Ocean Acidification Reduces Growth and Calcification in a Marine Dinoflagellate

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Abstract
Ocean acidification is considered a major threat to marine ecosystems and may particularly affect calcifying organisms such as corals, foraminifera and coccolithophores. Here we investigate the impact of elevated pCO2 and lowered pH on growth and calcification in the common calcareous dinoflagellate Thoracosphaera heimii. We observe a substantial reduction in growth rate, calcification and cyst stability of T. heimii under elevated pCO2. Furthermore, transcriptomic analyses reveal CO2 sensitive regulation of many genes, particularly those being associated to inorganic carbon acquisition and calcification. Stable carbon isotope fractionation for organic carbon production increased with increasing pCO2 whereas it decreased for calcification, which suggests interdependence between both processes. We also found a strong effect of pCO2 on the stable oxygen isotopic composition of calcite, in line with earlier observations concerning another T. heimii strain. The observed changes in stable oxygen and carbon isotope composition of T. heimii cysts may provide an ideal tool for reconstructing past seawater carbonate chemistry, and ultimately past pCO2. Although the function of calcification in T. heimii remains unresolved, this trait likely plays an important role in the ecological and evolutionary success of this species. Acting on calcification as well as growth, ocean acidification may therefore impose a great threat for T. heimii.

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Introduction
The oceans have taken up about one third of all CO2 emitted by anthropogenic activities since the onset of the industrial revolution [1–3]. This directly impacts seawater carbonate chemistry by increasing concentrations of CO2 and bicarbonate (HCO3−), decreasing concentrations of carbonate (CO32−) and a lowering of pH [4]. The acidification of ocean waters might impact marine life, notably calcifying organisms that use inorganic carbon to produce a calcium carbonate (CaCO3) shell. Calcifying organisms play an important ecological and biogeochemical role in marine ecosystems, evident from extensive coral reefs and vast calcite deposits found in geological records. Ocean acidification has been shown to reduce calcification of various key calcifying organisms such as corals [5], foraminifera [6], and coccolithophores [7,8]. Little is yet known about the general responses of calcareous dinoflagellates [9], and no study so far investigated the impact of ocean acidification on their calcification.

Dinoflagellates feature a complex life-cycle that often includes formation of cysts. In some species, these cysts are made of calcite and can contribute substantially to the ocean carbonate flux in certain regions [10–12]. Thoracosphaera heimii, the most common calcareous dinoflagellate species in present-day ocean, is autotrophic and occurs typically in subtropical and tropical waters [13–15]. The main life-cycle stage of T. heimii comprises coccoid vegetative cells with a calcium carbonate shell, so-called vegetative cysts [16,17]. Although the term cyst is most often used for long-term resting stages that are typically produced after sexual reproduction, in T. heimii this term is used for its coccoid vegetative cysts. Cysts of T. heimii can be commonly found in the fossil record in sediments dating back to the Cretaceous [18]. Therefore, T. heimii cysts may serve as potential proxy for reconstructing the past climate. For instance, Sr/Ca ratios have been shown to correlate well with sea surface temperatures [19], but also the oxygen and carbon isotopes trapped in the cysts could provide useful proxies.

The oxygen isotopic composition (δ18O) of calcite was found to be strongly controlled by the temperature and the δ18O of the seawater in which the organism calcifies [20–22]. In abiotic precipitation experiments, the δ18O of calcite is mainly a function of the δ18O and speciation of dissolved inorganic carbon (DIC), where dissolved CO2 is heavier with respect to 18O than HCO3− and CO32− [23,24]. Similarly, the carbon isotopic composition (δ13C) of calcite is predominantly controlled by the δ13C of DIC, yet dissolved CO2 is depleted with respect to...
$^{13}$C relative to HCO$_3^-$ and CO$_2^-$ [21,25]. In unicellular calcifiers like coccolithophores and T. heinii, calcification occurs intracellularly in specialized vesicles [16,26,27]. Therefore, the inorganic carbon used for calcification by these organisms must be derived from the intracellular inorganic carbon (Ci) pool. Consequently, changes in $^{13}$CO$_2$ and $^{13}$C of calcite should resemble changes in the intracellular Ci pool and may provide insights in the physiological processes underlying calcification and organic carbon production.

Comparable to coccolithophores, ocean acidification likely reduces calcification in T. heinii as well. Furthermore, increasing concentrations of CO$_2$ are expected to alter the stable carbon and oxygen isotopic composition of T. heinii cysts. To test these hypotheses, we grew T. heinii at a range of CO$_2$ levels and followed its responses in growth and calcification. Besides the assessment of $^{18}$O and $^{13}$C in T. heinii as a proxy, we use its isotopic composition as a tool to understand processes involved in organic carbon production and calcification. Transcriptomic analyses were applied to reveal mechanisms underlying the observed responses.

