Eco-energetic consequences of evolutionary shifts in body size

Abstract
Size imposes physiological and ecological constraints upon all organisms. Theory abounds on how energy flux covaries with body size, yet causal links are often elusive. As a more direct way to assess the role of size, we used artificial selection to evolve the phytoplankton species Dunaliella tertiolecta towards smaller and larger body sizes. Within 100 generations (c. 1 year), we generated a fourfold difference in cell volume among selected lineages. Large-selected populations produced four times the energy than small-selected populations of equivalent total biovolume, but at the cost of much higher volume-specific respiration. These differences in energy utilisation between large (more productive) and small (more energy-efficient) individuals were used to successfully predict ecological performance (r and K) across novel resource regimes. We show that body size determines the performance of a species by mediating its net energy flux, with worrying implications for current trends in size reduction and for global carbon cycles.

Keywords
Allometry, artificial selection, evolutionary size shift, experimental evolution, geometric biology, metabolism, net energy flux, primary production, scaling.


INTRODUCTION
Size imposes fundamental physical and chemical constraints on individuals by mediating rates of energy utilisation (West et al. 2001, 2002; Brown et al. 2004). Specifically, increasing body mass usually decreases the mass-specific energy acquisition and use of a species (Brown et al. 2004). From an ecological prospective, body size influences competitive ability (Werner & Gilliam 1984), resource utilisation (De Roos & Persson 2013) and the ecological niche of a species (Bergmann 1847; Roff 1986). From an evolutionary prospective, virtually all life-history traits covary with body size (Peters 1983; Werner 1988; Reznick et al. 1990). For example, smaller species tend to grow faster (Savage et al. 2004) and have shorter life spans (Marba et al. 2007). As such, body size is perhaps the most fundamental trait in biology (De Roos et al. 2003).

The past 20 years have focused on the role of size in affecting the energy use of a species. A host of theories have been proposed for how size affects energy use, from bacteria to whales, with intense debate about the mechanistic drivers underlying broad-scale patterns (Brown et al. 2004; Anderson & Jetz 2005; Loeuille & Loreau 2006). Yet, for all the speculation about how size affects energy use, tests of how size influences energy acquisition are surprisingly rare (De Roos & Persson 2013). Of those studies that do examine the effect of size on energy acquisition, few tend to simultaneously account for energy use. It seems obvious that the productivity of an organism (via growth or reproduction) will depend on how size affects both energy acquisition and use – that is, the net energy flux. Yet, our understanding of size-dependent net energy flux remains surprisingly poorly resolved. This knowledge gap has taken on new significance given current trends in body size reduction across a range of taxa worldwide (Allendorf & Hard 2009).

Humans are driving species to smaller body sizes, both directly and indirectly, with unknown consequences (Allendorf & Hard 2009; Traill et al. 2014; Eikeset et al. 2016). Fishing and hunting are promoting the evolution of smaller body sizes, because larger individuals are targeted specifically and because higher mortality rates can select for earlier maturation at smaller sizes. For example, the heavily exploited fish species of Atlantic cod (Gadus morhua) has evolved to reproduce sooner and grow less, because fishing pressure dramatically decrease the chances of survival beyond a single round of reproduction (Olsen et al. 2004; Swain et al. 2007). Humans can also indirectly cause body size shifts via predator removal (Roy et al. 2003; Brodin & Johansson 2004; Preisser & Orrick 2012). Finally, global temperature increases are reducing body sizes, particularly of aquatic organisms (Daufrere et al. 2009; Gardner et al. 2011; Forster et al. 2012). In particular, phytoplankton species are decreasing in cell size in response to increasing temperatures (Atkinson et al. 2003; Daufrere et al. 2009; Polovina & Woodworth 2012). Phytoplankton fix around half the carbon on the planet (Field et al. 1998) but the consequences of size shifts for energy flows and global carbon cycles remain unknown. Overall, the cascading influences of changes in body size are poorly understood, but if body size affects the net energy flux of a species, these changes are likely to substantially alter the flow of energy through populations, communities and ecosystems.

