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Phenotypic consequences of 1,000 generations of selection at elevated CO_2 in a green alga

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Estimates of the effect of increasing atmospheric CO_2 concentrations on future global plant production rely on the physiological response of individual plants or plant communities when exposed to high CO_2 (refs 1–6). Plant populations may adapt to the changing atmosphere, however, such that the evolved plant communities of the next century are likely to be genetically different from contemporary communities[7–12]. The properties of these future communities are unknown, introducing a bias of unknown sign and magnitude into projections of global carbon pool dynamics. Here we report a long-term selection experiment to investigate the phenotypic consequences of selection for growth at elevated CO_2 concentrations. After about 1,000 generations, selection lines of the unicellular green alga *Chlamydomonas* failed to evolve specific adaptation to a CO_2 concentration of 1,050 parts per million. Some lines, however, evolved a syndrome involving high rates of photosynthesis and respiration, combined with higher chlorophyll content and reduced cell size. These lines also grew poorly at ambient concentrations of CO_2. We tentatively attribute this outcome to the accumulation of conditionally neutral mutations in genes affecting the carbon concentration mechanism.

Plant growth depends on CO_2 concentration[1,2], which is expected to rise from current levels of about 400 parts per million (p.p.m.) to between 700 and 1,000 p.p.m. during the next century[3]. In response, global plant productivity in forests[4], grasslands[5], agroecosystems[6] and other ecosystems is expected to increase. Projections of future net primary productivity are complicated by synchronous changes in temperature and other factors, but most models predict increases in the land–atmosphere and ocean–atmosphere fluxes from current values of >-2 petagrams (Pg) C per year to about -5 Pg C per year[3]. This process is likely to be complicated by shifts in the species composition of plant communities[7], and more fundamentally by evolutionary changes within plant populations. In the very long term, this may involve the extinction of some groups and the radiation of others[8], but within a few hundred generations most plant populations may adapt to the increased supply of inorganic carbon. Selection experiments with plants have demonstrated a variety of responses[9–12], but have been limited to fewer than ten generations. The long-term response to selection and the properties of populations adapted to elevated CO_2 remain unknown, and constitute an important limit on our ability to predict future plant productivity.

We used a microbial model system in which large population size and short generation time make it possible to evaluate evolutionary change caused by the spread of novel mutations over hundreds of generations. *Chlamydomonas reinhardtii* is a unicellular green alga that has been extensively used to study the physiology and genetics of photosynthesis[13]. It possesses a carbon-concentrating mechanism (CCM), which increases the concentration of CO_2 near the active site of ribulose 1,5-bisphosphate carboxylase–oxygenase (Rubisco), in common with most other eukaryotic microalgae that have been studied[14]. We set up ten isogenic selection lines from each of two ancestral genotypes, half being grown at ambient CO_2 (ambient lines) and half at a concentration that increased from ambient to 1,050 p.p.m. over about 600 generations and was then maintained at this level for a further 400 generations (high lines). At least 10^5 cells per line were transferred for 125 transfers in a buffered, nutrient-rich medium. The history of these lines thus emulates the conditions that photosynthetic organisms are likely to experience during the next century or so, with respect to CO_2 levels alone.

The physiological effect of elevated CO_2 concentration is expected to be an increase in photosynthesis, causing an increase in growth. Net photosynthesis in the ambient lines increased by about 30% when they were grown at high CO_2 (Fig. 1a). The ambient lines diverged through time so that by the end of the experiment they varied significantly in the rate of photosynthesis (one-way analysis of variance (ANOVA): $F_{9,18} = 9.0$, $P < 0.001$) when grown at ambient CO_2 concentrations. The high lines had normal rates of photosynthesis at ambient CO_2, which increased by more than 50% as an average over all lines at high CO_2. However, this effect was very inconsistent: one group of high lines had low rates whereas a second group had very high rates of photosynthesis at high CO_2 concentration (Fig. 1a). This distinction was not related to the identity of the ancestor, and represented significantly more divergence in photosynthetic rates than was seen in the ambient lines ($F_{1,16} = 10.5$, $P = 0.005$).

