



Correlated responses to selection for different cell size in *Chlamydomonas reinhardtii* using divergent evolutionary pathways

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Received: 1 March 2023 / Revised: 18 April 2023 / Accepted: 22 April 2023 / Published online: 18 May 2023
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Abstract

Cell size is an important trait that is often correlated with organisms' survival under different thermal environments, and in the case of phytoplankton, it is also related to survival under different light irradiance. While smaller phytoplankton thrive under warm and high light (summer conditions), larger cells seem to be more efficient under cool and low light conditions (winter conditions). In this study, we used different evolutionary pathways, nitrogen depletion and size selection, to select for differences in average cell diameter in the single-celled alga *Chlamydomonas reinhardtii*. We tested whether there was a size-related fitness (growth rate) advantage across two light intensities and two temperatures; environmental effects that have dramatic effects on algal growth. Under these environmental conditions we then tested how the evolutionary pathway to reduced cell size affected fitness and photophysiology. The results suggest that the relationship between size and growth rate in *C. reinhardtii* is strongly correlated with changes in the photosynthetic apparatus in different sized cells. Large-selected cells evolved to have smaller light-harvesting antennae, making them less susceptible to photodamage. However, they accumulate larger amounts of reactive oxygen species, potentially due to their lack of antioxidant carotenoids. In addition, the maximum growth rate was reduced, potentially due to their reduced ability to repair PSII due to lower metabolic rates. Similar responses were observed in lines that evolved to have smaller cells under nitrogen depletion. The results demonstrate that different evolutionary pathways to reduced cell size result in different changes in metabolism, with N-depleted and Small-selected cell lineages having similar sizes, but thriving differently under winter/summer conditions. With large production systems in mind, it is possible to assume that the choice of strains selected using adaptative laboratory evolution or direct selection on size can affect the overall production yield and carbon capture according to the seasons and possibly other abiotic parameters.

Keywords Light-harvesting · ROS · F_v/F_m · Microalgae · Abiotic stress · Experimental evolution

Introduction

Among single celled organisms cell size is a fundamentally important trait; from the dynamics of phytoplankton blooms to experimental evolution under future climate change scenarios, size is frequently correlated with the ability of a particular species or lineages to survive in those environments (Brennan et al. 2017). It has been observed within species that cells become smaller with warmer temperatures (Peter and Sommer 2012) and within communities there is a shift

to smaller species (Morán et al. 2010). Ecological studies of phytoplankton document the prevalence of small cells (picoplankton, cyanobacteria and small flagellates) in warmer waters, while in cold waters larger cells (particularly large diatoms) predominate (Gonçalves-Araujo et al. 2012). The observed relationship between size and temperature aligns phytoplankton, and consequently microalgae, with Bergmann's rule and Temperature-Size Rules (TSR) (Sommer et al. 2017; Legacy et al. 2021). These environmental effects may have effects even within a species where it is possible to have great divergence in cell size; the cell length of *Dunaliella salina*, for example, can vary between 2.8 to 40 μm (Borovkov et al. 2019).

Microalgae cell size is strongly linked to light utilization (Raven 1998). Light is crucial for photosynthesis and microalgae survival; however, the light stage of photosynthesis

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produces superoxide and other reactive oxygen species (ROS), with increased light being strongly correlated with ROS production (Ugya et al. 2020). There are two kinds of photosystems (PS) in higher plants and green algae, each is associated with a type of reaction centre (RCI and RCII). While RCI has a [Fe-S]-type terminal electron acceptor, RCII has a quinone-type terminal electron acceptor (Baymann et al. 2001). In both reaction centres, the primary electron donor is a pair of chlorophyll-*a* (chl-*a*) compounds (Barber 1987). The pair of chl-*a* absorb light with a wavelength of 680 nm (P680) in the RCII and 700 nm in the RCI (Brown 1987). Under the same light intensity cells containing more chl-*a* produce more ROS (Pospíšil 2016). The accumulation of excessive ROS under stress conditions can lead to loss of protein function, pigment co-oxidation, lipid peroxidation, loss of membrane integrity, cumulative oxidative damage to DNA and RNA, leading ultimately to cell death (Ledford and Niyogi 2005; Dowling and Simmons 2009; Foyer et al. 2017). Microalgae have two groups of molecules to avoid ROS damage, the first consists of antioxidant enzymes such as superoxide dismutases, glutathione reductase, catalases and ascorbate peroxidases. The second group, which is more effective in the prevention of ROS production, comprises of non-enzymatic molecules such as ascorbates, flavonoids, carotenoids, glutathione, tocopherols and phenols (Rezayian et al. 2019). To avoid excessive ROS in the presence of high light intensity, microalgae produce carotenoids, such as lutein and zeaxanthin, which have a photoprotective function achieved by collecting excessive irradiance and energy (Niyogi et al. 1997; Sewelam et al. 2016). Another defence mechanism against the damage caused by ROS is the production of lipid droplets rich in triglyceride (TAG) at the chloroplast (Miao et al. 2019) (Foyer 2018). Furthermore, differential regulation in gene expression might also be involved, as many LHC-like genes are induced due to high-light exposure (Förster et al. 1999; Kozioł et al. 2007). However, LHC and chlorophyll gene expression decreases under stress (Arora et al. 2018). Temperature is also known to increase ROS in microalgae mainly through enzymatic lipid peroxidation (Prasad et al. 2016).

It has been observed in marine diatoms that small cells have larger light-harvesting antennae, however, they also have higher metabolic rates associated with the repair of photosystem II after the photosystem has been damaged by excessive light (Key et al. 2010). Larger species however, are less susceptible to photodamage due to smaller light-harvesting antennae and lower energetic costs under high light irradiances, especially when resources are limited (Key et al. 2010). When several pigment molecules are aggregated the light absorbed is reduced; a phenomenon called the “package effect”. The package effect increases with cell size and also increases for colonies and multicellular organisms, reducing light absorption with increasing size and cell accumulation (Beardall et al. 2009). Thus, smaller cells have a reduced package effect and

are exposed to a greater risk of photodamage than larger cells. As the package effect is reduced in small cells, they also have reduced energy costs for photon acquisition and increased growth compared to large cells (Raven 1998).

Size-selected cells of *Dunaliella tertiolecta* that Malerba et al. (2018a, b), Malerba and Marshall (2019a, b) evolved over 400 generations, obtained large-selected cells 2427% larger than the small-selected cells. They noticed that large-selected populations had higher photosynthesis and respiration rates per cell and were more productive than small cells. On the other hand, small-selected cells presented higher metabolic rates per cell volume. In their studies, larger cells had higher fitness (growth rates) under optimal conditions, while smaller cells had an advantage when resource availability was lower, but larger cells showed higher carrying capacities in all regimes. Under warm conditions, small-selected *D. tertiolecta* had increased fitness, while large cells decreased, mainly because large cells are more vulnerable to reactive oxygen species (Malerba and Marshall 2019b).

