reaching a little above 2 are likely to include qI or qZ [45,46], although the limited time frame for the protocol does not allow us to rule out contributions from longer-lived qE. It is also important to consider that the fraction of light
energy dissipated by the NPQ, i.e. $\Phi_{NPQ}$, will also depend on the fraction of PSII in open states [37], which will also be impacted by ambient and fluctuating light, $T_{leaf}$ and other factors.

As with LEF, a simple linear effects model (electronic supplementary material, table S1B) showed strong interactions between $T_{leaf}$ and $PAR_{amb}$ on NPQ$_{amb}$, and the corresponding GMM analysis identified four clusters (electronic supplementary material, figure S5). Cluster 1, which encompassed the lowest range of $PAR_{amb}$ values, showed strong dependence on $PAR_{amb}$, with no significant dependence on $T_{leaf}$. The remaining clusters showed either dependence solely on $T_{leaf}$ (cluster 4) or co-dependence on $PAR_{amb}$ and $T_{leaf}$ (clusters 2 and 3). Because GMM clustering suggests that $T_{leaf}$ and $PAR_{amb}$ can interact or act independently, depending on conditions, we excluded the linear effects models and focused on GMM for analyses of the remaining parameters.

For the analysis of NPQ$_{high}$ (electronic supplementary material, figure S6), we used the clusters found for NPQ$_{amb}$ (electronic supplementary material, figure S5), allowing us to directly compare changes in correlations among parameters within each cluster [41]. Cluster 1, which encompassed the lowest range of $PAR_{amb}$ values, showed strong dependence of NPQ$_{high}$ on both $PAR_{amb}$ and $T_{leaf}$. This pattern of

![Figure 3](https://royalsocietypublishing.org/doi/abs/10.1098/rsos.211102)
dependencies was in contrast to that for cluster 1 for NPQamb, which showed dependence solely on PARamb.

At a higher range of PARamb (cluster 3), NPQhigh showed significant dependence solely on Tleaf, again in contrast to the corresponding cluster for NPQamb, which showed dependencies on both PARamb and Tleaf. Overall, compared with NPQamb, NPQhigh showed increased dependence on Tleaf in all clusters, suggesting that it is more substantially controlled by metabolic or physiological factors (see Discussion).

3.4. The redox state of QA

Figure 4 shows the dependencies of QA redox state (qL) on PAR and Tleaf. qL measured at PARamb (qLamb, figure 4a), was relatively constant (ranging from about 0.3 to 0.75) across PARamb, with somewhat higher values at both extremes of PARamb. Lower leaf temperatures appeared to be associated with lower qL values, over the entire range of PARamb, although the effect was particularly pronounced at low light. By contrast, qL measured at 10 s of high light (qLhigh, figure 4b) showed strong dependence on PARamb, ranging from near zero (fully reduced QA) at low PARamb to almost one (fully oxidized) at higher PARamb. Again, low Tleaf appeared to correlate with lower qL-high.
throughout the range of PAR\textsubscript{amb}. Strikingly, as shown in figure 4, the high light treatment induced two distinct effects: at low PAR\textsubscript{amb} and/or T\textsubscript{leaf}, it induced a net reduction of QA, while it had the opposite effect at higher PAR\textsubscript{amb} and T\textsubscript{leaf}.

GMM clustering for qL\textsubscript{amb}, PAR\textsubscript{amb} and T\textsubscript{leaf} (electronic supplementary material, figure S7) identified four distinct clusters. In cluster 2, which encompasses points at low PAR\textsubscript{amb}, significant associations were observed only between qL\textsubscript{amb} and PAR\textsubscript{amb}. Clusters 1, 3 and 4 (at higher PAR\textsubscript{amb}) showed co-dependencies between qL\textsubscript{amb} and both PAR\textsubscript{amb} and T\textsubscript{leaf}. GMM clustering for qL\textsubscript{high}, PAR\textsubscript{amb} and T\textsubscript{leaf} showed five distinct clusters (electronic supplementary material, figure S8). Clusters 1, 2 and 5, which encompassed generally lower ranges for PAR\textsubscript{amb} and T\textsubscript{leaf} showed qL\textsubscript{high} dependencies on both PAR\textsubscript{amb} and T\textsubscript{leaf}. Clusters 3 and 4 (generally with higher PAR\textsubscript{amb} and T\textsubscript{leaf} values) showed only dependencies on T\textsubscript{leaf}. The overall pattern of cluster behaviour was similar to that observed with respect to NPQ\textsubscript{amb} and NPQ\textsubscript{high}.

