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Dissertation

zur Erlangung des Doktorgrades
Dr. rer. nat.
der Mathematisch-Naturwissenschaftlichen
Fakultät der Christian-Albrechts-Universität
zu Kiel

vorgelegt von
Kai Tim Lohbeck

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Rapid evolution of a key phytoplankton species to ocean acidification

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Contents

1 Zusammenfassung

Die extensive Nutzung fossiler Brennstoffe erhöht stetig den Kohlenstoffdioxidgehalt der Atmosphäre. Dieses überschüssige Kohlenstoffdioxid (CO_2) wird zum Teil von den Ozeanen aufgenommen und verursacht dort ein Absinken des pH-Wertes des Meerwassers. Dieses als Ozeanversauerung bezeichnete Phänomen stellt eine ernstzunehmende Bedrohung für viele Meeresorganismen dar und könnte weitreichende Konsequenzen für marine Nahrungsnetze und biogeochemische Stoffflüsse haben.

Das Phytoplankton steht an der Basis der marinen Nahrungsnetze und gilt als treibende Kraft der marinen Stoffkreisläufe. Daher sind mögliche Auswirkungen der Ozeanversauerung auf diese Organismengruppe von besonderer Bedeutung. Eine wichtige und zugleich besonders empfindliche Phytoplanktongruppe bilden die zu den Haptophyten zählenden Coccolithophoriden. Diese mit winzigen Kalkplättchen bedeckten Einzeller zeigen unter Ozeanversauerung zumeist verminderte Wachstums- und Kalkbildungsraten.

Unser gegenwärtiges Wissen über die Auswirkungen der Ozeanversauerung auf Meeresorganismen im Allgemeinen basiert fast ausschließlich auf Kurzzeitstudien, wohingegen eine mögliche evolutionäre Anpassung in der Regel vernachlässigt wird. Einzellige Planktonorganismen haben jedoch meist beachtliche Populationsgrößen und kurze Generationszeiten, was eine gute Grundvoraussetzung für schnelle evolutionäre Anpassung darstellt. An diesem Punkt setzt meine Doktorarbeit an, in der ich untersucht habe, ob die weltweit wichtigste einzellige Kalkalge, *Emiliania huxleyi*, durch evolutionäre Anpassung auf Ozeanversauerung reagieren kann.

Hierzu habe ich in einem Langzeitselektionsexperiment replizierte Algenpopulationen erhöhten CO_2 Bedingungen ausgesetzt und auf evolutionäre Anpassung untersucht. Auf diese Weise konnte ich zeigen, dass *E. huxleyi* in der Lage ist, sich innerhalb eines Jahres, einer für den Klimawandel relevanten Zeitskala, an die Ozeanversauerung anzupassen. Angepasste Algenpopulationen zeigten in alle Replikaten eine ähnliche partielle Wiederherstellung der Wachstums- und Kalzifizierungsraten, ein Hinweis auf phänotypische Konvergenz, wobei die genetische Basis jedoch unbekannt blieb.

Die genetische Basis der Anpassung an den hohen CO_2-Gehalt habe ich anschließend genauer untersucht, indem ich die angepassten Populationen einer neuen Umgebung ausgesetzt habe. Unter diesen Bedingungen zeigten die einzelnen Replikate der angepassten Populationen deutliche Unterschiede in ihren Wachstumsraten. Dies deutet darauf hin, dass die Anpassung an den hohen CO_2-Gehalt die Folge verschiedener Mutationen in den einzelnen Replikaten ist. Diese Mutationen werden in einer neuen Umgebung durch ihre unterschiedlichen pleiotropischen Effekte sichtbar und können somit indirekt nachgewiesen werden. Pleiotropische Effekte können auch bei der Anpassung von natürlichen Po-

pulationen eine wichtige Rolle spielen, nämlich dann, wenn verschiedene Selektionsdrücke gleichzeitig auftreten und dadurch das Potential zur Anpassung eingeschränkt wird.

Abschließend habe ich zelluläre Prozesse mit möglicher Beteiligung an der Hemmung und adaptiven Wiederherstellung von Wachstum und Kalzifizierung unter Ozeanversauerung untersucht. Hierzu habe ich eine Genexpressionsanalyse von 10 Kandidatengenen mit möglicher Funktion bei der Regulation des pH-Wertes, bei Kohlenstoffaufnahme und -transport sowie Kalzifizierung und Photosynthese durchgeführt. Die Expressionsanalyse dieser Gene hat neue Hinweise geliefert, dass unter Ozeanversauerung die Beinträchtigung der zellulären pH-Regulation eine wichtige Rolle bei der Schädigung, aber auch bei der evolutionären Anpassung von Wachstum und Kalzifizierung spielt.

Meine Ergebnisse lassen darauf schließen, dass zukünftige Phytoplanktonpopulationen durch evolutionäre Anpassung außerhalb des Reaktionsbereichs heutiger Algen reagieren werden. Somit könnte evolutionäre Anpassung dazu beitragen, die Funktionalität mikrobieller Prozesse an der Basis mariner Nahrungsnetze sowie der Stoffkreisläufe in einem sich rasant verändernden Ozean aufrecht zu erhalten. Ich betrachte es daher als äußerst wichtig, dass evolutionäre Prozesse in zukünftigen Studien über die Auswirkungen des Klimawandels auf einzellige Organismen berücksichtigt werden. Welche Bedeutung evolutionären Prozessen unter natürlichen Bedingungen tatsächlich zukommt, ist ebenfalls eine wichtige Frage, mit der sich zukünftige Forschung auf diesem Gebiet befassen sollte.

2 Summary

Ocean acidification, the ongoing drop in seawater pH due to uptake of excess fossil fuel derived carbon dioxide (CO_2) by the surface ocean, may seriously impair many marine organisms with likely consequences for food webs and biogeochemical cycles.

How marine phytoplankton will respond to ocean acidification is a highly relevant topic in marine sciences because phytoplankton forms the basis of marine food webs and constitutes a major driver of biogeochemical cycles. One important phytoplankton group, which is particularly sensitive to ocean acidification, are coccolithophores, members of the haptophytes with calcified platelets covering their cells. Most coccolithophores show decreased growth and calcification rates when exposed to acidified seawater conditions.

Our present understanding of the impacts of ocean acidification on marine organisms in general relies almost exclusively on short-term studies, while the potential for evolutionary adaptation is mostly neglected. Planktonic algae have usually large population sizes and short generation times which makes them particularly prone for rapid adaptation to environmental change. This was the starting point for my doctoral project where I investigated the potential for evolutionary adaptation to ocean acidification in the world's single most important calcifying phytoplankton species, the coccolithophore *Emiliania huxleyi*.

I performed a long-term selection experiment in which I exposed replicate populations to elevated CO_2 conditions and tested for adaptation. As a proof of principle I was able to show that adaptive evolution in a key phytoplankton species is possible and potentially fast enough to act on climate change relevant time scales. Partly restored growth and calcification rates were consistently observed in all independently evolved replicate populations. These findings suggest genetic adaptation that resulted in convergent phenotypes of replicate populations. However, the underlying genetic basis remained unknown.

In order to investigate the genetic basis of adaptation to high CO_2 I challenged CO_2 adapted populations in a novel environment. If high CO_2 adaptation involved different mutations in replicate populations, then these mutations may become apparent via different pleiotropic effects in a novel environment. I found divergent pleiotropic effects, suggesting different genetic bases of high CO_2 adaptation and a potential role for pleiotropy to constrain adaptation of natural *E. huxleyi* populations to ocean acidification.

Finally, I investigated cellular processes involved in inhibition and adaptive restoration of growth and calcification under ocean acidification that may help to unravel the genetic basis of adaptation to elevated CO_2 in *E. huxleyi*. I analyzed expression changes in 10 candidate genes relevant to pH regulation, carbon transport, calcification and pho-

tosynthesis in CO_2 adapted and control populations. This analysis revealed supporting evidence that cytosolic pH regulation is involved in inhibition but also in adaptive restoration of growth and calcification under ocean acidification.

Taken together, this work demonstrates that future phytoplankton populations have the potential to respond outside the range of contemporary genotypes by adaptation. Adaptive evolution may thus contribute to maintain the functionality of microbial processes at the base of marine food webs in the face of ocean change. I consider it very important to include adaptive processes into future studies that address the effects of global change on microbes. Future work should also investigate the extent of such adaptation and its significance compared to ecological effects, such as compositional change of communities, under natural conditions.

3 Introduction

3.1 Ocean Change in the Anthropocene

The oceans have been subject to constant change throughout millions of years. However, since the onset of industrialization the rate of change has increased considerably (Hönisch et al. 2012). Major contributing processes are extensive burning of fossil fuels, large scale deforestation and cement production that lead to increased atmospheric carbon dioxide (CO_2) concentrations which affect the earth's climate, oceans and ecosystems on a global scale (Crutzen 2002; Tyrrell 2011). These human induced changes will be visible in the future stratigraphic record and therefore this period is termed Anthropocene (Crutzen 2002). In the light of the ecological, biogeochemical and socio-economic consequences associated with these rapid perturbations, it is an important as well as challenging task to understand and reliably predict future earth- and ocean responses to global change.

3.2 Increasing Atmospheric Carbon Dioxide and Seawater Carbonate Chemistry

Anthropogenic carbon dioxide emissions progressively increase CO_2 concentrations in the atmosphere where it acts as a major greenhouse gas and promotes an increase in global mean temperature (IPCC 2007). Pre-industrial atmospheric CO_2 levels of about 280 parts per million (ppm) have already increased to contemporary levels of about 390 ppm (Tans 2009). Earth system models project a further increase of atmospheric CO_2 levels to 700-1000 ppm until the end of the 21st century (IPCC 2007). As the atmosphere is in constant equilibrium with the surface ocean, a considerable amount of this excess CO_2 ends up in the ocean where it causes a shift in the carbonate chemistry equilibrium (Wolf-Gladrow et al. 1999; Sabine et al. 2004).

When CO_2 dissolves in seawater it reacts with water (H_2O) and forms carbonic acid (H_2CO_3) that immediately dissociates into bicarbonate (HCO_3^-) and further into carbonate (CO_3^{2-}) by releasing two protons (Equation 1). Note that carbonic acid represents a negligible proportion that is chemically indistinguishable from CO_2 and therefore the concentrations of CO_2 and H_2CO_3 are usually combined and expressed as the hypothetical carbon species CO_2* (Dickson 2007).

$$CO_2^* + H_2O \overset{K_1}{\rightleftharpoons} HCO_3^- + H^+ \overset{K_2}{\rightleftharpoons} CO_3^{2-} + 2H^+ \quad (1)$$

This chemical equilibrium constitutes the carbonate system of seawater. The equilibrium concentrations of CO_2, HCO_3^-, and CO_3^{2-} can be calculated using the temperature, salinity and pressure dependent stoichiometric equilibrium constants K_1 and K_2 (e.g. Roy et al. 1993; Equation 2 and 3).

$$K_1 = \frac{[HCO_3^-][H^+]}{[CO_2]} \tag{2}$$

$$K_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} \tag{3}$$

The carbonate system of seawater is by definition extended with the conceptual parameters dissolved inorganic carbon (DIC, Equation 4) and total alkalinity (TA, Equation 5; Zeebe and Wolf-Gladrow 2001). DIC represents the sum of all dissolved inorganic carbon species while TA is defined as the amount of excess hydrogen ion equivalents to excess proton acceptors (Dickson 2007).

$$DIC = [CO_2^*] + [HCO_3^-] + [CO_3^{2-}] \tag{4}$$

$$\begin{aligned} TA = {} & [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] + [HPO_4^{2-}] + 2[PO_4^{3-}] \\ & + [SiO(OH)_3^-] + [NH_3] + [HS^-] - [H^+] - [HSO_4^-] - [HF] - [H_3PO_4] \end{aligned} \tag{5}$$

It results that the carbonate system of seawater is characterized by 6 parameters: $[CO_2]$, $[HCO_3^-]$, $[CO_3^{2-}]$, pH, DIC and TA. Measuring 2 out of these 6 parameters enables calculation of the other 4 parameters and therefore a complete determination of the seawater carbonate system (at a given temperature, salinity and pressure).

This illustrates that an increase in atmospheric CO_2 that equilibrates with the surface ocean will result in profound changes to all parameters of the seawater carbonate system. Furthermore, also biological processes affect the carbonate system of seawater.

Photosynthetic carbon fixation lowers DIC by fixation of CO_2 into organic carbon compounds while biogenic calcite production, e.g. in coccolithophores, reduces DIC and TA by precipitating Ca^{2+} and HCO_3^- to $CaCO_3$ and H^+.

Models project that increased atmospheric CO_2 levels of 700-1000 ppm will reduce the average global ocean surface pH by 0.14-0.35 units (Orr et al. 2005). Such projections are based on coupled climate-carbon cycle models (IPCC 2007). Nevertheless, a sample calculation nicely illustrates the underlying changes in seawater carbonate chemistry.

For instance, if we assume a contemporary atmospheric CO_2 concentration of 392 ppm and a surface ocean that has a total alkalinity of 2350 μmol kg^{-1} seawater and is in equilibrium with the atmosphere, the total amount of dissolved inorganic carbon is 2108 μmol kg^{-1} seawater and is partitioned into 1 % CO_2, 91 % HCO_3^- and 8 % CO_3^{2-} at a pH of 8.14. A projected increase of the atmospheric CO_2 concentration to 1000 ppm results in increased dissolved inorganic carbon of 2262 μmol kg^{-1} seawater, partitioned into 2 % CO_2, 94 % HCO_3^- and 4 % CO_3^{2-} and a lowered pH of 7.78 (calculated using CO_2SYS, Lewis and Wallace 1998; K_1 and K_2 from Roy et al. 1993; temperature = 15°C; salinity = 35; pH is reported on the free scale).

The ocean is therefore an appreciable sink for excess atmospheric CO_2. Indeed, currently the ocean takes up about 30 % of the annual fossil fuel derived carbon dioxide emissions and ultimately may sequester up to 90 % of man-made CO_2 (Sabine et al. 2004). At a first glance the ocean appears to act as a giant buffer by sequestering large amounts of CO_2. However, a closer look reveals that the ocean may respond in a more complex way than anticipated especially from a long-term perspective (Riebesell et al. 2009; Tyrrell 2011). Another issue is, as exemplified above, that the uptake of excess atmospheric CO_2 by the surface ocean leads to increased CO_2 and HCO_3^- availability, accompanied by a decrease in CO_3^{2-} concentration and a drop in pH. This process is dubbed ocean acidification (Caldeira and Wickett 2003) and has severe consequences for marine ecosystems and biogeochemical cycles (Feely et al. 2004; Fabry et al. 2008; Doney et al. 2009; Riebesell et al. 2009; Riebesell and Tortell 2011).

3.3 Biological Impacts of Ocean Acidification

Ongoing ocean acidification affects various marine organisms, especially those producing calcium carbonate shells or skeletons such as calcifying plankton, benthic molluscs, echinoderms, coralline algae and corals (Fabry et al. 2008; Doney et al. 2009; Riebesell and Tortell 2011).

Of particular interest are the effects of ocean acidification on marine phytoplankton. Although restricted to the sunlit surface layer of the ocean, these tiny algae are responsible for about half of all global primary production (Field et al. 1998). They are phylogenetically diverse unicellular photoautotrophs that passively drift with the ocean currents (Falkowski et al. 2004). The most abundant groups are cyanobacteria, coccolithophores, diatoms and dinoflagellates, which play a key role at the basis of marine food webs and in biogeochemical cycles (Falkowski 2012).

Coccolithophores are characterized by the production of biogenic calcite platelets termed coccoliths (Paasche 1968). This group appears to be particularly sensitive to ocean acidification (Riebesell et al. 2000). While most phytoplankton groups seemingly benefit from increased carbon availability that alleviates carbon limitation of photosynthesis, coccolithophores show decreased growth and calcification rates (Riebesell and Tortell 2011). They contribute a large proportion to marine primary production and carbon export to the deep ocean, especially by the putative ballasting effect of their calcite scales on sinking organic particles (Armstrong et al. 2002). A potential decline of coccolithophore abundance in an acidified future ocean may therefore have consequences for marine food web dynamics and the carbon cycle (Rost and Riebesell 2004; Riebesell et al. 2007; Riebesell and Tortell 2011).

3.4 The Coccolithophore *Emiliania huxleyi*

The coccolithophore *Emiliania huxleyi* (Lohmann 1902; Hay and Mohler 1967; Chapter 5, Fig. 1) is considered to be the single most abundant coccolithophore species in the ocean and has become a well-studied model organism in biological oceanography (Westbroek et al. 1989; Paasche 2002). *E. huxleyi* probably diverged from *Gephyrocapsa* about 270,000 years ago (Thierstein et al. 1977) and since, has persisted throughout two glacial-interglacial cycles and became dominant about 70,000 years ago (Bijma et al. 2001).

In contemporary oceans *E. huxleyi* has a nearly global distribution, except of polar waters (Westbroek et al. 1989). Under favorable environmental conditions it can form vast blooms that can be seen from space when innumerous calcite platelets reflect the sun light and turn the ocean surface milky (Westbroek et al. 1989; Chapter 5, Fig. 2). Currently, 6 morphotypes that differ in size and coccolith morphology have been identified (Young et al. 2003), while little is known about population structure, genetic diversity and reproduction.

The life cycle of *E. huxleyi* is complex, it includes the commonly studied non-motile, calcite scale bearing diploid phase and a motile but non-calcified organic scale bearing

haploid phase. Rare observations of non-calcified diploid cells can most likely be attributed to mutant cells that lost the ability to calcify (Klaveness 1972; Paasche 2002). Both life cycle stages can reproduce asexually, which is the frequently observed mode of reproduction under laboratory conditions while under natural conditions sexual reproduction is probably also common (Paasche 2002; Iglesias-Rodriguez et al. 2006).

Under bloom conditions rapid asexual proliferation was proposed to be the dominant mode of reproduction, however large standing genetic variation even at the end of bloom events suggests an important role of sexual reproduction (Medlin et al. 2000; Iglesias-Rodriguez et al. 2006). Alternatively, natural genetic diversity may be reduced at the end of bloom events, while examinations failed to detect this reduction due to insufficient sampling of the existing diversity. Moreover, as the authors quantified genetic diversity by neutral genetic markers, it also remains to be resolved if such neutral genetic diversity allows any inferences on functional genetic diversity at the population level.

Strain specific differences in phenotypic traits among isolates from geographically distinct populations suggest underlying functional genetic differences between populations and indicate limits to gene flow, local adaptation and probably even cryptic speciation in *E. huxleyi* (Brand 1982; Iglesias-Rodriguez et al. 2006; Buitenhuis et al. 2008; Langer et al. 2009; Hagino et al. 2011).

Calcification in *E. huxleyi* is a highly regulated intercellular process that takes place in Golgi-derived compartments, the coccolith vesicles (Mackinder et al. 2010). Intracellular precipitation of calcium carbonate requires uptake and transportation of large amounts of calcium and bicarbonate into the coccolith vesicle and produces one mole protons per mole calcium carbonate (Brownlee and Taylor 2004; Mackinder et al. 2010). Excess protons from calcification end up in the cytosol where they have to be removed via voltage gated proton channels to maintain cytosolic pH homeostasis (Suffrian et al. 2011; Taylor et al. 2011).

Ocean acidification inhibits growth and calcification in most investigated *E. huxleyi* isolates (Riebesell and Tortell 2011). Bach et al. (2011) identified the decline in seawater pH and not the concomitant changes in carbonate concentration as causative process. Lowered seawater pH interferes with the plasma membrane potential and results in a malfunction of voltage gated proton channels. As excess protons from calcification are not readily removed, cytosolic pH regulation fails and in turn inhibits growth and calcification (Suffrian et al. 2011; Taylor et al. 2011). However, the underlying pH sensitive components, their interplay and mode of inhibition by lowered cytosolic pH remain to be identified.

Numerous short-term studies have demonstrated these negative effects of ocean acidification on *E. huxleyi* (Riebesell et al. 2000; Riebesell and Tortell 2011). While physiolog-

ical responses to ocean acidification under controlled laboratory conditions are reasonably well understood (Zondervan 2007; Rost et al. 2008), we hardly know anything about the potential for adaptive evolution and consequent effects on competitive fitness and biogeochemically relevant phenotypic traits under natural conditions.

3.5 Adaptive Evolution to Ocean Change

One question of immediate concern is whether or not marine organisms can adapt fast enough to prevent large scale regime shift or even extinction, and which traits would confer increases in fitness under global change (Reusch and Wood 2007; Bell and Collins 2008; Hoffmann and Sgro 2011).

