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Life at elevated CO_2 modifies the cell composition of *Chromera velia* (Chromerida)

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ABSTRACT

We investigated the response to high CO_2 of *Chromera velia*, a photosynthetic relative of apicomplexan parasites that is possibly involved in symbiotic associations with scleractinian corals. The inorganic C content in the proximity of the symbiotic algal cells within the tissues of scleractinians is disputed. According to some authors, it is very high. A higher C content in the endodermal tissues of scleractinians than in the external environment may have favoured the constitution of symbiosis with organisms such as *Symbiodinium* and *Chromera* that have a type II Rubisco, which is intrinsically ill suited to low CO_2 environments. We thus cultured *C. velia* at the very high inorganic C estimated by some authors and assessed its growth and photosynthetic performance. We also evaluated whether these conditions affected C allocation and elemental stoichiometry in *C. velia* cells by state-of-the-art Fourier transform infrared spectroscopy and total reflection X-ray fluorescence spectrometry in combination with more traditional biochemical and physiological techniques. Our results demonstrated that *C. velia* was capable of coping with very high CO_2, which even stimulated biomass production and increased N, P, Mn, Fe and Zn use efficiency. Growth at elevated CO_2 changed the stoichiometric relationships among elements in *C. velia* cells, but had no effect on the relative abundance of the main organic pools. The high CO_2 in the animal tissue surrounding the photosynthetic cells may therefore facilitate *C. velia* life in symbiosis.

KEY WORDS C allocation; carbon concentrating mechanisms (CCMs); elemental stoichiometry; FTIR; nutrients; organic composition; symbiosis

Introduction

Chromera velia is an alga (*sensu* Raven & Giordano 2014) isolated in 2001 from Sydney Harbor (Moore *et al.*, 2008) which is believed to live in symbiotic association with at least three different species of scleractinian corals (Moore *et al.*, 2008; Cumbo *et al.*, 2013). Recently, the new phylum Chromerida was proposed in order to accommodate *C. velia* and its relative *Vitrella brassicaformis* (Moore *et al.*, 2008; Oborník *et al.*, 2012). The genome of *C. velia* (Woo *et al.*, 2015) revealed that this alga represents a link between the parasitic Apicomplexa (e.g. *Toxoplasma* spp., *Plasmodium* spp.), which are non-photosynthetic organisms with a relic, unpigmented plastid (apicoplast), and their photosynthetic predecessors (Linares *et al.*, 2014; Woo *et al.*, 2015). It is believed that the chromerids share their photosynthetic ancestors with dinoflagellates (Janouškovec *et al.*, 2010; Petersen *et al.*, 2014; Woo *et al.*, 2015), among which *Symbiodinium* is a symbiont of scleractinians. In contrast to the symbiosis between *Symbiodinium* and scleractinian corals, which has been thoroughly studied since its first identification (Brandt, 1881), the exact nature of the association between *C. velia* and stony corals has not been fully elucidated. The establishment of a symbiosis between free-living *C. velia* cells and larvae of the corals *Acropora digitifera* and *A. tenuis* (with the alga located both in the endoderm and in the ectoderm of the hosts) has been reported (Cumbo *et al.*, 2013), confirming the potential for an endosymbiotic behaviour of *C. velia*. Although the symbiotic relationship between *C. velia* and its hosts appears very likely, there is scant information on its location in the animal tissue. While *Symbiodinium* cells are well known to reside in the endodermal cells of the coral, within symbiosomes, the location of *C. velia* cells in the animal host is unclear. However, the fact that *C. velia* was first isolated from the cnidarium *Plesiastrea versipora* by using an isolation procedure similar to that used to extract *Symbiodinium* cells from their hosts (Moore *et al.*, 2008), suggests that the two organisms have a similar location within corals.

