Do larger individuals cope with resource fluctuations better? An artificial selection approach

Martino E. Malerba1,2, Maria M. Palacios1,2 and Dustin J. Marshall1,2

1Centre for Geometric Biology, School of Biological Sciences, and 2School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia

MEM, 0000-0002-7480-4779; MMP, 0000-0002-5450-674X

Size determines the rate at which organisms acquire and use resources but it is unclear what size should be favoured under unpredictable resource regimes. Some theories claim smaller organisms can grow faster following a resource pulse, whereas others argue larger species can accumulate more resources and maintain growth for longer periods between resource pulses. Testing these theories has relied on interspecific comparisons, which tend to confound body size with other life-history traits. As a more direct approach, we used 280 generations of artificial selection to evolve a 10-fold difference in mean body size between small- and large-selected phytoplankton lineages of *Dunaliella tertiolecta*, while controlling for biotic and abiotic variables. We then quantified how body size affected the ability of this species to grow at nutrient-replete conditions and following periods of nitrogen or phosphorous deprivation. Overall, smaller cells showed slower growth, lower storage capacity and poorer recovery from phosphorous depletion, as predicted by the ‘fasting endurance hypothesis’. However, recovery from nitrogen limitation was independent of size—a finding unanticipated by current theories. Phytoplankton species are responsible for much of the global carbon fixation and projected trends of cell size decline could reduce primary productivity by lowering the ability of a cell to store resources.

1. Introduction

The size of an organism and its use of resources are tightly linked [1–3]. Body size drives much of the variation in metabolism and, thus, in energy use [4], such that size evolution is linked to resource use in most species, including humans [5–7]. On one hand, because smaller individuals have lower absolute resource requirements, it is often argued that low resource conditions favour smaller individuals both within and among species [8,9]. On the other hand, larger individuals often have lower mass-specific metabolic rates and should, therefore, be more able to sustain higher biomasses for a given level of resources [4,10,11]. Larger organisms may be better at converting nutrients into energy [12], exploit more types of resources [13] and pay fewer mass-specific costs of transportation [14] than smaller organisms. Thus, the optimal body size for a species should depend on the net-use of resources in the environment. For example, the ‘supply–demand’ model predicts that an organism grows to use as much *per capita* resources as possible, but without reaching a size that requires more resources of what the environment can constantly provide [15].

Resource levels are rarely constant in nature but fluctuate in time and space, which may exert selection on body size. When fluctuations happen on a time scale longer than an organism’s life, there is often little directional selection on body size but rather periodic oscillations [16]. Conversely, more rapid fluctuations have the potential to induce strong selecting pressures toward either smaller or larger organisms, because size can mediate the capacity to store resources and tolerate periods of nutrient limitation [17]. For example, larger mammals allocate greater percentages of their body weight into stored
resources (adipose tissues) relative to smaller species [18,19]. Also, smaller animals typically must feed more often than larger animals, partly because of their faster mass-specific metabolic rates [20]. Hence, short-term resource fluctuations strongly affect the fitness of an organism, but it is still unclear how unpredictable nutrient regimes can influence the body size evolution of a species.

Different ecological theories have proposed contrasting explanations on which body size should be favoured under different nutrient regimes. Millar & Hickling [20] proposed the ‘fasting endurance hypothesis’ (FEH), which predicts that larger species can endure longer periods of fasting because of their greater ability to accumulate and store resources and their higher digestion efficiency. Thus, larger body sizes will be favoured under fluctuating and unpredictable resource regimes. By contrast, smaller species should be favoured under chronically low, but more stable, resource regimes because of their lower absolute nutrient requirements [18,21,22]. The FEH is intuitively appealing but classic ‘r-K selection’ theory (RKS) by Wilson & MacArthur [23] predicts the opposite: smaller organisms are better competitors for resource pulses (r-selection), because of their more rapid population growth rates, shorter generation times and higher mass-specific biomass production [24–26]. Conversely, larger species are better competitors during periods of stable nutrients and can maintain positive growth under resource shortage (K-selection). Also the ‘metabolic theory of ecology’ (MTE) predicted on how body size should mediate the ability of an organism to cope with fluctuating nutrient regimes. Specifically, species use resources at a rate that is proportional to their metabolic rates, which scale with body mass at M to a constant as the metabolic size-scaling exponent. Because organisms store resources proportionally to their size (M2), then body mass and metabolic rate both determine the starvation resistance of a species (as M2/M2 = M−2) [4,27], which can also be reconciled to the theory of ‘dynamic energy budget’ models [28]. Hence, MTE would predict larger organisms with lower mass-specific metabolic rates to grow slowly but to tolerate longer periods of nutrient shortage. Overall, FEH, RKS and MTE predicted different ways in which unpredictable resource regimes during an organism’s lifetime should influence the evolution of body size in a species.

