Long-Term Survival of *Chlamydomonas Reinhardtii* During Conditional Senescence

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Research Article

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Abstract

Chlamydomonas reinhardtii undergoes conditional senescence when grown in batch culture due to nutrient limitation. Here we explored plastid and photo-physiological adaptations in *Chlamydomonas reinhardtii* during a long-term ageing experiment by methodically sampling them over 22 weeks. Following exponential growth, *Chlamydomonas* entered an extended declining growth phase where cells continued to divide, though at a lower rate. Ultimately this ongoing division was fueled by the recycling of macromolecules, that was obvious in the rapidly declining protein and chlorophyll content in the cell during this phase. This process was sufficient to maintain a high level of cell viability as the culture entered stationary phase. Beyond that, cell viability starts to plummet. During the turnover of macromolecules after exponential growth that saw RuBisCO levels drop, the LHClI antenna was relatively stable. This, along with the upregulation of the light stress-related proteins (LHCSR), contributes to an efficient energy dissipation mechanism to protect the ageing cells from photooxidative stress during the senescence process. Ultimately, viability dropped to about 7% at 22 weeks in a batch culture. We anticipate that this research will help further understand the various acclimation strategies carried out by *Chlamydomonas* to maximize survival under conditional senescence.

Introduction

Senescence is the age-dependent deterioration process at the cellular, tissue, organ, or organismal level, ultimately leading to death. In plants, senescence is illustrated by the dramatic color changes that occur in the fall in temperate regions of the world. It is not a chaotic and unorganized breakdown but rather an orderly, active loss of normal function (Lim and Nam 2007). While this process is well studied in plants, the molecular responses associated with senescence in unicellular microorganisms is not well established.

Despite being “immortal” when grown in favourable conditions, symmetrically dividing unicellular organisms are known to undergo conditional senescence as a result of starvation-induced conditions (Nyström 2003; Lillie and Pringle 1980). In bacteria, conditional senescence leads to a reduction in cell size, often a loss in membrane integrity, and a drop in the overall rate of protein synthesis (Nyström 2003), but also the induction of a distinct set of proteins involved in stress responses (Kolter et al. 1993). Similarly, when yeast enter stationary phase and start senescence, the cells undergo a variety of changes to enhance longevity, including changes in the cell wall composition, storage carbohydrate accumulation, and induction of stress-related genes (Werner-Washburne et al. 1993). This indicates that unicellular microorganisms do age under the appropriate environmental conditions (Florea 2017).

Photosynthetic microorganisms also show signs of conditional senescence. In dinoflagellates and diatoms, there is a loss in chlorophyll during chloroplast degradation, similar to plant senescence (Messer and Ben-Shaul 1972; Louda et al. 1998). During conditional senescence in green microalgae, thylakoid

membranes become more compacted and with reduced density, chloroplast size declines, and there is an accumulation of lipid globules (McLean 1968; Humby et al. 2013), which also commonly occurs in leaves during senescence (Hurkman 1979). The microalga, *Spongiochloris typica*, acquires thickened cell walls as they enter stationary phase as a prelude to the formation of resting cells (McLean 1969). Indeed, some microalgae can undergo a transition to stress-resistant forms such as resting cells or cysts, that are arrested in the cell cycle as an alternative program to conditional senescence (Ellegaard and Ribeiro 2018a).

Conditional senescence in photosynthetic microorganisms is a unique challenge because of the potential damage from excess light. If cultures become nutrient-depleted while the light conditions remain the same, this increases the chances of a photooxidative stress. The reduction of reductant-consuming pathways, like reproduction and catabolic reactions, can lead to a build-up metabolic intermediates and can cause an indirect light stress. This stress can lead to an increase in reactive oxygen species (ROS) production. This energy would have to be diverted to other pathways or the energy effectively dissipated to reduce the cell from inadvertently producing ROS. While this ROS stress would certainly damage proteins and lipids if produced in excess of the detoxification capabilities of the cell, the unique combination of stressors during conditional senescence could produce a unique ROS signature that may signal the cells to acclimate appropriately (Choudhury et al. 2017). In the case of microalgae during conditional senescence, cells under high-light stress rapidly depleted PSII complexes and induced a pH-dependent quenching mechanism focused on detached LHCII antenna in a way that differs from a similar stress under nutrient-replete conditions (Meagher et al. 2021). Overall, during conditional senescence the photosynthetic apparatus is being disassembled and recycled on a large scale, yet certain components are maintained to have an ongoing photoprotection potential that could affect longevity.