Chromera velia has been the object of genomic studies that have provided evidence for some of the peculiarities of this organism. The chloroplast of *C. velia*, which was most likely inherited from red algae, contains genes also from the green lineage (Woehle *et al.*, 2011), although the extent of this contribution is disputed (Burki *et al.*, 2012). The interesting and yet

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unresolved evolutionary history of the *C. velia* plastid also emerges from its somewhat unusual genome organization: genes that encode core photosynthesis proteins (e.g. psaA, AtpB) are fragmented and the fragments are independently processed; other plastidial genes are much larger than their counterparts in other organisms (Janouškovec *et al.*, 2013; Woo *et al.*, 2015).

The physiology of *C. velia* has been investigated with reference to only a few processes. In this alga, the systems involved in light acquisition and utilization showed rather unusual characteristics (Moore *et al.*, 2008; Kotabová *et al.*, 2011, 2014; Pan *et al.*, 2012; Tichy *et al.*, 2013; Bína *et al.*, 2014). For instance, Kotabová *et al.* (2014) showed that *C. velia*, if exposed to red light, produced specific light harvesting complexes, with red-shifted absorption, but rapidly reversed to its normal light-harvesting complexes when light quality changed. Also, tetrapyrrole (thus also chlorophyll) biosynthesis is rather distinctive (Kořený *et al.*, 2011).

Little work has been conducted on the way *C. velia* fixes carbon (Janouškovec *et al.*, 2010; Quigg *et al.*, 2012). In the work of Quigg and collaborators (2012), ^{14}C was used to asses CO_2 fixation and a strong dependence of CO_2 fixation on the light regime was observed. This study, however, was not designed to investigate *C. velia* responses to different CO_2 and total inorganic C (C_i) concentrations.

Various authors (e.g. Beer *et al.*, 2000; Cai *et al.*, 2016) used microsensors/microelectrodes for spatially resolved determinations of solute concentrations in coral tissue, but the complexity of the measurements did not afford conclusive results. Furla *et al.* (2000) tried to calculate the actual CO_2 concentration within coral tissues and estimated that it was several orders of magnitude higher (147 000 µmol l^{-1} bicarbonate) than in the external medium; this would be the consequence of the activity of a CO_2 concentrating mechanism (CCM) operated by the animal (Allemand *et al.*, 1998; Leggat *et al.*, 2002; Bertucci *et al.*, 2013). High CO_2 in the external environment has been proposed to exert a negative influence on coral symbioses (Pêcheux, 2002; Anthony *et al.*, 2008). It is possible to speculate that high C_i within coral endoderm favours symbiosis, as is the case for instance for the green alga *Coccomyxa* sp., which thrives in the C_i-rich environment provided by its lichen symbiosis, but is unable to grow at ambient CO_2 concentration (Palmqvist *et al.*, 1997).

Assuming that a similar situation occurs in all scleractinian symbioses with photosynthetic organisms, *C. velia* could also be subject to elevated CO_2 concentration when in its symbiotic stage. The fact that *C. velia*, like *Symbiodinium*, is equipped with a type II Rubisco (Janouškovec *et al.*, 2010), which is especially sensitive to changes in the CO_2/O_2 ratio (Raven *et al.*, 2012, 2014), can make these organisms especially responsive to the CO_2 enrichment associated with life within corals. Preliminary experiments suggest that a CO_2 concentrating mechanism (CCM; Giordano *et al.*, 2005) is present in free-living *C. velia* cells (Giordano, Prasil & Pierangelini, personal communication). CCMs, although not very active, have also been detected in evolutionarily related dinoflagellates (Leggat *et al.*, 2002; Ratti *et al.*, 2007). The influence of modulation of CCM activity on C allocation has been demonstrated in the model organism *Chlamydomonas reinhardtii* (Memmola *et al.*, 2014). The down-regulation of the CCM in the elevated CO_2 environment of the holosymbiont and the energy savings it brings may thus contribute to a change in the C allocation pattern in *C. velia*. It has been shown for a number of organisms that life at elevated CO_2 may increase the use efficiency of light and nutrients such as N and Fe (i.e. less light, N and Fe are required for the same C fixation; see Beardall & Giordano, 2002; Raven *et al.*, 2011; Giordano & Ratti, 2013). This is expected to alter C allocation and elemental stoichiometry within the cell.

