Long-term dynamics of adaptive evolution in a globally important phytoplankton species to ocean acidification

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Marine phytoplankton may adapt to ocean change, such as acidification or warming, because of their large population sizes and short generation times. Long-term adaptation to novel environments is a dynamic process, and phenotypic change can take place thousands of generations after exposure to novel conditions. We conducted a long-term evolution experiment (4 years = 2100 generations), starting with a single clone of the abundant and widespread coccolithophore *Emiliania huxleyi* exposed to three different CO2 levels simulating ocean acidification (OA). Growth rates as a proxy for Darwinian fitness increased only moderately under both levels of OA [+3.4% and +4.8%, respectively, at 1100 and 2200 μatm partial pressure of CO2 (Pco2)] relative to control treatments (ambient CO2, 400 μatm). Long-term adaptation to OA was complex, and initial phenotypic responses of ecologically important traits were later reverted. The biogeochemically important trait of calcification, in particular, that had initially been restored within the first year of evolution was later reduced to levels lower than the performance of nonadapted populations under OA. Calcification was not constitutively lost but returned to control treatment levels when high CO2–adapted isolates were transferred back to present-day control CO2 conditions. Selection under elevated CO2 exacerbated a general decrease of cell sizes under long-term laboratory evolution. Our results show that phytoplankton may evolve complex phenotypic plasticity that can affect biogeochemically important traits, such as calcification. Adaptive evolution may play out over longer time scales (>1 year) in an unforeseen way under future ocean conditions that cannot be predicted from initial adaptation responses.

INTRODUCTION

About half of global primary production is contributed by marine phytoplankton (1), microscopic algae that form the base of marine food webs and play a major role in global biogeochemical cycles. They turn inorganic carbon and minerals into particulate organic matter that may eventually be transported into the deep ocean through sedimentation, a process known as the biological carbon pump (2). Coccolithophores, one of the important phytoplankton groups in contemporary oceans, are important contributors to the ballasting of organic particles (3). This is because their cells are covered by minute calcite platelets, coccoliths, which have a much higher density than seawater. Like many marine calcifying organisms, coccolithophores suffer from ocean acidification (OA) (4), resulting from the dissolution of excess anthropogenic CO2 in ocean waters (5). A wealth of short-term experiments where coccolithophores were exposed to OA conditions revealed an immediate response pattern of decreased calcification and growth rates (6, 7). Recent evolution experiments demonstrated, in line with evolutionary theory and results in model microbes, adaptation to OA and warming in the globally important coccolithophore *Emiliania huxleyi*, both through genotypic selection in multiclonal assays and through de novo mutations (8, 9). The above studies ran for approximately 1 year (>500 asexual generations). From evolutionary model species, such as *Escherichia coli* and yeast, it is well known that adaptive evolution is a dynamic long-term process even in the simplest experiments, designed with a single clone (or genotype) as the starting “population.” For example, adaptation to novel conditions through selective sweeps may be delayed by clonal interference (10, 11), adaptive improvements may be sudden and stepwise when mutations are rare (12), and consecutive beneficial mutations may depend on one another, introducing lineage-specific historical contingency (13). Hence, further phenotypic changes can occur over several thousand generations. For example, in *E. coli*, an adaptation to citrate utilization arose 31,500 generations after initial exposure to glucose minimal medium that was citrate-enriched (13). A recent 15-year time series reports evidence for adaptive changes of natural phytoplankton communities to yearly variation in environmental conditions (temperature and irradiance) (14).