How microalgae like *Chlamydomonas* survive and extend lifespan under chronic, nutrient depleted conditions is poorly understood. While there are many nutrient-depletion approaches that focus on individual nutrients over a short-time frame, conditional senescence is different in the time-frame and order of physiological and biochemical changes in the cell. Even in nutritional depletion studies of *Chlamydomonas*, there are differences in the response to individual and combined nutrient stresses (Kamalanathan et al. 2016; Yang et al. 2018). Presumably in conditional senescence, the combination of nutrient stresses is even more complex, though less well defined and dependent of the exact growth conditions. The objective of our study is to follow the long-term physiological and photo-physiological changes in *Chlamydomonas reinhardtii* during conditional senescence in a batch culturing system that is commonly used in many laboratories. We followed established senescence markers such as chlorophyll content, protein levels, and photochemical efficiency to examine the general approach to survival under these growth conditions. Our aim is to better understand strategies used by *Chlamydomonas reinhardtii* at extending their lifespan during conditional senescence and to use this baseline study to further explore the determinants of longevity under conditional senescent conditions.

Materials And Methods

Culturing and induction of Conditional Senescence

Chlamydomonas reinhardtii strain CC-125 was used for these experiments. A culture of CC125 was inoculated in TAP (Tris-Acetate-Phosphate) (Harris 2013) and grown for four days under low light on an orbital shaker at 30 rpm, ensuring that the culture remained at or below $5\text{-}6 \times 10^6$ cells ml^{-1} . The starting culture was then diluted with fresh TAP to a final density of 3×10^5 cells ml^{-1} and transferred to forty, 50 ml Erlenmeyer flasks with 22 ml of culture. This approach avoided any lag phase (Phase 1) from the growth curve that one typically observes when cultures are inoculated directly from plates or older cultures. This reduced variability in the timing of conditional senescence. The flasks were covered with a foam stopper and the top wrapped in aluminum foil. The flasks were then set in continuous low light of about $70 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 22°C on an orbital shaker at 30 rpm for a period of 152 days. Sampling was done on the 4th, 11th, 25th and, 40th day post inoculation (DPI) and every two weeks after that until day 152.

Cell abundance and viability

Cells were counted by hand using a Neubauer ultraplane ($1/400$ SQ. MM) hemocytometer under a light microscope and the viability assessed using SYTOX® orange nucleic acid (Life technologies, CA, USA) as described by Meagher et al (2021).

Cumulative survival curves were calculated by multiplying the percent viability of the one sampling day with the viability of the sampling day that it precedes. However, because dead cells don't decay immediately and can potentially be counted as dead in successive timepoints, we applied a correction factor to better estimate a survival curve. The total number of dead cells counted at a time point was corrected by accounting for the cell decay rate, determined from the slope of cell abundance curve post-stationary (from the curve in Fig. 1A). Beyond stationary, the maximum number of cells were used in the calculation of viability with the assumption that there was limited growth after that point, and only cell death, which is obviously a simplification but a reasonable assumption.

Chlorophyll quantification and fluorescence

Chlorophyll was extracted from 0.5 ml of culture in 80% acetone according to Meagher et al (2021). For fluorescence, samples were kept in the dark for about 15 minutes then 0.5ml of sample was filtered onto a 25 mm glass microfiber filters (GF/C™, Whatman™, GE healthcare life sciences, UK). The chlorophyll fluorescence was then measured using a PAM101 pulse amplitude modulated fluorometer (Walz, Germany). The samples were exposed to a saturating light pulse (0.6s) of $2500\text{--}3000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ to “close” the PSII reaction center to measure the maximum fluorescence in the dark-adapted state (F_m). The samples were then exposed to actinic light of $700 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 13 pulses to assess their ability to deal with short-term light stress. The actinic light was then turned off and the recovery process was followed for about 20 minutes in the dark.