Adaptation is a population-level process that increases mean population fitness, i.e. the intrinsic rate of growth, as a consequence of natural selection. Natural selection implies that some individuals have phenotypes that contribute relatively more offspring to the next generation, they are positively selected. This concept was introduced by Charles Darwin in his pioneering work "On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life" (Darwin 1859). However, at this time there was no valid concept of heredity. Only the consolidation of Darwin's theories with later discoveries in classical and molecular genetics resulted in the "Modern Evolutionary Synthesis", which attributes advantageous phenotypes to carry gene variants that confer fitness benefits. The concept of heritability ties genotypic changes to the observed phenotypic fitness increase. Hence adaptation is accompanied with genotype frequency changes from one generation to another. In summary, adaptive evolution is the change of heritable phenotypic traits from generation to generation towards increased mean population fitness (Futuyama 2007).

Ultimately, all genetic variation is the result of mutations, random changes on the DNA that can affect single nucleotides, lead to insertions or deletions, and to the complete loss, duplication or re-arrangement of genes. Existing variation may be recombined by means of sexual reproduction, the second principal mechanism of generating variation (Futuyama 2007). In experimental evolution the set-up can either allow for multiple genotypes or a single genotype to start with. In the latter case, only spontaneous and random emergence of mutations in the genetic material can be the source of variation.

The rate at which new mutations enter a population is influenced by many factors, such as the effective population size (i.e. the number of individuals that contribute offspring to the next generation), the rate of reproduction, the mutational target (i.e. genome size), specific properties of the replication machinery, and also external muta-

genic forces. Most mutations will be selectively neutral and are only affected by random genetic drift. This means that they cannot spread quickly. Those mutations that are deleterious will not survive for long and disappear. The proportion of mutations that are beneficial to fitness is difficult to estimate (Keightley and Lynch 2003). Mutation accumulation experiments in diploid yeast revealed that about 6 % of new mutations are beneficial to fitness in this model system (Joseph and Hall 2004). A new beneficial mutation first needs to escape accidental loss via random genetic drift in order to spread in a population. Once established, the rate at which a beneficial mutation increases in frequency in a population depends on the selective advantage this mutation confers and the effective size of the population (Desai et al. 2007).

The second important mechanism to generate and also to maintain variation is sexual recombination of parental alleles, variants of a single gene, to form new recombinant genotypes. Therefore, in sexual reproducing populations the frequency of sexual reproduction events and sexual selection are important factors that affect genotype diversity and frequency (Futuyama 2007).

These two mechanisms are the main forces to generate and maintain genetic diversity in sexually reproducing populations, while in asexually reproducing populations the generation and maintenance of variation relies on mutations and lateral gene transfer. For instance, in bacterial populations there exist several potential pathways for lateral exchange of genetic material (Ochman et al. 2000). Genome sequencing studies revealed a modular organization of bacterial genomes, involving a core genome, the part of the genetic material that is common to all genotypes of a group, which is extended by variable clusters of genes that differ remarkably between genotypes, together making up the pan genome. This suggests a high relevance for lateral gene transfer to generate new genetic combinations with important implications for evolutionary processes and phylogeny in prokaryotes (Syvanen 2012).

This illustrates that an estimate of the rate of new genetic variants to occur and spread in a population depends on many parameters and assumptions. However, fast reproduction and large population size promote the chance for new, better adapted genotypes to arise and increase in frequency on climate change relevant time scales, enabling mean population fitness increase and adaptation to ocean change (Elena and Lenski 2003; Bell and Collins 2008).

Because marine phytoplankton populations usually have large population sizes and fast reproduction rates that apparently results in high standing genetic variation to be common in natural populations (Medlin et al. 2000; Rynearson and Armbrust 2005; Iglesias-Rodriguez et al. 2006), this group has large potential for rapid adaptation to ongoing ocean acidification (Bell and Collins 2008).

Surprisingly, the impact of projected future ocean change on marine organisms, including phytoplankton, is almost exclusively evaluated based on physiological short-term experiments, while the potential for evolutionary adaptation is widely neglected (Collins 2011). This gap has probably two main reasons: i) outside the field of evolutionary biology adaptive processes are often considered to be restricted to geological time scales and hence beyond the temporal scale of contemporary climate change and ii) long-term selection experiments entail daunting logistical challenges and unpredictable risks to break down that make them unattractive compared to short-term experiments.

In experimental evolution, innumerable microbial selection experiments are evident that adaptive processes can be studied in real time (Lenski and Travisano 1994; Elena and Lenski 2003; MacLean and Bell 2003; Buckling et al. 2009). Model organisms are usually fast reproducing microbes such as *Escherichia coli*, *Pseudomonas*, and *Saccharomyces*, that are easy to cultivate and provide a broad range of genomic and recombinant technologies (Elena and Lenski 2003; Buckling et al. 2009). However, most experiments did not aim at a realistic ecological context, nor have most of the chosen organisms a prominent ecological, let alone biogeochemical role.

The marine phytoplankton species *Emiliania huxleyi* combines large population size and fast reproduction with an important ecological and biogeochemical role of global significance, making it well suited to venture a long-term selection experiment to test for adaptation to ongoing ocean change on a reasonable time scale for a doctoral project.

3.6 Thesis Outline

The results from my doctoral thesis are structured in three publications, which are presented in chapters 4, 5 and 6. The key findings and future research perspectives that resulted from this work are discussed in chapter 7.

In the first publication (Chapter 4), I explore the potential of a key marine phytoplankton species to adapt to ocean acidification. Using a long-term microbial selection experiment I exposed replicate single- and multi-clone populations of the coccolithophore *E. huxleyi* for 500 generations to elevated CO_2 conditions and tested for adaptation. Growth and calcification rates that are inhibited by high CO_2 were partly restored in high CO_2 adapted populations. Novel mutations and genotypic selection on pre-adapted genotypes were identified as key mechanisms of adaptation.

In the second publication (Chapter 5), I investigate the genetic basis of adaptation to high CO_2 by challenging CO_2 adapted single-clone derived populations in novel environments. I observed phenotypic divergence that can be used as a proxy for different

novel mutations which become apparent via divergent pleiotropic effects in a novel environment. This finding suggests that there are multiple evolutionary trajectories to high CO_2 adaptation in *E. huxleyi* and that pleiotropy may constrain adaptation of natural populations to ocean acidification.

In the third publication (Chapter 6), I used an alternative way to address the genetic basis of phenotypic change and investigated gene expression in 10 selected candidate genes in CO_2 adapted single-clone populations from the long-term selection experiment. I found supporting evidence that cytosolic pH regulation is a cellular target to inhibition but also a site of adaptive restoration of growth and calcification in *E. huxleyi* cells facing an acidified ocean.

4 Publication I

4.1 Adaptive evolution of a key phytoplankton species to ocean acidification

Authors: Kai T. Lohbeck, Ulf Riebesell, and Thorsten B. H. Reusch

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Adaptive evolution of a key phytoplankton species to ocean acidification

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Ocean acidification, the on-going enrichment of marine waters with fossil fuel carbon dioxide and associated drop in seawater pH, may seriously impair marine calcifying organisms, with likely consequences for biodiversity and ecosystem functioning. Our present understanding of the sensitivities of marine life to ocean acidification is based primarily on short-term CO_2 exposure experiments. Phytoplankton species with short generation times, in particular, may be able to respond to environmental alterations via adaptive evolution. Here, we present data on two 500-generation selection experiments starting with populations founded by either a single or multiple clones of the world's single most important calcifying organism, the coccolithophore Emiliania huxleyi. Compared to populations kept at ambient pCO_2 (400 µatm), those selected at elevated pCO_2 (1100 and 2200 µatm) revealed higher growth rates in both, the single- and multi-clone experiment when tested under ocean acidification. Calcification was partly restored, with rates that were up to 50% higher in adapted compared to non-adapted cultures. Our results suggest a role for contemporary evolution to maintain the functionality of microbial processes at the base of marine food webs and as driver of biogeochemical cycles in the face of global change.

At present, the ocean takes up about one-third of fossil fuel CO_2 emissions and eventually will sequester up to 90% of anthropogenic CO_2 (refs 1,2). When CO_2 dissolves in seawater, it forms carbonic acid, which increases seawater acidity and decreases carbonate ion concentration and carbonate saturation[2]. These changes in seawater chemistry, dubbed "ocean acidification"[3], increasingly impact marine organisms[4,5] and ecosystems[6,7,8]. Most prominently affected are species that build their cell walls, shells, scales or skeletons from calcium carbonate[1,4,6]. A case in point are coccolithophores (Prymnesiophyceae), a group of unicellular microalgae thriving in the sunlit surface layer of the ocean, that are among the most productive calcifying organisms in the sea[9]. Under favourable light and nutrient conditions, coccolithophores may form extensive blooms. Those are even visible from space, since light reflection from their delicate calcite platelets (coccoliths) turns the surface ocean milky. Vast areas of the ocean floor covered with coccolith-derived sediments are testimony to their long-term role in the oceanic carbon cycle[9]. According to one prominent hypothesis, coccoliths ballast organic aggregates and fecal pellets, thereby accelerating their sinking to deeper waters and thus critically contribute to carbon export from surface waters to the ocean interior[10]. Like many other marine calcifiers, coccolithophores are sensitive to ocean acidification, with most studies showing a decline in growth and calcification rate and an increase in coccolith malformation at elevated CO_2 levels[11,12,13]. Most studies on the effects of ocean acidification on marine organisms, including coccolithophores, have been short-term (<1 yr), and none tested for evolutionary adaptation[14], a major unknown when attempting to predict future impacts of ocean acidification on marine life[15,16]. Because populations of coccolithophores reproduce quickly and have large population sizes, they should be particularly prone to respond to ocean changes via adaptive evolution[16,17,18,19]. Such rapid evolutionary adaptation has previously been shown in microbial selection experiments on genetic model species exposed to novel environmental conditions[20,21,22].

E. huxleyi single- and multi-clone selection experiment

To test whether marine phytoplankton can adapt to ocean acidification, we conducted laboratory selection experiments with the coccolithophore *Emiliania huxleyi*, a bloom-forming microalgae found from mid- to high latitudes in both hemispheres[9]. We designed two experiments, one with replicated populations assembled from equal contributions of 6 clones or genotypes (multi-clone experiment), and one based on replicates founded by a single genotype (single-clone experiment). The multi-clone experiment was designed to provide standing genetic variation that would be readily available to genotypic selection[23], while in the single-clone experiment starting with one haphazardly chosen genotype, evolutionary adaptation requires novel mutations. Both experiments used freshly isolated genotypes from Bergen, Norway, and ran in batch cultures over ~500 asexual generations under continual slow rotation at ambient (400 µatm), medium (1100 µatm) and high (2200 µatm) pCO_2 levels. The medium CO_2-treatment represented a level projected for the beginning of the next century[24]. The high level served as a proof of principle representing a sufficiently strong selective pressure. Although falling outside the range of projected oceanic CO_2 concentrations, it is within the range of values occurring temporarily in coastal areas under upwelling of O_2-deficient water[25]. Well before reaching the stationary phase exactly 10^5 cells were transferred to the next batch cycle in order to keep the mutational target large and genetic drift effects low. Maximal cell densities at the end of each 5-d batch cycle were 1.5 x 10^5 mL^{-1} which corresponded to a maximal drawdown of dissolved inorganic carbon (DIC) of 6.5%.

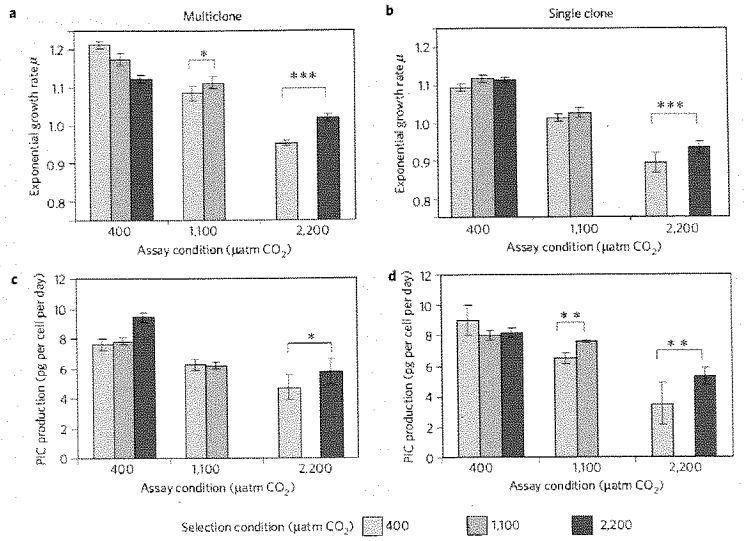

Figure 1 | Phenotypic responses to ~500 generations of selection in *E. huxleyi*. Replicate cultures (n=5) were either founded by six (left panels) or a single clone (right panels). **a, b,** mean exponential growth rate (±1s.d.). **c, d,** mean production rate of particulate inorganic carbon (PIC). Adaptation to medium (1100 μatm) and high (2200 μatm) partial pressure of carbon dioxide (pCO_2) were assessed in 2-way ANOVAs (selection x assay conditions), followed by planned contrasts among CO_2-selected vs. ambient-selected population after one full batch cycle of acclimation. Contrasts were only performed under the assay conditions of elevated CO_2 and when the interaction "selection x assay condition" was significant. For particulate inorganic carbon production in the single clone experiment, a Welch ANOVA was performed due to unequal variances, followed by a Wilcoxon planned comparison. *P≤0.05; **P≤0.01; ***P≤0.001.

Evolutionary responses of exponential growth rates

Increased CO_2 levels resulted in sustained lower growth and calcification rates in line with previous findings[11,12,13]. Lower population growth rates translated into ~530 asexual generations at ambient, 500 generations at medium and 430 generations at high CO_2 levels during the experimental time interval of ~1 yr. Because any freshly isolated microbial population will be subject to selection to general laboratory conditions, phenotypic changes in response to selection were always tested relative to populations selected at ambient CO_2 refs[20,21], rather than comparing phenotypes at the start and the end of the experiment. These reciprocal assay experiments were conducted under non-competitive exposure to CO_2 enrichment. The salient test for adaptation involved the comparison of populations adapted to elevated CO_2 conditions with those grown for long-term under ambient CO_2 (the selection conditions), both tested under elevated CO_2 (the assay condition). In order to control for physiological acclimation, an entire batch cycle of ~8 asexual generations was performed in the assay conditions before assessing adaptation.

In both experiments *E. huxleyi* populations adapted to ocean acidification and showed significantly increased exponential growth rates under elevated CO_2 compared to controls that were propagated for the same time under ambient CO_2 (**Fig. 1a, b**). Because in our experiments algal growth was terminated well before reaching the stationary phase, exponential growth rates are directly related to Darwinian fitness, i.e. the number of offspring produced[22]. In nature, for example under competitive conditions of a phytoplankton bloom, fitness will contain additional components[17]. Accordingly, multi-clonal populations selected under elevated CO_2 showed fitness increases [as quotient of the exponential growth rate[22]] of 2.6% (medium CO_2-selection) and 7% (high CO_2-selection). Note that due to its logarithmic properties, a 7% fitness difference would translate into a 3-fold difference in cell numbers after 14 days of exponential growth, for example during a phytoplankton bloom. However, the experimental period of ~1yr (500 asexual generations) was apparently insufficient to fully restore growth rates to values found under ambient CO_2 via adaptive evolution (**Fig. 1a, b**).

Next, correlated responses to selection were examined, i.e. the performance of populations selected at elevated CO_2 when exposed back to their ancestral environment. Accordingly, we compared high CO_2-selected populations to those selected at ambient CO_2, both subjected to ambient assay conditions. We observed negative correlated responses to selection, with medium- and high-CO_2 selected lines growing worse under ambient conditions (**Fig. 1a**; ANOVA, significant interaction "selection x assay condition": $F_{1,16}$= 21.6, P =0.0002 [data set ambient + medium CO_2]; $F_{1,16}$= 322.7, P <0.0001 [data set ambient + high CO_2]). The presence of the six experimental clones throughout the experiment was examined semi-quantitatively using microsatellite genotyping and diverged consistently among treatments (**Fig. 2**). Clone #75 remained in the ambient, clones #75 and 41 in the medium, and clone #62 in the high CO_2-treatment, while all other clones fell below our

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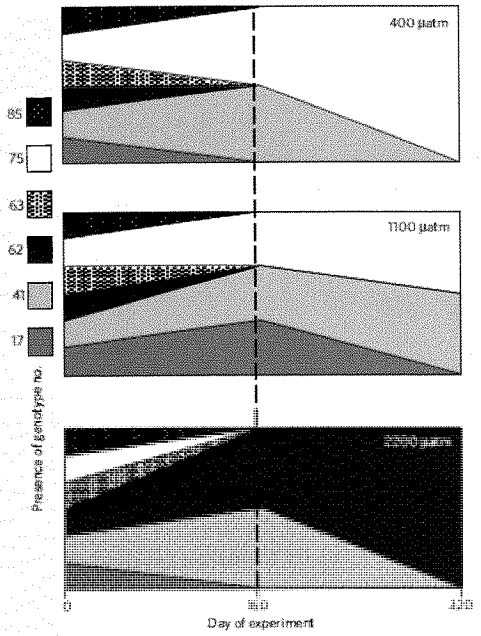

Figure 2 | Time course of the presence of *E. huxleyi* genotypes in the multi-clone experiment. All three CO_2-treatments started with the same mixture of six genotypes. Genotypes were identified via polymerase chain reaction of diagnostic alleles at five microsatellite marker loci[35]. For each genotype, at least one diagnostic microsatellite allele was present. As threshold, 5% of the peak height of the respective allele at the experimental start was chosen. The combinatorial likelihood for a single (ambient + high CO_2), or the same two genotypes (medium CO_2) remaining in all 5 replicates due to a random process were $P<0.0001$.

detection threshold. The likelihood that such consistent clone sorting would happen by chance was very small ($P<0.001$). One mechanism of adaptive change was thus selection among genotypes, the type of standing genetic variation present within asexually reproducing populations.

Interestingly, in the single-clone experiment, high CO_2-selected populations performed better under all CO_2-environments, as demonstrated by a non-significant interaction "selection x assay condition", and a significant positive effect of CO_2-selection (**Fig. 1b**; ANOVA, $F_{1,16}= 16.5$, $P=0.0009$, [data set ambient + medium CO_2]; $F_{1,16}= 17.3$, $P=0.0007$ [data set ambient + high CO_2]). Here, mean fitness increase was 1.8 and 3.3% for the populations selected at medium and high CO_2 conditions, respectively, compared to the respective controls. That populations originating from a single clone and selected in a novel environment also perform better under ancestral conditions compared to controls, i.e. positive correlated responses to selection, is well known from evolution experiments[26]. Several processes may be responsible including universal fitness benefits of mutations under the novel selection regime via pleiotropy[27], and non-transitive competitive interactions among two or more mutant genotypes ("clonal interference")[28].

Correlated traits responding to selection at elevated CO_2

Although we selected on exponential growth rates as one important fitness component, another prime interest in oceanography and biogeochemistry is how production rates of particulate inorganic carbon (PIC) present in the coccoliths changed during selection, given its importance for particle ballasting[10]. One expectation was that coccolith formation under elevated CO_2 becomes more costly[29,30], leading to its gradual reduction either via genetic decay or direct selection for lower calcification rates. On the contrary, both multi- and single-clone populations selected at elevated CO_2 revealed higher calcification rates than control populations when tested in a high CO_2 environment. Calcification rates were thus partly restored, with 22% (multi-clone assay, 2200 µatm pCO_2 selection), and 17 and 51% (single-clone assay at 1100 and 2200 µatm pCO_2 selection, respectively) more PIC production compared to ambient CO_2-selected controls (**Fig. 1c,d**). Between both experiments, the sign of the correlated response in PIC production differed, with CO_2-selection having a general positive effect in the multi-clone experiment (**Fig. 1c**, ANOVA, $F_{1,16}= 8.2$, $P=0.011$ [data set ambient + medium CO_2 selection], $F_{1,16}= 5.41$, $P=0.03$ [data set ambient + high CO_2 selection]). In contrast, PIC production correlated negatively in the single-clone experiment (**Fig. 1d**, Welch ANOVA for unequal variances, subsequent pair-wise comparisons of elevated vs. ambient CO_2-selected populations in reciprocal condition, all $P\leq0.0127$). That we observed negative and positive correlations of the same traits (calcification rate) as response to selection for elevated CO_2 among sub-experiments seems puzzling. However, since the influence of many versus single founding genotypes is confounded among both experiments, we cannot decide whether genotype identity or diversity per se was responsible for the differences observed in the correlated response. Notwithstanding, it is noteworthy that in 3 out of 4 cases, there were significant increases of calcification rates compared to non-adapted controls under CO_2 enrichment (**Fig. 1c,d**). Interestingly, when tested in their respective selection environment, PIC production between the 400 (ambient) and 1100 µatm pCO_2-selection treatment was not statistically significant in the single clone experiment (Welch ANOVA, pairwise Wilcoxon-test, $Z = 1.46$, $P = 0.143$), suggesting a complete restoration of PIC production in the single- but not the multi-clone experiment. The outcome of our selection experiment also suggests a functional importance of calcification for growth rates and competitive fitness in *E. huxleyi*[12,31].