Based on all of the above, we hypothesize that life at a CO_2 concentration similar to that found in coral symbiotic tissues would influence the organic and elemental composition of *C. velia* cells by increasing the resource use efficiency and by altering the pattern of C allocation to organic pools.

Materials and methods

Cultures

Semi-continuous cultures of *C. velia* RM12 obtained from the laboratory of O. Prašil (Institute of Microbiology ASCR, Centrum Algatech, Třeboň, Czech Republic) were grown in 250 ml Erlenmeyer flasks containing 150 ml of f/2 medium (Guillard & Ryther, 1962). The cultures were maintained at 25°C and continuously illuminated with photosynthetically active radiation (PAR) at an irradiance of 200 µmol photons $m^{-2}s^{-1}$. Two different CO_2 regimes were imposed by continuously bubbling the cultures with either atmospheric air (400 ppmv CO_2) or air enriched with CO_2 (25 000 ppmv CO_2), at a flow rate of 400 ml min^{-1}. The CO_2 in the elevated CO_2 treatment was sufficiently high to be in the approximate range suggested by Furla *et al.* (2000) for the endoderm of corals, without having to change the amount of buffer and the growth pH used for the low CO_2 treatment. The gas mixtures were obtained with a flow-mass gas mixer (IN-FLOW, Bronkhorst Hi-Tech, Ruurlo, the Netherlands); the flow rate was controlled through the software Flow-bus DDE 4.6 (Bronkhorst Hi-Tech, Ruurlo, the Netherlands). In order to verify whether the growth media were at equilibrium with the gas phase, we measured the total dissolved inorganic carbon in the media (Table 1) with an infrared gas analyser (Li-840A, Li-Cor Biosciences, Lincoln, Nebraska, USA), after

Table 1. Inorganic carbon speciation in media equilibrated with gas phases containing either 400 or 25 000 ppmv CO_2.

nominal $[CO_2]$ ppmv	measured $[CO_2]$ µatm	pH NBS	DIC µmol kg^{-1}	HCO_3^- µmol kg^{-1}	CO_3^{2-} µmol kg^{-1}	CO_2 µmol kg^{-1}	TA µmol kg^{-1}
400	399[a] (36.5)	8.11[a] (0.09)	2411[a] (507)	2108[a] (390)	291[a] (119)	11.5[a] (1.05)	2791[a] (641)
25 000	24 795[b] (2247)	7.96[a] (0.03)	101 933[b] (2665)	92 455[b] (2634)	8766[b] (539)	713[b] (64.6)	110 063[b] (2728)

The calculations were made with the CO_2SYS program, using media temperature, salinity, phosphate, pH and dissolved inorganic C (DIC) concentration as inputs. The equilibrium constant of Mehrbach *et al.* (1973) refitted by Dickson & Millero (1987) were chosen. Data are shown as the means (± SD) of three replicates. Different superscript letters indicate significantly different means. TA = Total Alkalinity.

conversion of all inorganic carbon to CO_2 by acidification. We then calculated the equilibrium values for CO_2 and the overall carbonate chemistry, for the pH and the salinity of the cultures, using the CO_2Sys free software (Lewis & Wallace, 1998) (Table 1).

The dilution rates for the semi-continuous cultures were determined based on the growth rate of batch cultures maintained in otherwise equal conditions.

Cell numbers, cell size and dry weight

Cell numbers and mean cell volume were determined with an automatic cell counter (CASY TT, Innovatis AG, Reutlingen, Germany). The cell dry weight was determined according to Ratti *et al.* (2011).

Elemental stoichiometry

The elemental composition of *C. velia* was determined with an elemental analyser (Costech International S.p. A., Pioltello, Milano, Italy) for C and N, and with a Total Reflection X-ray Fluorescence (TXRF) spectrometer (Picofox S2, Bruker Nano GmbH, Berlin, Germany) for P, S, Cl, K, Ca, Mn, Fe and Zn (Fanesi *et al.*, 2014).