Here, we evolved replicate populations derived from a single clone of *E. huxleyi* for 2100 asexual generations (4 years) to ambient [400 μatm partial pressure of CO2 (Pco2)] and two elevated Pco2, simulating different levels of OA (5). We chose a medium level projected for the end of this century for a worst-case CO2 emission scenario (1100 μatm) (15) and a high proof-of-principle treatment (2200 μatm Pco2). Already now, these levels are temporarily found in coastal waters under upwelling of oxygen-deficient waters (16). Upon initial findings of a partial restoration of calcification in *E. huxleyi* (8), we were particularly interested in how calcification played out in subsequent years. Another important question was whether adaptive evolution would lead to complete restoration of algal performance and fitness under OA compared to nonadapted controls when both were assayed under OA conditions.
RESULTS

Over 2100 asexual generations, mean exponential growth rates in all treatments increased with time [daily growth rates (µ) per generation: ambient CO2: 6.56 × 10⁻³; medium CO2: 4.33 × 10⁻³; high CO2: 7.60 × 10⁻³; autoregressive-moving average models with exogenous variables (ARMAX) model with significant autocorrelation terms, all trends P < 0.001], with no detectable difference between OA treatments (Fig. 1A). In contrast, cell diameter decreased over time in all treatments, again with no difference among treatment levels (ARMAX model, all trends P < 0.001; Fig. 1B). Thus, cells had between 12 and 22% less volume at the end of year 4 compared to the starting genotype. These background differences remained the same, especially in the high CO2 selection treatment, namely, there was a 12 to 22% general decrease in cell volume across treatments, which is given by the difference between control populations (always measured under ambient CO2 environment through reciprocal assay experiments relative to control populations that, at the same time, had evolved under ambient CO2 conditions (17). In all assay experiments, populations were acclimated for at least one full batch cycle (seven to eight asexual generations) to their novel condition. Assay experiments were conducted at five time points (approximately 500, 1000, 1200, 1600, and 2100 asexual generations) and also included a test of the correlated response, the back-exposure of populations evolved under elevated CO2 to ambient conditions (fig. S1).

We focus here on the time course of the adaptive response (Fig. 2), which is given by the difference between control populations (always depicted as a black thin line) relative to populations that were allowed to long-term adapt to OA, both tested under OA conditions. In all assay experiments, CO2-selected population grew faster than nonadapted ones when assayed under both medium and high CO2 [medium CO2 selection: F₁,₈ = 43.92, P = 0.0002, repeated-measures analysis of variance (rmANOVA); high CO2 selection: F₁,₈ = 53.72, P < 0.0001, rmANOVA; Fig. 2, A and B]. Growth rate adaptation increased over time under medium CO2 selection (time × selection: F₄,₃₂ = 2.941, P = 0.035, rmANOVA). Under high CO2 selection, the adaptation effect was continually present and did not increase further, nor was there a significant fluctuation of the adaptive response [time × selection: F₄,₃₂ = 1.991, P = not significant (ns), rmANOVA]. Except for the first assay experiment after approximately 500 generations, medium CO2- and high CO2–selected populations grew slower compared to controls under ambient CO2 (Fig. S1A), thus revealing a cost to adaptation.

Next, we focused on ecologically important cell traits that were not directly subjected to selection, such as cell sizes and elemental quotas, but may be correlated with asexual fitness. For cell size, the phenotype changed in the course of the 4-year experiment. Although medium CO2– and high CO2–selected cells were initially larger, they became smaller after 1400 generations relative to control populations (time × CO2: medium CO2 selection: F₄,₃₂ = 11.44, P < 0.0001, rmANOVA; time × high CO2 selection: F₄,₃₂ = 10.89, P < 0.0001, rmANOVA; Fig. 2, C and D), although the main effect CO2 selection was not significant (medium: F₁,₈ = 0.762, P = ns, rmANOVA; high: F₁,₈ = 0.593, P = ns, rmANOVA). Note that cell size changes associated with adaptation to elevated CO2 are superimposed by a general trend toward smaller cells in all treatments (Fig. 1B).