Coping with daily resource fluctuations is particularly important for phytoplankton species [29]. These aquatic microorganisms are responsible for much of the global primary production and their activity depends on rapid changes in available nutrients, especially nitrogen and phosphorous [30,31]. Yet, it is still unclear as to which body size is at an advantage following a new nutrient pulse. Specifically, smaller cells can grow faster and process newly available nutrients more rapidly (‘velocity’ specialists), whereas larger cells can take up nutrients faster, accumulate more nutrients and maintain positive growth rates for longer periods between resource pulses (‘storage’ specialists) [32–34]. Both ecological strategies could prevail under pulsed-nutrient conditions or, alternatively, different strategies can coexist as there are multiple pathways to maintain fitness under fluctuating conditions [34,35]. However, most considerations on body size and resource utilization have been at the among-species level, making causal inference difficult.

Among-species comparisons of the covariance between body size and resource utilization are a natural and powerful first step for understanding patterns in nature. Such approaches have the advantage of comparing taxa that vary many orders of magnitude in size [4,36,37]. However, they necessarily confound other life-history traits that covary with size (e.g. growth rate, metabolic rate, generation time, productivity), hampering efforts to unequivocally determine causal effects of body size on performance under different resource regimes [38]. For example, smaller species tend to have faster mass-specific metabolic rates, shorter generation times and grow faster than larger species. But is this because of size, generation time, metabolism or some other phylogenetically covarying factor? Thus, correlation of traits among-species complicates efforts to examine the potential of body size alone as a driver of performance across resource regimes. Worse, Heusner [39] and Kozlowski and Weiner [40] showed that interspecific allometries can originate simply from artificial combinations of subset allometries and per se do not reflect any functional relationship.

Perhaps the most direct way to investigate the effects of body size is to start from an ancestral population and use artificial selection to engineer genetically based differences in size. To this end, we used artificial selection to evolve a 10-fold difference in body size between small-selected and large-selected lineages of the green microalga species Dunaliella tertiolecta, by selecting for size twice a week for 276 generations (646 days). In this study, we aimed to evaluate the consequences of size-shifts on the growth performance of this species under different resource regimes. Specifically, we quantified the ability of cells in each size-selection treatment to convert newly available resources into biomass at nutrient-replete conditions or following a week of either nitrogen (N) or phosphorous (P) depletion, as the two most common limiting nutrients in phytoplankton communities. Previous studies of nutrient limitation in this species showed that both N and P can be stored intracellularly to maintain cell division in periods of insufficient external nutrients, but to different degrees. Internal P stores can sustain growth for up to a week, while N stores only for approximately 2–3 days [41,42]. Furthermore, the photosynthetic efficiency of many microalgal species, including D. tertiolecta, declines to a greater extent following P limitation compared to N limitation [43,44]. Finally, D. tertiolecta returns to normal growth rates sooner after periods of N depletion (less than 24 h) compared to P depletion (approximately 3–4 days; [45,46]). Thus, in our experiment we would expect P limitation to show more severe and long-lasting effects on population dynamics than N limitation.

