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SUPPLEMENTARY INFORMATION

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Lohbeck *et al.*: Adaptive evolution of a key phytoplankton species to ocean acidification

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Supplementary methods S1: Full Material and Methods

Cell isolation and culturing

Clonal cultures were obtained by single cell isolation from a natural *E. huxleyi* bloom in Raunefjorden near Bergen, Norway in May 2009. Algal cultures were verified to derive from diploid single cells by microsatellite genotyping (described below). Experimental cultures were grown in 0.2 µm sterile filtered (Whatman Polycap 75AS) artificial seawater (ASW, ref 40) supplemented with nutrients to 64 µmol kg^{-1} nitrate, 4 µmol kg^{-1} phosphate (nutrient ratio after ref 41), trace metals and vitamins according to f/8 adapted from ref 42, 10 nmol kg^{-1} selenium after ref 43 and 2 mL kg^{-1} sterile filtered North Sea water to exclude any limitations by micronutrients. Cultures were grown in 250 mL Schott Duran square flasks filled with a minimum headspace to a volume of 310 mL. Culture flasks were continuously rotated (0.5 rpm) in a Sanyo MLR-351 light cabinet at 15°C and a photon flux density of 150±10 µmol m^{-2} s^{-1} under a 16:8 light:dark cycle. All culture and media preparation work was done under the clean bench using sterile glassware, pipette tips and a peristaltic pump with sterile silicon hoses.

Carbonate system manipulations and measurements

The carbonate system was set up by adding 2380 µmol bicarbonate per kg ASW, yielding total alkalinity (TA) and dissolved inorganic carbon (DIC) concentrations of 2380 µmol kg^{-1}. Prior to inoculation ASW media were aerated for 24 h using a controlled CO_2 gas

mixing system at the desired treatment levels of 400, 1100 and 2200 µatm pCO_2. To minimize evaporation gases were saturated in humidity by prefixed gas wash bottles. After CO_2 manipulations ASW media were carefully pumped with minimal gas exchange into the respective culture flasks using a sterile silicon hose. One extra flask per treatment was prepared for DIC measurement. Flasks were immediately closed and stored in the dark at 15°C until inoculation and DIC measurements, respectively. The carbonate chemistry was determined by DIC and TA measurements. DIC samples were taken for all CO_2 levels from each individual batch cycle and measured colorimetrically[44] using a SOMMA autoanalyzer. Total alkalinity was periodically measured from all CO_2 levels by open-cell acidimetric titration using a Metrohm Basic Titrino 794. CO_2 partial pressure values in the culture media were calculated from DIC and total alkalinity with the software CO2SYS (ref 45) using appropriate solubility constants[46]. Average culture pCO_2 values (±1 s.d.) were calculated from averaging DIC and TA measured prior to inoculation and considering the draw-down from POC and PIC measurements. They were 361.6 µatm (±28), 1067.2 µatm (±102) and 2021.0 µatm (±279), for ambient, medium and high treatments, and thus, not significantly different from the desired treatment levels which are therefore given in the figures.

Experimental procedures

Selection experiment Experimental multi-clone populations were founded by equal contribution in cell number from 6 founder genotypes which were acclimatized to experimental CO_2 conditions for 11 days prior to mixing. The multi-clone experiment was initiated with 1.67×10^4 cells from each genotype, while the single-clone experiment only received 10^5 cells from the randomly chosen founder genotype #62 (all n=5). Both

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experiments ran in parallel under identical conditions. Every 5 days, a serial transfer of exactly 10^5 cells initiated the next batch cycle. Cell counts and diameter measurements were performed in triplicate using a Beckman Coulter Z2 Particle and Size Analyzer. Cell counts were always performed at the same time of the day. The variation among the triplicate cell counts within replicate cultures was very small (mean coefficient of variation 1.1%, range 0.2%-3.3%). Daily exponential growth rates (μ) were calculated from cell densities according to $\mu = (\ln t_1 - \ln t_0)/d$, where t_1 and t_0 are cell concentrations at the beginning and end of incubations, respectively and $d = 5$ is the duration of one batch cycle in days. Preliminary experiments verified cultures to follow constant exponential growth over a 5-d batch cycle and therefore the use of an averaged growth rate as direct fitness measure in this experimental setup.