Responses of correlated traits to selection, such as cell size, particulate organic carbon (POC) per cell and the PIC:POC ratio were more complex, but all measured traits responded either generally to selection at elevated CO_2, or selection responses depended on the assay environment (**Fig. 3a-h**). Cell size suffered a sustained decrease under high CO_2 conditions. This decrease was partly reverted under adaptation to high (multi-clone) and medium (single-clone) CO_2 selection conditions, always compared to the respective controls (**Fig. 3a,b, supplementary**

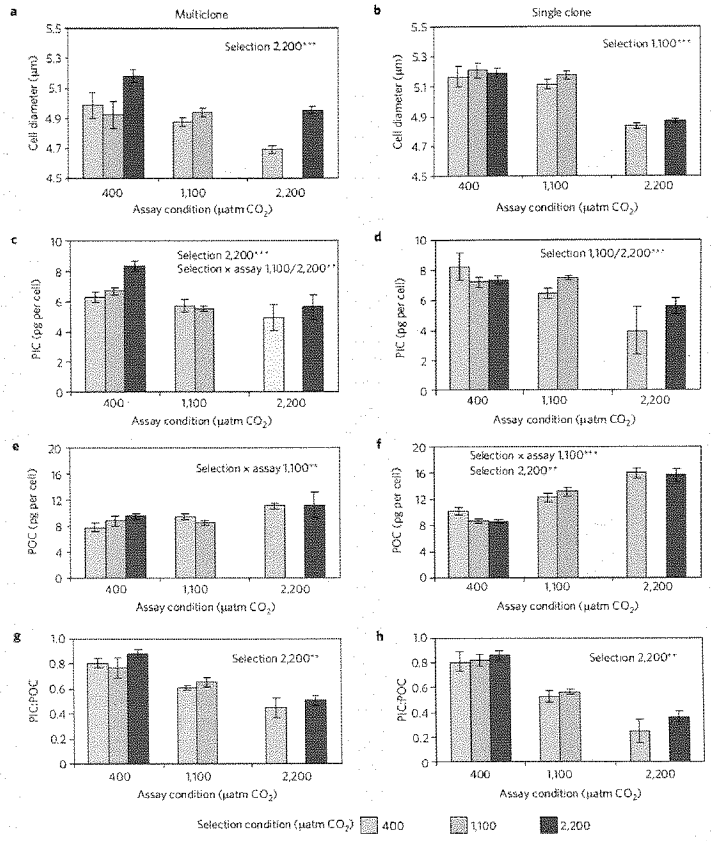

Figure 3 | Response of correlated traits after selection to elevated CO_2 levels in the coccolithophore *E. huxleyi*. **a, b**, Mean cell diameter (means ±1 s.d., n=5) in the multi- and single clone experiment. **c, d**, particulate inorganic carbon per cell (PIC), **e, f**, particulate organic carbon per cell (POC), **g, h**, PIC:POC ratio. Statistically significant results of main and interaction effects 500 generations of selection under the respective CO_2 environment (1100 or 2200 µatm) are given in each panel (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). For further statistical details see supplementary Tables S1, S2.

tables S1, S2). The multi-clone cultures selected at elevated CO_2 even completely restored their cell size relative to cultures selected under ambient conditions when tested under elevated CO_2. Particulate inorganic carbon (PIC) on a per cell basis mostly increased in adapted populations relative to non-adapted controls, as a response to high (multi-clone) and medium and high (single-clone) CO_2 selection conditions (**Fig. 3c, d**). When assayed at both, 1100 and 2200 µatm, cells fixed more particulate organic carbon (POC) per cell under elevated CO_2 irrespective of the previous selection environment. Those rates were only little affected by adaptation to elevated CO_2, but rather by pronounced correlated responses of evolved lines exposed to the ancestral ambient environment (**Fig. 3e, f**). Overall, there was a general positive effect of selection to restore PIC:POC-ratios in high CO_2 levels in both experiments relative to controls adapted to ambient CO_2, although this effect was statistically significant only at 2200 µatm (**Fig. 3g, h, Table S1, S2**). As ocean acidification possibly increases the energetic cost of biogenic calcification[4], a partial restoration of calcification rate imposes energetic trade-offs on coccolithophore cells that may not be visible in a nutrient and light saturated laboratory environment. Longer sustained experiments with additional limiting factors and careful consideration of possible trade-offs are needed to reveal the absolute amount of possible phenotypic evolution as a response to ocean acidification.

Mechanisms of adaptation to ocean acidification

Previous studies found no adaptive responses of the haploid freshwater algae *Chlamydomonas reinhardtii* to selection at elevated CO_2. Rather, as a correlated response, reduced growth was found when exposing high-CO_2 selected populations back to the ambient environment, suggesting a degeneration of carbon concentrating mechanisms by conditionally neutral mutations[20]. In contrast, our study identified direct, positive adaptation to elevated CO_2 levels in a calcifying marine phytoplankton species. We identified genotypic selection as one immediate mechanism of population-level adaptation in the multi-clone experiment. The adaptive responses observed in the single-clone experiment can only be explained by the emergence and partial fixation of advantageous new mutations. The rate of adaptation was in line with other laboratory selection experiments in diploid microbes that were conducted over some hundreds of asexual generations (**supplementary table S3**) and match population

genetic expectations (**supplementary note S1**). It has been argued that evolution is slow in diploid microalgae compared to haploid species because mutations must be partially or completely dominant to produce a phenotype in diploid *E. huxleyi*[14,32]. However, a direct comparison of adaptation rates in haploid and diploid yeast strains revealed only small, non-significant differences under comparable population sizes and generation times as employed in our single-clone experiment[32,33] (and **supplementary table S3**). Interestingly, replicates within treatments evolving under elevated CO_2 were not more divergent in terms of their phenotypes than those under ambient conditions. Accordingly, variances in growth rates were statistically not different from each other (Bartlett-test of variance homogeneity, all $P>0.1$). Nevertheless, the beneficial mutations underlying adaptation in the different replicates probably have a different genetic basis[21]. Such phenotyic convergence is often observed in microbial evolution experiments, in particular if there are functional constraints to adaptation[34]. Interestingly, clone #62 that by chance was selected as founder for the single-clone experiment (**Fig. 2**), prevailed in the multi-clone experiment under high CO_2 selection conditions. Because the presence of 5 other genotypes constitutes a different selection environment within the multi- compared to the single-clone experiment, it is not surprising that mean values of growth and calcification vary due to competition and clonal interference[21,27].

Ecological and biogeochemical implications

We deliberately chose a simple selection protocol and exposed asexually reproducing populations immediately into new, CO_2-enriched environments. Our intention was to make results as comparable as possible to the many other microbial selection experiments and short-term physiological assessments of ocean acidification[13]. It is clear that the genetic diversity of natural phytoplankton populations will by far exceed the diversity in our multi-clone experiment[35,36], let alone in the single-clone approach that started from a single founding genotype. Moreover, sexual reproduction will probably further enhance within-population additive genetic variance and thus, the possible rate of evolutionary adaptation[37]. While the frequency of sexual reproduction and recombination is unknown for natural *E. huxleyi* populations, the diversity of genotypes even in bloom situations[35] suggests abundant sexual reproduction in the wild. Coccolithophores play an important role for ocean productivity and the export of particulate carbon to the deep sea[9,10,15,31]. Hence, the swift adaptation processes observed here have the potential to affect food web dynamics and biogeochemical cycles on time-scales of a few years, thus surpassing predicted rates of ongoing global change including ocean acidification. Since experimental evolution experiments only reveal the potential for adaptation they need to be scrutinized against field observations. A recent study reports surprisingly high coccolith mass in an *Emiliania huxleyi* population off Chile in high CO_2 waters[38] that suggest across-population variation in calcification, in line with findings of rapid microevolution identified here. While the macro-evolutionary trends and associated ecological niches among the major eukaryotic phytoplankton groups are reasonably well understood[39], our results provide a starting point to examine the potential of within-species adaptive evolution in marine microbes.

Methods

Experimental cultures were founded with freshly isolated *Emiliania huxleyi* clones and grown in sterile filtered artificial seawater media under continuous rotation at 15°C and 150±10 µmol m^{-2} s^{-1} photon flux density under a 16:8 light:dark cycle. The seawater carbonate system was set up by bicarbonate addition and subsequent aeration using a controlled CO_2 gas mixing system. Carbonate chemistry was determined by dissolved inorganic carbon (DIC) and total alkalinity (TA) measurements. Average culture CO_2 partial pressure values were calculated from DIC and TA measurements and drawdown estimates.

The multi-clonal selection experiment was initiated with an equal contribution of 1.67×10^4 cells from six genotypes, while the single-clone experiment received 10^5 cells from one randomly chosen genotype. Both experiments were run in parallel by serial transfer of 10^5 cells every 5 days. Cell counts were performed in triplicate after each batch cycle and exponential growth rates were calculated for each replicate. We tested for adaptation to elevated CO_2 concentrations after 320 days (~500 asexual generations). Populations grown at ambient CO_2 were compared to populations selected at high CO_2, in both, the ambient and elevated CO_2 assay environment. As response variables, growth rates, cell diameter, particulate inorganic and organic carbon (PIC, POC) per cell and their production rates were assessed. Genotype presence in the multi-clone experiment was assessed with genotype-specific alleles in at least one microsatellite locus, using five *E. huxleyi* specific primers[35] after 0, 160 and 320 days. Statistical analyses used two-way analysis of variance (ANOVA), in combination with planned contrasts or Welch-ANOVA with subsequent Wilcoxon tests when variances were heterogeneous.

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Author contributions

T.B.H.R. conceived the project, all authors designed the experiment, K.T.L. performed the experiment. All authors analyzed and interpreted the data and wrote the manuscript.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper on www.nature.com/naturegeoscience. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence should be addressed to T.B.H.R. and requests for materials should be addressed to K.T.L.

4.2 Additional Information - Publication I

Author contributions

This chapter is published in a scientific journal under multiple authorship. My contribution to this work is described below.

Title: Adaptive evolution of a key phytoplankton species to ocean acidification

Authors: Kai T. Lohbeck, Ulf Riebesell, and Thorsten B. H. Reusch

Published in: Nature Geoscience 5:346-351 (2012)

Author contributions: TBHR conceived the project, all authors designed the experiment, KTL performed the experiment. All authors analyzed and interpreted the data and wrote the manuscript.

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5 Publication II

5.1 Functional genetic divergence in high CO_2 adapted *Emiliania huxleyi* populations

Authors: Kai T. Lohbeck, Sinead Collins, Ulf Riebesell, and Thorsten B. H. Reusch

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SPECIAL SECTION

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FUNCTIONAL GENETIC DIVERGENCE IN HIGH CO_2 ADAPTED *EMILIANIA HUXLEYI* POPULATIONS

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Predicting the impacts of environmental change on marine organisms, food webs, and biogeochemical cycles presently relies almost exclusively on short-term physiological studies, while the possibility of adaptive evolution is often ignored. Here, we assess adaptive evolution in the coccolithophore *Emiliania huxleyi*, a well-established model species in biological oceanography, in response to ocean acidification. We previously demonstrated that this globally important marine phytoplankton species adapts within 500 generations to elevated CO_2. After 750 and 1000 generations, no further fitness increase occurred, and we observed phenotypic convergence between replicate populations. We then exposed adapted populations to two novel environments to investigate whether or not the underlying basis for high CO_2-adaptation involves functional genetic divergence, assuming that different novel mutations become apparent via divergent pleiotropic effects. The novel environment "high light" did not reveal such genetic divergence whereas growth in a low-salinity environment revealed strong pleiotropic effects in high CO_2 adapted populations, indicating divergent genetic bases for adaptation to high CO_2. This suggests that pleiotropy plays an important role in adaptation of natural *E. huxleyi* populations to ocean acidification. Our study highlights the potential mutual benefits for oceanography and evolutionary biology of using ecologically important marine phytoplankton for microbial evolution experiments.

KEY WORDS: Adaptation, experimental evolution, genetic divergence, ocean acidification, phytoplankton, pleiotropy.

Experimental evolution has revolutionized evolutionary biology, providing the unique opportunity to investigate evolutionary processes in real time. Model systems for experimental evolution studies are typically unicellular microbes that are easy to cultivate, reproduce quickly, and provide a range of genomic and recombinant technologies, so that organisms such as *Escherichia coli*, *Pseudomonas*, and *Saccharomyces* are the microbes of choice for most experimental evolution (Elena and Lenski 2003; Buckling et al. 2009). Although experimental evolution has made inroads as an applied science for medically relevant organisms such as *Pseudomonas* (e.g., Perron et al. 2006), most research is still done using organisms where we know little about their ecology outside the lab. In contrast, a model system with a clear ecological role that is also studied in the context of biogeochemical consequences of global climate change provides the opportunity to use microbial experimental evolution to address contemporary questions of pressing ecological and socioeconomic relevance.

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Figure 1. Scanning electron micrograph of an *Emiliania huxleyi* cell covered with calcite scales. Image provided by L. T. Bach, GEOMAR, credits to the Institute for Geosciences, University of Kiel.

Potential model systems with well-studied ecology include marine phytoplankton; prokaryotic and eukaryotic microbes that are responsible for about 50% of the global primary production (Field et al. 1998). One question of immediate concern is how future marine phytoplankton communities are likely to respond (including the possibility of adaptive evolution) to a warmer, more stratified, and more acidic ocean of the coming decades (Riebesell et al. 2009; Collins 2012; Reusch and Boyd this issue). The coccolithophore *Emiliania huxleyi* (Fig. 1) is a prominent model organism in biological oceanography. This microscopic alga thrives under a wide range of environmental conditions, with a ubiquitous distribution, except for polar waters, and has the ability to form extensive blooms (Paasche 2002) that are large enough to make them visible from space (Fig. 2). These characteristics suggest an important role of *E. huxleyi* for marine primary production and carbon cycling (Westbroek 1989). Besides photosynthetic carbon fixation, coccolithophores possess a second biogeochemically relevant trait—they produce minute calcite scales that can accelerate the export of organic matter from the surface into the deep ocean and thereby also affect the oceanic carbon cycle (Armstrong et al. 2002). Increasing atmospheric concentrations of fossil fuel derived CO_2 changes ocean chemistry; it leads to a lowering in seawater pH termed ocean acidification (Caldeira and Wickett 2003). This in turn, may negatively affect growth and calcite production in coccolithophores, which has reinforced the attention given to *E. huxleyi* (Riebesell et al. 2000; Riebesell et al. 2009).

While short-term physiological responses to changes in various environmental parameters are reasonably well studied in *E. huxleyi* (Paasche 2002; Zondervan 2007), long-term experiments that investigate evolutionary responses are needed to predict future responses of marine phytoplankton to global change (Riebesell 2011; Collins 2012; Lohbeck et al. 2012). This is where experimental evolution and biological oceanography meet to open a new and promising interdisciplinary research area with the potential to benefit both fields (Reusch and Boyd this issue).

To investigate the evolutionary response of *E. huxleyi* to elevated CO_2, we carried out an experimental evolution experiment with freshly isolated genotypes from a coastal, temperate site near Bergen, Norway (Lohbeck et al. 2012). After the first 500 generations of asexual growth, we found 3.3% increase in fitness and 51% restoration in calcification rate in high CO_2 adapted *E. huxleyi* populations relative to ambient CO_2 selected control populations when tested under high CO_2 conditions. Based on laboratory experiments, adaptive evolution in this key phytoplankton species appears possible and swift enough to keep pace with global climate change scenarios, even in the absence of sex, migration, or other mechanisms that may speed up adaptation in natural populations.

Interestingly, replicate selection lines revealed strikingly similar phenotypes in terms of direct and correlated responses to selection under CO_2 enrichment. All five replicate populations independently revealed similar fitness increases relative to ambient CO_2 selected control populations, with within-group variances being similar between adapted and control populations. There are two possible explanations for such phenotypic convergence. First, the same mutations may have occurred and increased in frequency throughout all five adapting replicates (Wichman et al. 1999; Woods et al. 2006). Alternatively, different mutations characterize phenotypically convergent replicate populations (Travisano et al. 1995; Ostrowski et al. 2008). If so, the pleiotropic effects of these new allelic variants are likely to be different in another, novel environment. Phenotypic divergence then can be taken as indirect evidence for a different genetic basis of CO_2-adapted phenotypes (Travisano et al. 1995; MacLean and Bell 2003; Ostrowski et al. 2008). Such functional genetic divergence can constrain evolution in the face of additional stressors and therefore be a key variable when assessing phenotypic responses to a multitude of complex selective forces natural phytoplankton populations will encounter in the future ocean (Boyd 2011).

To distinguish among these alternatives, we used two "challenge" assays to resolve to what extent the populations founded from a single genotype had diverged during adaptation to the high CO_2 environment. If the replicate populations have not diverged genetically, they would have the same phenotypes in the novel environment. If, on the other hand, they have diverged genetically, it is expected that their phenotypes in some novel environments differ, either due to divergent pleiotropic effects of adaptive mutations fixed during selection at high CO_2, or to replicate-specific genetic variation that is neutral at high CO_2 but that affects fitness in a novel environment. We used high-light and low-salinity

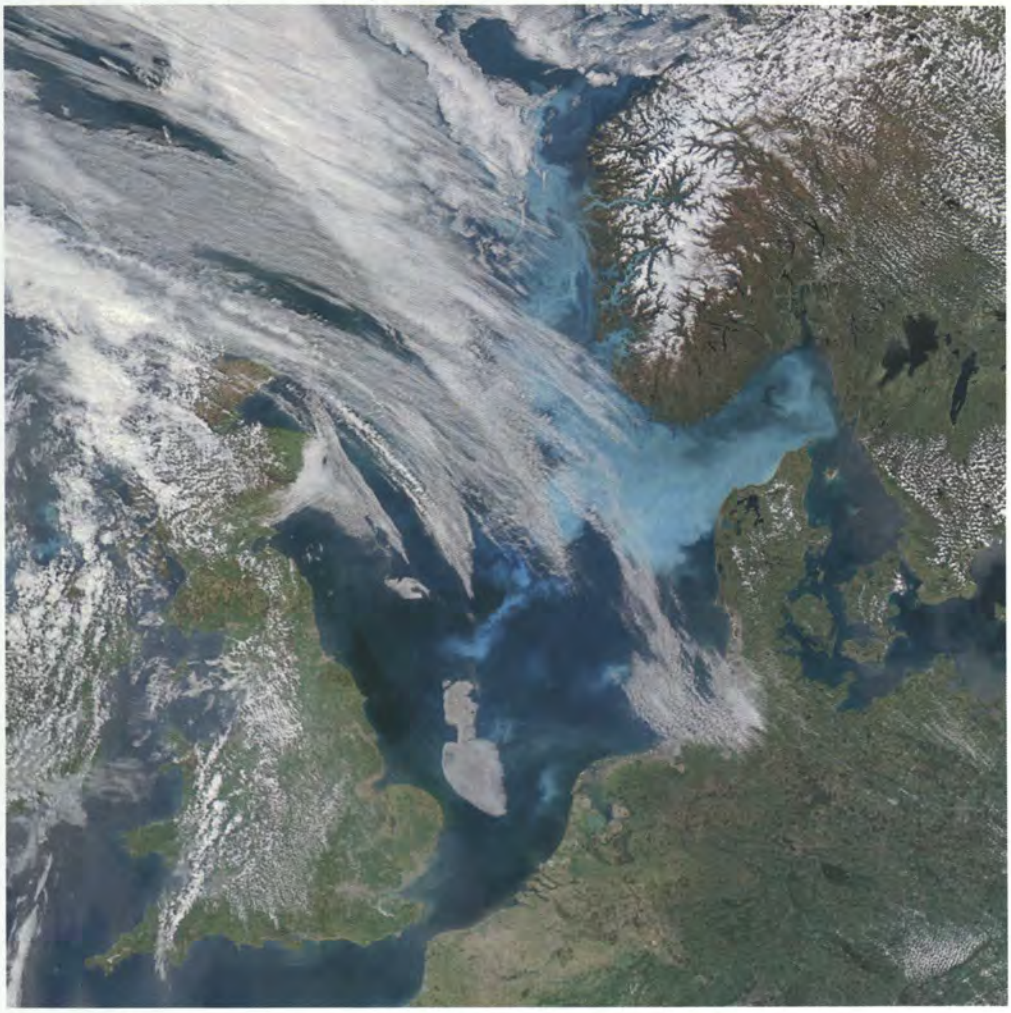

Figure 2. Satellite image of an *Emiliania huxleyi* bloom off the Norwegian coast. The light blue color, caused by increased light reflection due to the calcite platelets produced by the cells, marks the extension of the bloom. Image provided by the SeaWiFS Project, NASA/Goddard Space Flight Center and GeoEye.

environments that were not experienced by the populations during the high CO_2 selection experiment, to test for functional genetic divergence in high CO_2 adapted populations that may affect responses to other environmental changes.