Fluorescence parameters calculated after 5 mins into the actinic light period included the quantum yield

of NPQ ($\phi_{NPQ} = \frac{F_s}{F_m'} - \frac{F_s}{F_m}$); the quantum yield of non-regulated energy dispersion of PSII,

($\phi_{NO} = \frac{F_s}{F_m}$), which represents all the heat dissipating mechanisms that are non-photo-protective; and

the quantum efficiency of PSII ($\phi_{PSII} = \frac{F_m' - F_s}{F_m'}$), which represents the redox state of PSII (Kramer et al. 2004). Also, we calculated the non-photochemical quenching using the classic equation

($NPQ = \frac{F_m - F_m'}{F_m'}$) and also the corrected NPQ (Tietz et al. 2017) was also used to account for the possibility of unrelaxed quenching according, as implemented by Meagher et al (2021).

Protein extraction and analyses

Total cell protein was isolated from 5ml of culture. Processing of the cells, quantitation of the proteins, fractionation of the proteins on 12% SDS-Polyacrylamide gels, and Western blotting were done as described by Meagher et al (2021).

Results

Declining Growth Phase-Phase 3

The experiment was designed so that measurements started just as the cells were exiting exponential phase (Phase 2), which occurred at about 4 days post-inoculation (Fig. 1a). However, cells continued to grow for at least 40 days in batch culture at a reduced growth rate of 0.038 doublings per day, compared to about 1 doubling per day from 0–4 days; a phase we call the declining growth phase (DGP – phase 3, Fig. 1a). At the end of the DGP the final cell abundance was 1.6×10^7 cell ml⁻¹. There were no significant differences in cell viability in this phase ($p < 0.01$), with over 95% of the cells being viable. Cumulative survival was estimated at 90% over the entire DGP (Fig. 1b).

The DGP marked the beginning of the degradation processes where total chlorophyll levels per viable cell dropped significantly by about 44%, from a high of 5.5 to 3.2 pg Chl cell ml⁻¹ at day 40 ($p < 0.01$, Fig. 2a), though there was no change in the ratio of chlorophyll a to b (Fig. 2b). Total cell protein levels dropped significantly by over 70% on a viable cell basis (Fig. 3A, $p < 0.01$). RuBisCO, one of the most abundant proteins in the cell, showed large declines during this phase (Fig. 3B). The major light harvesting complex proteins also declined, but by a small amount, and remained otherwise stable through the DGP. The stress-related protein LHCSR, however, increased substantially during the DGP, as expected (Fig. 3B).

Fluorescence parameters used to assess light-usage efficiency in the cells, showed changes in how excess light was handled by the cell. There were no significant changes in the maximum quantum efficiency of PSII (F_v/F_m) or the F_o/F_m parameter during the declining growth phase (Fig. 4). However,

when faced with a light stress, PSII efficiency (ϕPSII) is zero, meaning that the excess light is being dissipated rather than being used to drive photosynthesis (Fig. 5a). The proportion of light dissipated by non-photochemical quenching (ϕNPQ) increases significantly during the DGP (Fig. 5b), but a portion of the light is still dissipated by non-regulated means (ϕNO) (Fig. 5c). We also calculated NPQ 5 minutes into actinic light exposure using the classic equation based on the Stern-Volmer relationship ($\text{NPQ} = (F_m - F_m')/F_m'$). This NPQ calculation peaked at day 25 cultures and remained high for the remainder of the DGP (Fig. 6, supplementary material), similar to the ϕNPQ calculation of Karmer et al (2004). The higher NPQ capacity roughly correlates with the induction of LHCSR during the DGP.