Assay experiment We tested populations for adaptation to CO_2-selection at elevated CO_2 concentrations after 310 days of exponential growth, translating into 527 (ambient), 496 (medium) and 434 (high CO_2) asexual generations in the multi-clone experiment. After 320 days, we performed an analogous assay in the single-clone experiment, corresponding to 544 (ambient), 512 (medium) and 448 (high CO_2) mitotic divisions. A simultaneous performance of both assay experiments (single and multi-clone) was not possible due to logistical limitations, and therefore was separated in time by about two weeks. Since experimental conditions can never be replicated across time points and media preparations, any quantitative comparisons between treatment means from these different experiments are not permissible.

Populations grown at ambient CO_2 were compared to populations selected at high CO_2, in both, the ambient and elevated assay environment. This resulted in two 2x2 factorial

designs of (i) [ambient vs. medium CO_2 selection condition] x [ambient vs. medium CO_2 assay condition] (ii) [ambient vs. high CO_2 selection condition] x [ambient vs. high CO_2 assay condition]. As response variables, exponential growth rates, cell diameter, particulate inorganic (PIC) and organic carbon (POC) per cell and their production rates were assessed. In order to ensure physiological acclimation, all measurements of the adaptation assay were taken in a 2^{nd} batch cycle.

DNA extraction and microsatellite genotyping

Clone identity in the multi-clone experiment was followed over time by microsatellite genotyping. We used polymerase chain reaction (PCR) with *E. huxleyi* specific primers amplifying the microsatellite loci P01F08, P02E11, P02B12, P01E05 and P02E09 (ref 35). For DNA extraction, 1.5 mL of well-mixed culture suspension was centrifuged for 30 min at 5000 rpm. The supernatant was discarded and the remaining pellet frozen at -20°C. DNA extraction was performed using a DNeasy Blood & Tissue kit according to the manufacturer's protocol (Qiagen). PCR products were analyzed by capillary electrophoresis on an ABI 3130xL genetic analyzer. Allele size scoring against the internal lane standard ROX350 was performed using the software GeneMarker v. 1.85 (SoftGenetics). We used genotype-specific alleles in at least one microsatellite locus for each genotype. Since allele sizes and hence amplification effectivity varied across genotypes, PCR using microsatellite loci is only semi-quantitative. Hence, we implemented a 5% threshold of the respective peak height at the onset of the experiment, to identify the absence or presence of individual genotypes in multi-clone populations after 0, 160 and 320 days.

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Elemental composition and production rates

For quantification of particulate organic carbon (POC) and particulate inorganic carbon (PIC) 95 and 190 mL culture suspension, respectively, were filtered (<100 mbar) onto pre-combusted Whatman glass fiber filters (GF/F). All filtrations were performed at the same time of the day, 3.5 h after the beginning of the light phase and within a narrow time window of less than 2 h to prevent artifacts due to intrinsic dial cycling. Filters were handled with acetone cleaned forceps and quickly stored in combusted glass Petri-dishes at -20°C until further processing. After thawing, POC filters were incubated for 2 h under 37% HCl fume to remove inorganic carbon. All filters were dried for 12 h at 60°C and packed into tin caps for subsequent measurement. Cellular carbon and nitrogen content was assessed using an isotope ratio mass spectrometer (IRMS) in combination with a Hekatech Euro EA elemental analyzer. POC and PIC cell quotas and production rates were standardized to cell numbers. Production rates $cell^{-1}$ were calculated by multiplying with the exponential growth rates of each culture.

Statistical analyses

Statistical analyses used univariate analysis of variance (ANOVA) performed using JMP v. 9.0 (Statsoft Inc.). Planned contrasts for assessing adaptation were performed under the elevated CO_2 conditions and only when in an initial ANOVA the interaction "selection x assay condition" was statistical significant. Unless otherwise indicated, data were untransformed. Variance homogeneity was assessed using Levene's test. The normality of residuals was visually inspected. When variance homogeneity could not be achieved

with data transformation, as was the case for PIC production rates and cell contents in the single-clone experiment, Welch-ANOVAs were performed with subsequent Wilcoxon-tests for comparing treatment means.

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Supplementary Table S1: Analysis of variance on direct and correlated responses of cell traits to selection in *Emiliania huxleyi* in the multi-clone experiment. Given are the results of analysis of variances (ANOVAs) and subsequent planned contrasts in mean cell diameter, particulate inorganic (PIC) and organic carbon (POC) per cell, and the ratio PIC:POC. Contrasts to test for adaptation under elevated CO_2 conditions were only performed when the interaction "selection x assay condition" was statistically significant, otherwise "na". General effects of the ~500-generation selection treatment, as well as significant "assay x selection" interactions are highlighted in **boldface**. For mean trait values see Fig. 3.

