# Circadian and diel regulation of photosynthesis in the bryophyte *Marchantia polymorpha*

David Cuitun-Coronado[1,2], Hannah Rees[3], Anthony Hall[3], Luíza Lane de Barros Dantas[1], Antony N. Dodd[1*]

1. Department of Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Norwich, UK.

2. School of Biological Sciences, University of Bristol, Bristol, UK.

3. Earlham Institute, Norwich Research Park, Norwich, UK.

* Corresponding author: antony.dodd@jic.ac.uk

Main text: 4772 words. Abstract: 170 words.

## Abstract

Circadian rhythms are 24-hour biological cycles that align metabolism, physiology and development with daily environmental fluctuations. Photosynthetic processes are governed by the circadian clock in both flowering plants and cyanobacteria, but it is unclear how extensively this is conserved throughout the green lineage. We investigated the contribution of circadian regulation to photochemistry in *Marchantia polymorpha*, a liverwort that diverged from flowering plants early in the evolution of land plants. First, we identified in *M. polymorpha* the circadian regulation of several measures of photosynthetic biochemistry (delayed fluorescence, the rate of photosynthetic electron transport, and non-photochemical quenching of chlorophyll fluorescence). Second, we identified that light-dark cycles increase the robustness of the 24 h cycles of photosynthesis in *M. polymorpha*, which might be due to the masking of underlying circadian rhythms of photosynthesis by light-dark cycles. Finally, we used a pharmacological approach to identify that chloroplast translation might be necessary for clock control of light harvesting in *M. polymorpha*. We infer that the circadian regulation of photosynthesis might be well-conserved amongst terrestrial plants.

## Introduction

The rotation of the earth on its axis causes 24-hour cycles in environmental conditions such as light and temperature. These diel cycles are thought to have selected for the evolution of circadian clocks within several Kingdoms of life (Dodd *et al.* 2005, Eelderink-Chen *et al.* 2021, Millar 2016, Ouyang *et al.* 1998, Spoelstra *et al.* 2016). Circadian rhythms are self-sustaining biological cycles that have a period of about 24 h, which are thought to provide a biological measure of the time of day. These rhythms coordinate and sequence processes around the diel cycle, whilst ensuring that responses to environmental cues are appropriate for the time of day.

Circadian rhythms in plants are generated by a molecular oscillator that is formed from a series of transcription-translation feedback loops, which has a predominance of negative feedback steps (Hsu and Harmer 2014). The oscillator is entrained to environmental cues such as light and temperature, which align the phase of the rhythm with the day/night cycle so that it provides an accurate biological measure of the time of day. There is circadian regulation of a variety of key aspects of the physiology of flowering plants, such as stomatal opening, light harvesting, $CO_2$ fixation and starch metabolism (Dakhiya and Green 2022, Dakhiya *et al.* 2017, Dodd *et al.* 2005, Graf *et al.* 2010). Furthermore, the circadian oscillator contributes to the photoperiodic control of flowering time (Hayama and Coupland 2003, Park *et al.* 1999) and the fitness of plants (Dodd *et al.* 2005, Green *et al.* 2002). This means that circadian regulation has a pervasive influence upon physiology, metabolism, and development (Hotta *et al.* 2007, Millar 2016), reflecting the close functional relationship between plants and their fluctuating environments.

Circadian regulation of photosynthesis has been reported in a variety of flowering plants, including *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, *Phaseolus vulgaris*, *Kalanchoë daigremontiana* and *Mesembryanthemum crystallinum* (Dakhiya *et al.* 2017, Davies and Griffiths 2012, Dodd *et al.* 2003, Dodd *et al.* 2004, Dodd *et al.* 2005, Gould *et al.* 2009, Hennessey and Field 1991, Wyka *et al.* 2005). Aspects of photosynthetic physiology that are circadian regulated include the rate of $CO_2$ assimilation, oxygen evolution, and measures of photosynthetic light harvesting reported using chlorophyll fluorescence analysis (Dakhiya *et al.* 2017, Hennessey and Field 1991, Litthauer *et al.* 2015, Schweiger *et al.* 1964). There are also circadian rhythms in stomatal opening, which are functionally separable from rhythms of $CO_2$ assimilation, because rhythms of assimilation continue when the intercellular partial pressure of $CO_2$ is held at a constant level to remove the influence of rhythmic stomatal opening upon assimilation (Hennessey and Field 1991). In addition to flowering plants, aspects of metabolism associated with photosynthesis are circadian regulated in some cyanobacteria and algae (Cano-Ramirez *et al.* 2018, Mitsui *et al.* 1986, Samuelsson *et al.*

1983, Schneegurt *et al.* 1994, Schweiger *et al.* 1964). Despite this evidence for the circadian regulation of photosynthesis, the mechanisms and evolution of this process remain less well understood.

The pervasiveness of circadian regulation of photosynthesis within flowering plants and unicellular photosynthetic organisms might suggest that this process is conserved throughout multicellular plant life, with a potentially ancient evolutionary origin. One approach to examine this idea is to investigate plant species that diverged from flowering plants at a relatively early stage of land plant evolution, because this can establish whether the mechanism might originate from a common ancestor. As a model to investigate this question, we selected the bryophyte liverwort *Marchantia polymorpha*. *M. polymorpha* is thought to have diverged from flowering plants about 400 million years ago (Delwiche and Cooper 2015, Kohchi *et al.* 2021), with liverwort-like plants occurring earlier in the fossil record than other land plant forms such as vascular plants (Edwards *et al.* 1995). *M. polymorpha* is a useful model to investigate questions concerning the evolution of circadian regulation because it has a circadian oscillator that shares some components with flowering plants (Lagercrantz *et al.* 2021, Linde *et al.* 2017), there is circadian regulation of a subset of the transcriptome and the position of its thallus lobes (Lagercrantz *et al.* 2021, Lagercrantz *et al.* 2020), and it is a relatively tractable system that can be used in experimental designs comparable to models such as Arabidopsis. Whilst circadian rhythms and some aspects of circadian clock structure have been identified in *M. polymorpha*, it is not yet known whether the regulation of photosynthesis represents a conserved output from its circadian system.

Here, we used *M. polymorpha* as tool to investigate three questions concerning the conservation of circadian regulation of photosynthesis in multicellular plants. First, we investigated whether there is circadian regulation of photosynthesis in *M. polymorpha*, to determine whether this process is conserved in a species that diverged from vascular plants at a relatively early stage of land plant evolution. Second, we investigated whether a circadian clock in *M. polymorpha* might influence the daily timing of a measure of

photosynthesis under cycles of light and dark, to determine whether circadian regulation might contribute to the timing of photosynthesis under diel cycles. Finally, we investigated whether pharmacological approaches might provide insights into the mechanisms that underlie the circadian rhythms of photosynthesis.

## Results

### *Circadian rhythms of photosynthesis in M. polymorpha*

We used two proxies of photosynthetic activity to investigate circadian rhythms of photosynthesis in *M. polymorpha*. These were delayed chlorophyll fluorescence (DF), and prompt fluorescence (PF) measured using the pulse amplitude modulation (PAM) method (Dakhiya and Green 2022, Gould *et al.* 2009, Maxwell and Johnson 2000). When illuminated leaves are transferred to darkness, the potential for electrons to move down the electron transport chain is removed, and energy within the system is instead re-emitted as light and heat. The light emitted within the nanosecond range after the lights turn off is referred to as prompt fluorescence (Dakhiya and Green 2022, Maxwell and Johnson 2000). Light emitted within the millisecond to second range following lights-off is referred to as DF (Gould *et al.* 2009, Rees *et al.* 2019, Strehler and Arnold 1951). Both DF and certain features of PF are circadian-regulated in a range of flowering plants and some algae (Cano-Ramirez *et al.* 2018, Dakhiya and Green 2022, Gould *et al.* 2009, Gyllenstrand *et al.* 2014). One exception appears to be spruce trees (*Picea abies*), where the DF rhythm damps rapidly in accordance with a rapidly damping circadian oscillator (Gyllenstrand *et al.* 2014). Features of PAM fluorescence that oscillate with a circadian rhythm include the apparent quantum yield of PSII (YII), a measure of non-photochemical quenching of chlorophyll fluorescence within PSII (NPQ), and the estimated photosynthetic electron transport rate (ETR) (Cano-Ramirez *et al.* 2018, Dakhiya and Green 2022, Dakhiya *et al.* 2017). Taken together, rhythms of DF and PAM metrics describe circadian regulation of processes of photochemistry over time, as greater energy loss as heat (NPQ) and light (DF) indicate lower quantum yields (Murchie and Lawson 2013).

We investigated whether free-running circadian rhythms of DF occur in *M. polymorpha* under either conditions of constant light (LL) or constant darkness (DD). Rhythmic thalli were identified using Metacycle ($q < 0.001$; period between 18 and 34 hours). Under constant light conditions, 63% of thalli had circadian rhythms of DF that persisted for 4-5 days, and damped over time (Fig 1a, b; Table 1). The mean period of these rhythms under LL was 25.50 h ± 0.52 h (mean ± s.e.m.).

