Cell size, photosynthesis and the package effect: an artificial selection approach

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Summary

- Cell size correlates with most traits among phytoplankton species. Theory predicts that larger cells should show poorer photosynthetic performance, perhaps due to reduced intracellular self-shading (i.e. package effect). Yet current theory relies heavily on interspecific correlational approaches and causal relationships between size and photosynthetic machinery have remained untested.
- As a more direct test, we applied 250 generations of artificial selection (c. 20 months) to evolve the green microalga Dunaliella tertiolecta (Chlorophyta) toward different mean cell sizes, while monitoring all major photosynthetic parameters.
- Evolving larger sizes (> 1500% difference in volume) resulted in reduced oxygen production per chlorophyll molecule – as predicted by the package effect. However, large-evolved cells showed substantially higher rates of oxygen production – a finding unanticipated by current theory. In addition, volume-specific photosynthetic pigments increased with size (Chla+b), while photo-protectant pigments decreased (β-carotene). Finally, larger cells displayed higher growth performances and Fv/Fm, steeper slopes of rapid light curves (α) and smaller light-harvesting antennae (c or φ) with higher connectivity (ρ).
- Overall, evolving a common ancestor into different sizes showed that the photosynthetic characteristics of a species coevolves with cell volume. Moreover, our experiment revealed a trade-off between chlorophyll-specific (decreasing with size) and volume-specific (increasing with size) oxygen production in a cell.

Introduction

Living organisms have evolved a seemingly endless range of geometries. Yet, at a fundamental level, all sizes and shapes conform to certain physical laws and principles to optimise the flux of resources and energy (Beardall et al., 2009; Niklas & Cobb, 2017). Differing by as much as nine orders of magnitude, the cell size of single-celled phytoplankton species strongly covaries with virtually all aspects of their physiology and ecology, especially photosynthetic performance (Litchman & Klausmeier, 2008; Maranon, 2015; Ward et al., 2017). For instance, smaller cells usually have a higher mass-specific photosynthetic rate than larger cells – with a cell-specific size-scaling between ½ and 1 (Finkel et al., 2004; Lopez-Urrutia et al., 2006; Maranon, 2008; Malerba et al., 2017).

Cell size also correlates with most light harvesting characteristics among phytoplankton species. For example, smaller cells usually have higher chlorophyll density per unit mass (Agusti, 1991; Finkel et al., 2004; Wu et al., 2014), higher light absorption relative to their volume (Agusti, 1991), higher absorption per unit chlorophyll (Finkel et al., 2004), higher carbon-dense biomass (Maranon et al., 2013), lower carbon-to-nitrogen ratio (Maranon et al., 2013) and are more efficient at absorbing photons (Wilmel et al., 2014). Phytoplankton alone are responsible for most of the photosynthesis in the oceans and half of that on the planet (Field et al., 1998; Maranon, 2015). Unsurprisingly, understanding the basic rules which regulate the physiology and ecology of phytoplankton species has been a major goal in biology for many decades (Banse, 1976; Berner et al., 1989; Agusti, 1991), but such efforts have taken on a new urgency as it appears that climate change could alter the size structure of phytoplankton as temperatures increase (Daufresne et al., 2009; Gardner et al., 2011; Forster et al., 2012).

The optical principle explaining why so many photosynthetic parameters correlate with cell size is the ‘package effect’. Specifically, all other things being equal, the efficiency of pigment molecules to harvest photons decreases as cell volume increases (Kirk, 1975, 1994; Raven, 1984; Raven & Beardall, 2016). Proposed over 30 yr ago, the package effect is today recognized as a convincing explanation for why smaller species should be superior competitors for light than larger species (Morel & Bricaud, 1981; Geider et al., 1986; Geider & Osborne, 1987; Berner et al., 1989; Finkel & Irwin, 2000). Mechanistic models show that a decline in chlorophyll-specific absorption is due to...
increasing self-shading among pigments, as photons penetrate throughout a cell (Finkel & Irwin, 2000; Key et al., 2010). Moreover, several size-related trends of the photosynthetic characteristics of a species can be seen as evolutionary adaptations to minimize the package effect (Geider et al., 1998; Finkel & Irwin, 2000; Ross & Geider, 2009). First, larger species usually develop lower pigment densities than smaller species (Geider et al., 1986; Agusti, 1991; Finkel & Irwin, 2000). Second, larger species have fewer chlorophyll pigments associated with each reaction centre in the thylakoid membrane (i.e. a smaller light-harvesting antenna; Mauzerall, 1986; Suggett et al., 2009; Key et al., 2010; Kirst et al., 2014; Suggett et al., 2015). Third, for a given concentration of ultraviolet radiation, larger cells are less susceptible to UVB damage due to the longer path length and increased potential for photon absorption, which also reduces the need for protective ‘sunscreen’ pigments such as β-carotene (Garcia-Pichel, 1994; Key et al., 2010). All these common adaptations of larger phytoplankton species are thought to optimize photosynthetic performance by reducing light gradients and enabling deeper light penetration in the cell (Raven, 1984, 1998).