Because of the ambivalent theory of FEH, RKS and MTE regarding the relative advantages of different sizes under resource fluctuations, we have contrasting a priori predictions as to which size should best respond to a nutrient pulse. Investigating how body size mediates the response of resource-deprived individuals to a pulse of nutrients can help distinguish among predictions from competing models. Specifically, the FEH would predict larger cells to be more buffered against periods of nutrient limitation, whereas RKS predicts smaller cells with faster generation time to better take advantage of a nutrient pulse. Finally, MTE would predict tolerance to nutrient deprivation to be negatively correlated with the mass-specific metabolic rate of an organism. Mass-specific metabolic rates generally decrease with size, but phytoplankton often show different metabolic scaling [47,48]. Specifically, the mass-specific
metabolic rate of this species increases with size [49,50]. Thus, applying the general principles of MTE to our species yields the prediction that larger cells should grow faster but be less tolerant to nutrient depletion compared to smaller cells.

2. Material and methods

(a) Study species and culturing conditions

We used cultures of the green microalga *D. tertiolecta* (Butcher) that had experienced 276 generations of artificial selection for either smaller or larger cell sizes (see [49,50] for more details). This cosmopolitan, fast-growing species was originally sourced from the Australian National Algae Culture Collection (ANACC; strain code CS-14). Cultures were reared in standard F/2 medium (without silica) prepared with 0.45 µm filtered seawater [51] and kept in a temperature-controlled room at 21 ± 2°C with a 14–10 h day–night cycle at a light intensity of 150 µM m⁻² s⁻¹ using low-heat 50 W LED flood lights (Power-lite™, Nedlands Group, Bedfordale, Australia). Samples were not axenic, but bacterial loads in the cultures were kept to minimal levels by resuspending cells in autoclaved medium twice a week and by handling samples using sterile materials under a laminar-flow cabinet (Gelman Sciences Australia, CF235, NATA certified).

The experiment had three phases (see figure 1 for experimental design). First, we evolved the species toward different cell volumes (‘artificial selection’ phase). Second, we placed cells in either nutrient-replete or nutrient-deplete resource conditions before starting trials (‘pre-trial nutrient condition’ phase). Third, we started trials by monitoring population density after resuspending cells from the pre-trial condition into fresh F/2 medium (‘trial growing condition’ phase; figure 1).

(i) Phase 1: artificial selection

For a detailed description of artificial selection protocols see Malerba et al. [50]. Briefly, artificial selection was achieved through differential centrifugation. The method relies on larger cells sinking and forming a pellet at the bottom of test tubes at lower forces compared to smaller cells, which instead will remain in solution. On 25 April 2016, 72 cultures were inoculated from the same mother culture into aseptically 75 cm² plastic cell culture flasks (Corning™, Canted Neck, Nonpyrogenic). Since then, lineages have been selected twice a week, each Monday and Thursday: 30 lineages were large-selected, 30 small-selected and 12 were the control. Control cultures experienced identical conditions (including centrifugation) without being size-selected. At the end of selection, all cultures were reincubated into standard F/2 medium. Every Thursday, cultures were moved to newly sterilized cell culture flasks. We were concerned that our technique for selecting cells of larger size could inadvertently select for rounder cells. However, after 300 generations, we observed little covariance between cell size and cell shape (electronic supplementary material, figure S1). Moreover, previous work showed no difference in cell carbon density among size-selection treatments [49] and comparable swimming performance between small-selected and large-selected cells [50].

For this experiment, cells were sampled after 276 generations of artificial selection (646 days). To remove any environmental effects and non-genetic phenotypic differences, all experimental cultures were grown for three generations (a week) under common garden, neutral selection (i.e. with no centrifugation) before exposing cultures to pre-trial nutrient conditions. Following neutral selection, mean cell area was calculated after measuring at least 200 cells with optical light microscopy at 400× after staining with Lugol’s iodine at 2% with software ImageJ and Fiji (version 2.0; [52]). Cell biovolume was calculated assuming prolate spheroid shape, as recommended for this species by Sun and Liu [47].

**Figure 1.** Experimental design. Ten phytoplankton cultures of *D. tertiolecta* were independently size-selected for 276 generations (figure 2), subjected to a week under either nutrient-replete, nitrogen (N)-deplete or phosphorous (P)-deplete conditions and algal growth was monitored daily for each lineage under nutrient-replete conditions in three independent well plates. During trials, population dynamics of 270 cultures were monitored for 11 days.