3.5. P700 redox state

Figure 5 shows the extent of oxidized P\textsubscript{700}\textsuperscript{+} (P\textsuperscript{+}), based on the DIRK of absorbance changes at 810 nm. P\textsubscript{700}\textsuperscript{+} at PAR\textsubscript{amb} (P\textsuperscript{+}\textsubscript{amb}, figure 5\textit{a}), after 10 s of high light (P\textsuperscript{+}\textsubscript{high}, figure 5\textit{b}) and the light-induced difference (P\textsuperscript{+}\textsubscript{high-amb}, figure 5\textit{c}). The extent of P\textsuperscript{+}\textsubscript{amb} was nearly linearly related to PAR\textsubscript{amb}. Increasing the light resulted in higher P\textsuperscript{+} values (P\textsuperscript{+}\textsubscript{high}), indicating that, in all cases, PSI became more oxidized at high light. The extent of the light-induced oxidation was dependent on PAR\textsubscript{amb} with lower extents at low PAR\textsubscript{amb}, and a peak at about 200–300 µmol photons m\textsuperscript{-2} s\textsuperscript{-1}. The decrease at higher PAR\textsubscript{amb} was probably due to the accumulation of pre-oxidized P\textsubscript{700} prior to the high light treatment.

The full extent of P\textsuperscript{+}\textsubscript{high} was relatively constant over the conditions, suggesting that high light was able to nearly fully oxidize P\textsubscript{700}. However, there was a slight trend to lower P\textsuperscript{+}\textsubscript{high} at the highest PAR\textsubscript{amb} or T\textsubscript{leaf}, suggesting that total oxidizable PSI may have decreased at high light or temperatures, perhaps reflecting accumulation of PSI photodamage or electron sink limitations. Consistent with these general trends, GMM analyses of P\textsuperscript{+}\textsubscript{amb}, PAR\textsubscript{amb} and T\textsubscript{leaf} identified four distinct clusters (electronic supplementary material, figure S9), with dependencies on either PAR\textsubscript{amb} by itself (clusters 3 and 4), or both PAR\textsubscript{amb} and T\textsubscript{leaf} (clusters 1 and 2). GMM clustering for P\textsuperscript{+}\textsubscript{high} identified five distinct clusters (electronic supplementary material, figure S10), that showed a positive dependency of P\textsuperscript{+}\textsubscript{high} on either PAR\textsubscript{amb} (cluster 1), or T\textsubscript{leaf} (cluster 5), or a small, negative dependence on T\textsubscript{leaf} (cluster 3).

3.6. EC\textsubscript{St} and thylakoid pmf

Figure 6 shows dependencies of relative thylakoid pmf, estimated by normalized EC\textsubscript{St} measurements, at ambient PAR (EC\textsubscript{St}\textsubscript{amb}, figure 6\textit{a}) and after 10 s exposure to high light (EC\textsubscript{St}\textsubscript{high}, figure 6\textit{b}). The high light-induced differences (EC\textsubscript{St}\textsubscript{high-amb}) are shown in figure 6\textit{c}. EC\textsubscript{St}\textsubscript{amb} showed strong, positive correlations with PAR\textsubscript{amb}, similar to the responses of NPQ\textsubscript{amb} (figure 3\textit{a}) and P\textsuperscript{+}\textsubscript{amb} (figure 5\textit{a}). EC\textsubscript{St}\textsubscript{high} values were, in general, larger than EC\textsubscript{St}\textsubscript{amb}, resulting in positive values for EC\textsubscript{St}\textsubscript{high-amb}. At low PAR\textsubscript{amb}, EC\textsubscript{St}\textsubscript{high} showed high variability, suggesting that the response is strongly dependent on other factors, but appeared to saturate (flatten) at higher PAR\textsubscript{amb}. These behaviours were reflected in EC\textsubscript{St}\textsubscript{high-amb}, which showed strong variability at lower PAR\textsubscript{amb} or T\textsubscript{leaf}, peaked at about 50–100 µmol photons m\textsuperscript{-2} s\textsuperscript{-1}, and saturated at higher PAR\textsubscript{amb}.