Under conditions of constant darkness, 78% of thalli had circadian rhythms of DF (Table 1; Metacycle $q < 0.001$). However, there was considerable variability between the rhythm of individual thalli, so the mean DF signal from rhythmic thalli had little overt rhythmicity (Fig. 1c, d). In contrast, under cycles of 12 h light and 12 h dark, 100% of thalli were rhythmic (Metacycle $q < 0.001$), and the rhythms were closely synchronized between individual thalli (Fig. 1e, f). Under all three conditions, the rhythm of DF had a peak phase around dusk or subjective dusk, with the mean phase significantly earlier under LD cycles (around 10 h after dawn) compared with constant light or darkness (around 12 h after dawn) (Fig. 1g). This is generally consistent with the phase of rhythms of DF in Arabidopsis, whereas the phase of the rhythm is later in some cereals (Gould *et al.* 2009). The mean period of the rhythm of DF was the same under all conditions tested (Fig. 1h), and the amplitude was much greater under light/dark cycles than under conditions of constant light or constant darkness (Fig. 1i). We reasoned that this greater amplitude might reflect a masking of the underlying circadian rhythm by direct effects of the light/dark cycle upon the biochemistry of DF, or alternatively the rhythms of individual thalli might be better synchronized with each other under LD compared with the other conditions, as is suggested by comparing the DF rhythms of individual thalli under the three conditions (Fig. 1b, d, f).

To investigate this further, we compared the robustness of rhythms of DF under the three light conditions using the parameter of relative amplitude error (RAE), which is derived from analysis of the data by fast Fourier transform-non linear least squares method (FFT-NLLS). RAE provides a measure of the robustness of the oscillation, representing the ratio of the

amplitude error to the amplitude, with a value of 0 indicating a robust and non-damping rhythm, and a value of 1 indicating an absence of rhythmicity, or very noisy data. This identified significantly more robust oscillations of DF under LD cycles compared with constant conditions (Fig. 1j). The mean RAE of rhythms of DF for thalli under LL was 0.46 ± 0.01, which might suggest a slightly lower robustness of the rhythm compared to rhythms of DF in *Arabidopsis* seedlings under the same conditions (RAE= 0.31 – 0.44, depending on accession) (Rees *et al.* 2021). Comparing all rhythmic thalli, those thalli under LD cycles had a narrower range of periods relative to LL and DD conditions, under which individual thalli had a considerable range of period lengths (Fig. 1k). This suggests that whilst a proportion of thalli were rhythmic under conditions of constant light or darkness (Table 1), the rhythm of DF varied considerably between thalli and had lower rhythmic robustness compared with under LD conditions (Fig. 1k). This supports the notion that light input masks or maintains the daily rhythm of DF in *M. polymorpha*, and that these circadian rhythms might have considerable plasticity.

To extend our DF data, we also measured circadian rhythms of photosynthetic activity in *M. polymorpha* using the PAM chlorophyll fluorescence method. Under the conditions of constant blue actinic light within the PAM instrument, there were circadian rhythms in the estimated rate of photosynthetic electron transport (ETR) (Fig. 2a, b) and a measure of non-photochemical quenching of chlorophyll fluorescence within PSII (NPQ) (Fig. 2c, d). These rhythms had a period between 27 h and 28 h (28.10 h ± 0.15 h (ETR), 27.55 h ± 0.38 h (NPQ)) in *M. polymorpha* (Fig. 2b, d). These period estimates are longer compared with the period estimates obtained using DF (Fig. 1h). This might be caused by the different light conditions used with the two methods, because the period of some circadian-regulated processes in *M. polymorpha* depends upon the light intensity (Linde *et al.* 2017). ETR and NPQ oscillated with opposing phases (Fig. 2a, c), which is consistent with other reports (Cano-Ramirez *et al.* 2018) and expected because these measures represent competing fates for light energy absorbed by leaves.

Together with the measurements of DF, we conclude that free-running circadian rhythms of several photosynthetic outputs occur in *M. polymorpha* under constant light conditions. The DF data suggest that free-running rhythms are less robust and more variable under constant darkness, but remain detectable in a substantial proportion of thalli (Table 1). Under light/dark cycles, the similarity of the period estimate to the zeitgeber cycle length (24 h; Fig. 1k), combined with the robustness of individual rhythms (Fig. 1j, k), suggests that under cycles of light and dark, the rhythm of DF is influenced strongly by the light/dark cycle. This indicates that under light/dark cycles, the underlying circadian oscillation (Fig. 1a) is either driven by the light/dark cycle, or the underlying circadian rhythm is masked by the light/dark cycle. This is consistent with the notion that the light/dark cycle has a strong effect upon the diel regulation of the *M. polymorpha* circadian oscillator and its physiological outputs (Linde *et al.* 2017).

*Contribution of circadian regulation in* M. polymorpha *to the timing of rhythms of delayed fluorescence under zeitgeber cycles*

Because we identified that the zeitgeber cycle strongly influences the daily oscillation of DF in *M. polymorpha* (Fig. 1a-f), we wished to investigate the extent of light *versus* clock control of delayed fluorescence in *M. polymorpha* under light/dark cycles. To achieve this, we used an experimental approach whereby the phase of the rhythmic process was monitored under a range of zeitgeber cycle lengths (T cycles; e.g. T24 = 12 h light / 12 h dark; T28 = 14 h light / 14 h dark). This can identify effects of circadian regulation upon the timing of a process under diel cycles, because the circadian oscillator can influence the phase of the process, producing systematic changes in the phase of either the entire waveform (Aschoff 1960, Eelderink-Chen *et al.* 2021, Roenneberg *et al.* 2005), or parts of the rhythm (Dodd *et al.* 2014). We found that oscillations of DF occurred under diel cycles of T20 to T28 (Fig. 3a-d), and the period length of DF matched the T cycle length closely (20.09 h ± 0.06 h (T=20), 22.44 h ± 0.07 h (T=22), 24.36 h ± 0.07 h (T=24) and 28.17 h ± 0.06 h (T=28), measured using FFT-NLLS in Biodare2). When the phase was normalized to the length of the T cycle,

we found that the T cycle length had a significant influence upon the phase, such that the phase was relatively late under the longest T cycle (Fig. 3e). In general, a circadian oscillator would be expected to cause relatively earlier phases under longer T cycles (Aschoff 1960), including in plants (Dodd *et al.* 2014). This did not occur within our experiment (Fig. 3e), where instead the phase followed the zeitgeber cycle length (Fig. 3a-e). This suggests that the effect of the zeitgeber cycle masked the underlying effects of clock control under these conditions.

*Abrogation of circadian rhythms of photosynthesis in* M. polymorpha *by a potential inhibitor of chloroplast translation*

Whilst the canonical circadian oscillator underlies the daily timing of photosynthesis (Dodd *et al.* 2004, Dodd *et al.* 2005), the mechanisms by which the circadian oscillator regulates photosynthesis remain poorly understood. One approach to identify potential cellular processes associated with circadian rhythms of photosynthesis is to use a pharmacological strategy, because several chemicals inhibit specific processes within chloroplasts. We combined a pharmacological approach with the monitoring of circadian rhythms of chlorophyll fluorescence to identify candidate processes associated with the circadian regulation of photosynthesis.

First, we tested whether an inhibitor of chloroplast translation can attenuate circadian rhythms of chlorophyll fluorescence in *M. polymorpha*. Lincomycin is an antibiotic that binds to the 50S subunit of bacteria-like ribosomes (Chang and Weisblum 1967) and is thought to inhibit chloroplast but not mitochondrial protein synthesis (Sullivan and Gray 1999, Zhao *et al.* 2018). We supplemented the growth media with a range of lincomycin concentrations (25 µg $ml^{-1}$, 50 µg $ml^{-1}$ and 100 µg $ml^{-1}$, and a vehicle control), and monitored rhythms of photosynthesis using the PAM fluorescence method (Fig. 4a-l; Fig. S1a-d). There was a dose-dependent effect of lincomycin on the amplitude of circadian rhythms of the estimated rate of photosynthetic electron transport (ETR) and NPQ (Fig. 4f, l; Fig. S1b, d), such that the amplitude of these parameters were reduced by greater lincomycin concentrations (Fig.

4f, l; Fig. S1b, d)). Furthermore, the RAE of circadian rhythms of ETR was significantly greater in the presence of lincomycin compared with the vehicle control (Fig. 4e; Fig. S1a). The effect of lincomycin on the RAE of rhythms of NPQ was less consistent across two experiments (Fig. 4k; Fig. S1c), suggesting that lincomycin might have a greater effect upon the rhythmic robustness of ETR compared with NPQ. These measures of photosynthesis are ratiometric, so the decreased amplitude is unlikely to be caused by a lower fluorescence signal in the presence of lincomycin. Whilst we cannot exclude the possibility of off-target effects of lincomycin in *M. polymorpha*, our data suggest that chloroplast translation might be required to maintain circadian rhythms of photosynthetic light harvesting.

