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Gene expression changes in the coccolithophore *Emiliana huxleyi* after 500 generations of selection to ocean acidification

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Coccolithophores are unicellular marine algae that produce biogenic calcite scales and substantially contribute to marine primary production and carbon export to the deep ocean. Ongoing ocean acidification particularly impairs calcifying organisms, mostly resulting in decreased growth and calcification. Recent studies revealed that the immediate physiological response in the coccolithophore *Emiliana huxleyi* to ocean acidification may be partially compensated by evolutionary adaptation, yet the underlying molecular mechanisms are currently unknown. Here, we report on the expression levels of 10 candidate genes putatively relevant to pH regulation, carbon transport, calcification and photosynthesis in *E. huxleyi* populations short-term exposed to ocean acidification conditions after acclimation (physiological response) and after 500 generations of high CO₂ adaptation (adaptive response). The physiological response revealed down-regulation of candidate genes, well reflecting the concomitant decrease of growth and calcification. In the adaptive response, putative pH regulation and carbon transport genes were up-regulated, matching partial restoration of growth and calcification in high CO₂-adapted populations. Adaptation to ocean acidification in *E. huxleyi* likely involved improved cellular pH regulation, presumably indirectly affecting calcification. Adaptive evolution may thus have the potential to partially restore cellular pH regulatory capacity and thereby mitigate adverse effects of ocean acidification.

1. Introduction

Marine phytoplankton plays a key role in the ocean's food webs and biogeochemical cycles [1]. One group of particular interest are coccolithophores (Haptophyta, Prymnesiophyceae), unicellular marine algae that are characterized by their ability to produce delicate calcite scales [2]. *Emiliana huxleyi* is considered to be the most abundant coccolithophore in contemporary oceans [3]. This species complex [4] forms vast blooms and contributes significantly to global biogenic calcite production [5]. Excess anthropogenic CO₂ that equilibrates with the surface ocean results in a drop of seawater pH, termed ocean acidification [6]. This phenomenon represents a major threat to many marine organisms, especially for those producing calcium carbonate structures [7–9], with likely consequences for marine carbon fluxes and ecosystem functioning [10,9]. 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 mostly to decrease under more acidic seawater conditions [9]. While there is a profound understanding of short-term responses [11–13], the potential for evolutionary adaptation has only recently been addressed [14].

In a 500 generations selection experiment, we demonstrated evolutionary adaptation to ocean acidification in single-clone-derived *E. huxleyi* populations, indicating the potential for adaptation to counteract adverse

effects found in short-term experiments [14]. While we found partial restoration of growth and calcification rates in high CO₂-adapted populations, the underlying adaptive changes at the cellular level and their genetic bases remain to be explored. That we observed calcification to recover along with growth, although we did not directly select for calcification, indicates a close linkage among both traits. Biogenic calcite production in *E. huxleyi* and other coccolithophores is considered to be a highly regulated cellular process [15] with large ecological and biogeochemical implications [3,16]. Still, our knowledge of the underlying molecular mechanisms as well as the biological function of coccolith production is limited [11,15,17]. Experimental evolution approaches can be very useful to uncover functional links among fitness and individual traits [18]. In this study, we combine molecular genetic techniques and the previous laboratory selection experiment to gain insights into adaptive changes at the cellular level, underlying partly restored growth and calcification rates that we observed in high CO₂-adapted *E. huxleyi* populations.

The environmental factor causing inhibition of growth and calcification rates in *E. huxleyi* under ocean acidification conditions is the lowered seawater pH that is associated with changes in dissolved inorganic carbon (DIC) concentration [19], although the pH sensitivity of *E. huxleyi* can be modulated by CO₂ and bicarbonate availability [20]. Suffrian *et al.* [21] demonstrated that *E. huxleyi* has poor abilities to regulate cytosolic pH as the surrounding seawater acidifies, while Taylor *et al.* [22] identified voltage-gated proton channels that serve to quickly release excess protons produced in calcification from the cytosol. Seawater acidification is supposed to disrupt the regulation of these channels by interfering with the plasma membrane potential, and thereby affects cytosolic pH regulation [22]. Microarray-based transcription profiling in *E. huxleyi* further supports that ocean acidification impairs signal transduction and ion transport and revealed rearrangement of carbon and energy fluxes within and across compartments [23]. Taken together, these results suggest that altered carbon metabolism and pH regulation play a key role in *E. huxleyi* cells exposed to lowered seawater pH. Therefore, these cellular processes may serve as a proxy for adaptive changes that have caused restored growth and calcification rates in the CO₂ selection experiment.