The divergence between cell sizes and their characteristics can be applied to different microalgae production systems (photobioreactors and open ponds), that are used to create a plethora of biomolecules and provide services such as wastewater treatment and CO₂ fixation (Lubián et al. 2000; Spolaore et al. 2006; Chisti 2007; Schenk et al. 2008; Pruvost et al. 2022). Using adaptive evolution coupled with species size divergence, it may be possible to improve strains according to specific environmental circumstances. Summer conditions usually promote higher biomass accumulation, however, energy conversion through chlorophyll-*a* is less effective because there is too much light, and the cells cannot use all the available energy (Sutherland et al. 2014). In such conditions the use of large cells could be beneficial, increasing productivity and having a more efficient use of light by the microalgae culture. Conversely, in winter, energy conversion is more efficient because most of the available photons are used, but biomass accumulation is lower due to lower light availability (Perrine et al. 2012; Sutherland et al. 2014). Hence, for production systems using natural light there is too much light energy to fix the available carbon in summer, and not enough light for adequate carbon fixation in winter. Selecting the best adapted and most productive strain is a crucial step when establishing a large-scale culture (Chisti 2007). For example, there has been recent advances in the isolation of strains for conditions previously considered inappropriate for outdoor cultivation, such as the low temperatures and low luminosity in Nordic countries (Cheregi et al. 2019). Understanding how divergence in cell size interacts with different temperatures and light intensity is crucial for selecting adequate species and strains according to the environmental conditions. In addition, it might be possible to alternate different strains through the season to have a positive effect on biomass accumulation, and carbon capture and consequently improve the feasibility of the cultivation system.

In this study, we used two distinct approaches to select for the difference in average cell diameter in the model species *Chlamydomonas reinhardtii* (Harris 2001). We selected *Chlamydomonas* lines of three different sizes using centrifugation and we evolved strains of reduced cell diameter under nitrogen depletion for over 1400 generations. We assayed growth rate, cell diameter, pigment concentration, ROS and the maximum potential quantum efficiency of Photosystem II to assess the adaptation of different cell sizes in different light intensities and temperatures using a factorial experimental design. We then compared changes in fitness and photosynthetic response to different environmental conditions in small cell lines generated by the different evolutionary pathways to see whether they responded the same way or differently. This allowed us to assess whether the evolutionary pathway of cell size reduction affected the light-harvesting complex, metabolism and ROS accumulation.

Methods

Strains

Eight strains of *Chlamydomonas reinhardtii* (cc-124, cc-125, cc-1690, cc-1692, cc-2931, cc-2932, cc-2935, cc-2936) were acquired from the Chlamydomonas Resource Centre (<https://www.chlamycollection.org/>) in 2013 and kept in Tris–acetate-phosphate medium (TAP) (Gorman and Levine 1965).

Selection procedures

Size diverged

Strains cc-1690 and cc-1692, in five replicates each, were split into three selection regimes (Small, Control and Large) and kept in TAP medium throughout the experiment. To select for small cells, 8 mL cultures were centrifuged for 3 min at 120 rpm and the supernatant was transferred to a new tube, this was repeated one more time. To select large cells, 8 mL cultures were centrifuged for 3 min at 90 rpm, the supernatant was discarded, and the cells were resuspended in 4 mL TAP medium. This step was repeated once. The control lines were centrifuged once at 120 rpm and once at 90 rpm for 3 min, but nothing was discarded. After this, cells were counted using a Countess II Automated Cell Counter, without Trypan Blue staining solution, and transferred to new media in cell density of 5×10^5 cells mL⁻¹ in a total volume of 8 mL. Cultures were kept in a room with a controlled temperature (25 ± 2 °C), a photoperiod of 12:12 light/dark cycle and photon flux density (PFD) of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under "cool white" fluorescent light. This selection procedure was performed two times a week for 85 weeks in total, which is equivalent to approximately 1030 generations under selection.

Nitrogen depleted

We also selected for small cell size using nitrogen depletion (Fae Neto et al. Unpublished). In this selection experiment, we used a single replicate of each of eight strains (cc-124, cc-125, cc-1690, cc-1692, cc-2931, cc-2932, cc-2935, cc-2936) and maintained both a reduced nitrogen treatment and control lines. The strains were kept in a room with a controlled temperature (25 ± 2 °C), a photoperiod of 12:12 light/dark cycle and photon flux density (PFD) of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under "cool white" fluorescent light. We had a control group in TAP medium (TAP lines), while the nitrogen restricted group (N50 lines) was in an identical medium but which contained only 50% of the nitrogen. Cells were passed twice a week in 14 mL tubes with 8 mL of culture with an initial density of 5×10^5 cells mL⁻¹. Each of the eight strains was represented as a single lineage in each treatment and maintained under selection for at least 1490 vegetative generations. The number of generations was estimated using an average of 1.87 generations per day, calculated from the cell counts to pass the cultures twice a week.

Experimental design

TAP lines, N50 lines, Small, Control and Large lines, were transferred to fresh TAP media and put in four different controlled conditions, *Cold* (15 °C) or *Hot* (30 °C), *High Light* (210 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or *Low Light* (35 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). There were 8 replicates of TAP, 8 replicates of N50 and 30 size diverged cell lines (10 replicates of each size). The 46 distinct evolutionary lines were each split into four and one of the four allocated to each assay condition. In total there were 184 replicates representing 46 lineages across four treatments. The treatments were setup on two identical refrigerated incubators with internal lights (Thermoline Tril-200-DL) and a photoperiod on a 12:12 light/dark cycle. The cell lineages were transferred twice for acclimation before the experiment. The experiment ran for six days in mixotrophic conditions. Cell density, average cell diameter (μm) and pigments were measured daily. Because of the large number of samples and volume needed for the assays, the ROS analysis and PAM-fluorometry measurements were performed only once for each replicate and on different days for each environment, aiming to be as close as possible to the end of the exponential growth phase (*Hot* and *High Light* environment, day 3; *Cold* and *High Light*, day 4; *Hot* and *Low Light* environment, day 5; *Cold* and *Low Light* environment, day 6).

The number of cells and average cell diameter (μm) were measured using a CountessII Automated Cell Counter without using Trypan Blue staining solution, until the culture reached the end of the exponential growth phase. The specific growth rate (μ , day⁻¹) was calculated according to Barsanti and Gualtieri (2014) with the following equation:

$$\mu = \frac{\ln(N_{T_f}/N_{T_0})}{T_f - T_0}$$

where N_{T_f} is the cell density (cells mL⁻¹) at time T_f (end of the exponential growth phase) and N_0 is the cell density at time T_0 (start of the experiment).

Total carotenoids, chlorophyll-*a* (chl-*a*) and *b* (chl-*b*) concentrations were estimated using a microplate absorbance reader (Bio-Rad, iMark) using the methodology proposed by Haire et al. (2018). The absorbance of cell cultures in vivo was measured at 470, 650, 680 and 750 nm, and pigment concentrations were estimated according to the following equations (Haire et al. 2018):

$$\begin{aligned}\text{Chl-}a(\mu\text{g mL}^{-1}) &= 12.25(A_{680}) - 2.79(A_{650}) \\ \text{Chl-}b(\mu\text{g mL}^{-1}) &= 21.5(A_{650}) - 5.1(A_{680}) \\ \text{Total carotenoid}(\mu\text{g mL}^{-1}) &= \\ [1000(A_{470}) - 1.82(\text{Chl-}a) - 85.02(\text{Chl-}b)]/198\end{aligned}$$

The absorbance at 750 nm was used to correct all absorbance values for turbidity. Only data from the end of the exponential growth phase (the timepoint used to calculate maximum growth rate) was used in the data analysis.