Materials and Methods

CELL ISOLATION AND CULTURING

Clonal cultures were founded by single cell isolation from a natural *E. huxleyi* bloom in Raunefjorden, Norway in May 2009 and verified to originate from a single diploid cell by microsatellite genotyping as described in Lohbeck et al. (2012). We used nonaxenic cultures, which is a common approach for *E. huxleyi* culture experiments, to allow for long-term batch culturing over many years. To exclude the presence of a significant bacteria fraction, we regularly performed automated cell counts and microscopy throughout the experiments.

Artificial seawater (ASW) was produced as in Kester et al. (1967) with the exception of 2400 μmol bicarbonate kg^{-1} ASW addition (Merck, Germany), resulting in a total alkalinity (TA) of 2444 μmol kg^{-1} at a salinity of 35 (ASW_{35}). Low-salinity ASW

(ASW_{15}) was produced by adding 1.334 kg MilliQ water kg^{-1} ASW_{35} and subsequent addition of 1314 μmol bicarbonate kg^{-1} low salinity ASW, resulting in ASW with a salinity of 15 (ASW_{15}) and a TA of 2367 μmol kg^{-1} ASW_{15}. All ASW media were 0.2-μm sterile filtered (Whatman Polycap 75AS, GE Healthcare, UK) and supplemented with 64 μmol kg^{-1} nitrate, 4 μmol kg^{-1} phosphate (nutrient ratio after Redfield 1958), trace metals and vitamins according to f/8 adapted from Guillard and Ryther (1962), 10 nmol kg^{-1} selenium after Danbara and Shiraiwa (1999), and 2 mL kg^{-1} sterile filtered North Sea water to exclude any limitations by micronutrients. Initial nutrient concentrations were sufficient to ensure that under all final cell densities observed in the experiments an adequate remaining nutrient concentration prevents any nutrient limitation effects.

Prior to inoculation, ASW media were aerated for 24 h using a controlled CO_2 gas mixing system at the treatment levels of 400 and 2200 μatm pCO_2. Evaporation was minimized by prefixed gas wash bottles filled with MilliQ water to saturate gases with humidity. After CO_2 manipulations, ASW media were carefully pumped into the respective culture flasks using a sterile silicon hose to ensure minimal gas exchange. For each treatment, two extra flasks were prepared for dissolved inorganic carbon (DIC) and TA measurements.

Experimental cultures in the CO_2 selection and assay experiments were grown under the same conditions as given here for the challenge experiments but under continuous rotation. Details are described in Lohbeck et al. (2012). In the challenge experiments, cultures were grown in 250-mL Duran square flasks (Schott, Germany) filled with 310 mL ASW medium leaving a minimum headspace. Cultures were incubated in a RUMED Light Thermostat 1301 (Rubarth Apparate GmbH, Germany) at 15°C and a photon flux density of 155 ± 2 μmol photons m^{-2} s^{-1} under a 16:8 light:dark cycle. All culture flasks were manually rotated 10 times, twice a day (10 and 17 h).

EXPERIMENTAL PROCEDURES

Replicate selection lines were propagated for 750 and 1000 generations under ambient (400 μatm) and high (2200 μatm) pCO_2 and assayed under reciprocal CO_2 conditions using the same protocol as in Lohbeck et al. (2012). To account for technical variation in the challenge experiments, triplicate subreplicates of each individual replicate population were inoculated into an acclimation batch cycle prior to an assay batch cycle in their respective control and challenge environments. We applied one full acclimation cycle (approximately six to eight cell divisions) prior to the assay cycle to account for nongenetic effects after transfer into a new environment. Growth in novel environments (high light, low salinity) was used to detect functional genetic divergence. Populations were grown in the CO_2 environment that they had been selected at for either 750 or 1000 generations. Populations selected at high CO_2 are referred to as "high CO_2 populations" and those selected at ambient CO_2 are referred to as "ambient CO_2 populations." Each population was then subjected to a novel environment that is not obviously connected to CO_2 levels.

Cell densities were measured in triplicate using a Z2 Particle and Size Analyzer (Beckman Coulter, Brea, CA). Exponential growth rates (μ) were calculated from cell densities where $\mu = (\ln N_1 - \ln N_0)/d$, and N_1 and N_0 are cell concentrations at the beginning and end of a batch cycle and d is the duration of the batch cycle in days. Experimental cultures were started with a population size of 100,000 cells per flask (323 cells/mL). In both challenge experiments, control populations from ambient and high CO_2 selection lines were grown in five-day batch cycles (155 μmol photons m^{-2} s^{-1}, salinity 35). High light challenged populations (800 μmol photons m^{-2} s^{-1}, salinity 35) were grown in four-day batch cycles and salinity challenged populations in six-day (400 μatm pCO_2, salinity 15) and 12-day (2200 μatm pCO_2, salinity 15) batch cycles, respectively. Throughout the experiments, all populations grew exponentially and never reached the stationary phase.

CARBONATE CHEMISTRY MEASUREMENTS

Carbonate chemistry was determined by DIC and TA measurements. DIC samples were taken for all treatment groups prior to inoculation and measured with an AIRICA system (Marianda, Germany). TA was measured from all ASW batches by open-cell acidimetric titration using a Basic Titrino 794 (Metrohm, Switzerland). CO_2 partial pressure in the culture media was calculated from DIC and TA using the software CO2SYS (Lewis and Wallace 1998) with solubility constants after Roy et al. (1993). Average culture pCO_2 values of 351 ± 12 and 2065 ± 103 μatm (ambient and high CO_2 treatments) were calculated from DIC and TA measured prior to inoculation and the draw-down estimate from final cell numbers following the approach of Bach et al. (2011). Under these conditions, the batch culture environment does not constitute a constant but a slightly decreasing CO_2 environment due to DIC drawdown during population growth that accounted for a maximum DIC drawdown of 7.6%.

We were not able to measure DIC samples taken from the high-light challenge experiment, but the average culture CO_2 values were almost certainly within the range set up by the CO_2 gas system for two reasons. First, we have more than 1000 generations of culture work experience using this CO_2 gas mixing system and so far the expected CO_2 levels have always been confirmed by DIC and TA measurements. Second, growth rate is a sensitive indicator for CO_2 levels in this culture media. We prepared control and challenged media from the same aerated ASW batch and found growth rates of control populations to be absolutely in line with expectations for ambient CO_2 culture media. As such, the average culture CO_2 values from all experiments were unlikely to

differ significantly from the desired treatment levels of 400 and 2200 μatm pCO_2 that are given in the figures.

STATISTICAL ANALYSES

To test for heterogeneity of variances among replicates within the challenge experiment, we first performed an overall nested analysis of variance with replicate population nested into the respective treatment combinations. In the challenge experiments, the treatment structure was a factorial composed of the factor combination high versus low CO_2 selection × normal/high light challenge, or normal/low salinity challenge, respectively. Only if the replicate lines were heterogeneous at $\alpha < 0.05$ did we proceed with pairwise comparisons of the variances within the same selection treatment (ambient or high CO_2) among control and challenge. To this end, we used a direct *F*-test comparing the within-treatment variances in numerator (larger variance) and denominator (smaller variance). The variances were formulated using the five subreplicate means per treatment combination. Hence, numerator and denominator have 4 degrees of freedom each. The hypothesis being tested was that only high CO_2 adapted replicate populations would diverge in their variance under challenge conditions.

Results

ASSAY EXPERIMENTS AFTER 500, 750 AND, 1000 ASEXUAL GENERATIONS

Here, we show the results of the second year of selection of *E. huxleyi* populations under high CO_2 conditions simulating future ocean acidification. Although there was a direct response to selection at high CO_2 during the first year of the experiment (0–500 generations), no further increases in fitness relative to ambient CO_2 selected populations were detected in the second year (after 750 and 1000 generations) (Figs. 3, 4). Direct responses were measured as the growth rate of the high CO_2 adapted populations relative to the ambient CO_2 adapted populations in a high CO_2 assay environment. In contrast, correlated responses, measured as growth rate of high CO_2 adapted populations assayed in the ancestral ambient CO_2 environment, were found to have increased by 4% relative to ambient CO_2 selected populations after 500 generations but showed a decrease by 3% relative to ambient CO_2 selected populations after 750 and 1000 generations, respectively (Figs. 3, 4).

Consistent with earlier assessments from reciprocal transplants after 500 generations (Lohbeck et al. 2012), we found low within-treatment variances in exponential growth rate among replicate selection lines also after 750 and 1000 generations, indicating phenotypic convergence in all selection lines under high CO_2 selection (Fig. 3).

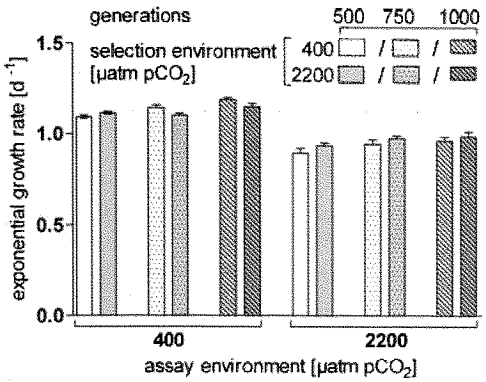

Figure 3. Mean exponential growth rate (±1 SD) of replicate high CO_2 adapted (gray background) and ambient CO_2 adapted (white background) *Emiliania huxleyi* populations ($N = 5$) tested in the respective assay environment after 500 (blank), 750 (dotted), and 1000 (striped) generations.

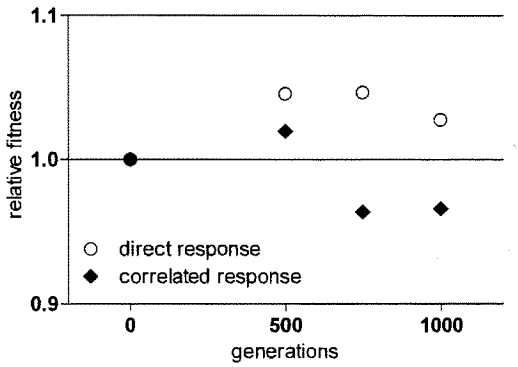

Figure 4. Mean fitness in direct and correlated response of high CO_2 selected populations relative to ambient CO_2 selected populations ($N = 5$) at the start of the experiment and after 500, 750, and 1000 generations. Fitness ratios are meant to illustrate the time course of direct and correlated response based on fitness data shown in detail in Figure 3. No error bars were calculated for the depicted fitness ratios because the evaluation of statistically significant effects was based on the treatment means, not the ratios. Direct response is the fitness of high CO_2 selected populations relative to the fitness of ambient CO_2 selected populations in the environment they were selected for (high CO_2). Correlated response is the fitness of high CO_2 selected populations relative to ambient CO_2 selected populations in any other environment they were not selected for (e.g., ambient CO_2).

CHALLENGE EXPERIMENTS

Populations transferred into a novel high-light environment showed an increased mean fitness by 3% under ambient and 7% under high CO_2, respectively (Fig. 5A). There was no significant

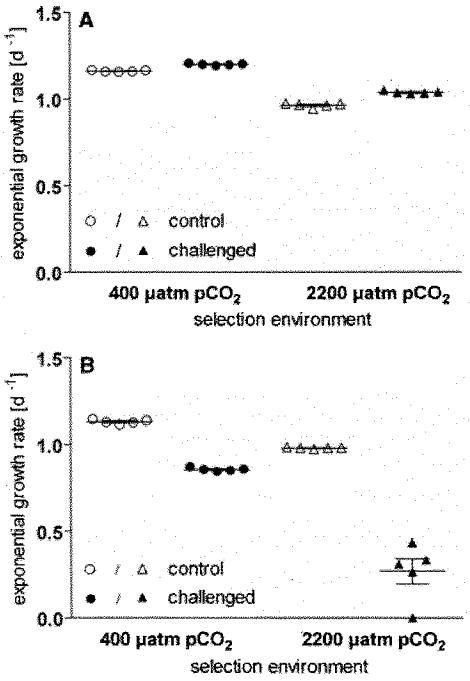

Figure 5. Mean exponential growth rate ($N = 3$) of all individual replicate populations ($N = 5$), and their overall mean (horizontal line ±1 SD) selected under ambient CO_2 (circles) and high CO_2 (triangles) when tested under (A) control light (open symbols) and high-light challenge (closed symbols) and (B) control salinity (open symbols) and low-salinity challenge (closed symbols) assay conditions. Note that SD values (horizontal thick line) are so small that they are collapsed with the overall mean (thin horizontal line).

within-treatment heterogeneity among the four treatment combinations ambient versus high CO_2 selection × challenge versus control (nested ANOVA, $F_{3,16} = 1.865$, $P = 0.056$). Because the P-value was close to statistical significance, we also compared the among treatment variances pairwise across selection environments. We found no significant difference of within-treatment variance between the challenge and control treatments in either the ambient or the high CO_2 selected populations (all $P > 0.4$). Here, the high-light challenge did not detect any functional genetic divergence.

In contrast, the low-salinity challenge revealed that the high CO_2 selected lines had diverged genetically. Selection lines transferred into a novel low-salinity environment showed a consistent decrease in fitness by an average of 24% in the ambient CO_2 treatment and of 67% in the high CO_2 treatment (Fig. 5B). The combination of high CO_2 and low salinity showed an additive effect on mean fitness. When grown under lower salinity, a significant within-treatment variance indicated that phenotypes were no longer convergent (nested ANOVA, $F_{3,16} = 19.94$, $P < 0.0001$). We identified which within-treatment/among replicate variance produced this heterogeneity. The within-treatment variance of populations selected at ambient CO_2 levels was not significantly different among challenged and control treatments (direct F-test to compare variances, $F_{4,4} = 1.41$, $P > 0.1$), but the high CO_2 adapted populations had a much greater variance in the challenge treatment than in their respective control treatments (direct F-test to compare variances, $F_{3,4} = 249$, $P << 0.0001$). In line with this, under low-salinity challenge, all three subreplicates from one high CO_2 selected population failed to reach sufficient cell numbers for cell counting and inoculation within the acclimation cycle of 12 days. Growth rates of zero (populations failed to grow within the 12-day batch cycle) were included in the graph (Fig. 5B), but were omitted from the statistical analysis. Because it neglects the most extreme phenotype seen in the low-salinity environment, the homogeneity of the fitness response is overestimated in the salinity challenge × CO_2 adaptation treatment, making our statistical test for detecting functional genetic divergence conservative.

Discussion

Replicate populations of the globally important marine phytoplankton species *E. huxleyi* were grown for 1000 generations under ambient and elevated CO_2 levels, representing a contemporary control and a future ocean acidification scenario. Reciprocal transplants revealed adaptation to ocean acidification in populations selected under high CO_2. Over the experiment, all replicate populations in a treatment group showed similar fitness responses, indicating phenotypic convergence, although it was not known whether this reflected underlying genetic convergence. Using an experimental approach that utilizes the hypothetical pleiotropic role of novel, fitness conferring mutations (Travisano et al. 1995; MacLean and Bell 2003; Ostrowski et al. 2008), we demonstrated functional genetic divergence underlying adaptation to high CO_2 in single clone derived *E. huxleyi* populations. Although no functional genetic divergence was detected in a novel high-light environment, we found strikingly divergent phenotypes only among the populations adapted to high CO_2, but not ambient CO_2 levels, when challenged with low salinity.

DIRECT AND CORRELATED RESPONSE TO HIGH CO_2

The dynamics of adaptation to high CO_2 in *E. huxleyi* populations using de novo variation happened in two phases during our selection experiment. Over the first 500 generations, we observed rapid adaptation characterized by a marked increase in fitness in high CO_2 selected populations, after which fitness remained constant for the following 500 generations. This adaptive pattern suggests that high CO_2 exerts strong selective pressure on

E. huxleyi populations, and that initially beneficial mutations (either few mutations of large effect or many mutations of small effect) were common and rose to high frequencies within the populations quite rapidly (≤500 generations). Because there was no detectible rise in fitness in the second half of the experiments, one could conclude that further beneficial mutations are rare or even absent on this genetic background. If further rare beneficial mutations that cause fitness increases relative to the ancestor exist, then we expect those mutations to eventually occur in a subset of the high CO_2 selected populations (Blount et al. 2008). Because it is very unlikely that rare mutations would simultaneously occur in all five replicate populations, this would lead to divergent direct responses to selection.

Although no obvious change in the direct response to selection occurred between generations 500 and 1000, we observed changes in the correlated response of high CO_2 selected populations. Because these changes occurred in all replicate populations and were relatively rapid, and the variance in fitness within populations did not increase (direct F-test of variance comparison to compare variances, $P > 0.5$), they are consistent with continued adaptive evolution rather than a relaxation of selection pressure. The pattern seen here may indicate temporary stasis, slow-paced fitness increase not detectable on the short time scales investigated, or nontransitive increases in fitness, despite continued genomic adaptation. A similar pattern has also been reported by Lenski and colleagues (Lenski and Travisano 1994; Barrick et al. 2009) during the first few thousand generations of their *E. coli* selection experiment.

PHENOTYPE DIVERGENCE IN A NOVEL ENVIRONMENT

Phenotype convergence was observed during 1000 generations CO_2 selection in the diploid marine phytoplankton species *E. huxleyi*. Convergent phenotypes may either result from the same sets of mutations being independently acquired in all replicate populations or from different mutations that produce a similar phenotype (Lenski et al. 1991; Travisano et al. 1995). If more than one beneficial mutation exists on this genetic background, and there is no hypermutable region or genetic switch involved, then it is unlikely that the same mutations occur twice, let alone in all five replicate populations (Woods et al. 2006). In this case, the expectation is that different evolutionary trajectories have resulted in similar phenotypic adaptations to high CO_2, but divergent genetic bases for adaptation, in replicate populations. If this is true, pleiotropy has the potential to constrain adaptive evolution in natural *E. huxleyi* populations in the face of the complex environmental changes associated with an acidifying ocean.

To further investigate the underlying basis for phenotypic convergence, we exposed replicate populations already adapted to ambient or high CO_2 to two novel environments. In a novel environment where fitness relies on sets of genes not under selection in the high CO_2 environment, conditionally neutral mutations and pleiotropic interactions can be detected by an increased variance in fitness among replicate populations (Travisano et al. 1995; Ostrowski et al. 2008). However, the coincidence of a conditionally neutral mutation and a beneficial mutation shortly thereafter in the same cell lineage appears unlikely.

The two novel environments used here produced different outcomes. Exposure to a novel high-light environment did not reveal increased variance in fitness among replicate populations, indicating that no functional genetic divergence had occurred in genes involved in responses to non-stressful high-light levels that are within the acclimation capacity of genotypes of the study populations (Paasche 2002). In contrast, exposure to a stressful environment resulted in significant increase in variance in fitness among high CO_2 selected populations but not among ambient CO_2 selected populations. This indicates that different mutations have become fixed or frequent in the populations that have adapted to high CO_2, and that several different genetic solutions exist to the problem of adaptation to ocean acidification in *E. huxleyi* populations (Travisano et al. 1995; Ostrowski et al. 2008).

We used a stressful environment that is probably at the far end of natural environmental conditions experienced by coastal *E. huxleyi* genotypes (Paasche et al. 1996). However, more moderate salinity changes are likely to occur regularly in the coastal environment and will be important to adaptation of coastal *E. huxleyi* populations to future ocean changes. The marked fitness decrease in populations exposed to low salinity and high CO_2, relative to the fitness decrease under low salinity and ambient CO_2, illustrates the importance of additive interactions of these two environmental factors. Complex changes of multiple environmental parameters can exert a very different selective force than anticipated from single factor selection experiments and, in addition to our results on pleiotropic effects, potentially play an important role in the adaptive evolution of natural *E. huxleyi* populations to ocean acidification. In particular, the range of adaptive responses to elevated CO_2 may be constrained by having to maintain the ability to deal with fluctuations in salinity. This may be especially important for calcifying phytoplankton populations that live in or close to estuaries with widely fluctuating salinity levels (Paasche et al. 1996).

That we could not find divergent pleiotropic effects in the high light but in the low-salinity environment may have several explanations. First, the two challenge experiments were performed about 250 generations apart from each other. Although we cannot rule out an effect of time, this explanation appears unlikely because we had already found a marked adaptive response in all replicates at the 500 generations mark. The different outcome of the chosen novel environments to produce divergent phenotypes may also be attributed to qualitative differences between the

environments. The low-salinity environment may fall outside the boundaries for an environmental parameter for which the population has acquired adaptive plasticity in the form of acclimation whereas the high-light environment does not (Alpert and Simms 2002; Paasche 2002). In consequence, the low-salinity environment is stressful, sensu decreasing Darwinian fitness, whereas the benign high-light environment actually increases fitness in our experiment. Depending on the cause of the pleiotropy involved, it may only become apparent in a stressful environment. We would expect this to be the case for trade-offs that are the result of energy allocation, for example. Alternatively, the inability to detect divergent pleiotropy may reflect the absence of such pleiotropic mutations in genes relevant to the high-light environment. The same genes may be under selection in the ambient and high-light environments. This assumption would further suggest a very limited linkage of genes relevant to CO_2 and light adaptation in *E. huxleyi*. The reciprocal assay environments used to test for adaptation in the CO_2 selection experiment may also be considered as novel environments. That we did not find increased variance when transplanting high CO_2 selected populations into ambient CO_2 may indicate, similar to the latter explanation brought for high light, that the same genes are relevant to fitness under high and ambient CO_2 and therefore adaptation to one CO_2 environment results in a homogeneous response in a certain range of other CO_2 environments.