Organic composition

The organic composition of *C. velia* was investigated by means of Fourier transform infrared (FTIR) spectroscopy, using a Tensor 27 spectrometer (Bruker Optics GmbH, Ettlingen, Germany). Preparation of samples, FTIR spectra acquisition and analysis were conducted according to Domenighini & Giordano (2009). The relative abundance of lipids and carbohydrates was determined semi-quantitatively as in Palmucci *et al.* (2011). Peterson's (1977) assay was used to determine the absolute amount of cell proteins. Since *C. velia* cells are known to be difficult to break, different lysis methods were compared (i.e. incubation in lysis media containing detergents, vitrification in liquid nitrogen followed by mechanical grinding, rapid N_2 decompression). Complete cell lysis and the highest protein recovery were attained with N_2 decompression, using a pressure bomb (Parr Instrument, Moline, Illinois, USA) (Supplementary fig. S1). This cell breakage procedure was therefore used.

Carbonic anhydrase (CA) activity

The activity of carbonic anhydrases was assayed on intact cells and crude extracts, obtained according to Ratti *et al.* (2007). The intracellular extract was further subdivided into soluble and insoluble fractions following the procedure described in Karlsson *et al.* (1995). Intact cells and the soluble and insoluble fractions of the cell homogenates were assayed independently for carbonic anhydrase activity; the assays were conducted according to Wilbur & Anderson (1948) and later modification by Miyachi *et al.* (1983). Intact cells or cell extracts were added to an assay mixture that contained 25 mM phosphate buffer (pH 8.36) and 10 µM $ZnCl_2$ (Giordano & Maberly, 1989; Ratti *et al.*, 2007). After the addition of MilliQ water saturated with CO_2, we recorded the pH changes over time (Recorder 56, Perkin-Elmer, Waltham, USA) and then calculated the time required for the pH to decrease by one unit. In the control assays, intact cells were substituted with equal volumes of phosphate buffer, while crude extracts were substituted with equal volumes of boiled cell extract.

Statistics

All experiments were conducted on at least three distinct algal cultures. The statistical significance of mean differences was determined by *t*-tests using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA), setting the significance level at $p < 0.05$.

Results

Growth rates, cell size and cell dry weight

No differences in the specific growth rate were detected between low and high CO_2-grown cells in batch cultures (Table 2). The lowest growth rate measured in the batch cultures (0.16 d^{-1}) was imposed to all semi-continuous cultures by daily dilutions of the culture medium.

Semi-continuously cultivated *C. velia* cells exposed to a gas phase containing 25 000 ppmv CO_2 did not differ in the mean cell volume from the cells grown at 400 ppmv CO_2, but had a significantly higher dry weight ($p = 0.0079$; Table 2).

Table 2. Specific growth rate (μ), cell volume, cell dry weight, C production rate, cell protein content and carbonic anhydrase activity of *Chromera velia* acclimated to either 400 or 25 000 ppmv CO_2.

Growth [CO_2] in the gas phase ppmv	μd^{-1}	Cell volume μm^3	Dry weight pg $cell^{-1}$	C production rate pg $cell^{-1}$ d^{-1}	Protein content pg $cell^{-1}$	Carbonic anhydrase activity Wilbur-Anderson units		
						external	internal (insoluble)	internal (soluble)
400	0.16[a] (0.01)	203[a] (19.8)	126[a] (22.2)	9.82[a] (1.91)	16.5[a] (3.19)	0.12[a] (0.96)	0.39[a] (0.29)	4.17[a] (1.98)
25 000	0.17[a] (0.02)	192[a] (21.7)	212[b] (20.8)	21.0[b] (4.58)	33.4[b] (3.50)	0.04[a] (0.38)	0.23[a] (0.57)	4.11[a] (1.08)

The standard deviations are shown in parentheses. Means that are statistically different are identified by different superscript letters; equal letters indicate not significantly different means (n = 3 for all the parameters except the carbonic anhydrase activity, for which n > 5).