Reactive Oxygen Species (ROS) analysis

The method used was based on the oxidation of the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH₂-DA) to the fluorescent 2',7'-dichlorofluorescein (DCF) by the ROS present in the algal culture (Dao and Beardall 2016). The extent of oxidation is proportional to the ROS level in the cell. The number of cells was standardised to 1×10^6 cells mL⁻¹ and washed three times with TRIS-HCl. Cells were incubated with 4 mL of TRIS-HCl and 1 mL of DCFH₂-DA for 90 min at 37 °C. Samples were centrifuged, washed with TRIS-HCl, and resuspended in 3 mL of TRIS-HCl. The cells were lysed using a probe sonicator (Branson Sonifier-B12) with the sample placed in an ice bath to avoid overheating for 1 min. The cells were centrifuged, and the supernatant was transferred to a 3 mL cuvette and analysed in a SpectraMax M5 spectrofluorometer (excitation 485 nm; emission 525 nm). ROS results are in relative fluorescence units (RFU).

PAM-fluorometry

Photosynthetic performance was assayed through the measurement of the maximum potential quantum efficiency of Photosystem II (PSII) with a pulse-modulated amplitude fluorimeter (PAM), Mini-PAM (Walz GmbH, Germany and Win-Control software). For these measurements 6 mL of each sample was placed in 14 mL tubes. The fluorescence readings were performed with the optical fibre sensor slightly introduced into the sample. Samples were left in the dark

for 20 min to relax NPQ and keep the PSII reaction centres open before measurements (White et al. 2011). The minimum fluorescence (F_0 for dark-adapted samples) and maximum fluorescence (F_m) were measured. The photosynthetic potential quantum efficiency was calculated using $F_v/F_m = (F_m - F_0) / F_m$ (Genty et al. 1989). The higher the value of this ratio, the greater photosynthetic efficiency.

Data analysis

The analyses were made separately for each of the three sections, using R (Team R C 2020). The first section (Part 1) compares only the size of the five different *Selection Histories* (N50, TAP, Small, Control and Large). The analyses in Part 2 compares the responses of the size-diverged cells (through centrifugation) to growth in different light and temperature environments in a factorial design. Finally, Part 3 assesses the difference in responses to changes in the environment between Small-selected lines and N50 lines. Strains N50 and Small have divergent evolutionary histories, to make comparisons about the effects of *Light* and *Temperature* on them, we analysed the difference (Δ) between the selected strains and their respective control groups, TAP and Control. Positive values indicate that the selected strains had higher values than their control group, if there is no interaction involving *Selection History* the cell lineages behaved in the same way across the treatments. This approach allows us to determine in what way N50 cells diverge relative to Small, the absolute values for which are reported in “Part 2”.

We used linear mixed-effects models with package *lme4* (Bates et al. 2015) to test the effects of *Selection History*, *Light*, *Temperature*, and their interaction on each measured trait. Because we have different strains that generally represent the variation in *Chlamydomonas*, we considered strains as a random effect in our analyses (Arnqvist 2020). The mating type was not significant in any of the models and therefore was removed from the model. Models with different random effects were tested using the Akaike information criterion, and the model with the lowest AIC was chosen. The model was coded in R as follows:

```
Model <- lmer (Trait ~ Light * Temperature * Selection History
+ (1 + Light * Temperature | Selection History) :
Strain : Replicate), data)
```

The distribution of residuals was checked through Q-Q plots and Shapiro-Wilk tests. We performed randomisation analyses to confirm our model when the model's residuals were not normally distributed. For the randomisation analysis, we first shuffled the value of the variable of interest between the fixed factors but kept it within their strain and replicate and ran a linear model with the mixed values. We did the shuffling and modelling a hundred times for each variable and compared the *F* values of the randomised data to the ones from the original data. *P* rand is the proportion of times the observed *F* value is less than the *F* value for randomised data (for a given fixed effect). Post hoc comparisons were made

using *lsmeans* (Lenth 2016) with "Sidak" adjustment. The full Anova table of the models can be found in the supplementary material (Tab. S1). The figures were produced using *ggplot2* in R (Wickham 2016), and a confidence interval (CI) of 84%, such that non-overlapping error bars are indicative of significance, was used on the graphics based on Julious (2004).

Results

Part 1. Size divergence

There was a significant effect ($F_{[4,41]} = 25.3$, $p = 0.0001$) of *Selection History* on average cell diameter (Fig. 1). These differences show greatest divergence across the lineages selected by centrifugation and no significant differences between the Control group and TAP lines, nor between N50 and Small-selected cells or N50 and TAP.

Part 2. Effects of temperature and light conditions on cells diverged through direct size selection

Maximum growth rate

Focussing only on the size diverged cells; when analysing maximum growth rate (Day^{-1}), there were significant differences between *Light* environments ($F_{[1,9]} = 32.8$, $p = 0.0003$; Fig. 2), with higher growth rates under *High*

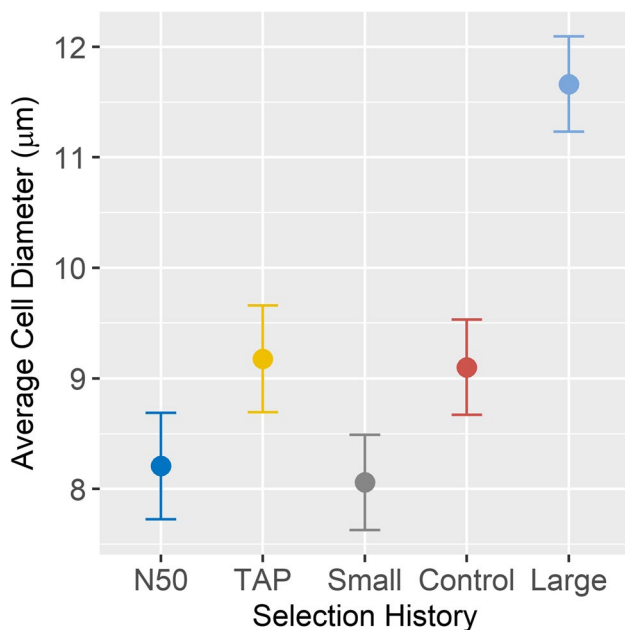


Fig. 1 Average cell diameter, measured at the end of exponential growth phase, of the different Selection Histories (N50, TAP, Small, Control and Large) reared mixotrophically in TAP media. Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