Perhaps the most direct way to assess how size affects energy acquisition and usage is to engineer genetic differences in mean body size and evaluate its consequences for the net energy flux and fitness. We used artificial selection to generate differences in body size in the phytoplankton species Dunaliella tertiolecta (Chlorophyta) and measured traits at multiple times throughout the evolution of this species (i.e. generations 27, 70 and 103). We assessed different traits at multiple time points (rather than at the same generation) partly because
some of our hypotheses emerged from earlier tests, and also because some of our assays took days to perform, which meant that inevitably we could not conduct all of the assays at the same time point. We quantified how size shifts influenced the capacity of the evolved lineages to both acquire and use energy, as well as other ancillary traits. Then, we used these data to generate a priori predictions about vital ecological rates under different resource regimes. Finally, we tested these predictions by estimating key demographic rates across a resource gradient in later generations. We found that we can successfully predict the ecological dynamics of the evolved organisms based on size-dependent differences in energy acquisition and usage.

METHODS

Model species

The cosmopolitan, fast growing green alga Dunaliella tertiolecta (Butcher) was chosen as model species due to its intermediate cell size (relative to other phytoplankton species) and its capacity to grow well in laboratory conditions. Monoclonal batch cultures were sourced from the Australian National Algae Culture Collection (ANACC; strain code CS-14) and reared in standard F/2 algal media (without silica), prepared with 0.45 filtered seawater (Guillard 1975). All cultures used in the experiment were kept in a temperature-controlled room at 21 ± 2 °C with a 14–10 h day–night cycle at a light intensity of 150 µmol quanta m⁻² s⁻¹. All cultures were handled aseptically with sterile material under a laminar-flow cabinet (Alternative Environmental Solutions fitted with high-efficiency particulate arresting filter, Australia Standards 4260, National Association of Testing Authorities certified).

Cell size evolution

Artificial selection on cell size was achieved through differential centrifugation. This method relies on larger cells 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. On the 25th of April 2016, 72 independent 50 mL cultures were inoculated from the same mother culture into aseptic 75 cm² plastic cell culture flasks (Corning®, Canted Neck, Nonpyrogenic, Merck, Darmstadt, Germany): 30 lineages for large selection, 30 for small selection, and 20 control cultures. Experimental design and neutral selection

Cell size determination

Mean cell volumes were analysed regularly every 3 generations for the first 12 generations, and every 15 generations thereafter. Mean cell volumes were calculated after measuring at least 200 cells with optical light microscopy from randomly selected lineages and after allowing three generations of neutral selection (i.e. without any centrifugation) before collecting photos. A 1 mL aliquot of culture was stained with Lugol’s iodine at 2%. Photomicrographs were taken at 400× after loading 5 µL onto a slide gently covered with a cover slip. Cell area was calculated with ImageJ and Fiji (Schindelin et al. 2012) and cell biovolume was calculated assuming prolate spheroid shape, as recommended for this species by Sun & Liu (2003).

Staining cells with Lugol iodine solution can in some species lead to cell shrinkage (Montagnes et al. 1994). However, pilot studies showed no sign of shrinkage between samples of live cells and cells that were stained immediately ($F_{1,170} = 1.55$, $P = 0.21$) and between 0, 5 and 7 days after Lugol staining ($F_{1,128} = 0.97$, $P = 0.33$).

Experimental design and neutral selection

Phenotypic characterisation of the lineages started after 27 generations of size-selection, when a consistent difference between treatments emerged ($F_{2,69} = 23.47$, $P < 0.001$): at this stage, cell volumes were 10% larger in large-evolved lines and 2% smaller in small-evolved cells, compared to control lineages (Fig. 1). Also, the mean density of single cells, calculated as cell weight divided by cell volume, did not differ among treatments (see Appendix S1). Prior to the assays, a 10 mL aliquot was sampled from each of the 72 evolving lines (30 small, 30 large and 12 control) and fertilised with 40 mL autoclaved fresh media. To remove any environmental conditioning from selection protocols, experimental cultures were grown for three generations (a week) under neutral selection (i.e. with no centrifugation). Cells were maintained under nutrient-replete conditions during neutral selection, by adding new media 3 days before and the same day of the assays. All assays were carried out after standardising all lineages to the same blank-corrected optical density value of 0.27, a level of Mondays and Thursdays. Every Thursday, all cultures were moved to newly sterilised flasks.