Elemental cell quotas and stoichiometry

The C content of *C. velia* was higher at high CO_2, regardless of the basis on which the results were expressed (p = 0.0035 on a cell-basis, Table 3; p = 0.0002 on a dry weight-basis; Fig. 1). The C production rate per cell was more than two times higher at 25 000 ppmv CO_2 (p = 0.0176; Table 2).

The N and P contents, on a dry weight basis (pg element/pg dw), were significantly lower at high CO_2 (p < 0.0001; p = 0.0066, respectively) (Fig. 1); the cell quotas of these elements were unaffected by the CO_2 treatment (Table 3). The S content was unaffected by CO_2, both per cell and per unit of dry weight (Table 3, Fig. 1).

The contents of Cl, Ca, Mn, Fe and Zn were significantly higher at low CO_2, on a dry weight basis (Fig. 1), but their content per cell was the same at the two growth regimes (Table 3).

The C/N, C/P, C/S and N/P atomic ratios were significantly lower at 400 ppmv CO_2 (Fig. 2), with the greatest difference between the two CO_2 treatments (2.7-fold) for the C/P ratio. The ratio between P and S, instead, was lower at 25 000 ppmv CO_2 (Fig. 2).

Organic composition

All FTIR absorbance ratios (lipids/proteins, carbohydrates/proteins, carbohydrates/lipids) were unaffected by the CO_2 in the growth medium (Table 4), indicating that all three pools did not vary or varied together as a function of growth conditions. The absolute protein content per unit of biomass (pg protein/pg of dry weight) and the carbohydrate/protein, lipid/protein and carbohydrate/lipid ratios were not statistically different between high CO_2- and low CO_2-grown cells (Supplementary table S1). The protein content per cell was significantly higher in high CO_2-grown cells than in their low CO_2-grown counterpart (p = 0.0035; Table 2).

Carbonic anhydrase activity

Although various carbonic anhydrase genes are present in the *C. velia* genome (Woo *et al.*, 2015), they have, when expressed, moderate activity. No external carbonic anhydrase activity was observed when we measured the activity on intact cells, under all culture conditions (Table 2). Intracellular carbonic anhydrase activity was detected solely in the soluble fraction of both low CO_2- and high CO_2-grown cells, with no significant differences between the two treatments (Table 2).

Discussion

Growth responses to elevated CO_2

Coral reefs became prominent only late in their evolutionary history, probably in the Eocene (40–30 million years ago; Perrin, 2002), after the global decline in the CO_2/O_2 ratio of the late Cretaceous

Table 3. Cell quotas of C, N, P, S, Cl, K, Ca, Mn, Fe and Zn in *Chromera velia* cultivated at either 400 (low CO_2) or 25 000 (high CO_2) ppmv CO_2.

Element	Cell quota pg $cell^{-1}$		Mass ratio g (g P^{-1})		Molar ratio mol (mol $P)^{-1}$	
	Low CO_2	High CO_2	Low CO_2	High CO_2	Low CO_2	High CO_2
C	61.0[a] (11.1)	125[b] (14.3)	180[a] (32.6)	393[b] (56.8)	464[a] (84.1)	1014[b] (147)
N	14.0[a] (2.51)	14.4[a] (1.23)	41.3[a] (7.40)	45.4[a] (7.43)	91.4[a] (16.4)	100[a] (16.4)
P	0.41[a] (0.17)	0.31[a] (0.11)	–	–	–	–
S	0.26[a] (0.04)	0.36[a] (0.13)	0.82[a] (0.06)	1.21[b] (0.05)	0.79[a] (0.06)	1.17[b] (0.04)
Cl	0.14[a] (0.07)	0.13[a] (0.19)	0.48[a] (0.14)	0.61[a] (0.76)	0.42[a] (0.12)	0.53[a] (0.66)
K	0.15[a] (0.05)	0.19[a] (0.08)	0.57[a] (0.12)	0.62[a] (0.32)	0.45[a] (0.10)	0.49[a] (0.25)
Ca	0.20[a] (0.10)	0.20[a] (0.14)	0.71[a] (0.26)	0.72[a] (0.54)	0.55[a] (0.20)	0.56[a] (0.42)
Mn	0.02[a] (0.01)	0.01[a] (0.00)	0.04[a] (0.00)	0.04[a] (0.01)	0.02[a] (0.00)	0.02[a] (0.00)
Fe	0.22[a] (0.13)	0.18[a] (0.11)	0.54[a] (0.08)	0.56[a] (0.11)	0.30[a] (0.05)	0.31[a] (0.06)
Zn	0.05[a] (0.02)	0.06[a] (0.04)	0.12[a] (0.01)	0.22[a] (0.18)	0.06[a] (0.00)	0.10[a] (0.08)