### Materials and Methods

#### Experimental Set-up

Cells of Thraustochytrium heinii RCC1512 (formerly AC214; Roscoff Culture Collection) were grown as dilute batch cultures in 2.4 L air-tight borosilicate bottles. Population densities were kept low at all times (<1,000 cells mL$^{-1}$) in order to keep changes in carbonate chemistry minimal (i.e. <3.5% with respect to DIC; Table S1). Filtered natural seawater (0.2 μm) was enriched with metals and vitamins according to the recipe for f/2-medium, except for FeCl$_3$ (1.9 μmol L$^{-1}$), H$_2$SeO$_4$ (10 nmol L$^{-1}$), and NiCl$_2$ (6.3 nmol L$^{-1}$). The added concentrations of NO$_3^-$ and PO$_4^{3-}$ were 100 μmol L$^{-1}$ and 6.25 μmol L$^{-1}$, respectively. Cultures were grown at a lightdark cycle of 16:8 h and an incident light intensity of 250±25 μmol photons m$^{-2}$ s$^{-1}$ provided by daylight lamps (Lumilux HO 54W/965, Osram, München, Germany). Bottles were kept at 15°C and placed on a roller table to avoid sedimentation. Prior to inoculation, the culture medium was equilibrated with air containing 150 atm CO$_2$ (future scenarios assuming unabated emissions).

#### Sampling and Analyses

Prior to the experiments, cells were acclimated to the respective CO$_2$ concentrations for at least 21 days, which corresponds to >5 cell divisions. Experiments were run for 8 days and included >3 cell divisions. Cell growth was monitored by means of triplicate cell counts daily or every other day with an inverted light microscope (Axiovert 40C, Zeiss, Germany), using 0.5–2 ml culture suspension fixed with Lugol’s solution (2% final concentration in mQ$_2$). Cell counts included determination of vegetative cysts, i.e. cells containing cell material, and empty shells. Because empty shells also contain inorganic carbon, the total number of cysts was used for estimating inorganic carbon quota, while only vegetative cysts were included in the growth rate estimations. From each biological replicate, growth rates were estimated by means of an exponential function fitted through the number of vegetative cysts over time, according to:

$$N_t = N_0e^{at}$$

(1)

where $N_t$ refers to the population density at time $t$ (in days), $N_0$ to the population density at the start of the experiment, and $a$ to the growth rate (Fig. S1).

For total alkalinity (TA) analyses, 25 mL of culture suspension was filtered over glass-fibre filters (GF/F, ~0.6 μm pore size, Whatman, Maidstone, UK) and stored in gas-tight borosilicate bottles at 3°C. Duplicate samples were analysed by means of potentiometric titrations using an automated TitroLine burette system (SI Analytics, Mainz, Germany). pH was measured immediately after sampling with a pH electrode (Schott Instruments, Mainz, Germany), applying a two-point calibration on the NBS scale prior to each measurement. For DIC analyses, 4 mL culture suspension was filtered over 0.2 μm cellulose-acetate filters, and stored in headspace free gas-tight borosilicate bottles at 3°C. Duplicate samples of DIC were analysed colorimetrically with a QuAAtro autoanalyzer (Seal Analytical, Mequon, USA). Carbonate chemistry (Table S1) was assessed by total alkalinity (TA) in combination with pH/InS and temperature and salinity, using the program CO$_2$sys [28]. For the calculations, an average phosphate concentration of 6.4 μmol L$^{-1}$ was assumed, the dissociation constant of carbonate acid was based on Mehrbach et al. [29], refit by Dickson and Millero [30]. The dissociation constant of sulfuric acid was based on Dickson [31].

To determine the isotopic composition of DIC ($^{13}$CDIC), and the water ($^{18}$Owater), 4 mL of culture suspension was sterilized over 0.2 μm cellulose-acetate filters and stored at 3°C. Prior to analyses, 0.7 mL of sample was transferred to 8 mL vials. For determination of $^{13}$CDIC, the headspace was filled with helium and the sample was acidified with three drops of a 102% H$_2$PO$_4$ solution. For determination of $^{18}$Owater, the headspace was flushed with helium containing 2% CO$_2$, CO$_2$ and O$_2$ isotopic composition in the headspace were measured after equilibration using a GasBench-II coupled to a Thermo Delta-V advantage isotope ratio mass spectrometer with a precision of <0.1% [32].