Hypotheses such as the package effect rely heavily on interspecific comparisons of cells of different sizes. While they are informative, such comparisons necessarily confound other traits that covary with cell size: growth rate, pigment content/diversity ratio, extrinsic or intrinsic proteins of photosystem II, activities of various enzymes, chemical composition, genome size and other life-history traits. As a result, species of similar size can differ by more than one order of magnitude in their photosynthetic performance (Moloney & Field, 1989; Finkel et al., 2004, 2010). Worse, trait covariance with size makes it difficult to determine which trait is driving the underlying differences in photosynthetic performance among species (Niklas, 1997; Litchman & Klausmeier, 2008; Maranon, 2015). Are species of a certain size because of specific features of their photosynthetic apparatus, or is the light-harvesting apparatus able to adjust to the size of the cell? Hence, while it is an important first step, interspecific studies provide limited insights into the contribution of cell size to photosynthetic performance. An alternative approach to more reliably establish cause-and-effect relationships would be to change only the size of a species and to examine if other characteristics coevolve as predicted by current theory.

Perhaps the most direct way to examine the effect of size on the photosynthetic performance of phytoplankton species is to engineer genetic differences in mean cell size via artificial selection and evaluate its consequences. Artificial selection experiments have often been used to investigate the heritability and evolutionary potential of many macroscopic model species (Tobler & Nijhout, 2010; Voje et al., 2014; Bolstad et al., 2015), but have been utilised more rarely in photosynthetic microorganisms. The aim of the present study was to assess how photosynthetic characteristics change as cells are evolved toward different sizes (measured as the mean cell volume within a culture), and if these changes are consistent with theoretical expectations of optimizing light capturing and minimizing the package effect of larger cells. To this end, we extended an artificial selection programme (Malerba et al., 2018) to 250 generations of artificial selection (c. 20 months) to generate differences of over 1500% in the cell volume of the green microalga Dunaliella tertiolecta (Chlorophyta). While Malerba et al. (2018) documented differences in energy use among our cell lineages, this study looks at the physiological underpinnings of size-dependent photosynthetic rates, by unpacking the mechanistic drivers leading to different energy utilizations. As cells evolved to different sizes, we repeatedly assessed all major photosynthetic parameters: pigment content (Chla and b, β-carotene), photosynthetic oxygen production, maximum quantum yield, the initial slope of the rapid light curve, antenna size and the connectivity of the alpha centres of photosystem II (PSII α). We also measured population growth rates. We found that small-evolved cells showed greater rates of chlorophyll-specific oxygen production – as predicted by the package effect. However, large-evolved cells upregulated their volume-specific Chla and b concentrations and improved their photosynthetic production, as well as their growth performance – a finding that was not anticipated by current theory.

Materials and Methods

Model species

As a model species, we chose the cosmopolitan, fast growing marine green alga Dunaliella tertiolecta (Butcher). This species has intermediate cell volumes (relative to other phytoplankton species) and grows well under laboratory conditions. Monoclonal batch cultures were sourced from the Australian National Algae Culture Collection (ANACC; strain code CS-14) and reared in standard autoclaved f/2 algal medium (without silica), prepared with 0.45 μm filtered seawater (Guillard, 1975). All experimental cultures were grown at 21 ± 2°C with a 14 h : 10 h, day : night cycle, at a light intensity of 150 μmol quanta m−2 s−1. Bacterial load in the cultures was kept to minimal levels by resuspending cells in autoclaved media twice a week and by handling all samples with sterile material in a laminar-flow cabinet (CF23S, NATA certified; Gelman Sciences, Melbourne, Vic., Australia).

Cell size evolution

For a detailed description of the artificial selection protocols see Malerba et al. (2018). Briefly, artificial selection on cell size was achieved through differential centrifugation. The method relies on cells larger in volume sinking and forming a pellet at the bottom of a test tube at lower centrifugal forces compared to smaller cells, which instead will remain in the supernatant. A total of 72 lineages were created and kept independent throughout the evolutionary experiment: 30 lineages were selected for smaller cell volumes, 30 for larger cell volumes, and 12 as control. Control cultures experienced identical conditions (including centrifugation) but no size was selected for. Starting on 25 April 2016, selection routines were repeated twice a week, every Monday and Thursday. Day–night cycles are known to synchronize the circadian rhythm of phytoplankton populations, with cell division occurring mostly at night. To control for the influence of photocycle on selection outcome, we carried out artificial selection in...
the morning on newly divided cells. Pilot studies showed that, on average, cells divide three times a week, which means between one and two times before each round of selection. In this way, the selection frequency also allowed a substantial opportunity for natural selection on traits providing a fitness benefit at the newly evolved cell volumes. At the end of each selection round, all cultures were reinoculated into fresh f/2 medium at comparable bio-volume densities. Every Thursday, cultures were moved to newly sterilized 75 cm³ plastic cell culture flasks (Canted Neck, Nonpyrogenic; Corning Inc., Corning, NY, USA).

