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PUBLISHED ONLINE: 14 SEPTEMBER 2014 | DOI: 10.1038/NCLIMATE2379
nature climate change

Adaptation of a globally important coccolithophore to ocean warming and acidification

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Although ocean warming and acidification are recognized as two major anthropogenic perturbations of today's oceans we know very little about how marine phytoplankton may respond via evolutionary change. We tested for adaptation to ocean warming in combination with ocean acidification in the globally important phytoplankton species *Emiliania huxleyi*. Temperature adaptation occurred independently of ocean acidification levels. Growth rates were up to 16% higher in populations adapted for one year to warming when assayed at their upper thermal tolerance limit. Particulate inorganic (PIC) and organic (POC) carbon production was restored to values under present-day ocean conditions, owing to adaptive evolution, and were 101% and 55% higher under combined warming and acidification, respectively, than in non-adapted controls. Cells also evolved to a smaller size while they recovered their initial PIC:POC ratio even under elevated CO_2. The observed changes in coccolithophore growth, calcite and biomass production, cell size and elemental composition demonstrate the importance of evolutionary processes for phytoplankton performance in a future ocean.

Marine phytoplankton, a diverse group of photoautotrophic microbes thriving in the world's oceans, generate about half of the global primary production[1,2]. They form the basis of marine food webs and play a major role in the Earth's biogeochemical cycles[2]. Consequently, observed and projected changes in marine primary productivity associated with surface ocean warming[3] are of deep concern for the functioning of the Earth system. Warming may directly impact phytoplankton physiology and productivity[4] and increase growth rates in temperate regions[5]. At the community level, higher temperatures will increase heterotrophy by shifting the balance between photosynthetic and respiratory processes[1,6]. Indirect effects, such as enhanced thermal stratification, will constrain nutrient availability and ultimately limit primary productivity in the sunlit ocean surface layer[7,8]. Ocean warming is progressing along with another major perturbation of today's oceans, the dissolution of excess atmospheric CO_2 in surface waters[9]. This phenomenon, termed ocean acidification[10], improves carbon availability to primary producers, while at the same time decreasing growth and biomineralization in calcifying species[11], including our study species *Emiliania huxleyi*[12,13]. Complex interactions between CO_2 concentration and temperature have recently been reported in physiological experiments with different phytoplankton species[14–16]. For example, under high temperatures, the optimal CO_2 level for calcification increases substantially in *E. huxleyi* well beyond ambient CO_2 levels found today[14]. How these interactions play out over longer timescales is largely unknown[17,18].

Recently, the possibility of evolutionary rescue of populations or species from global climate change has become a major area of research[18–22]. The most direct approach to address possible adaptive responses are evolution experiments[23]. Although planktonic microbes lend themselves to such approaches, owing to their short generation time and large population size[18,22,24], there are very few long-term evolution experiments (hundreds of generations) testing marine phytoplankton responses to global change (but see refs 13,25), and none tested for thermal adaptation.

Here, we present data from a long-term (roughly 460 asexual generations) evolution experiment that tested for temperature adaptation in factorial combination with increased CO_2 concentration (ocean acidification) in the globally important coccolithophore *E. huxleyi*. This species is a calcifying haptophyte (Prymnesiophyceae) that forms calcium carbonate platelets (coccoliths) and plays an important role in the global carbon cycle[26,27]. *E. huxleyi* is considered to be the single most important calcifying algae in the world's ocean, with blooms that can be seen from outer space[26].

Experimental asexual populations were founded in May 2009 from a single cell isolated from a natural phytoplankton assemblage in the coastal waters off Bergen. Temperature selection was initiated in February 2013 by duplicating each of five replicate populations that had been maintained in semi-continuous batch cultures at 15.0 °C and three defined p_{CO_2} levels (400 µatm = ambient, 1,100 µatm = medium, 2,200 µatm = high p_{CO_2}) for about three years (Supplementary Fig. 1). Whereas the medium level intended to simulate an end-of-the century worst-case scenario, the high concentration corresponds to the highest future level of ocean acidification[9]. To initiate high-temperature selection, populations were subjected to 1 °C d^{-1} increments to a final temperature of 26.3 °C, a temperature at the upper tolerance limit at which *E. huxleyi* divides at a similar rate as in 15.0 °C. Pilot experiments

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at 26.3, 26.5 and 27.0 °C revealed that growth rates decline rapidly above 26.3 °C (Supplementary Table 1), while the chosen experimental temperature is well within the environmental range for this widely distributed species[28]. Our experiment thus addressed the potential for adaptation to a stressful high temperature at the upper tolerance limit under ambient, medium and high CO_2 conditions. Replicated populations were kept for one year under temperature selection, along with controls that remained at 15.0 °C. To disentangle the effects of sequential and simultaneous adaptation to two stressors, we also founded new selection lines based on populations that had been kept at 15.0 °C and ambient CO_2 and exposed them to simultaneous high-temperature and high-CO_2 selection, creating a fourth treatment (hereafter $2{,}200_{simult}$, Supplementary Fig. 1).