We used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to study the regulation of 10 selected candidate genes that report on key metabolic pathways. On the one hand, we studied the relative gene expression levels in non-adapted *E. huxleyi* populations exposed to medium (1100 µatm) and high (2200 µatm) pCO₂ compared to ambient (400 µatm) pCO₂ control conditions. On the other hand, we investigated gene expression levels in populations adapted for 500 generations to medium and high pCO₂ compared to non-adapted control populations when both were assayed under medium and high pCO₂ conditions, respectively.

With this approach we aim to reveal links between the observed adaptive responses in growth and calcification and underlying metabolic changes at the cellular level. Although gene expression is also a phenotype, such an analysis provides a step forward towards the genetic basis of adaptation to ocean acidification in the globally important coccolithophore *E. huxleyi*.

2. Material and methods

(a) Experimental design and procedures

The gene expression analysis presented in this study is based on samples taken from the previously published CO₂ selection experiment [14]. Detailed information regarding experimental design, culturing and sampling are available in the electronic supplementary material, section S2, of Lohbeck *et al.* [14].

In brief, replicated ($N = 5$) populations of *E. huxleyi* (Lohmann) Hay and Mohler were kept in semi-continuous batch cultures under ambient (400 µatm), medium (1100 µatm) and high (2200 µatm) pCO₂ conditions and assayed after 544 (ambient), 512 (medium) and 448 (high CO₂) mitotic divisions. As response variables, exponential growth rates, cell diameter, particulate inorganic and organic carbon per cell and their production rates were assessed [14].

Here, we focus on the corresponding gene expression response of 10 selected candidate genes putatively relevant to pH regulation, carbon transport, calcification and photosynthesis. The overall analysis comprises two different categories of gene expression responses: on the one hand, the physiological response after acclimation (for brevity hereafter 'physiological response') that is based on the comparison of populations transferred from ambient into medium or high CO₂ to their respective control populations that are kept under ambient CO₂; on the other hand, the adaptive response that is based on the comparison of medium and high CO₂-adapted populations to ambient CO₂-adapted control populations when assayed under medium or high CO₂, respectively.

(b) RNA sampling and extraction

For RNA sampling, 250 ml of 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 rinsed off the filter with 500 µl RNAlater (Qiagen), pipetted in 1.5 ml Eppendorf cups and placed on ice to allow cells to settle out for 2 h before samples were frozen at -20°C until further processing. RNA was extracted using the RNeasy kit (Qiagen) following the manufacturer's protocol and quantified with a Qubit fluorometer (Life technologies). RNA samples were stored at -80°C until further processing.

(c) Candidate genes

Genes of interest (GOI) were chosen from the literature [20,24–27] based on their putative role in pH regulation, carbon transport, calcification and photosynthesis. In particular, we investigated genes coding for a putative bicarbonate transporter belonging to the solute carrier 4 family (AEL1), a putative cytosolic α carbonic anhydrase (αCA), a putative membrane-associated δ 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 CO₂-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-1,5-bisphosphate carboxylase-oxygenase (Rubis-CO, RB). A complete list of all candidate and endogenous reference genes (ERGs), their putative functions, primer details, amplicon sizes and corresponding references can be found in the electronic supplementary material, table S1.