EXPERIMENTAL EVOLUTION IN A MARINE ALGA

One objective of our selection experiment was to incorporate an established model system from biological oceanography, the coccolithophore *E. huxleyi*, into microbial experimental evolution. We used a modified microbial selection experiment spanning 1000 generations of selection to investigate adaptive evolution in this globally important phytoplankton species to ongoing ocean acidification.

Interdisciplinary studies, assessing adaptive evolution in marine phytoplankton species to climate change scenarios, should be more widely used to fill the data gap commonly masked by extrapolating short-term physiological studies to predict phytoplankton responses in a future ocean (Riebesell et al. 2009; Collins 2012; Reusch and Boyd this issue). New model systems also hold the opportunity for evolutionary biology to test for generality of concepts beyond the limited group of classical model organisms. In particular, diploid eukaryotes from the marine phytoplankton provide important study systems for evolutionary biology because they represent deeply divergent polyphyletic groups (Falkowski et al. 2004) that can be used to study fundamental evolutionary concepts. For example, the bulk of comparative studies on the consequences of diploidy versus haploidy for the rate of adaptation come from yeast (e.g., Zeyl et al. 2003) while phylogenetically deeply divergent eukaryotic (diploid and haploid) and prokaryotic (haploid) phytoplankton species may allow for more systematic and inclusive tests of the role of ploidy for adaptation. Finally, there are also obvious drawbacks of using marine algae in experimental evolution under climate change scenarios. Most challenging are probably the sophisticated and labor-intensive culturing work and seawater carbonate chemistry manipulations and measurements that by far exceed space and time requirements of classical model species such as *E. coli* or yeast. Nonetheless, considering the need for reliable estimates of future ocean responses to ongoing climate change (Bell and Collins 2008; Riebesell et al. 2009; Boyd et al. 2010), the gain in value from an ecological and socioeconomical perspective makes such applied evolution using marine microbes worthwhile.

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5.2 Additional Information - Publication II

Author contributions

This chapter is published in a scientific journal under multiple authorship. My contribution to this work is described below.

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6 Publication III

6.1 Adaptation to ocean acidification in *Emiliania huxleyi* involves genes putatively relevant to pH regulation and bicarbonate transport

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Manuscript is prepared for submission to a scientific journal

Adaptation to ocean acidification in *Emiliania huxleyi* involves genes putatively relevant to pH regulation and bicarbonate transport

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Coccolithophores are unicellular marine autotrophs that not only fix organic carbon by photosynthesis but also produce biogenic calcite scales. As such, they contribute substantially to marine primary production and carbon export to the deep ocean. On-going ocean acidification, the drop of seawater pH due to uptake of anthropogenic carbon dioxide by the surface ocean, impairs calcifying marine organisms. When exposed to lowered seawater pH, the coccolithophore *Emiliania huxleyi* commonly shows decreased growth and calcification rates, whereas our understanding of pH sensitive cellular processes, their mode of inhibition and adaptive restoration is still limited. Here we report on the expression of 10 candidate genes in *E. huxleyi* populations adapted for 500 generations to high CO_2 compared to non-adapted control populations that were kept under ambient CO_2. We found a physiological (short-term) response in 8 out of 10 genes that corresponds with high CO_2 induced decrease of growth and calcification rate. Moreover, high CO_2 adapted populations revealed an adaptive (long-term) response in 3 genes potentially relevant to pH regulation and bicarbonate transport that agrees with restored growth and calcification rate. Our analysis provides new evidence that cytosolic pH regulation is a primary target but also a site of adaptation under ocean acidification. These findings contribute to our understanding of cellular mechanisms involved in inhibition of growth and calcification as well as evolutionary adaptation to ocean acidification in *E. huxleyi*.

Marine phytoplankton plays a key role in the ocean's food webs and biogeochemical cycles (Falkowski 2012). One group of particular interest are coccolithophores (Haptophyta, Prymnesiophyceae), unicellular marine algae that are characterized by their ability to produce delicate calcite scales, the coccoliths (Paasche 1968). *Emiliania huxleyi* is considered to be the most abundant coccolithophore species in contemporary oceans (Westbroek et al. 1989). It forms vast blooms and contributes about half of the global biogenic calcite production (Westbroek et al. 1993). Excess fossil fuel derived CO_2 equilibrates with the surface ocean and results in a drop of seawater pH, termed ocean acidification (Caldeira and Wickett 2003). This phenomenon represents a major threat for many marine organisms, especially for those producing calcite structures (Orr et al. 2005; Fabry et al. 2008; Riebesell and Tortell 2011), with likely consequences for marine carbon fluxes and ecosystem functioning (Riebesell et al. 2009; Riebesell and Tortell 2011). Coccolithophores are no exception. Numerous short-term studies have focused on the physiology of *E. huxleyi* and other coccolithophores and shown growth and calcification rates to decrease in most species under acidified seawater conditions (Riebesell and Tortell 2011). While there is a profound understanding of short-term responses (Paasche 2002; Zondervan 2007; Rost et al. 2008), the potential for evolutionary adaptation has only recently been addressed (Lohbeck et al. 2012a). In a 500 generations selection experiment we investigated the adaptive potential of freshly isolated *E. huxleyi* clones to ocean acidification. We found about 3 % increase in fitness and 50 % restoration of calcification rate in single-clone

derived high CO_2 adapted populations when compared to control populations under ocean acidification conditions. We concluded that adaptive evolution in this key phytoplankton species is possible and potentially swift enough to keep pace with global climate change (Lohbeck et al. 2012a). Interestingly, after 500 generations of high CO_2 selection calcification rate was independently restored across all high CO_2 adapted populations, even though our artificial selection regime targeted growth rate only.

Biogenic calcite production in *E. huxleyi* and other coccolithophores is a highly regulated cellular process (Mackinder et al. 2010) with large ecological and biogeochemical implications (Westbroek et al. 1989; Milliman 1993). However, our knowledge on the underlying molecular mechanisms as well as its ecological relevance is still limited (reviewed in Paasche 2002; Mackinder et al. 2010; Raven and Crawfurd 2012). Various hypotheses on the ecological role of calcification have been discussed, including mechanical protection against grazing or virus attack, buoyancy regulation, modification of intracellular light levels, high light protection and a carbon concentration mechanism to fuel photosynthesis, with no consensus emerging to date (reviewed in Raven and Crawfurd 2012).

As challenging as to resolve the ecological function of calcification is to work out by which means lowering seawater pH actually inhibits calcification and growth in *E. huxleyi*. A promising approach seems to investigate pH sensitive cellular processes, their interplay and mode of inhibition at low seawater pH (Bach et al. 2011; Suffrian et al. 2011; Taylor et al. 2011). Here we present data on the physiological (short-term) and the adaptive (long-term) expression responses of 10 selected candidate genes using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). We sampled replicate populations that were selected for 500 asexual generations under elevated and control CO_2 conditions and then tested under reciprocal CO_2 assay conditions (Lohbeck et al. 2012a). Our approach covers a range of genes involved in bicarbonate uptake and transport, pH regulation, calcium transport and binding as well as photosynthetic carbon fixation. In particular, we have investigated the expression of genes coding for a putative bicarbonate transporter from the solute carrier 4 family (AEL1), a putative cytosolic alpha carbonic anhydrase (αCA), a putative membrane associated delta carbonic anhydrase (δCA), a putative calcium/proton exchanger (CAX3), a putative vacuolar type two-sector proton pump (ATPVc/c'), a putative plasma membrane type proton pump (PATP), a putative sodium/proton exchanger (NhaA2), a low CO_2 induced gene of unknown function found in *E. huxleyi* (LCIX), a calcium binding protein associated with coccolith polysaccharides in *E. huxleyi* (GPA), and the large subunit of ribulose bisphosphate carboxylase-oxygenase (Rubis-CO, RB).

The regulatory response of a given gene under ocean acidification will crucially depend on its specific function in *E. huxleyi*. As the specific functions of our candidate genes are largely unknown for *E. huxleyi*, we can only speculate on consequences and expectations of expression changes under ocean acidification (Mackinder et al. 2011). Nevertheless, we can formulate some general hypotheses. Up-regulation in the short-term response can be indicative for a cellular response to counteract negative effects of ocean acidification. Hence, it may identify gene products that are directly affected by ocean acidification. In contrast, down-regulation may identify gene products involved in cellular processes that are negatively affected by their dependence on impaired components or actively down-regulated due to resource reallocation under ocean acidification. In the latter cases, down-regulation reflects the adjustment of the respective gene product to an altered physiological state of the cell caused by lowered seawater pH. In the adaptive response, regulation of a gene that was altered by a beneficial mutation can be in any direction, depending on the function of the gene and the effect of the mutation. However, it appears unlikely that a beneficial mutation occurred in one of the 10 candidate genes. Therefore, a regulatory adaptive response will most likely represent a restored molecular phenotype that corresponds with restored growth and calcification rates observed in adapted populations (Lohbeck et al. 2012a). It further may point to a closely linked cellular process that is affected by a beneficial mutation and hence involved in the adaptive response of *E. huxleyi* to ocean acidification.

By investigating this diverse set of candidate genes, underlying key cellular processes that are potentially relevant to pH regulation, carbon acquisition and transport, calcification and

photosynthesis (Corstjens et al. 1998; von Dassow et al. 2009; Richier et al. 2011; Mackinder et al. 2011), we aim to improve our understanding on the molecular mechanisms relevant to inhibition and adaptive restoration of growth and calcification rate in *E. huxleyi* under ocean acidification.

Methods

Cell isolation and culturing - The gene expression analysis performed in this study is based on RNA samples taken from the reciprocal assay experiment after 500 generations of CO_2 selection described in Lohbeck et al. (2012a). Detailed information regarding experimental design, culturing and sampling can be found in the supplementary methods section S1 of Lohbeck et al. (2012a).

In brief, clonal cultures were founded by single cell isolation and confirmed to originate from a single diploid cell by microsatellite genotyping (Lohbeck et al. 2012a). Experimental populations were grown in 250 ml Schott Duran square flasks filled with 310 ml artificial seawater medium in a Sanyo MLR-351 light cabinet at 15°C, 150±10 µmol $m^{-2}s^{-1}$ photon flux density, 16:8 h light:dark cycle and continuous rotation.

Artificial seawater (ASW) was produced as in Kester et al. (1967) with the exception of 2380 µmol bicarbonate kg^{-1} ASW addition (Merck) and subsequent 0.2 µm sterile filtration (Whatman Polycap 75AS, GE Healthcare) and addition of 64 µmol kg^{-1} nitrate, 4 µmol kg^{-1} phosphate (nutrient ratio after Redfield 1958), trace metals and vitamins according to f/8 adapted from Guillard and Ryther (1962), 10 nmol kg^{-1} selenium after Danbara and Shiraiwa (1999) and 2 ml kg^{-1} sterile filtered North Sea water to exclude any limitations by micronutrients. The applied nutrient concentrations were sufficient to prevent nutrient limitation. ASW media were aerated with CO_2 enriched air for 24 h at the treatment levels of 400, 1100 and 2200 µatm pCO_2 using a controlled CO_2 gas mixing system. Evaporation was minimized by preceding gas wash bottles filled with MilliQ water. Aerated ASW media were carefully pumped into the culture flasks using a sterile silicon hose to minimize gas exchange.

Carbonate chemistry measurements - Seawater carbonate chemistry was determined by dissolved inorganic carbon (DIC) and total alkalinity (TA) measurements. DIC samples were taken for all treatment groups prior to inoculation and measured colorimetrically using a SOMMA autoanalyzer. TA was measured from all ASW batches by open-cell acidimetric titration using a Metrohm Basic Titrino 794. CO_2 partial pressure in the culture media was calculated from DIC and TA measured prior to inoculation and the draw-down estimate from final cell numbers and organic and inorganic carbon quotas using the software CO2SYS (Lewis and Wallace 1998) with solubility constants after Roy et al. (1993). Average culture pCO_2 values of 361.6 µatm (±28), 1067.2 µatm (±102) and 2021.0 µatm (±279) for ambient, medium and high CO_2 treatments differed not significantly from the desired treatment levels which are therefore used in the text and figures.

Experimental procedures - In the selection experiment replicated populations were propagated for 500 generations under ambient (400 µatm), medium (1100 µatm) and high (2200 µatm) pCO_2 conditions by serial transfer of exactly 10^5 cells every 5 days. Cell densities and diameters were measured in triplicate with a Beckman Coulter Z2 Particle and Size Analyzer. Measurements were always performed at the same time of the day. Exponential growth rates µ were calculated from cell densities according to Equation 1 where N_1 and N_0 are cell concentrations at the beginning and end of a batch cycle and d is the duration of the batch cycle in days.

$$\mu = (\ln N_1 - \ln N_0)/d \quad \textit{(Eq. 1)}$$

Throughout the experiments all populations grew exponentially and never reached the stationary phase. In the assay experiment populations were tested for adaptation to high CO_2-selection after 544 (ambient), 512 (medium) and 448 (high CO_2) mitotic divisions. Populations grown at ambient CO_2 were compared to populations grown at high CO_2, in both, the ambient and high CO_2 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] and (ii) [ambient vs. high CO_2 selection

condition] x [ambient vs. high CO_2 assay condition]. One full acclimation cycle (~6-8 cell divisions) was applied prior to the assay cycle to account for non-genetic effects after transfer into a new environment. As response variables, exponential growth rates, cell diameter, particulate inorganic (PIC) and organic carbon (POC) per cell and their production rates were assessed and published in Lohbeck et al. (2012a). Here we focus on the corresponding gene expression response of 10 genes putatively involved in pH regulation, calcification and photosynthesis.

RNA sampling, extraction and reverse transcription - For gene expression analysis 250 ml cell suspension were filtrated onto 0.8 µm polycarbonate filters (GE Healthcare) immediately after culture flasks were taken out of the light cabinet. Cells were flushed off the filter with 500 µl RNAlater (Qiagen) and pipetted in 1.5 ml Eppendorf cups that were placed on ice to allow cells to settle out for about 2 h and then were frozen at -20°C until further processing. RNA was extracted using an RNeasy kit (Qiagen) following the manufacture's protocol. RNA extractions were verified using a Qubit fluorometer (Life technologies). RNA samples were stored at -80°C until further processing. Reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen) following the manufacture's protocol.

Candidate genes - Genes of interest (GOI) were chosen from promising candidate genes potentially involved in pH regulation, calcification and photosynthesis from the literature. A complete list of all candidate genes and endogenous reference genes (ERGs), their putative functions, primer details, amplicon sizes and corresponding references are given in Table 1.

qRT-PCR - The amount of target gene mRNA was explored by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) on a StepOne Plus Cycler (Applied Biosystems) using the Fast Cyber Green QPCR Master Mix (Applied Biosystems). Amplification efficiency was assessed by linear regression of cycles to cross a fixed threshold from the standard curves of a six step dilution series ranging from 1:4 to 1:1024. Efficiencies of 81 to 98 % were calculated from the slopes ranging from -3.4 to -3.9 with $R^2 > 0.98$. All plates were run in technical triplicates. The variance among triplicates was inspected and individual outliers were removed when the variance was > 0.3. Non-reverse-transcription controls and non-template controls were run in parallel to the genes of interest on each plate and were always found to be negative. We considered α-Tubulin (αTUB), Actin (Actin) and Elongation factor 1 (EFG1) as potential endogenous reference genes (ERGs). However, only α-Tubulin showed sufficient stability over all treatments and was therefore used as ERG while the others were excluded.

The negative difference in cycles to cross the threshold value (-ΔCT) between the ERG and the respective GOI were calculated for all 5 replicate populations and technical replicates individually according to Equation 2. Then mean -ΔCT values were calculated for all 5 individual replicate populations from -ΔCT values of the technical replicates. These mean -ΔCT values were used for subsequent statistical analysis as described below. For graphical depiction −ΔΔCT values were calculated for each individual replicate population and its corresponding control population according to Equation 3. Then −ΔΔCT means and standard errors that are shown in the graphs were calculated from these 5 individual replicate −ΔΔCT values. Fold expression change values given in the text were calculated from mean −ΔΔCT values. Asterisks in the plots indicate significant differences of mean -ΔCT values underlying the depicted −ΔΔCT value.

$$-\Delta CT = CT_{ERG} - CT_{GOI} \quad (Eq.\ 2)$$

$$-\Delta\Delta CT = (-\Delta CT_{treatment}) - (-\Delta CT_{control}) \quad (Eq.\ 3)$$

Table 1: List of genes of interest (GOI) and endogenous reference genes (ERGs) from *Emiliania huxleyi* investigated in this study. Gene name as used in the text, full name, putative function, primer name, primer sequence, amplicon size and reference are given.

Gene name	Full name	Putative function	Primer name	Primer sequence 5'-3'	Amplicon size	Reference
EFG1	Elongation Factor 1	endogenous reference gene	EFG1_F	GCT GGA AGA AGG ACT TTG TTG	101	Mackinder et al. 2011
			EFG1_R	TCC ACC AGT CCA TGT TCT TC		
Actin	Actin	endogenous reference gene	Actin_F	GAC CGA CTG GAT GGT CAA G	96	Mackinder et al. 2011
			Actin_R	GCC AGC TTC TCC TTG ATG TC		
αTUB	α Tubulin	endogenous reference gene	αTUB_F	GCA TCG CCG AGA TCT ACT C	84	Bach et al. *in prep.*
			αTUB_R	TCG CCG ACG TAC CAG TG		
RB	Rubisco	Gene coding for large subunit of RUBISCO	RB_F	CAA TCG GTC ACC CAG ATG GTA	100	Bruhn et al. 2010
			RB_R	GCG ATA TAA TCA CGG CCT TCG		
AEL1	Anion Exchanger Like 1	Bicarbonate transporter, SLC4 family	AEL1_F	TTC ACG CTC TTC CAG TTC TC	102	Mackinder et al. 2011
			AEL1_R	GAG GAA GGC GAT GAA GAA TG		
αCA	α Carbonic Anhydrase 2	Alpha carbonic anhydrase	αCA2_F	AGA GCA GAG CCC TAT CAA CA	134	Richier et al. 2011
			αCA2_R	TCG TCT CGA AGA GCT GGA A		
δCA	δ Carbonic Anhydrase	Delta carbonic anhydase	δCA_F	ACG AGC ACG AGA TGT TCA AG	87	Bach et al. *in prep.*
			δCA_R	TCT CGC CAA CCA TCA TCT C		
CAX3	Ca^{2+}/H^{+} exchanger 3	Ca2+/H+ exchangers, similar to CAX family	CAX3_F2	CTC CTC TGC GTC TTT GCA T	90	Mackinder et al. 2011
			CAX3_R2	GAG GGC GGT GAT GAG GTA		
ATPVc'/c	Vacuolar-type H^{+} pump	Vacuolar H+-ATPase, V0, subunit c/c'	ATPV_F	TAC GGC ACT GCA AAG TCT G	83	Mackinder et al. 2011
			ATPV_R	ACG GGG ATG ATG GAC TTC		
PATP	Plasma membrane type H^{+} pump	P type H+-ATPase	PATP_F	GAG CAC AAG TTC CTC ATC GTC	105	Bach et al. *in prep.*
			PATP_R	CAC GTC GGC CTT CTT GAG		
NhaA2	Na^{+}/H^{+} exchanger 2	Na+/H+ antiporter	NhaA2_F	CTC GTC TGC TAT GGC ATC TC	80	Bach et al. *in prep.*
			NhaA2_R	GTT GCT CGC GTC CAT TC		
LCIX	Low CO_2 induced gene	Protein in Emiliania huxleyi 457793	LCIX_F	CAG CAG TCG TGG CTC AAG	94	Bach et al. *in prep.*
			LCIX_R	CGT AAG CGA CGT GGA TCA G		
GPA	Ca^{2+} binding protein	Calcium-binding protein in Emiliania huxleyi	gpaBR_F	AGG CCT TCT CCA GCA TCA T	70	Richier et al. 2009
			gpaBR_R	GTT CAG CGT GCT CTC CGA G		

Statistical analyses - Statistical analyses were performed using R 2.15.1 (R Core Team 2012). All statistical analyses were done on -ΔCT data. We applied a factorial design with two missing treatment combinations, since we did not assay populations evolved at 1100 µatm pCO_2 under 2200 µatm pCO_2 and *vice versa*. Consequently, the -ΔCT data set for each gene of interest was split and analyzed by 2x2-factorial analyses of variance (ANOVA). Planned contrasts for assessing adaptation were performed only when in the initial 2-factorial ANOVA either the main effect "selection condition" or the interaction "selection x assay condition" was statistical significant. To account for multiple testing P-values were corrected using false discovery rate control after Benjamini and Hochberg (1995). Variance homogeneity was verified using Levene's test and normality of residuals was tested using the Shapiro-Wilk test.

