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Continuous Monitoring of Growth Detects Photoperiod–Dependent Oscillations in Growth rates in *Chlorella vulgaris*

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Running Title: Growth oscillations in *Chlorella vulgaris*
ABSTRACT

The green alga, *Chlorella vulgaris*, exhibits minimal capacity to adjust exponential growth rates in response to photo flux density (PFD) when monitored on a discontinuous basis. We hypothesized that modulation of maximum growth rates in *Chlorella vulgaris* by PFD is a photoperiod-dependent phenomenon. The use of the photobioreactors to monitor continuous growth allowed us to detect repetitive daily oscillations in growth which were photoperiod-dependent. The rate of change in optical density (OD735) during the daily light period was 2-fold greater in cells grown at 28°C and either a PFD of 2000 or 150 μmol m⁻² s⁻¹ when *C. vulgaris* was grown under a daily light:dark cycle. Concomitantly, oscillations of the chlorophyll fluorescence parameters paralleled the oscillations observed in growth rate. When cultures were shifted from a 12h photoperiod at low light to continuous light (CL), the growth oscillations disappeared. In contrast, oscillations in the fluorescence parameters persisted even after the shift from a 12 h photoperiod to CL. We suggest that the nocturnal catabolism of starch reserves in conjunction with changes in cellular volume coupled with the diurnal changes in DNA content quantified by changes in Vybrant Green fluorescence yield indicate that these growth oscillations reflect synchronized cellular division in *C. vulgaris* that is not evident when growth is assayed discontinuously.

KEY WORDS: cell size, *Chlorella vulgaris*, growth, oscillations, photoperiod
INTRODUCTION

Photoautotrophic metabolism, development and growth are tightly regulated by the prevailing environmental conditions. Light quality and quantity, as well as photoperiod, together with temperature and nutrient availability influence photoautotrophic morphology and the timing of developmental phases (Venediktov et al. 1981; Dodd et al. 2014a; Jin and Zhu 2019; Moraes et al. 2019). Among these environmental cues, day length provides the most reliable indicator of diurnal and seasonal progression due to its high degree of predictability on a daily and yearly level. The ability to anticipate changes in the light environment accurately, and coordinate metabolism and growth to external light:dark cycles, has been demonstrated to confer a selective advantage by enhancing fitness in *Arabidopsis thaliana* (Kulheim et al. 2002; Dodd et al. 2005).

The circadian clock is a series of interconnected transcriptional feedback loops that provides internal measures of time that serve to synchronize photoautotrophic physiology with the external light:dark cycle by regulating downstream targets. Clock-controlled biological oscillations demonstrating circadian rhythms exhibit periods of approximately 24 h that persist under continuous light (CL) (McClung 2006; Harmer 2009). Light and temperature signals serve as the major regulators used to entrain the clock to the environment (Eckardt 2005; Salome and McClung 2005). The circadian clock has been implicated in the regulation of photosynthesis, both nuclear and plastid-localized gene transcription, starch metabolism as well as cellular division (Dodd et al. 2005; Graf and Smith 2011; Staiger et al. 2013; Dodd et al. 2014b).

Asexual cellular division by cell fission in green algae has been suggested to be an adaptation to a naturally occurring light environment characterized by light:dark cycles such that the light period is maximally exploited to drive photoautotrophic growth (Venediktov et al. 1981; Bišová and Zachleder 2014). Reproduction by multiple fission events (n), where each mother cell gives rise to $2^n$ daughter cells is shared among the chlorophyte algae (Kirk 2004).

A cell cycle consists of a growth phase ($G_1$ phase), followed by a DNA replication (or synthesis) phase (S phase), a secondary growth phase ($G_2$ phase) and mitosis (M phase) which is closely followed by fission or cellular division. Mitchison (1971) proposed that this cycle proceeds as two coordinated events consisting of growth ($G_1$ phase) and a DNA replication-division sequence composed of DNA replication (S phase), the $G_2$ growth phase and nuclear division (M phase) closely followed by cytokinesis. Alternative models suggest that progression of the cell cycle from the major growth phase to a reproductive mode characterized by the DNA
replication and cellular division sequence is initiated either by signals generated by the circadian clock (Edmunds and Adams 1981; Homma and Hastings 1989; Makarov et al. 1995; Lüning et al. 1997) or following attainment of a critical point marked by the acquirement of a critical cellular volume (Vítová et al. 2011a,b; Bı şová and Zachleder 2014).

It has previously been reported that *C. vulgaris* appears to exhibit minimal capacity to adjust exponential growth rates which is associated with a minimal response of either light saturated rates of photosynthesis and carbon metabolism to a range of continuous growth light (CL) intensities (Savitch et al. 1996; Wilson and Hün er 2000). In terrestrial plants, growth under CL is typically associated with a marked decrease in photosynthetic capacity and growth reflecting feedback inhibition of photosynthesis in the absence of a dark period (Stessman et al. 2002; van Gestel et al. 2005; Sysoeva et al. 2010; Velez-Ramirez et al. 2011). We hypothesized that modulation of maximum growth rates in *C. vulgaris* by photon flux density (PFD) is a photoperiod-dependent phenomenon. To test this hypothesis, *C. vulgaris* cultures were grown at a constant temperature of 28 °C under either a 24 h photoperiod (CL, control), an 18 h photoperiod or a 12 h photoperiod at either high light (HL; 2000 µmol photons m⁻² s⁻¹) or low light (LL; 150 µmol photons m⁻² s⁻¹).

**MATERIALS AND METHODS**

*Cell culture conditions*

Cultures of *Chlorella vulgaris* Berjiermick (UTEX 265) were grown axenically in Bold’s basal media (Nichols and Bold 1965) with modifications according to Maxwell et al. 1994. Cultures were grown as batch cultures in 400 mL capacity Photobioreactor cultivation vessels (FMT 150) (Photon System Instruments, Hogrova, Czech Republic) and aerated with sterile, humidified air. The temperature and light regimes were regulated by the photobioreactor control system which maintained a temperature of 28 °C ± 1 °C and CL PFD of either 150 (28/150) or 2000 µmol photons m⁻² s⁻¹ (28/2000) supplied by an equal combination of red and blue light emitting diodes. Cells were grown under either CL (24 h photoperiod), an 18 h photoperiod or 12 h photoperiod at both LL (28/150) or HL(28/2000).