Mean exponential growth rates in treatments subjected to high temperature increased rapidly under all high temperature-CO_2 treatment combinations, with rates between 0.025 and 0.037 batch cycle^{-1} (Fig. 1a, all slopes highly significant in an autoregressive time series analysis, $P < 0.0001$, Supplementary Table 2). In contrast, 15.0 °C treatments maintained their growth rates throughout one year (Fig. 1b, none of the slopes significant). Pair-wise tests revealed that warming resulted in similar rates of fitness increase in all three CO_2 levels (Supplementary Table 3). The populations that evolved for one year in response to high-temperature and high-CO_2 concentration simultaneously ($2{,}200_{simult}$) had a significantly higher rate of fitness increase than the treatment subjected to high temperature only, and to the populations under medium levels of ocean acidification followed by further temperature selection (Supplementary Table 3).

At the end of the one-year temperature selection phase, we conducted a reciprocal assay experiment[29] in which temperature-adapted asexual populations were compared against the respective non-adapted control populations under high temperature, and vice versa (Supplementary Fig. 1). Note that we define 'population' here as entirely asexually reproducing individuals that can generate diversity and adaptive divergence only by mitotically derived mutations, because sexual recombination in *E. huxleyi* cannot be induced in the laboratory. We tested for temperature adaptation only within all three CO_2 selection environments that were maintained during the assay experiment, because the inclusion of ocean acidification as additional factorial treatment levels in the assay phase of the experiment would have resulted in a prohibitively large number of replicates. In the assay experiment, a duplicate of each of the five replicate populations of each selection treatment was slowly (1 °C d^{-1}) transferred to the higher or lower temperature, respectively, in addition to control assays that remained at their long-term temperature (Supplementary Fig. 1). To verify that the observed phenotypic changes were stable over time we conducted four subsequent batch cycles in addition to an initial (first) acclimation cycle. No significant effect of batch cycle on growth rates was found (four-way analysis of variance (ANOVA), interaction #batch cycle*evol_temp*assay_temp*CO_2_level not significant), hence all data presented were taken in assay experiment cycle #5 after 30–35 asexual divisions under the respective assay condition.

High-temperature-adapted populations grew significantly better at high temperature than non-temperature-adapted populations in all CO_2 conditions (Fig. 2a; ANOVA, log-transformed growth rates, interaction evol_temp*assay_temp: $F_{1,48} = 56.99$, $P < 0.0001$, any interaction with CO_2 level not significant). There was also a pronounced correlated response—a growth decline of high-temperature-adapted populations compared to those selected in 15.0 °C when assayed under 15.0 °C. This suggests that the entire thermal reaction norm shifted to higher temperatures, although confirming this would require further study with an increased number of intermediate temperature levels.