Results

In this study we investigated the expression of 10 candidate genes in *E. huxleyi* populations adapted to ambient, medium and high CO_2 levels when assayed under their respective selection and reciprocal CO_2 conditions (Lohbeck et al. 2012a). The analysis comprises two different categories of gene expression responses. On the one hand, the physiological (short-term) response that is based on the comparison of populations transferred from ambient into medium and high CO_2 to their control populations that are kept under ambient CO_2. On the other hand, the adaptive (long-term) response that is based on the comparison of medium and high CO_2 adapted populations to ambient CO_2 adapted control populations when assayed under medium or high CO_2, respectively.

The physiological (short-term) response was characterized by stronger differences in mean expression levels than the adaptive response. Most investigated genes were significantly down-regulated in populations assayed under medium and high CO_2 relative to their respective control populations assayed under ambient CO_2 (Fig. 1). The gene expression response was consistent with decreased growth and calcification rates found under medium and high CO_2 conditions (Lohbeck et al. 2012a). A significant down-regulation

under medium and high CO_2 conditions relative to control conditions was found in the genes coding for a putative bicarbonate transporter (AEL1), a putative vacuolar proton pump (ATPVc/c'), a putative calcium/proton exchanger (CAX3), a delta carbonic anhydrase (δCA) and a putative sodium/proton exchanger (NhaA2). These genes were about 1.5 to 1.8 fold down-regulated under medium and about 2 to 5 fold down-regulated under high CO_2 conditions. Genes coding for an alpha carbonic anhydrase (αCA), a putative membrane associated proton pump (PATP) and RubisCO (RB) showed a significant down-regulation in high CO_2 assayed populations only (2 to 6 fold), while genes coding for the calcium binding protein (GPA) and a low CO_2 induced protein (LCIX) showed no significant response at all (results from statistical analyses are given in *Suppl. Table S1*).

In the adaptive response mean transcript levels were highly variable among replicate populations for almost all genes we investigated, in particular among the high CO_2 adapted populations. Nevertheless, a general pattern was that all but one gene (GPA) revealed higher mean expression levels (1.1 – 2.5 fold up-regulation) in high CO_2 adapted compared to control populations. However, statistical significance was only observed for 3 out of 10 genes (Fig. 2). We identified a significant up-regulation in genes coding for the putative bicarbonate transporter AEL1 and the two putative proton pumps ATPVc/c' and PATP in elevated CO_2 adapted populations relative to ambient CO_2 adapted control populations. AEL1 and ATPVc/c' were significantly up-regulated about 1.5 fold only in medium CO_2 adapted populations, while a similar but non-significant response was found in high CO_2 adapted populations (ANOVA, significant main effect "selection environment": $F_{1,16}$=10.7, P=0.0048 [AEL1]; $F_{1,16}$=7.54, P=0.0143 [ATPVc/c']; planned contrasts "selection x assay condition": $F_{1,16}$= 10.08, P=0.0059 [AEL1]; $F_{1,16}$= 6.06, P=0.0255 [ATPVc/c']). PATP was significantly up-regulated about 2 fold only in high CO_2 adapted populations while a weaker response in medium CO_2 adapted populations failed statistical significance (ANOVA, significant interaction "selection x assay condition": $F_{1,16}$=10.06, P=0.0059; planned contrasts: "selection x assay condition": $F_{1,16}$=5.94, P=0.0269; further results from statistical analyses are given in *Suppl. Table S1*).

Discussion

We performed a qRT-PCR based candidate gene approach to investigate gene expression in single-clone derived *Emiliania huxleyi* populations selected for 500 generations under ambient (400), medium (1100), and high (2200 µatm) pCO_2 conditions and assayed under their respective selection and reciprocal CO_2 condition (Lohbeck et al. 2012a). Populations adapted to elevated CO_2 showed up to 3 % higher growth and 50 % restoration of calcification rate compared to non-adapted control population when tested under elevated CO_2 (Lohbeck et al. 2012a). Here we report on the corresponding expression changes of 10 genes with potential relevance to key cellular processes such as pH regulation, calcification, photosynthesis, and ultimately growth. We analyzed the physiological (short-term) response of ambient CO_2 adapted populations transferred into elevated CO_2 relative to their control populations assayed under ambient CO_2. This response is driven by the different CO_2 assay environments populations were exposed to. Moreover, we explored the adaptive (long-term) response of elevated CO_2 adapted populations relative to ambient CO_2 adapted control populations when assayed under elevated CO_2. The adaptive response is measured under the same assay conditions and compares replicated populations with a different adaptation history. In contrast to the short-term response, the magnitude of expression change as function of different adaptation history is generally lower because

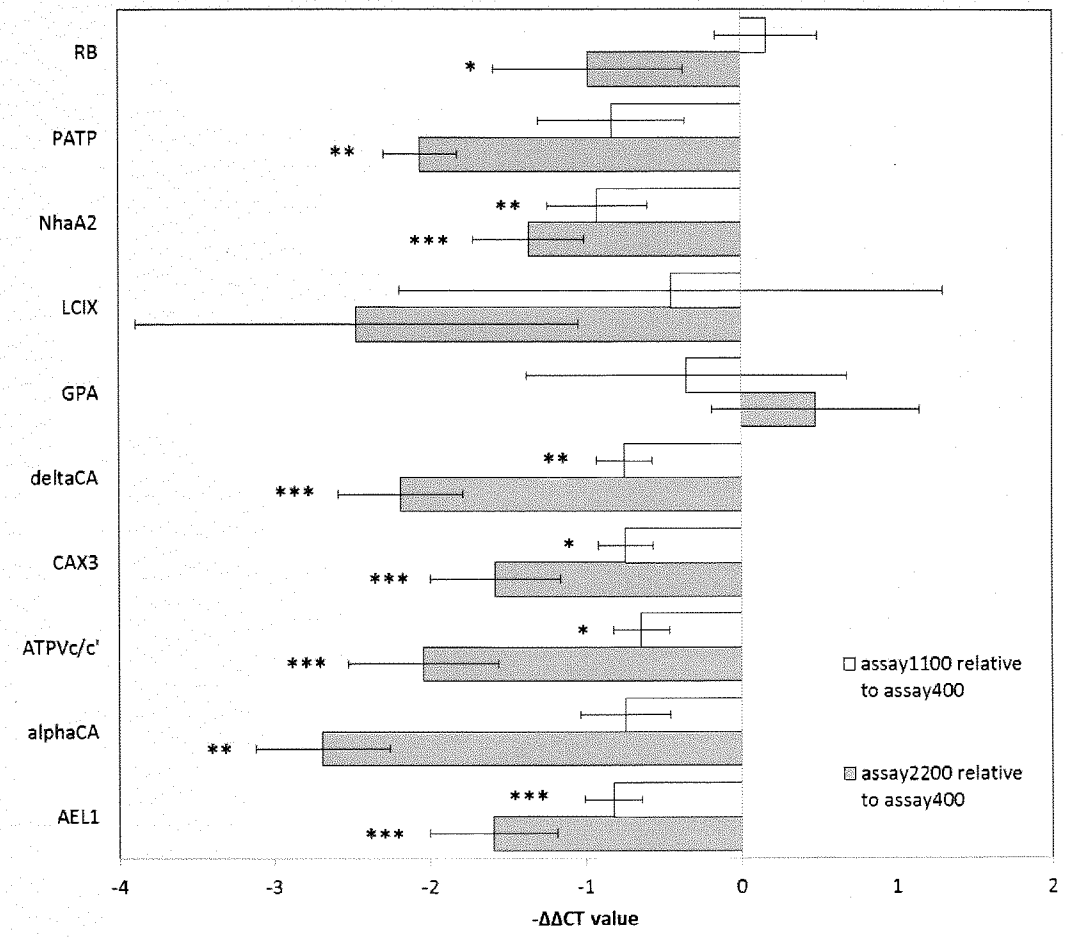

Figure 1: Physiological (short-term) response in relative gene expression of 10 candidate genes in replicate populations (n=5) when tested under medium (1100 µatm) and high (2200 µatm) pCO_2 conditions. Differences in mean gene expression levels are indicated as $-\Delta\Delta CT$ values ± standard error of the mean. White bars indicate gene expression of control populations assayed at 1100 µatm pCO_2 relative to control populations assayed 400 µatm pCO_2. Grey bars indicate gene expression of control populations assayed at 2200 µatm pCO_2 relative to control populations assayed at 400 µatm pCO_2. Significance levels are indicated: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

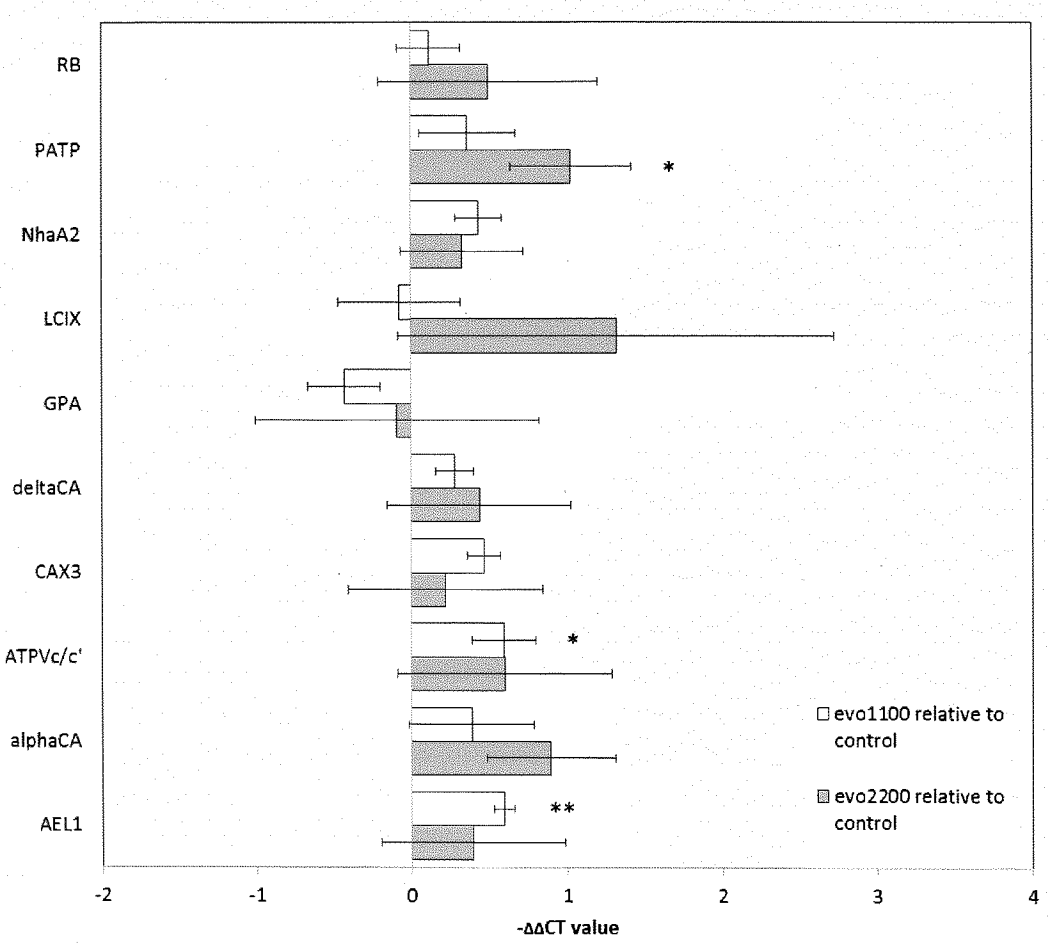

Figure 2: Adaptive (long-term) response in relative gene expression of 10 candidate genes in replicate CO_2 adapted *E. huxleyi* populations (n=5). Differences in mean gene expression levels between adapted and control populations are indicated as $-\Delta\Delta CT$ values ± standard error of the mean. White bars indicate gene expression in populations adapted to 1100 µatm pCO_2 relative to control populations adapted to 400 µatm pCO_2 when tested at 1100 µatm pCO_2. Grey bars indicate gene expression in populations adapted to 2200 µatm pCO_2 relative to control populations adapted to 400 µatm pCO_2 when tested at 2200 µatm pCO_2. Significance levels are indicated: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

it is exclusively controlled by the new genetic background of adapted relative to non-adapted populations. While we do not have any comparable data on the adaptive response, the physiological response of decreased growth and calcification rate under ocean acidification conditions is in line with the response of most *E. huxleyi* isolates investigated to date (Riebesell and Tortell 2011).

In the short-term response we found 8 out of 10 investigated genes to be down regulated under elevated CO_2 conditions, whereas the high CO_2 treatment consistently resulted in a stronger down-regulation than the medium CO_2 treatment (Fig. 1). This regulatory pattern agrees with a concomitant decrease in growth and calcification rate (Lohbeck et al. 2012a). Though, without precise knowledge on the location and function of the respective gene products in *E. huxleyi*, an allocation to any specific cellular process appears difficult.

Among the down-regulated genes were all 3 candidate genes relevant to carbon acquisition and transport, namely the putative bicarbonate transporter (AEL1) and the two putative carbonic anhydrases (αCA and δCA). However, αCA was significantly down-regulated only under high CO_2. These genes could be involved in carbon supply to calcification as well as photosynthesis. That we found a regulatory response that goes into the same direction as calcification rate but not photosynthetic carbon fixation (Lohbeck et al. 2012a) could support a proposed role of AEL1 in bicarbonate supply to calcification (von Dassow et al. 2009; Mackinder et al. 2011). Photosynthetic carbon supply may either involve another pathway or be switched to CO_2 rather than bicarbonate under high CO_2 conditions.

Moreover, all 4 genes potentially involved in pH regulation, namely genes coding for the putative vacuolar proton pump (ATPVc/c'), the putative calcium/proton exchanger (CAX3), the putative sodium/proton exchanger (NhaA2) and the putative membrane associated proton pump (PATP) showed decreased expression under elevated CO_2. Such a response supports the important role of cytosolic pH regulation in inhibition of growth and calcification under ocean acidification (Suffrian et al. 2011; Taylor et al. 2011). Down-regulation of AEL1, ATPVc/c' and CAX3 is likely linked to decreased calcification rate as Mackinder et al. (2011) could show a positive correlation between calcification and the regulatory response of these genes by comparing isogenic calcified and non-calcified *E. huxleyi* populations.

We found a significant down-regulation under high CO_2 but no significant expression change under medium CO_2 in the gene coding for the large subunit of RubisCO (RB). Down regulation of RB translates into lower enzyme abundance, however must not necessarily indicate decreased photosynthetic carbon fixation that also depends on the CO_2 concentration at the active site of RubisCO. Increased CO_2 availability under ocean acidification may therefore increase RubisCO's carboxylase activity and allow efficient photosynthesis with less enzyme units compared to ambient CO_2 conditions.

The genes coding for the calcium binding protein (GPA) that may be involved in calcification and a low CO_2 induced protein of unknown function (LCIX) showed no significant expression change at all. GPA codes for a protein supposed to be involved in calcium binding and coccolith formation (Corstjens et al. 1998), though contrasting findings in transcript levels from calcifying and non-calcifying *E. huxleyi* clones question a straight forward association of GPA and calcification. Von Dassow et al. (2009) performed an analysis of expressed sequence tags (ESTs) from genetically identical non-calcifying haploid and calcifying diploid *E. huxleyi* clones and found GPA to be expressed in both life stages. Richier et al. (2009) used a qRT-PCR based approach and found GPA to be expressed much stronger in diploid than in haploid *E. huxleyi* cells. Using the same technique Mackinder et al. (2011) found a

negative correlation between GPA expression and calcification and Richier et al. (2011) did not find any expression change in this gene under elevated CO_2 conditions. However, they also did not find any decrease in calcification in their experiments under CO_2 levels that are comparable to our medium CO_2 treatment, which is in contrast to decreased calcification rates found in the isolates we have investigated.

The overall short-term response in gene expression represents the molecular phenotype of acclimated *E. huxleyi* populations under elevated CO_2. A prominent down-regulation but no significant up-regulation in the investigated genes suggests that the respective gene products are not directly affected by ocean acidification. If this was the case, we would expect an up-regulation response to mitigate inhibitory effects of ocean acidification. In contrast, that we observed a marked down-regulation response most likely points to an adjustment of the gene product level to the altered physiological state of the cell caused by ocean acidification. Allocating these gene products to specific cellular process will help to identify potential molecular target sites of ocean acidification in *E. huxleyi*.

That we found restoration of growth and calcification rate in replicate high CO_2 adapted populations is most likely the result of novel beneficial mutations that differ among replicate selection lines (Lohbeck et al. 2012a; Lohbeck et al. 2012b). We have demonstrated such divergent genetic bases of high CO_2 adaptation by challenging replicate CO_2 adapted populations in a novel environment (Lohbeck et al. 2012b). On the one hand, variation in gene expression response may therefore partly reflect different genetic backgrounds resulting from specific mutations in each replicate population. On the other hand, a prominent mean response may indicate that the respective gene products are similarly affected by different mutations among replicate populations, resulting in a convergent molecular phenotype.

We found large variation in gene expression for most investigated genes among replicate populations. Variation was most obvious in the adaptive response of putative pH regulation, carbon transport and calcification related genes AEL1, ATPVc/c' CAX3, δCA, GPA and NhaA2 in high CO_2 adapted populations. This pattern applied mostly to high CO_2 adapted populations, while adaptive changes appeared more uniform in medium CO_2 adapted populations (Fig. 2). Note that in the challenge experiment, designed to uncover hidden genetic variation in replicate populations, we only considered high CO_2 adapted populations (Lohbeck et al. 2012b).

In order to conclusively assess the individual phenotypic reaction of each replicate population, further sub-replication would have been necessary. As we were not able to increase the level of replication we can only speculate that the high variance in expression change resulted from different evolutionary trajectories of individual populations. We cannot rule out technical variation in culturing and sampling procedures to contribute to the observed variation. At least technical variation of triplicate samples in the qRT-PCR assay was small, making a significant contribution to observed variation unlikely. Interestingly, we did not find the overall variance of gene expression responses to differ significantly between ambient and elevated CO_2 adapted populations. This finding may question the role of divergent genetic bases of replicate high CO_2 adapted populations to be the main driver of observed variance in the gene expression response. This issue has to be addressed in future work with an appropriate level of replication.

To explore the relation of candidate gene regulation to beneficial mutations and the resulting phenotypic changes described earlier (Lohbeck et al. 2012a), we focused on significant adaptive mean responses in the genes AEL1, ATPVc/c' and PATP. AEL1, coding for a putative bicarbonate transporter, and ATPVc/c', coding for a putative vacuolar type proton pump, were both significantly up-

regulated only in medium CO_2 adapted populations. In high CO_2 adapted populations we found the same trend in the mean response, while large variation may point to different evolutionary trajectories in replicate populations as discussed above. The gene coding for a putative plasma membrane type proton pump (PATP) was significantly up-regulated only in high CO_2 adapted populations. In medium CO_2 adapted populations we found an obvious trend of up-regulation that failed statistical significance. In contrast to the latter interpretation, lower variance observed for PATP suggests a common adaptive response that was not strong enough to reach significance under the weaker selective pressure of medium CO_2 compared to high CO_2.

These three genes are promising candidates to identify cellular processes that are restored in adapted relative to non-adapted populations under ocean acidification conditions. Strikingly, two out of three genes are proton pumps, likely involved in cellular pH regulation and potentially also in calcification. Mackinder et al. (2011) proposed the vacuolar proton pump (ATPVc/c') may serve in active proton transport into Golgi derived vesicles while the plasma membrane associated proton pump (PATP) may acidify compartments of the peripheral endoplasmatic reticulum. Here protons may be used in exchange for calcium ions with the external medium to enrich this compartment with calcium to enable calcification at a later stage (Mackinder et al. 2011). The third gene that showed a significant adaptive response is the putative bicarbonate transporter from the solute carrier 4 family (AEL1). Notably, next to its proposed function in bicarbonate supply to calcification in *E. huxleyi*, transporters from this protein family are known to act as base transporters in the bicarbonate/carbonate buffer system in eukaryotic cells, a key determinant to cytosolic pH (Pushkin and Kurtz 2006).