Speed and duration of centrifugation routines were determined through pilot studies, to maximise selection differential while diluting biomass to approximately 20% of the initial density. Pilot studies on the selection differential protocol detected a symmetrical shift for both larger and smaller cells of 10% between cell size before and after selection. Furthermore, a preliminary analysis showed that centrifuging cultures for 5 min from 20 to $425 \times g$ (500 to 2000 rpm) did not alter the growth rate of the species compared to samples that were not centrifuged ($F_{4.53} = 0.55$, $P = 0.7$). As cells started responding to selection throughout the experiment, centrifugation time and speed were gradually adjusted, to maintain a symmetric selection differential and comparable dilution rates.
Mean cell volume (µm$^3$)

![Graph showing mean cell volume over generations for Small, Control, and Large treatments.](image)

**Figure 1** Effect of artificial selection on mean cell volume for size-selection treatments of small, control and large (±1SE). Net energy flux (Fig. 2a), chlorophyll concentration (Fig. 3a), sinking assays (Fig. 3b), swimming trials (Fig. 3c and d) and cell density (see Appendix S1) were measured at the 27th generation, while growth performance (Fig. 2c) at the 103th generation, and neutral selection assays (Appendix S3) at the 103th generation. Each point on the graph was calculated after measuring at least 200 cells from independent lineages. Photos on the right were taken with light microscopy after 103 generations: panoramic view of lugol-stained cells at 400X, and close-up of unstained single cells at 600X.

**Dark and light metabolism**

The rate of oxygen consumption ($\text{VO}_2$) was measured at 19 °C with 8 × 24-channel PreSens Sensor Dish Reader (SDR; AS-1 Scientific Wellington, New Zealand) with methods adopted from Malerba et al. (in press), White et al. (2011) and Warkentin et al. (2007). Before the experiment, all SDR were calibrated with air-saturated (AS) seawater (100% AS) and water containing 2% sodium sulphite (0% AS). After three generations of neutral selection, cultures were collected in exponential phase and placed in 5 mL sealed vials being careful to remove any air pocket from inside the vials. Biomass that was tested to work well with all experimental protocols.

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was monitored daily (at the same time of the day) until reaching carrying capacity (7 days). Pilot studies showed that the daily evaporation within a well plate was very low (c. 1% per day) and was therefore not included in the analysis. Estimates for maximum exponential growth (r) and carrying capacity (K) were calculated for each lineage by fitting standard logistic growth models to daily time-series of optical density from three independent cultures with non-linear least squares techniques (function nls in R). Models that failed to converge or where residuals showed clear lack of fit were excluded from further analyses (i.e. 1 lineage for small and 2 for large, out of 72 lineages in total).

Additional assays

All lineages were also analysed for chlorophyll content, sinking rates, swimming speed and density of the cells. All assays followed standard methods (see figure legends for more details): volume-specific chlorophyll content (Fig. 3a), sinking rates (Fig. 3b), swimming performance (Fig. 3c and d) and mean density of a cell (see Appendix S1).

Statistical analysis

All analyses were carried out in R (R Core Team 2016). Non-linear logistic models were calibrated using package nlme with the function nls (Pinheiro et al. 2016; R Core Team 2016). Linear models were then used to assess the effects of the size-selection (i.e. small, control and large) on each variable of interest: population growth (parameters r and K), light and dark metabolism (parameters Pmax, Ec and a), cell density, chlorophyll content, swimming speed, swimming distance and sinking rate. Data transformations were employed whenever the residuals lacked normality. Also, in cases where treatments displayed unequal variances, treatment-specific variance coefficients were included in the model (varIdent function in R).