Next, we tested the hypothesis that an inhibitor of chloroplast transcription can attenuate circadian rhythms of photosynthesis in *M. polymorpha.* In Arabidopsis, mutants of a circadian-regulated sigma submit of plastid encoded plastid RNA polymerase (PEP) known as SIG5 can alter the phase of circadian rhythms of delayed fluorescence (Noordally *et al.* 2013). Therefore, we reasoned that chloroplast transcription by PEP might contribute to circadian rhythms of photosynthesis in *M. polymorpha*. To test this, we supplemented the growth media with rifampicin, which is thought to inhibit chloroplast transcription by one form of PEP (Pfannschmidt and Link 1997, Surzycki 1969). We measured circadian rhythms of ETR and NPQ in the presence of several concentrations of rifampicin (Fig. 4m-x; Fig. S2g-l). Across two experiments, there were no consistent differences caused by the range of rifampicin concentrations tested, upon the circadian rhythm of the estimated rate of electron transport or NPQ, under these experimental conditions (Fig. 4q, r, w, x; Fig. S1e, f, g, h).

## Discussion

Here, we present evidence for the circadian regulation of two proxies for photosynthetic activity in *M. polymorpha*. Our experiments cannot prove that circadian rhythms of photosynthesis occurred in early land plants, but the presence of these rhythms in both bryophytes and angiosperms suggests that they might be well-conserved. Given that reporters of photosynthetic activity are also circadian regulated in aquatic photosynthetic

organisms such as cyanobacteria, uni- and multicellular algae (Cano-Ramirez *et al.* 2018, Hastings *et al.* 1961, Okada *et al.* 1978, Schneegurt *et al.* 1994, Schweiger *et al.* 1964, Sorek and Levy 2012, Sorek *et al.* 2013, Sweeney and Haxo 1961), our study suggests that the circadian regulation of photosynthesis was conserved from aquatic ancestors during the terrestrialization of plants. One interpretation is that circadian regulation of photosynthesis confers a selective advantage to all photosynthetic life, although the nature of the advantage and degree of selection pressure for this might depend upon the ecological niche occupied by the organism (Hellweger *et al.* 2020).

Our experiments identified that under constant light conditions, the period of the rhythm of delayed fluorescence was not uniform through our experiments, with considerable variation between individual replicate thalli (Fig. 1a-c). In comparison to *M. polymorpha*, the circadian rhythm of DF in Arabidopsis and other flowering plants is relatively stable, with a period of about 24 h (Gould *et al.* 2009). Other reporters for circadian rhythms in *M. polymorpha* are also less robust than in flowering plants. For example, rhythms of some promoter-luciferase reporters damp quite rapidly and present a range of periods across replicates, and this difference in robustness is evident when circadian rhythms in *M. polymorpha* and Arabidopsis are compared (Linde *et al.* 2017). One potential explanation for this difference between *M. polymorpha* and flowering plants might relate to circadian oscillator structure (Linde *et al.* 2017). The circadian oscillator in *M. polymorpha* has been proposed to include a smaller number of components than the oscillator of Arabidopsis, with greater levels of oscillator complexity thought to confer robustness to perturbation (Shalit-Kaneh *et al.* 2018, Troein *et al.* 2009). Therefore, the less robust circadian oscillations of photosynthesis that we report here might arise from lower levels of redundancy within a less complex circadian clock in *M. polymorpha* compared with Arabidopsis. An alternative explanation for this difference might relate to differences in the strength of coupling of circadian rhythms between cells and across tissues between Arabidopsis and *M. polymorpha*. Although there is weak intercellular coupling of circadian rhythms in Arabidopsis (Wenden *et al.* 2012),

differences between the circadian period of individual cells means that the rhythms of cell populations become progressively more desynchronized over time in the absence of intercellular coupling, leading to an apparent damping of the rhythm (Gould *et al.* 2018, Paajanen *et al.* 2021). Therefore, the rhythms might damp more rapidly in *M. polymorpha* if intercellular coupling of circadian rhythms is weaker in *M. polymorpha* compared with Arabidopsis, especially when combined with a potentially less robust circadian oscillator within each cell. This could explain why individual thalli appear to desychronize under free running conditions (Fig. 1b), whereas under zeitgeber cycles the tissues become resynchronized on a daily basis (Fig. 1f). The idea of relatively unstable circadian rhythms in the bryophytes is also supported by DF data from *Physcomitrium patens*, which is arrhythmic under constant light and has a noisy rhythm under low light conditions (Gyllenstrand *et al.* 2014).

The thallus of *M. polymorpha* lacks stomatal pores for gas exchange and, instead, has air pores in its surface that are formed by a channel passing through a cylindrical structure of 16 cells (Jones and Dolan 2017, Shimamura 2016). Unlike the stomata of flowering plants, the air pores of *M. polymorpha* are thought to be static structures that do not adjust in response to environmental cues. Therefore, circadian rhythms of the reporters of photosynthesis described here might be due entirely to the circadian regulation of the biochemical reactions of photosynthesis, rather than changes in the rate of $CO_2$ and $O_2$ exchange with the atmosphere. Nevertheless, we cannot rule out alterations in conductivity to gas exchange through diel or circadian changes in cell turgor or potential “motor cells” within liverwort air pores (Walker and Pennington 1939). Such changes might influence the measures of photosynthesis that we used, because the presence of the pores and air spaces within the thallus appears to reduce its resistance to $CO_2$ diffusion (Green and Snelgar 1982).

The reduction in robustness of rhythms of ETR caused by lincomycin could suggest that chloroplast translation is necessary for circadian rhythms of this reporter of photosynthesis. Lincomycin inhibits the synthesis of PSII D1, PSII D2 and RbcS proteins, and the expression

of photosynthesis-associated nuclear encoded genes (PhAnGs) in angiosperms (Bachmann *et al.* 2004, Karpinska *et al.* 2017, Mulo *et al.* 2003). Perhaps inhibiting the expression of these proteins prevents or reduces PSII repair, which disrupts circadian cycles of the rate of electron transport by interfering with the electron transport process. Whilst this suggests that protein synthesis in chloroplasts is necessary for the circadian regulation of photosynthesis, it does not identify that this is a rate-limiting regulatory step that underlies the clock control of the process. It also supports the idea that *M. polymorpha* represents an informative model for investigation of mechanisms underlying the circadian regulation of physiology, particularly considering the range of gene-editing tools available.

Whilst the apparent reduction of robustness of circadian rhythms in *M. polymorpha* might increase the susceptibility of rhythms in *M. polymorpha* to environmental perturbation (Linde *et al.* 2017), it is alternatively possible that a circadian oscillator with greater plasticity might confer selective advantages. For example, plasticity of circadian regulation of the daily photosynthetic cycle of CAM plants, and of carbohydrate metabolism in Arabidopsis, are both thought to allow better alignment between metabolic processes and daily cycles of energy availability (Borland *et al.* 2011, Dodd *et al.* 2002, Graf *et al.* 2010, Webb *et al.* 2019). In comparison, very little is known about diel cycles of metabolism in *M. polymorpha*, or how circadian regulation might confer fitness to *M. polymorpha*. To provide a better context for understanding these findings, it might be valuable in future to investigate the regulation and adaptive functions of the circadian system in *M. polymorpha* under conditions more representative of its natural habitat, or in naturally occurring populations of *M. polymorpha*.

## Materials and Methods

### *Plant material and growth conditions*

Tissue of *Marchantia polymorpha* subsp. *ruderalis* (Tak-1 background) was cultivated under sterile conditions, using half-strength Gamborg B5 media containing 0.8% (w/v) agar at pH 5.8 (Solly et al. 2017). Cultivation occurred in Panasonic MLR-352 growth chambers, under

cycles of 12 h light / 12 h darkness, at 19 °C, under 100 µmol photons $m^{-2}$ $s^{-1}$ of white light. For inhibitor experiments, lincomycin (Alfa Aesar) or rifampicin (Melford Laboratories Ltd) were added for the PAM chlorophyll fluorescence experiments at concentrations of 25, 50 and 100 µg/ml, with a dimethylsulfoxide (DMSO) vehicle control where necessary.

For DF experiments, gemmae (Shimamura 2016) were positioned on nutrient agar and cultivated for 14 days prior to the start of the experiment. For PAM fluorescence experiments, pieces of 21-day old thallus were cut into square sections under sterile conditions and allowed to regenerate for 6 days. Preparation of the thallus as square shapes prevented hyponasty during these experiments. For both DF and PAM experiments, plants were entrained under conditions described above prior to imaging.