From this perspective, all three genes that revealed a significant adaptive response have the potential to be involved in cytosolic pH regulation and hence may identify this process as a primary target of ocean acidification in *E. huxleyi*. This interpretation is in line with recent results from cell physiological studies. Under ocean acidification conditions, the lowering of seawater pH and not the associated changes in dissolved inorganic carbon was identified as the causative agent that inhibits growth and calcification in *E. huxleyi* (Bach et al. 2011). Suffrian et al. (2011) have demonstrated convincingly that *E. huxleyi* has poor abilities to regulate cytosolic pH as the surrounding seawater acidifies. Taylor et al. (2011) identified voltage gated proton channels that serve to quickly release excess protons produced in calcification from the cytosol. Lowered seawater pH most likely disrupts the regulation of these channels by interfering with the plasma membrane potential and thereby affects cytosolic pH regulation (Taylor et al. 2011). The authors propose that calcification may be down regulated to relieve the cell from additional proton load as the cytosol acidifies. Under these conditions, the restoration of calcification in our high CO_2 adapted populations is likely to be caused by an adaptive response that restored the ability to regulate cytosolic pH under acidified seawater conditions rather than a direct adaptive response to the process of calcification.

We conclude that the gene products of the investigated candidate genes are probably not directly affected but relevant to cellular processes that are inhibited by ocean acidification. Further, we found new evidence that the genes AEL1, ATPVc/c' and PATP are involved in restoration of growth and calcification rate in *E. huxleyi* populations adapted to ocean acidification. This finding supports the scenario that cytosolic pH regulation is a primary cellular target to ocean acidification and a promising starting point to

further explore the molecular mechanisms behind the adverse effects of ocean acidification to *E. huxleyi*.

Acknowledgements

We thank Jana Meyer and Katrin Beining for laboratory assistance; Luke Mackinder for advice in candidate gene selection and qRT-PCR procedures. This project was funded by the German Federal Ministry of Education and Research; coordinated program 'BIOACID'.

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6.2 Additional Information - Publication III

Author contributions

This chapter is prepared for publication in a scientific journal under multiple authorship. My contribution to this work is described below.

Title: Adaptation to ocean acidification in *Emiliania huxleyi* involves genes putatively relevant to pH regulation and bicarbonate transport

Authors: Kai T. Lohbeck, Ulf Riebesell, and Thorsten B. H. Reusch

Author contributions: TBHR conceived the project, all authors designed the experiment, KTL performed the experiment. All authors analyzed and interpreted the data and wrote the manuscript.

7 Synthesis

This chapter summarizes the key findings that have resulted from my doctoral project and addresses how they advance our knowledge of marine phytoplankton responses to projected ocean change. Further, I discuss potential implications of my results for pelagic communities in general, ecosystem functioning and biogeochemical cycling and close with a section on future research perspectives.

7.1 Experimental Evolution and Ocean Acidification Research

Ocean acidification affects various marine organisms. While primary producers in general may benefit from increased carbon availability, organisms producing calcium carbonate shells or skeletons such as coccolithophores and other calcifying plankton, benthic molluscs, echinoderms, coralline algae and corals are mostly impaired (Fabry et al. 2008; Doney et al. 2009; Riebesell and Tortell 2011). Our present understanding of the impacts of ocean acidification on marine organisms is almost exclusively based on short-term experiments, while the potential for adaptation is mostly neglected (Collins 2011a). Therefore, one question of immediate concern is whether or not marine organisms can adapt fast enough to keep pace with ongoing ocean change (Reusch and Wood 2007; Bell and Collins 2008; Hoffmann and Sgro 2011). As phytoplankton has large population sizes and fast reproduction rates, this group appears to be particularly well suited to test the adaptive potential to ocean change (Reusch and Wood 2007; Collins 2011a). This is where experimental evolution and biological oceanography meet and open a new interdisciplinary research direction with large potential for mutual benefits, especially in the light of an urgent need to predict future responses of marine organisms with ecological, biogeochemical and socio-economic implications (Reusch and Boyd 2013).

7.2 The Adaptive Potential of *Emiliania huxleyi* to Ocean Acidification

In order to explore the adaptive potential of a key phytoplankton species to ocean acidification I exposed replicate populations derived from either a single or multiple genotypes of the coccolithophore *Emiliania huxleyi* for 500 generations to elevated CO_2 conditions and tested for adaptation. The experimental design is illustrated in Figure 1. The common short-term response of *E. huxleyi* when exposed to elevated CO_2 is a decrease in growth and calcification rate (Riebesell et al. 2000; Riebesell and Tortell 2011). While I

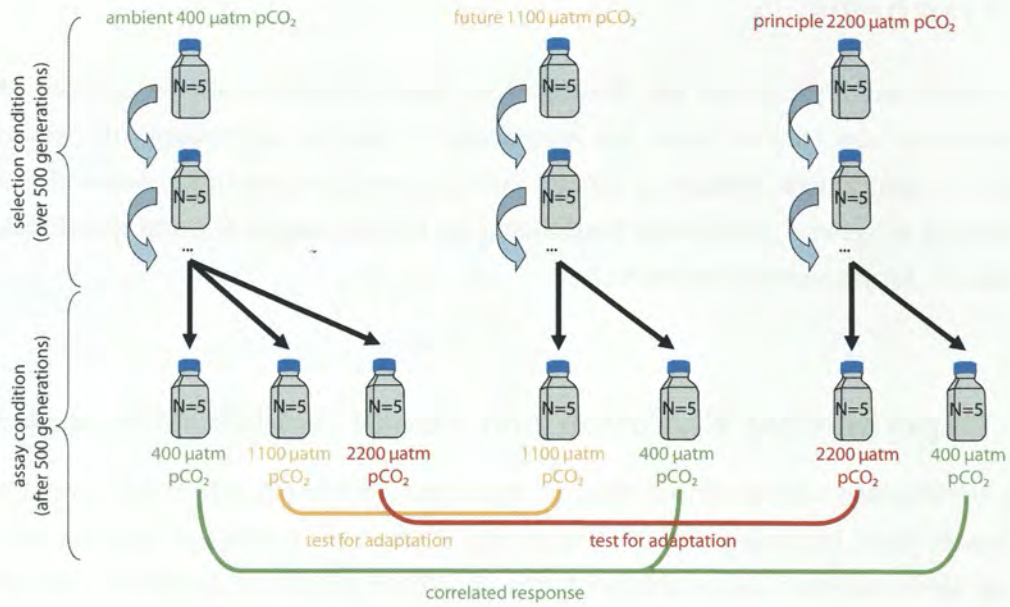

Figure 1: Experimental design of the long-term selection experiment. Replicate populations (N=5) were selected over 500 generations under ambient (400 μatm), future projected (1100 μatm), and high (2200 μatm) pCO_2 conditions, the latter serving as a proof of principle. In a reciprocal assay experiment we tested for the direct adaptive response to elevated CO_2 and the correlated response when adapted populations were exposed back to ambient CO_2.

observed this in the beginning of the experiment, I found partial restoration of growth and calcification rate in populations adapted to increased CO_2 levels after 500 generations. With this experiment, I was able to demonstrate for the first time that adaptive evolution in a key phytoplankton species is possible and potentially fast enough to act on climate change relevant time scales (Chapter 4).

I identified two mechanisms of adaptation to elevated CO_2 concentrations: i) genotypic selection as a mechanism of population-level adaptation in populations composed of multiple genotypes, and ii) appearance of advantageous new mutations that spread and eventually became fixed in populations derived from a single genotype (Figure 2).

Genotypic selection in the multi-clone populations describes the selective sorting of initially six genetically distinct clones via differences in their asexual fitness (exponential growth rate). Note that the competitive performance of clones is not necessarily related to their performance in single-genotype cultures (Collins 2011b). In all five replicate populations the same clones prevailed under the respective CO_2 treatment conditions.

Standing genetic variation in the experiment was small (six genotypes) compared to the apparently vast genetic diversity found in natural *E. huxleyi* populations (Medlin et al. 2000; Iglesias-Rodriguez et al. 2006). Large genetic diversity in natural popula-

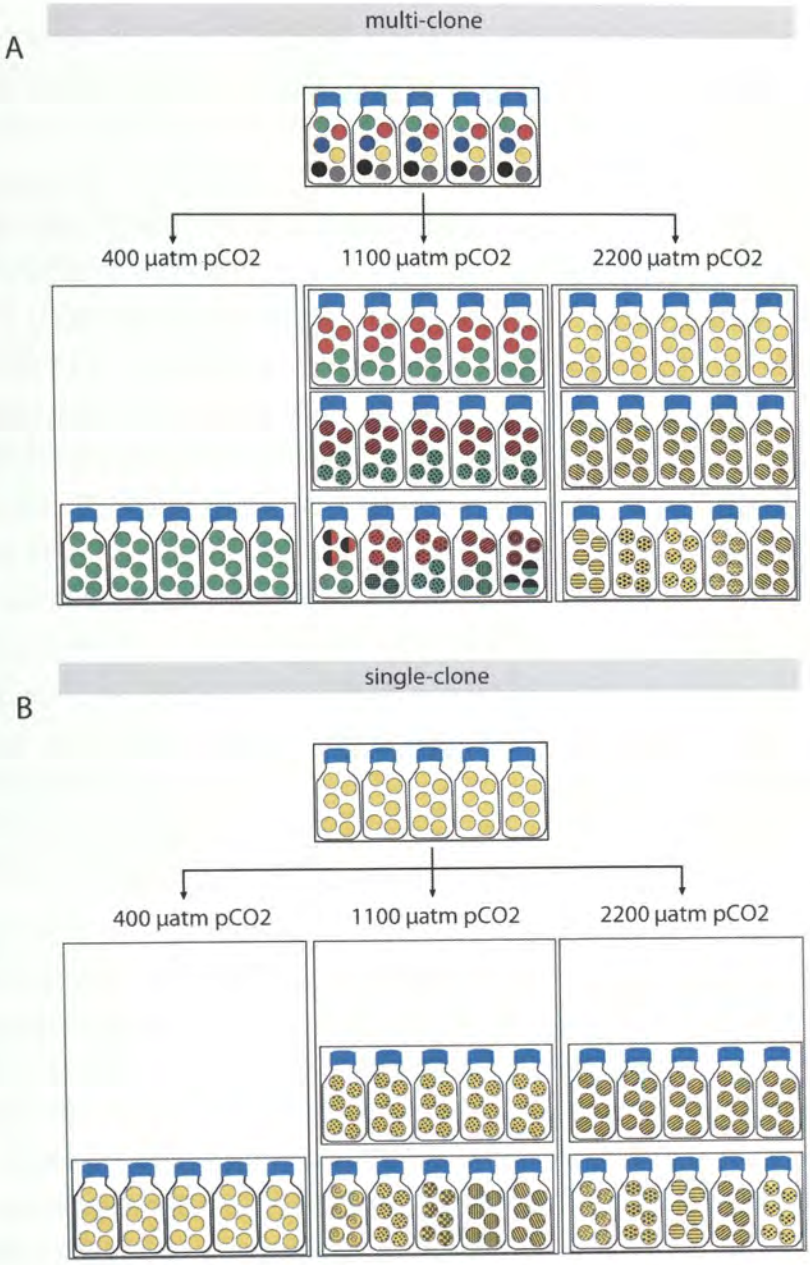

Figure 2: Illustration of the mechanisms of adaptation observed in the selection experiments. Multiple boxes per treatment illustrate potential scenarios underlying the observed responses. In all experiments we used 5 replicates (indicated by 5 flasks). The multi-clone experiment (A) started with equal contributions of 6 genotypes (indicated by different colors at the beginning of the experiment), while the single-clone experiment (B) started with only one genotype. We observed adaptation to elevated CO_2 in both experiments. In the multi-clone experiment adaptation resulted from selective sorting of genotypes (indicated by colors remaining after 500 generations) and potentially also by beneficial mutations (indicated by different patterns). In contrast, in the single-clone experiment, adaptation could only result from novel beneficial mutations (indicated by different patterns). In both experiments, either the same beneficial mutations could have occurred in all replicate populations or, different mutations could have occurred in individual populations (indicated by homogeneous and heterogeneous patterns).

tions suggests an important role of sexual reproduction that increases within-population additive genetic variance and creates a high potential for evolutionary adaptation (Kaltz and Bell 2002). In contrast, the mode of reproduction in the CO_2 selection experiment was asexual and did not generate new recombinant genotypes. That I found a prominent role of genotypic selection, even with such a small number of genotypes, emphasizes the important role that this adaptive mechanism will probably have in natural populations.

The observed adaptation in single-clone derived populations suggests that beneficial mutations occurred and rose to fixation over 500 generations of CO_2 selection. This is consistent with evolutionary theory when assuming reasonable mutation frequencies, associated fitness benefits and fixation times (Chapter 9.1, Supplementary Note S1). Phenotypic variance among elevated CO_2 adapted populations was not higher than among those adapted to ambient CO_2. Such phenotypic convergence points to functional constrains of adaptation (Travisano et al. 1995). Convergent phenotypes may have resulted either from the same mutations that must have been acquired independently by all replicate populations or by different mutations that have resulted in a similar phenotype (Lenski et al. 1991; Travisano et al. 1995). The likelihood that the same mutations occurred independently in all replicates is negligible (Woods et al. 2006). Consequently, different advantageous mutations are expected to make up different genetic bases of high CO_2 adaptation in replicate populations (Elena and Lenski 2003).

In order to test this assumption and further explore the genetic basis of high CO_2 adaptation in single-clone populations I challenged CO_2 adapted populations in a novel environment, where phenotypic divergence can be used as a proxy for different mutations underlying high CO_2 adaptation in replicate populations. The underlying concept requires that different mutations which are beneficial to fitness under high CO_2 conditions have divergent pleiotropic effects on fitness in the novel environment (Travisano et al. 1995). In fact, I found divergent pleiotropic effects in high CO_2 adapted but not in control populations and concluded that multiple evolutionary trajectories to high CO_2 adaptation are possible in *E. huxleyi*. These results illustrate that pleiotropy may constrain adaptation to ocean acidification in natural populations that have to face a far more complex environment (Chapter 5). At present, it is impossible to predict whether or not adaptation to one environmental factor (e.g. ocean acidification) facilitates or impedes adaptation to another stressor, say temperature. If pleiotropy is abundant, then there are even no universal answers to such a question. This is another case in point to highlight the relevance of evolutionary processes to predict future responses of marine phytoplankton to ocean change.

Ultimately, an understanding of the genetic basis of adaptation is needed to reliable predict the effects of ocean change on population viability (Reusch and Wood 2007;

Coelho et al. 2012). As complementary approach to get closer to the genetic bases of the observed phenotypic changes in CO_2 adapted *E. huxleyi* populations, I performed a candidate gene expression analysis that covered the physiological short-term and the adaptive long-term gene expression response. Different expression levels in CO_2 adapted and control populations may help to identify particular genes under selection in an acidified ocean (Whitehead and Crawford 2006; Reusch and Wood 2007). The success of a candidate gene approach relies mainly on the selection of appropriate candidate genes. Based on earlier work by Mackinder et al. (2011) and Bach et al. (2013), I chose 10 candidate genes relevant to pH regulation, carbon transport, calcification and photosynthesis. I found down-regulation of eight candidate genes in the short-term response and up-regulation of three candidate genes in the adaptive response to ocean acidification. My results suggest that pH regulation is involved in the inhibition and adaptive restoration of growth and calcification under ocean acidification (Chapter 6). This is in line with the outcome from recent cell-physiological studies where low seawater pH was found to inhibit specific proton channels from releasing excess calcification derived protons to the cell exterior (Suffrian et al. 2011; Taylor et al. 2011). Finally, this study adds another important step towards a comprehensive understanding of the molecular mechanisms involved in inhibition and adaptive restoration of growth and calcification in *E. huxleyi* cells facing an acidified ocean.

7.3 Future Phytoplankton Responses to Ocean Change

In my doctoral thesis I have demonstrated that the coccolithophore *E. huxleyi*, a key phytoplankton species of global relevance, has the potential to adapt to ocean acidification on climate change relevant time scales. It seems plausible that such a potential for adaptive evolution is common among fast reproducing marine microbes (Elena and Lenski 2003). However, this assumption needs to be validated for other important phytoplankton species and selection regimes in experimental evolution experiments. Unfortunately, to date there are only few comparable experiments that have investigated the adaptive response of eukaryotic phytoplankton to ocean change.

Crawfurd et al. (2011) exposed populations of the marine diatom *Thalassiosira pseudonana* for 100 generations to elevated CO_2 conditions and did not observe any adaptation to high CO_2. In this study there was probably not enough time for beneficial mutations to occur and become fixed in the population. Moreover, high CO_2 did not inhibit growth in *T. pseudonana* and consequently may provide a much weaker selective pressure than in *E. huxleyi*. The strength of selective pressure can affect the adaptive

potential of a species in a given time. This is because it influences the selective advantage of beneficial mutations and the rate at which they can establish in a population (Desai et al. 2007).

Another long-term CO_2 selection experiment was done by Collins and Bell (2004) who exposed the unicellular freshwater alga *Chlamydomonas reinhardtii* for 1000 generations to increased CO_2 concentrations. High CO_2 selected algae showed no specific adaptations to high CO_2 but poor growth when exposed back to ambient CO_2. The authors speculated that conditionally neutral mutations in genes relevant to the carbon concentration mechanism (CCM) may be responsible.

Although *C. reinhardtii* is a freshwater alga that experiences much stronger pH variations in its natural environment compared to marine algae, there may be some useful analogies. Most marine phytoplankton, except from coccolithophores, apparently benefit from increased carbon availability which alleviates carbon limitation of photosynthesis (Riebesell and Tortell 2011). Consequently, phytoplankton species which are not negatively affected by low pH can benefit from increased carbon availability. On short time scales, algae which rely on diffusive CO_2 uptake and those with inefficient CCMs are likely to benefit most from elevated CO_2 availability and gain a selective advantage over other algae that have costly CCMs, with a declining competitive value under ocean acidification (Rost et al. 2008). However, on longer time scales an evolutionary response will probably result in degradation and finally loss of CCMs as this trait is no longer under positive selection, again altering the competitive fitness of such algae.

Future phytoplankton populations will be shaped by adaptive evolution and respond to changing ocean conditions outside the range of contemporary genotypes. It remains to be resolved to what extent relative abundances of individual phytoplankton species or even entire functional groups, such as coccolithophores, will be altered and how this in turn will affect marine primary production, ecosystem functioning and biogeochemical cycles.

7.4 Broader Implications of Adaptive Evolution to Ocean Acidification

Ocean acidification induced changes in phenotypic traits of individual phytoplankton species, as well as changes in community composition, will have a large potential for cascading effects on higher trophic levels, ecosystem functioning and biogeochemical cycles (Riebesell and Tortell 2011).

Elemental stoichiometry was found to be affected by ocean acidification in several phytoplankton species. Carbon to nitrogen and nitrogen to phosphorus quotas in cyanobacteria and eukaryotic phytoplankton revealed that ratios either remained unaffected or strongly increased, suggesting implications for cycles of major nutrient elements in the ocean (Hutchins et al. 2009). Adaptive evolution in marine microbes may help to restore elemental stoichiometry of cells, given that these traits are relevant to fitness in a future ocean. Changes in phytoplankton elemental stoichiometry will also affect the elemental stoichiometry of organic matter which is sinking into the deep ocean, and thereby potentially affect carbon export (Riebesell et al. 2009).

Next to carbon export, increased organic carbon to nitrogen and phosphorus quotas reduce the nutritious value of phytoplankton cells to higher trophic levels and hence may affect marine food web dynamics (Doney et al. 2009). Moreover, Rossoll et al. (2012) have shown that under ocean acidification the fatty acid composition of the diatom *Thalassiosira pseudonana* becomes detrimental to growth and reproduction of the copepod *Acartia tonsa* feeding on this alga. Future phytoplankton selection experiments should therefore also consider the potential for adaptive restoration of elemental cell stoichiometry and fatty acid composition.

Ocean acidification has also direct detrimental effects on marine organisms that have much slower reproduction rates compared to unicellular phytoplankton (Orr et al. 2005; Doney et al 2009). For instance, multicellular calcifying marine organisms such as pteropods will probably have much less potential to adapt on climate change relevant time scales. Unfortunately, experimental evolution experiments in species that reproduce slowly and are difficult to cultivate are currently not possible. An alternative approach is to assess functional genetic diversity in natural populations to evaluate the adaptive potential of such organisms based on existing genetic variation (Reusch and Wood 2007).

7.5 Future Research Perspectives

In the preceding chapters I have demonstrated the potential of the coccolithophore *E. huxleyi* to adapt to elevated CO_2 conditions and identified selection on clonal genetic diversity and emergence of novel mutations as key mechanisms of adaption. However, the underlying genetic basis of high CO_2 adaptation remains to be explored.

I plan to unravel the genetic basis of high CO_2 adaptation in replicate single-clone *E. huxleyi* populations in a follow up project that involves whole genome re-sequencing of adapted and control populations on a next-generation sequencing platform (Araya et al. 2010; Dettman et al. 2012). With this approach I aim to identify genes and genomic

regions under selection in the microcosm based CO_2 selection experiment presented here. Moreover, I plan to extend the candidate gene expression analysis presented in chapter 6 by a whole transcriptome profiling approach, using next generation RNA sequencing technologies (Vera et al. 2008; Wang et al. 2009). This will provide a comprehensive picture of the molecular phenotype and likely help to identify genes and regulatory pathways involved in adaptation to ocean acidification in *E. huxleyi*. Moreover, such an analysis will shed light on general physiological and molecular mechanisms that are affected by low seawater pH.