The mass and molar ratios between each of these elements and P are also shown. The standard deviations are shown in parentheses (n = 3, except for Cl, K, Ca, Mn, Fe and Zn cell quotas, for which six replicates were averaged). Different superscript letters indicate means that are statistically different; equal letters identify not significantly different means.

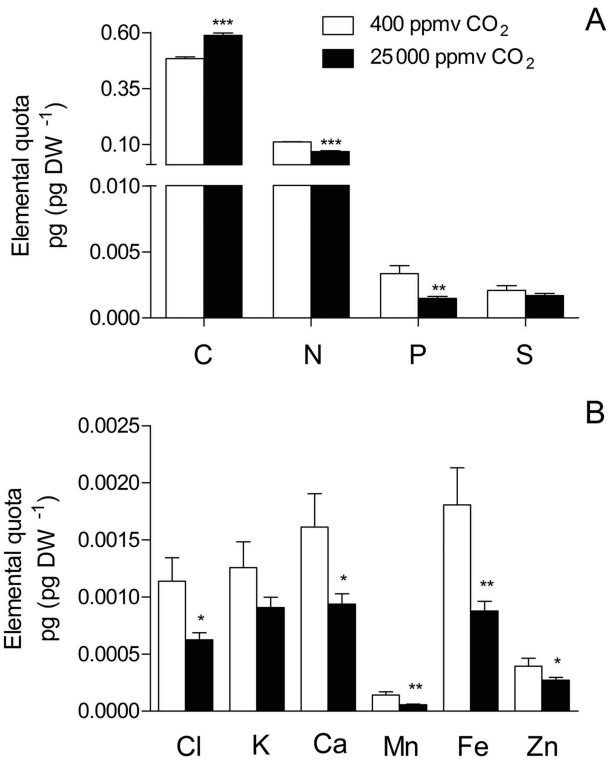

Fig. 1. Elemental composition of *Chromera velia* acclimated to either 400 or 25 000 ppmv CO_2. (**A**) C, N, P and S content per unit of dry weight; (**B**) Cl, K, Ca, Mn, Fe and Zn content per unit of dry weight. Asterisks indicate that the differences between means (n = 3) are significant (***: P-value < 0.001; **: P-value from 0.001 to 0.01; *: P-value from 0.01 to 0.05).

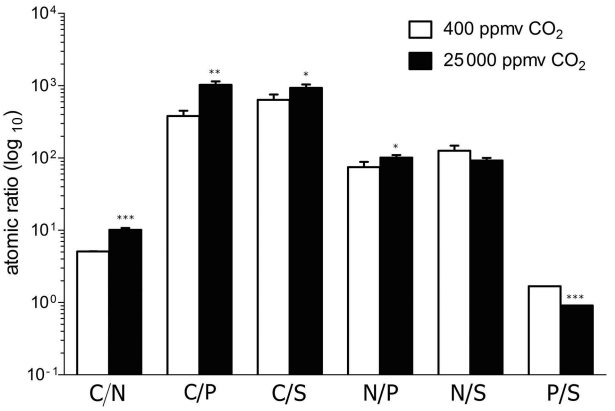

Fig. 2. C, N, P and S atomic ratios in *Chromera velia* cells acclimated to 400 or 25 000 ppmv CO_2. Asterisks indicate that the value at high CO_2 was significantly different from the corresponding value at low CO_2 (n = 3). (***: P-value < 0.001; **: P-value from 0.001 to 0.01; *: P-value from 0.01 to 0.05).