(ii) Phase 2: pre-trial nutrient status

Following three generations of neutral selection, a subsample of 10 size-selected lineages per size-selection treatment were resuspended into either nutrient-replete (standard fresh F/2 medium) or nutrient-deplete conditions (modified F/2 medium where either phosphorous or nitrogen was omitted) and loaded into 24-well plates (Corning® polystyrene, flat bottom, with lid, sterile, non-treated, Sigma-Aldrich). All 90 samples were grown for a week: nutrient-replete samples grew exponentially, while nutrient-depleted samples soon reached a stable biomass density and stopped dividing.

(iii) Phase 3: growing conditions during trials

As pre-trial conditions of nutrient-deplete and nutrient-replete cultures produced different population densities, before starting trials all lineages were resuspended into standard F/2 medium and standardized to the same blank-corrected optical density (750 nm), which is a reasonable proxy for total biovolume in a culture (electronic supplementary material, figure S2). Inevitably, however, standardizing cultures to the same total biovolume also meant starting the experiment with large-selected populations having fewer cells than small-selected populations—this difference was unavoidable given our manipulation of size. However, pilot studies showed biovolume to be a better predictor of resource use in our system, compared to cell number—for instance, lineages of different size-selection treatments reach similar maximum biovolumes but widely different maximum population densities (compare figure 3b with electronic supplementary material, figure S6).

After standardizing for optical density, three 250 µl replicate cultures for each size-selected lineage at each nutrient condition were allocated into three independent 96 well plates, after randomizing the position within the well plate (yielding a total of 270 cultures). Low-heat 50 W LED flood lights (Power-lite™, Nedlands Group) were used to provide a light intensity of 150 µM m⁻² s⁻¹ (14–10 h day–night cycle) while minimizing changes in temperature. Pilot study showed that evaporation in the wells was low (approx. 1% per day). Nonetheless, outer wells (where evaporation was higher) were not used for cultures but were filled with water, so that internal humidity would reduce evaporation from inner wells. The blank-corrected optical density (750 nm) of all 270 cultures was monitored daily (at the same time into the photoperiod) for 11 days.

Linear calibration curves were used to convert from units of blank-corrected optical density to units of total biovolume (µm³ µl⁻¹), calculated as the mean cell volume (µm³ cell⁻¹) of a culture multiplied by its population density ($R^2 = 0.88$, $F_{1,60} = 468.5$, $p < 0.001$; electronic supplementary material, figure S2). Samples were grown in well plates under identical conditions to experimental cultures. Every day for 10 days,
were loge-transformed to reduce heteroscedasticity when fitting biovolume over time for each culture. Values of total biovolume were used nonlinear growth models to describe changes in total biovolume with different parametrizations (electronic supplementary material, figure S3). Furthermore, extracting the maximum predicted shape of the model fit (not from the model coefficients), r max, unit: day −1), which represents the maximum growth rate of the population. Because r max and K are extracted from the shape of the best-fitting model to quantify the dynamics of each culture across size-selection treatments and pre-trial nutrient status (electronic supplementary material, figure S3). From each nonlinear curve, we extracted the maximum predicted value of total cell biovolume (K; units µm 2 µl −1). From the first derivative of the curve, we extracted the maximum rate of increase (r max, unit: day −1), which represents the maximum growth rate of the population. Because r max and K are extracted from the shape of the model fit (not from the model coefficients), these parameters are comparable even when estimated from models with different parametrizations (electronic supplementary material, figure S3). Furthermore, extracting r max and K from the best-fitting line ensures that their values describe dynamics observed in the cultures, avoiding extrapolating model dynamics beyond the range validated by data. In this way, K is highly

Figure 2. Mean cell volumes of size-selection treatments of D. tertiolecta (a) across generations and (b) at the time of the experiment (generation 276) as a function of pre-trial nutrient condition. (Online version in colour.)