Experimental design

Photosynthetic traits were measured after 50, 100 and 200 generations of size-selection (4, 8 and 16 months, respectively). Growth performances were measured after 150 generations (12 months). For each experiment, 12 randomly selected lineages were sampled and analysed for each size-selection treatment. To remove any environmental conditioning from the selection protocols, all experimental cultures were grown for three generations (a week) under neutral selection (i.e. with no centrifugation) before collecting any data. Cells were maintained under nutrient-replete conditions during neutral selection, by adding new medium 3 d before and on the same day of the assays. All assays were carried out after standardizing all lineages to the same blank-corrected optical density, as we had previously shown that this represents a reasonable proxy for biovolume (Malera et al., 2018).

Cell size

The mean cell volume of each lineage was calculated after measuring at least 200 cells with optical light microscopy at ×400, after staining with Lugol’s iodine at 2%. Cell area was measured with IMAGEJ and FIJI (Schindelin et al., 2012) and cell biovolume was calculated assuming a prolate spheroid shape, as recommended for this species by Sun & Liu (2003).

Flow-cytometric analysis

The total number of cells per µl in each culture was measured by flow cytometer (FlowCore, BD LSRII; BD Biosciences, Franklin Lakes, NJ, USA) using CountBright absolute counting beads (Thermo Fisher, Waltham MA, USA) as internal standards in each sample. Mean flow-cytometric optical properties of single cells were also recorded (using a blue laser at 488 nm), as forward scatter, side scatter and red fluorescence (using a 650–670 nm detection filter). Optical variables were represented by the means of the cytometric histograms of the cell populations normalized by the mean values of the CountBright beads, as described in Malera et al. (2016, 2018). Flow-cytometric techniques were also used to quantify DNA content and ploidy level, by staining live cells with Hoechst fluorescence dye and checking the distribution of the fluorescence histogram (laser excitation at 405 nm and fluorescence measurement at 460 nm; Jayat & Ratinaud, 1993).

Ash-free dry weight

Ash-free dry weights were measured using standard protocols (Zhu & Lee, 1997; Malera et al., 2012). A 15 ml aliquot of each culture was gently filtered through filter papers (Whatman GF/C, diameter 47 mm, precombusted overnight at 550°C) and dried for 2 d in an oven at 100°C (UNB 400; Memmert, Schwabach, Germany). Dry weights were measured to the closest 0.1 mg after cooling for 30 min in a desiccation chamber with silica beads. The samples were then placed in a muffle furnace (Lindberg/Blue M828; Thermo Fisher) at 550°C for 24 h and weighted a second time. The ash-free dry weight of each sample was calculated as the difference between weight after drying oven and weight after muffle furnace. All values were correcting for salt content by subtracting the mean ash-free dry weight from seven samples with the same volume of medium without cells (salt blanks).

Pigment analysis

A 4 ml aliquot of culture from each lineage was concentrated and resuspended in 1 ml of ethanol (ACS reagent grade >99.9%; Sigma–Aldrich) and stored overnight at 4°C. After ensuring there was a colourless particulate matter and successful pigment extraction, absorbance spectra were measured from 400 to 700 nm at 1 nm steps with a SPECTROstar® Nano microplate reader (BMG Labtech, Aylesbury, UK), after loading three 250 µl samples per lineage into 96-well plates (Corning® polystyrene, flat bottom, nontreated; Sigma-Aldrich). The concentrations of Chla, Chlb and β-carotene were calculated using the spectral deconvolution method described in Thran et al. (2015). Briefly, the method uses Gaussian functions to reconstruct (using non-negative linear models) the total absorbance spectrum of a sample, as a weighted sum of all its individual pigment spectra. Finally, the chlorophyll-specific oxygen production (fmol O₂ d⁻¹ mg⁻¹) was calculated by dividing the per-cell daily net oxygen production (fmol O₂ d⁻¹ per cell) by per-cell Chla+b content (µg per cell).

PSII fluorescence measurements

Chlorophyll fluorescence measurements were performed using a Phyto-PAM phytoplankton analyser (Heinz Walz, Effeltrich, Germany) with the software PHYTOWin v.3 (Heinz Walz). All samples were resuspended in fresh medium and standardized at the same optical density (at 750 nm). After 15 min of dark acclimatization, minimum (F₀) and saturating (Fm) fluorescence values were measured. Maximum quantum yield of photosynthetic energy conversion in PSII (Fm/F₀) was then calculated, as (Fm – F₀)/Fm. We also used rapid light curves to quantify the initial slope (α) of the increase in relative electron transport rate (rETR) as a function of light exposure, proportional to the efficiency of a cell to transform incident photons into photosynthetic energy (Liang et al., 2006; Zhang et al., 2017). The α parameter was estimated with PHYTOWin by exposing each culture to 18
light irradiances (0–600 μmol m$^{-2}$ s$^{-1}$) and fitting a Platt curve between RETR and the light intensity.

The effective absorption cross-sectional area (hereafter referred to as antenna size: $\sigma_{\text{PSII}}$) and the connectivity ($\rho$) of the $\alpha$ component of the alpha centres of photosystem II (PSII $\alpha$) were estimated using Flash Fluorescence Induction (FFI) curves with a double-modulation fluorometer (Photon Systems Instruments, Brno, Czech Republic). Cultures were dark-acclimatized for 5 min before being exposed to a single saturating turnover flash. The fluorescence increase of the FFI curve was then analysed as described by Nedbal et al. (1999) and Pierangelini et al. (2014). All fluorescence values were blank corrected.