Optical density (OD) was measured continuously at 680 and 735 nm by a densitometer integrated into the photobioreactor. Maximum fluorescence (F_M') of photosystem II reaction...
centres and steady-state fluorescence (Fs) at the growth PFD were measured using a fluorometer integrated into the photobioreactor system. Both optical density and Chl a fluorescence measurements were taken at 30 minute intervals and were automatically recorded by the photobioreactor control system.

As an additional control, the growth pattern of *C. vulgaris* in photobioreactors was compared to our previous published method of discontinuous growth of *C. vulgaris* cultures in 150 mL capacity pyrex tubes suspended in temperature controlled aquaria which maintained a temperature of 28 °C and bubbled with sterile air (Maxwell et al. 1994). During cultivation in growth tubes, cells were illuminated by a bank of white fluorescent lights (Sylvania T12 daylight) which supplied growth light of either 150 or 2000 µmol photons m⁻² sec⁻¹. Tube-grown cells were aerated with sterile air. Samples were removed at 24 h intervals and growth was assayed as change in optical density at 750 nm. Growth rate was calculated from data during the exponential growth phase at either 28/150 or 28/2000,

**Growth rate**

Cellular growth rates were measured as a change in light scattering at 735 nm measured at 30 minute intervals via the integrated densitometer. Specific growth rates were calculated for the exponential growth phase using natural log transformed absorbance readings at 735 nm as \( \mu = \ln(N_1/N_0)/(t_1-t_0) \) where \( \mu \) is the pseudo-first order growth constant (days⁻¹), and \( N_0 \) and \( N_1 \) represent optical density at 735 nm at time 0 (\( t_0 \)) and time 1 (\( t_1 \)), respectively (Wood et al. 2005). Doubling time was calculated as \( \ln2/\mu \) (Wood et al. 2005).

**Cell size**

Cell size was estimated using a PhytoCyt Flow Cytometer (C6) equipped with a 488 nm argon laser (Turner, California, USA). Forward scatter (FSC) was used as an indicator of relative cell diameter; FSC is light from the illumination beam that has been deflected at a small angle as it passes throughout the cell suspension and is proportional to cell size as described in detail by Givan (2001, 2010) and Picot et al. (2012). A flow cytometry size calibration kit with nonfluorescent size calibration standards of 1, 2, 4, 6, 10 and 15 µm were used to determine cell
size following the instruction provided by the manufacturer (Molecular Probes, Eugene, Oregon, USA).

**DNA stain and cell cycle tracking**

Samples were either removed from the photobioreactor immediately prior to the end of the light period as well as immediately prior to the end of the dark period during exponential growth for cells grown under either an 18 h or 12 h photoperiod, or were collected during exponential growth for cells grown under CL. VybrantDyeCycle Green stain was used to estimate DNA mass (Molecular Probes, Eugene, Oregon, USA). Background fluorescence was measured on 50 µL of unstained cells using the 488 nm excitation and 530/30 emission wavelengths (Molecular Probes, Eugene, Oregon, USA). DNA content was estimated in stained cells using the instructions provided by the manufacturer; 4 µL of dye was added to 1 mL cells for a final concentration of 10 µM and incubated at 37 °C for 30 min in the dark (Molecular Probes, Eugene, Oregon, USA). A 50 µL sample of the stained cells was then measured using the PhytoCyt Flow Cytometer (Turner, California, USA) where the Vybrant DyeCycle Green:DNA complex was analyzed using the 488 nm excitation and green emission (~520 nm) wavelengths (Molecular Probes, Eugene, Oregon, USA). The normalized fluorescence signal was calculated as the difference between the mean fluorescence signal of stained and unstained cells.

**Carbohydrate analysis**

Carbohydrate analysis was conducted on exponentially growing cells harvested by centrifugation at 5,000 x g for 5 min and stored at -80 °C until analysis. Samples were collected from the Photobioreactor immediately prior to the end of the light period as well as immediately prior to the end of the dark period for cells grown under either an 18 h or 12 h photoperiod; or under CL, mid-log phase cells harvested during exponential growth. Pigments were extracted from thawed cells with 80% (v/v) ethanol until cells were completely pigment free. Total starch content was quantified using the Megazyme total starch assay kit (Megazyme, Wicklow, Ireland) and total sucrose content was quantified using the Sigma sucrose assay kit (Sigma-Aldrich, USA).
Missouri, USA) according to the manufacturers' recommendations according to Lee et al. (2013) for microalgal samples.

**Statistical analysis**

Doubling times in photobioreactor or growth tube-grown cells of *C. vulgaris* grown at 28/150 and 28/2000 under CL were compared using a two-way analysis of variance (ANOVA) using growth PFD and cultivation vessel type as explanatory variables. Doubling time, rate of change in absorbance at 735 nm and rate of starch consumption for cells grown at both 28/150 and 28/2000 in photobioreactors under either CL, an 18 h photoperiod or 12 h photoperiod were compared using a two-way ANOVA using growth PFD and photoperiod as explanatory factors. Two-way ANOVAs were conducted using the statistical software package R version 3.0.2 and followed by Tukey's Honest Significant Different (HSD) post hoc tests.

Three-way repeated ANOVAs were conducted on diel changes in cellular volume, starch content and sucrose content for cells grown at both 28/150 and 28/2000 under either an 18 h or 12 h photoperiod. Tukey's HSD post hoc tests were performed on significant single factor and two factor interactions. The significant three-way interaction for change in cellular volume was examined by running two-way repeated measures ANOVAs for each PFD separately with time as the within subject factor and photoperiod as the between subject factor. All repeated measures ANOVAs were conducted using the statistical software package R version 3.0.2. Prior to each test, the data were visually inspected for normality and homoscedasticity. A value of \( p < 0.05 \) was considered significant throughout.