The analytical methods consisted of three successive steps (see electronic supplementary material, figure S3 for a graphical summary of the statistical methods). First, we chose the best-fitting model among five candidates to best describe changes in total biovolume over time for each culture. Second, we used the best-fitting model to estimate population growth parameters (i.e. growth rates, maximum biovolume reached) to quantify the dynamics of each culture. Third, we used linear mixed-models to evaluate the influence of size-selection and pre-trial nutrient history on each population growth parameter (electronic supplementary material, figure S7 for more details on each treatment).

(b) Statistical analysis
The analytical methods consisted of three successive steps (see electronic supplementary material, figure S3 for a graphical summary of the statistical methods). First, we chose the best-fitting model among five candidates to best describe changes in total biovolume over time for each culture. Second, we used the best-fitting model to estimate population growth parameters (i.e. growth rates, maximum biovolume reached) to quantify the dynamics of each culture. Third, we used linear mixed-models to evaluate the influence of size-selection and pre-trial nutrient history on each population growth parameter (electronic supplementary material, figure S7 for more details on each treatment).

(i) Step 1: fitting non-linear growth models
We used nonlinear growth models to describe changes in total biovolume over time for each culture. Values of total biovolume were loge-transformed to reduce heteroscedasticity when fitting models [48]. The qualitative shapes of the time-series changed considerably across treatments: some followed saturating trajectories, while others showed sinusoidal shapes, sometimes decreasing after reaching maximum population densities. To best capture these different features, four candidate models were fitted to each time-series: a Gompertz-type sinusoidal growth model (i.e. three-parameter Gompertz curve), a logistic-type sinusoidal growth model with lower asymptote forced to 0 (i.e. three-parameter logistic curve), a more general logistic-type sinusoidal growth model with non-zero lower asymptote (i.e. four-parameter logistic curve) and a modified Gompertz-type sinusoidal growth model including population decline after reaching maximum biovolumes (i.e. four-parameter Gompertz-like curve including mortality; see electronic supplementary material, figure S4 for more details on each growth model). A saturating growth model (i.e. two-parameter Michaelis–Menten curve) was initially tried as a candidate model, but was never favoured by Akaike Information Criteria (AIC) [53] model selection and was therefore removed. For more details on the ‘Gompertz with mortality’ model, see Werker & Jaggard [54], while for all other models see Paine et al. [48]. At each combination of size-selection and pre-trial nutrient treatments, growth models were fitted to the three replicate time-series (located on different well plates) of loge-transformed total biomass (residual degree of freedom from each growth model between 29 and 31). AIC [53] were used to determine which growth model best described the dynamics of a culture. Successful convergence was ensured for all best-fitting models.

(ii) Step 2: extract population growth parameters—r max and K
Two population growth parameters were extracted from the shape of the best-fitting model to quantify the dynamics of each culture across size-selection treatments and pre-trial nutrient status (electronic supplementary material, figure S3). From each nonlinear curve, we extracted the maximum predicted value of total cell biovolume (K; units µm 2 µl −1). From the first derivative of the curve, we extracted the maximum rate of increase (r max, unit: day −1), which represents the maximum growth rate of the population. Because r max and K are extracted from the shape of the model fit (not from the model coefficients), these parameters are comparable even when estimated from models with different parametrizations (electronic supplementary material, figure S3). Furthermore, extracting r max and K from the best-fitting line ensures that their values describe dynamics observed in the cultures, avoiding extrapolating model dynamics beyond the range validated by data. In this way, K is highly
correlated with the model coefficient quantifying the upper asymptote of the model ($\gamma_{\text{max}}$) and $r_{\text{max}}$ with the coefficient quantifying the maximum steepness of the model ($s$). As a confirmatory step, we also estimated these parameters after fitting non-parametric spline curves (instead of population growth models)—all conclusions remained qualitatively similar (data not shown). Finally, we used prediction intervals to ensure that the uncertainty around each estimate was low (less than 0.8% and less than 10% for $K$ and $r_{\text{max}}$ respectively) and was safe to omit without altering any of the conclusions.