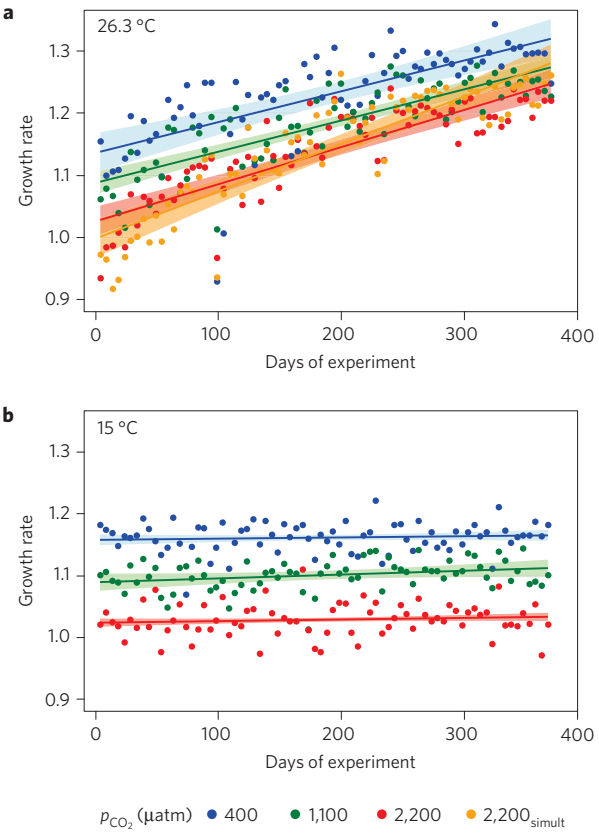

Figure 1 | Time course of exponential growth rates in *Emiliania huxleyi* over one year subjected to different combinations of temperature and CO_2 concentration. Two temperatures, 26.3 °C (**a**) and 15.0 °C (**b**) were used in combination with three levels of CO_2 concentration simulating ocean acidification. Growth rates were calculated every five days. Fitted lines are based on an autoregressive moving average model that incorporates significant autocorrelation terms, shaded areas depict ±95% prediction intervals. All lines at 26.3 °C reveal highly significant slopes (Supplementary Table 2), whereas none of the slopes is significant at 15.0 °C. Pair-wise tests of slopes are given in Supplementary Table 3.

Effects on cell size, cell quotas and production

Next, we analysed changes in cell size as an important 'master trait' affecting nutrient acquisition, sinking velocity and the trophic role of plankton[30,31]. In addition to the established physiological decreases of cell size within species[31] we observed significant reductions in cell diameter associated with temperature adaptation across CO_2 treatments, regardless of whether or not the coccosphere was included in the volume calculation (Fig. 2b and Supplementary Fig. 2a). The size reduction due to adaptation to warming added to the immediate, physiological decline in cell size on high-temperature exposure in all but the highest CO_2 condition (ANOVA; response cell diameter; assay_temp: $F_{1,48} = 320.5$, $P < 0.0001$, evol_temp $F_{1,48} = 27.52$, $P < 0.0001$). Size reduction after selection to warming was stronger for cell sizes excluding the coccosphere, with cells having 15% less volume when assayed under high temperature compared to non-adapted controls (Supplementary Fig. 2a, decalcified cell diameter, evol_temp $F_{1,48} = 39.76$, $P < 0.0001$). Cell size and growth rates were strongly inversely correlated, but this relationship was entirely driven by the experimental treatments (analysis of covariance, covariate cell diameter, categorical factors evol_temp, assay_temp, CO_2_level, and their interactions, not significant).

As shown previously, the immediate physiological response to elevated CO_2 is an increase of particulate organic carbon (POC) con-