**RESULTS**

*Growth of Chlorella vulgaris under CL*

Figure 1 shows representative growth curves for cultures of *Chlorella vulgaris* grown in Photobioreactors at either (A) 28/150 or (B) 28/2000. Growth was measured as a change in cell density, monitored at 735 nm (OD\(_{735}\)), over time using a densitometer integrated into the photobioreactor system (Figure S1). Change in cell density in cultures of *C. vulgaris* grown at either 28/150 or 28/2000 under CL exhibited a typical sigmodial growth pattern with distinct lag, exponential growth and stationary phases (Figures 1 and S2). Typical growth curves for *C. vulgaris* grown in photobioreactors at 28/150 and 28/2000 under CL resembled the representative growth patterns of cells assayed discontinuously in growth tubes under the same
growth light and temperature regimes. The doubling times for photobioreactor-grown cultures as well as growth tube cultures of *C. vulgaris* grown under CL appeared to be independent of growth PFD (Table 1); however, photobioreactor grown cultures exhibited a 2-fold longer doubling time relative to cells grown in growth tubes (Table 1) (irradiance, $F_{1,16} = 3.1, p = 0.0979$; cultivation vessel, $F_{1,16} = 40.5, p < 0.0001$; irradiance*cultivation vessel, $F_{1,16} = 0.5, p = 0.475$).

The photobioreactor system simultaneously measured change in chlorophyll (Chl) content over time at 680 nm (Figures 1 and S2). The increase in Chl abundance closely paralleled the increase in optical density for cultures of *C. vulgaris* grown at either 28/150 or 28/2000 under CL (Figure 1). The integrated fluorometer additionally measured changes in Chl a fluorescence induction over time (Figure 2). Since nearly identical patterns were obtained for F_M' and F_S at both the red and blue excitation (Figure S3), the response for the blue light only is presented (Figure 2). Increases in F_M', the maximum light-adapted Chl a fluorescence (Figure 2; closed symbols), and F_S, steady-state Chl a fluorescence (Figure 2; open symbols), were measured in cultures grown at both 28/150 and 28/2000 under CL that paralleled the increase in OD (Figure 1).

**Effect of photoperiod on cell growth**

Figures 1 and 2 show representative diurnal growth dynamics for cultures of *C. vulgaris* grown at either 28/150 or 28/2000, respectively, under varying photoperiods. The overall sigmodial growth patterns exhibited by cells of *C. vulgaris* grown under either an 18 h or 12 h photoperiod were similar to those observed under CL (Figure 1). However, growth under either an 18 h or 12 h photoperiod exhibited transient oscillations in OD_735 at both growth PFDs. At both 28/150 and 28/2000 there was a rapid rise in OD_735 during the light period followed by a marked decline in optical density that corresponded with the start of the dark period (Figure 3). Oscillations in OD_735 occurred with a period of approximately 24 h and appeared to be independent of the growth PFD. However, the amplitude of these oscillations did dampen as the cells approached the stationary growth phase (Figures 1 and 2). Similar oscillation patterns were observed for Chl accumulation (Figure 1) as well as Chl a fluorescence (Figure 2).

Doubling times remained relatively constant at about 24 h across the range of photoperiods and growth PFD (PFD, $F_{1,24} = 3.1, p = 0.092$; photoperiod, $F_{2,24} = 0.9, p = 0.404$;
PFD*photoperiod, $F_{2,24} = 0.2, p = 0.828$) (Table 2; Table 4). However, closer examination of the rate of change in OD$_{735}$ over time during the light period was approximately 2-fold greater in C. vulgaris grown at 28/2000 compared to cells grown at 28/150 when compared at the same photoperiod (PFD, $F_{1,8} = 13.9, p = 0.00575$; photoperiod, $F_{1,8} = 0.01, p = 0.924$; PFD*photoperiod, $F_{1,8} = 0.447, p = 0.523$) (Table 3). Thus, contrary to previous results discontinuous growth of C. vulgaris in standard tubes (Maxwell et al. 1994; Wilson and Hüner 2000), PFD does appear to modulate growth rate in C. vulgaris grown in the photobioreactors. However, this is evident during the light period only.

When cells of C. vulgaris grown under a daily light:dark cycle were shifted to CL at the same growth PFD and temperature, the oscillations in both OD$_{735}$ (Figure 4A) and OD$_{680}$ (Figure 4B) were dampened immediately following the transfer. In contrast, the daily oscillations in Chl a fluorescence continued to persist in the absence of a photoperiod for several cell divisions (Figure 4C).

**Physiological basis underlying growth oscillations**

An important advantage in the use of the photobioreactors is the capability of continuous monitoring of growth rates by light scattering (OD$_{735}$) which showed that Chlorella vulgaris undergoes regular, photoperiod-dependent transitions in growth rate over time (Figures 1 to 4). What cellular properties could account for such changes in light scattering properties of C. vulgaris cultures? Three possibilities include: (1) changes in starch grains, (2) modulation of cell size and (3) cell fission. We examined each of these three possibilities.

**Effect of photoperiod on starch content**

Since starch granules are opaque, the presence or absence of these granules in chloroplasts may contribute to the photoperiod-dependent changes in the light scattering properties of the cells. To test this, total starch content was assayed biochemically. There was a significant decrease in total starch content on a per cell basis during the daily dark period (time, $p < 0.0001$, Table 4) (Figure 5A). Total starch content decreased by 88 and 86% by the end of the dark period in C. vulgaris grown at 28/150 under an 18 h and 12 h photoperiod, respectively, while starch content decreased by 95 and 92% in cells grown at 28/2000 under an 18 h and 12 h photoperiod, respectively (Figure 5A). However, there were no significant effects of either the
growth PFD or the duration of the photoperiod on total starch content (PFD, \( p = 0.261 \); photoperiod, \( 0 = 0.327 \), Table 4) (Figure 5A and Table S1).