(iii) Step 3: assess the effect of size-selection and pre-trial nutrient status on growth parameters

Linear mixed models were used to estimate the effects of size-selection (i.e. small, control or large) and pre-trial nutrient status (i.e. nutrient-replete, phosphorus-deplete or nitrogen-deplete conditions before starting growth trials) for each population growth parameter. The fully parameterized model structure was $y \sim Size + N\text{-hist} \times \text{Size} + \text{ID} + (1|\text{Lineage}) + (1|\text{Well Position})$, where $y$ is a population growth parameter (either $r_{\text{max}}$ or $K$), $\text{Size}$ is the evolutionary size-selection treatment (i.e. small, large, or control), $N\text{-hist}$ is the nutrient regime of the culture before starting trials (i.e. nutrient-replete, phosphorus-deplete or nitrogen-deplete), a $\text{Size} \times N\text{-hist}$ interaction, and the initial total biovolume density in the culture ($ID$). Lineage identity ($\text{Lineage}$) and the position of the sample within the well plate (Well Position) were included in the model as random effects. AIC was used to select the best-fitting model among all combinations of nested structures. Standard model validation with diagnostic plots of model residuals was examined to ensure goodness of fit, normality, homoscedasticity and absence of influential observations or outliers. We also used AIC to evaluate the improvement in goodness of fit when adding single or combinations of treatment-specific variance coefficients in the model (varident function in R). Finally, when assessing differences among subgroups within the best-fitting model, if the confidence intervals are not overlapping, the difference was judged to be statistically significant.

All analyses were carried out in R [55] using packages nlme [56], lme4 [57] and plyr [58] for model fitting, MuMIn [59] for model selection and ggplot2 [60] and cowplot [61] for plotting.

3. Results

(a) Cell size

After 276 generations of artificial selection, the mean cell volume of large-selected cells (8295 µm$^3$) was more than 10 times greater than small-selected cells (730 µm$^3$) and 2.7 times larger than control cells (2226 µm$^3$; figure 2a). On average, the mean cell volume of small-selected lineages decreased in size by 2.19 µm$^3$ per generation ($F_{1,24} = 18.465$, $p < 0.001$), while large-selected lineages increased by 24.8 µm$^3$ per generation ($F_{1,24} = 100.09$, $p < 0.001$) and control cultures did not change in size ($F_{1,23} = 2.15$, $p = 0.156$; figure 2a).

Exposing cells to either nitrogen-deplete or phosphorus-deplete conditions led to an increase in volume for all size-selection treatments. But while small-selected and large-selected cells increased on average by 32% and 12%, respectively, control cultures increased by 96% (figure 2b). The shape of the cells was also influenced by both size-selection and pre-trial nutrient status (electronic supplementary material, figure S5).

(b) Max. growth rate ($r_{\text{max}}$)

Overall, $r_{\text{max}}$ values were higher in large-selected lineages than small-selected lineages, with control cells showing intermediate values (figure 3a). But there was a significant interaction between size-selection treatment and pre-trial nutrient history ($F_{1,54} = 3.1981$, $p = 0.0198$; electronic supplementary material, table S1). Large-selected cells grew at the same (relatively high) $r_{\text{max}}$ regardless of resource regime (figure 3a), whereas control cells showed a decrease in $r_{\text{max}}$ of 17% between nutrient-replete and P-deplete conditions (figure 3a), and there was no difference between nutrient-replete and N-deplete conditions (figure 3a). Similarly, smaller cells did not show significant differences in $r_{\text{max}}$ between
N-replete and N-deplete cells, while P-deplete cells grew 21% slower than N-replete cells (figure 3a).

