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

Kai T. Lohbeck1,2, Ulf Riebesell2 and Thorsten B. H. Reusch1

1Evolutionary Ecology of Marine Fishes, and 2Biological Oceanography, GEOMAR Helmholtz Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

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 Emiliania 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]. Emiliania 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 µM) and high (2200 µM) pCO2 compared to ambient (400 µM) 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 carbon 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 filtered 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 −80 ºC 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 ºC 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 family 4 family (AE1L), a putative cytosolic α-carboxy-anhydrase (aCA), a putative membrane-associated δ-carboxy-anhydrase (6CA), 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 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 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 (αTUB), Actin (Actin) and Elongation factor 1 (EFG1) as potential ERGs. However, only αTUB 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.

\[
- \Delta CT = CT_{ERG} - CT_{GOI}
\]  
\[
\text{Fold expression change} = \pm 2^{((-\Delta CT_{\text{Treatment}}) - (-\Delta CT_{\text{Control}}))}
\]

(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 μatm pCO₂ under 2200 μatm pCO₂ and vice versa. As we were particularly interested in the assay × evolution environment interactions, this resulted in two × 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 × 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 CO₂ selection. The physiological response is the difference in gene expression between ambient CO₂-adapted populations transferred into elevated CO₂ relative to ambient CO₂-adapted populations that remained at ambient CO₂. It is driven by the CO₂ assay environment and informative on the acclimated short-term response of a contemporary E. huxleyi clone exposed to elevated CO₂ conditions after approximately six to eight generations of acclimation. The adaptive response is the difference in gene expression of medium or high CO₂-adapted populations relative to ambient CO₂-adapted populations, serving as control, when both are assayed under medium or high CO₂, 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 CO₂ conditions.

(a) Physiological response
We first focus on the physiological response (figure 1) where we observed a significant 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 CO₂ models) and within-subjects interactions (control versus high CO₂ 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 CO₂ 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 αCA ($p = 0.002$) and βCA ($p = 0.001/0.001$). Note that αCA was significantly downregulated only under high CO₂. 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 CO₂. Note that PATP was significantly downregulated only under high CO₂. 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 CO₂ ($p = 0.012$) does not necessarily imply decreased primary production. Photosynthetic carbon fixation strongly depends on the CO₂ concentration at the active site of RB. Increased CO₂ availability under ocean acidification may therefore increase RB’s carboxylase activity and allow efficient photosynthesis with fewer enzyme units compared with ambient CO₂ conditions. In the physiological response, only the genes coding for the calcium-binding protein (GPA) and a low CO₂-induced protein
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 CO₂ 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, CO₂ was sufficiently available. Increased proton concentrations likely disrupted cellular pH regulation and thereby interfered with calcification [21,22]. Decreased calcification under CO₂-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 CO₂ constant while the other carbonate system parameters varied accordingly and observed a response to CO₂ and bicarbonate but not to pH in these genes. Following their interpretation, increased CCM activity in our ambient CO₂ treatments compared to the medium and high CO₂ treatments could have resulted in a relative downregulation of the candidate genes under medium and high CO₂ conditions. However, as we found a stronger regulatory response in high compared to medium CO₂, this would imply higher CCM activity in the medium compared with the high CO₂ treatment. Given the high CO₂ 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 CO₂ treatment but different bicarbonate and CO₂ 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 (binominal 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 CO₂ selected replicate populations in a stressful salinity environment [31]. We found large variation only among high CO₂-adapted replicates, which was indicative of different genetic
bars indicate gene expression in populations adapted to 2200 m
ences in mean gene expression levels between CO2-adapted and control populations are illustrated as fold expression change
2200 p ¼ ned contrasts 'selection (approx. twofold) only in high CO2-adapted populat-
medium CO2 model) or among-subjects multivariate effects
found that either within-subjects interactions (control versus
adaptive response by repeated measures MANOV As and
show marked divergence in the salinity challenge experiment.
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 CO2 model) or among-subjects multivariate effects
(control versus high CO2 model) were significant (MANOVA,
p ¼ 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 CO2-adapted populations, while a
similar but non-significant response was found in high CO2-
adapted populations (ANOVA, significant main effect ‘selection
environment’: p ¼ 0.005 (AEL1); p ¼ 0.014 (ATPVc/c’); planned
contrasts ‘selection 1 assay condition’: p ¼ 0.006 (ATPVc/c’); p
¼ 0.026 (AEL1); p ¼ 0.026 (ATPVc/c’)). PATP was significantly upregulated
(approx. twofold) only in high CO2-adapted populations,
while a weaker response in medium CO2-adapted populations
failed statistical significance (ANOVA, significant interaction
‘selection 1 assay condition’: p ¼ 0.006; planned
contrasts: ‘selection 1 assay condition’: p ¼ 0.027). Detailed
results from statistical analyses are given in the electronic
supplementary material, table S3.

Strikingly, two promising candidate genes that were rest-
ored 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 bicar-
bonate 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 cel-
lar pH regulation as a primary cause of depressed growth and
calcification in *E. huxleyi* under ocean acidification [21,22].

In the CO2 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 CO2 [33]. By contrast, we observed a direct positive
response of calcification rate to CO2 selection. Consequently,
calcification may either be relevant to fitness or geneti-
cally or phenotypically correlated to a fitness-relevant trait
in our selection experiment. Correlated traits in general are
particularly interesting for assessing the potential for evolu-
tional 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 ben-
eficial 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

Figure 2. Adaptive response in relative gene expression of 10 candidate genes in replicated (N ¼ 5) medium and high CO2-adapted *E. huxleyi* populations. Differences in mean gene expression levels between CO2-adapted and control populations are illustrated as fold expression change ± s.e.m. Pale bars indicate gene expression in populations adapted to 1100 μatm pCO2, relative to control populations adapted to 400 μatm pCO2 when tested under 1100 μatm pCO2. Dark bars indicate gene expression in populations adapted to 2200 μatm pCO2, relative to control populations adapted to 400 μatm pCO2 when tested under 2200 μatm pCO2. Significance levels are indicated: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
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 \textit{E. huxleyi} to ocean acidification at the gene expression level. Although there are other recent long-term studies on gene expression in \textit{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 \textit{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 \textit{E. huxleyi} to an acidifying ocean.

Acknowledgement. We thank Jana Meyer and Katrin Beining for laboratory assistance; Luke Mackinder for advice in candidate gene selection and qRT-PCR procedures.

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

Funding statement. This project was financially supported by the German Federal program ‘BIOACID’ (Biological Impacts of Ocean Acidification).