### Light and dark metabolism

The rate of oxygen evolution (VO$_2$) was measured for all lineages after 200 generations of size-selection at 19°C with a 4 × 24-channel PreSens Sensor Dish Reader (SDR; AS-1 Scientific, Wellington, New Zealand). All methods were adapted from Malerba et al. (2017, 2018). The metabolic rates of this species at earlier generations can be found in Malerba et al. (2018). Before the experiment, all SDRs were calibrated with air-saturated (AS) sea-water (100% AS) and water containing 2% sodium sulfite (0% AS). The lineages were placed in 5 ml sealed vials, being careful to remove any bubbles from the inside of the vials. Sodium bicarbonate (2 mM) was added to the medium to ensure photosynthesis was not limited by carbon availability. At least three vials were used as blanks and filled with filtered supernatant in each SDR. All vials were then placed horizontally under the light source, to avoid cell deposition on the top of the oxygen sensor at the base of the vial. Samples were randomly allocated to the top row of each SDR, while blanks were placed in the bottom row (pilot studies showed there was no row effect on blank measures). VO$_2$ measurements were taken in the dark under a saturating light regime (300 μmol quanta m$^{-2}$ s$^{-1}$), each replicated four times. Previous studies (Malerba et al., 2018) and more recent pilot studies have shown that such light intensities were enough to saturate cell photosynthetic rates for all size-selected treatments. Rates of light and dark metabolism for each sample were calculated from linear rates of O$_2$ change over time. Metabolism was then standardized to total cell biovolume (i.e. cell number × mean cell biovolume).

### Population growth rates

Maximum growth rate and carrying capacity were quantified from 10 randomly selected lineages per size-selection treatment after 150 generations of artificial selection. More methodological details can be found in Malerba et al. (2018). Briefly, after three generations of neutral selection, the optical densities (750 nm) of three 250 μl independent cultures per lineage in separate 96-well plates were monitored with SPECTROstar®Nano (BMG labtech) until they reached carrying capacity (c. 7 d). Optical density values were converted to units of total biovolume (using log-linear calibration curves) and fitted with logistic growth curve models to estimate max. growth rate (r) and carrying capacity ($K$) for each lineage.

### Scanning election microscopy (SEM)

Cells were sampled after 250 generations of artificial selection and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature. Cells were washed three times in cacodylate buffer (10 min each) and fixed in 1% tetroxide in buffer solution for 1 h at room temperature. After three washes in distilled water (10 min each), 200 μl of fixed cells were incubated on polyester coverslips (Nunc™Thermanox™ Coverslips) for 30 min. Cells were dehydrated through a graded ethanol series (30%, 50%, 70%, 90% and 100%, 10 min each), followed by two more washes in 100% ethanol (30 min each). Coverslips were then placed in mesh baskets in fresh ethanol. Cells were dried on coverslips using a Bal-Tec CPD 030 (Bal-Tec, Los Angeles, CA, USA). Coverslips were mounted on aluminium SEM stubs using double-sided sticky carbon tabs. Samples were gold coated using a Bal-Tec SCD 005. Finally, cells were viewed in a Nova NanoSEM 450 scanning electron microscope.

### Statistical analysis

All analyses were carried out in R (R Core Team, 2018). Logistic growth curve models were used to quantify population growth rates [nls package in R]. Ordinary least-squares linear models were used to assess the effects of cell volume ($\mu$m$^3$) on each variable of interest: C-content (g per cell), fluorescent measurements ($F_i/F_m$, alpha, and antenna size), light and dark metabolism (fmol O$_2$ per cell), pigment content (mg per cell), optical density, flow-cytometric cell optical properties (forward scatter, side scatter and red fluorescence) and population growth performance (maximum growth rate and carrying capacity). For the analysis of log-log size-scaling relationships, traits were deemed to scale allometrically with cell volume if the slope coefficient did not include 1 in its 95% confidence interval; otherwise the relationship was considered not significantly different from isometry. While other studies have also used Reduced Major Axis (RMA) to calculate size-scaling exponents, Kilmer & Rodriguez (2017) recommended against this technique and in favour of ordinary least-squares linear models when the uncertainty in the x-axis (i.e. mean cell volume) is lower than that on the y-axis. Because our estimates of mean cell size were precise (CV < 5%), size-scaling relationships were analysed with ordinary least-square linear models. Data transformations were employed whenever the residuals departed from normality. In addition, in cases where treatments displayed unequal variances, treatment-specific variance coefficients were included in the model (VARIADENT function in R). A blocking variable describing whether the culture was measured at generation 50, 100 or 200 was added in the model to control for differences due to instrument calibrations among rounds of data collection across the experimental period (> 1 yr). Finally, evolutionary rates of cell volume were quantified using Haldane units, as $d/(SD \times t)$ where $d$ is the difference of log$_e$-transformed cell volumes between two means, SD is the pooled standard deviation of all log$_e$-transformed cell volumes and $t$ is the generation interval (see Supporting Information Fig. S1B).
One Haldane unit is defined as a change by a factor of one standard deviation per generation (Haldane, 1949; Gingerich, 1993).