*PAM chlorophyll fluorescence measurement*

Circadian time-series of chlorophyll fluorescence measurements were obtained using an IMAGING-PAM M series chlorophyll fluorescence imaging system (Walz GmbH, Effeltrich, Germany), using a protocol described previously (Cano-Ramirez *et al.* 2018). Briefly, rapid light curves (RLCs) were obtained at 2 hour intervals, with the plants maintained under constant blue light at other times during the timecourse. Each RLC was acquired over a period of 6 minutes, which was preceded by a 20 minute period of dark adaptation. For the acquisition of each RLC, a saturating light pulse (845 µmol $m^{-2}$ $s^{-1}$) was applied, and followed by an increase in actinic light across 16 steps, at 20 second intervals, to a maximum of 789 µmol $m^{-2}$ $s^{-1}$. After acquiring the RLC, samples were held under continuous blue light (15 µmol $m^{-2}$ $s^{-1}$) until the next measurement cycle. PAM chlorophyll fluorescence parameters were measured at 108 µmol $m^{-2}$ $s^{-1}$ actinic light on the RLC, which was similar to the growth and entrainment conditions (100 µmol $m^{-2}$ $s^{-1}$).

*Measurement of delayed fluorescence*

Delayed fluorescence analysis was performed as described previously (Gould *et al.* 2009, Rees *et al.* 2019). This involved image capture with Retiga LUMO cameras (QImaging,

Canada), fitted with Xenon 0.95/25 mm lenses (Scheneider-Kreuznach, Germany). Plants were illuminated with mixed red/blue LED arrays (approx. 60 µmol $m^{-2}$ $s^{-1}$) under the control of the µManager Software (v1.4.19, Open Imaging) and an Arduino Uno microcontroller board. The camera and LEDs were mounted in growth chambers maintained at 19 °C (Sanyo MIR-553). The camera settings for all experiments were Binning = 4, Gain = 1, and Readout-Rate=0.650195 MHz, 16 bits. Three control scripts were used for DF measurement in *Marchantia polymorpha*. The constant light (LL) script was a regime of 59 minutes of light followed by one minute of image acquisition, the constant darkness (DD) script was 54 minutes of darkness followed by five minutes of light and one minute of image acquisition, and the neutral day (LD) script was an alternating combination of LL and DD scripts that swapped every half T-cycle. Exposure of plants to 5 minutes of light before image acquisition under both continuous darkness and the dark period of LD cycles was necessary to obtain the DF signal, and might be perceived by plants as a low fluence response equivalent to an average PAR of approximately 3 µmol $m^{-2}$ $s^{-1}$ over each hour. All experiments started at entrained dawn, and finished after seven days of imaging. T-cycle experiments employed the LD scripts, but using daily periods of 20 h (10L/10D), 22 h (11L/11D) and 28 h (14/14D), respectively. For analysis, images were imported into FIJI (version 1.53c) and regions of interest were selected and extracted using the multi-measure plugin to measure integrated density of each region. The DF data were baseline and amplitude (BAMP) de-trended using Biodare2, and a moving average was used to smooth traces from individual thalli.

*Data analysis*

Rhythmic features of the data were identified using the Fast Fourier Transform-Non-linear least squares method (FFT-NLLS) algorithm within the Biodare2 suite (Zielinski *et al.* 2014), and also with Metacycle (Wu *et al.* 2016). Statistical analysis on derived data was conducted using IBM SPSS Statistics v24 and SigmaPlot v14 for PAM chlorophyll fluorescence and DF data. Graphs were prepared using R, with a combination of the ggplot2, scales, rhesape2, cowplot, png, grid, RColorBrewer, wesanderson, colorspace, ggpubr, ggrepel, and

patchwork packages. Heat maps were created using the R package Pheatmap. A 5h moving average was applied to DF and PAM fluorescence data before analysis (Gould *et al.* 2009).

**Acknowledgements**

We thank our funders: UKRI-BBSRC Institute Strategic Programme (ISP) Genes in the Environment (BB/P013511/1), Core Strategic Programme Grant 901 (Genomes to Food Security BB/CSP1720/1) and its workpackage 902 BBS/E/T/000PR9819 (Regulatory Interactions and Complex Phenotypes), Designing Future Wheat ISP (BB/P016855/1; 904 BBS/E/T/000PR9783 (WP4 Data Access and Analysis), and Conacyt (Mexico) (CVU: 510730, Scholarship: 472284). We thank Jill Harrison (Bristol), Ryuichi Nishihama (Kyoto) and Takayuki Kohchi (Kyoto) for advice about experimentation with *M. polymorpha*, and Martha Merrow (LMU), Zheng Eelderink-Chen (LMU) and Jasper Bosman (Hanze Univ.) for advice about data analysis and interpretation.

**Experiment 1**

| Condition | Number of thalli | Number of rhythmic thalli | Rhythmic thalli (%) |
|---|---|---|---|
| LL | 50 | 33 | 66 |
| DD | 50 | 42 | 84 |
| LD | 50 | 50 | 100 |

**Experiment 2**

| Condition | Number of thalli | Number of rhythmic thalli | Rhythmic thalli (%) |
|---|---|---|---|
| LL | 50 | 30 | 60 |
| DD | 50 | 36 | 72 |
| LD | 50 | 50 | 100 |

**Experiments combined**

| Condition | Number of thalli | Number of rhythmic thalli | Rhythmic thalli (%) |
|---|---|---|---|
| LL | 100 | 63 | 63 |
| DD | 100 | 78 | 78 |
| LD | 100 | 100 | 100 |

**Table 1.** Comparison of number of *M. polymorpha* thalli having a circadian or diel rhythm of delayed fluorescence under each light condition, from two independent experiments. Data were classified as rhythmic when the Metacycle q-value was < 0.001 and period lengths between 18 h and 34 h. The abbreviations LL, DD and LD represent conditions of constant light, constant darkness and 12 h light / 12 dark cycles, respectively.

## Figure legends

**Figure 1.** Circadian and diel rhythms of delayed fluorescence of *Marchantia polymorpha* thallus under (a, b) constant light, (c, d) constant darkness, and (e, f) light/cycles. (a, c, e) indicates the mean DF signal from thalli that were categorized as rhythmic after filtration with Metacycle (*q*-value < 0.001; period between 18 h and 34 h). (b, d, f) Heat maps summarize DF intensity of 50 individual and randomly selected thalli across two experimental repeat experiments under (b) constant light, (d) constant dark and (f) light/dark cycles. Data in (a, c, e) are smoothed with a 5-hour moving average, baseline and amplitude de-trended, and normalized to the mean signal. On heat maps, colour values are scaled per row using Z-score normalization. (g) Mean circular phases relative to dawn (CT0) of rhythmic thalli under each condition (constant light (LL; red), constant dark (DD; blue) and diel cycles (LD; orange) calculated using FFT-NLLS. (h) Mean period, (i) amplitude, and (j) RAE for rhythmic thalli under each condition, derived from FFT-NLLS analysis of data. Statistical significance of differences between conditions are indicated by letters above each bar (ANOVA with Tukey's post-hoc test, $p < 0.05$), error bars show SEM. (k) period estimate and relative amplitude error (RAE) of individual thalli under three light conditions, obtained using FFT-NLLS. Data derive from 100 individual thalli, across two independent experimental replicates, for each condition.

**Figure 2.** Circadian rhythms of modulated chlorophyll fluorescence in thallus of *Marchantia polymorpha*. Under constant blue light conditions, time-course analysis of (a, b) the estimated rate of photosynthetic electron transport rate (ETR), and (c, d) non-photochemical quenching of PSII fluorescence (NPQ). (a, c) are the normalized signal level for each chlorophyll fluorescence parameter, processed using amplitude and baseline detrending and normalized to the mean (using Biodare2). (b, d) compare the estimated period and relative amplitude error (RAE) of the rhythmic thalli, with period, RAE and the presence of rhythmicity quantified using FFT-NLLS (using Biodare2). n = 20 independent thalli.

**Figure 3.** Analysis of the contribution of circadian regulation to the rhythms of delayed chlorophyll fluorescence under light/dark cycles. DF rhythms were quantified under T cycles of lengths of (a) 20 h, (b) 22 h, (c) 24 h and (d) 28 h, and the mean intensity plotted. (e) The phase was estimated from each time-series and normalized to a 24 h T cycle length, to evaluate the phase relationship between the rhythm of DF and the zeitgeber cycle. A strong contribution of the circadian clock to the timing of the rhythm under light/dark cycles would be expected to drive a relatively earlier phase under longer T-cycles, which (e) is not the case. This suggests that the effect of the light dark cycle masks the effect of circadian regulation of this proxy for photosynthesis in *M. polymorpha*. n = 150 thalli per T cycle (T20: 126 of 150 points rhythmic; T22: 127 of 150 points rhythmic; T24: 122 of 150 points rhythmic T28: 129 of 150 points rhythmic). Phase estimates obtained using FFT-NLLS, with data amplitude and baseline detrended. Only rhythmic thalli were included in FFT-NLLS analysis. In (e), different letters indicate significantly different mean phases (one-way ANOVA, where $p < 0.05$ based on Tukey's post-hoc test).