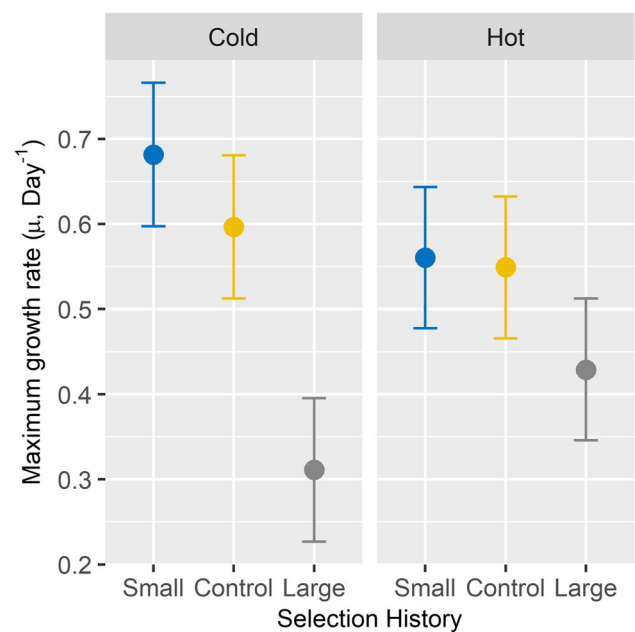


Fig. 2 Maximum growth rate for the size diverged lines reared mixotrophically in TAP medium in the Cold (15 °C) and Hot (30 °C) treatments. Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

Light, as expected (figure not reported). There was a significant difference ($F_{[1,9]} = 8.22$, $p = 0.018$) between the *Temperature* treatments, with generally higher values of growth rates under *Hot* conditions (Fig. 2). The large-selected cells showed significantly ($F_{[2,8]} = 17.7$, $p = 0.0011$) lower growth rates compared to Control and Small lineages (Fig. 2). Although the interaction *Temperature* $\times$ *Selection History* was marginally non-significant ($F_{[2,8]} = 4.11$, $p = 0.0591$), Control and Large lineages presented a tendency to have a slightly higher growth rate under *Hot* conditions. However, the small-selected lines thrived under *Cold* conditions.

Reactive oxygen species

Temperature $\times$ *Selection history* had a significant effect ($F_{[2,8]} = 8.23$, $p = 0.0114$) on reactive oxygen species (Fig. 3). *Control* and *Large*-selected cells accumulated more ROS under *Hot* conditions when compared to *Cold* conditions. On the other hand, small cells did not show significant differences in the amount of ROS accumulated by cells under *Cold* and *Hot* conditions. There was also a higher accumulation of ROS under *High Light* ($F_{[1,9]} = 341$, $p = 0.0001$) and with no other interactions.

Potential quantum photosynthetic efficiency of photosystem II

There was a significant *Temperature* $\times$ *Selection History* interaction ($F_{[2,24]} = 25.5$, $p = 0.0001$; Fig. 4) on F_v/F_m .

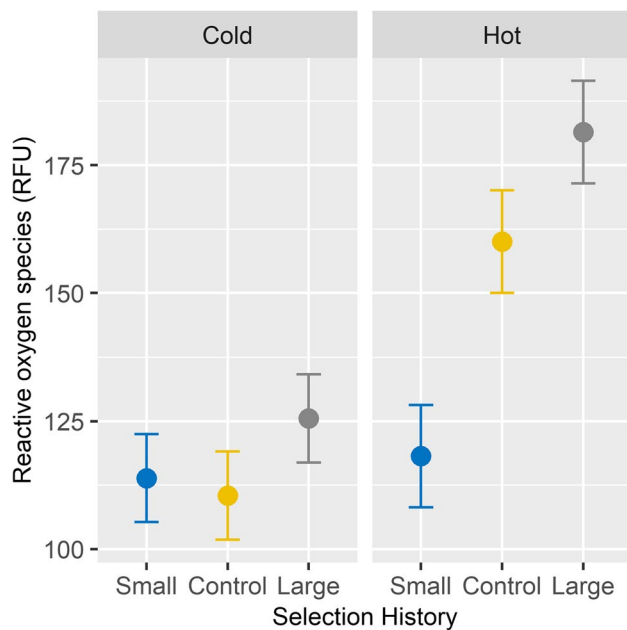


Fig. 3 Reactive oxygen species (RFU) per 10^6 cells for the size diverged lines reared mixotrophically in TAP medium in Cold ($15\text{ }^{\circ}\text{C}$) and Hot ($30\text{ }^{\circ}\text{C}$). Measurements were taken as close as possible to the end of exponential growth phase, Hot and High Light on day three, Cold and High Light on day four, Hot and Low Light on day five and Cold and Low Light on day six. Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

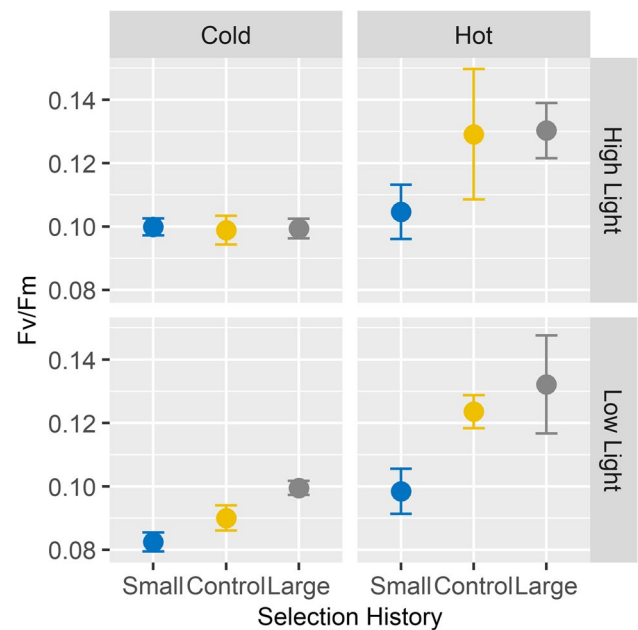


Fig. 4 F_v/F_m for the size diverged lines reared mixotrophically in TAP medium, in High Light ($180\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) and Low Light ($35\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$), Cold ($15\text{ }^{\circ}\text{C}$) and Hot ($30\text{ }^{\circ}\text{C}$). Measurements were taken as close as possible to the end of exponential growth phase, Hot and High Light on day three, Cold and High Light on day four, Hot and Low Light on day five and Cold and Low Light on day six. Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

Under *Hot* conditions, Control and Large cell lines had higher F_v/F_m values (higher photosynthetic efficiency) than the Small cell lines, however, these differences were not apparent under *Cold* conditions. There was also a *Light* $\times$ *Selection History* interaction ($F_{[2,23]} = 4.44$, $p = 0.023$; Fig. 4). Under *Low Light* irradiance, the small-selected had significantly lower efficiency than the other cell lines. In addition, there was a significant *Light* $\times$ *Temperature* interaction effect ($F_{[1,88]} = 11.3$, $p = 0.0011$), with higher values under *Hot Temperature* and *High Light* than under *Cold Temperature* and *Low Light*.

Pigment concentration

There was a significant three-way *Light* $\times$ *Temperature* $\times$ *Selection History* interaction ($F_{[2,8]} = 8.37$, $p = 0.0109$) for the total concentration of pigments ($\text{mg}\cdot\text{L}^{-1}$, Fig. 5). Under *Cold* temperature and *High Light* conditions, there were no significant differences between *Selection History*, while under *Cold* temperature and *Low Light*, Large-selected cells showed a higher concentration of pigments. For Large and Control lines the pigment concentration is elevated in *High Light* and *Hot* conditions. In contrast, the pigment concentration for small cells is affected by *Light* intensity, but not by higher temperatures.