The growth rate of cultures grown at elevated CO_2 was correlated with their photosynthetic rate among the ambient lines, but not among the high lines (Fig. 1b). The physiological effect of CO_2 on photosynthesis was reflected by growth in pure culture, where the maximal rate of increase (Fig. 1c) and the limiting density (Fig. 1d) of both the ambient and the high lines are enhanced substantially by high CO_2. However, there was no indication of a parallel evolutionary response: by the end of the selection experiment, the high lines had not become specifically adapted to growth at high CO_2; their growth at high CO_2 being no greater than, and perhaps even less than, the growth of the ambient lines. There was nevertheless an indirect response: the growth of some high lines was markedly impaired at ambient CO_2 concentrations where two of the lines could scarcely be propagated. This result was supported by the outcome of competition assays in which the selection lines were mixed with standard genetically marked strains and the change in frequency during growth in culture recorded (Table 1). The high lines had considerably lower competitive ability at ambient CO_2, where three of them (including the two with strongly reduced growth in pure culture) were such weak competitors that they were consistently eliminated by the tester strains within 10–15 generations. They were, however, no more successful than the ambient lines at high CO_2. In short, 1,000 generations of selection at high CO_2 concentrations had caused no increase in growth at high CO_2, whereas growth at ambient CO_2 was often considerably reduced.

Photosynthesis is linearly related to respiration in the dark among lines at ambient CO_2; this relationship is the same for ambient and high lines (Fig. 2a). It has been shown in *Chlamydomonas* that post-illumination rates of O_2 consumption provide a

good estimate of the rates of respiratory O_2 consumption during the preceding light period[15]. The same correlation is expressed at high CO_2, but respiration rates are on average greater for the high lines, because some high lines have extremely high respiration rates, whereas others have normal rates (Fig. 2b). Two of the three high lines that had high photosynthetic rates also had very high respiration rates. This will reduce the effectiveness of photosynthesis, yet it cannot alone account for the lack of increase in growth, because our measurements of net photosynthesis include photorespiration. In these cases, another process, such as increased leakage of fixed carbon from cells, must also be involved. The evolutionary response is in the opposite sense to the physiological response, which for elevated CO_2 concentrations is to induce lower rates of dark respiration in C_3 land plants[2].

Both chlorophyll content and cell size responded to selection at elevated CO_2 concentrations. The physiological response to increased CO_2 is an increase in chlorophyll *a* content, seen in both the ambient and high lines. In the ambient lines, the average increase in chlorophyll content per cell is about 28%. The high lines, however, show the same inconsistent effect as with rates of photosynthesis (Fig. 3a): those lines with very high photosynthetic rates at high CO_2 also have very large increases in chlorophyll content at high CO_2. The physiological effect of high CO_2 is a marked increase in cell volume, both in ambient and in high lines (Fig. 3b). The high lines have smaller cells than the ambient lines, regardless of the concentration of CO_2. The effect is considerable, amounting to an average reduction of 22% in volume. The evolutionary response is thus of comparable magnitude but opposite in sign to the physiological response. The evolution of reaction norms in this way, so as to mitigate unfavourable physiological responses to extreme environments, has often been observed in long-term selection experiments[16].

In addition to a markedly reduced ability to grow at ambient concentrations of CO_2, the high lines also had a lower limiting density at high CO_2, suggestive of either a lowered affinity for CO_2, or a higher per-cell requirement for inorganic carbon, attributable to higher internal organic carbon content, an increase in respiration

Table 1 **Competitive fitness**

Selection environment	Assay environment	Competitive fitness (±s.e.)	Extinct
High CO_2	High CO_2	−0.682 ± 0.306	0
High CO_2	Ambient CO_2	−1.231 ± 0.325	3
Ambient CO_2	High CO_2	−0.292 ± 0.325	1
Ambient CO_2	Ambient CO_2	−0.386 ± 0.290	0

Fitness of all lines was measured against a common marked strain. Selection and marked lines were inoculated in approximately equal numbers at the beginning of the assay. Fitness is calculated such that a fitness of 0 indicates fitness equal to the marker. There is a marginally significant effect of selection on competitive fitness (ANOVA: $F = 3.92$, $P = 0.056$). When a selection line was completely out-competed by the marked strain by the first time point, it was assumed to be present at the limit of detection (1/200); the number of lines becoming 'extinct' is recorded in the final column.