(d) Quantitative reverse transcriptase polymerase chain reaction

Reverse transcription was performed using the QuantiTect reverse transcription kit (Qiagen) following the manufacturer's protocol. The amount of transcribed target gene mRNA was measured on a StepOne Plus Cyclor (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 standard curves of a six-step dilution series ranging from 1:4 to 1:1024. Efficiencies were calculated from the slopes (all $R^2 > 0.98$) to 81–98%. All plates were run in technical triplicates. The variance among triplicates was inspected and individual outliers were excluded when the variance was more than 0.3. Non-reverse transcription controls and non-template controls were run in parallel on each plate and were always negative. We considered α -Tubulin (α TUB), Actin (Actin) and Elongation factor 1 (EFG1) as potential ERGs. However, only α TUB showed sufficient stability over all treatments and was therefore used as ERG.

The negative differences in cycles to cross the threshold value ($-\Delta$ CT) between the ERG and the respective GOI were calculated for all five replicate populations and technical replicates individually according to equation (2.1). Mean $-\Delta$ CT values were calculated for all five individual replicate populations from $-\Delta$ CT values of the technical replicates. For graphical depiction, fold expression change values were calculated according to equation (2.2). Asterisks in the plots indicate significant differences of mean $-\Delta$ CT values underlying the depicted fold expression change values.

$$-\Delta\text{CT} = \text{CT}_{\text{ERG}} - \text{CT}_{\text{GOI}} \quad (2.1)$$

$$\text{Fold expression change} = \pm 2^{((-\Delta\text{CT}_{\text{treatment}}) - (-\Delta\text{CT}_{\text{control}}))} \quad (2.2)$$

(e) Statistical analyses

Our experimental design is an incomplete factorial design with two missing treatment combinations, because we did not assay populations evolved at 1100 $\mu\text{atm } p\text{CO}_2$ under 2200 $\mu\text{atm } p\text{CO}_2$ and *vice versa*. As we were particularly interested in the assay \times evolution environment interactions, this resulted in two 2×2 full factorial datasets that were analysed separately. First, in order to assess the multivariate signal of all 10 genes in the physiological and the adaptive response, we subjected $-\Delta$ CT values of all genes to 2×2 factorial multivariate analyses of variance (MANOVA) in a repeated measures model. The MANOVAs contained both among-subjects multivariate effects and within-subjects effects. When repeated measures MANOVAs were significant, we proceeded with 2×2 factorial analyses of variance (ANOVA) to assess which genes exactly responded in the physiological and the adaptive assays and if there were interactions among assay and evolution environment. Planned contrasts for assessing adaptation were performed only when in the initial two-factorial ANOVA either the main effect ‘selection condition’ or the interaction ‘selection \times assay condition’ was statistical significant. To account for multiple testing, p -values were corrected using false discovery rate control after Benjamini & Hochberg [28]. Variance homogeneity was verified using Levene’s test and normality of residuals was tested using the Shapiro–Wilk test. We also used an exact binominal test to inspect general up- or downregulation patterns within treatments and a direct F test to compare variances between the physiological and the adaptive response groups. Statistical analyses were performed using JMP v. 9.0 (Statsoft Inc.) for multivariate, and R v. 2.15.1 [29] for univariate analyses.

3. Results and discussion

This study considered two different types of gene expression responses, the physiological response after acclimation and the adaptive response after 500 generations of CO_2 selection. The physiological response is the difference in gene expression between ambient CO_2 -adapted populations transferred into elevated CO_2 relative to ambient CO_2 -adapted populations

that remained at ambient CO_2 . It is driven by the CO_2 assay environment and informative on the acclimated short-term response of a contemporary *E. huxleyi* clone exposed to elevated CO_2 conditions after approximately six to eight generations of acclimation. The adaptive response is the difference in gene expression of medium or high CO_2 -adapted populations relative to ambient CO_2 -adapted populations, serving as control, when both are assayed under medium or high CO_2 , respectively. It is measured under the same assay conditions and compares populations with a different adaptation history. In contrast to the physiological response, the magnitude of expression change as a function of different adaptation histories is generally expected to be lower because it is exclusively controlled by the new genetic background of populations adapted to elevated CO_2 conditions.