Experimental evolution experiments are an important tool but ultimately can only demonstrate the potential for adaptation. Microcosm based results need to be tested in field studies to draw conclusions on the pace and relevance of adaptive processes under natural conditions. Presently it is unclear how such experiments translate into natural settings. Under natural conditions organisms face complex changing environments and interactions with other species that may slow down or even prevent adaptation to rapid environmental change (De Mazancourt et al. 2008).

In another future research project I plan to study the role of genotypic selection on within-population genetic variation in natural *E. huxleyi* populations enclosed by CO_2 manipulated mesocosms. Large scale, free floating mesocosm devices, such as the Kiel Offshore Mesocosms (KOSMOS), enable researchers to enclose and manipulate tens of cubic meters of seawater and offer the unique opportunity to study the responses of enclosed ecosystems under near natural conditions (Riebesell et al. 2008). I aim to follow functional genetic diversity changes of enclosed *E. huxleyi* populations using single nucleotide polymorphisms (SNPs) for a population-level genotyping approach, i.e. temporal genome scan. Based on population wide functional genotype frequencies, this assay will enable the detection of genomic sites under selection, such as selective sweeps, that will be mapped on the *E. huxleyi* genome and contribute to identify the genetic basis of high CO_2 adaptation. Such temporal genome scans should be particularly powerful if the same genomic regions under selection re-appear consistently as a function of treatment. Technically, it is currently unclear how large the fraction of *E. huxleyi* DNA has to be to successfully sequence sufficient DNA fragments for mapping and subsequent SNP calling using a backbone genome.

Whole genome re-sequencing and transcriptome profiling of high CO_2 adapted single-clone derived *E. huxleyi* populations will probably reveal the genetic bases of adaptation to high CO_2 in replicate populations of this particular clone. Combining these data with population-level functional genetic diversity changes and regions under selection in natural *E. huxleyi* populations will most likely allow a first estimate of the pace and relevance of adaptive evolution to ocean acidification in natural *E. huxleyi* populations.

An important challenge outside the scope of my work will be the parameterization of functional genetic diversity and adaptive plasticity in ecosystem models, which currently reduce the large genetic variability of genotypes and species into unified functional groups without the ability to adapt to imposed ocean change (Blackford 2010).

Finally, ocean change is not only ocean acidification. Models project atmospheric CO_2 levels of 700-1000 ppm to result in a global mean temperature increase of $2-4°C$ until the end of the 21^{st} century (IPCC 2007). Such a temperature rise will lead to increased stratification and de-oxygenation of the surface ocean and thereby modify the environmental conditions experienced by marine phytoplankton (Boyd 2011). In future experiments multifactorial designs will be needed to investigate phenotypic and genotypic plasticity of phytoplankton to multiple environmental parameters that may become relevant in a projected future ocean. Such experiments will not only enable us to identify synergistic or antagonistic effects of ocean change in contemporary genotypes, they will also reveal constraints to adaptation, such as demonstrated by pleiotropy in chapter 5, and thereby significantly contribute to a better understanding of phytoplankton responses in the future ocean.

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9 Appendix

9.1 Supplementary Material - Publication I

Title: Adaptive evolution of a key phytoplankton species to ocean acidification

Authors: Kai T. Lohbeck, Ulf Riebesell and Thorsten B. H. Reusch
Published in Nature Geoscience 5:346-351 (2012)

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

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Lohbeck et al. Rapid adaptive evolution of a key phytoplankton species to ocean acidification

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} seleniumDanbara, 1999 #3054} 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

p.3

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[43] 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 44) using appropriate solubility constants[45]. 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-clonal 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-clonal 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

p.4

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. 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

p.5

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 2nd 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-clonal populations after 0, 160 and 320 days.

p.6

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, 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

p.7

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.

References full material and methods

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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 (nominator) and 16 (denominator) degrees of freedom.

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

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 (nominator) and 16 (denominator) degrees of freedom.

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

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

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[§]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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Desai, M. M., Fisher, D. S. & Murray, A. W. The Speed of Evolution and Maintenance of Variation in Asexual Populations. *Current Biology* **17**, 385-394 (2007).

Goddard, M. R., Godfray, H. C. J. & Burt, A. Sex increases the efficacy of natural selection in experimental yeast populations. *Nature* **434**, 636-640 (2005).

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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

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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 $U_b \sim 10^{-5} - 10^{-6}$. 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 $U_b \sim 10^{-7}$ 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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Supplementary data set S1: Experimental data on exponential growth rates and calcification rates of single- and multi-clone experiment. Given are mean exponential growth rates, particulate inorganic (PIC) and organic carbon (POC) per cell +/- 1 standard deviation from multi-clone and single-clone assay experiments performed after ~500 generations CO2 selection treatment.

multi-clone experiment

growth rate	evo environment [µatm CO2]	test environment [µatm CO2]	growth rate [1/d]	sd
	400	400	1.213393271	0.010342995
	400	1100	1.086400621	0.019580701
	400	2200	0.953698042	0.006871969
	1100	400	1.175232106	0.016789871
	1100	1100	1.114159232	0.015358333
	2200	400	1.124893761	0.009787234
	2200	2200	1.020920253	0.011203285

PIC/cell	evo environment [µatm CO2]	test environment [µatm CO2]	PIC per cell [µg]	sd
	400	400	6.26E-06	3.68221E-07
	400	1100	5.73442E-06	4.16542E-07
	400	2200	4.93997E-06	8.8046E-07
	1100	400	6.67972E-06	2.33904E-07
	1100	1100	5.53551E-06	1.95036E-07
	2200	400	8.37958E-06	3.53141E-07
	2200	2200	5.61139E-06	8.33882E-07

POC/cell	evo environment [µatm CO2]	test environment [µatm CO2]	POC per cell [µg]	sd
	400	400	7.82501E-06	6.87708E-07
	400	1100	9.44192E-06	4.88242E-07
	400	2200	1.11018E-05	4.07877E-07
	1100	400	8.7884E-06	7.97811E-07
	1100	1100	8.50508E-06	3.12259E-07
	2200	400	9.49609E-06	4.49982E-07
	2200	2200	1.11399E-05	1.98405E-06

single-clone experiment

growth rate	evo environment [µatm CO2]	test environment [µatm CO2]	growth rate [1/d]	sd
	400	400	1.092635088	0.009601501
	400	1100	1.013614595	0.00961977
	400	2200	0.893954739	0.026460999
	1100	400	1.117560777	0.010731632
	1100	1100	1.026821433	0.011847412
	2200	400	1.114185594	0.007234575
	2200	2200	0.934771977	0.01668177

PIC/cell	evo environment [µatm CO2]	test environment [µatm CO2]	PIC per cell [µg]	sd
	400	400	8.23724E-06	9.07022E-07
	400	1100	6.42763E-06	3.68837E-07
	400	2200	3.94477E-06	1.59523E-06
	1100	400	7.195E-06	3.1175E-07
	1100	1100	7.44206E-06	1.46793E-07
	2200	400	7.33466E-06	2.58437E-07
	2200	2200	5.64481E-06	5.39892E-07

POC/cell	evo environment [µatm CO2]	test environment [µatm CO2]	POC per cell [µg]	sd
	400	400	1.02446E-05	5.435E-07
	400	1100	1.22623E-05	5.66634E-07
	400	2200	1.6057E-05	8.11697E-07
	1100	400	8.78252E-06	3.59375E-07
	1100	1100	1.31368E-05	5.88341E-07
	2200	400	8.59593E-06	2.63876E-07
	2200	2200	1.56825E-05	1.00202E-06

9.2 Supplementary Material - Publication II

Title: Functional genetic divergence in high CO_2 adapted *Emiliania huxleyi* populations

Authors: Kai T. Lohbeck, Sinead Collins, Ulf Riebesell and Thorsten B. H. Reusch
Published in Evolution 10.1111/j.1558-5646.2012.01812.x (2012)

Lohbeck *et al.* 2012 Functional genetic divergence in high CO2 adapted *Emiliania huxleyi* populations

Supplementary Table S1: Exponential growth rate of replicate (N=5) high CO2 adapted (selection environment = 2200 µatm pCO2) and ambient CO2 adapted (selection environment = 400 µatm pCO2) populations tested in the respective assay environment (assay environment = 400 or 2200 µatm pCO2) after 500, 750, and 1000 generations.

generations	selection environment	assay environment	population	growth rate µ
	[µatm pCO2]	[µatm pCO2]	no.	[1/d]
500	400	400	1	1.1023
500	400	400	2	1.0972
500	400	400	3	1.0814
500	400	400	4	1.0990
500	400	400	5	1.0832
500	2200	400	1	1.1075
500	2200	400	2	1.1221
500	2200	400	3	1.1057
500	2200	400	4	1.1192
500	2200	400	5	1.1165
500	2200	2200	1	0.9095
500	2200	2200	2	0.9547
500	2200	2200	3	0.9428
500	2200	2200	4	0.9356
500	2200	2200	5	0.9314
500	400	2200	1	0.9079
500	400	2200	2	0.8969
500	400	2200	3	0.9228
500	400	2200	4	0.8901
500	400	2200	5	0.8521

generations	selection environment	assay environment	population	growth rate µ
	[µatm pCO2]	[µatm pCO2]	no.	[1/d]
750	400	400	1	1.1683
750	400	400	2	1.1338
750	400	400	3	1.1305
750	400	400	4	1.1467
750	400	400	5	1.1356
750	2200	400	1	1.0955
750	2200	400	2	1.1094
750	2200	400	3	1.0911
750	2200	400	4	1.1146
750	2200	400	5	1.0976
750	2200	2200	1	0.9490
750	2200	2200	2	0.9933
750	2200	2200	3	0.9883
750	2200	2200	4	0.9829
750	2200	2200	5	0.9660
750	400	2200	1	0.9519
750	400	2200	2	0.9325
750	400	2200	3	0.9573
750	400	2200	4	0.9527
750	400	2200	5	0.9341

generations	selection environment	assay environment	population	growth rate µ
	[µatm pCO2]	[µatm pCO2]	no.	[1/d]
1000	400	400	1	1.1724
1000	400	400	2	1.1789
1000	400	400	3	1.1698
1000	400	400	4	1.2020
1000	400	400	5	1.2277
1000	2200	400	1	1.1808
1000	2200	400	2	1.1568
1000	2200	400	3	1.1519
1000	2200	400	4	1.1403
1000	2200	400	5	1.1180
1000	2200	2200	1	1.0093
1000	2200	2200	2	0.9751
1000	2200	2200	3	1.0253
1000	2200	2200	4	0.9675
1000	2200	2200	5	0.9719
1000	400	2200	1	0.9780
1000	400	2200	2	0.9473
1000	400	2200	3	0.9828
1000	400	2200	4	0.9578
1000	400	2200	5	0.9495

Supplementary Table S2: Exponential growth rate of sub-replicated populations selected under ambient CO2 (selection environment = 400 µatm pCO2) and high CO2 (selection environment = 2200 µatm pCO2) when tested under control light (treatment = control) and high light challenge (treatment = challenge).

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
control	400	1	1	1.1651
control	400	1	2	1.1621
control	400	1	3	1.1728
control	400	2	1	1.1548
control	400	2	2	1.1590
control	400	2	3	1.1613
control	400	3	1	1.1552
control	400	3	2	1.1641
control	400	3	3	1.1509
control	400	4	1	1.1519
control	400	4	2	1.1561
control	400	4	3	1.1690
control	400	5	1	1.1593
control	400	5	2	1.1773
control	400	5	3	1.1631

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
challenge	400	1	1	1.2246
challenge	400	1	2	1.1996
challenge	400	1	3	1.1792
challenge	400	2	1	1.1945
challenge	400	2	2	1.2113
challenge	400	2	3	1.2035
challenge	400	3	1	1.2050
challenge	400	3	2	1.1829
challenge	400	3	3	1.2061
challenge	400	4	1	1.2112
challenge	400	4	2	1.1987
challenge	400	4	3	1.2105
challenge	400	5	1	1.1879
challenge	400	5	2	1.2011
challenge	400	5	3	1.1899

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
control	2200	1	1	0.9659
control	2200	1	2	0.9718
control	2200	1	3	0.9683
control	2200	2	1	0.9718
control	2200	2	2	0.9679
control	2200	2	3	0.9511
control	2200	3	1	0.9767
control	2200	3	2	0.9741
control	2200	3	3	0.9758
control	2200	4	1	0.9529
control	2200	4	2	0.9459
control	2200	4	3	0.9375
control	2200	5	1	0.9816
control	2200	5	2	0.9717
control	2200	5	3	0.9592

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
challenge	2200	1	1	1.0407
challenge	2200	1	2	1.0382
challenge	2200	1	3	1.0259
challenge	2200	2	1	1.0283
challenge	2200	2	2	1.0390
challenge	2200	2	3	1.0297
challenge	2200	3	1	1.0497
challenge	2200	3	2	1.0598
challenge	2200	3	3	1.0367
challenge	2200	4	1	1.0228
challenge	2200	4	2	1.0429
challenge	2200	4	3	1.0191
challenge	2200	5	1	1.0407
challenge	2200	5	2	1.0526
challenge	2200	5	3	1.0265

Supplementary Table S3: Exponential growth rate of sub-replicated populations selected under ambient CO2 (selection environment = 400 µatm pCO2) and high CO2 (selection environment = 2200 µatm pCO2) when tested under control salinity (treatment = control) and low salinity challenge (treatment = challenge). Note that all 3 sub-replicate populations of high CO2 selected and salinity challenged population no. 1 failed to grow. These populations were included in the graph but were omitted from the statistical analysis.

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
control	400	1	1	1.1371
control	400	1	2	1.1473
control	400	1	3	1.1557
control	400	2	1	1.1348
control	400	2	2	1.1416
control	400	2	3	1.1401
control	400	3	1	1.1374
control	400	3	2	1.1259
control	400	3	3	1.1235
control	400	4	1	1.1227
control	400	4	2	1.1353
control	400	4	3	1.1203
control	400	5	1	1.1240
control	400	5	2	1.1114
control	400	5	3	1.1100

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
challenge	400	1	1	0.8461
challenge	400	1	2	0.8632
challenge	400	1	3	0.8517
challenge	400	2	1	0.8551
challenge	400	2	2	0.8411
challenge	400	2	3	0.8497
challenge	400	3	1	0.8588
challenge	400	3	2	0.8570
challenge	400	3	3	0.8578
challenge	400	4	1	0.8693
challenge	400	4	2	0.8684
challenge	400	4	3	0.8739
challenge	400	5	1	0.8456
challenge	400	5	2	0.8420
challenge	400	5	3	0.8437

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
control	2200	1	1	0.9779
control	2200	1	2	0.9901
control	2200	1	3	0.9691
control	2200	2	1	0.9848
control	2200	2	2	0.9857
control	2200	2	3	0.9682
control	2200	3	1	0.9866
control	2200	3	2	0.9805
control	2200	3	3	0.9859
control	2200	4	1	0.9772
control	2200	4	2	0.9664
control	2200	4	3	0.9717
control	2200	5	1	0.9761
control	2200	5	2	0.9845
control	2200	5	3	0.9755

treatment	selection environment	population	sub-replicate	growth rate µ
	pCO2 [µatm]	no.	no.	[1/d]
challenge	2200	1	1	0.0000
challenge	2200	1	2	0.0000
challenge	2200	1	3	0.0000
challenge	2200	2	1	0.3177
challenge	2200	2	2	0.2805
challenge	2200	2	3	0.3311
challenge	2200	3	1	0.4519
challenge	2200	3	2	0.4288
challenge	2200	3	3	0.4179
challenge	2200	4	1	0.3080
challenge	2200	4	2	0.3379
challenge	2200	4	3	0.3569
challenge	2200	5	1	0.2591
challenge	2200	5	2	0.2653
challenge	2200	5	3	0.2710

9.3 Supplementary Material - Publication III

Title: Adaptation to ocean acidification in *Emiliania huxleyi* involves genes putatively relevant to pH regulation and bicarbonate transport

Authors: Kai T. Lohbeck, Ulf Riebesell and Thorsten B. H. Reusch
Manuscript prepared for submission

Lohbeck et al. - Adaptation to ocean acidification in Emiliania huxleyi involves genes putatively relevant to pH regulation and bicarbonate transport

Supplementary Table S1: Analyses of variance on -ΔCT data from ambient, medium and high CO_2 adapted *Emiliania huxleyi* populations after ~500 generations. Given are the results of 2x2 factorial analyses of variances (ANOVAs) and subsequent planned contrasts on mean -ΔCT values. Planned contrasts for assessing adaptation were performed only when in the initial 2-factorial ANOVA either the main effect "selection condition" or the interaction "selection x assay condition" was statistical significant. All F-ratios have 1 (nominator) and 16 (denominator) degrees of freedom. For mean -ΔCT values see fig. 1 and 2.

Gene of interest	Data sets used in ANOVAs	Selection condition	Assay condition	Selection x assay condition	Planned contrasts
AEL1	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=10.713 *P*=0.0048 *F*=0.3755 *P*=0.5486	*F*=25.427 *P*=0.0001 *F*=28.1428 *P*<0.0001	*F*=1.480 *P*=0.2414 *F*=0.8616 *P*=0.3671	*F*=10.078 *P*=0.0059 *na*
alphaCA	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.3148 *P*=0.5825 *F*=0.6997 *P*=0.4152	*F*=1.9074 *P*=0.1862 *F*=22.5955 *P*=0.002	*F*= 0.0993 *P*=0.7567 *F*=1.2277 *P*=0.2842	*na* *na*
ATPVc/c'	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=7.5456 *P*=0.0143 *F*=1.0998 *P*=0.3099	*F*=9.3006 *P*=0.0076 *F*=35.2723 *P*<0.0001	*F*=0.5409 *P*=0.4727 *F*=0.9355 *P*=0.3478	*F*= 6.063 *P*=0.0255 *na*
CAX3	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=2.4809 *P*=0.1348 *F*=0.0020 *P*=0.9646	*F*=10.4546 *P*=0.0052 *F*=27.1082 *P*<0.0001	*F*=1.3923 *P*=0.2553 *F*=0.5907 *P*=0.4533	*na* *na*
deltaCA	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.2249 *P*=0.6418 *F*=0.3248 *P*=0.5766	*F*=15.4993 *P*=0.0012 *F*=64.2940 *P*<0.0001	*F*=2.3241 *P*=0.1469 *F*=1.6305 *P*=0.2198	*na* *na*
GPA	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.0016 *P*=0.9682 *F*=0.0168 *P*=0.8986	*F*=1.3600 *P*=0.2606 *F*=0.4948 *P*=0.4919	*F*=0.3940 *P*=0.5390 *F*=0.0001 *P*=0.9908	*na* *na*
LCIX	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.0449 *P*=0.8349 *F*=0.1202 *P*=0.7334	*F*=0.4996 *P*=0.4899 *F*=2.5409 *P*=0.1305	*F*=0.0825 *P*=0.7776 *F*=1.1782 *P*=0.2938	*na* *na*
NhaA2	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.6470 *P*=0.4330 *F*=0.0074 *P*=0.9323	*F*=12.1306 *P*=0.0031 *F*=35.9695 *P*<0.0001	*F*=2.4499 *P*=0.1371 *F*=3.1210 *P*=0.0964	*na* *na*
PATP	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.0988 *P*=0.7573 *F*=0.0757 *P*=0.7867	*F*=0.9505 *P*=0.3441 *F*=14.0222 *P*=0.0018	*F*=1.7188 *P*=0.2084 *F*=10.0578 *P*=0.0059	*na* *F*=5.940 *P*=0.0269
RB	400-400/1100-400 vs. 400-1100/1100-1100 400-400/2200-400 vs. 400-2200/2200-2200	*F*=0.6023 *P*=0.4490 *F*=1.7555 *P*=0.2038	*F*=0.1986 *P*=0.6619 *F*=8.1453 *P*=0.0115	*F*=0.0800 *P*=0.7809 *F*=0.0561 *P*=0.8158	*na* *na*

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11 Eidesstattliche Erklärung

Hiermit bestätige ich, dass die vorliegende Arbeit mit dem Titel:

Rapid evolution of a key phytoplankton species to ocean acidification

von mir selbstständig angefertigt wurde.

Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren.

Ich habe keine als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Teile dieser Arbeit wurden als Manuskripte in wissenschaftlichen Fachzeitschriften veröffentlicht: Publikation I in Nature Geoscience mit Ulf Riebesell und Thorsten B. H. Reusch als Koautoren, Publikation II in Evolution mit Ulf Riebesell, Sinead Collins und Thorsten B. H. Reusch als Koautoren.

Kiel, im Januar 2013

Kai Tim Lohbeck