As expected, the reduction in starch reserves during the dark period coincided with an increase in cellular sucrose content (time, \( p < 0.0001 \), Table 4) (Figure 5B). Sucrose content increased by approximately 60% by the end of the dark period at both the 18 h and 12 h photoperiods for cells grown at either 28/150 or 28/2000 (Figure 5B). Furthermore, the interaction between growth PFD and photoperiod on cellular sucrose content was significant (PFD*photoperiod, \( p = 0.026 \), Table 4) (Figure 5B). The length of the photoperiod only had a significant effect on cellular sucrose content at low light; there was an approximately 3.6-fold decrease in sucrose content at the 12 h photoperiod relative to the 18 h photoperiod at 28/150 (Tukey's HSD \( p = 0.0015 \)) while there was no effect of photoperiod at 28/2000 (Tukey's HSD \( p = 0.232 \)) (Figures 5B and Table 4).

**Effect of photoperiod on cellular volume**

Changes in cell size may additionally account for the transient, photoperiod-dependent oscillations in OD\(_{735}\). To test this, diel changes in cellular volume were measured in cultures of *C. vulgaris* grown at both 28/150 and 28/2000 under either an 18 h or 12 h photoperiod; cell size was measured immediately prior to the end of the light period (open bars) as well as immediately prior to the end of the dark period (closed bars) (Figure 6A). The response of cellular volume was dominated by a three-way interaction (irradiance*photoperiod*time, \( p = 0.043 \), Table 4). At low light (28/150), there was no significant change in cellular volume during the dark period (time, \( p = 0.072 \), Table 4) (Figure 6A). However, at high light, while there was a significant 30% decrease in cellular volume during the dark period in *C. vulgaris* grown under an 18 h photoperiod (Tukey's HSD \( p = 0.0069 \)), there was no significant change in cell volume in cells grown at high light but a 12 h photoperiod (PFD*time, \( p = 0.041 \), Tukey's HSD \( p = 0.628 \), Table 4) (Figure 6A). However, there was a shift in the distribution of cell sizes such that there was a greater abundance of cells with a smaller diameter, estimated by flow cytometry as forward scatter (FSC), at the end of a dark period relative to the end of the light period (Figure 6B). Thus, while there was not significant effect of dark exposure on the mean cell volume under all
conditions, there was shift in the distribution of cell sizes that may not be reflected by changes in
the mean cell volume (Figure 6B).

Cell cycle tracking

Photoperiod-dependent cell division could also account for the observed transient
oscillations in OD\textsubscript{735}. To test this, the change in cellular DNA content was assayed using a cell
membrane-permeable fluorescent dye. A stably low Vybrant green fluorescence signal was
observed during the light period followed by a 4-fold increase in the fluorescence signal at the
light-to-dark transition in \textit{C. vulgaris} grown under a daily photoperiod (Figure 7A). The
fluorescence signal returned to baseline levels by the end of the dark period (Figure 7A).
Furthermore, a 4-fold increase in cellular volume appeared to precede the increase in the DNA
fluorescence signal by 6 h (Figure 7B).

DISCUSSION

Contrary to the original hypothesis, the mean specific growth rates of photobioreactor-
grown cultures of \textit{C. vulgaris} were not only independent of growth PFD but also photoperiod
(Tables 1 and 2). Thus, consistent with previous reports for \textit{C. vulgaris} (Wilson and Hüner
2000), this species appears unable to up-regulate growth rate in response to increased growth
PFD. However, the use of the photobioreactors allowed us to detect significant daily oscillations
in growth rates. Although we did not detect an effect of PFD on the absolute mean growth rate
during the exponential growth phase at either 28/150 or 28/2000, the rate of change in OD\textsubscript{735}
over time during the daily light period was 2-fold greater in cells grown at 28/2000 relative to
those grown at 28/150 when \textit{C. vulgaris} was grown under a daily light:dark cycle (Tables 3 and
4). This is analogous to studies in \textit{Chlamydomonas reinhardtii} which demonstrated an increased
growth rate with increased PFD (Vítová et al. 2011\textsuperscript{a,b}). However, in \textit{C. vulgaris} this positive
relationship between growth rate and PFD appears to be limited to periods of light exposure only
(Table 3), since the mean specific growth rates during the exponential growth phase of \textit{C.
\textit{vulgaris} were ultimately independent of both growth PFD and photoperiod (Table 2).

We propose that the daily oscillations in OD\textsubscript{735} observed during growth and development
under various photoperiods are reflective of synchronization of the cell cycle by the light:dark
cycle. During growth under an alternating light:dark cycle, the majority of cells appear to be in
the G\textsubscript{0}/G\textsubscript{1} phase of the cell cycle during the light period as characterized by a relatively constant
but low DNA fluorescence signal where the relatively low fluorescence signal would correspond to cells in the G0/G1 phases. There was an approximately four-fold increase in the DNA fluorescence signal corresponding to increased DNA content in mitotic cells that occurred at the light-to-dark transition during growth under a 12 h light:dark cycle (Figure 7A) which has been demonstrated to immediately precede nuclear and cell division in green algae (Bišová and Zachleder 2014). This marked increase in the fluorescence signal at the end of the daily light period corresponded with a shift in the distribution of cells such that there was a greater proportion of high fluorescence cells, corresponding to cells with an increased DNA content in the G2/M phase of the cell cycle, at the end of the light period relative to cells measured at the end of the dark period (Figure S4). This is in contrast to cells grown under CL where cells were equally distributed between the G0/G1 and G2/M phases (Figure S4) characteristic of an unsynchronized population of cells. Furthermore, the 4-fold increase in cellular volume that preceded the increase in DNA cellular content (Figure 7B) is characteristic of the attainment of a critical size during cell cycle progression that precedes a commitment to divide in green algae (Bišová and Zachleder 2014).