**Figure 4.** Amplitude of circadian rhythm of chlorophyll fluorescence parameters is reduced by the inhibitor of chloroplast translation lincomycin. (a-f) Effect of range of concentrations of lincomycin upon the circadian oscillation of the estimated rate of photosynthetic electron transport. (g-l) Effect of range of concentrations of lincomycin upon the circadian oscillation of non-photochemical quenching of chlorophyll fluorescence. (m-r) Effect of range of concentrations of rifampicin upon the circadian oscillation of the estimated rate of photosynthetic electron transport. (s-x) Effect of range of concentrations of rifampicin upon the circadian oscillation of non-photochemical quenching of chlorophyll fluorescence. (e, f, k, l, q, r, w, x) Relative amplitude error, period and amplitude were estimated using FFT-NLLS with amplitude and baseline detrending. (e, k, q, w) Different letters next to data points indicate significant differences in period (upper case)) and RAE (lower case), with data plotted ± s.e.m. (f, l, r, x). Analyzed with one-way ANOVA, where $p < 0.05$ based on Tukey's post-hoc test; n = 25 thalli.

**Figure S1.** Amplitude of circadian rhythm of chlorophyll fluorescence parameters is reduced by the inhibitor of chloroplast translation lincomycin. This is a second direct repeat of the experiment in Fig. 4. (a-d) Effect of range of concentrations of lincomycin upon the circadian oscillation of (a, b) the estimated rate of photosynthetic electron transport and (c, d) non-photochemical quenching of chlorophyll fluorescence. (e-h) Effect of range of concentrations of rifampicin upon the circadian oscillation of (e, f) the estimated rate of photosynthetic electron transport and (g, h) non-photochemical quenching of chlorophyll fluorescence. (a, c, e, g) Different letters next to data points indicate significant differences in period (upper case) and RAE (lower case), with data plotted ± s.e.m. (b, d, f, h) Different letters indicate significantly different mean phases. Analyzed with one-way ANOVA, where $p < 0.05$ based on Tukey's post-hoc test; n = 25 thalli.

## References


**Aschoff, J.** (1960) Exogenous and endogenous components in circadian rhythms. *Cold Spring Harbor Symposia on Quantitative Biology*, **25**, 11-28.

**Bachmann, K.M., Ebbert, V., Adams Iii, W.W., Verhoeven, A.S., Logan, B.A. and Demmig-Adams, B.** (2004) Effects of lincomycin on PSII efficiency, non-photochemical quenching, D1 protein and xanthophyll cycle during photoinhibition and recovery. *Functional Plant Biology*, **31**, 803-813.

**Borland, A.M., Barrera Zambrano, V.A., Ceusters, J. and Shorrock, K.** (2011) The photosynthetic plasticity of crassulacean acid metabolism: an evolutionary innovation for sustainable productivity in a changing world. *New Phytologist*, **191**, 619-633.

**Cano-Ramirez, D.L., Saskia de Fraine, T., Griffiths, O.G. and Dodd, A.N.** (2018) Photosynthesis and circadian rhythms regulate the buoyancy of marimo lake balls. *Current Biology*, **28**, R869-R870.

**Chang, F.N. and Weisblum, B.** (1967) The specificity of lincomycin binding to ribosomes. *Proceedings of the National Academy of Sciences*, **6**, 836-843.

**Dakhiya, Y. and Green, R.** (2022) Detection and analysis of circadian rhythms via prompt chlorophyll fluorescence. In *Plant Circadian Networks: Methods and Protocols* (Staiger, D., Davis, S. and Davis, A.M. eds). New York, NY: Springer US, pp. 33-45.

**Dakhiya, Y., Hussien, D., Fridman, E., Kiflawi, M. and Green, R.** (2017) Correlations between circadian rhythms and growth in challenging environments. *Plant Physiology*, **173**, 1724-1734.

**Davies, B.N. and Griffiths, H.** (2012) Competing carboxylases: circadian and metabolic regulation of Rubisco in C3 and CAM *Mesembryanthemum crystallinum* L. *Plant, Cell & Environment*, **35**, 1211-1220.

**Delwiche, Charles F. and Cooper, Endymion D.** (2015) The evolutionary origin of a terrestrial flora. *Current Biology*, **25**, R899-R910.

**Dodd, A.N., Borland, A.M., Haslam, R.P., Griffiths, H. and Maxwell, K.** (2002) Crassulacean acid metabolism: plastic, fantastic. *Journal of Experimental Botany*, **53**, 569-580.

**Dodd, A.N., Dalchau, N., Gardner, M.J., Baek, S.-J. and Webb, A.A.R.** (2014) The circadian clock has transient plasticity of period and is required for timing of nocturnal processes in Arabidopsis. *New Phytologist*, **201**, 168-179.

**Dodd, A.N., Griffiths, H., Taybi, T., Cushman, J.C. and Borland, A.M.** (2003) Integrating diel starch metabolism with the circadian and environmental regulation of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. *Planta*, **216**, 789-797.

**Dodd, A.N., Parkinson, K. and Webb, A.A.R.** (2004) Independent circadian regulation of assimilation and stomatal conductance in the *ztl*-1 mutant of Arabidopsis. *New Phytologist*, **162**, 63-70.

**Dodd, A.N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd, J.M., Millar, A.J. and Webb, A.A.R.** (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science*, **309**, 630-633.

**Edwards, D., Duckett, J.G. and Richardson, J.B.** (1995) Hepatic characters in the earliest land plants. *Nature*, **374**, 635-636.

**Eelderink-Chen, Z., Bosman, J., Sartor, F., Dodd, A.N., Kovács, Á.T. and Merrow, M.** (2021) A circadian clock in a nonphotosynthetic prokaryote. *Science Advances*, **7**, eabe2086.

**Gould, P.D., Diaz, P., Hogben, C., Kusakina, J., Salem, R., Hartwell, J. and Hall, A.** (2009) Delayed fluorescence as a universal tool for the measurement of circadian rhythms in higher plants. *The Plant Journal*, **58**, 893-901.

**Gould, P.D., Domijan, M., Greenwood, M., Tokuda, I.T., Rees, H., Kozma-Bognar, L., Hall, A.J.W. and Locke, J.C.W.** (2018) Coordination of robust single cell rhythms in

the Arabidopsis circadian clock via spatial waves of gene expression. *eLife*, **7**, e31700.

**Graf, A., Schlereth, A., Stitt, M. and Smith, A.M.** (2010) Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. *Proceedings of the National Academy of Sciences*, **107**, 9458-9463.

**Green, R.M., Tingay, S., Wang, Z.-Y. and Tobin, E.M.** (2002) Circadian rhythms confer a higher level of fitness to Arabidopsis plants. *Plant Physiology*, **129**, 576-584.

**Green, T.G.A. and Snelgar, W.P.** (1982) A comparison of photosynthesis in two thalloid liverworts. *Oecologia*, **54**, 275-280.

**Gyllenstrand, N., Karlgren, A., Clapham, D., Holm, K., Hall, A., Gould, P.D., Källman, T. and Lagercrantz, U.** (2014) No time for spruce: Rapid dampening of circadian rhythms in *Picea abies* (L. Karst). *Plant and Cell Physiology*, **55**, 535-550.

**Hastings, J.W., Astrachan, L. and Sweeney, B.M.** (1961) A persistent daily rhythm in photosynthesis. *Journal of General Physiology*, **45**, 69-76.

**Hayama, R. and Coupland, G.** (2003) Shedding light on the circadian clock and the photoperiodic control of flowering. *Current Opinion in Plant Biology*, **6**, 13-19.

**Hellweger, F.L., Jabbur, M.L., Johnson, C.H., van Sebille, E. and Sasaki, H.** (2020) Circadian clock helps cyanobacteria manage energy in coastal and high latitude ocean. *The ISME Journal*, **14**, 560-568.

**Hennessey, T.L. and Field, C.B.** (1991) Circadian rhythms in photosynthesis: Oscillations in carbon assimilation and stomatal conductance under constant conditions. *Plant Physiology*, **96**, 831-836.

**Hotta, C.T., Gardner, M.J., Hubbard, K.E., Baek, S.J., Dalchau, N., Suhita, D., Dodd, A.N. and Webb, A.A.R.** (2007) Modulation of environmental responses of plants by circadian clocks. *Plant, Cell & Environment*, **30**, 333-349.

**Hsu, P.Y. and Harmer, S.L.** (2014) Wheels within wheels: the plant circadian system. *Trends in Plant Science*, **19**, 240-249.

**Jones, V.A.S. and Dolan, L.** (2017) Mp*WIP* regulates air pore complex development in the liverwort *Marchantia polymorpha*. *Development*, **144**, 1472-1476.

**Karpinska, B., Alomrani, S.O. and Foyer, C.H.** (2017) Inhibitor-induced oxidation of the nucleus and cytosol in Arabidopsis thaliana: implications for organelle to nucleus retrograde signalling. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **372**, 20160392.

**Kohchi, T., Yamato, K.T., Ishizaki, K., Yamaoka, S. and Nishihama, R.** (2021) Development and molecular genetics of *Marchantia polymorpha*. *Annual Review of Plant Biology*, **72**, 677-702.