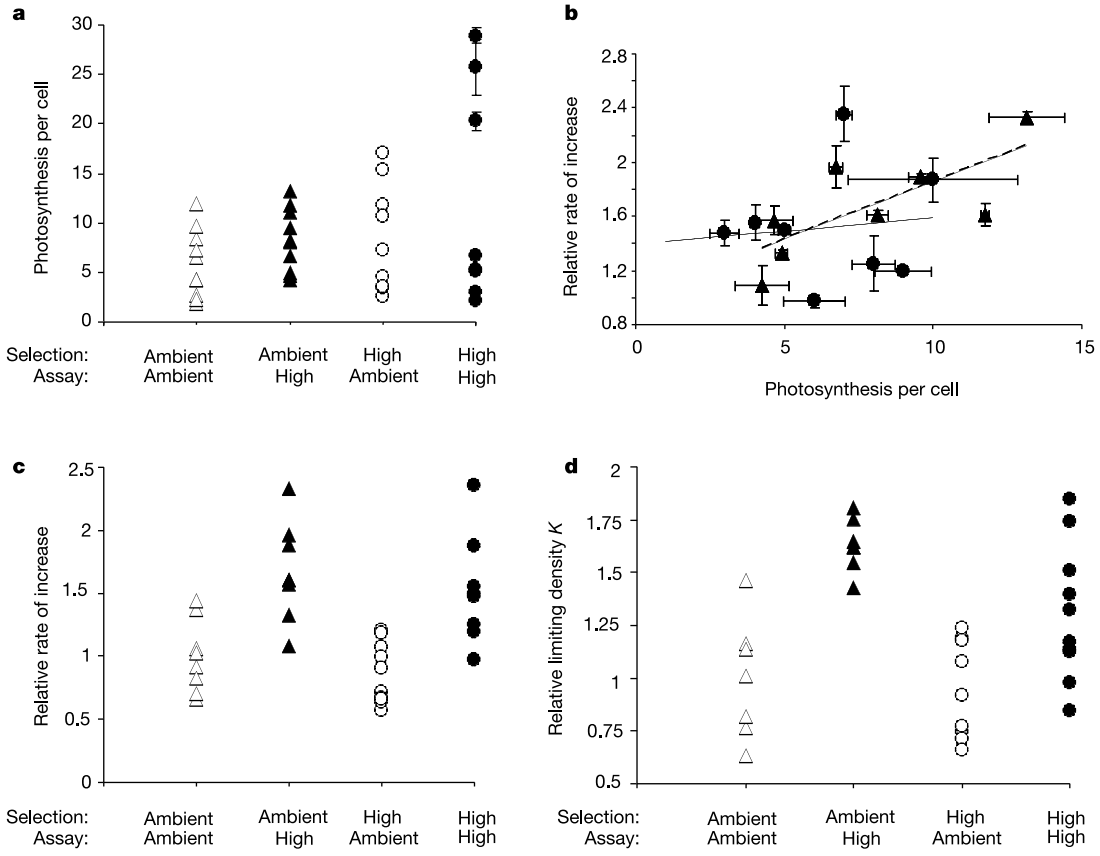

Figure 1 Response to selection at elevated CO_2 concentrations. Symbols designate conditions of selection (ambient, triangle; high, circle) and assay (ambient, open; high, filled). **a**, Photosynthetic rates measured as O_2 evolution per cell. Points are means based on two independent replicates; average s.e.m. 0.61 (range 0.02–2.85). Error bars (±1 s.e.) are shown explicitly for the three selection lines with exceptionally high rates of photosynthesis at elevated CO_2. **b**, Relationship between growth rate and photosynthesis at high CO_2 for ambient lines (upper regression, $P = 0.038$) and high lines (lower regression, not significant). Error bars are ±1 s.e. **c**, Pure culture growth rates. All values are calculated relative to the average growth rate of ambient lines growing at ambient CO_2 concentrations. Lines show increased growth rates at high CO_2 ($F = 33.6$, $P < 0.0001$). Points are means based on three independent replicates; average s.e.m. 0.096 (range 0.01–0.51). **d**, Limiting densities. All values are calculated relative to ambient lines growing at ambient CO_2. High lines have significantly lowered carrying capacities than do ambient lines (effect of selection: $F = 5.1$, $P = 0.03$; effect of assay: $F = 32.5$, $P < 0.0001$). Points are means based on three independent replicates; average s.e.m. 0.116 (range 0.01–0.25).

or increased loss of carbon from cells. Under ambient CO_2 conditions, *Chlamydomonas* and other microalgae concentrate inorganic carbon through an energy-requiring process that keeps Rubisco saturated with CO_2[17–19]. When the external concentration of CO_2 is increased, mutations in downregulated or unexpressed CCM genes might be neutral in the high-CO_2 environment, despite being deleterious in the ancestral environment. If the CCM were compromised in some way, the evolved lines would show a decreased affinity for inorganic carbon, resulting in a decreased limiting density. A lowered affinity for inorganic carbon without a necessary decrease in steady-state photosynthesis at high CO_2 is seen in some *Chlamydomonas* high-CO_2-requiring mutants where components of the CCM are inactivated[20–22]. We tentatively attribute the outcome of selection in our experiment to the accumulation of conditionally neutral mutations that are deleterious when expressed in more stringent conditions. This class of mutation has previously been shown to explain antagonistic indirect responses to selection in microbial selection experiments[23,24].