The results of this study are consistent with early work by Tamiya and colleagues in synchronous cultures of green algae which demonstrated that the separation of mother cells following mitosis was confined to the dark periods during growth under daily light:dark cycles (Hase et al. 1959; Morimura 1959; Tamiya and Morimuta 1961; Morimura et al. 1964). Similarly, mitosis occurs predominately during dark period in the macroalgal species Ulva pseudocurvata (Titlyanov et al. 1996) and Porphyra umbilicus (Lüning et al. 1997), as well as the unicellular green alga Chlamydomonas reinhardtii (Goto and Johnson 1995) during growth and development under various photoperiods. The temporal separation of photosynthesis and photosynthetic carbon metabolism during the day and DNA replication-division during the night has been suggested to confer an evolutionary advantage by allowing for optimization of growth during the light period when energy is readily available (Bišová and Zachleder 2014).

In addition to growth rate, starch content additionally appears insensitive to both photoperiod and growth PFD (Figure 5A and Table S1). Therefore, consistent with the previous studies using CL (Savitch et al. 1996), we conclude C. vulgaris exhibits a minimal capacity to upregulate absolute metabolic sink capacity. While there was a significant decrease in starch content during the dark period at both the 18 h and 12 h photoperiods when cells were grown at
either 28/150 or 28/2000, the apparent consumption rate of starch during the daily dark period was 2-fold lower when *C. vulgaris* was grown under a 12 h photoperiod relative to an 18h photoperiod at both PFDs (Table S2). This suggests that photoperiod may modify carbohydrate metabolism in *C. vulgaris*. Similarly, growth under a short-day photoperiod has been demonstrated to decrease the rate of starch degradation during the dark relative to growth under a long-day photoperiod in *A. thaliana* which is believed to prevent pre-mature depletion of starch reserves during the night (Gibon et al. 2009; Moraes et al. 2019). Although the mechanisms regulating transient starch formation and degradation in green algae are not well understood, the corresponding increase in sucrose content during the dark period is consistent with the suggested mechanism of feedback inhibition from carbohydrate metabolism (Lepisto and Rintamaki 2012).

Studies in *C. reinhardtii* indicate starch is required both to supply energy for the metabolic demands of the cell during the dark period as well as for use as a vital energy supply for cellular division as starch reserves were consumed in this species during cellular division in the light where the energy required for division could be supplied directly by photosynthesis (Vítová et al. 2011b). This suggests a close relationship between the cellular energy stores and growth rate. We therefore suggest that the apparent inability to up-regulate growth rate in *C. vulgaris* in response to increased growth PFD may be ultimately reflective of limitations at the level of the capacity to increase starch reserves.

Despite a reasonable correlation between OD$_{735}$ and cell counts during growth under CL (Figure S2) it appears OD$_{735}$ is influenced by other optical, or light scattering properties of the cells, including cell size as well as the presence of highly light scattering carbohydrate reserves such as starch. The results for diel changes in cell size, DNA content and carbohydrate content appear consistent with recent models of cellular division in green algae (Venediktov et al. 1981; Bišová and Zachleder 2014) and indicate that oscillation in OD$_{735}$ are likely reflective of cell cycle progression in *C. vulgaris*. Diurnal cell division has been observed in multiple species of microalgae (Nelson and Brand 1979). Many models account for this periodicity in cell division by suggesting the cell cycle, or cell cycle machinery, is regulated by an endogenous circadian clock (Edmunds and Adams 1981; Homma and Hastings 1989). However, we suggest a model of cell division regulated by an endogenous clock is likely not applicable to *C. vulgaris* as the diel oscillations in OD$_{735}$ disappeared immediately upon a shift to CL (Figure 4). This is in direct contrast to the green alga *Nannochloropsis gaditana* (Braun et al. 2014) as well as the
cyanobacterium *Cyanothece* sp. (Nedbal et al. 2008) where photobioreactor-grown cultures of *N. gaditana* and *Cyanothece* sp. both exhibited similar diel oscillations in OD$_{735}$ during growth and development under a 12 h photoperiod; however, unlike *C. vulgaris*, these oscillations in OD$_{735}$ persisted upon a shift to CL (Nedbal et al. 2008; Braun et al. 2014). This likely indicates that regulation of growth by light and photoperiod in photoautotrophic microbes is species specific.

The apparent immediate synchronization of cellular division in *C. vulgaris* in response to changes in light availability following a shift to CL (Figure 4) supports a model for direct regulation of the cell cycle by the environmental light:dark cycle. Recently, the cell cycle in *C. reinhardtii* (Vítová et al. 2011a,b) and the multicellular algal *Ulva compressa* (Kuwano et al. 2008; Kuwano et al. 2014) have been demonstrated to be regulated independently of an endogenous clock. Alternative models propose that cell cycle progression is regulated directly by the light:dark cycle (Vitová et al. 2011a,b; Bišová and Zachleder 2014; Kuwano et al. 2014). These models propose that cell volume increases in the light period using the energy supplied by photosynthesis until a critical size, termed the commitment point, is reached and DNA replication and cell division occur (Vitová et al. 2011a,b; Bišová and Zachleder 2014; Kuwano et al. 2014).