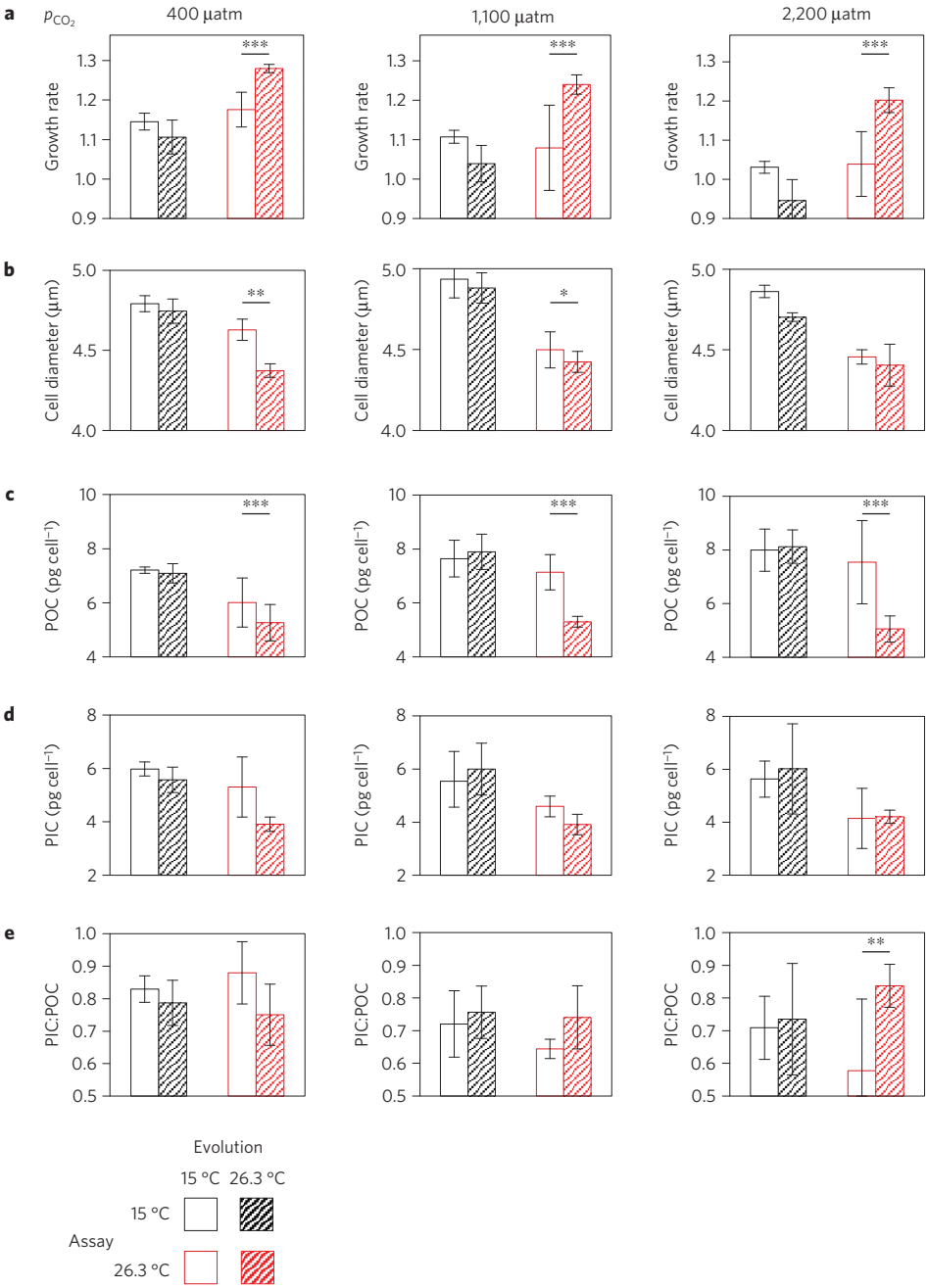

Figure 2 | Evolutionary adaptation in *Emiliania huxleyi* after one year of temperature selection (26.3 versus 15.0 °C) in combination with three CO_2 levels simulating ocean acidification. Mean trait values (±1 s.d., $n=5$) of temperature-adapted versus non-adapted (hatched versus open bars, respectively) populations of *E. huxleyi* are depicted in three different CO_2 environments, when assayed at cold and warm temperatures (black versus red bars, respectively). **a**, Exponential growth rate. **b**, Cell diameter. **c**, Particulate organic carbon per cell (POC cell^{-1}). **d**, Particulate inorganic carbon per cell (PIC cell^{-1}). **e**, Ratio of PIC:POC. Significant results of planned contrasts after ANOVA are depicted only for temperature adaptation by horizontal lines and asterisks (*$0.05 \geq P > 0.01$, **$0.01 \geq P > 0.001$, ***$P < 0.001$). Complete ANOVA results are given in Supplementary Table 5.

tent[32] (Fig. 2c). We can confirm this at medium and high CO_2 levels for populations assayed at 15.0 °C. However, high-temperature-adapted treatments at all CO_2 levels revealed a pronounced decline in POC cell quotas under high temperatures in the assay when compared to the non-temperature-adapted replicates, in particular under medium and high CO_2 conditions (ANOVA; POC cell^{-1}; interaction evol_temp*assay_temp $F_{1,47}=28.11$, $P<0.0001$). This response was even stronger when standardizing POC cell quotas to the decreasing decalcified cell volume in warm-adapted populations (Supplementary Fig. 2b). Here, POC cell^{-1} under temperature adaptation was up to 30% lower under medium and high CO_2 when assayed under high temperature (ANOVA, evol_temp*assay_temp*CO_2_level: $F_{2,47}=8.37$, $P=0.0008$) as compared to non-temperature-adapted populations.

For the particulate inorganic carbon (PIC) content of the cells only immediate temperature- and CO_2 driven declines but no significant evolution-related effects were observed (Fig. 2d; ANOVA, log-transformed PIC cell^{-1}; assay_temp: $F_{1,47}=48.59$, $P<0.0001$). For the specific weight and, hence, the ballasting effect of single coccolithophore cells, the PIC:POC ratio is an important parameter[33]. Under ambient CO_2 the evolutionary response of PIC:POC to temperature was a decrease, whereas under long-term