(a) Physiological response

We first focus on the physiological response (figure 1) where we observed an overall downregulation in eight out of 10 target genes in both treatment comparisons (binominal test, $p < 0.001$), a pattern consistent with decreased growth and calcification under ocean acidification conditions. This finding supports the selection of our target genes and suggests a tight link between the regulation of these genes and cellular processes underlying growth and calcification. The multivariate signals of all genes were assessed by repeated measures MANOVAs. We found that among-subjects multivariate effects (control versus medium/high CO_2 models) and within-subjects interactions (control versus high CO_2 model) were significant (MANOVA, $p = 0.042 / < 0.001 / < 0.001$; electronic supplementary material, table S2). Therefore, we proceeded with univariate tests to investigate the regulation of each individual candidate gene.

We found significant downregulation under medium/high CO_2 conditions in three genes presumably relevant to inorganic carbon acquisition and transport, namely the putative bicarbonate transporter AEL1 ($p < 0.001 / < 0.001$) and the two putative carbonic anhydrases α CA ($p = 0.002$) and δ CA ($p = 0.001 / < 0.001$). Note that α CA was significantly downregulated only under high CO_2 . These genes are likely involved in carbon supply to calcification and photosynthesis.

Moreover, four genes potentially involved in ion transport and pH regulation, namely genes coding for the putative vacuolar proton pump ATPVc/c’ ($p = 0.008 / < 0.001$), the putative calcium/proton exchanger CAX3 ($p = 0.005 / < 0.001$), the putative sodium/proton exchanger NhaA2 ($p = 0.003 / < 0.001$) and the putative membrane-associated proton pump PATP ($p = 0.012$) showed decreased expression levels under medium/high CO_2 . Note that PATP was significantly downregulated only under high CO_2 . Assuming that these genes are relevant to cellular pH regulation, they are not involved in a compensatory response to counteract lowered intracellular pH. Poor abilities to regulate cytosolic pH [21] probably indicate that such a response is absent in *E. huxleyi*.

Downregulation of RubisCO (RB) under high CO_2 ($p = 0.012$) does not necessarily imply decreased primary production. Photosynthetic carbon fixation strongly depends on the CO_2 concentration at the active site of RB. Increased CO_2 availability under ocean acidification may therefore increase RB’s carboxylase activity and allow efficient photosynthesis with fewer enzyme units compared with ambient CO_2 conditions. In the physiological response, only the genes coding for the calcium-binding protein (GPA) and a low CO_2 -induced protein