The particulate organic carbon (POC) content of the cells decreased over time in both selection treatments as a consequence of elevated CO2. Compared to short-term exposed controls, we observed a decrease of POC content under both medium and high CO2 selection (medium CO2 selection: F₁,₇ = 18.13, P = 0.0038, rmANOVA; high CO2 selection: F₁,₆ = 17.45, P = 0.0058, rmANOVA; Fig. 2, E and F), with the interaction with time being nonsignificant in both cases. Except for a non-significant increase at 1000 generations, the general decrease in POC is also apparent for all three selection treatments under ambient CO2 (correlated response; fig. S1C). Hence, the general laboratory conditions may have selected for cells with less organic carbon content. Because there was a 12 to 22% general decrease in cell volume across treatments, we also standardized cell quota by cell volume. The general picture remained the same, especially in the high CO2 selection treatment, namely, we found an additional decrease in the treatments long-term adapted to elevated CO2 (medium CO2 selection: F₁,₇ = 6.47, P = 0.0384, rmANOVA; high CO2 selection: F₁,₆ = 12.42, P = 0.0124, rmANOVA; fig. S2, A and B).
We were particularly interested in how cell quotas in particulate inorganic carbon (PIC) would change throughout the selection experiment. We expected that, given sufficient time, the observed partial restoration of PIC cell quotas under OA compared to controls after 500 generations ($t^{8}$) would be completely restored in both medium and high CO2 selection treatments. Contrary to expectations, PIC cell quotas markedly decreased after generation 1000 to be lower in populations adapted to high CO2 than in nonadapted controls when subjected to OA conditions ($time \times CO_2$: medium CO2 selection: $F_{4,28} = 4.85, P = 0.0042$, rmANOVA; high CO2 selection: $F_{4,24} = 6.79, P = 0.0008$, rmANOVA; Fig. 2, G and H). In response to medium and high CO2 selection, CO2-adapted populations displayed 21 and 22% lower PIC, respectively, compared to the physiological decline of PIC in the control populations under medium and high CO2 (all at 2100 generations; Fig. 2, G and H). This pattern also remained after we standardized PIC content on cell volume to compensate for the general decrease in cell size over time ($time \times CO_2$: medium CO2 selection: $F_{4,28} = 3.95, P = 0.0115$, rmANOVA; high CO2 selection: $F_{4,24} = 5.58, P = 0.0025$, rmANOVA; fig. S2, C and D). In terms of PIC/POC ratio, we found an interaction of time and CO2 selection in the high, but not in the medium, CO2 selection ($time \times CO_2$: medium: $F_{4,28} = 0.3378, P = ns$, rmANOVA; high: $F_{4,24} = 6.36, P = 0.0012$, rmANOVA; Fig. 2, I and J).

For the final assay experiment at generation 2100, we plotted population-wise reaction norms for important parameters under ambient and elevated CO2 levels to address the evolution of plasticity in traits other than fitness itself that accompanied adaptation (details of statistical analysis are in table S2; Fig. 3, A and B). After 4 years, corresponding to ~2100 asexual generations, we observed an increase of...
are indicated by asterisks (*0.05 ≥ P > 0.01; **0.01 ≥ P > 0.001; ***P < 0.001).

The reaction norms of the POC quota of cells after CO2 selection revealed a strong “overshooting” response of POC cell quota of non-adapted populations under medium (+20% POC; Fig. 3C) and high CO2 assay conditions (+19%; Fig. 3D) compared to high CO2–adapted ones. However, slopes of the reaction norms in response to assay CO2 were only significantly different among ambient CO2–selected versus medium CO2–selected populations (interaction selection × assay CO2: P = 0.011, two-way ANOVA; Fig. 3C and D). Also, medium CO2–adapted populations restored their POC quotas under high CO2 to levels observed in controls under ambient CO2 (planned contrast, P = ns; Fig. 3C), whereas there was still 11% more POC per cell for the high CO2–selected replicates tested under high CO2 compared to the ambient control (planned contrast, P = 0.0368; Fig. 3D).