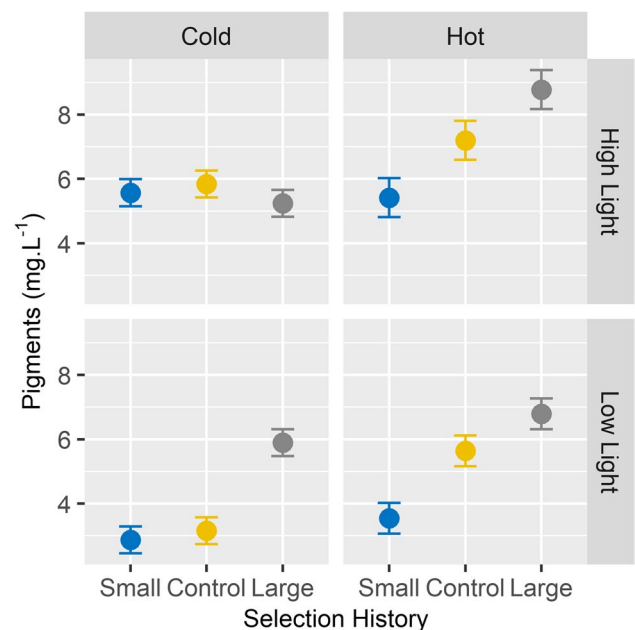


Fig. 5 Pigments measured at the end of exponential growth phase for the size diverged lines reared mixotrophically in TAP media, in High Light ($180\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) and Low Light ($35\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$), Cold ($15\text{ }^{\circ}\text{C}$) and Hot ($30\text{ }^{\circ}\text{C}$). Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

Pigment ratios

The interaction *Temperature* × *Selection History* had a significant effect ($F_{[2,8]}=6.8$, $p=0.0188$) on the ratio between carotenoids and chlorophyll-*a* (car/chl-*a*, Fig. 6). Small cells in a *Hot* environment presented higher car/chl-*a* than the small cells under *Cold* conditions. However, this pattern was not significant for Control and Large *Selection Histories*, which had similar values between the two *Temperatures*. In addition, under *Cold* and *Low Light* conditions, Large-selected cells presented the lowest values observed.

Part 3. The effects of divergent evolutionary pathways to reduced cell size on fitness and photophysiology

Cell size

Small-selected and N50, had no absolute differences in average cell diameter, as seen in “Part 1”. However, the different *Selection Histories* responded differently compared to their control groups in response to *Temperature* ($F_{[1,16]}=13.3$, $p=0.0021$; Fig. 7). The N50 lines decreased more than Small lineages in *Cold* conditions, while Small lines decreased more in *Hot* conditions. Cell size is differentially sensitive to *Temperature*.

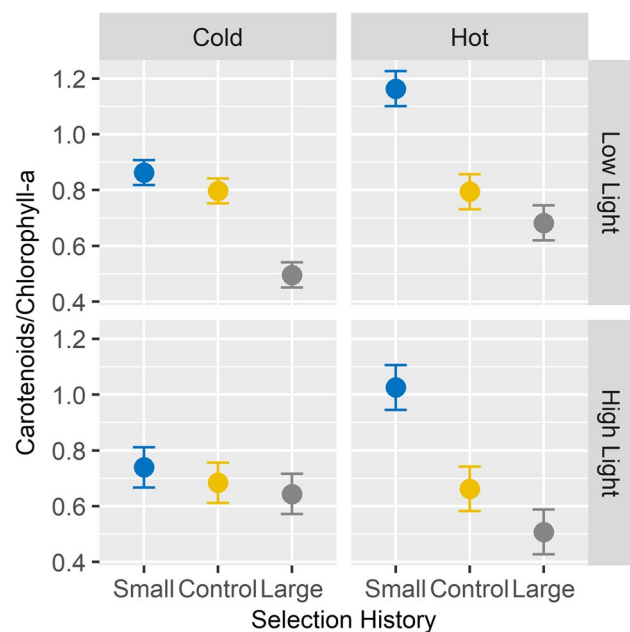


Fig. 6 Carotenoids/chlorophyll-*a* measured at the end of exponential growth phase for the size diverged lines reared mixotrophically in TAP media in High Light ($180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and Low Light ($35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), Cold (15°C) and Hot (30°C). Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

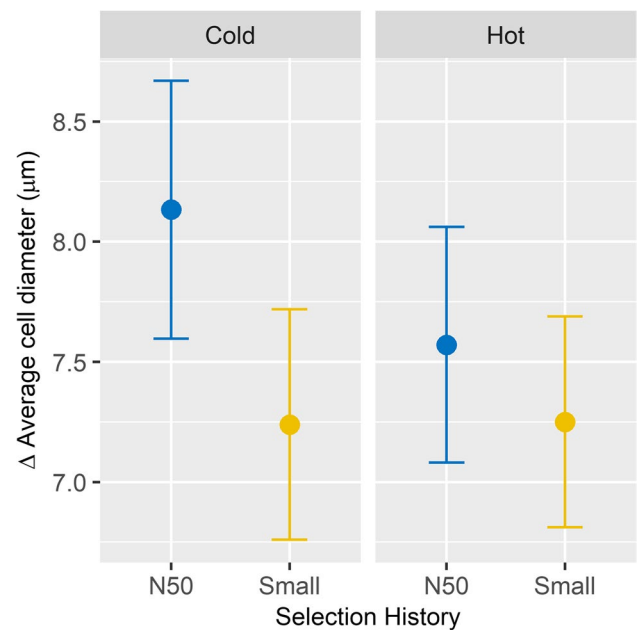


Fig. 7 Difference in average cell diameter (μm), measured at the end of exponential growth phase, for the Small-selected cell lines (compared to Control) and N50 cell lines (Compared to TAP) reared mixotrophically in TAP media in the Cold (15°C) and Hot (30°C). Mean $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

Growth rates

There were no differences in the sensitivity to *Light* ($F_{[1,16]}=0.0064$, $p=0.937$) and *Temperature* ($F_{[1,16]}=2.45$, $p=0.137$). Hence, in terms of growth rate, N50 similarly responds to the environment in the same way as Small-selected.

Pigment concentration

For the total pigment concentration, the three-way interaction was nearly significant ($F_{[1,16]}=4.44$, $p=0.0513$) and all of the two-way interactions were significant (*Light* × *Temperature* $F_{[1,16]}=7.36$, $p=0.015$; *Temperature* × *Selection History* ($F_{[1,16]}=10.7$, $p=0.0047$; *Light* × *Selection History* ($F_{[1,16]}=6.3$, $p=0.0232$; Fig. 8). The N50 cell lines had significantly higher differences compared to their TAP control lines than the Small-selected to their Control group in *Cold* conditions, accumulating more pigments than the TAP lines. The difference in pigment concentration was higher for N50 cells under both *Light* regimes. However, Small-selected cells have a more considerable difference between the two *Light* regimes.