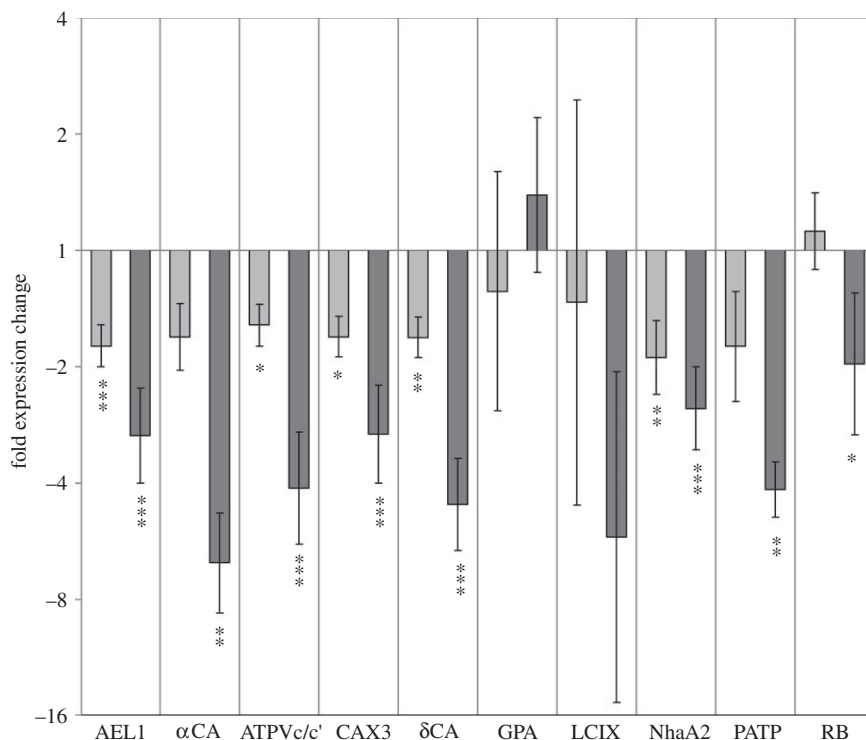


Figure 1. Physiological response in relative gene expression of 10 candidate genes in replicated ($N = 5$) ambient $p\text{CO}_2$ ($400 \mu\text{atm}$)-adapted control populations when tested under medium ($1100 \mu\text{atm}$) and high ($2200 \mu\text{atm}$) $p\text{CO}_2$ conditions. Differences in mean gene expression levels are illustrated as fold expression change \pm s.e.m. Pale bars indicate relative gene expression of control populations assayed at $1100 \mu\text{atm}$ $p\text{CO}_2$ relative to control populations assayed at $400 \mu\text{atm}$ $p\text{CO}_2$. Dark bars indicate gene expression of control populations assayed at $2200 \mu\text{atm}$ $p\text{CO}_2$ relative to control populations assayed at $400 \mu\text{atm}$ $p\text{CO}_2$. Significance levels are indicated: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

of unknown function (LCIX) showed no significant expression change at all. Detailed results from statistical analyses are given in the electronic supplementary material, table S3.

These findings agree well with a prominent role of impaired cytosolic signal transduction, ion transport and pH regulation and cellular carbon flux reallocation to cause lowered growth and calcification rates in *E. huxleyi* under ocean acidification conditions [21–23]. Bach *et al.* [20] investigated a largely overlapping set of candidate genes and reported upregulation of these same genes under low DIC conditions. The authors proposed a role of these genes in a carbon-concentrating mechanism (CCM) that operates on bicarbonate to meet cellular carbon demands under low DIC. Under such conditions, calcification is supposed to be downregulated in order to ensure sufficient carbon supply to photosynthesis. By contrast, under DIC-saturated conditions CO_2 seems to be the primary carbon source for photosynthesis [30]. Consequently, calcification will be the main bicarbonate consumer and drive the regulation of those CCM-related genes.

In our simulated ocean acidification treatments, CO_2 was sufficiently available. Increased proton concentrations likely disrupted cellular pH regulation and thereby interfered with calcification [21,22]. Decreased calcification under CO_2 -saturated conditions may then have resulted in the concomitant downregulation of CCM-related genes. However, the relative contribution of decreased bicarbonate demand by photosynthesis and restricted calcification cannot be disentangled in our study.

Bach *et al.* [20] used a combination of carbonate system manipulations that allowed keeping either pH or CO_2 constant while the other carbonate system parameters varied accordingly and observed a response to CO_2 and bicarbonate

but not to pH in these genes. Following their interpretation, increased CCM activity in our ambient CO_2 treatments compared to the medium and high CO_2 treatments could have resulted in a relative downregulation of the candidate genes under medium and high CO_2 conditions. However, as we found a stronger regulatory response in high compared to medium CO_2 , this would imply higher CCM activity in the medium compared with the high CO_2 treatment. Given the high CO_2 availability in both treatments, such a response seems unlikely and would contradict the CCM properties reported by Bach *et al.* [20]. Alternatively, the carbonate chemistry manipulations used in Bach *et al.* [20], with similar pH levels compared with our medium CO_2 treatment but different bicarbonate and CO_2 concentrations, had probably affected calcification and candidate gene regulation differently compared with the ocean acidification scenario we applied in this study.

(b) Adaptive response

The adaptive response (figure 2) revealed a remarkably consistent pattern of upregulation when summarizing the results of all genes tested in both treatment comparisons (binomial test, $p = 0.003$) and was in line with partly restored growth and calcification rates observed in Lohbeck *et al.* [14] after 500 generations of adaptive evolution.