Stationary Phase—Phase 4:

Stationary phase would normally be defined by the lack of additional cell growth, though it is questionable whether that definition would accurately describe *Chlamydomonas* cultures under these conditions. Nevertheless, Phase 4 was defined by an apparent lack of increase in cell abundance, though it wasn't always straight-forward to define at what point the cell number plateaued. However, there are other physiological parameters that help define this stage between days 40 and 68. The onset of stationary phase coincides with smaller reductions in the mean cell viability over 4 weeks, but the variability at this stage was such that the differences were not significant (Fig. 1a). Cumulative survival, however, was different from the DGP where the day 68 average cumulative survival was at 0.57 (Fig. 1b). The general cell decline continued in stationary: total chlorophyll levels drop an additional 30% (60% decline from exponential levels) and total protein content per cell reaches its lowest levels of 2.3 pg per viable cell, a significant 84% drop from exponential phase (Fig. 3a). Amongst these declines in total protein, the LHCSR antenna proteins are more-or-less stable (Fig. 3b). Somewhat unexpectedly, the amount of LHCSR in the cell transiently drops at the end of stationary phase (day 68, Fig. 3b).

Under relatively low-light conditions, stationary phase is where there are clearly significant changes in the photosynthetic apparatus. There is a drop in the maximum quantum efficiency of PSII in stationary phase, reaching a low of 0.3 at day 68 (Fig. 4a). This drop in F_v/F_m is driven by an increase in the F_o , which is obvious by the increase in the F_o/F_m value starting at day 54 and remaining high throughout stationary (Fig. 4b). There are apparent variations in the way excitation energy is handled in stationary phase with declines in ϕNPQ and a corresponding increase in ϕNO (Fig. 5b, c). However, using the classic NPQ equation, the NPQ returns to low levels. Because this could be due to residual quenching following dark adaptation, thus underestimating the true F_m , we calculated corrected NPQ (Tietz et al 2017), which showed that NPQ levels remain high during this phase (Fig. 6, supplementary material).

Death Phase—Phase 5.

The march of death for *Chlamydomonas* begins in earnest after 68 days in culture with an exponential decline in viability that continues to day 124. By day 124, only 15% of the cells are viable, with the rate of cell death between day 68 and 124 being roughly 2.5×10^5 cells per day (Fig. 1a). Beyond day 124, the cells remaining die at a slower rate and by day 152, about 7% of the cells in the culture remain viable.

Cumulative survival at the end of this experiment (day 152) was 0.012 (Fig. 1b). By the start of the death phase, the protein levels per viable cell are at their lowest but gradually start to rise, and by the end of the death phase reach a level that is roughly 4 times higher than cells in exponential. This late increase is potentially an artifact since it is difficult to account for residual protein from dead cells inflating the estimates. During this phase chlorophyll levels gradually drop approximately 80% from exponential phase maximum, such that the culture looks white (Fig. 2a). While the concentration of LHClI proteins remained fairly constant up to day 96, beyond that, the levels of the protein declined until only trace amounts were present at day 138. The Chl a/b ratio after day 96 was around 1.5, implying that most of the remaining chlorophyll was likely associated with the antenna rather than reaction centers, though this wasn't directly assessed (Fig. 2B). LHCSR, however, rebounded from its transient decline between days 96 and 110, a period that coincided with drops in LHClI content (Fig. 3B). LHCSR levels declined to lower levels on days 124 and 138. Beyond day 138, protein samples were challenging to work with and proteins did not migrate well on gels, so they were abandoned.

During death phase, the fluorescence characteristics remain similar for the cells in stationary phase. The high F_o/F_m remained a feature along with the suppressed F_v/F_m (Fig. 4a,b). ϕNPQ and corrected NPQ calculations suggest that the remaining cells continued to have a capacity for dissipation of light with the remaining LHClIs and LHCSR proteins in the membrane. Fluorescence signatures beyond day 138 were difficult to resolve owing to the exceedingly low levels of chlorophyll, so interpretation of these values is limited.