(c) Maximum biovolume reached (K)
Control cultures mostly reached higher total biovolume densities than small-selected and large-selected cultures, regardless of resource regime (K; figure 3b). As for $r_{max}$, there was a significant interaction between size-selection treatment and pre-trial nutrient history ($F_{1,4} = 5.25, p = 0.0012$; electronic supplementary material, table S1). Specifically, small-selected cultures showed a 25% decrease in K from nutrient-replete to both N- and P-deplete conditions (figure 3b). Conversely, control cultures showed no difference between nutrient-replete and P-deplete, but 13% lower K for N-deplete cultures (figure 3b). Similarly, populations of large-selected cells showed no significant difference between nutrient-replete and P-deplete, but N-deplete cultures had 16% lower K (figure 3b; see electronic supplementary material, figure S6 for K converted from units of $\mu m^3 \mu l^{-1}$ into cells $\mu l^{-1}$).

(d) N and P storage capacity
When we explored how cell size affected storage capacity, we found intracellular P of larger cells to support greater subsequent biomass production in P-free environments than smaller cells, whereas intracellular N could generate equivalent production among cell sizes in N-free environments (electronic supplementary material, figure S7). Moreover, smaller cells reached equivalent total biovolumes in N- and P-free environments (overlapping green and light blue points in electronic supplementary material, figure S7). In contrast, control and larger cells could support greater total biovolume in P-free relative to N-free environments (light blue points higher than green points).

4. Discussion
The growth rate of larger cells was least affected by previous periods of nutrient depletion, which is consistent with the ‘fasting endurance hypothesis’ (FEH). Specifically, cultures recovering from a week (approx. 3 generations) of nutrient limitation responded to a new nutrient pulse with $r_{max}$ rates that were comparable to those of nutrient-replete cultures. Also consistent with FEH, the maximum total biovolume (K) of small-selected cultures showed the biggest decrease when recovering from nutrient-deplete periods (25% lower than nutrient-replete conditions), while control and large-selected cultures did not show negative effects after phosphorous (P)-deplete conditions. However, only P depletion showed size-related effects on the growth of this species that are consistent with theory. In contrast, cells recovering from nitrogen (N) depletion showed equivalent $r_{max}$ reduction in K regardless of cell size.

(a) Recovery from nutrient depletion
The FEH was conceived to explain the role of a single nutrient reserve (fat content) in the evolution of body size in mammals and birds [20]. However, relationships between body size and nutrient storage are common to a wide range of other taxonomic groups, including marine phytoplankton [62,63]. Our study simultaneously considered the role of size in two types of limiting nutrient reserves (nitrogen (N) and phosphorous (P)) and revealed that different limiting resources interacted with size in different ways—a finding unanticipated by current theory. Our results showed that larger cells could better recover from P depletion than smaller cells, while N limitation was not size-dependent. These findings suggest that larger cells are better buffered against periods of P limitation due to greater internal storage. But not all cellular materials are equivalent with regard to internal stores. For example, cells can rapidly convert P-rich phospholipids into P-free membranes to recover new P for essential metabolic activities, as opposed to P that is more permanently stored as RNA or DNA molecules [64,65]. Similarly, chlorophyll is a more readily available source of nitrogen compared to carotenoid pigments [66,67].

Our results on N and P storage capacity also showed that intracellular P of larger cells could support greater subsequent biomass production in P-free environments than smaller cells, whereas N storages generated an equivalent biomass regardless of cell size. This finding provides further evidence that larger phytoplankton cells are at an advantage in environments that are frequently P-limited, whereas the same is untrue for N-limitation. The greater size-dependency of intracellular P reserves is likely because P makes up a smaller proportion of the structural composition of a cell, and can, therefore, accumulate to a greater degree than N content [68]. As a result, phytoplankton species usually show more flexibility in C:P ratio than C:N and 2–5 times greater ratios of storage capacity to subsistence quota for P than N [68,69]. Finally, our results are also in agreement with the meta-analysis by Edwards, Thomas and coworkers [63], which detected a greater size-dependency in P-related phytoplankton utilization traits (i.e. maximum nutrient uptake rate, half-saturation constant, minimum subsistence quota) than N-related traits (although with substantial variability).