Pigment ratios

There were no interaction effects with *Selection History* on the stress response of the cell lines as indicated by the

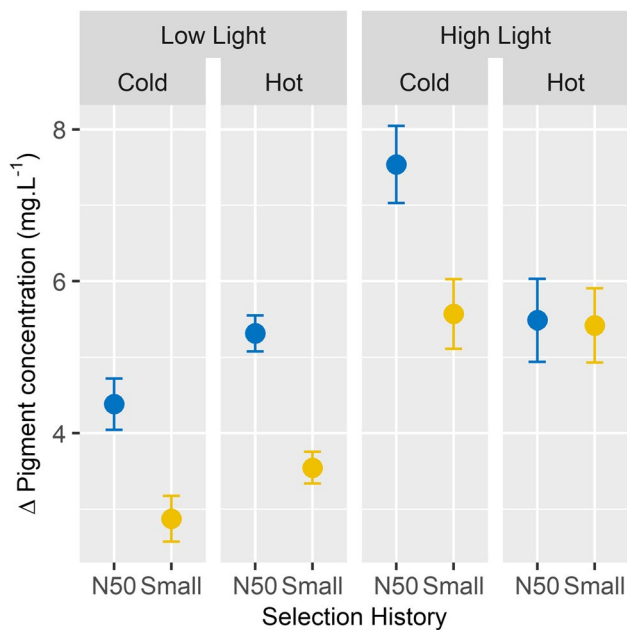


Fig. 8 Differences in pigments concentrations measured at the end of exponential growth phase for the Small-selected cell lines (compared to Control) and N50 cell lines (compared to TAP) reared mixotrophically in TAP media in High Light ($180 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and Low Light ($35 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), Cold (15°C) and Hot (30°C). Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

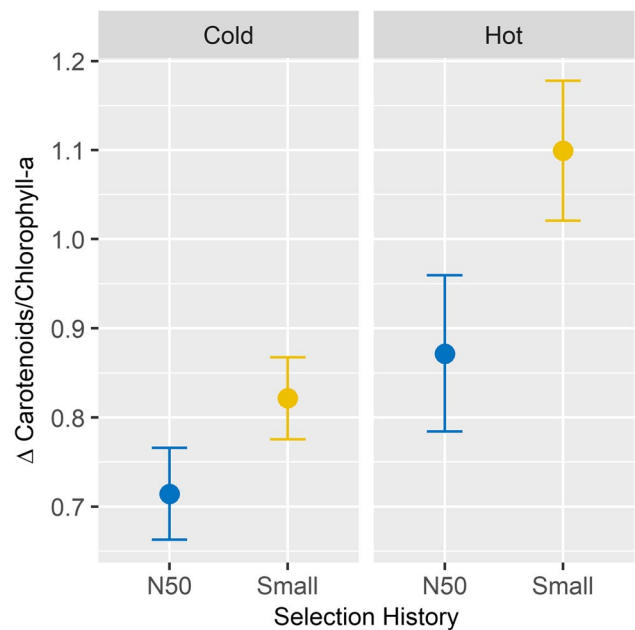


Fig. 9 Difference in carotenoids/chlorophyll-a measured at the end of exponential growth phase for the Small-selected (compared to Control) and N50 cell lines (compared to TAP) reared mixotrophically in TAP media in the Cold (15°C) and Hot (30°C). Mean $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

car/chl-*a* ratio. However, the significant *Selection History* effect ($F_{[1,16]} = 19.6$, $p = 0.0004$) shows that N50 cells were more stress tolerant with smaller differences in comparison to their control lineage (Fig. 9), with a higher proportion of carotenoids by chl-*a* found for small-selected cell lines. There were also significant effects of *Temperature* ($F_{[1,16]} = 20.4$, $p = 0.0004$), with higher values of car/chl-*a* in the Small and N50 lineages than in their respective controls under *Hot* conditions.

ROS

There was a significant interaction of *Temperature* $\times$ *Selection History* ($F_{[1,16]} = 18.3$, $p = 0.0006$; Fig. 10) for ROS accumulation. Under *Cold* conditions, N50 and Small did not show significant differences in ROS from their control. However, under *Hot* conditions, N50 cell lines had increased values of ROS when compared to TAP lines, while Small-selected cells presented lower values than the Control group.

Potential quantum efficiency of photosystem II

The divergence in F_v/F_m was greater between N50 and its control group ($F_{[1,16]} = 32$, $p = 0.0001$; Fig. 11), but this difference did not vary by *Temperature* and *Light*.

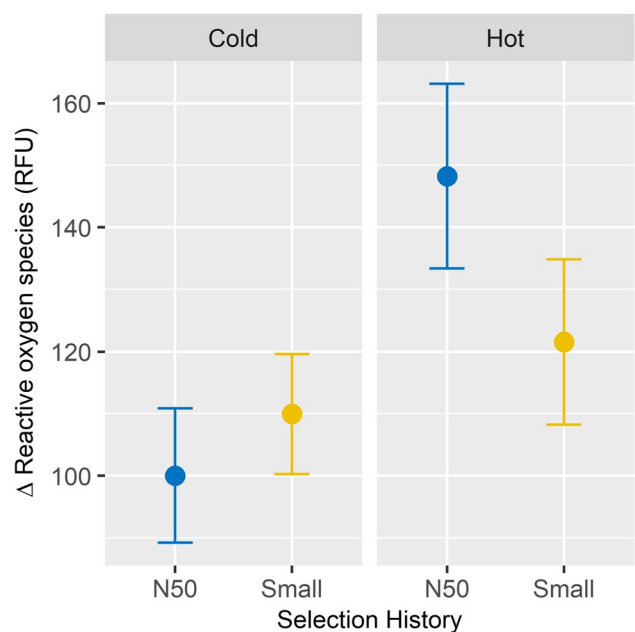


Fig. 10 Difference in Reactive oxygen species (RFU) per 10^6 cells for the Small-selected (compared to Control) and N50 cell lines (compared to TAP) reared mixotrophically in TAP media in Cold (15°C) and Hot (30°C) conditions. Measurements were taken as close as possible to the end of exponential growth phase, Hot and High Light on day three, Cold and High Light on day four, Hot and Low Light on day five and Cold and Low Light on day six. Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

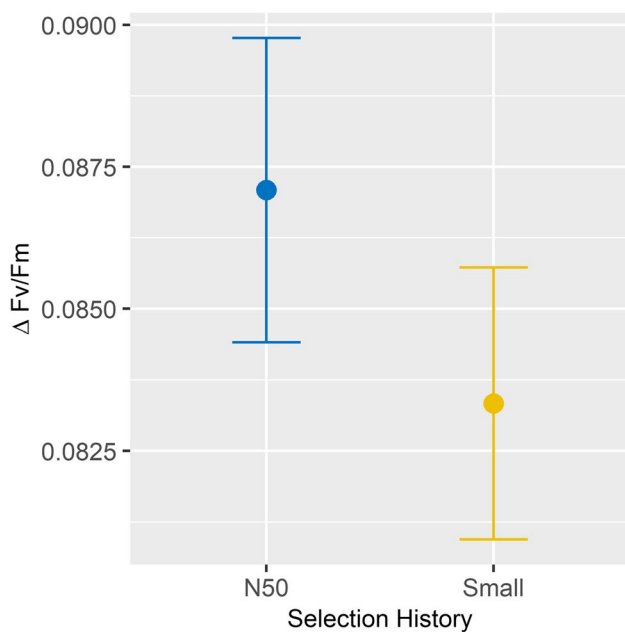


Fig. 11 Differences in F_v/F_m measured at the end of exponential growth phase for the Small-selected cell lines and N50 cell lines reared mixotrophically in TAP media in High Light ($180 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and Low Light ($35 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), Cold (15°C) and Hot (30°C). Measurements were taken as close as possible to the end of exponential growth phase, Hot and High Light on day three, Cold and High Light on day four, Hot and Low Light on day five and Cold and Low Light on day six. Estimated marginal means $\pm$ CI (84%), error bars that do not overlap are significantly different ($p < 0.05$)