GMM analysis of EC\textsubscript{St}\textsubscript{amb} identified five distinct clusters (electronic supplementary material, figure S11). The cluster at the lowest range of PAR\textsubscript{amb} (cluster 1) showed dependence primarily on PAR\textsubscript{amb}. The remaining clusters showed positive correlations between EC\textsubscript{St}\textsubscript{amb} and PAR\textsubscript{amb} but negative correlations with T\textsubscript{leaf}. By contrast, GMM of EC\textsubscript{St}\textsubscript{high} (electronic supplementary material, figure S12) showed almost no dependence on either PAR\textsubscript{amb} or T\textsubscript{leaf} except at the lowest PAR\textsubscript{amb} (cluster 1) which showed negative correlations with PAR\textsubscript{amb} and positive correlations with T\textsubscript{leaf}.

4. Discussion

4.1. Using PhotosynQ and MultispeQ to sample and resolve the effects of environmental fluctuations on photosynthetic processes

The MultispeQ measurements described above were designed to explore the photosynthetic responses of plants in a natural, fluctuating environment. In this type of field experiment, it is not possible to control
all variables. Rather, the strategy was to ‘sample’ responses under as many conditions as practical, while recording key metadata so that subsequent analyses can assess the impacts of various environmental fluctuations. Thus the observed trends may reflect both primary and acclimatory factors that change (or accumulate) over different time scales. Correlations that appear in such analyses can be used to test, at least to some extent, certain models, though it is important to note that more controlled experiments will be needed to fully determine cause–effect relationships, as discussed below.

A major outcome of the experiment is that, despite the fact that measurements were made over many plants, times, etc., clear patterns of responses emerged that allow us to make some broad conclusions about the responses of photosynthesis to ambient and rapidly changing light. For example, the majority of NPQ_{high} was found, in general, to be rapidly reversible, suggesting that qE was the major contributor; at lower PAR_{amb}, that majority of NPQ_{high} was rapidly induced (figure 3c), while at higher PAR_{amb} pre-existing NPQ was rapidly recoverable (figure 3e).

Another important trend was the suppression of the LPs of both LEF (figure 2) and NPQ (figure 3) under some conditions, particularly under lower PAR_{amb} and/or T_{leaf}. Further, strong decreases in LEF_{high} were not always accompanied by compensatory increases in NPQ_{high}, implying that the

Figure 5. The light and temperature dependencies of the redox state of P700^{+}. The redox state of P700 was measured using DIRK at 810 nm absorbance change (a) under ambient light (P^{+}_{amb}), (b) at 10 s of high light (P^{+}_{high}), and (c) the change in P^{+} between high and ambient PAR (P^{+}_{high-amb}) as a functions of the square root of ambient PAR.
productive and photoprotective LPs can be simultaneously suppressed under certain conditions, a situation that is likely to promote the formation of ROS and photodamage (see also below), with important implications for understanding the environmental robustness of photosynthesis [29].

4.2. Disentangling interacting environmental impacts on photosynthetic processes

A key challenge to the field experiment approach is in teasing apart effects from different environmental factors, especially considering that such factors may be co-dependent or interact with each other in complex ways. For example, in visual inspection, most of the parameters show apparent dependencies on both PAR_{amb} and T_{leaf} (e.g. figures 2–6) but, because increases in T_{leaf} are often correlated with increases in PAR_{amb}, the effects of the two parameters may have been coincidental. It may also be that the environmental parameters interacted in complex ways, e.g. high PAR_{amb} may have exacerbated the effects of low T_{leaf}. To address these issues, we applied an approach based on GMM to identify

Figure 6. The light and temperature dependencies of the thylakoid pmf probed using ECS signal. The pmf was measured using ECS (a) under ambient light (ECS_{amb}), (b) at 10 s of high light (ECS_{high}), and (c) the change in ECS between high and ambient PAR (ECS_{high-amb}) as a functions of the square root of ambient PAR.
clusters representing distinct interactions among parameters. The approach is unsupervised, thus eliminating potential bias, while allowing us to test for changes in the environmental dependencies among multiple environmental parameters (electronic supplementary material, figures S3–S12).

Analysis of GMM clusters implied that most parameters were dependent on both PAR_{amb} and T_{leaf}, and at least under some conditions these effects are independent, or that one of the two factors predominates. Thus, the effects cannot be explained simply by coincidences between increased PAR and temperatures. Moreover, the non-rectilinear shapes of the clusters suggest that the effects of PAR_{amb} and T_{leaf} were interactive, e.g. changes in T_{leaf} modulated the effects of PAR_{amb} and vice versa. Overall, these interactions are in line with well-known temperature and PAR dependence of photosynthesis, but this type of analyses can reveal the specific combination of conditions that induce distinct behaviours, allowing for assessments of the involvement of specific mechanisms (see below) and to identify genotypic or management impacts on crop resilience and productivity.