**Lagercrantz, U., Billhardt, A., Rousku, S.N., Leso, M., Reza, S.H. and Eklung, D.M.** (2021) *DE-ETIOLATED1* has a role in the circadian clock of the liverwort *Marchantia polymorpha*. *New Phytologist*, **232**, 595-609.

**Lagercrantz, U., Billhardt, A., Rousku, S.N., Ljung, K. and Eklund, D.M.** (2020) Nyctinastic thallus movement in the liverwort *Marchantia polymorpha* is regulated by a circadian clock. *Scientific Reports*, **10**, 8658.

**Linde, A.-M., Eklund, D.M., Kubota, A., Pederson, E.R.A., Holm, K., Gyllenstrand, N., Nishihama, R., Cronberg, N., Muranaka, T., Oyama, T., Kohchi, T. and Lagercrantz, U.** (2017) Early evolution of the land plant circadian clock. *New Phytologist*, **216**, 576-590.

**Litthauer, S., Battle, M.W., Lawson, T. and Jones, M.A.** (2015) Phototropins maintain robust circadian oscillation of PSII operating efficiency under blue light. *The Plant Journal*, **83**, 1034-1045.

**Maxwell, K. and Johnson, G.N.** (2000) Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany*, **51**, 659-668.

**Millar, A.J.** (2016) The intracellular dynamics of circadian clocks reach for the light of ecology and evolution. *Annual Review of Plant Biology*, **67**, 595-618.

**Mitsui, A., Kumazawa, S., Takahashi, A., ikemoto, H., Cao, S. and Arai, T.** (1986) Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature*, **323**, 720-722.

**Mulo, P., Pursiheimo, S., Hou, C.-X., Tyystjärvi, T. and Aro, E.-M.** (2003) Multiple effects of antibiotics on chloroplast and nuclear gene expression. *Functional Plant Biology*, **30**, 1097-1103.

**Murchie, E.H. and Lawson, T.** (2013) Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of Experimental Botany*, **64**, 3983-3998.

**Noordally, Z.B., Ishii, K., Atkins Kelly, A., Wetherill Sarah, J., Kusakina, J., Walton Eleanor, J., Kato, M., Azuma, M., Tanaka, K., Hanaoka, M. and Dodd Antony, N.** (2013) Circadian control of chloroplast transcription by a nuclear-encoded timing signal. *Science*, **339**, 1316-1319.

**Okada, M., Inoue, M. and Ikeda, T.** (1978) Circadian rhythm in photosynthesis of the green alga *Bryopsis maxima*. *Plant and Cell Physiology*, **19**, 197-202.

**Ouyang, Y., Andersson, C.R., Kondo, T., Golden, S.S. and Johnson, C.H.** (1998) Resonating circadian clocks enhance fitness in cyanobacteria. *Proceedings of the National Academy of Sciences*, **95**, 8660.

**Paajanen, P., Lane de Barros Dantas, L. and Dodd, A.N.** (2021) Layers of crosstalk between circadian regulation and environmental signalling in plants. *Current Biology*, **31**, R399-R413.

**Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A. and Nam, H.G.** (1999) Control of circadian rhythms and photoperiodic flowering by the Arabidopsis *GIGANTEA* gene. *Science*, **285**, 1579-1582.

**Pfannschmidt, T. and Link, G.** (1997) The A and B forms of plastid DNA-dependent RNA polymerase from mustard (Sinapis alba L.) transcribe the same genes in a different developmental context. *Molecular and General Genetics MGG*, **257**, 35-44.

**Rees, H., Duncan, S., Gould, P., Wells, R., Greenwood, M., Brabbs, T. and Hall, A.** (2019) A high-throughput delayed fluorescence method reveals underlying differences in the control of circadian rhythms in *Triticum aestivum* and *Brassica napus*. *Plant Methods*, **15**, 51.

**Rees, H., Joynson, R., Brown, J.K.M. and Hall, A.** (2021) Naturally occurring circadian rhythm variation associated with clock gene loci in Swedish *Arabidopsis* accessions. *Plant, Cell & Environment*, **44**, 807-820.

**Roenneberg, T., Dragovic, Z. and Merrow, M.** (2005) Demasking biological oscillators: Properties nad principles of entrainment exemplified by the *Neurospora* circadian clock. *Proceedings of the National Academy of Sciences*, **21**, 7742-7747.

**Samuelsson, G., Sweeney, B.M., Matlick, H.A. and Prézelin, B.B.** (1983) Changes in Photosystem II account for the circadian rhythm in photosynthesis in *Gonyaulax polyedra*. *Plant Physiology*, **73**, 329-331.

**Schneegurt, M.A., Sherman, D.M., Nayar, S. and Sherman, L.A.** (1994) Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. *Journal of Bacteriology*, **176**, 1586-1597.

**Schweiger, E., Wallraff, H.G. and Schweiger, H.G.** (1964) Endogenous circadian rhythm in cytoplasm of Acetabularia: Influence of the nucleus. *Science*, **146**, 658-659.

**Shalit-Kaneh, A., Kumimoto, R.W., Filkov, V. and Harmer, S.L.** (2018) Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions. *Proc Natl Acad Sci U S A*, **115**, 7147-7152.

**Shimamura, M.** (2016) *Marchantia polymorpha*: Taxonomy, phylogeny and morphology of a model system. *Plant and Cell Physiology*, **57**, 230-256.

**Solly, J.E., Cunniffe, N.J. and Harrison, C.J.** (2017) Regional growth rate differences specified by apical notch activities regulate liverwort thallus shape. *Current Biology*, **27**, 16-26.

**Sorek, M. and Levy, O.** (2012) The effect of temperature compensation on the circadian rhythmicity of photosynthesis in Symbiodinium, coral-symbiotic alga. *Scientific Reports*, **2**, 536.

**Sorek, M., Yacobi, Y.Z., Roopin, M., Berman-Frank, I. and Levy, O.** (2013) Photosynthetic circadian rhythmicity patterns of *Symbiodium*, the coral endosymbiotic algae. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20122942.

**Spoelstra, K., Wikelski, M., Daan, S., Loudon, A.S.I. and Hau, M.** (2016) Natural selection against a circadian clock gene mutation in mice. *Proceedings of the National Academy of Sciences*, **113**, 686.

**Strehler, B.L. and Arnold, W.** (1951) Light production by green plants. *J Gen Physiol*, **34**, 809-820.

**Sullivan, J.A. and Gray, J.C.** (1999) Plastid translation is required for the expression of nuclear photosynthesis genes in the dark and in roots of the pea *lip1* mutant. *The Plant Cell*, **11**, 901-910.

**Surzycki, S.J.** (1969) Genetic functions of the chloroplast of of *Chlamydomonas reinhardi*: Effect of rifampicin on chloroplast DNA-dependent RNA polymerase. *Proceedings of the National Academy of Sciences*, **63**, 1327-1334.

**Sweeney, B.M. and Haxo, F.T.** (1961) Persistence of a photosynthetic rhythm in enucleated Acetabularia. *Science*, **134**, 1361.

**Troein, C., Locke, J.C.W., Turner, M.S. and Millar, A.J.** (2009) Weather and seasons together demand complex biological clocks. *Current Biology*, **19**, 1961-1964.

**Walker, R. and Pennington, W.** (1939) The movements of the air pores of *Preissia quadrata* (Scop.). *New Phytologist*, **38**, 62-68.

**Webb, A.A.R., Seki, M., Satake, A. and Caldana, C.** (2019) Continuous dynamic adjustment of the plant circadian oscillator. *Nature Communications*, **10**, 550.

**Wenden, B., Toner, D.L.K., Hodge, S.K., Grima, R. and Millar, A.J.** (2012) Spontaneous spatiotemporal waves of gene expression from biological clocks in the leaf. *Proceedings of the National Academy of Sciences*, **109**, 6757-6762.

**Wu, G., Anafi, R.C., Hughes, M.E., Kornacker, K. and Hogenesch, J.B.** (2016) MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics*, **32**, 3351-3353.

**Wyka, T.P., Duarte, H.M. and Luttge, U.E.** (2005) Redundancy of stomatal control for the circadian photosynthetic rhythm in *Kalanchoë daigremontiana* Hamet et Perrier. *Plant Biology*, **7**, 176-181.

**Zhao, X., Huang, J. and Chory, J.** (2018) *genome uncoupled1* mutants are hypersensitive to norflurazon and lincomycin. *Plant Physiology*, **178**, 960-964.

**Zielinski, T., Moore, A.M., Troup, E., Halliday, K.J. and Millar, A.J.** (2014) Strengths and limitations of period estimation methods for circadian data. *PLOS ONE*, **9**, e96462.