As the cultures aged, it was increasingly difficult to distinguish between intact cells and cell debris. Therefore, to verify the accuracy of our data in the ageing cultures, we also determined the number of viable cells using the viable plate counts method (VPC). We performed the VPC on the samples as from day 138. There were no significant differences between the data assessed using SYTOX® Orange and the data obtained from the VPC method ($P = 0.130$, data not shown). Another issue with such long-term cultures is they can be susceptible to evaporation, even though there are foam stoppers and aluminum foil covering them. We estimated that there was 57 μ l of water lost through evaporation per day. This could skew the cell abundance slightly toward higher values in the older cultures, but with the cell viability estimates, that remains a minor complication.

Discussion

This study was initiated after finding an old culture of *Chlamydomonas* from a long-departed student (about a year earlier) on a shaker was completely bleached (and presumed dead) yet recovered when plated out on fresh media. The strategy for surviving this length of time in the absence of nutrients, and presumably an ongoing stress, brought up many interesting questions about the long-term survival under adverse conditions. Understanding how *Chlamydomonas* ages in batch culture and its strategy for survival is not only a relevant ecological question, but one relevant for biotechnology for the production of high-value products in cell culture (Scranton et al. 2015).

We would normally predict that *Chlamydomonas* doesn't age under benign, nutrient-sufficient conditions, continuing to divide akin to an immortal cell line. Budding yeast (*Saccharomyces cerevisiae*), can also be propagated indefinitely, but individuals within these asymmetrically dividing unicells do eventually age and die. The budding yeast is a model system for investigating both replicative lifespan (number of cell divisions before senescence) and chronological lifespan (longevity in a non-dividing state) (Denoth Lippuner, Julou, and Barral 2014). While a chronological lifespan concept is more appropriate for *Chlamydomonas*, the symmetrically dividing fission yeast (*Schizosaccharomyces pombe*) may be a better system to compare concepts of ageing in microalgae. In fission yeast, there is no evidence of ageing in cells grown under favourable conditions; however, if these yeast cultures are stressed, evidence of cellular ageing can be detected (Coelho et al 2013). In particular, heat shock protein-associated protein aggregates are asymmetrically distributed between "symmetrically" dividing cells such that there is an old and young product of cell division (Coelho et al. 2013; 2014). The younger of these cells would be free of the protein aggregates, putting it on the path to recovery. Even the bacterium, *Escherichia coli*, that apparently divides symmetrically, the offspring can differentially possess new or old cell components that can affect its subsequent growth rate (Stewart et al. 2005; Lindner et al. 2008). While there are other studies looking at ageing in unicellular organisms (Florea 2017), it's unknown whether something similar occurs with *Chlamydomonas* cells during cell division. However, culture heterogeneity has been described where there are different populations of cells with distinct growth-rates in the same culture (Damodaran et al. 2015), which may suggest that there is some type of ageing occurring in these cells even though these symmetrically dividing cells were grown under favourable conditions.

Our system deals more with conditional senescence, where cells senesce as nutrient levels are depleted and there is a transition from favourable to unfavourable conditions. It's under these conditions where cell division is limited that we would predict cellular ageing to accelerate. The transition to conditional senescent conditions is first evidenced by the changing growth rate as cells run out of nutrients. This declining growth phase where cells continued to grow, though at a much-reduced rate, lasted for about 40 days. The declining growth phase is able to support limited growth by recycling internal macromolecules. In particular, protein levels per cell decline by close to 70%. This is a long-observed pattern in bacteria where protein levels decline upon entry into stationary phase when other stored nutrients are depleted (Wanner and Egli 1990). Similar protein declines are also observed in *Chlamydomonas reinhardtii* during nitrogen, phosphorus, or sulphur deprivation experiments (Cakmak et al. 2012; Kamalanathan et al. 2016). While part of the mechanism for this reduction in protein during senescence is dilution through cell division (Meagher et al. 2021), the activation of autophagy is likely an essential process regulating the turnover of proteins to enhance nutrient recycling. Evidence for the importance of autophagy in senescence includes the induction of ATG8 in *Chlamydomonas* cultures approaching stationary phase (Pérez-Pérez, Florencio, and Crespo 2010; Meagher et al. 2021). ATG8 is a ubiquitin-like protein that is conjugated to a phospholipid in a step required for autophagosome formation (Mizushima, Yoshimori, and Ohsumi 2011; Pérez-Pérez and Crespo 2010). Autophagy was found required for turnover of ribosomal proteins and regulation of lipid metabolism (Couso et al. 2018), so this process is important for metabolic restructuring in the cell. This nutrient recycling likely supported a limited cell division, but it