At the end of each experiment, cultures were harvested for analyses of particulate organic carbon (POC) and related isotopic composition ($^{13}$CPOC), total particulate carbon (TPC), isotopic composition of the calcite ($^{13}$Ccalcite and $^{18}$Ocalcite), and for the Scanning Electron Microscope (SEM). For POC and TPC analyses, 250–500 mL cell suspension was filtered over pre-combusted GF/F filters (12 h, 500°C) and stored at −25°C in precombusted Petri dishes. Prior to POC measurements, 200 μL of 0.2 N analytical grade HCl was added to the filters to remove all particulate inorganic carbon (PIC), and filters were dried overnight. POC, $^{13}$CPOC, and TPC were analysed in duplicate on an Automated Nitrogen Carbon Analyser mass spectrometer (ANCA-SL 20–20, SerCon Ltd., Crewe, UK). PIC was calculated as the difference in carbon content between TPC and POC. $^{13}$Ccalcite and $^{18}$Ocalcite were measured with a Thermo Scientific MAT253 coupled to a Kiel IV carbonate preparation device. Analytical stability and calibration was checked routinely by analyzing NBS19 and IAEA-CO1 carbonate standards. Reproducibility (Kiel IV and MAT253) was <0.05% and <0.03% for $^{18}$O and $^{13}$C, respectively.

For SEM analyses, 50 mL culture suspension was filtered over a 0.8 μm polycarbonate filter and dried overnight at 60°C. Filters were fixed on aluminium stubs, sputter-coated with gold-palladium using an Emscope SC500 Sputter Coater (Quorum Technologies, Ashford, UK), and viewed under a FEI Quanta FEG 200 scanning electron microscope (FEI, Eindhoven, the Netherlands). From each replicate, a total of >200 cysts were counted and assessed as complete or incomplete.
Isotopic Fractionation

Isotopic fractionation during organic carbon production and calcification was calculated based on the carbon isotopic composition of the cellular organic carbon, cellular inorganic carbon and DIC, and the oxygen isotopic composition of the calcite and seawater, respectively. The carbon isotopic composition is reported relative to the PeeDee belemnite standard (PDB):

$$\delta^{13} C_{\text{sample}} = \left( \frac{^{13}C/^{12}C_{\text{sample}}}{^{13}C/^{12}C_{\text{PDB}}} - 1 \right) \times 10^3 \tag{2}$$

The isotopic composition of CO2 ($\delta^{13} C_{\text{CO2}}$) was calculated from $\delta^{13} C_{\text{DIC}}$ using a mass balance relation according to Zeebe and Wolf-Gladrow [24], applying fractionation factors between CO2 and HCO3$^-$ from Mook et al. [33] and between HCO3$^-$ and CO3$^{2-}$ from Zhang et al. [34]. The isotopic fractionation during POC formation ($\varepsilon_p$) was calculated relative to $\delta^{13} C_{\text{CO2}}$ according to Freeman and Hayes [35]:

$$\varepsilon_p = \frac{\delta^{13} C_{\text{CO2}} - \delta^{13} C_{\text{POC}}}{1 + \delta^{13} C_{\text{POC}} \times 10^{-3}} \tag{3}$$

The carbon isotopic fractionation during calcite formation ($\varepsilon_k$) was calculated relative to $\delta^{13} C_{\text{DIC}}$:

$$\varepsilon_k = \frac{\delta^{13} C_{\text{DIC}} - \delta^{13} C_{\text{PIC}}}{1 + \delta^{13} C_{\text{PIC}} \times 10^{-3}} \tag{4}$$

The oxygen isotopic composition in the calcite is also reported relative to the PDB standard:

$$\delta^{18} O_{\text{calcite}} = \left( \frac{^{18}O/^{16}O_{\text{calcite}}}{^{18}O/^{16}O_{\text{PDB}}} - 1 \right) \times 10^3 \tag{5}$$

The oxygen isotopic composition in DIC ($\delta^{18} O_{\text{DIC}}$) was determined using the oxygen fractionation factor between DIC, calculated after Zeebe and Wolf-Gladrow [24], and water ($\alpha_{\text{DIC}-\text{H}_2\text{O}}$), calculated after Zeebe [36], with temperature corrected fractionation factors from Beck et al. [37]. The isotopic composition of DIC ($\delta^{18} O_{\text{DIC}}$) was calculated according to:

$$\delta^{18} O_{\text{DIC}} = \left( \frac{\delta^{18} O_{\text{H}_2\text{O}+10^3}}{\alpha_{\text{DIC}-\text{H}_2\text{O}}} - 1 \right) \times 10^3 \tag{6}$$

Transcriptomic Analyses

For RNA extraction, 500 mL of culture suspension was concentrated to 50 mL with a 10 μm mesh-sized sieve, and subsequently centrifuged at 15°C for 15 min at 4000 g. Cell pellets were immediately mixed with 1 mL 60°C TriReagent (Sigma-Aldrich, Steinheim, Germany), frozen with liquid nitrogen and stored at −80°C. Subsequently, cell suspensions were transferred to a 2 mL cryovial containing acid washed glass beads. Cells were lysed using a BIO101 FastPrep instrument (Thermo Savant, Illkirch, France) at maximum speed (6.5 m s$^{-1}$) for 2×30 s, with an additional incubation of 5 min at 60°C in between. For RNA isolation, 200 μL chloroform was added to each vial, vortexed for 20 s and incubated for 10 min at room temperature. The samples were subsequently centrifuged for 15 min at 4°C with 12,000 g. The upper aqueous phase was collected by 20 min centrifugation at 4°C and 12,000 g. The pellet was washed twice, first with 70% ethanol and afterwards with 96% ethanol, air-dried and dissolved with 100 μl RNase free water (Qiagen, Hilden, Germany). The RNA sample was further cleaned with the RNeasy Kit (Qiagen) according to manufacturer's protocol for RNA clean-up including on-column DNA digestion. RNA quality check was performed using a NanoDrop ND-100 spectrometer (PezLab, Erlangen, Germany) for purity, and the RNA Nano Chip Assay with a 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany) was performed in order to examine the integrity of the extracted RNA. Only high quality RNAs (OD260/OD280 > 2 and OD250/OD280 < 1.0) as well as RNA with intact ribosomal peaks (obtained from the Bioanalyzer readings) were used for microarrays.