At low PAR_{amb}, we expect steady-state photosynthesis to be predominantly light-limited, and thus the effects of T_{leaf} should be low. As light increases, downstream biochemistry should become increasingly limiting. Because downstream energy storage and metabolic processes are likely to be more temperature dependent than photochemistry, this shift may allow us to distinguish between these types of limitations. Such behaviours are apparent in many of the measured parameters, e.g. LEF_{amb}, which was not substantially dependent on T_{leaf} at low PAR_{amb}, but became co-dependent on PAR_{amb} and T_{leaf} at higher PAR_{amb} (figure 2a; electronic supplementary material, figure S3), consistent with a progressive shift from light-limitation to assimilation-limitation. Similarly, NPQ_{amb} was solely dependent on PAR_{amb} in the cluster at low PAR_{amb}, but became increasingly dependent on T_{leaf} as PAR_{amb} increased (figure 3a). This shift is consistent with a control of NPQ_{amb} by PAR (at low PAR_{amb}) and downstream metabolic processes, particularly at higher PAR_{amb}, e.g. due to regulation of the ATP synthase activity or cyclic electron flow (CEF) [47].

By contrast, LEF_{high} and NPQ_{high} showed much greater dependence on T_{leaf}, and these differences were more pronounced when the high light was imposed on leaves at low PAR_{amb} and T_{leaf} i.e. the opposite of what was seen for LEF_{amb} and NPQ_{amb}. Interestingly, the LEF_{high} rates achieved in leaves exposed to lower PAR_{amb} were strongly suppressed below the maximum LEF_{amb} values measured at higher PAR_{amb} (compare figure 2a,b). This behaviour suggests that the suppression of LEF_{high} occurs when abrupt increases in light overwhelm the activation of downstream energy storage and metabolic processes. This is generally consistent with observations that the activities of metabolic enzymes are regulated to match the availability of energy from the light reactions, which involve a large suite of co-regulatory processes, as extensively reviewed elsewhere, (e.g. [16,47–53]), but that these responses lag behind the changes in light. The in situ LP measurements afforded by MultispeQ show that these situations are very likely to occur under many field situations.

These results also imply that accurate estimates of LEF, NPQ and other photosynthetic parameters under natural conditions will require measurements under ambient light, because sudden changes in PAR can lead to severe perturbations in photosynthetic limitations or regulation. Attempts to ‘simplify’ field experiments by setting PAR to some constant value will lead to strong perturbations and the measured values will reflect these perturbations. The effects are vividly demonstrated by the opposite dependencies of Phi_{2amb} and Phi_{2high} on PAR_{amb} (figure 2d), and validate the use of the PAR matching feature of the MultispeQ instrument. Nevertheless, as shown here, the effects of these perturbations can be informative, but care must be taken in extrapolating to the non-perturbed state. It is also important to keep in mind that the rates of acclimatization may vary substantially between species, and that these may be assessed by performing more intensive experiments with variable high light and dark recovery times.

### 4.3. Mechanisms for controlling the light potentials of LEF and NPQ using MultispeQ field data

The rapid reversal of NPQ_{amb} and NPQ_{high} over 10 s of dark indicated that, under our conditions, a large fraction of NPQ is in the form of qE (figure 3b,c), and thus dependent on lumen acidification and subsequent pH-dependent responses. It is important to note, though, that residual NPQ will contribute to decreases in photochemical efficiency, and that the extent of these effects will also be impacted by other factors, including the redox state of QA [37]. Lumen acidification can be controlled by changes in proton influx (through changes in LEF and CEF), proton efflux through the ATP synthase and the partitioning of pmf into electric field (Δψ) and ΔpH components, which in turn, are impacted by metabolic status, as proposed earlier [15,39]. Here, we explore the possible mechanistic bases for these effects, by comparing the correlations among MultispeQ measurements.
Scheme 1 illustrates three basic mechanistic models describing proposed processes that can limit the LPs of photosynthetic and photoprotective mechanisms. The models make qualitative predictions about how the actions of each mechanistic model will impact correlations between measured photosynthetic parameters, and thus can be used as a framework for interpreting the field data introduced in Results. The expected effects on the measured parameters are summarized in scheme 1, which shows specific effects of each model.