also extended lifespan during conditional senescence— cell viability remained high during the declining growth phase.

It's clear that the duration and characteristics of the declining growth phase is variable depending on how the cells are grown, and perhaps the strain used. For instance, Humby et al. (2103) used a cell wall-less strain under low gas-exchange conditions and observed a DGP that was very short; cells entered stationary shortly after the exponential phase. Under active bubbling for maximum gas exchange using the same strain as in this study (CC125), the declining growth phase lasted for at least 10 days following the exit from logarithmic growth (Meagher et al. 2021). The declining growth phase is often ignored or not identified in many culturing systems. In fact, these extended declining growth phases are a hallmark of batch culture growth of bacteria (Wanner and Egli 1990) and fungi (Borrow et al. 1964; Vrabl et al. 2019). It's been proposed that entry in this phase is due to a limiting nutrient that curbs cell growth, the slope of which gets lower as other nutrients become depleted (Monod 1949; Vrabl et al. 2019). This is likely the case in the variation we observed in different studies, where there is depletion of CO₂, acetate, nitrogen, phosphorous, or a micronutrient. It's clear that by altering the culturing conditions, particularly changing the potential for gas exchange, the progression of the standard growth curve can shift dramatically.

Chlorophyll degradation/dilution in conditional senescence starts shortly after exiting exponential phase and continues through the declining growth, stationary, and death phases. The degradation of chlorophyll during senescence is a common response in photosynthetic organisms and the result of activation of several enzymes that break down chlorophyll to a variety of catabolites (Hörtensteiner 2006), and in some ways resembles the loss of chlorophyll during nutrient deficiency (Plumley and Schmidt 1989; Kamalanathan et al. 2016). Under stress conditions when the normal sink capacities are limited, chlorophyll can be a potential cellular photo-toxin when the absorbed light energy is diverted inappropriately to oxygen, leading to the production of reactive oxygen species (ROS) (Hörtensteiner and Kräutler 2011). The large and rapid drop in RuBisCO content after exiting exponential growth phase (Fig. 3B) is indicative of a lost sink capacity and the risk associated with maintaining the light harvesting machinery. Excess light in the absence of an appropriate sink could lead to an oxidative stress where ROS can damage macromolecules (Apel and Hirt 2004). It's interesting that our earlier study using a cell wall-less strain showed no decline in chlorophyll levels as cells aged in a more closed culturing system, despite the fact that thylakoid membrane density and chloroplast size declined dramatically (Humby et al. 2013). While this was unexpected, we proposed that chlorophyll may have accumulated in the enlarging oil bodies as cells aged. However, it's possible that this was an artifact of the cell wall-less strain that was used. These cells lyse shortly after death, perhaps leaving plastid fragments containing chlorophyll that were still harvestable in later steps given the more accelerated senescence timeline.