Data accessibility

All data and codes generated in this study are available in Dryad (doi: 10.5061/dryad.642j9). Also contained in the Dryad file are the photos from optical microscopy and scanning electron microscopy of the size-evolved cells.

Results

Cell size evolution

Size-selection on a common ancestor of D. tertiolecta led to increasing differences in the mean cell volume between small- and large-selected lineages (Figs 1, 2). After 50, 100 and 200 generations, the mean cell volume among large-selected cultures was on average 81%, 142% and 248% larger than that of small-selected cultures, respectively (Fig. 1). The range of size differences among lineages increased throughout generations: 130% after 50 generations, 230% after 100 generations and 500% after 200 generations (Fig. 1). At generation 250, the two lineages with the smallest and largest mean cell volumes spanned by as much as 1567% (from 854 to 14 241 μm³; Fig. 1). Control lineages recorded intermediate sizes, but remained more similar in volume to small-selected than to large-selected lineages (Fig. 1). Cell size trajectories showed significant trends over time for both small- (generalized additive models: \( F_{\text{eff},2.11} = 7.61, P < 0.001 \)) and large-selected lineages (\( F_{7.07} = 20.42, P < 0.001 \)), but not for control lineages (\( F_{6.48} = 1.91, P = 0.06 \)). Moreover, there was a positive linear correlation between the mean and variance of log-transformed cell volumes among lineages (\( r_{5.2} = 10.57, P < 0.001, r = 0.83 \)), with lineages of larger cells showing more size variability than small-selected and control cultures. Finally, all conclusions remained consistent when analysing median cell volumes instead of mean cell volumes among lineages.

Evolutionary rates of cell volume over time were highest for large-selected lineages (increasing by 0.02 standard deviations per generation) than the control (0.003 SD per generation) and small (−0.003 SD per generation lineages (\( F_{2.15} = 196.18, P < 0.001 \); Fig. S1B). The range of cell volumes in the ancestral population (at generation 0) showed very little overlap with the range of size-selected lineages after 250 generations of selection (Fig. S1A). In addition, there was no difference in mean (\( F_{1.10} = 2.41, P = 0.14 \)) and median (\( F_{1.10} = 2.04, P = 0.18 \)) DNA content among size-selected treatments, with the majority of cells in haploid stage (data not shown).

Consistently with observed increases in cell volume, all optical properties measured from single cells using flow cytometry (i.e. forward scatter, side scatter and red fluorescence) systematically increased with cell size (Fig. S2). Finally, there was a positive relationship between the mean cell volume of a lineage and the per-cell optical density at 750 nm recorded with spectrophotometry (Fig. S3).

Carbon mass

The carbon (C) mass of individual cells spanned from 1.5 to 10 ng C per cell (Fig. 3). Interestingly, the 95% confidence interval for the size-scaling exponent largely overlapped 1, indicating a proportional (isometric) relationship between cell volume and cell C mass (Fig. 3). Below we use cell volume as proxy for cell size, as it can be measured more accurately and precisely than C mass. However, the isometric scaling between cell volume and cell C mass indicates that the same qualitative results can be obtained from standardizing for cell C mass.

Pigment composition

Per-cell chlorophyll content varied by up to two orders of magnitude among size-selected cells, from 7 to 295 pg per cell for Chl a and from 0.6 to 100 pg per cell for Chl b (Fig. 4a,b). By contrast, β-carotene increased by almost fourfold, from 8 to 32 pg per cell (Fig. 4c). Size-scaling for Chl a and b were both hyper-allometric (size-scaling exponent significantly > 1), while β-carotene increased hypo-allometrically (size-scaling exponent significantly < 1; Fig. 4a–c); as cells increased in size, volume-specific chlorophyll content increased while β-carotene content decreased. On average, the volume-specific pigment concentration of cells in the small-selected treatment recorded 16% and 23% less Chl a and b, respectively, but also a 36% increase in β-carotene, compared to cells in the large-selected treatment (Fig. 4d–f). Pigment composition was statistically indistinguishable between cells in small-selected and control treatments (Fig. 4d–f).

Photosynthetic characteristics

Both the maximum PSII quantum yield (\( F_{\text{v}}/F_{\text{m}} \)) and the initial slope (α) of the rapid light curve increased with size, with large-selected lineages recording up to 22% and 36% higher \( F_{\text{v}}/F_{\text{m}} \) and α values than small-selected lineages, respectively (Fig. 5a,b; Table 1). By contrast, the size of the photosynthetic antenna (\( \sigma_{\text{PSII}} \)) decreased with cell size, with large-selected lineages showing a 16% decrease in mean antenna size compared to small-selected lineages (Table 1). Finally, there was an inverse relationship between the size (\( \sigma_{\text{PSII}} \)) and the connectivity (ρ) of the antennae in PSII α: smaller antennae of larger cells showed higher ρ than the larger antennae of smaller cells (Fig. S4).