**Model 1: PSI acceptor-side limitations** (scheme 1, Model 1) where lack of NADP\(^+\), ferredoxin or other PSI acceptors prevent further LEF. We expect this limitation to result in accumulation of electrons throughout the electron transfer chain, thus resulting in net reduction of QA (decreasing qL) and P\(_{700}^+\) (decreasing the 810 nm absorbance signal). The decreases in proton fluxes associated with back-up of electrons may, in addition, prohibit rapid, light-induced increases in pmf, lumen acidification and qE responses.

**Model 2: Increased NPQ** (scheme 1, Model 2) should decrease delivery of excitation energy to PSII (but not to PSI), resulting in net oxidation of QA (increasing qL) and P\(_{700}^+\) (increased 810 nm DIRK signal). Under some conditions, the NPQ will be rapidly induced by increased pmf and lumen acidification followed by activation of qE, which should be visible as increased NPQ\(_{\text{high-amb}}\). Under other conditions, e.g. at higher PAR\(_{\text{amb}}\) and elevated pmf, the NPQ may already have been induced. If this NPQ is in the form of rapidly reversible qE, it should substantially decay during the 10 s dark recovery period, resulting in increased NPQ\(_{\text{high-rec}}\). More slowly induced or relaxing forms of NPQ, including qI, qZ and long-lived qE, may be also present prior to and throughout the experiment. The forms should register as increases in NPQ\(_{\text{rec}}\), but not in NPQ\(_{\text{high-amb}}\) or NPQ\(_{\text{high-rec}}\), but given that the high light and recovery periods were only 10 s long, our results do not allow us to distinguish among these possible forms.

**Model 3: Photosynthetic control** (PCON, scheme 1, Model 3). PCON results from the slowing of PQH\(_2\) oxidation at the cytochrome b\(_{6}f\) complex as the lumen becomes acidified. If PCON occurs without activation of qE, we expect a net reduction of QA (decreasing qL) but a net oxidation of P\(^+\) (increasing the 810 nm absorbance signal).

The qE and PCON models can be further subdivided \[15,18\]. In most cases, we expect lumen acidification accompanied by elevated pmf, reflected in an increased ECSt signal, which can be induced by increased proton influx into the lumen, due to increased LEF, increased CEF, or decreased conductivity of the thylakoid to protons (\(g_{H^+}\)) by slowing the ATP synthase, all of which can contribute to change in pmf under fluctuating light \[11,18,54,55\]. Alternatively, lumen acidification can also be associated with an increase in the fraction of pmf that is stored as \(\Delta \psi\) and \(\Delta \psi\) [10,16,56]. In this case, acidification may occur with little or no increases in total pmf, or the rates of proton influx [57], though the current field-based data do not allow us to directly distinguish these possibilities.
These models, while not mutually exclusive, will tend to counteract each other, at least within a particular leaf. For instance, PSI acceptor-side limitations will tend to inhibit electron flow, thus decreasing proton flux and $pmf$ generation. On the other hand, the generation of $pmf$ will tend to slow electron flow (through Models 2 or 3), thus preventing the build-up of electrons on PSI electron acceptors. However, it is important to note that, in a survey-type experiment like ours, photosynthesis in different leaves may be limited by distinct processes, and thus any collection of samples may reflect various combinations of the above models.

4.4. Testing models for limitations in light potentials

By plotting MultispeQ parameters against each other, we can test for more detailed patterns of behaviours predicted by the above models. Figure 7 shows that $P^+_{700\text{ high-amb}}$ (high light-induced $P^+_{700}$...
oxidation) was positively correlated with light-induced increases in \( \text{pmf} \) (\( \text{ESC}_{\text{high-amb}} \)). Under all conditions, increasing \( \text{PAR}_{\text{amb}} \) to \( \text{PAR}_{\text{high}} \) resulted in a net oxidation of \( \text{P}_700^+ \), i.e. \( \text{P}^+_{\text{high-amb}} \) was consistently positive. This behaviour is consistent with Models 2 (NPQ) or 3 (PCON), both of which predict a decrease in delivery of electrons from PSII to PSI. By contrast, we did not see evidence for high light-induced net reduction of \( \text{P}_700^+ \), i.e. values of negative \( \text{P}^+_{\text{high-amb}} \) implying that Model 1 was not a major limitation to LEF LP. This does not exclude Model 1 from limiting photosynthesis in different species and conditions, as has been proposed to be important in chilling sensitive plants [58] as well as under pulse light [59] or in mutants that sufficiently acidify the lumen and activate PCON [21,23]. The apparent avoidance of Model 1 (or prevalence of Models 2 and 3) behaviour may reflect the ‘tuning’ of the light reactions to prevent the accumulation of reduced electron acceptors of PSI associated with photodamage [23], and the associated \( \text{O}_2 \) caused by build-up of electrons on PSI [60].