For calcification (assessed as PIC cell quota), populations selected for 4 years to OA showed markedly different reaction norms and, hence, evolved a different pattern of phenotypic plasticity (interaction selection × assay CO2: P = 0.022 and 0.0033 for medium and high selection, respectively). Despite the loss of calcification under OA assay conditions, CO2–selected populations increased their PIC quota when transferred back into ambient CO2 (+25% for medium CO2 selection, Fig. 3E; +58% for high selection, Fig. 3F; planned contrast, both P < 0.001), which was then indistinguishable from control populations under ambient CO2. As a result of both changes in POC and PIC cell quota reaction norms, the PIC/POC ratio changed, which, in turn, determines the specific mass and, hence, the contribution to enhanced sinking velocity of organic matter by an individual coccolithophorid cell (Fig. 3, G and H). Here, for the high CO2–selected populations only, the PIC/POC ratio was significantly reduced after long-term adaptation under high CO2 while overcompensated upon reexposure to ambient CO2 (Fig. 3H).

DISCUSSION

Our experiment is the first to describe long-term adaptation for a few thousand generations in any marine microbial species, including phytoplankton (19). Before the experiment, we expected the previously identified adaptation response in growth rates after 1 year (8) between treatments subjected to long-term OA versus controls to increase. However, the adaptation response did only increase marginally with time for the low, but not the high, CO2 levels, suggesting either some fundamental constraints to adaptation to OA or that waiting times for required rare mutations were exceedingly long. This was different for important correlated traits, such as elemental cell quotas, in particular, the inorganic and organic carbon content of individual cells, where responses partly reverted between years 1 and 4. Hence, even 1 year of evolutionary change reported earlier (8) may only cover a transient response in these traits when compared to longer time intervals. These complex long-term dynamics have also been found in selection experiments with model microbes (11, 13, 20). Whereas these species lack an immediate ecological or biogeochemical significance, our results have implications for marine primary production and carbon sequestration, given the important role of phytoplankton in general and that of *E. huxleyi* as the world’s most abundant calcifying phytoplankton species in particular (21). Specifically, we show that long-term evolution can exacerbate the immediate physiological decline in calcification, which is assessed here as PIC cell quotas. This reverted the initial notions after 1 year of adaptation to OA, which revealed partial rescue...
of calcification (8). The reverse response under OA adaptation observed here would decrease the ballasting effect of an individual coccolithophorid cell adapted to OA. If extrapolated to the ocean, this, in turn, may have negative consequences for the ocean’s biological carbon pump, along with the general abundance of coccolithophores in a phytoplankton community (3).

**Mutational dynamics in an asexual diploid**

How these changes in the adaptive dynamics came about is currently elusive. It may be that genotypes that calcified less under high CO2 levels, and thus were already “plastic” with respect to producing calcite plates, arose within the first year of experimental duration. However, they only rose to (near) fixation during the subsequent years because of competition with other genotypes carrying other favorable mutations owing to clonal interference (22). Recent experiments in a yeast model species under asexual reproduction and diploidy have revealed rampant genetic hitchhiking and clonal competition during the course of simple evolution experiments (11), leading to unpredictable time lags and nonsynchronous adaptation processes. Alternatively, mutations affecting the regulation of calcification may have a smaller mutational target and are thus much less common, meaning that the waiting time to such mutations is longer than 1 year.

To assess the contributions of initially rare genotypes and to follow their dynamics, one would have to take many subreplicates during the run time of the experiment, which was prohibited by the large logistical effort to grow *E. huxleyi* in appreciable population sizes in the laboratory. Growth rate is the parameter we selected for and directly reflects the Darwinian fitness in our asexually reproducing batch populations (18).