454-libraries were constructed by Vertis biotechnologie AG (http://www.vertis-biotech.com/). From the total RNA samples poly(A)+ RNA was isolated, which was used for cDNA synthesis. First strand cDNA synthesis was primed with an N6 randomized primer. Then 454 adapters were ligated to the 5’ and 3’ ends of the cDNA, and the cDNA was amplified with 19 PCR cycles using a proof reading polymerase. cDNA with a size range of 500–900 bp was cut out and eluted from an agarose gel. The generated libraries were quantified with an RL-Standard using the Quantifi-Fluor (Promega, Mannheim, Germany). The library qualities were assessed using the High Sensitivity DNA chip on the Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). For all sequencing runs 20×10$^7$ molecules were used for the emulsion PCR that were carried out on a MasterCycler PCR cycler (Eppendorf, Hamburg, Germany). The following enrichment was performed according to the manufacturer’s instructions. Sequencing was performed with the GS Junior Titanium Sequencing Kit under standard conditions. The 454 Sequencing System Software version 2.7 was used with default parameters, i.e., Signal Intensity filter calculation, Primer filter, Valley filter, and Base-call Quality Score filter were all enabled.

Statistical Analysis

Normality was confirmed using the Shapiro-Wilk. Variables were log-transformed if this improved the homogeneity of variances, as tested by Levene's test. Significance of relationships between variables and concentration of CO2 and CO3$^{2-}$ were tested by means of linear regression. Significance treatments was tested using one-way ANOVA, followed by post hoc comparison of the means using Tukey’s HSD ($\alpha = 0.05$) [38].

Results

Increasing concentrations of CO2 cause a strong decline in growth (Fig. 1A), which decreases by up to 53% over the investigated CO2 range (Table S2). Although the total carbon quota (TPC) is not affected by CO2 (Table S2), the organic carbon quota (POC) gradually increases while the inorganic carbon quota (PIC) shows a substantial decrease (Fig. 1B). Consequently, the PIC:POC ratio strongly decreases with increasing concentrations.
of CO₂ (Fig. 1C), showing a decrease of ~54% from the lowest to the highest CO₂ treatment (Table S2).

The reduced degree of calcification is also evident from the cyst morphology. In the lowest CO₂ treatment, the majority of cysts shows a fully closed and completed calcite structure (Fig. 2A–C). At the highest CO₂ concentration, however, calcification of most cysts is incomplete (Fig. 2D–H). Some cysts show initial stages of calcification, indicated by typical square pores (Fig. 2E,F) [16]. In other cysts, the numerous crystallization sites remain unconnected showing clear cavities in the calcite structure (Fig. 2G,H). These cavities likely cause the collapse of many cysts upon filtration (Fig. 2D, white arrows). With increasing concentrations of CO₂, the number of completed cysts dramatically decreases from ~98% at the lowest CO₂ treatment towards ~18% at the highest CO₂ treatment (Fig. 1D).

Carbon isotope fractionation responds strongly to the applied CO₂ treatments, showing an increase in \( \delta^{13}C \) and a decrease in \( \delta^{18}O \) with increasing \( p_{CO_2} \) (Fig. 3A). In other words, the organic carbon fraction of the cells becomes depleted in \(^{13}C\) while the inorganic carbon fraction (i.e. the calcite) increases its \(^{13}C\) content. Furthermore, the calcite also becomes \(^{18}O\)-enriched, indicated by the increase in \( \delta^{18}O_{calcite} \) with increasing \( p_{CO_2} \) (Fig. 3B). As dissolved CO₂ is heavier than HCO₃⁻ and CO₃²⁻ [24], increasing CO₂ levels cause \( \delta^{18}O_{DIC} \) to increase (Fig. 3B). Yet, changes are relatively small and the \( \delta^{18}O_{DIC} \) remains close to that of HCO₃⁻, which is the dominant inorganic carbon species. To permit comparison with previous findings, \( \delta^{18}O_{calcite} \) values were corrected for the \( \delta^{18}O \) of water (−0.52 ± 0.07 \%) and plotted as a function of CO₃²⁻ concentration (Fig. 3C). Calcite \( \delta^{18}O \) decreases strongly with increasing concentrations of CO₃²⁻, and the slope is similar to the one reported for another T. heinii strain (RCC1511) [9].