Table 4. FTIR absorbance ratios for the main organic pools in *Chromera velia* cells acclimated to 400 or 25 000 ppmv CO_2.

Growth [CO_2] in the gas phase	Lipids/ Proteins	Carbohydrates/ Proteins	Carbohydrates/ Lipids
400	0.35 (0.07)	2.61 (0.27)	7.62 (0.75)
25,000	0.36 (0.14)	2.28 (0.46)	6.67 (1.32)

No significant differences were detected between the two culture conditions (n = 3).

(Royer *et al.*, 2001; Beerling *et al.*, 2002; Berner *et al.*, 2003; Beerling & Berner, 2005). It is interesting that this occurred at approximately the same time as the spreading of C4 plants on land (Edwards *et al.*, 2001) and the radiation of microalgal taxa equipped with CCMs in the oceans (Giordano *et al.*, 2005). Coral symbioses almost exclusively involve dinoflagellates and, as has been recently discovered (Moore *et al.*, 2008), chromerids; these are the only groups of eukaryotic photolithotrophs that contain type II Rubisco, an enzyme that is rather ineffective in discriminating in favour of CO_2 and against O_2 (Read & Tabita, 1994). These organisms may have found refuge in the animal tissue, where the availability of inorganic carbon can be much higher than in the water column (Furla *et al.*, 2000), as an alternative to the development of the energy-dependent CCMs that allow many marine algae to cope with low CO_2 availability (Giordano *et al.*, 2005; Raven *et al.*, 2005). Consensus exists on the concomitance of the rise to prominence of coral reefs and the establishment of algal symbioses (see Stanley, 2003 for a discussion on the origin of corals and coral reefs), although the presence of zooxanthellae in Palaeozoic corals of the order Rugosa has also been proposed, based on rather indirect evidence (Hill, 1981) and in contrast with other authors (e.g. Coates & Jackson, 1987).

Whether *C. velia* possesses a biophysical CCM, which increases the CO_2 concentration in the proximity of Rubisco through the action of inorganic carbon transporters and the rapid equilibration of the different C_i forms mediated by carbonic anhydrases, and/or a biochemical CCM, which relies on the temporary incorporation of inorganic carbon into organic intermediates and their subsequent decarboxylation (Giordano *et al.*, 2005), is currently unknown. The finding that the internal carbonic anhydrases of *C. velia*, according to our results, are not inducible by changes in the environmental CO_2 concentration suggests that *C. velia* does not possess a biophysical CCM, which usually involves CAs (Giordano *et al.*, 2005). However, the presence of a CCM that does not make use of inducible CAs cannot be excluded (Giordano *et al.*, 2005). There is some evidence supporting the presence, in the genome of *C. velia*, of genes encoding proteins similar to those involved in biochemical CCMs in C3 and C4 plants; a plastid decarboxylase has also been found *in silico* (Giordano, Ewe & Prasil, personal communication). Genomic evidence, however, is hard to directly translate to function, especially considering the very common occurrence of potential C4 genes in most aquatic autotrophs (Raven & Giordano, 2017). The existence of rather weak CCMs has been

observed in the dinoflagellates, a neighbouring taxonomic group to Chromerida (Leggat *et al.*, 1999; Ratti *et al.*, 2007); the relative ineffectiveness of these CCMs (in terms of their ability to concentrate CO_2 in the proximity of Rubisco) may be another reason why dinoflagellates, and possibly chromerids, were prone to enter symbiotic associations.