Discussion

The importance of small size under stress, the advantage of larger cells when there is no resource limitation and the effects of different light and temperature conditions on resource acquisition, metabolism and size, are well described in the literature (Andersen et al. 2016). These differences are true for size differences within species for *D. tertiolecta* (Malerba et al. 2017a), among diatom species (Key et al. 2010) and in phytoplankton succession (Gonçalves-Araujo et al. 2012). Here, our interest was to determine whether different cell sizes of *C. reinhardtii* would be advantageous in simulated winter (Low Light and Cold temperature) and summer conditions (High Light and Hot temperature). In addition, we examined whether two different evolutionary pathways for achieving reduced average cell diameter varied in their responses to changes in environmental conditions. The results show that large cells presented lower growth rates than small cells in all the environmental conditions, however, they seem to accumulate more pigments. Further, even though N50 cells and Small-selected cells are of similar size, they had clear differences in the characteristics of their photophysiology.

Part 1. Size divergence

The size selection protocols were efficient in evolving populations with diverged and stable average cell diameters. Stability is demonstrated by their persistent divergence even after acclimation for two weeks without any selection. Among the centrifugally size-diverged lineages (Small, Control and Large), there was a similar size difference between Control and Large as between Control and Small. This contrasts with Malerba et al. (2017b), where there was a larger size difference between control and large than between control and small. One hypothesis proposed by Malerba et al. (2017b) for the asymmetrical divergence they observed was that the experimental light conditions ($150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 14:10 light:dark cycle) would be an additional selection pressure favouring large cells. In our experiment, the light intensity during selection was much less ($60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 12:12 photoperiod). We did not observe an interaction between light intensity and *Selection History*, but there was a generalised effect of *High Light* intensity on average cell diameter, with increased cell size under *High Light*. While resources may limit the increase in cell size, the limitation for the decrease in cell size was probably the minimum space necessary to accommodate the organelles without affecting functionality (Raven 1998; Andersen et al. 2016). It is conceivable that both N50 and small-selected cells are at the lower boundary of possible sizes for the species. The main reason for reduced size in nitrogen-depleted cells is the need for nitrogen in protein synthesis and chlorophyll synthesis. In addition, a higher surface-area-to-volume ratio is beneficial under low nutrient conditions (Key et al. 2010; Andersen et al. 2016). Differences between N50 and Small are discussed in more detail below.

Part 2. Effects of temperature and light conditions on cells diverged through direct size selection

The lowest values of growth rate observed in all environments was expected to be found in Large-selected cells because of their surface-area-to-volume ratio (Rubner's rule). The rule suggests that growth rates decrease with increasing cell size due to the constraint between metabolic rate, nutrient acquisition and diffusion (Kagami and Urabe 2001). In our experiment, this relationship was less clear under *Low Light* conditions. When comparing *Cold* and *Hot* environments, while the maximum specific growth rate of large cells increased in *Hot* treatment compared to *Cold*, small cells showed a reduction in growth rate. Large-selected cells showed higher total pigment concentration in all environments except for *Cold* and *High Light* conditions. Hence, even though large cells have a lower maximum growth rate under both *Temperature* conditions, they accumulate more

biomass. By selecting larger cells with lower growth rates, it is possible that we also selected populations with lower death rates, because there is a trade-off between high maximum growth rates and mortality (Flynn 2009). These populations would outcompete fast growing cells under suboptimal conditions. Because we started our experiments using the same number of cells and not biomass or biovolume, it is possible that selection for large cell size also selected for reduced nutrient availability. We did not find a significant interaction between *Light* intensity and the *Selection History* for most of the measured traits, including maximum growth rate, average cell diameter or total biovolume. This was surprising since large cells are expected to benefit from more resources (Van Oostende et al. 2015). It is possible that such interaction was not observed because of the vast difference between *Low Light* and *High Light* intensities (35 to 210 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), with the *Low Light* being a limiting factor for all the *Selection Histories*.

Under *Cold* conditions, control lines had similar values of biomass to small lines. However, under *Hot* conditions, control lines had similar values to Large-selected cells and Large-selected cells had a particular advantage in both *Temperatures*. It is important to note that our experiment does not test the competition between those strains. We found evidence that larger cells are more vulnerable to ROS, especially under warmer conditions, supporting the findings by Malerba and Marshall (2019b). Warmer temperatures increase cell metabolism, increasing the need to acquire more resources. In this case reducing cell size is beneficial since it increases the surface area-to-volume ratio, allowing cells to compensate for the increased demand for resources. As cell size increases, the surface area-to-volume ratio decreases and the average transport distance within the cell increases, so diffusion becomes increasingly inadequate to maintain constant solute concentrations throughout the cell's cytosol (Kagami and Urabe 2001). One of the advantages of small cells is the facilitation of intracellular transportation, which can be more efficient through diffusion (Raven et al. 1999; Beardall et al. 2009).

Reducing cell size does not improve the light use efficiency as indicated by F_v/F_m . While self-shading might be reduced, there was no apparent improvement in photosynthesis. On the contrary, large and control cells had higher efficiency than smaller cells under *Hot* conditions, while in *Cold* and *Low Light* conditions (winter conditions), large cells were more efficient. In addition, the pigments results suggest that large cells are a better option for biomass production. Ban et al. (2019) used ARTP to mutate *Chlamydomonas* selected cells with truncated antennae, and the strain showed higher light use efficiency and increased average cell size compared with wild-type. In addition, they observed increased activity of genes encoding for the light-harvesting complex proteins. My results agree with their study, with Large-selected cells presenting smaller antennae, as demonstrated by the *car/chl-a*

values, while still being more efficient in using light. Similar results were observed for two mutants of *Chlorella sorokiniana* (TAM-2 and TAM-4) isolated after UV mutagenesis and screened for low maximum fluorescence of *chl-a* (Cazzaniga et al. 2014). Their study showed higher *chl-a/chl-b* and lowered *car/chl-a* for the mutants than the wild type, and they suggest a depletion of light-harvesting complexes. The *Chlorella* mutant strains showed improved light penetration and photosynthetic activity, increasing the light-to-biomass conversion efficiency (Cazzaniga et al. 2014). In addition, in mutated cells with truncated light-harvesting complex, Polle et al. (2000) observed that *chl-a* can replace *chl-b*.