Overall, the behaviours seen in figure 7 are consistent with restrictions in electron flow to PSI imposed by increases in \( \text{pmf} \), most likely through the acidification of the thylakoid lumen. In the case of Model 2 (rapid NPQ), this would be related to the induction of qE, while in Model 3 (PCON), this could be related to slowing of electron flow at the cytochrome \( b_{6f} \) complex.

Figure 8a further investigates this behaviour by plotting the dependence of high light-induced changes in \( \text{P}_700^+ \) (\( \text{P}^+_{\text{high-amb}} \)) with changes in \( \text{QA} \) redox state (\( \text{qL}_{\text{high-amb}} \)). The expected theoretical changes in measurable parameters upon activation of the three models are indicated by the coloured boxes in the figure, and can be related to Models 1–3 in scheme 1:

- **Model 1** (violet box) predicts net reduction of \( \text{P}_700 \) (\( \text{P}^+_{\text{high-amb}} < 0 \)) and net reduction of \( \text{QA} \) (\( \text{qL}_{\text{high-amb}} < 0 \)).
- **Model 2** (red box) predicts net oxidation of \( \text{P}_700 \) (\( \text{P}^+_{\text{high-amb}} > 0 \)) and net oxidation of \( \text{QA} \) (\( \text{qL}_{\text{high-amb}} > 0 \)).
- **Model 3** (blue box) predicts net oxidation of \( \text{P}_700 \) (\( \text{P}^+_{\text{high-amb}} > 0 \)) but net reduction of \( \text{QA} \) (\( \text{qL}_{\text{high-amb}} < 0 \)).

We observe behaviours consistent with both Models 2 and 3, suggesting that the behaviour of the system changed with conditions. Note that the boxes in figure 8a represent ‘pure’ behaviours, and it is possible that the effects of a particular mechanism may be intermediate, e.g. the responses may be limited by a combination of reduction of \( \text{QA} \) and increased NPQ.

Figure 8b plots the dependence of \( \text{NPQ}_{\text{high-amb}} \) which can be attributed to light-induced qE changes, on light-induced \( \text{pmf} \) changes (\( \text{ESC}_{\text{high-amb}} \)). A generally positive correlation was observed between \( \text{NPQ}_{\text{high-amb}} \) and \( \text{ESC}_{\text{high-amb}} \) but with high variability, especially at higher values. Applying the clustering obtained for figure 8a on top of the data in figure 8b, we see that this variability can be explained by the environmental conditions and the modes of behaviours. Specifically, we see clear evidence for condition-dependent suppression of rapid activation of qE in response to increases in \( \text{pmf} \). Particularly, the sensitivities of \( \text{NPQ}_{\text{high-amb}} \) to \( \text{ESC}_{\text{high-amb}} \), as indicated by the slopes in figure 8b, were smallest in clusters 1 (slope \( \sim 1.6 \)) and 2 (slope \( \sim 17.7 \)), which comprise those with Model 3-like behaviour and occurred at low \( \text{T}_{\text{leaf}} \) and \( \text{PAR}_{\text{amb}} \) values. Higher sensitivities of \( \text{NPQ}_{\text{high-amb}} \) to \( \text{ESC}_{\text{high-amb}} \) were seen for clusters 3 (slope \( \sim 28.1 \)) and 4 (slope \( \sim 35.1 \)), which comprised those associated with Models 2 and intermediate, and occurred at higher \( \text{T}_{\text{leaf}} \) and \( \text{PAR}_{\text{amb}} \) values.