RESULTS

Cell size evolution

After 100 generations of selection there was a fourfold difference in cell volume between large-selected and small-selected lineages of *Dunaliella tertiolecta* (Fig. 1). We found that large-selected lines grew more than small-selected lines shrunk, despite symmetrical artificial selection regimes (Fig. 1). Cell volume increased at a rate of 9.6% per 10 generations in large-selected cells (F1,10 = 26.45, P < 0.001), while it decreased at 5% per 10 generations in small-selected cells (F1,10 = 20.31, P < 0.001). Control cells were of intermediate sizes between small and large cells, without showing any trend in size over time (F1,9 = 4.216, P = 0.07).

Energy acquisition and usage

All of our measures of energy acquisition and usage are from samples at the 27th generation of size-selection and are here reported in volume-specific terms, so that large and small cells can be compared directly. Overall, large-selected cells were substantially more efficient at converting photons into energy, but also displayed higher rates of dark respiration (Fig. 2a). The maximum photosynthetic rate reached in large-selected cells was up to three times higher compared to small-selected and control cells (Pmax,l in eqn 2; asymptote in Fig. 2a). Also, the rate of energy production was twice as high in large-selected cells compared to the other two treatments (z1 in eqn 2; initial slope in Fig. 2a). The compensation light intensity (Ec,l in eqn 2; X-intercept in Fig. 2a) was lower in large compared to small and control cells, that is, large-selected cells needed less light to achieve ‘break even’ energy production. Finally, rates of dark respiration were twice as high in large cells compared to small and control cells (Y-intercept in Fig. 2a). The superior ability of large-evolved cells to produce energy at higher light conditions was consistent with their higher volume-standardised chlorophyll concentration (Fig. 3a). Later analysis on energy acquisition, energy usage and chlorophyll concentration of cells size-selected for more than 103 generations confirmed that the same qualitative differences detected at the 27th generation remained, mostly with differences among size treatments strengthening over time.

Predicting and testing productivity across environmental gradients

After measuring the net energy flux of size-selected cells at each light condition (Fig. 2a), we calculated the volume-specific daily net energy budget (i.e. photosynthesis during the day minus respiration at night) across a light gradient, varying in both light intensity and photoperiod (Fig. 2b). Under low energy conditions (i.e. low light intensities and/or short light durations), we predicted a priori that lower respiratory costs of small-selected cells would lead to superior overall daily energy budgets compared to control and large cells (blue region in Fig. 2b). Conversely, we predicted that the higher photosynthetic capacities of large-selected cells would yield the highest productivity in high energy environments (i.e. high light intensities and/or extended light exposures; red region in Fig. 2b). These predictions were later confirmed empirically. When grown in low-light environments, small-selected cells showed faster exponential growth rates than control and large-selected cells (Fig. 2c). At intermediate lights, rates were statistically indistinguishable among size classes (Fig. 2c). Under high light regimes, large-selected cells displayed 77 and 45% faster exponential growth rates than control and small cells respectively (Fig. 2c).

An unexpected finding was that small-selected cells achieved much lower total biovolumes at carrying capacity (Fig. 2c). We predicted that decreasing light intensity amplified the relative importance of lower maintenance costs of smaller organisms compared to greater energy capacity of larger organisms (Fig. 2a). But lower energy needs of smaller sizes came at a cost: smaller cells reached lower total biovolumes at carrying capacity (by as much as 40%), regardless of resource regimes (Fig. 2c).

Sinking rate and cell motility

Volume-standardised sinking rates did not show statistically significant differences among size-selected treatments.
This was because smaller cells showed slower per-cell sinking rates ($F_{2,34} = 3.48$, $P = 0.04$), but proportionally to their volume all treatments showed equal per-volume sinking rate. This result is consistent with comparable density of individual cells among size treatments (Fig. S1). Also, cells that were not size-selected showed higher (volume-standardised) swimming performances than small- and large-selected cells, with faster speeds (Fig. 3c) and for longer travelled distances (Fig. 3d).