To sustain high growth rates under optimal conditions, cells must genetically adapt to tolerate and metabolise excessive ROS produced by increased photosynthesis (Lindberg and Collins 2020). One of the reasons large cells did not have higher growth rates might be that they have a low capacity to deal with excessive ROS. Larger cells frequently have lower pigment content per cell volume and smaller antennae size. This enables lower metabolic costs towards PSII repair, as the amount of photodamage is reduced. On the other hand, smaller species invest in higher metabolic rates to repair PSII, promoting higher pigment content per cell volume and larger light-harvesting antennae with faster electron transport. Under conditions where metabolic rates are reduced, for example under low temperatures, larger cells would have an advantage as their photosystem does not rely on high metabolic rates to repair (Key et al. 2010). Chloroplasts have a complex system that allows photosynthesis to be performed efficiently, even in resource-limited conditions, including a wide range of antioxidants involved in controlling redox. The excessive production of reactive oxygen species (ROS) in the chloroplast under heat stress can damage DNA, proteins and lipids, subsequently inhibiting the repair mechanisms of PSII (Mussnug et al. 2007). Microalgae reared under low light conditions and then exposed to high light, present reduced F_v/F_m and growth rate because cells have a low ability to dissipate excess light energy (Yarnold et al. 2015). Under high irradiance, the reduction of light-harvesting pigments enables cells to reduce their basal ROS (Cirulis et al. 2013).

Part 3. The effects of divergent evolutionary pathways to reduced cell size on fitness and photophysiology

The comparison of lineages with different evolutionary histories is an important tool in evolutionary analysis and is achieved through the comparative method by using pairwise comparisons between lineages, which are related, but differ in the expression of some traits of interest (Harvey and Pagel 1992). Here we use this principle to control for differences in the evolutionary backgrounds of our lineages (e.g. strains, time under selection, etc.) by subtracting the treatment means from

their respective controls. This yields a measure of the response to the assay conditions compared to their control. N50 cells compared to similar-sized cells (Small cells) show lower fitness in both *Light* environments. The evolution of small cells under nitrogen-depleted conditions promoted cells with lower car/chl-*a* content and higher total pigments, this suggests a decrease in cell size and the light-harvesting complex (Merzlyak et al. 2007) that would make cells more efficient under high light intensities. However, there is a trade-off, as they are more susceptible to photodamage. In this process under conditions with an increase in metabolism, for example under *Hot* conditions, there is also the accumulation of ROS inside the cells which is reflected in their growth rate (Erickson et al. 2015). Smaller organisms have higher metabolic rates, higher growth rates, higher mass-specific nutrient uptake and a higher photosynthetic rate. N50 cells have small antennae sizes, similar to large cells, which could be an advantage under high light environments, making N50 less susceptible to photodamage. However, the lack of carotenoids affects the reduction of ROS, especially in warm conditions, where metabolic rates are increased and the growth capacity of N50 cells is compromised. In addition, temperature significantly increases protein synthesis, increasing the demand for nitrogen (Toseland et al. 2013). As N50 cells are adapted to survive with lower nitrogen, their metabolism might be faster than small cells.

In a previous study (Fae Neto et al. [Unpublished](#)), we hypothesised that N50 cells' smaller light-harvesting antennae efficiency under *High Light* conditions would be higher than their control group (TAP). However, here we see that when compared to similar-sized cells, with the main difference being the photosynthetic apparatus, the N50 cells probably were strongly adapted to the environmental conditions in which they were selected (25 °C, 12:12 photoperiod, $\sim 50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). The lack of auxiliary pigments makes growing under *Low Light* conditions more difficult for N50 cells. An important point to remember is that our experiments were performed using TAP as a medium, and in that case the presence of acetate has a substantial impact, especially when analysing the photosystem response. Acetate is known to interact with PSII and force a circular electron flow instead of a linear electron flow, inducing a partial reduction of the plastoquinone pool. Therefore, cells grown in TAP tend to have lower F_v/F_m (Polle et al. 2000). This is especially important when testing strains under different light intensities, as the cells reared in TAP tend to be less prone to photoinhibition and photodamage (Roach et al. 2013). In this case, our assays might have underestimated the effects of photoinhibition and photodamage. In addition, the presence of acetate in the media used to evolve N50 lineages is possibly an additional evolutionary pressure. Acetate provides an alternative carbon source, reducing the pressure on the photosystem for carbon supply (Heifetz et al. 2000). Similarly, cells that evolved smaller light-harvesting

complexes would be more dependent on acetate and have a faster degradation of the photosystem (Chapman et al. 2015). Metabolic pathways related to the use of acetate in the Calvin-Benson cycle (e.g. malate and gluconeogenic precursors) are also suggested to be upregulated adding to the inhibition of linear electron flow (Saint-Sorny et al. 2022). The supplementation with an alternative carbon source on mixotrophic microalgae culture is known to increase starch and lipid accumulation in *C. reinhardtii*, especially under nitrogen stress (Smith and Gilmour 2018). Strains of the commercially important microalgae *Chlorella* and *Scenedesmus* have also been cultured mixotrophically with higher biomass accumulation than when grown autotrophically (Koller et al. 2012; Chen et al. 2017). An important constraint in growing microalgae mixotrophically is the reduced CO_2 fixation rates prohibiting the use of such cultures as carbon capture mechanisms (Subramanian et al. 2016). However, it becomes an advantage when microalgae are used to treat wastewater rich in organic carbon sources, providing an important environmental service (Koller et al. 2012).

Conclusion

In this study we showed that the relationship between size and growth rate for the model species *C. reinhardtii* strongly correlates with changes in the photosynthetic apparatus. As evidenced by the lower car/chl-*a*, Large-selected cells have smaller light-harvesting antennae making them less susceptible to photodamage. However, large cells accumulate more ROS, probably due to the lack of carotenoids acting as antioxidants. In addition, since large cells have lower metabolic rates to repair PSII, the maximum growth rate is reduced. Similar effects happen with N50 cells, which evolved to have smaller light-harvesting antennae due to nitrogen stress (Fae Neto et al. [Unpublished](#)). Under the experimental conditions in this study, N50 lineages showed similar behaviour to large cells compared to small-selected cells. Thus, our study provides evidence that cell photophysiology is of key importance for understanding the variation in growth rate and that differences in evolutionary backgrounds can change the relationship between growth rate and cell size under different environmental conditions. In addition, from a microalgae production perspective, even though Large-selected cells presented lower growth rates they show a clear advantage in pigment accumulations in every scenario, with the exception of in *Cold* and *High Light* conditions, where *Cold* and *High Light* conditions are prevalent such as altitude, or latitude or where cooling systems are in operation, N50-selected lineages might be more productive than larger cells. But before making a decision it would be important to have a full biochemical profile of those strains to align with the cultivation purposes.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10811-023-02978-1>.

Acknowledgements We are indebted to Danica McCorquodale for establishing and maintaining the selection lines over numerous generations.

Author contribution Wladimir A Fae Neto: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing—original draft, Writing—review & editing. Ryan Dosselli: Methodology, Writing—review & editing. W. Jason Kennington: Conceptualization, Formal analysis, Visualization, Supervision, Writing—review & editing. Joseph L. Tomkins: Conceptualization, Resources, Writing—review & editing, Funding acquisition, Visualization, Supervision.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions This work was supported by the Australian Research Council grant DP170100554 (JLT). Wladimir Angelino Fae Neto was supported by the Australian Government Research Training Program (RTP) Scholarship.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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