Oscillations in OD$_{680}$, a measure of Chl concentration, were also detected that paralleled the response of OD$_{735}$ to the daily light:dark cycle where there was a steady increase in OD$_{680}$ during the light period and decrease during the dark period (Figure 1). Consistent with the immediate dampening of oscillations on OD$_{735}$ in CL, oscillations in OD$_{680}$ also immediately disappeared following a transfer to CL (Figure 4). The strictly diurnal increase in OD$_{680}$ suggests that Chl biosynthesis is positively regulated by light in *C. vulgaris*. The light-dependent photoreduction of protochlorophyllide to chlorophyllide is catalyzed by the enzyme protochlorophyllide oxidoreductase (POR) (Reinbothe et al. 2010). However, a second structurally unrelated dark operative POR enzyme found in photoautotrophic bacteria, algae and gymnosperms is capable of catalyzing this reaction in the dark (Reinbothe et al. 2010). Although the genes encoding the subunits of this dark operative, or light independent, POR enzyme have been detected in *C. vulgaris* (Gabruk et al. 2012), we speculate that the strictly diurnal increase in OD$_{680}$ may indicate that *C. vulgaris* does not express this enzyme under these conditions. Interestingly, circadian oscillations in δ-aminolevulinic acid (ALA), an early precursor to Chl biosynthesis, have been observed in *Hordeum vulgare* (Beator and Kloppstech 1993; Kruse et al.
1997). However, the strict diurnal increases in both OD\textsubscript{735} and OD\textsubscript{680} suggests that growth as well as Chl accumulation is light-dependent in \textit{C. vulgaris} providing an environmental control to coordinate Chl biosynthesis to periods of light exposure.

While oscillations in OD\textsubscript{680} and OD\textsubscript{735} were immediately dampened following a transfer from growth under a light:dark cycle to CL, oscillations in both F\textsubscript{M}' and F\textsubscript{S} persisted upon a shift to CL (Figure 4). This indicates PSII photochemistry may oscillate independently of cellular division in \textit{C. vulgaris}. Similar circadian oscillations in PSII quantum yield (\(\Phi_{\text{PSII}}\)) have been detected in \textit{Kalanchoe daigremontiana} using modulated Chl \textit{a} fluorescence (Wyka et al. 2005).

Circadian regulation of light harvesting capacity was first described in algae (Sweeney and Haxo 1961). Circadian oscillations in photosynthetic oxygen evolution (Sweeney and Haxo 1961) and electron transport (Mackenzie and Morse 2011) have since additionally been detected in algae. Early work by Sweeney and colleague (1961) demonstrating oscillations in photosynthesis occur in anucleated \textit{Acetabularia} (Sweeney and Haxo 1961) was later followed by the finding that oscillations in peroxiredoxin redox state occur independently of the nuclear-encoded circadian oscillator in \textit{Ostreococcus tauri} (O’Neill et al. 2011). The persistent oscillations in Chl\textit{a} fluorescence under CL in the absence of apparent rhythms in cellular division may also indicate the presence of plastid-autonomous circadian rhythms. However, more work is required to confirm this in \textit{C. vulgaris}.

\textit{C. vulgaris} under an alternating light:dark cycle exhibits oscillations in OD\textsubscript{735}, OD\textsubscript{680} and Chl \textit{a} fluorescence that are not observed during growth at development under CL. We suggest that the nocturnal decrease in OD\textsubscript{735} principally reflects changes in the light-scattering, or optical properties of the cells, including a decrease in cell size and consumption of starch reserves. However, a limitation of our study is our inability to clearly distinguish between the contributions of changes in cell size versus consumption of starch reserves to the optical properties of the Chlorella suspension. Further work is required to overcome this limitation. However, we suggest that the nocturnal catabolism of starch reserves in conjunction with the diurnal changes in DNA content and cellular volume indicate that these oscillations reflect synchronized cellular division in \textit{C. vulgaris}.

ACKNOWLEDGMENTS
N.P.A.H. acknowledges the financial support of the NSERC Discovery Grants programme, the Canadian Foundation for Innovation and the Canada Research Chairs programme.

REFERENCES


Table 1. Comparison of growth characteristic for *C. vulgaris* cultivated in either photobioreactors or growth tubes. Cells were grown to mid-log phase at either 150 or 2000 µmol photon m⁻² sec⁻¹ under continuous growth light at 28 °C. Numbers under growth regime indicate growth temperature (°C) / PFD (µmol photons m⁻² s⁻¹). The doubling times of photobioreactor-grown cells as well as tube-grown cells of *C. vulgaris* were compared using a two-way ANOVA followed by a Tukey's HSD post hoc test; means not connected by the same letter were statistically different at *p* < 0.05. Values represent mean ± SEM; *n* = 5.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Photobioreactor</th>
<th>Growth regime</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling time (h)</td>
<td>20.3 ± 0.5^a</td>
<td>25.6 ± 1.2^a</td>
<td>12.8 ± 0.8^b</td>
</tr>
</tbody>
</table>
Table 2. Growth rates of *C. vulgaris* grown at 150 and 2000 µmol photon m⁻² sec⁻¹ at 28 °C under either continuous light, an 18 h photoperiod or 12 h photoperiod. Doubling times were measured in exponentially growing cultures. Numbers under growth regime indicate growth temperature (°C) / PFD (µmol photons m⁻² s⁻¹) followed by the length of light (L) and dark (D) periods in a 24 h cycle. Values represent mean ± SEM; *n* = 5. Means for either growth rate or doubling time were compared using a two-way ANOVA; means not connected by the same letter were significantly different at *p* < 0.05.

<table>
<thead>
<tr>
<th>Growth regime</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28/150</td>
<td></td>
</tr>
<tr>
<td>24L:0D</td>
<td>20.3 ± 0.5ᵃ</td>
</tr>
<tr>
<td>18L:6D</td>
<td>24.8 ± 1.4ᵃ</td>
</tr>
<tr>
<td>12L:12D</td>
<td>24.1 ± 0.6ᵃ</td>
</tr>
<tr>
<td>28/2000</td>
<td></td>
</tr>
<tr>
<td>24L:0D</td>
<td>25.6 ± 1.2ᵃ</td>
</tr>
<tr>
<td>18L:6D</td>
<td>27.4 ± 1.3ᵃ</td>
</tr>
<tr>
<td>12L:12D</td>
<td>26.9 ± 1.3ᵃ</td>
</tr>
</tbody>
</table>
Table 3. Change in light scattering at 735 nm (OD$_{735}$) over time during a daily light period for *C. vulgaris* grown at 150 and 2000 µmol photon m$^{-2}$ sec$^{-1}$ at 28 °C under either an 18 h photoperiod or 12 h photoperiod. Numbers under growth regime indicate growth temperature (°C) / PFD (µmol photons m$^{-2}$ s$^{-1}$) followed by the length of light (L) and dark (D) periods in a 24 h cycle. Values represent mean ± SEM; $n$ = 3. Means were compared using a two-way ANOVA followed by a Tukey's HSD post hoc test; means not connected by the same letter were significantly different at $p < 0.05$.