Light and dark metabolism

The photosynthetic oxygen production of a cell scaled hyper-allometrically with its cell volume at 1.23 (95% CI of size-scaling exponent > 1; Fig. 6a). Specifically, for a 525% increase in cell volume corresponded to a disproportionally higher (889%) increase in oxygen production (Table 1). The mean size-scaling relationship of dark respiration was hyper-allometric at 1.2; the metabolism increases by 754% for a 525% increase in cell size (Table 1). While the size-scaling exponent for dark respiration does not exclude an isometric scaling with 95% statistical confidence (CI includes 1; Fig. 6b; Table S1), there are several lines of
evidence showing that an increase in sample size and further evolution may confirm the hyper-allometric pattern (as for other rates of oxygen evolution): (1) the lower estimate for the 95% confidence interval for the parameter best-estimate of the size-scaling of dark respiration is very close to 1 (0.97), (2) the parameter best-estimate is substantially higher than 1 (1.19) and (3) isometric scaling is excluded with 93% confidence (Fig. 6b; Table S1).

Using a 14 h : 10 h, day : night photoperiod, we can quantify the daily photosynthetic production of a cell as the difference between oxygen produced during the day-phase minus oxygen consumed during the night-phase. The daily net oxygen production scaled hyper-allometrically at 1.25 (95% CI of size-scaling exponent > 1; Fig. 6c; Table S1): large-selected cells are 525% larger than small-selected cells but produced 889% more oxygen (Table 1).

All rates of oxygen production scaled hypo-allometrically (between 0.73 and 0.76) with the concentration of light-harvesting pigments (i.e. Chl \(a\) and \(b\)) of a cell (Fig. 6d–f; Table S1). Hence, the efficiency of a pigment to produce oxygen decreased – by as much as 41% – as the concentration of pigments increased within a cell (Table 1). In the same way, standardizing the net oxygen production of a cell by its pigment concentration showed a negative relationship with the size of the cell (Fig. S5).

**Population growth rate**

After 150 generations of artificial selection, large-selected lineages showed faster maximum growth rates (\(F_{2,27} = 37.05, P<0.001\)) and higher carrying capacities (\(F_{2,27} = 1739, P<0.001\)) compared to both small-selected and control lineages, respectively (Fig. S6). Population growth rates measured in previous generations are reported in Malerba et al. (2018).

**Discussion**

In this study we showed that all traits measured for the green microalga *D. tertiolecta* coevolved in response to selection on cell size. In Malerba et al. (2018), we reported the first 100 generations for this model system – here we have extended this program for 150 more generations. While cell carbon mass increased proportionally to the cell volume (isometric size-scaling), most other properties did not (allometric size-scaling). For instance, volume-specific concentrations of Chl \(a\) and \(b\) increased as cells evolved to larger sizes (hyper-allometric size-scaling), while volume-specific \(\beta\)-carotene decreased (hypo-allometric size-scaling). Overall, we found that smaller cells are 41% more efficient at producing oxygen per unit chlorophyll – as predicted by the package effect – but larger cells have 60–100% more chlorophyll per unit volume and their per-cell oxygen production increased by 889% compared to smaller cells. Consistently, larger cells showed a 22% increase in maximum quantum yield (\(F_{v}/F_{m}\)) and a 36% increase in the initial slope of the rapid light curve (\(\alpha\)). Furthermore, larger cells displayed smaller effective absorbance cross-section (\(\sigma_{\text{PSII}}\)), but higher connectivity (\(\rho\)) of PSII \(\alpha\). Finally, large-selected cultures showed significantly faster maximum growth rates and higher carrying capacities compared to small-selected and control populations, which is consistent with the results from earlier generations reported in Malerba et al. (2018). Hence, by evolving a common ancestor to different sizes, we showed that...
altering the size of a cell profoundly alters many fundamental traits of algal physiology and ecology.

Coevolution of photosynthetic characteristics with size

We only exerted artificial selection on the cell volume, but found that many other traits systematically also coevolved with this property. As such, our findings provide strong evidence that body size is an important evolutionary driver for many photosynthetic characteristics of a species. Even after 200 generations of selection (c. 16 months), cells of similar size showed similar photosynthetic characteristics – further evidence that it is size (not generation, nor time) driving many of the physiological characteristics in our study system. In particular, our results showed that large cells can still produce more photosynthetic energy than small cells, even though their chlorophyll-specific oxygen production decreases with size. These results suggest that adaptive evolution can more than compensate for the inevitable self-shading associated with an increase in cell size. Perhaps the high-light regime we used in the laboratory was the reason for these results: by reducing the costs of intracellular light penetration at increasing cell sizes, larger cells may need to dissipate less excess excitation energy as heat through nonphotochemical quenching mechanisms, compared to smaller cells (Dubinsky & Stambler, 2009). Similarly, growing our cells in a nutrient-rich medium may have favoured the fitness of cells with more intracellular space and higher capacity for biosynthesis (Raven, 1998; Maranon, 2015).