The transcriptome indicates substantial gene regulation in response to changes in carbonate chemistry, with a total of 9701 genes being expressed (Fig. S2). The expression of the majority of genes was treatment specific, amounting to 3183, 2704, and 2176 genes in the low, present-day and high CO₂ treatments, respectively (Fig. S2). Interestingly, the number of expressed genes to which a function could be assigned by comparison with public databases was highest in the low and present-day CO₂ treatment (~22%), and lowest in the high CO₂ treatment (~13%). The expressed genes from each treatment are differentially distributed over different ‘eukaryotic orthologous groups’ (KOGs; Fig. S3 and Table S3). Although the total number of expressed genes is largely comparable between treatments, different sets of genes within the KOGs are expressed. About 55% of the number of expressed and annotated genes in each treatment are associated to the KOGs ‘Translation, ribosomal structure and biogenesis’, ‘Signal transduction mechanisms’, ‘Posttranslational modification, protein turnover and chaperones’, and ‘Energy production and conversion’ (Fig. S3). Expression of genes associated to the latter two categories increased in response to increasing \( p_{CO_2} \). In contrast, expression of genes involved in ‘Inorganic ion transport and metabolism’ decreased in the high CO₂ treatment (Fig. S3).

We therefore investigated the genes involved in ion transport and inorganic carbon acquisition in more detail (Fig. 4; Table S4). We observed a substantial regulation of genes associated to vacuolar Ca²⁺ and H⁺ transport, including P-type Ca²⁺ ATPases, Ca²⁺/Na⁺ exchangers (NCX1), Ca²⁺/H⁺ antiporters (VCX), and vacuolar H⁺ ATPases (V-ATPase). In particular, the relative expression of genes associated to NCX and V-ATPase decreases

**Figure 1.** Effect of increasing CO₂ concentrations on growth and calcification. (A) Specific growth rate, (B) PIC and POC, (C) PIC:POC ratio, and (D) fraction of completed cysts. Solid lines indicate linear regressions (n = 12) with (A) \( R^2 = 0.94, P < 0.001 \), (B) POC: \( R^2 = 0.35, P = 0.042 \), and PIC: \( R^2 = 0.66, P = 0.001 \), (C) \( R^2 = 0.70, P < 0.001 \), and (D) \( R^2 = 0.98, P < 0.001 \).
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from the low to the high CO₂ treatment (Fig. 4). Similarly, the relative expression of genes associated to carbonic anhydrases (CA) and aquaporins decreases with increasing pCO₂. In the present-day CO₂ treatment, we observed expression of a gene associated to an SLC4 family anion exchanger (AE), most likely responsible for the transport of HCO₃⁻ into the cell (Fig. 4) [39]. This gene was expressed in neither the low nor the high CO₂ treatment. An SLC26 family SO₄²⁻/HCO₃⁻/C₂O₄²⁻ anion exchanger (SAT-1) was yet another exclusive expression of a gene only found in the low CO₂ treatment. The potential role of this anion exchanger in Ci acquisition by phytoplankton remains to be elucidated.

Discussion

Growth and Carbon Production

Our results show considerable impacts of elevated pCO₂ on T. heimi, with strong decreases in its growth rate and degree of calcification (Fig. 1, 2). Despite the increase in organic carbon quota (POC), the overall biomass production decreases substantially with increasing pCO₂ (Table S2). Higher availability of CO₂ has been shown to promote phytoplankton growth and carbon production [40,41]. Such CO₂ responses are typically associated to the poor catalytic properties of RubisCO, which is characterized by low affinities for its substrate CO₂. Increasing concentrations of CO₂ are however accompanied by a reduction in pH, which may have consequences for calcification. For the most common coccolithophore Emiliania huxleyi, lowered pH in fact hampers calcification while elevated pCO₂ stimulates biomass production, causing a reallocation of carbon and energy between these key processes [42,43]. This flexibility may explain why growth in E. huxleyi is typically not affected by ocean acidification [44]. In T. heimi, however, we observed a strong decrease in calcification, in biomass production as well as in growth. Apparently, T. heimi lacks the ability to efficiently reallocate cellular carbon between pathways and maintain growth relatively unaffected. Our data furthermore suggests that calcification plays a fundamental role in its growth, life cycle and hence survival. Recent findings have shown that growth and calcification by E. huxleyi may, at least partly, recover from ocean acidification as result of evolutionary adaptation [45]. Whether or not T. heimi exhibits such capabilities of adaptive evolution can only be answered from long-term incubations over hundreds of generations [46].