<table>
<thead>
<tr>
<th>Growth regime</th>
<th>ΔOD$_{735}$ / h</th>
</tr>
</thead>
<tbody>
<tr>
<td>28/150</td>
<td></td>
</tr>
<tr>
<td>18L:6D</td>
<td>0.19 ± 0.05$^b$</td>
</tr>
<tr>
<td>12L:12D</td>
<td>0.16 ± 0.13$^b$</td>
</tr>
<tr>
<td>28/2000</td>
<td></td>
</tr>
<tr>
<td>24L:0D</td>
<td>0.30 ± 0.03$^a$</td>
</tr>
<tr>
<td>18L:6D</td>
<td>0.32 ± 0.05$^a$</td>
</tr>
</tbody>
</table>
Table 4. Results for statistical analysis (three-way repeated measures ANOVA) for *C. vulgaris* grown at 150 and 2000 µmol photons m\(^{-2}\) sec\(^{-1}\) under either an 18 h or 12 h photoperiod at 28 °C. *I*, irradiance; *PP*, photoperiod, *T*, time.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Source of variation</th>
<th>Result</th>
<th>Conclusion (post hoc test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Starch</strong></td>
<td><em>I</em></td>
<td>(F_{1,8} = 23.2, p = 0.261)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>PP</em></td>
<td>(F_{1,8} = 17.3, p = 0.327)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T</em></td>
<td>(F_{1,8} = 76.1, p &lt;0.0001)</td>
<td>EL &gt; ED</td>
</tr>
<tr>
<td></td>
<td><em>I</em>T</td>
<td>(F_{1,8} = 0.07 p = 0.793)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>PP</em>T</td>
<td>(F_{1,8} = 0.3, p = 0.590)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>I</em>PP</td>
<td>(F_{1,8} = 30.2, p = 0.205)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>I</em>PP*T</td>
<td>(F_{1,8} = 1.3, p = 0.294)</td>
<td></td>
</tr>
<tr>
<td><strong>Sucrose</strong></td>
<td><em>I</em></td>
<td>(F_{1,8} = 405.9, p &lt;0.0001)</td>
<td>HL &gt; LL</td>
</tr>
<tr>
<td></td>
<td><em>PP</em></td>
<td>(F_{1,8} = 91.3, p &lt;0.0001)</td>
<td>PP18h &gt; PP12h</td>
</tr>
<tr>
<td></td>
<td><em>T</em></td>
<td>(F_{1,8} = 161.3.2, p &lt;0.0001)</td>
<td>ED &gt; EL</td>
</tr>
<tr>
<td></td>
<td><em>I</em>T</td>
<td>(F_{1,8} = 26.9, p = 0.0008)</td>
<td>HL:ED &gt; HL:EL &gt; LL:ED &gt; LL:EL</td>
</tr>
<tr>
<td></td>
<td><em>PP</em>T</td>
<td>(F_{1,8} = 6.7, p = 0.0320)</td>
<td>18h:ED &gt; 18h:EL = 12h:ED &gt; 12h:EL</td>
</tr>
<tr>
<td></td>
<td><em>I</em>PP</td>
<td>(F_{1,8} = 7.4, p = 0.026)</td>
<td>HL18h = HL12h &gt; LL18h &gt; LL12h</td>
</tr>
<tr>
<td></td>
<td><em>I</em>PP*T</td>
<td>(F_{1,8} = 3.4, p = 0.102)</td>
<td></td>
</tr>
<tr>
<td><strong>Cell volume</strong></td>
<td><em>I</em></td>
<td>(F_{1,8} = 63.8, p &lt;0.0001)</td>
<td>HL &gt; LL</td>
</tr>
<tr>
<td></td>
<td><em>PP</em></td>
<td>(F_{1,8} = 20.9, p = 0.002)</td>
<td>PP18h &gt; PP12h</td>
</tr>
</tbody>
</table>

https://mc06.manuscriptcentral.com/botany-pubs
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>$F_{1,8}$</th>
<th>p</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$T$</td>
<td></td>
<td>22.8</td>
<td>0.001</td>
<td>EL &gt; ED</td>
</tr>
<tr>
<td>$I^*T$</td>
<td></td>
<td>0.04</td>
<td>0.852</td>
<td></td>
</tr>
<tr>
<td>PP*$T$</td>
<td></td>
<td>0.2</td>
<td>0.649</td>
<td></td>
</tr>
<tr>
<td>$I^*PP$</td>
<td></td>
<td>3.4</td>
<td>0.104</td>
<td></td>
</tr>
<tr>
<td>$I^<em>PP</em>T$</td>
<td></td>
<td>5.8</td>
<td>0.043</td>
<td></td>
</tr>
</tbody>
</table>

See two-way ANOVAs for LL and HL, respectively.