Costs and benefits of cell size

Evolving our study species toward different cell sizes revealed a trade-off between more efficient (small-selected) and more productive (large-selected) cells, with respect to their photosynthetic performance. Specifically, by becoming smaller, cells developed a greater efficiency at producing oxygen with each chlorophyll molecule (+41%); by becoming larger, cells massively increased their volume-specific chlorophyll $a$ (+847%) and $b$ (+1075%) concentrations and upregulated their total daily photosynthetic production, both per-volume (+69%) and per-cell (+889%). However, this superior photosynthetic performance of larger cells also comes at a cost. For instance, larger cells also substantially increased their oxygen demand in the dark (+791%). Following this, we would predict that low light regimes would disproportionately affect the fitness of larger cells compared to smaller cells (as was already shown for this species in Malerba et al. (2018)). In addition, the higher photosynthetic performances of larger cells coincided with a substantial increase in chlorophyll synthesis and with an improvement in growth performance. Given that chlorophyll molecules are highly rich in nutrients such as nitrogen and iron (Turpin, 1991), we would also predict that nutrient regimes impairing the synthesis of pigment would reduce net oxygen production more in larger than smaller cells. Finally, $\beta$-carotene is an important nonenzymatic antioxidant (as well as a light harvesting component) and it plays a major role in protecting the photosynthetic apparatus against photo-oxidative damage by deactivating reactive oxygen species (ROS) formation, in Dunaliella sp. (Fu et al., 2013; Kim et al., 2013) and most other photosynthetic organisms (Frank & Cogdell, 1996). As such, the faster rates of electron turnover could make larger cells more vulnerable to ROS build-up. Overall, our results indicate that the costs and benefits of cell size are case-specific and must consider the interplay between physiology, ecology and environment.

Cell size, package effect and pigment concentrations

We used the approach described in Raven (1984) to quantify the predicted package effect solely based on cell size and pigment content. As cells evolved from 800 to 5000 $\mu$m$^3$ in volume, we would expect that pigments in larger cells would absorb up to 200% fewer photons compared to smaller cells (Fig. S7 and Method S1). Instead, our observations showed only a 41% decrease (Table 1). Thus, larger cells appeared to have evolved compensatory mechanisms to maximize photon harvesting while minimizing the negative consequences of the package effect. For instance, large-selected cells showed adaptations in their light-harvesting machinery that were consistent with both algae and plants adapting to low-light conditions: increased photosynthetic pigments (mainly Chl$a$ and $b$), decreased photo-protectant...
pigments (e.g. β-carotene), increased Chl a to β-carotene ratios, and a reduction in effective absorption cross-section area in PSII α (Givnish, 1988; Mussgnug et al., 2007; Dubinsky & Stambler, 2009; Kirst et al., 2014). Moreover, it is likely that larger cells reduced the impact of the package effect by altering not just the size but also the number of reaction centres in PSII α – another common light adaptation strategy (Falkowski & Owens, 1980; Richardson et al., 1983).

Size and nutrient storage

Phytoplankton species with larger cell sizes are placed at an advantage because of their ability to continue growth even after nutrient concentrations are limited, by relying on internal nutrient storage (or cell ‘quota’). Although we did not measure nutrient quota, there is some evidence showing that the intensity of the cell flow-cytometric red fluorescence is proportional to the cell nitrogen quota (Malerba et al., 2016). Here we found that the intensity of red fluorescence emitted from larger cells is up to an order of magnitude higher than in smaller cells (Fig. S2C). This suggests an increased ability to store limiting nutrients, which could lead to important advantages in evolving larger cell sizes, especially under fluctuating nutrient regimes (Grover, 1991; Holt, 2008; Barton et al., 2013). In contrast, we would also anticipate parallel costs from increasing cell size, in particular increased sinking rates, reduced swimming performances and higher nutrient requirements (Maranon, 2015; Grover, 2017; Malerba et al., 2018). Testing competitive interactions between size-evolved treatments could provide an insight into the size-mediated mechanisms which maintain phytoplankton diversity.

Artificial selection vs macro-evolutionary patterns

While our results mostly confirmed the theory of the package effect, some of findings on the role of size from our artificial selection program were not consistent with among-species allometric studies. In particular, we found here that large-selected cells produced more photosynthetic energy per unit volume compared to small-selected cells (i.e. size-scaling exponent of > 1). However, many previous among-species studies showed that size-specific primary production and respiration decreased with size (i.e. size-scaling exponent < 1; Brown et al., 2004; Finkel et al., 2004; Lopez-Urrutia et al., 2006). Furthermore, Malerba et al. (2017) analysed energy use among 21 phytoplankton species using the same laboratory conditions and the same instruments as those here and found photosynthetic energy flux to scale at < 1 with cell size. These contrasting findings between within- and
Fig. 4 Size-scaling relationships for per-cell (a) chlorophyll (Chl\textsubscript{a}), (b) Chl\textsubscript{b}, and (c) \(\beta\)-carotene as a function of mean cell volume, and per-volume (d) Chl\textsubscript{a}, (e) Chl\textsubscript{b}, and (f) \(\beta\)-carotene as a function of the size-selection treatment of \textit{Dunaliella tertiolecta}. (a–c) Solid lines (± 95\% confidence intervals) indicate the fit of the allometric model (a, \(F\textsubscript{3,89} = 242, P < 0.001\); b, \(F\textsubscript{3,89} = 241, P < 0.001\); c, \(F\textsubscript{3,89} = 192, P < 0.001\)), whose mean estimates (± 95\% confidence intervals) and coefficients of determination are also reported. (d–f) Black circles indicate the mean volume-standardized pigment content (± 95\% confidence intervals) among all independent size-selected lineages (coloured symbols). All model results were significant (d, \(F\textsubscript{2,90} = 4.63, P = 0.012\); e, \(F\textsubscript{2,90} = 6.63, P = 0.002\); f, \(F\textsubscript{2,90} = 22.31, P < 0.001\)). Letters indicate groupings following Tukey’s post hoc test.