Transcriptomic analyses reveal a substantial regulation of genes in response to elevated pCO₂. Even though no major shift in the relative distribution of expressed genes to the functional categories (KOGs) is induced by the treatments, T. heimi uses different sets of genes within these categories. There is a slight increase in the expression of genes associated to signal transduction and posttranslational modifications upon elevated pCO₂, and a decrease in the expression of genes involved in inorganic ion transport (Fig. S3), suggesting that T. heimi readjusts its transcriptome on several levels when grown under different pCO₂. Many phytoplankton species have the ability to deal with changes in CO₂ availability by regulating their so-called carbon concentrating mechanism (CCMs) [47–49]. T. heimi also appears to regulate its proteome towards changes by down-regulating genes involved in CA and aquaporins under elevated pCO₂, and by up-regulating these genes under lowered pCO₂ (Fig. 4). CA accelerates the equilibrium between CO₂ and HCO₃⁻, and can be located both intra- and extracellularly. From our results it remains unclear whether T. heimi expresses intra- or extracellular CA. Yet, in both cases CA plays a key role in the CCM, as it replenishes the CO₂ around RubisCO (intracellular) or the carbon source being depleted in the boundary layer due to active uptake (extracellular) [49,50]. Aquaporins have been suggested to play a role in CO₂ transport [47,51], which is supported by the observed CO₂-
dependency in our expression patterns. Besides CO₂ also HCO₃⁻ is often transported into the cell, which will facilitate the high intra-cellular CO₂ requirements imposed by RubisCO. Indeed, T. heimii expresses genes associated to putative HCO₃⁻ transporters at both low and present-day pCO₂, but not at high pCO₂ (Fig. 4).

Our results thus suggest a down-scaling of the CCM in T. heimii under elevated pCO₂, which possibly makes energy available for other processes as it has been observed in other species [43,52]. Yet it seems that neither the down-scaling of the CCM nor an extensive regulation of the transcriptome can compensate for the adverse effects of elevated pCO₂ on growth and calcification in T. heimii.

Calcification and Isotope Fractionation
Calcification in T. heimii was strongly affected by elevated pCO₂. Along with a reduction in the degree of calcification (Fig. 1B,C), also the morphology of T. heimii cysts was influenced (Fig. 2). With elevated pCO₂ the number of completed cysts dramatically decreased and the number of collapsed cysts increased. The completed calcite structures predominant at low and present-day pCO₂, whereas the incomplete calcite structures, prevailing under high pCO₂, resemble those of young cells [16,26]. The incomplete cysts in our experiments, however, often contain an opening through which the cell has left for division, being indicative for mature cells. Thus, cells remained either in the cyst too short for completing the calcite structure, the calcite cyst was directly affected by the low pH of the water, and/or cells reduced their calcification rates. Since growth rates were strongly reduced upon elevated pCO₂, it seems unlikely that cells remained in the cyst stage too short for completion of the cyst, as could be expected under enhanced growth rates. Although pH in our highest CO₂

Figure 3. Effect of increasing CO₂ concentrations on the stable isotope composition. (A) δ¹³C fractionation of organic carbon (εp) and calcite (εk), (B) δ¹⁸O composition of calcite (δ¹⁸Ocalcite) and DIC (δ¹⁸ODIC), and (C) relationship between the oxygen isotopic composition of calcite (δ¹⁸Ocalcite-water) in Thoracosphaera from this study (open diamonds) and from Ziveri et al. [9] (grey diamonds). Horizontal lines in (B) indicate δ¹⁸O values for HCO₃⁻ and CO₃²⁻, and dashed line indicates trend of curve. Solid lines indicate linear regressions (n=12) with (A) εp: R² = 0.75, P<0.001, and εk: R² = 0.90, P<0.001, (B) δ¹⁸Ocalcite: R² = 0.76, P<0.001, and (C) This study: R² = 0.99, P<0.001, and Ziveri et al. (2012), (n=7): R² = 0.95, P<0.001.

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Figure 4. Effect of elevated pCO₂ on gene regulation. Number of readings found for genes associated to ion transport and Ci acquisition in the 150 µatm and 1400 µatm CO₂ treatments relative to the present-day (380 µatm) CO₂ treatment.

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treatment was close to 7.6, the water still remained supersaturated with respect to calcite (i.e. an \( \Omega_{\text{calcite}} > 1.2 \); Table S2), and calcite dissolution seem unlikely to have caused the incomplete and cavities in the calcite structure (Fig. 2). Thus, the large number of affected \( T. \) heimii cysts at elevated \( pCO_2 \) seems mainly to be a result of reduced calcification rates by the cells.