**Dynamics of correlated cell traits**

How other traits respond to CO2 selection depends upon the genetic architecture (for example, genetic correlations) and on the contribution of any particular trait to fitness (that is, its fitness function). Parameters such as cell size are probably correlated with cell division and thus exponential growth rates of the batch cultures to a certain extent (23). In our experiment, the decrease in cell volume of up to 22% (Fig. 1B) observed in all treatments was mainly driven by our selection regime of sequential batch cultures, favoring maximal exponential growth rates and thus resulting in smaller cells. When subtracting the decline in cell volume attributable to selection for fast growth, assay experiments revealed that there is still an additional decrease of POC, which is caused by selection to increased CO2 (cf. an absolute difference of 1.6 and 1.8 pg POC per cell among medium and high selected treatments and controls in Fig. 2, E and F, during both final time points). These findings contrast the adaptive responses we observed after 500 generations (8) of nearly similar POC cell quotas. At this point, we can only speculate that prolonged selection under nutrient-replete culture conditions may have favored an altered cellular carbon storage strategy, resulting in smaller cells containing less organic carbon compared to the ancestral population. This is particularly interesting in the light of short-term physiological responses to OA, which are characterized by an increase in organic storage compounds, including lipids and glucons (24). More detailed physiological assessments along the time course of experimental evolution are clearly warranted to dissect the cellular mechanisms, costs, and constraints determining the observed phenotypic responses. However, note that the specific time course of adaptation, correlated responses, and trait evolution may be contingent upon the particular genotype (clone #62) that was chosen to initiate the experiment (25). Even if the genetic starting material is completely identical, historical chance events may produce idiosyncratic outcomes of evolution when “rewinding the evolutionary tape” (13). We can only speculate whether or not other starting genotypes would have evolved a similar plastic response. What is clear, is that the sensitivity to OA in terms of calcification declines already varies among existing genotypes (26).

**Possible mechanisms driving PIC changes**

How coccolith formation and, hence, the PIC cell quota are linked to fitness still remains elusive (27). The biogenic precipitation of calcium carbonate is an energy-demanding process, with energetic costs expected to increase under elevated CO2 (28). When initiating the long-term selection experiment with *E. huxleyi*, we expected a reduction in calcification as one adaptation mechanism to OA. This was based on the assumption that a selective pressure to maintain coccolith formation is absent in our enemy-free batch culture system. The initial partial restoration of PIC quotas and calcification rates during the first year of experimental evolution (8) refuted those ideas. However, the subsequent evolutionary dynamics reported here is now consistent with these initial expectations after about generation 1000. The ability to calcify was not constitutively lost in high CO2-adapted populations, but we observed the evolution of a flexible response, that is, phenotypic plasticity. Although we found a complete restoration of PIC quotas under ambient CO2 elevated CO2 selection resulted in a further decrease of PIC, and this decrease was significantly lower than the immediate physiological response of control populations to OA.

**The evolution of phenotypic plasticity**

Phenotypic plasticity describes how the same genotype gives rise to different phenotypes in response to different environments (29). One important aspect of studying phenotypic plasticity is to investigate how different genotypes of a particular phytoplankton species respond to higher CO2 levels. To address how phytoplankton growth and photosynthesis rates are affected by higher dissolved inorganic carbon (DIC) availability associated with increasing CO2 is important to predict primary productivity in future ocean conditions, particularly in noncalcifying species (30). Coccolithophores only benefit from increased DIC availability at relatively low levels (*E. huxleyi* up to 600 µatm PCO2) (31). If the net CO2 effect on growth rates is negative under higher OA levels (beyond 1000 µatm PCO2), as in *E. huxleyi*, plastic buffering may extend the range of tolerances and keep organismal function even under OA (32). Here, we found that an energetically costly trait, namely, biogenic calcification (21, 28), is reduced because of long-term selection under high CO2 conditions, whereas it is almost unchanged when high CO2-adapted populations are back-exposed to the ancestral condition (ambient CO2 concentration). Thus, the high CO2-adapted replicates have evolved a pattern of phenotypic plasticity in the high CO2 environment (33), which is consistent with an adaptive reduction of calcification when it is costly. They now reveal two distinct phenotypes—one with reduced and one with “normal” PIC cell quotas—as a function of the assay CO2 environment (=correlated response). In a selection experiment with increased CO2 in the freshwater alga *Chlamydomonas*, conditionally deleterious mutations were believed to have accumulated in the high CO2 selection lines, leading to lower growth when reexposed to ambient CO2 because of deterioration of carbon-concentrating mechanisms (CCMs) (34). Hypothetically, such a loss of CCM may
also be the mechanism leading to costs of CO₂ adaptation in the medium CO₂– and high CO₂–selected replicates past generation 1000. In contrast, during the first 500 generations, we have to invoke the accumulation of gain-of-function mutations that were only visible when comparing control to adapted populations in the novel environment. They probably involved regulatory regions because the phenotypic effects were reversible and only visible in the high CO₂ assay environment.