**DISCUSSION**

Confronting *a-priori* predictions with empirical data is perhaps the most robust way to test a hypothesis. In this study, we first evolved cells towards smaller and larger sizes compared to the ancestral condition, estimated size-specific net energy production across size classes, and used these estimates to generate predictions about vital ecological rates across a resource gradient. Finally, we tested these predictions by measuring demographic rates across different resource conditions. Our results showed that we can successfully predict ecological dynamics solely based on the interplay between size-specific energy production, energy usage and resource availability. We found that larger sizes increased the volume-specific energy production of a cell by improving photosynthesis, but also increased volume-specific respiration rates in the dark. Consequently, larger cells showed faster population growth rates under higher resource regimes, but were inferior to smaller
cells at lower resource regimes. However, larger cells consistently reached higher carrying capacities than smaller cells at all resource regimes—a discovery that is harder to explain. Other key traits, such as swimming speed and travelling distance, showed highest performance at control cells, perhaps because they maintained an optimal ratio of cell size to length of the swimming flagella. These results showed context-dependent costs and benefits of different cell sizes, which suggest natural limits on cell size evolution, and that current cell size is the product of conflicting selection pressures due to variable resource regimes in nature. Also, by imposing size-selective pressure on a common ancestor, we provided direct empirical evidence on the causal relationships between the body size of a species, and its energy flux and demographic rates. Finally, all assays were carried out after standardising cultures to the same total biovolume, as to also standardise for resource requirements among treatments. This standardisation necessarily confounds population densities (i.e. small-selected cultures have more cells), but in our system, biovolume drives dynamics more than the density of cells—population growth slows down more at equivalent biovolumes than at equivalent cell densities, indicating that total biovolume is a better predictor of total population resource requirements among size treatments.
Faster evolution towards larger sizes

Despite the application of symmetrical selection pressures for more than 100 generations of size-selection, cell volume increased two times faster in large-selected cells, than it decreased in small-selected cells. This trend of faster evolution towards larger sizes is opposite to studies on other organisms, which instead found mutational variance to be biased towards reducing sizes (Azevedo et al. 2002; Estes et al. 2005; Ostrow et al. 2007). We propose three (non-mutually exclusive) hypotheses to explain the higher success of evolving larger cells relatively to smaller cells. First, the fact that large cells performed better in high resource environments (i.e. 150 μmol quanta m⁻² s⁻¹ and 14:10 light cycle falls in red region of Fig. 2b) suggests our culturing conditions imposed a lower volume-specific daily net energy budget on smaller cells relative to larger cells. Thus, our experimental conditions inadvertently represented an additional selection pressure for increased size, conflicting with our selection regime towards smaller individuals. A second hypothesis is that faster evolutionary rates towards larger sizes may be due to larger developmental scope for increasing rather than decreasing cell volume. Many components within a cell are non-scalable and reducing the intracellular volume can potentially limit the overall functionality of a cell (Schmidt-Nielsen 1984; Maranon 2015). A third potential explanation for why cells evolved more towards larger sizes is that our methods to select for small cells favoured both smaller cells at mature stage and also younger cells at earlier ontogenetic stages. This latter group of cells will still have the potential to grow into larger cells, reducing the ultimate selection differential towards smaller sizes. Although a complete test of these hypotheses would require replicating the evolutionary experiment under different culture conditions (i.e. test if low light conditions induce faster evolution towards smaller sizes instead of larger) and with different selection protocols, we performed a preliminary test in which we analysed the rates of body size evolution after stopping artificial selection for size (see Appendix S3). Large-selected cells reared under relaxed artificial selection maintained their larger size only when exposed to high light levels, whereas low light regimes led to shrinkage of both large-selected and control cells (Appendix S3). These results support the first hypothesis that faster rates towards evolving larger sizes are due to a superior daily net energy budget. However, small-selected cells did not decrease their mean cell volume after being exposed to low light levels (Appendix S3), which also supports the second hypothesis of a lower boundary to cell size evolution.