Calcification in \( T. \) heimii likely takes place intracellularly in vesicles [16,26], comparable to coccolithophores [9,27]. Hence, the inorganic carbon needed for calcification is obtained from the intracellular inorganic carbon pool (\( C_i \)), which may deviate strongly from external conditions in terms of speciation as well as isotopic composition. We observed an increase of carbon isotope fractionation for organic carbon production (\( \epsilon_{\text{org}} \)), whereas it decreased for calcite formation (\( \epsilon_{\text{calc}} \)) in response to elevated \( pCO_2 \) (Fig. 3A). With a higher availability of \( CO_2 \), more of the intracellular \( C_i \) pool may be replenished by \( CO_3^- \), which is depleted in \( ^{13}C \) compared to \( HCO_3^- \). Consequently, Rubisco can fractionate against an isotopically lighter \( C_i \) pool and thus better express its preference for lighter \( ^{13}C \), which could explain the increasing \( \epsilon_{\text{org}} \). As a consequence, the intracellular \( C_i \) pool becomes enriched with \( ^{13}C \) by so-called Rayleigh distillation, which \textit{a priori} could explain the decrease in \( \epsilon_{\text{calc}} \). However, increased \( CO_2 \) availability in combination with a reduced organic carbon production should lead to a lowered Rayleigh distillation, and in fact decrease the enrichment of \( ^{13}C \) within the cell. Also, Rayleigh distillation should always feedback on \( CO_2 \) fixation as well as \( CaCO_3 \) precipitation, and thus cannot explain the opposing trends of fractionation in those processes.

The opposing \( CO_2 \) effects on \( \epsilon_{\text{org}} \) and \( \epsilon_{\text{calc}} \) can thus only be explained if both processes use \( C_i \) pools that are isotopically different. \( CO_2 \) fixation uses the \( C_i \) pool within the chloroplast, which is affected by the relative \( CO_2 \) and \( HCO_3^- \) fluxes, the \( CO_2 \) leakage as well as the intrinsic fractionation by Rubisco [53,54]. The \( C_i \) pool for calcification will mainly be controlled by the condition in the cytosol, which in turn is largely affected by the processes in the chloroplast. Discrimination of \( ^{12}C \) during fixation will lead to \( ^{13}CO_2 \) efflux from the chloroplast, causing the cytosolic \( C_i \) pool to be enriched with \( ^{13}CO_2 \). If this \( ^{13}CO_2 \) is prevented from fast conversion to \( HCO_3^- \) due to a lack of cytosolic CA activity, it could enter the calcifying vesicle by diffusion and be ‘trapped’ by the high \( pH \) resulting from proton pumping (Fig. 5). In fact, we do observe a higher \( \epsilon_{\text{org}} \) (i.e. more \( ^{13}CO_2 \) can accumulate) and lower overall CA activities under elevated \( pCO_2 \) (i.e. \( ^{13}CO_2 \) is not rapidly converted to \( HCO_3^- \)), which could have contributed to the opposing trends of \( ^{13}C \) fractionation during organic and inorganic carbon production. To fully understand the intriguing interplay between these processes and their \( ^{13}C \) fractionation, detailed measurements on the modes of \( C_i \) acquisition in \( T. \) heimii are needed.

The oxygen isotopic composition (\( \delta^{18}O \)) of calcite was also strongly affected in \( T. \) heimii, and increased by almost \( 6 \% \) over the investigated \( CO_2 \) range (Fig. 3B). Even though biologically mediated, precipitation of calcite is an abiotic process, which does not directly involve enzymatic reactions and thus mainly depends on the carbonate chemistry at the calcification site. Assuming negligible fractionation during the transport into the calcification vesicle, \( \delta^{18}O_{\text{calcite}} \) should therefore predominantly reflect the \( \delta^{18}O \) of the \( C_i \) species used for calcification. \( C_i \) species differ strongly in their \( \delta^{18}O \) values, ranging from lower values for \( CO_3^{2-} \) (\(-4.7 \% \)) and \( HCO_3^- \) (\(2.1 \% \)) to much higher values for \( CO_2 \) (\(11.2 \% \)) [24]. A previous study proposed a conceptual model to explain the \( \delta^{18}O \) dependence of \( T. \) heimii calcite and other unicellular planktonic calcifiers on seawater \( CO_2 \) concentration (Fig. 3C) [9]. The authors attribute the negative slope between \( \delta^{18}O \) and \( [CO_3^{2-}] \) to an increased contribution of \( HCO_3^- \) to the calcification vesicle. Also in our data, \( \delta^{18}O_{\text{calcite}} \) increases with increasing \( pCO_2 \), starting from values close to the \( \delta^{18}O \) of \( CO_2 \) towards those of \( HCO_3^- \) (Fig. 3B). As argued above, however, the \( C_i \) pool in the calcifying vesicle may also be increasingly influenced by \( CO_2 \), which is in line with the observed trends in \( \delta^{18}O_{\text{calcite}} \). Such a shift in \( C_i \) speciation may be an indication for a lowered intracellular \( pH \), which in fact could be the reason for the hampered calcification under elevated \( pCO_2 \) [55,56].