		Effect test[§]			
Trait	Selection condition (µatm CO_2)	Assay condition	Selection condition	Selection x assay condition	Adaptation contrast (selected at elevated CO_2 vs. selected at ambient) in elevated CO_2
Cell diameter	1100	F=2.37, P=0.143	F =0.003, P=0.9	F =4.49, P=**0.05**	P=0.14
	2200	F =116.9, P<0.0001	F =86.9, ***P*<0.0001**	F =1.92, P=0.185	na
PIC per cell	1100	F =34.87, P<0.0001	F =0.58, P=0.456	F =4.69, P=**0.046**	P=0.33
	2200	F =48.38, P<0.0001	F =22.43, P=**0.0002**	F =6.02, P=**0.026**	P=0.12
POC per cell	1100	F =6.15, P=0.025	F =0.02, P=0.98	F =12.5, P=**0.003**	P=0.0254
	2200	F =25.33, P=0.0001	F =3.05, P=0.099	F =2.79, P=0.11	na
PIC:POC	1100	F =50.93, P<0.0001	F =0.03, P=0.86	F =3.55, P=0.078	na
	2200	F =262.9, P<0.0001	F =9.72, P=**0.007**	F =0.19, P=0.66	na

[§]All F-ratios have 1 (numerator) and 16 (denominator) degrees of freedom.

Supplementary Table S2: Analysis of variance on direct and correlated responses of cell traits to selection in *Emiliania huxleyi* in the single-clone experiment. Given are the results of analysis of variances (ANOVAs) and subsequent planned contrasts in mean cell diameter, particulate inorganic (PIC) and organic carbon (POC) per cell, and the ratio PIC:POC. Contrasts to test for adaptation under elevated CO_2 conditions were only performed when the interaction "selection x assay condition" was statistically significant, otherwise "na". General effects of 500 vegetative generations of selection as well as significant "assay x selection-interactions" are highlighted in **boldface**. Mean trait values are given in **Fig. 3**.

		Effect test[§]			
Trait	Selection condition	Assay condition	Selection condition	Selection x assay condition	Adaptation contrast (selected at elevated CO_2 vs. selected at ambient) in elevated CO_2
Cell diameter	1100	F =3.29, P=0.088	F =5.36, P=**0.034**	F =0.19, P=0.67	na
	2200	F =284.4, P<0.0001	F =2.04, P=0.170	F =0.15, P=0.70	na
PIC per cell	1100	Welch ANOVA, $F_{3,7.8}$=10.99, P=**0.0035**			P=0.012
	2200	Welch ANOVA; $F_{3,7.7}$=19.16, P=**0.0006**			P=0.036
POC per cell	1100	F =185.9, P<0.0001	F =1.58, P=0.226	F =25.0, P<**0.0001**	P=0.017
	2200	F =410.2, P<0.0001	F =10.09, P=**0.006**	F =4.00, P=0.063	na
PIC:POC	1100	F =129.9, P<0.0001	F =1.52, P=0.23	F =0.29, P=0.59	na
	2200	F =281.9, P<0.0001	F =7.02, P=**0.018**	F =1.14, P=0.31	na

[§] All F-ratios have 1 (numerator) and 16 (denominator) degrees of freedom.

Supplementary Table S3: Rates of adaptation in seven evolution experiments using asexually propagated haploid and diploid yeast (*Saccharomyces cerevisiae*) Mean fitness increases of replicate cultures were taken from relevant figures. If possible, these were interpolated for the duration of the present study, i.e. 500 asexual generations. If experiments ran over shorter time intervals, these values were given. In all studies, selection lines were founded by a single cell/genotype, thus they are comparable to the single-clone experiment presented in Lohbeck et al.. In two studies, direct comparisons among diploid and haploid yeast strains were made.

Asexual population size	Selection regime	Mean fitness increase *s*/no generations[§]	Ploidy level	Study
3.4×10^9	General laboratory selection	0.10/250	haploid	Paquin & Adams 1983
1.7×10^9	General laboratory selection	0.09/250	diploid	Paquin & Adams 1983
1.4×10^4	Dextrose minimal medium	0.056/500	haploid	Zeyl *et al*. 2003
1.4×10^4	Dextrose minimal medium	0.05/500	diploid	Zeyl *et al*. 2003
4.6×10^6	Glucose limitation	0.12/500	diploid	Zeyl *et al*. 2005
$1.4 \times 10^3 - 3.5 \times 10^6$	General laboratory selection	0.042- 0.05/500	diploid	Desai *et al*. 2007
3×10^6	'Benign' vs. ‚harsh' environment: 0.2M NaCl, 37°C	0.55/300	diploid	Goddard *et al*. 2005

[§]The relative fitness increment in an asexual population is equal to the selection coefficient s. Assuming that the fitness of the ancestral or non-adapted genotype is 1, the fitness of an adapted culture is $1 + s$.