To assess what controlled the switch between Models 2 and 3, we performed GMM (using \( \text{qL}_{\text{high-amb}} \), \( \text{P}^+_{\text{high-amb}} \), \( \text{T}_{\text{leaf}} \) as inputs). Four distinct clusters were observed (see symbol colours, figure 8a). Intercuster comparisons show that points in clusters 1 and 2 fell exclusively in the region predicted for Model 3. Cluster 3 fell entirely within the region predicted for Model 2. Cluster 4 extended between these regions, possibly indicating contributions from both mechanisms. The clusters falling in the Model 3 region were associated with relatively low \( \text{T}_{\text{leaf}} \) (figure 8c) and \( \text{PAR}_{\text{amb}} \) (figure 8d), compared with those associated with Model 2 or intermediate behaviours, suggesting that Model 2 prevailed at higher \( \text{T}_{\text{leaf}} \) and/or \( \text{PAR}_{\text{amb}} \), while Model 3 prevailed at lower values. Within the GMM clusters (electronic supplementary material, figure S13), \( \text{qL}_{\text{high-amb}} \) was dependent predominantly on \( \text{T}_{\text{leaf}} \) (cluster 3), \( \text{PAR}_{\text{amb}} \) (cluster 1), or both (clusters 2 and 4). This dependence suggests that \( \text{T}_{\text{leaf}} \) and \( \text{PAR}_{\text{amb}} \) acted either independently or cooperatively, depending on conditions, affecting the propensity for photosynthesis to adopt Model 2 or 3 behaviours. As a first-order test of the robustness of these clusters by re-analysing randomly selected subpopulations of the data. As discussed in the legend to electronic supplementary material, figure S14, we obtained comparable results, i.e. that we would...
interpret in similar ways, with as few subpopulations as small as 25% of the full dataset, suggesting that the clustering approach was reasonably robust.

The data in figure 8 show that, at lower Tleaf and PAR, qE activation was suppressed despite light-induced increases in pmf, and that this behaviour was associated with accumulation of electrons on QA but oxidation of P700 (figure 8a), suggesting that, under these conditions, light-induced increases in ΔpH caused slowing of the cytochrome b$_6$f complex (PCON), but that the qE response lagged behind or was completely suppressed, leading to Model 3 behaviour. It is known that, initially after an abrupt increase in PAR, increased thylakoid pmf is stored as Δψ; conversion of Δψ to ΔpH is controlled by the movement of counterions across the thylakoid membrane, and protonation of lumenal proton buffering groups occurs over the seconds to tens of seconds time scale [25,61–63], and is dependent on the activities of various thylakoid ion transporters [9–11]. However, little is known about the natural diversity of Δψ/ΔpH balancing.

It has been shown that the lumen pH-dependencies of qE and PQH$_2$ oxidation by the cytochrome b$_6$f complex are tightly coordinated, so that increased lumen acidity activates photoprotection prior to

Figure 8. Relationships among measured parameters, predicted model behaviours and clustering. The relationships between light-induced changes in QA redox state and P700 redox state (a), and between rapidly inducible NPQ and thylakoid pmf (b) and the leaf temperature (c) and PAR (d) dependencies of Gaussian mixture models (GMM) clusters. Changes in P700$^+$ (P$_{+}^{\text{high-amb}}$), QA redox state (qL$_{\text{high-amb}}$), rapid changes in NPQ (NPQ$_{\text{high-amb}}$) and thylakoid pmf (ECS$_{\text{high-amb}}$) were measured as described in Material and Methods. Data were clustered using the GMM approach described in the text, resulting in four distinct clusters, designated by the blue, green, red and ochre symbol colours (see legend in a). In (b), the slopes for the relationship between NPQ$_{\text{high-amb}}$ and ECS$_{\text{high-amb}}$ were estimated by linear regression (slopes for clusters 1, 2, 3 and 4 were estimated to be 1.6, 17.7, 28.1 and 35.1, respectively). Panels (c,d) show distributions of (c) leaf temperatures (T$_{\text{leaf}}$) and (d) square root of ambient PAR for each cluster in (a,b).
PCON, presumably to prevent the accumulation of reduced QA [15]. However, these experiments were performed under more slowly changing (near steady-state) conditions in the laboratory, and our results suggest that this coordination can be defeated under real-world conditions in the field, especially when Tleaf is low and PAR fluctuates rapidly. This discoordination can have strong implications for photodamage, as it has been shown that high thylakoid pmf can greatly accelerate PSII recombination reactions, especially when QA is reduced, leading to \( \text{O}_2 \) production [28,29,32]. It thus seems reasonable to suggest that the shift from qE to PCON at low Tleaf will increase the rates of photodamage.

There are several possible mechanisms by which the response of qE can be uncoupled from increased pmf. Longer-term dependencies of NPQ on temperature have been reported under both field [64–66] and laboratory [67,68] conditions. The current work shows effects on rapid NPQ and LEF changes, which can be related to distinct mechanistic models. For example, it is known that the xanthophyll cycle is strongly temperature dependent, though the general observation is that zeaxanthin tends to accumulate at lower temperatures due to a slowing of the epoxidation of zeaxanthin [67–69]. Interestingly, we would expect the accumulation of zeaxanthin to augment, rather than suppress qE responses as we have observed in the current results. Lumen acidification may also be rate limiting for formation of qE. While rapid increase in light can result in nearly instantaneous increases in Δψ, formation of ΔpH and lumen acidification require counterion transport processes, which tend to be slow, and thus lumen acidification lags behind [25,29], and it is possible that this process is substantially slowed at low temperature. Other possible limitations include temperature-dependence of conformational rearrangement of antenna complexes following protonation of PsbS [70,71], which in turn may be related to the interactions among thylakoid proteins, lipids and ultrastructure [12,45,72,73]. The current data do not allow us to discriminate between these models, but the work suggests conditions and species under which such limitations occur, and how they may impact plant productivity or resilience.