The main result of our experiment is that we were unable to demonstrate specific adaptation to high CO_2 concentrations, because after 1,000 generations the high selection lines neither grew faster nor had a higher limiting density than did the ambient lines in the high CO_2 environment. Instead, the physiological response of all traits measured was attenuated or reversed in at least some of the high lines by the end of the selection experiment. This suggests that projecting future change on the basis of current physiological responses may be misleading, and should wherever possible be attempted in conjunction with an empirical knowledge of evolutionary responses. However, a more fundamental obstacle to precise forecasting is the uncertainty of the evolutionary response, which is a general feature of selection experiments[25–27]. We observed the evolution of two distinct syndromes with respect to carbon metabolism, defined by the ability to respond physiologically to changes in CO_2. One group of lines showed no change in chlorophyll *a* content, photosynthesis or respiration rates, and when growing at high CO_2 seemed to mimic control cells growing at ambient concentrations of CO_2. The second group of selection lines was more variable in its responses, but all lines within the group had elevated rates of photosynthesis when grown at high CO_2, although they could not channel the fixed carbon into growth. Two of the three evolved lines with elevated photosynthetic rates also had elevated respiration rates when grown at high CO_2 concentrations.

The outcome of our experiment suggests that over the next century many phytoplankton communities will evolve less efficient CCMs through the accumulation of conditionally neutral mutations, and will come to consist of smaller cells with broader ranges in photosynthesis and respiration rates than is currently seen. This would affect global processes by changing the rate of carbon turnover in aquatic and perhaps in terrestrial systems. The experimental system, however, is far from perfect in simulating the