The drop in the Chl a/b ratio in the death phase (after day 68), and the relatively stable maintenance of LHCII levels implies there is a preferential maintenance of the LHC antenna over reaction centres during conditional senescence (Humby et al. 2013; Meagher et al. 2021). This was also observed in the leaves of senescing *Arabidopsis* (Nath et al. 2013). The Chl a/b ratio started to decline at about 54 days post

inoculation, which corresponded with the drop in Fv/Fm and increase in the Fo/Fm, that remained high throughout stationary and death phases. As Meager et al (2021) argued, this is likely due to the detachment of the antenna from the PSII reaction centre, leading to an increase in the Fo, and subsequent drop in the Fv/Fm. In that study, the increase in the Fo/Fm was only apparent in a HL-stress culture in the DGP, not the low-light culture, indicative of a light-stress response. In this study, the Fo/Fm increase was pushed to the stationary phase under low-light conditions, indicating an indirect-light stress caused by limited sink capacity. Of course, LHCII is a major antenna complex having a dual function; light-harvesting and photo-protection (Natali and Croce 2015). LHCII is able to switch to a quenched conformation in light stress conditions thereby dissipating excess light in the form of heat (Tian et al. 2015). The abundance of LHCII well into the death phase could suggest its importance during conditional senescence, most likely in photo-protection that is triggered by LHCSR.

The ability of *Chlamydomonas* to deal with excess light changed throughout conditional senescence. When faced with an excess-light challenge, the quantum efficiency of PSII (ϕ PSII) was always close to zero, indicating the reaction centers were fully reduced and all of the incoming light was being dissipated when challenged with excess light. Initially, this energy was dissipated in a manner that was not photoprotective (high ϕ NO) when non-photochemical quenching (ϕ NPQ) capacity was low. It is indicative of cells that are unable to properly protect themselves from excess light (Klughammer and Schreiber 2008), that could invariably lead to photoinhibition. However, ϕ NPQ started to increase at day 11, reaching a maximum at 25 days post inoculation. ϕ NPQ corresponds to the fraction of energy dissipated in the form of heat via regulated, nonphotochemical quenching mechanisms (Kramer et al. 2004). This increase is very likely due to the upregulation of LHCSR at day 11, which is known to be a major player in NPQ in *Chlamydomonas* (Peers et al. 2009).

The stress-related LHC proteins (collectively LHCSR1 and 3) were notable in their upregulation in response to senescence, peaking shortly after exponential growth. This stress-related protein is upregulated during high-light stress conditions and responsible, in part, for the induction of non-photochemical quenching in *Chlamydomonas* (Peers et al. 2009). LHCSR also responds to a variety of stresses, such as nutrient limitation, not just light (Toepel et al. 2013), so its induction during senescence is not surprising and previously observed (Humby et al. 2013; Meagher et al. 2021). The induction of LHCSR likely facilitates the conversion of the antenna into light-quenching centers, acting as a photo-protective mechanism for the reaction centers (Girolomoni et al. 2019). LHCSR is able to sense pH variations in the thylakoid lumen and can reversibly switch its conformation from a light-harvesting one to a dissipative one (Peers et al. 2009). This would essentially assist in neutralizing the incoming light energy to minimize the production of ROS under these stress conditions. The transient decline in LHCSR levels at days 68 and 82 was very consistent between replicates, yet a rather curious response. It's possible that recovered nutrients from dying cells in stationary contributed to the decline, but otherwise, the signal triggering the reduction is unknown. However, the drop in LHCSR does roughly correlate to the transit decline of ϕ NPQ during these timepoints, and the increase in ϕ NO. This reinforces the known link of LHCSR with quenching potential (Peers et al. 2009).

This work highlights strategies used by *Chlamydomonas* to extend their lifespan under conditional senescence. Following the exit from exponential growth, cells maintain a low-level of cell division for an extended period through recycling macromolecules and reducing protein content and the size of the photosynthetic apparatus. During this time, photoprotective mechanisms are induced, and these remain high throughout the period, to minimize the production of reactive oxygen species. The prolonged maintenance of LHCII over a period of almost 124 days and the induction of LHCSR has an important role in this process. While we proposed a shift in the photo-protective mechanisms involving bulk fluorescence, LHCII could also potentially have a structural role in maintaining the integrity of the thylakoid membranes in ageing cultures, which might be essential for the recovery process when the conditions improve.