The process of calcification requires tight metabolic regulation, such as pH regulation and directed Ca²⁺ and HCO₃⁻ transport to the coccolith vesicle before the precipitation of calcite itself (28, 35). Under OA conditions, the regulation of the cytosolic pH is compromised and decreases (24, 36). Although not only critical for calcification, pH homeostasis is important for many metabolic processes. Initially, adaptation may have improved cellular pH regulation under high CO₂ conditions, and calcification may have partly been restored by passively following this adaptive regulatory response (37). The later decrease in calcification suggests that, beyond approximately 1000 generations, independent of putative further improvements in pH regulation (determining growth rates), calcification was also directly affected. From a cellular metabolic perspective, it seems likely that a complex process like calcification is deeply tangled with various other metabolic pathways, limiting the regulatory capacity of calcification for the sake of pH homeostasis. We can only speculate that, at some point beyond 1000 generations under high CO₂, calcification became less tightly coupled with other essential processes and, as a consequence, gained new regulatory flexibility. This could explain the novel patterns in phenotypic plasticity we have observed after 2100 generations.

Ecological implications
Care needs to be taken when translating our results from laboratory batch cultures to the natural system. For example, we always kept our cultures in exponential phase to maintain the desired treatment levels of OA, whereas in nature, population densities in bloom situations lead to much higher competition strength. In nature, trade-offs with other important functions of calcification and coccolith production [for example, grazer or viral defense (27, 38)] will most likely prohibit the evolution of phenotypic plasticity with respect to coccolith formation observed here. Also, by starting with a single genotype, we only allowed for novel mutations as driver of evolutionary adaptation, whereas genotypic selection will most likely be equally or even more important in nature (8, 39).

Experimental evolution fills the gap between paleontological studies of evolutionary changes (40) and field time series (14) by directly addressing the evolutionary dynamics on a time scale of months to years. Although evolution in response to OA can often only be observed throughout a single generation in other species (41, 42), phytoplankton species lend themselves to direct experimentation (39, 43). However, experimental control is likely to be traded off against realism. Our data demonstrate that caution is advised even in the simplest experimental setups, as responses after 1 year of adaptation may be transient. Clearly, more complex designs are highly warranted, for example, those that compare varying versus constant selection regimes (44). Also, experiments including competitors, pathogens, and predators are clearly desirable to simulate adaptive evolution under more realistic conditions as one component of phytoplankton community change in a future ocean (39, 45).

MATERIALS AND METHODS
The asexual populations in this experiment originated from a single cell, which is isolated in May 2009 from the coastal waters off Bergen, Norway [clone #62; (8)]. Populations were kept in artificial seawater [ASW; (46); medium [for details, see the study by Lohbeck et al. (8)]. To achieve a total alkalinity (TA) of 2380 μmol kg⁻¹, 0.19 g of bicarbonate per kilogram of ASW was added. CO₂ levels were manipulated by aerating the ASW medium for 24 hours at 15°C under saturated humidity with CO₂-enriched air before culture flasks were inoculated. The cultures were kept under nonaxenic condition because using antibiotics would have caused problems in maintaining the cultures over several years in exponential phase.