Multiple genes associated to calcification have been described for \( E. \) huxleyi and include genes associated to the regulation of inorganic ions [39,55–59]. Here we show that the expression of genes in \( T. \) heimii being involved in inorganic ion transport, in particular \( Ca^{2+} \) transport, decreased upon elevated \( pCO_2 \) (Fig. 4; Fig. S3). This decrease in ion transport is in line with the observed decrease in calcification, which is comparable to observations in \( E. \) huxleyi [39,59]. We also observed a strong \( CO_2 \) dependent regulation of the vacuolar \( H^+\text{-ATPases (V-ATPase). These pumps play a key role in generating } H^+ \text{ gradients and membrane voltage, which drive multiple transport processes [57,60]. As indicated from our data, } H^+\text{-ATPases seem to play an important role in calcification in } T. \) heimii, which is in agreement to observations for \( E. \) huxleyi and \( Pleurochrysis carterae \) [39,59,61]. Here we propose a conceptual model of calcification in \( T. \) heimii, which comprises some of the main processes described in this study (Fig. 5). Although many processes remain to be elucidated, this is a first step towards understanding the process of calcification in dinoflagellates.

**Paleo Proxies**

The \( \delta^{18}O \) isotopic composition of \( T. \) heimii cysts has been used for the reconstruction of past temperatures [22,62]. Indeed, \( \delta^{18}O \) changed linearly from about \(-1 \) to \(-4 \% \) with an increase in temperature from about 12 to 30°C. At the same time, however, \( pH \) decreased from about 8.4 to 7.9 in this study [22]. Hence, the observed changes in \( \delta^{18}O \) were most probably a result of both changes in temperature and seawater carbonate chemistry [see also 62]. Here we show remarkable changes in \( \delta^{18}O \) from about 0 to \(-5 \% \) with an increase in \( [CO_3^{2-}] \) from 50 to 260 \( \mu \text{mol L}^{-1} \), which is largely in agreement to an earlier study including a different \( T. \) heimii strain (Fig. 3C) [9]. Interestingly, the observed slopes of \( \delta^{18}O/[CO_3^{2-}] \) in both \( T. \) heimii strains are up to 10-fold steeper compared the coccolithophore \( Calcidiscus leptoporus \) and different foraminifera species [9,25,63]. Thus, the apparent \( ^{18}O \) fractionation during calcification in \( T. \) heimii is much more sensitive to changes in \( [CO_3^{2-}] \) as compared to other key planktonic marine calcifiers. The steep slope and negative correlation between \( \delta^{18}O \) and \( [CO_3^{2-}] \) observed in both \( T. \) heimii strains suggests that the \( \delta^{18}O \) in \( T. \) heimii cysts may be a good candidate to serve as a proxy for past \( CO_3^- \) concentrations in ocean waters. This relationship may provide an ideal asset, especially when combined with different \( \delta^{18}O/[CO_3^{2-}] \) slopes observed in for instance coccolithophores, which will exclude confounding effects of additional environmental parameters such as temperature. Ultimately, this proxy could be further developed for reconstructing past atmospheric \( pCO_2 \).

**Conclusion**

We observed a strong reduction in growth rate and calcification of \( T. \) heimii under elevated \( pCO_2 \). Although the function of calcification in \( T. \) heimii remains unresolved, it likely plays an important role in its ecological and evolutionary success. Acting on calcification as well as growth, ocean acidification may impose a great threat for \( T. \) heimii. Furthermore, the strong correlations
between the stable isotope composition and carbonate chemistry suggest a great potential of *T. heimii* cysts to be used as paleo proxy for reconstructing seawater carbonate chemistry and ultimately past atmospheric $p_{\text{CO}_2}$.

**Supporting Information**

**Figure S1** Population growth dynamics. Population densities in each replicate over time in the (A) 150 μatm, (B) 380 μatm, (C) 750 μatm, and (D) 1400 μatm $p_{\text{CO}_2}$ treatments. Lines indicate an exponential function fitted through the population densities ($n=8$) of replicate 1 (black), 2 (grey) and 3 (white), with (A) 1: $R^2 = 0.98$, p$<0.0001$, 2: $R^2 = 0.97$, p$<0.0001$, and 3: $R^2 = 0.97$, p$<0.0001$, (B) 1: $R^2 = 0.97$, p$<0.0001$, 2: $R^2 = 0.97$, p$<0.0001$, and 3: $R^2 = 0.92$, p$<0.0001$, (C) 1: $R^2 = 0.92$, p = 0.0007, 2: $R^2 = 0.96$, p$<0.0001$, and 3: $R^2 = 0.97$, p$<0.0001$, and (D) 1: $R^2 = 0.96$, p$<0.0001$, 2: $R^2 = 0.95$, p$<0.0001$, and 3: $R^2 = 0.91$, p = 0.0002.

**Figure S2** Number of expressed genes. Venn diagram of the number of expressed genes in the 150 μatm, 380 μatm, and 1400 μatm $p_{\text{CO}_2}$ treatments.

**Figure S3** Distribution of expressed genes grouped according to KOG. Values represent the number of genes expressed per KOG, relative to the total number of genes expressed in the respective treatment.
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