(b) Maximum growth rate ($r_{max}$)
In our experiment, populations of larger cells produced biomass at faster rates (high $r_{max}$) than an equivalent biovolume of smaller individuals, both after nutrient-replete and nutrient-deplete conditions. This result contradicts classic r-K selection theory (RKS), whereby smaller species should have faster population growth rates following a pulse of nutrients [70,71]. Instead, the ‘metabolic theory of ecology’ (MTE) predicts that the growth rate of a species is proportional to its energy use [72]. Previous studies on D. tertiolecta showed that smaller cells have lower volume-specific metabolic rates than larger cells [49,50], making the faster growth rates recorded here for larger cells consistent with MTE. Previous studies also showed that large-evolved cells of this species recorded higher volume-specific concentrations of photosynthetic pigments, higher max. quantum yields and greater light harvesting efficiencies [49], providing further evidence that the faster growth rate of larger cells is due to a greater size-specific energy flux.

(c) Maximum biovolume reached (K)
All size treatments reached comparable maximum total biovolumes under nutrient-replete conditions, but under nutrient-deplete conditions control cells reached greater biovolumes (K) than small- and large-selected cultures. There are two potential explanations for these findings. The first
A strong selecting pressure (e.g., artificial selection) is well-known to favor the evolution of specialized, monomorphic phenotypes and to reduce the niche breadth of a species [73–75]. As circumstantial evidence for a reduced tolerance to environmental changes in our size-selected lineages, we found cells of intermediate sizes (control cultures) to increase in volume twice as much (+100% change) than large- (+30%) or small-selected (+57%) cells after experiencing nutrient limitation, suggesting that control cells were far more phenotypically plastic. Increasing body size at the onset of nutrient limitation is a common response of many phytoplankton species when cell division is inhibited by lack of inorganic nutrients, allowing cells to divert photosynthetic energy toward intracellular energy storages (such as starch and lipids) [76–78]. Thus, control-lineage cells may have better fitness homeostasis under fluctuating resource conditions because they have retained greater degrees of cell size plasticity relative to our size-selected lines. A second potential explanation for control cells reaching higher K after N- and P-deprived conditions is that fluctuations in nutrient availability may impede stabilizing selection toward an intermediate body size of this species. In previous studies we have showed that large-selected lineages after 27 generations of artificial selection consistently reached higher K compared to control and small-selected cultures [50]. Large-selected cells at those earlier generations were more similar in size to present-day controls (1927 μm^3 versus 1478 μm^3) than to present-day large-selected cells (7311 μm^3). While confounded in time, these results imply an optimal cell size of *D. tertiolecta* at around 1500–2000 μm^3 in volume.

5. Conclusion

Evolving *D. tertiolecta* of different sizes showed that previous periods of phosphorous (P) depletion disproportionately impacted the maximum growth rate (r_max) and the maximum biovolume reached (K) of smaller individuals, as predicted by the ‘fasting endurance hypothesis’ (FEH). However, contrary to theory, the recovery from nitrogen (N) limitation was not size-specific, suggesting that the effects of body size on resource regime depended on the type of limiting resource. These results indicate that increasing size can promote the use of stored resources to supplement growth in unfavourable environments, but only when growth is P limited. Storing nutrients to maintain fast growth can substantially increase the long-term fitness of communities experiencing not only spatial but also temporal heterogeneity in resource levels [79], especially in aquatic environments [29, 80]. Phytoplankton species are responsible for a large part of the total carbon fixation on the planet [30]. However, increased temperatures and ocean acidification are leading to a shift in cell size in the phytoplankton community, either due to plasticity, adaptive evolution or species turnover [81–83]. If such size-shift toward smaller sizes will occur, our experiment suggests that they may lead to a reduction in total primary productivity when resources vary, with repercussions for global carbon cycles.

Data accessibility. All data, codes and cell microscopy photos generated in this study are available in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.4mh47ez [84].

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

Competing interests. We declare we have no competing interests.

Funding. We are particularly grateful to the Australian Research Council for financial support (grants DP110103529 to D.J.M.).

Acknowledgements. We thank Lucy Chapman for help with data collection and Dr Chris Grooming for suggestions on laboratory procedures. We would like to express our gratitude to Prof. John DeLong and an anonymous reviewer for their insightful comments.

References