Under our conditions, growth of free-living *C. velia* was substantially (more than double) stimulated by the elevated CO_2 in terms of overall organic C production, but not in terms of cell division (Table 2). This observation is further evidence of the fact that, at least in our growth conditions, if *C. velia* possess a CCM, it is not sufficient to saturate Rubisco at 400 ppmv CO_2. This contrasts with what has been found for the planktonic peridinin-containing dinoflagellate *Protoceratium reticulatum*, whose cell division rate increased with increasing CO_2, with a decrease in the cell dry weight (Pierangelini *et al.*, 2016). If elevated C_i strongly stimulated cell division within the host, the stability of the symbioses could be threatened (Kinzie & Chee, 1979; Taylor *et al.*, 1989; Baghdasarian & Muscatine, 2000), although other mechanisms for the control of algal proliferation, needed to maintain a balanced symbiotic relationship, are likely to be operated by the host (Falkowski *et al.*, 1993; Gates *et al.*, 1995; Smith & Muscatine, 1999). In this work, the use of free-living *C. velia* allowed us to isolate the effect of elevated C_i on growth from other animal-mediated mechanisms to control *C. velia* cell division. Whether this response to elevated C_i is an ancestral trait of *C. velia* or it was acquired after it became a symbiont of scleractinians is not known at this stage.

The growth response of *C. velia* also has implications for the growth rate hypothesis (GRH; Sterner & Elser, 2002), which assumes a relationship between P (as a component of ribosomal RNA) cell quota and growth rate. In *C. velia*, such a correlation exists in terms of cell division, but is absent if growth is considered in terms of biomass increase, suggesting that, in our case, more ribosomal P was required for cell division than for the observed biomass increase. The observed values of P content and growth rates also have a corollary in the assumption that, at low CO_2, P is not accumulated in storage compounds (such as polyphosphates); according to the GRH, protein production (pg protein day^{-1}) requires less P at high CO_2 than at low CO_2. A thorough discussion of our data in relation to the GRH and its implications is well beyond the aim of this paper; for a critical discussion on the applicability of the GRH to microalgae, the reader may refer to Flynn *et al.* (2010) and Giordano *et al.* (2015).

Impact of elevated C_i availability on C allocation and elemental composition

Chromera velia cells, similar to what was observed for the dinoflagellate *P. reticulatum* (Montechiaro & Giordano, 2010), exert a strict homeostatic control (Giordano, 2013) on the relative abundance of the main organic components (Supplementary table S1). The protein to carbohydrate, protein to lipid and carbohydrate to lipid ratios are unaffected by the CO_2 used for growth, although, in absolute terms, high CO_2-grown cells contained more protein (Table 2), and thus more carbohydrate and lipid, than low CO_2-grown cells. This was surprisingly coupled with a substantially higher C content (Table 3) and C/N (and C/P and C/S) ratio at elevated CO_2 (Fig. 2). This C allocation pattern and elemental stoichiometry indicate that the extra C of high CO_2-grown cells is not preferentially allocated, as might be expected, to carbohydrates or lipids (they should increase more than protein for this to be possible). One possible explanation could be the accumulation of inorganic N. However, ammonium accumulation has rarely been reported in algae (Pick *et al.*, 1991), while ammonium-related toxicity is a well-recognized phenomenon (ZoBell, 1935); therefore, it is unlikely that ammonium accumulation accounted for an appreciable quota of the N at low CO_2. Consequently, these results must be ascribed to either the accumulation of nitrate (Conover, 1975; DeManche *et al.*, 1979; Dortch, 1982) and/or the allocation of some of the N of low CO_2-grown cells to compounds in which the C/N ratio is lower than in proteins (see Supplementary fig. S2 for examples of such molecules). What this means in physiological terms is hard to explain, especially due to the many anomalies of *C. velia* (see the Introduction of this paper), which prevent easy comparisons to other algae. Future studies will hopefully cast further light on C allocation in *C. velia*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

M. Venuleo performed the experiments, analysed the results and contributed to writing the manuscript; O. Prášil contributed to data analyses and to writing the manuscript; M. Giordano designed the experiments, analysed the results and wrote the manuscript.

Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at https://doi.org/10.1080/09670262.2017.1376255

Supplementary table. S1. Protein content and relative abundance of lipids and carbohydrates in *Chromera velia* acclimated to either 400 or 25 000 ppm CO_2 (v/v), on a dry-weight basis.

Supplementary fig. S1. Protein content in *Chromera velia* cells acclimated to either 400 or 25 000 ppmv CO_2.

Supplementary fig. S2. C/N atomic ratio for the main N-containing compounds in marine phytoplankton calculated from chemical formula.

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