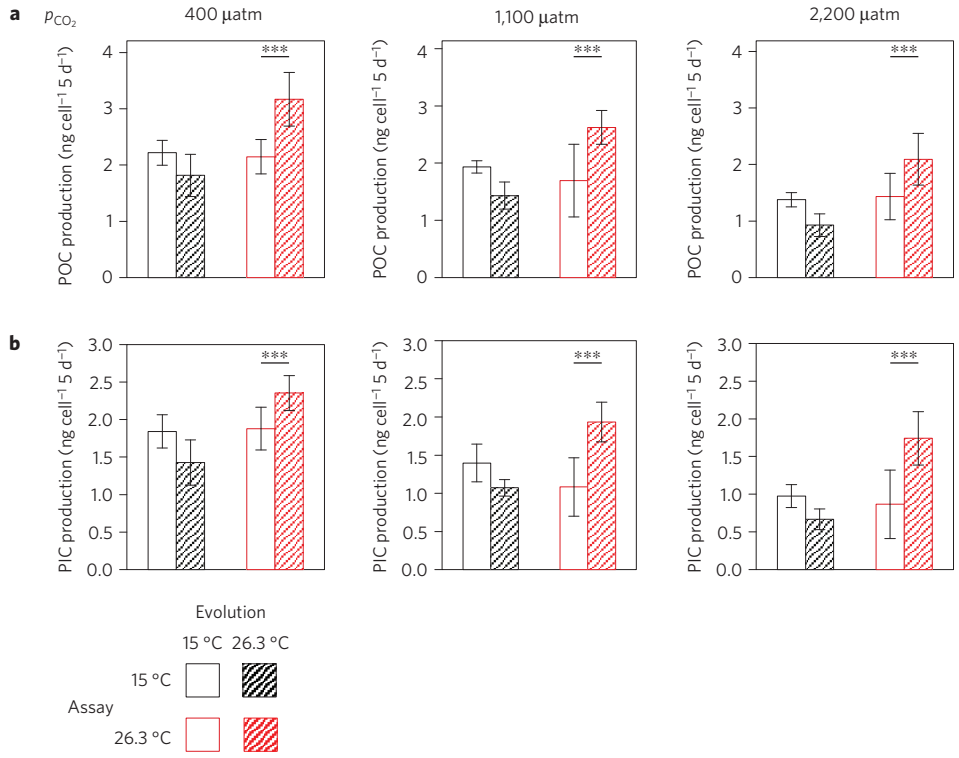

Figure 3 | Restoration of biomass production in *E. huxleyi* after one year of temperature selection. Given are mean values (±1 s.d., $n=5$) of production per cell over five days as a function of assay temperature and CO_2 environment in a fully factorial design. **a**, Particulate organic carbon. **b**, Particulate inorganic carbon. Further details as in Fig. 2. Complete ANOVA results are given in Supplementary Table 5.

medium/high CO_2 the ratio increased relative to non-adapted treatments (Fig. 2e, ANOVA, cubic-root transformed PIC:POC; evol_ temp*CO_2_level: $F_{2,48}=5.74$, $P=0.0058$) when assayed under warm conditions. Thus, counter-correlated trait changes of cell size, POC and PIC cell quotas cancelled out, with the net results that the PIC:POC ratio was up to 30% higher after temperature adaptation under high CO_2 compared to the temperature response without evolutionary adaptation. Importantly, there was a complete restoration of the initial PIC:POC ratio at 15.0 °C (leftmost versus rightmost bar in Fig. 2e, ANOVA planned contrast $P > 0.5$, not significant).

The organic nitrogen content of phytoplankton relative to organic carbon is an important predictor of nutritional quality to higher trophic levels[34]. We find that in temperature-adapted populations, the C:N ratio remained more stable when tested under warming, particularly under the highest CO_2 treatment (Supplementary Fig. 2c, log-transformed C:N ratio, evol_temp*assay_temp*CO_2_level: $F_{2,47}=3.85$, $P=0.028$). This suggests the maintenance of one aspect of nutritional quality under the combination high-temperature/high-CO_2 owing to adaptive evolution.

Although cells evolved to a smaller size and contained less carbon, overall production rates in our five-day experiment were substantially higher in the warm-adapted populations compared to controls. Our experiment simulated a coccolithophore bloom by keeping the cells continually in the exponential growth phase, inoculating new batch cycles every five days with exactly 10^5 cells. POC and PIC production (µgC cell^{-1} per 5-day cycle) were 52 and 101% higher, respectively (Fig. 3a,b, ANOVA; interaction evol_temp*assay_temp for PIC and POC both $P < 0.0001$). Five-day biomass production under the most stressful future ocean scenario (26.3 °C and 2,200 µatm p_{CO_2}) was about as high as under original conditions before initiation of the experiment, mirroring the restoration of PIC:POC ratios.