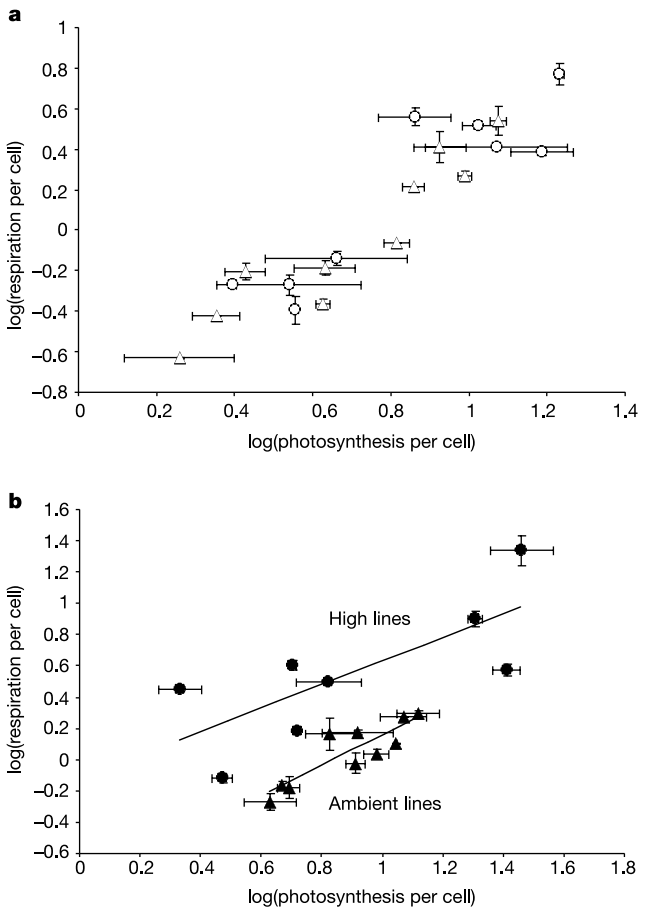

Figure 2 Relationship between photosynthesis and respiration rates at ambient (**a**) and high (**b**) CO_2 concentrations. Photosynthesis and respiration rates are measured as O_2 evolved s^{-1} $cell^{-1}$ and O_2 consumed s^{-1} $cell^{-1}$, respectively. Two high lines, which failed to grow at ambient CO_2, were excluded. Each point represents independent duplicate measurements of a single line; error bars are ± 1 s.e. Circles, high lines; triangles, ambient lines.

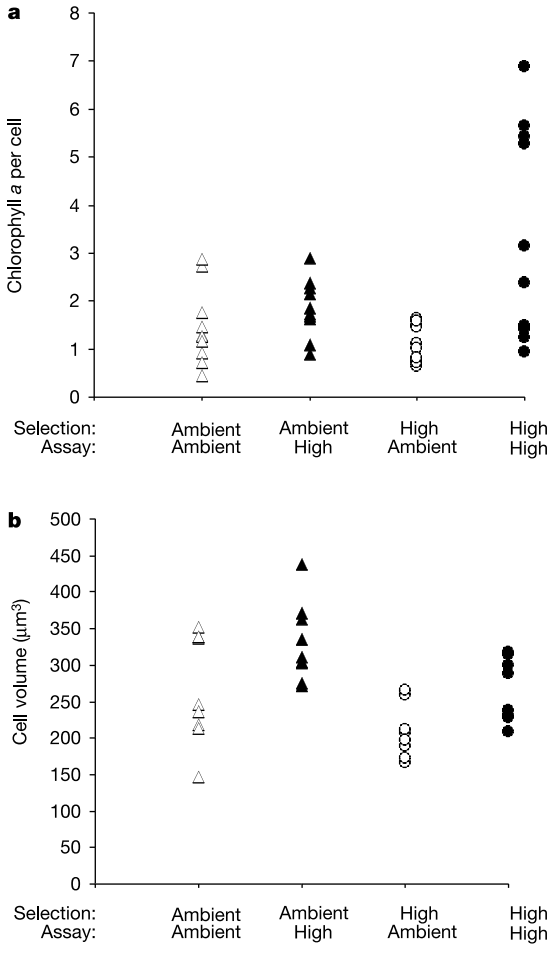

Figure 3 Correlated responses to selection at elevated CO_2. **a**, Chlorophyll *a* content per cell. Points are means of two measurements; average s.e.m. 0.116 (range 0.01–0.92). **b**, Cell volume. Each point represents average cell volume of a single replicate line. Averages were calculated from measurements of 200 cells (high lines) or 170 cells (ambient lines); average s.e.m. 1.53 (range 0.79–2.68). Two lines where single cells could not be accurately measured because of clumping were excluded.