Consistent recovery in expression of our target genes indicates convergence in the molecular phenotype. In an earlier experiment, we challenged ambient and high CO_2 selected replicate populations in a stressful salinity environment [31]. We found large variation only among high CO_2 -adapted replicates, which was indicative of different genetic

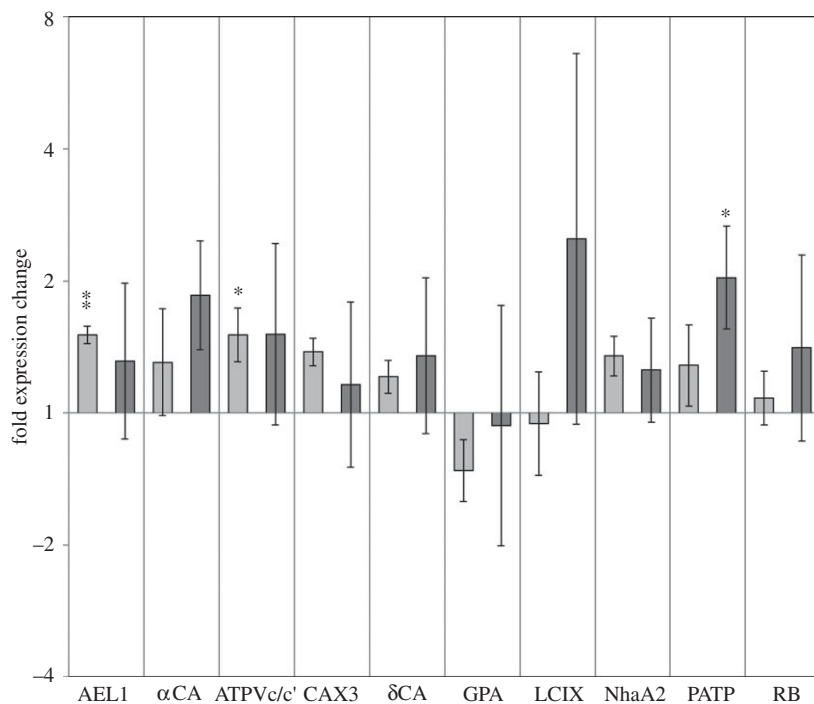


Figure 2. Adaptive response in relative gene expression of 10 candidate genes in replicated ($N = 5$) medium and high CO_2 -adapted *E. huxleyi* populations. Differences in mean gene expression levels between CO_2 -adapted and control populations are illustrated as fold expression change \pm s.e.m. Pale bars indicate gene expression in populations adapted to 1100 $\mu\text{atm } p\text{CO}_2$ relative to control populations adapted to 400 $\mu\text{atm } p\text{CO}_2$ when tested under 1100 $\mu\text{atm } p\text{CO}_2$. Dark bars indicate gene expression in populations adapted to 2200 $\mu\text{atm } p\text{CO}_2$ relative to control populations adapted to 400 $\mu\text{atm } p\text{CO}_2$ when tested under 2200 $\mu\text{atm } p\text{CO}_2$. Significance levels are indicated: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

backgrounds leading to the same high CO_2 adaptation. In the adaptive gene expression response, large within-treatment variation was most obvious in high CO_2 -adapted populations while gene expression changes in medium CO_2 -adapted populations were more uniform (figure 2). Interestingly, there was no significant difference in within-treatment variation between the adaptive and the physiological responses, although the latter was measured in replicated populations that did not show marked divergence in the salinity challenge experiment.

We assessed the multivariate signals of all genes in the adaptive response by repeated measures MANOVAs and found that either within-subjects interactions (control versus medium CO_2 model) or among-subjects multivariate effects (control versus high CO_2 model) were significant (MANOVA, $p = 0.002/0.046$; electronic supplementary material, table S2). Therefore, we proceeded with univariate tests to investigate the regulation of each individual candidate gene.