### LL

<table>
<thead>
<tr>
<th></th>
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<th>$F_{1,4}$</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>PP</td>
<td></td>
<td>3.4</td>
<td>0.139</td>
</tr>
<tr>
<td>$T$</td>
<td></td>
<td>5.9</td>
<td>0.072</td>
</tr>
<tr>
<td>PP*$T$</td>
<td></td>
<td>1.0</td>
<td>0.364</td>
</tr>
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</table>

### HL

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>$F_{1,4}$</th>
<th>p</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td></td>
<td>23.1</td>
<td>0.009</td>
<td>PP18h &gt; PP12h</td>
</tr>
<tr>
<td>$T$</td>
<td></td>
<td>57.8</td>
<td>0.002</td>
<td>EL &gt; ED</td>
</tr>
<tr>
<td>PP*$T$</td>
<td></td>
<td>5.8</td>
<td>0.041</td>
<td>PP18h:EL &gt; PP18h:ED = PP12h:EL = PP12:ED</td>
</tr>
</tbody>
</table>

ED, end dark; EL, end light; HL, high light; LL, low light; PP18h, 18h photoperiod; PP12h, 12h photoperiod.
FIGURE LEGENDS

Figure 1. Representative growth curves for cells of *C. vulgaris*. (A) Cells grown at 28 °C / 150 photons m\(^{-2}\) sec\(^{-1}\) or (B) 28 °C / 2000 photons m\(^{-2}\) sec\(^{-1}\) under either 24 h, 18 h or 12 h photoperiods in a 24 h cycle. An integrated densitometer measured change in cell density over time which was monitored as light scattering at 735 nm as well as change in chlorophyll mL\(^{-1}\) at 680 nm at 30 min intervals. Values represent temperature (°C) / growth PFD (µmol photons m\(^{-2}\) s\(^{-1}\)) followed by the length of the light (L) and dark (D) periods in a 24 h cycle.

Figure 2. Representative growth curves for cells of *C. vulgaris*. Cells grown at (A) 28 °C / 150 photons m\(^{-2}\) sec\(^{-1}\) or (B) 28 °C / 2000 photons m\(^{-2}\) s\(^{-1}\) under either 24 h, 18 h or 12 h photoperiods in a 24 h cycle. An integrated fluorometer measured light-adapted maximum Chl \(a\) 678 nm fluorescence (\(F_M\); closed circles) and the steady-state chlorophyll \(a\) fluorescence (\(F_S\); open circles) using blue excitation light at 30 minute intervals. Values represent temperature (°C) / growth PFD (µmol photons m\(^{-2}\) s\(^{-1}\)) followed by the length of the light (L) and dark (D) periods in a 24 h cycle.

Figure 3. Change in optical density and chlorophyll content over a three day period. Cultures of *C. vulgaris* were grown to mid-log phase at 28 °C /150 µmol photons m\(^{-2}\) sec\(^{-1}\) under either continuous light (CL), an 18 h photoperiod (18L:6D) or 12 h photoperiod (12L:12D). Optical density was measured at 735 nm while chlorophyll per mL was measured as absorption at 680 nm. Open bars represent the daily light period while closed bars represents the daily dark period in a 24 h cycle. Numbers in the panels represent the length of the light period (L) and dark period (D) in a 24 h cycle.

Figure 4. Effect of a shift to continuous light on photoperiod-dependent oscillations. Cells of *C. vulgaris* grown to mid-log phase at 28 °C /150 µmol photons m\(^{-2}\) sec\(^{-1}\) under a 12 h photoperiod were shifted to continuous light, indicated by an arrow, during the exponential growth phase. Change in (A) optical density (735 nm), (B) chlorophyll content per mL (680 nm) and (C) the
chlorophyll a fluorescence parameters F_M' and F_S were measured at 30 minute intervals. Trends were confirmed by 3 independent experiments.

Figure 5. Effect of photoperiod on starch and sucrose content during a daily dark period in C. vulgaris cells grown at either 150 or 2000 μmol photons m^{-2} s^{-1} under either an 18 h or 12 h photoperiod at 28 °C. Carbohydrate content was measured immediately following the end of a daily light period (open bars) and immediately following the end of the daily dark period (closed bars). Means not connected by the same letter are significantly different at p < 0.05. Data represent mean ± SEM; n = 3.

Figure 6. Effects of photoperiod on cell volume and cell size. (A) Change in cellular volume during a daily dark period. C. vulgaris was grown at either 150 or 2000 μmol photons m^{-2} s^{-1} at 28 °C under either a 12 h photoperiod (12L:12D) or 18 h photoperiod (18L:6D) photoperiod. Cellular volume was measured immediately prior the end of a daily light period (open bars) as well as immediately prior to the end of the daily dark period (closed bars). See Table 4 for statistical differences in cellular volume. Data represent mean ± SEM; n = 3. (B) Representative histograms illustrating the distribution of cell sizes, where cellular diameters was estimated using flow cytometry as forward scatter (FSC), take immediately prior to the end of the light period (end light) as well as prior to the end of the dark period (end dark).

Figure 7. Representative diel changes in cell density, cellular DNA content and cellular volume. C. vulgaris was grown to mid-log phase at 28 °C / 150 μmol photons m^{-2} s^{-1} under a 12 h photoperiod. (A) Daily changes in DNA fluorescence were assayed using flow cytometry as a function of time where time 0 h represents the start of a 12 h light period for cells grown under a 12 h photoperiod. (B) Cellular volume was measured using flow cytometry at the same time points. Trends were confirmed with independent biological replicates. The open bar under the graph represents the daily light period while the closed bar represents the daily dark period.
FIGURES

Figure 1
Figure 2
Figure 3

A – CL

B – 18L:6D

C – 12L:12D

Optical density (735 nm)

Time (days)
Figure 4

A. Optical density (735 nm)

B. Optical density (680 nm)

C. Fluorescence (a.u.)

Time (h)
Figure 5
Figure 6

(A) Cell volume (μm³)

- 18L:6D
- 12L:12D
- 18L:6D
- 12L:12D

- 28/150
- 28/2000

(B) Forward scatter x 10⁴ (FSC)

- End dark
- End light
Figure 7

Cell volume (µm³)

Normalized DNA fluorescence cell⁻¹ x 10⁻⁵

Time (h)

0h 3h 6h 12h 24h

0.0 1.0 2.0 3.0 4.0 5.0

0h 3h 6h 12h 24h

0 100 200 300 400 500