phytoplankton communities of oligotrophic ocean systems, and still less so for terrestrial plants. We have described phenotypes likely to result from changes in CO_2 concentrations alone; how changes in other variables, such as temperature, pH and nutrients modify these phenotypes remain to be seen. Selection experiments in more realistic systems will be necessary to validate the evolutionary response to global atmospheric change. □

Methods

Selection experiment

Ten replicate lines were founded from a single clone of *C. reinhardtii* M566B (laboratory isolate), and ten replicate lines were founded from a single clone of CC2344 (Chlamydomonas Genetics Center, Duke University). Five replicates from each clone were grown in an increasing CO_2 environment and five replicates from each clone were grown in an ambient CO_2 environment. The ambient CO_2 environment consisted of flasks being bubbled with air containing 430 p.p.m. CO_2 for the entire experiment. Lines in the high CO_2 treatment were initially grown in flasks being bubbled with air containing 430 p.p.m. CO_2, and CO_2 levels were raised steadily to 1,050 p.p.m. over the first 600 generations of the experiment. These lines were then grown at 1,050 p.p.m. CO_2 for a further 400 generations. Lines were propagated by batch culture grown in bubbled flasks containing 300 ml of Suoka high salt medium[28] (HSM) in a phytotron chamber under constant light ($800 \pm 20\,\mu mol\,m^{-2}\,s^{-1}$) at 25 °C. One millilitre (about 10^5 cells) was transferred every 3–4 days for approximately 1,000 generations for each replicate line.

Pure culture growth rates and limiting densities

Pure culture growth rates were measured in 384-well plates containing 90 μl HSM per well. Cultures were first acclimated (3–6 days), then diluted and transferred to assay plates. For the two evolved lines that often failed to grow at ambient CO_2 concentrations, several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays. The plates were grown in the same phytotron chamber as above at either 430 p.p.m. or 1,050 p.p.m. CO_2. Absorbance of each culture was measured every 24 h. Limiting densities were calculated from the maximum absorbance maintained by a culture. Values given are means of three independent replicates.

Competitive fitness assay

Competitive fitness was measured by inoculating 300 ml of HSM with equal volumes (approximately equal numbers) of acclimated selection line and a marked strain CC48 arg^- (from Chlamydomonas Genetics Center, Duke University). The flasks were grown in the same phytotron chamber as above, bubbled with either 430 p.p.m. or 1,050 p.p.m. CO_2. The cultures were sampled every 3 days and plated on HSM plus arginine plates. After colony growth, the plates were counted and then replica-plated onto HSM-only plates. The relative frequencies of marker and selection lines were calculated by difference. Dead (arginine-requiring) colonies were usually visible on the HSM-only plates. In cases where the selection lines were absent on plates, they were assumed to be present just below the detection limit of the assay, and were entered into the analysis as having a frequency of 0.005. Values given are means from three independent replicates.

Photosynthesis and respiration assays

Photosynthetic oxygen evolution and respiration (oxygen uptake in the dark) were measured in a Clark-type oxygen electrode illuminated at $800\,\mu mol\,m^{-2}\,s^{-1}$. Cultures were depleted of oxygen by bubbling with N_2/CO_2 at either 430 p.p.m. or 1,050 p.p.m. CO_2. Net photosynthesis (oxygen electrode output from illuminated cells) was used for analysis. Respiration was calculated from oxygen uptake in the dark immediately after a light period. Values given are means of two independent replicate measurements. Chlorophyll was determined by acetone extraction[28]. Values given are means of two replicate measurements from the same culture.

Cell measurements

Cells from acclimated liquid cultures were fixed and 200 (high lines) or 170 (ambient lines) cells measured under a microscope. Cell volume was calculated based on the shape of cells[29].

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Pack-MULE transposable elements mediate gene evolution in plants

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***Mutator*-like transposable elements (MULEs) are found in many eukaryotic genomes and are especially prevalent in higher plants[1–3]. In maize, rice and *Arabidopsis* a few MULEs were shown to carry fragments of cellular genes[4–6]. These chimaeric elements are called Pack-MULEs in this study. The abundance of MULEs in rice and the availability of most of the genome sequence permitted a systematic analysis of the prevalence and**