We identified significant adaptive mean responses in genes coding for the putative bicarbonate transporter (AEL1) and the two putative proton pumps (ATPVc/c', PATP). AEL1 and ATPVc/c' were significantly upregulated (approx. 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': $p = 0.005$ (AEL1); $p = 0.014$ (ATPVc/c'); planned contrasts 'selection \times assay condition': $p = 0.006$ (AEL1); $p = 0.026$ (ATPVc/c')). PATP was significantly upregulated (approx. twofold) only in high CO_2 -adapted populations, while a weaker response in medium CO_2 -adapted populations failed statistical significance (ANOVA, significant interaction 'selection \times assay condition': $p = 0.006$; planned contrasts: 'selection \times assay condition': $p = 0.027$). Detailed results from statistical analyses are given in the electronic supplementary material, table S3.

Strikingly, two promising candidate genes that were restored in populations adapting to ocean acidification were proton pumps, potentially involved in cellular pH regulation (ATPVc/c' and PATP). The third gene (AEL1) that showed a significant adaptive response codes for a putative bicarbonate transporter. Interestingly, next to its proposed function in bicarbonate supply to photosynthesis and 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 of cytosolic pH [32]. All three genes that revealed a significant adaptive response have the potential to be involved in cytosolic pH regulation. This interpretation agrees with the proposed disruption of cellular pH regulation as a primary cause of depressed growth and calcification in *E. huxleyi* under ocean acidification [21,22].

In the CO_2 selection experiment, we observed an adaptive response in calcification rate across all replicates, though we selected for growth rate only [14]. An energetically costly trait such as calcification may get lost if not contributing to fitness, as was shown in *Chlamydomonas* populations where carbon-concentrating abilities degenerated after selection under high CO_2 [33]. By contrast, we observed a direct positive response of calcification rate to CO_2 selection. Consequently, calcification may either be relevant to fitness or genetically or phenotypically correlated to a fitness-relevant trait in our selection experiment. Correlated traits in general are particularly interesting for assessing the potential for evolutionary adaptation to projected ocean changes [34] as they may either promote [35] or constrain [36] the potential for adaptation. We cannot rule out that calcification was beneficial to fitness in our artificial microcosm environment, although the emergence of non-calcifying mutant populations under laboratory conditions [25] suggests the opposite and questions a strong genetic correlation of calcification and

traits relevant to fitness under laboratory conditions. Thus, the most parsimonious explanation is that calcification is a phenotypically correlated trait and its restoration in CO₂-adapted populations was indirectly caused by a more general adaptive response that did not target the process of calcification itself but components of cellular pH regulation. This interpretation is also consistent with improved cytosolic pH regulation in high CO₂-adapted populations after 500 generations under acidified seawater conditions.

To our knowledge, this is the first study that provides information on the adaptive response of *E. huxleyi* to ocean acidification at the gene expression level. Although there are other recent long-term studies on gene expression in *E. huxleyi* [37], their design does not permit for a formal test of adaptation via reciprocal assay experiments, which precludes a direct comparison to our data.

4. Conclusion

Evolutionary adaptation in key phytoplankton species have only recently come into the focus of marine ecology and biogeochemistry. Such processes are of high relevance for a comprehensive understanding of how global change will affect marine ecosystem functioning and biogeochemical cycles [14,34,38]. We identified potential links between the

adaptive response in growth and calcification and underlying metabolic changes at the level of gene expression that suggest improved cellular pH regulation to be involved in the adaptive response of *E. huxleyi* to ocean acidification. Our approach illustrates that laboratory selection experiments can not only be applied to test for adaptation at the level of phenotypes, but may also serve to unravel underlying molecular mechanisms of key traits [34]. Moreover, combining laboratory selection experiments and molecular genetic techniques can identify genes involved in adaptation, here to high CO₂, and thereby provide insights into potential evolutionary trajectories. This work is a first step to approach the genetic basis of adaptation to ocean acidification, a key determinant in predicting the adaptive potential of natural populations of the globally important coccolithophore *E. huxleyi* to an acidifying ocean.

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Data accessibility. All data underlying this publication are available at the PANGEA data repository.

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