An important question is whether there are adaptations in the surviving cells that encourage long-term survival under nutrient depleted conditions. Certainly, sexual reproduction is activated in *Chlamydomonas* under nutrient stress and the resulting zygote (hypnozygotes or zygospores) is effectively a resting stage resistant to environmental stress (Daniel, Henley, and VanWinkle-Swift 2007; Ellegaard and Ribeiro 2018b). However, there are also non-sexual resting cells that are more comparable to this study. Many microalgae can form resting cells, cysts, or akinetes with a similar environmental stress resistance (Coleman 1983). In the green alga, *zygonema*, for instance, increasing age of the culture leads to the formation of what are called “pre-akinetes” that are the result of the accumulation of storage products and thickening of the cell wall. In *zygonema*, there is a metabolic restructuring that pushes metabolism toward production of storage products (Arc et al. 2020), something that also occurs in *Chlamydomonas* with age and nutrient deprivation (Siaut et al. 2011). We don’t have any clear evidence for formation of stress-resistant resting cells, or its equivalent. But, for instance, we did observe a gradual increase in protein per viable cell beyond 68 days. This may represent a collective partial recovery from scavenged nutrients from dying cells and perhaps these cells are more resistant to the stress condition due to some change in the cell wall or other intracellular structures. However, it is questionable whether this increase in protein is real or an artifact of the cell collection process where protein from dead cells could clearly be included, but it is nevertheless an intriguing question whether *Chlamydomonas* can produce something akin to a resting cell or akinete, indicating a programmed response.

Declarations

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DD conducted the research, analyzed data and participated in the writing of the manuscript. DGD designed the experiment, analyzed the data, and wrote the manuscript.

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Figures

Figure 1

Growth and survival of *Chlamydomonas* during long-term culturing. a) Cell abundance (left axis, filled circles) and cell viability assessed using SYTOX® Orange (right axis, open diamonds). b) Cumulative survival curve data showing the probability of a cell surviving to a specific day (each replicate shown). The growth phases identified are the exponential growth (2), the declining relative growth phase (3), the stationary phase, (4) and the death phase (5). $n=3$, error bars = 2SE. Means that do not share a letter are significantly different ($p<0.01$, ANOVA, Tukey Comparison)

Figure 2

Chlorophyll content of cells during long-term batch culture growth. a) The average chlorophyll a and b content in pg per viable cell: total chlorophyll (filled circles), chl a (grey circles), chl b (open circles).b) The average ratio of chlorophyll a/b, which reflects the ratio between the antenna and reaction centers. n=3, error bars =2SE. Photographs show the cultures at day 4 (bottom) and day 166 (top). Means that do not share a letter are significantly different ($p < 0.01$, ANOVA, Tukey Comparison)

a)

b)

Figure 3

Protein changes in *Chlamydomonas* during long-term batch culture growth. a) Total protein per viable cell (pg cell⁻¹). Offset graph shows the last three timepoints in the series .n=3, error bars =2SE. Means that do not share a letter are significantly different (p<0.01, ANOVA, Tukey Comparison) b) Western blots detecting total LHCII; RuBisCO, and the LHCSR protein. A representative Coomassie Brilliant Blue-stained acrylamide gel is shown on the lower panel

Figure 4

The fluorescence parameters of *Chlamydomonas* during long-term batch culture growth. A) Maximum quantum efficiency of PSII (Fv/Fm). B) The dark-adapted, minimum fluorescence (Fo) normalized to the maximal fluorescence (Fm) C). n=3, error bars =2SE. Means that do not share a letter are significantly different (p<0.01, ANOVA, Tukey Comparison)

Figure 5

The fluorescence parameters of *Chlamydomonas* during long-term batch culture growth when challenged with excess light. A) Fraction of light used in photochemistry (ϕ_{PSII}), B) fraction of light dissipated by non-photochemical quenching (ϕ_{NPQ}), and C) the fraction of light dissipated by other, non-regulated processes (ϕ_{NO}). $n=3$, error bars = $2SE$. Means that do not share a letter are significantly different ($p<0.01$, ANOVA, Tukey Comparison)

Supplementary Files

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