Taken together, both the restored PIC:POC ratio that determines the specific weight of a single cell of *E. huxleyi*, along with the increased biomass production of PIC may enhance the ballasting of coccolith-containing particles for transport to the deep ocean[27,33] as a consequence of adaptive evolution to warming.

Sequential versus simultaneous temperature selection

Adaptation to warming along with ocean acidification occurred independently of whether or not CO_2 selection had happened before temperature selection (sequentially), or at the same time. Replicate populations that were subjected to high temperature and CO_2 selection simultaneously (the $2{,}200_{simult}$ treatment) were compared to treatments with sequential adaptation (204 batch cycles CO_2-selection ≈1,500 generations, then for an additional temperature selection ≈460 generations, Supplementary Fig. 1). For most response variables, notably growth rate, we found only small and non-significant differences among the sequential and simultaneous treatments (Supplementary Table 4). An exception were PIC cell quotas, which were significantly smaller in the simultaneous versus sequential treatment, which also translated to a lower PIC:POC ratio. It is possible that the duration of high-CO_2 selection for the recovery of this trait was too short to yield the same adaptive outcome.

Adaptation to ocean warming and acidification

A largely open question is how novel selection regimes interact with one another and affect adaptive dynamics[35]. Experimentally, the scope for adaptation to multiple stressors has previously been addressed only in model microbes—for example, bacteria adapting to several antibiotics[36]—and remains an open question for global-change-associated selection. Hence, we compared adaptation to ocean acidification alone from a previous study[13], which was not directly tested in the current experiment, with adaptation to either warming or warming in combination with acidification

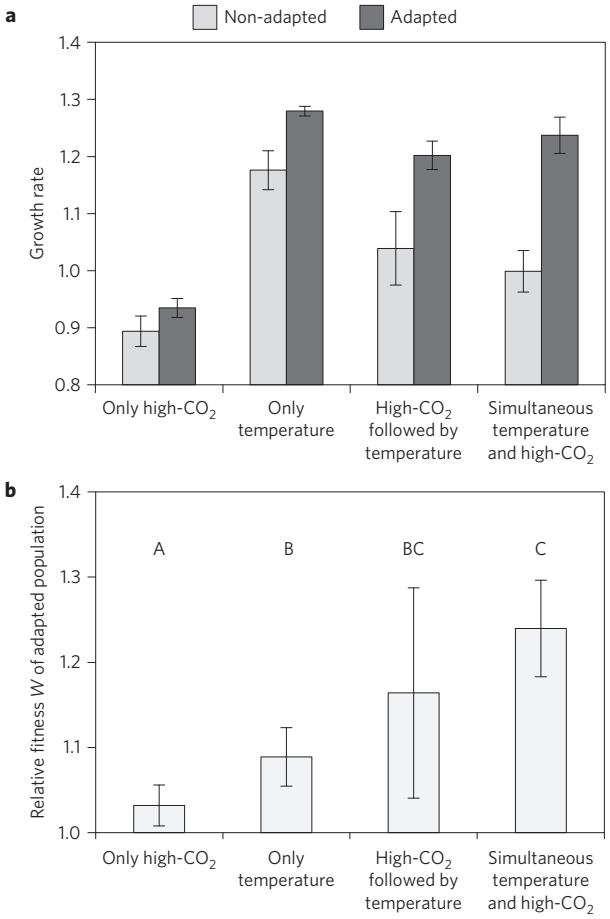

Figure 4 | Evolutionary adaptation in *Emiliania huxleyi* to ocean acidification and to temperature alone, and to a combination of both factors. Effect sizes of adaptation to ocean acidification alone (leftmost bar) are from ref. 13. **a**, Mean growth rates of adapted asexual populations ±1 s.d. (dark bars) relative to their respective control treatments (light bars). **b**, Resulting relative fitness increase (±1 s.d.) of the adapted treatment as the quotient of exponential growth rates of the adapted (dark bars) versus non-adapted (light bars) replicates. Mean values that are significantly different from one another are indicated by different capital letters on top of the relevant column (Tukey HSD post-hoc test ($\alpha = 0.05$) after one-way ANOVA).