Size evolution across resources

Our results provided direct empirical evidence for the importance of body size and resource levels in mediating the net energy assimilation of an organism. Pioneering studies have established that the productivity of a species can either increase (e.g. Sanderson et al. 1999) or decrease (e.g. Massie et al. 2010) with organismal body size (ontogenetic asymmetry; De Roos et al. 2013; Persson & de Roos 2013). Our results expand these findings, as they showed that the energy efficiency and productivity of an organism not only changed with its size but also with resource level (see also Ghedini et al. in press; Malerba et al. in press). Specifically, high light regimes disproportionately favoured the greater capacity of larger cells to create energy, while low light conditions benefited the lower maintenance costs of smaller cells. In nature, there are many examples where resource levels affect the size-structure of a population, by favouring the survival of either smaller or larger individuals: fish (Edeline et al. 2016), amphibian (Asquith & Vonesh 2012), colonial invertebrates (Ellison & Harvell 1989), molluscs (Smalley 1983), insects (Amarillo-Suarez et al. 2011) and plants (Bertrand et al. 2011). Here, we argue that the consequences of size reduction will also heavily depend on resource levels. If these same mechanisms were to be confirmed among other species, new assessments should be undertaken to evaluate the magnitude of the impacts that anthropogenic activities have on natural populations by increasing (e.g. nutrient eutrophication) or decreasing (e.g. habitat degradation) resource levels.

Size and temperature

Our results have interesting applicability to the widely observed negative covariance between body size and temperature (Daufresne et al. 2009; Gardner et al. 2011). Here, we showed that increasing cell size led to higher daily variation in energy use, by increasing both volume-specific rates of photosynthesis and respiration. At warmer temperatures, a smaller body size confers a fitness advantage in many uni- and multicellular organisms (Forster et al. 2011, 2013). Higher temperatures increase metabolic rates (Gillooly et al. 2001) and reducing body size might represent a strategy to limit fluctuations in energy budget between energy acquisition and energy utilisation. Large cells may simply be unable to store up sufficient energy during the day to make it through a dark, hot night. Alternatively, a reduction in body size at higher temperatures might be due to respiration being more temperature sensitive than energy acquisition processes. This latter hypothesis is supported by studies on both autotrophic and heterotrophic species (Coles & Jokiel 1977; Atkinson et al. 2003; Liang et al. 2013). In this case, the costs of larger body sizes increased more than the benefits.

Size and global carbon cycles

Our results have worrying implications for the impacts of global change on carbon fixation in the ocean. We find that simple intuitions about populations of identical biomass but different body sizes do not hold. For example, we calculated that a large cell of 5000 μm³ in volume can produce up to four times more energy than the combined production of five small cells of 1000 μm³ each. If increased temperatures select for smaller cell sizes, as seems likely (Peter & Sommer 2012), then our results suggest that these cells will become nutrient limited at much lower resource levels, decreasing productivity by as much as 40%.

In addition to rising temperatures, our findings suggest that also resource limitation can act as an additional selective pressure towards reduction in organismal size. Decreasing
resources amplified the relative importance of lower maintenance costs of smaller organisms compared to greater energy capacity of larger organisms. But the lower energy needs of smaller sizes came at a cost: here, we showed that smaller sizes reached much lower total biovolumes at carrying capacity, regardless of resource regimes. This may be because smaller organisms tend to have lower C:N or C:P ratios and can therefore produce less biovolume than larger organisms from the same limiting resources (Maranon et al. 2013; Maranon 2015). If these same relationships were to be confirmed for other species, future global warming scenarios will select for smaller organisms not just by increasing temperatures but also by reducing available resources. This represents an unanticipated ‘one-two punch’ for aquatic primary productivity – decreasing both production and carrying capacity under lower resource regimes. Climate change is predicted to increase ocean stratification, which will reduce the transfer of nutrients to the surface and limit primary productivity in the euphotic zone. Aquatic primary producers are responsible for nearly half of the biospheric net photosynthesis (Field et al. 1998), and an additional reduction in autotrophic biomass could act as a positive feedback by reducing the ocean ability to mitigate anthropogenic carbon emissions.

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AUTHORSHIP

All authors contributed to designing the study. MEM conducted the experiment and collected the data. MEM and DJM carried out statistical analyses. MEM wrote the initial draft of the manuscript, while DJM and CRW provided substantial feedback. All authors gave final approval for publication.

DATA ACCESSIBILITY

All data and codes generated in this study are available in Dryad (https://doi.org/10.5061/dryad.bc1gb). Also in the file are photos taken with light microscopy showing the different cell sizes among size-selected lineages.

REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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