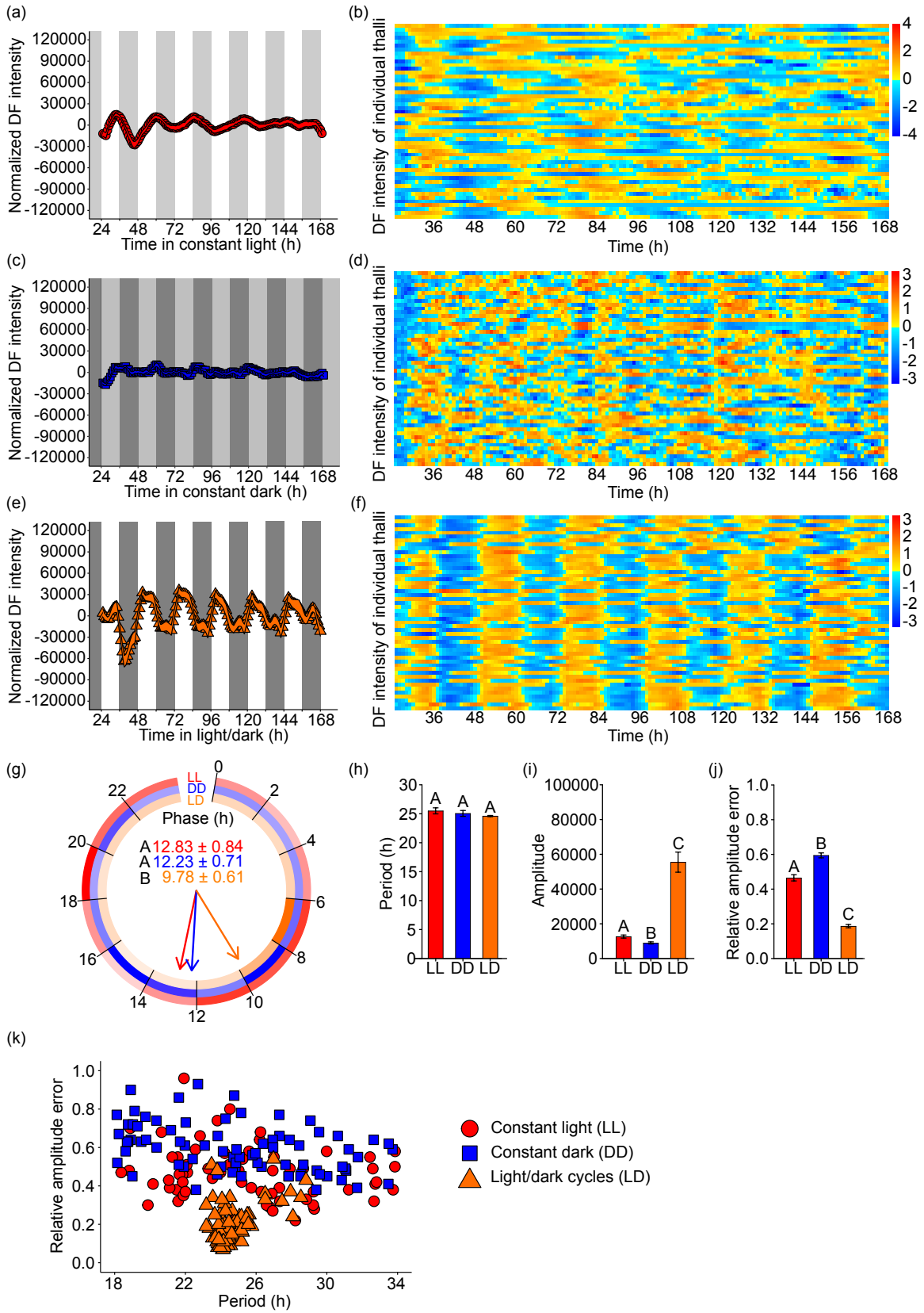


**Figure 1.** Circadian and diel rhythms of delayed fluorescence of *Marchantia polymorpha* thallus under (a, b) constant light, (c, d) constant darkness, and (e, f) light/cycles. (a, c, e) indicates the mean DF signal from thalli that were categorized as rhythmic after filtration with

Metacycle (*q*-value < 0.001; period between 18 h and 34 h). (b, d, f) Heat maps summarize DF intensity of 50 individual and randomly selected thalli across two experimental repeat experiments under (b) constant light, (d) constant dark and (f) light/dark cycles. Data in (a, c, e) are smoothed with a 5-hour moving average, baseline and amplitude de-trended, and normalized to the mean signal. On heat maps, colour values are scaled per row using Z-score normalization. (g) Mean circular phases relative to dawn (CT0) of rhythmic thalli under each condition (constant light (LL; red), constant dark (DD; blue) and diel cycles (LD; orange) calculated using FFT-NLLS. (h) Mean period, (i) amplitude, and (j) RAE for rhythmic thalli under each condition, derived from FFT-NLLS analysis of data. Statistical significance of differences between conditions are indicated by letters above each bar (ANOVA with Tukey's post-hoc test, $p < 0.05$), error bars show SEM. (k) period estimate and relative amplitude error (RAE) of individual thalli under three light conditions, obtained using FFT-NLLS. Data derive from 100 individual thalli, across two independent experimental replicates, for each condition.

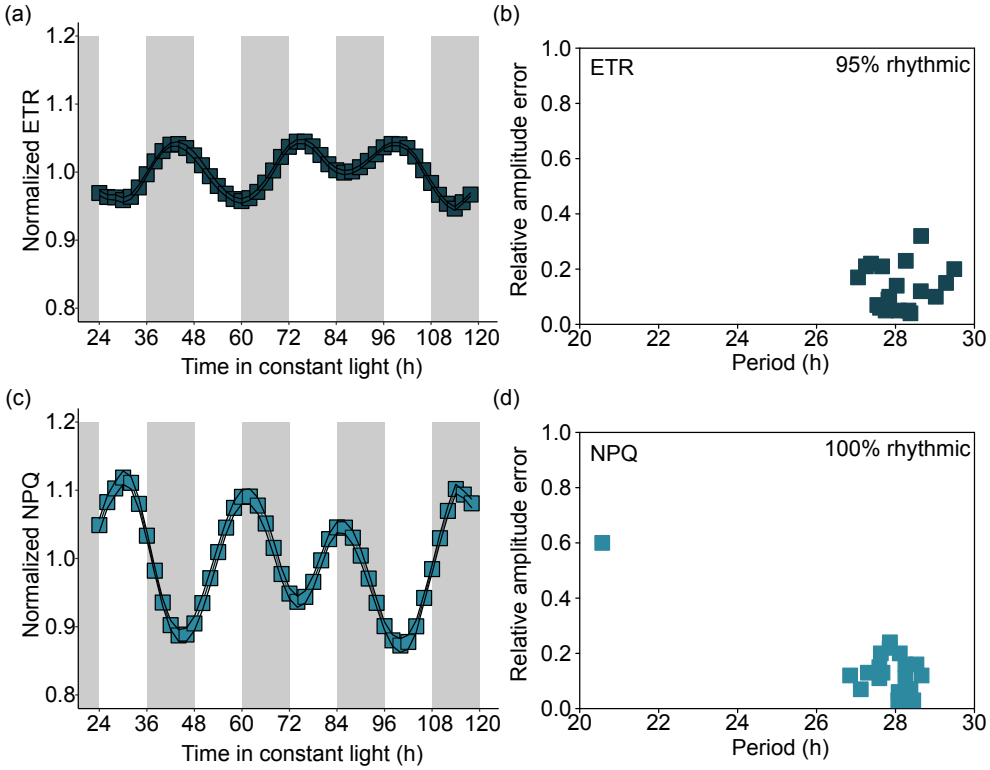


**Figure 2.** Circadian rhythms of modulated chlorophyll fluorescence in thallus of *Marchantia polymorpha*. Under constant blue light conditions, time-course analysis of (a, b) the estimated rate of photosynthetic electron transport rate (ETR), and (c, d) non-photochemical quenching of PSII fluorescence (NPQ). (a, c) are the normalized signal level for each chlorophyll fluorescence parameter, processed using amplitude and baseline detrending and normalized to the mean (using Biodare2). (b, d) compare the estimated period and relative amplitude error (RAE) of the rhythmic thalli, with period, RAE and the presence of rhythmicity quantified using FFT-NLLS (using Biodare2). n = 20 independent thalli.