(this study, for details see Supplementary Material and Methods). The magnitude of adaptation in growth rate to warming (under ambient CO_2) was roughly three times larger than adaptation to CO_2 (always at 15.0 °C). The adaptation response is composed of the relative fitness difference of the adapted (dark shaded bars) versus non-adapted populations (light shaded bars) when assayed under the relevant future ocean condition (Fig. 4a). The quotient of the exponential growth rates[37] denotes the relative fitness of the adapted populations (a) over the control populations (c) as $W_{ac} = \mu_a/\mu_c$ (Fig. 4b). We find significantly higher relative fitness in *E. huxleyi* in response to the combined stressors—warming and acidification (sequential adaptation)—than to acidification alone, whereas W associated with temperature adaptation alone was intermediate (Fig. 4b). Finally, we tested the adaptation response to the simultaneous challenge of warming and acidification using a further assay treatment in which replicate populations selected under ambient CO_2/low temperature had been immediately exposed to both stressors. Here, W of adapted versus non-adapted populations was largest, and higher compared to both single-factor adaptations (one-way ANOVA on randomly combined fitness quotients among adapted/non-adapted populations, subsequent Tukey HSD post-hoc test, Fig. 4b).

We thus observed no interference in the adaptation of a phytoplankton species to two major global-change-related stressors. This finding is of great ecological relevance, because adaptation to different stressors may also be antagonistic if there are negative trait correlations[38], here with respect to stress tolerance towards heat and CO_2. The faster fitness gains under simultaneous selection may be due to pervasive sign epistasis of (beneficial) mutations in microbial genomes[39]. Mutations that were fixed under only CO_2 selection may not confer any fitness advantages under selection for warming and 'lock' the evolutionary trajectory at a lower fitness. In contrast, under simultaneous selection, only mutations with a beneficial effect in both environments will rise to fixation.

Previously, a 500-generation evolution experiment revealed adaptation to increased CO_2 concentrations via novel mutations and via genotypic sorting. Both growth and calcification rates were partially restored[13]. Here, we observed an even faster adaptation to temperature in *E. huxleyi* that was largely independent of the CO_2 environment. As in many other phytoplankton species, sexual reproduction cannot be induced in *E. huxleyi*, hence our approach must ignore any recombination that will generate additional within-population diversity available to selection. In the light of ample within-population diversity in thermal reaction norms among phytoplankton species[5,28,40,41], our results are conservative, because all genetic diversity on which selection acted must have come from *de novo* mutations. In addition to one year of temperature selection during the thermal adaptation phase, neutral mutations relevant to temperature adaptation could have arisen in all 15 independent replicates (five at each of three p_{CO_2} values of 400/1,100/2,200 μatm) and maintained at a low frequency during the previous cultivation over roughly 1,500 generations (three years) at 15.0 °C (refs 42,43), partly explaining the high rate of adaptive evolution. An indirect challenge experiment revealed that the genetic basis for adaptation to ocean acidification must be different among replicate high-CO_2 selected populations[42]. As with the earlier study on adaptation to ocean acidification[42], the phenotypic convergence observed here for thermal adaptation among the replicates was remarkable, although the mutational basis is likely to be different owing to the independent cultivation of replicates for a total of about 2,000 asexual generations.

Ocean change and phytoplankton adaptation

Contrary to expectations, there were no apparent antagonistic effects when selecting for ocean acidification and high temperature simultaneously in the world's most abundant calcifying organism, *Emiliania huxleyi*. Rather, asexual population growth rates fully recovered even under the most stressful future ocean scenario of 26.3 °C and 2,200 μatm p_{CO_2} compared to treatments under ambient CO_2 concentration and a benign temperature of 15.0 °C. We can only speculate why adaptation to a high temperature at the upper thermal tolerance was up to six times faster than to elevated CO_2 concentration. As most enzymes are temperature dependent[4] there is possibly a larger mutational target for thermal than for CO_2 adaptation, accelerating the speed of adaptation, provided that the frequency of beneficial mutations is rate limiting. In the same vein, mutations for thermal adaptation may have, on average, a larger beneficial effect, in particular when the sample of mutational effects is larger[44].