References Supplementary Table S3

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Supplementary Note S1: Population genetic expectations of rates of adaptive evolution in asexual populations

The rate of adaptive evolution in asexual populations that are founded by genetically identical ancestors, such as the single-clone experiment in Lohbeck *et al*., depends on the rate and fixation dynamics of favourable mutations. While the underlying rate of novel beneficial mutations conferring adaptation is difficult to estimate, the population genetic process of a given favourable mutation to spread in an asexual population is well understood. Therefore, we will first determine the rate of fixation ('sweep time') of a genotype carrying a novel, favourable mutation (hereafter 'mutant' genotype) that has established and escaped genetic drift. In a second step, we will use the remaining time given our 500 generation experiment to evaluate if the required rate of beneficial mutations that confer adaptation under ocean acidification matches the available number of generations in which spontaneous mutations can occur (the 'waiting time').

Our batch culture approach entails dynamically fluctuating population sizes which need to be translated into a genetically effective population size proportional to the size of an ideal population of that size. Under periodical bottlenecks[1], this effective population size N_e is N_b x G x ln2, where N_b is the population size right after the bottleneck, and G the number of asexual generations between bottlenecks. This leads to $N_e = 1 \times 10^5 \times 8 \times \ln 2 = 5.77 \times 10^5$.

The beneficial mutation confers a selective advantage of s, which is defined as difference between both Malthusian parameters of the ancestral and the mutant genotype[2]. For simplicity, we assume that the mutant genotype carrying a novel, beneficial mutation will have a fitness increase of 1+ s due to complete dominance. Corrections can be made for incomplete dominance as fitness = $1 - hs$, where h is the dominance factor (h= 1 complete dominance, h = 0, completely recessive).

The time it takes an established mutant genotype to half-fixation, i.e. to a frequency that is markedly altering mean population fitness, is equal to $(1/s)$ ln $(N_e s)$(ref 2,3). Probable selection coefficients for early adaptive mutations are in the order of s = 0.05- 0.1 (ref 2,3, and examples in supplementary table S3). Selection coefficients are expected to be quite large and within this order of magnitude as ocean acidification represents a severe stress to coccolithophores. For our effective population size ($N_e = 5.77 \times 10^5$) this yields estimates for s = 0.05 and 0.1 of 205 and 110 generations, respectively, that are needed to increase mean population fitness by 2.5 and 5%, respectively. Since we have observed appreciable fitness increases over 500 asexual generations, the available waiting times for a novel mutant to occur by spontaneous mutation before the sweep are 295 and 390 generations, respectively.

As we now show, such a waiting time correlates with a reasonable parameter space for favourable mutation rates in populations of our size, and is more than sufficient to observe at

least one adaptive and fitness altering mutation in each replicate culture. The total input of mutations entering a population can be estimated from global per base pair mutation rates. Assuming conservatively, that the per site mutation rate per generation is in the order of 1 x 10^{-9} (which ignores other types of mutations such as insertions, deletions, duplications) and the haploid genome size of *E. huxleyi* is 1.68 x 10^{8} base pairs, we expect ~0.2 mutations per haploid genome per generation (*U)*, the overwhelming majority of which will be neutral.

The fraction of non-neutral mutations entering a population is generally unknown, but a reasonable assumption would be that 10^{-2} of all mutations are fitness altering, while maybe another 10^{-2} – 10^{-3} of these would be adaptive under ocean acidification conditions, thus U_b ~ 10^{-5} - 10^{-6}. Then we would observe novel fitness increasing mutations per generation per population in the order of $2N_eU_b$ = 1.15 –11.5 where N_e is 5.77 x 10^{5} (see above). The estimate for U_b agrees well with estimates of mutation accumulation lines in diploid yeast where it was estimated as 3 x 10^{-6} (ref 5). Note that the product N_eU_b attains similar values in several papers given in supplementary table S3 (e.g. ref 3).

Moreover, even under the conservative assumption that U_b ~ 10^{-7} we would still be in a parameter space where mutations conferring fitness benefits under ocean acidification in the order of s= 0.05 had sufficient time to occur and establish (i.e. ~100 asexual generations), and subsequently alter mean population (replicate culture) fitness in a measurable order of magnitude (i.e. increase of exponential growth rate by ~5%).

References supplementary note S1

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