Fig. 5 Relationships between cell volume and photosynthetic characteristics of \textit{Dunaliella tertiolecta}: (a) maximum quantum yield (\(F\textsubscript{v}/F\textsubscript{m}\), rel. units); (b) slope of relative electron transport rate (\(rETR\)) as a function of light exposure (\(\alpha\), rel. units); (c) effective absorption cross-sectional area of PSII \(a\) (\(r\textsubscript{PSII}; \mu\text{m}^2\)); (d) antenna connectivity parameter of PSII \(a\) (\(q\); rel. units). Solid lines (± 95\% confidence intervals) indicate the fit of the linear model (a, \(F\textsubscript{3,87} = 11.21, P < 0.001\); b, \(F\textsubscript{3,79} = 28.2, P < 0.001\); c, \(F\textsubscript{3,88} = 249.6, P < 0.001\); d, \(F\textsubscript{3,89} = 69.34, P < 0.001\)).
among-species suggest: (1) among-species comparisons may not reflect mechanistic relationships between traits because too many traits can vary simultaneously, and (2) that our experimental approach may have relaxed the selection against very large cells, by providing a high light environment and by not requiring cells to swim effectively to remain in the photic zone. Regardless, it
seems that inferences about interspecific size relationships do not reflect intraspecific relationships.

Caveats

Other than adaptive evolution, it is possible that pleiotropy and/or linkage disequilibrium could also have contributed to the coevolution of photosynthetic characteristics with body size (Lande, 1980; Hansen, 2006). If this was the case, our results may be explained by developmental or functional constraints rather than solely by natural selection on fitness-enhancing traits and may not be generalizable to other species. Unfortunately, there is no information on the genetic correlation (G matrix) between size and photosynthesis in the ancestral algal population, as the nature of the system (this algal species appears to reproduce asexually) makes such estimates difficult to obtain. Furthermore, the G matrix evolves over time and any estimate of genetic correlations in the ancestral, unselected population may not be informative 200 generations later (Steppan et al., 2002). We cannot rule out pleiotropy and/or linkage disequilibrium, but adaptive evolution seems to be the most important driver of trait coevolution, especially given an intervening round of natural selection. In this regard, we must let the reader decide for themselves what the likely drivers of coevolution were. Extending similar artificial selection programmes to other species would certainly help with confirming the causal effects of size on species traits, but, for now, our study remains the only one.

Conclusions

Global temperature increases are modifying ocean conditions and reducing body sizes among phytoplankton species, with likely cascading consequences on marine food web dynamics (Daufresne et al., 2009; Gardner et al., 2011; Forster et al., 2012). Our experiment showed that evolving larger cells of D. tertiolecta improved their growth performance and the oxygen production of the species, compared to small-evolved cells. If these same mechanisms were also found to apply to other species, we would expect a general reduction in the ability of phytoplankton biomass to fix carbon. However, it is very likely that these size-related differences in oxygen production were also dependent on experimental conditions, in particular nutrient and light regimes. Smaller cells are more efficient at producing oxygen per unit chlorophyll, which would make them better adapted to low-resource conditions. Future research will better resolve size-related effects across treatments of nutrient conditions, light regimes and temperatures but for now it seems likely that any shifts in size will have profound consequences for global carbon cycles.

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Author contributions

M.E.M., D.J.M. and J.B. designed the study. M.E.M., M.M.P. and Y.M.P.D. conducted the experiment and collected the data. M.E.M. carried out statistical analyses and wrote the initial draft of the manuscript, while all other co-authors provided substantial feedback. All authors gave final approval for publication.

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References


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Cell size distribution and rates of evolutionary change from the ancestral population (at generation 0) to 250 generations of artificial selection.

**Fig. S2** Changes in optical properties of size-selected cells as measured by flowcytometer (i.e. forward scatter, side scatter and red fluorescence).

**Fig. S3** Relationship between total optical density (750 nm) of the culture – standardized for population density – and mean cell volume of the culture.

**Fig. S4** Relationship between antenna size of PSII α (Å) and antenna connectivity parameter of PSII α.

**Fig. S5** Allometric relationships for chlorophyll-standardized (Chl a+b) per-cell O2 evolution rates as a function of cell volume.

**Fig. S6** Maximum growth rate and carrying capacity across size-evolved lineages after 150 generations of artificial selection.

**Fig. S7** Predicted photon harvesting efficiencies standardized per unit as a function of cell volume.

**Table S1** Summary of all allometric size-scaling relationships

**Methods S1** Quantifying the magnitude of the packaging effect on a cell.

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