The selection experiment was carried out in a semicontinuous batch culture system with five replicates each for ambient (400 μatm P₇C₅O₂, control), medium (1100 μatm P₇C₅O₂), and high (2200 μatm P₇C₅O₂) CO₂ levels. Three replicates were grown at 400 μatm control PAO were acclimated over one full batch cycle to the respective elevated CO₂ levels and vice versa. Growth rates and additional correlated traits were then measured after ~500, 1000, 1200, 1600, and 2100 asexual generations, treatments grown at 400 μatm control P₇C₅O₂ were acclimated over one full batch cycle to the respective elevated CO₂ levels and vice versa. Growth rates and additional correlated traits were then measured along with the long-term evolved treatments under medium and high CO₂ assay conditions (adaptive response). Replicates evolving at 1100 and 2200 μatm P₇C₅O₂, respectively, were also back-transferred to ambient CO₂ to assess the correlated response along with control replicates. The experimental design was incomplete because, for logistical reasons, the reciprocal exposure of 1100 and 2200 μatm P₇C₅O₂ was omitted. Dissolved inorganic carbon was measured colorimetrically using a SOMMA autoanalyzer or an AIRICA system (MARIANDA). P₇C₅O₂ values before inoculation were calculated from DIC and TA using the program CO2SYS (47). The drawdown of TA and DIC during the batch cycles was calculated from the total particular carbon measure-
day (~4 to 5 hours after start of light phase). POC filters were fumed with 37% HCl for 2 hours to remove inorganic carbon and then dried at 60°C for 12 hours. The measurements were performed with elemental analyzers. PIC was calculated by subtracting HCl-fumed POC from TPC values.

We estimated changes in terms of cell sizes and growth rates using autogeissergative moving average model (ARMAX) with transfer functions and autocorrelation terms if appropriate, according to

\[
\text{Response} = b_0 + b_1 \cdot d_1 + t + b_2 \cdot d_2 \cdot t + b_3 \cdot d_1 + \text{ARMA terms}
\]

where \(d_i\) in [0,1] is a dummy variable \(i\)th \(d_1 \cdot t_1 = 1\) if observation \(i\) at time \(t\) belongs to treatment 1 and \(d_1 \cdot t_1 = 0\); else, \(d_2 = 1 - (d_1 \cdot t_1)\) indicates whether \(d_1 \cdot t_1 = 1\) or not \(d_2 \cdot t_1 = 0\) the observation belongs to treatment 2. Hypotheses of significant slopes and treatment differences were assessed using conventional t and F tests. Note that, according to Eq. 1, dynamic effects are assumed to be the same in both of the samples. This assumption is justified theoretically because of the unified experimental design and is empirically corroborated by preliminary empirical tests.

For the statistical analysis of the reciprocal assay experiments, the data set was subdivided, for each level of elevated CO₂, into two parts to avoid singularities. In order to avoid data interdependencies of the same replicates measured at two different assay conditions and to accurately represent the distinct hypotheses, we analyzed the adaptive and correlated responses separately. For each subset, an rmANOVA including a sphericity test for the within-subject terms was conducted using a globalema assay of Emiliania huxleyi to ocean acidification and global warming, thesis, University of Kiel, Kiel, Germany (2016). 8. S. Lühr, Long-term adaptation of the coccolithophore Emiliania huxleyi to ocean acidification and global warming, thesis, University of Kiel, Kiel, Germany (2016).
Acknowledgments: We thank R. Ebbinghaus, K. Reining, J. Meyer, G. Faucher, S. Audritz, D. Gill, A. Ludwig, and K. Nachtigall for laboratory assistance and M. Gutowska for advice. Funding: This project was funded through the Cluster of Excellence “The Future Ocean” and the Bundesministerium für Bildung und Forschung (BMBF) project “Biological Impacts of Ocean Acidification” (BIOACID). Author contributions: L.S. and T.B.H.R. conceived the study; K.T.L. and L.S. performed the experiments; K.T.L., T.B.H.R., and L.S. analyzed the data; J.P.G. conducted the time series analyses; L.S. and T.B.H.R. wrote the manuscript; and all authors discussed and edited the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. Raw data were deposited in the World Data Center for Marine Environmental Sciences (WDC-MARE) (accession http://doi.pangaea.de/10.1594/PANGAEA.846062).

Submitted 17 November 2015
Accepted 15 June 2016
Published 8 July 2016
10.1126/sciadv.1501660

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Sci Adv 2 (7), e1501660.
DOI: 10.1126/sciadv.1501660