We show here that thermal niches in phytoplankton may be more evolutionarily flexible than previously thought[1], despite our experiment being ultimately based on asexual offspring of a single wild isolate. Clearly, it would have been desirable to use more than one genotype to address any effects of the specific genetic background of an experimental strain. Many evolution experiments with classical model organisms have identified evolutionary

patterns based on a single isolate that were later found to be representative for entire species[23]. Hence, we consider it unlikely that the adaptive capacity via novel mutations of our particular genotype is not representative of other *E. huxleyi* genotypes. The apparently uninterrupted growth rate increase during the one-year time interval observed here leaves it open as to when adaptation to temperature will reach a fitness plateau, as known from many other evolution experiments[37]. Our results complement several recent large-scale physiological experiments, field surveys and models[1,4,5,45] that have highlighted temperature as one prime factor determining plankton distribution patterns, ocean primary productivity and phytoplankton metabolic function. Controlled laboratory evolution experiments lack the realism of mesocosm experiments employing natural populations or field studies[29], but are currently the only direct test for adaptive change in most marine microbial species where the resurrection of ancient propagules from layered sediments is impossible[46]. That even the asexual offspring of a single isolate will considerably change their performance at high temperature within months to years owing to evolution illustrates that evolutionary processes need to be considered when predicting the effects of a warming and acidifying ocean on phytoplankton[47].

Methods

Experimental asexual populations of *Emiliania huxleyi* were founded in 2009 from a single cell isolated from a natural phytoplankton assemblage in the coastal waters off Bergen (Norway) at an average surface water temperature of 10.0 °C. Since then, five independent replicates of each CO_2 treatment were propagated under constant rotation (0.5 min^{-1}) at three levels of CO_2 partial pressure (p_{CO_2}) at 15.0 °C and at a photon flux density of 150 ± 15 µmol m^{-2} s^{-1} under a 16:8 light:dark cycle for 1,500 asexual generations. The desired p_{CO_2} levels (400, 1,100 and 2,200 µatm) were achieved by aerating the artificial seawater medium for 24 h at the target temperature under saturated humidity. Exactly 10^5 cells were transferred every five days, corresponding to 6–7 asexual generations per batch cycle. The maximal final cell densities did not exceed 2×10^5 ml^{-1}, such that the DIC drawdown was always <8%. Duplicates of the CO_2 selection lines were created on 8 February 2013 and slowly (1 °C d^{-1}) raised from 15.0 °C to 26.3 °C, a temperature at the upper thermal tolerance. A further selection treatment was subjected to high CO_2 and warming simultaneously, originating of duplicates from the original ambient CO_2/15.0 °C selection lines. Temperature selection lasted one year (437–478 asexual generations). In a final assay experiment, replicates were either exposed to their original temperature, or non-temperature adapted populations were exposed to high temperature, and vice versa. Before all measurements one acclimation batch cycle in addition to four further batch cycles were performed to ensure that only persistent phenotypic effects were present in the assay experiment. The cell density and diameter were measured in triplicate at every time transfer point using a Beckman Coulter Z2 particle and size analyser. The cell counts were always performed three hours after the onset of the light phase. Growth rates were calculated as $\mu = (\ln N_d - \ln N_0)/d$, where $N_{(0,d)}$ are cell concentrations and d the duration of the batch cycle in days. Changes in growth rates over time were analysed with an autoregressive moving average model with exogenous variables to test for significant trends. We measured cell density, cell diameter, total particulate carbon (TPC), particulate organic carbon (POC) and particulate organic nitrogen (PON). As well as measurements of calcified cell size, we decalcified cells with 10mM EDTA adjusted to pH 8.2 and repeated the size measurements within five minutes of EDTA addition. Culture suspension for the quantification of TPC and POC were vacuum filtered (<100 mbar) onto pre-combusted glass fibre filters. To prevent artefacts due to intrinsic diel cycling, all filtrations were performed at the same time of the day, four hours after lights on. All sampling was completed within roughly one hour. Response variables in the assay experiment were subjected to factorial ANOVA, followed by planned contrasts if appropriate.

Received 24 June 2014; accepted 20 August 2014; published online 14 September 2014

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Acknowledgements

We thank R. Ebbinghaus, K. Beining, J. Meyer, S. Audritz, D. Gill, A. Ludwig and K. Nachtigall for laboratory assistance. This project was funded by the Kiel Cluster of Excellence 'Future Ocean' and the BMBF project 'Biological Impacts of Ocean Acidification' (BIOACID). Comments by B. Matthiessen improved earlier versions of the manuscript.

Author contributions

L.S., T.B.H.R., U.R. and K.T.L. planned the experiment, L.S. conducted the experiment. L.S. collected data. J.P.G. contributed statistical analyses. L.S., K.T.L., M.A.G., U.R. and T.B.H.R. analysed and interpreted the results. T.B.H.R. wrote the paper.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints.
Raw data have been deposited in the data repository PANGAEA http://doi.pangaea.de/10.1594/PANGAEA.835341.
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Competing financial interests

The authors declare no competing financial interests.