5. Conclusion: current limitations and prospects for open science-led efforts to understand and improve photosynthesis

There are intense, ongoing efforts to improve photosynthesis, yet the importance of the responses of photosynthesis under fluctuating, real-world conditions are just now being recognized. In particular, we lack understanding of the extents and impacts of these responses, as well as their mechanisms and genomic control, which will be critical to achieving field-relevant improvements in efficiency and robustness, especially in a changing environment.

Here, we demonstrate methods and tools to assess the light responses of photosynthetic processes under real-world conditions, and use them to explore the factors that limit the capacity of plants to use or dissipate rapidly increased PAR. A major outcome is that, despite the complexities of field environments, clear behavioural patterns can be resolved, as long as the experiment contains a sufficient number of points taken over a large environmental space, and that includes environmental metadata. Such combinations of rapid measurements allowed us to test for various models over broad scales by looking for internally consistent relationships among the various measured parameters. For example, we observed no evidence for Model 1 (limitation at the acceptor side of PSI) behaviour in the current study, but we do not exclude the possibility in different species and/or different environmental conditions. The analysis supports the operation of Model 2, the rapid activation of NPQ resulting in net oxidation of QA and Model 3, the strong activation of PCON, resulting in accumulation of QA. We surmised that Model 2 behaviour would be the most photoprotective, while Model 3 type behaviour would probably lead to photodamage, though we do not have independent endpoint measurements (e.g. yield, growth rates, etc.) to validate that the propensity for Model 3 behaviour has long-term consequences. Further, the models are not exclusive, and there will almost certainly be cases, e.g. cluster 4 in figure 8, where intermediate behaviours will be apparent, either because of co-limitations among multiple processes or heterogeneity between chloroplasts in the leaf samples.

We also emphasize that the data presented here were intended to introduce the approaches and methods, and thus leave a number of questions unanswered, but set up the approach to further study. The origins of these effects may include several classes of processes [31,74] that may differ under different conditions [75], including induction of downstream assimilatory reactions and metabolic pools [76,77], downstream sink reactions [78], redox regulation [79,80], balancing between the production and consumption of ATP and NADPH [1,49], ion homeostasis and regulation of thylakoid...
pmf [25,81], low stomatal aperture that may lead to transient depletion of internal CO₂ levels. Distinguishing these will probably require more detailed phenotyping and biochemical [10,60,82] modelling [31] and genomics and genetics approaches [83].

The accessibility of the tools should allow larger numbers of researchers to answer these types of questions over a broader set of results. This approach was made possible by the combination of several open science advances. Collation of large amounts of data and metadata through the MultispeQ and PhotosynQ platforms [34], allowed us to explore the interdependencies of multiple responses and environmental conditions (metadata). The GMM methods allowed us to explore the interactions among multiple environmental parameters and photosynthetic responses, and test for the participation of distinct mechanistic models to limit the limitations to photosynthesis under field conditions, leading to the identification of distinct limitations in the rapid activation of NPQ and LEF at low temperature. Finally, making all tools, protocols and analytical methods available in directly usable forms, the project can be readily expanded to include multiple environments and species, as well as alternative models.

Data accessibility. Primary data are available on the photosynq.org site under the project ‘rapid-ps-responses-pam-ecst-npqt-mint-dmk’. Data cleaning and analysis code is available in a GitHub repository (https://github.com/protonzilla/Light-Potentials-in-Field).

The data are provided in electronic supplementary material [84].

Authors’ contributions. A.K. and D.M.K. designed the experiments. A.K. and H.T. conducted experiments. A.K., A.C., S.K. and D.M.K. analysed data. A.K., A.C., S.K., T.M. and D.M.K. contributed to the interpretations of data and writing the manuscript.

Competing interests. D.M.K. and S.K. are co-founders of PhotosynQ which maintains the PhotosynQ platforms and distributes and maintains the MultispeQ instruments. The current project was performed independently with no funding to or from the PhotosynQ organization.

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