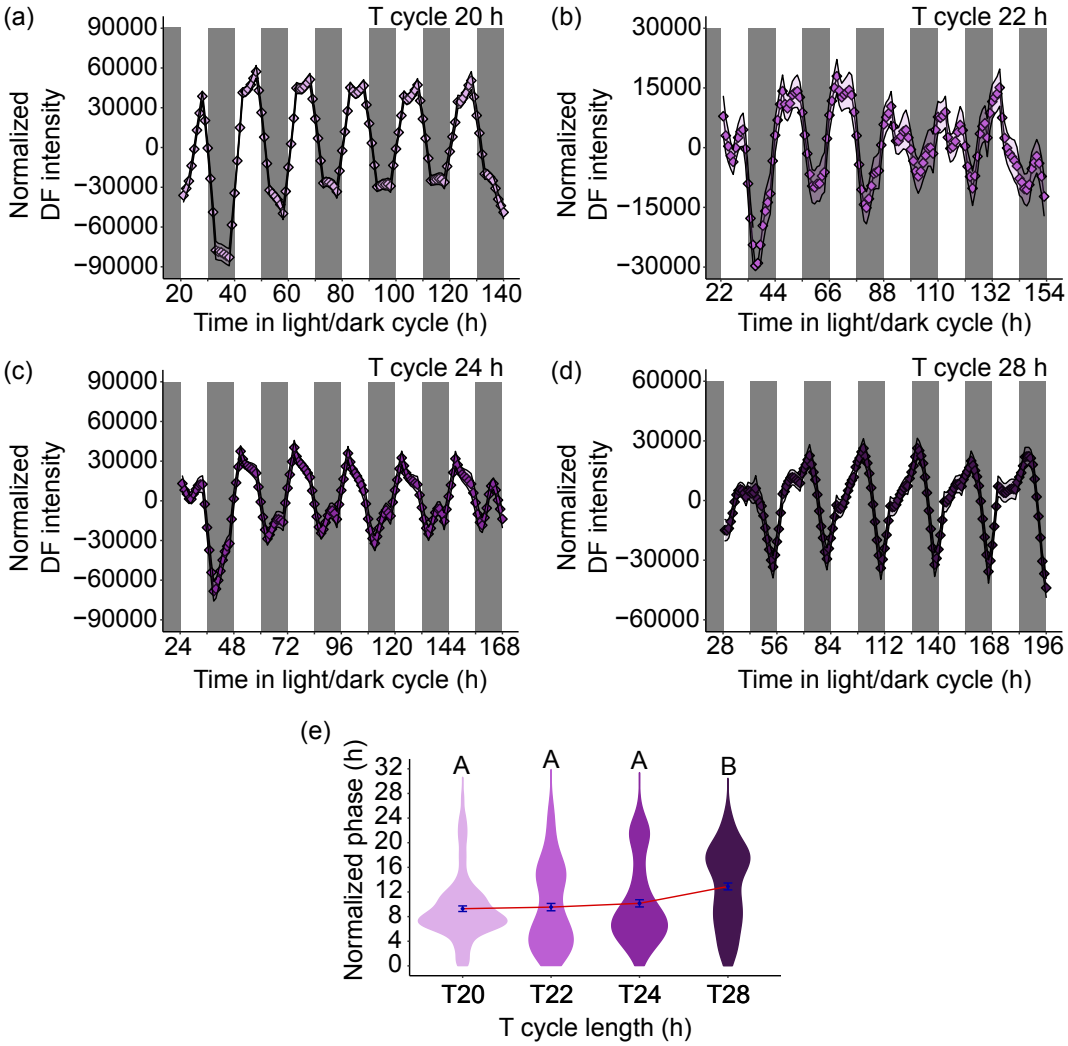


**Figure 3.** Analysis of the contribution of circadian regulation to the rhythms of delayed chlorophyll fluorescence under light/dark cycles. DF rhythms were quantified under T cycles of lengths of (a) 20 h, (b) 22 h, (c) 24 h and (d) 28 h, and the mean intensity plotted. (e) The phase was estimated from each time-series and normalized to a 24 h T cycle length, to evaluate the phase relationship between the rhythm of DF and the zeitgeber cycle. A strong contribution of the circadian clock to the timing of the rhythm under light/dark cycles would be expected to drive a relatively earlier phase under longer T-cycles, which (e) is not the case. This suggests that the effect of the light dark cycle masks the effect of circadian regulation of this proxy for photosynthesis in *M. polymorpha*. n = 150 thalli per T cycle (T20: 126 of 150 points rhythmic; T22: 127 of 150 points rhythmic; T24: 122 of 150 points rhythmic T28: 129 of 150 points rhythmic). Phase estimates obtained using FFT-NLLS, with data

amplitude and baseline detrended. Only rhythmic thalli were included in FFT-NLLS analysis. In (e), different letters indicate significantly different mean phases (one-way ANOVA, where $p < 0.05$ based on Tukey's post-hoc test).

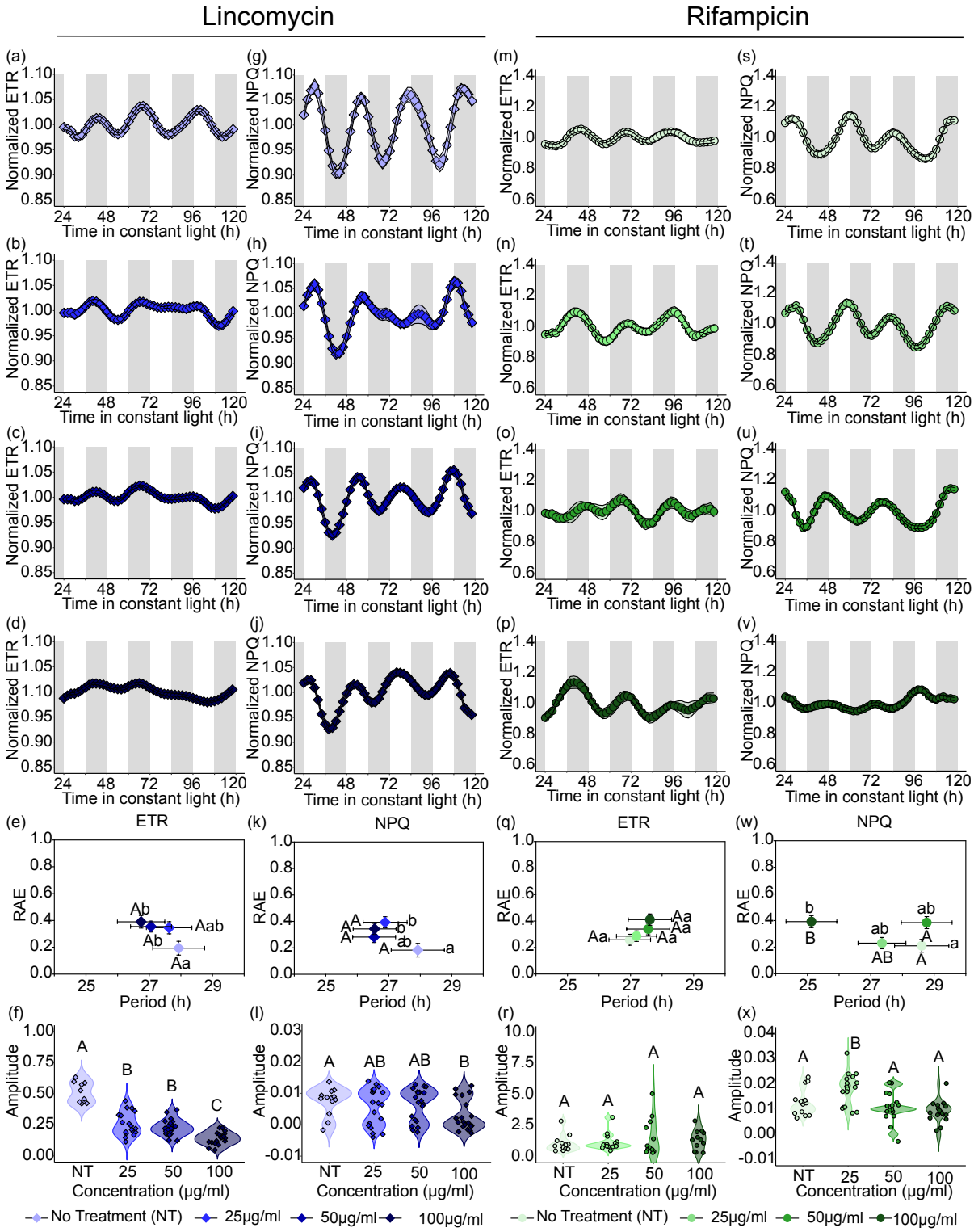


**Figure 4.** Amplitude of circadian rhythm of chlorophyll fluorescence parameters is reduced by the inhibitor of chloroplast translation lincomycin. (a-f) Effect of range of concentrations of lincomycin upon the circadian oscillation of the estimated rate of photosynthetic electron transport. (g-l) Effect of range of concentrations of lincomycin upon the circadian oscillation

of non-photochemical quenching of chlorophyll fluorescence. (m-r) Effect of range of concentrations of rifampicin upon the circadian oscillation of the estimated rate of photosynthetic electron transport. (s-x) Effect of range of concentrations of rifampicin upon the circadian oscillation of non-photochemical quenching of chlorophyll fluorescence. (e, f, k, l, q, r, w, x) Relative amplitude error, period and amplitude were estimated using FFT-NLLS with amplitude and baseline detrending. (e, k, q, w) Different letters next to data points indicate significant differences in period (upper case)) and RAE (lower case), with data plotted ± s.e.m. (f, l, r, x). Analyzed with one-way ANOVA, where $p < 0.05$ based on Tukey's post-hoc test; n = 25 thalli.