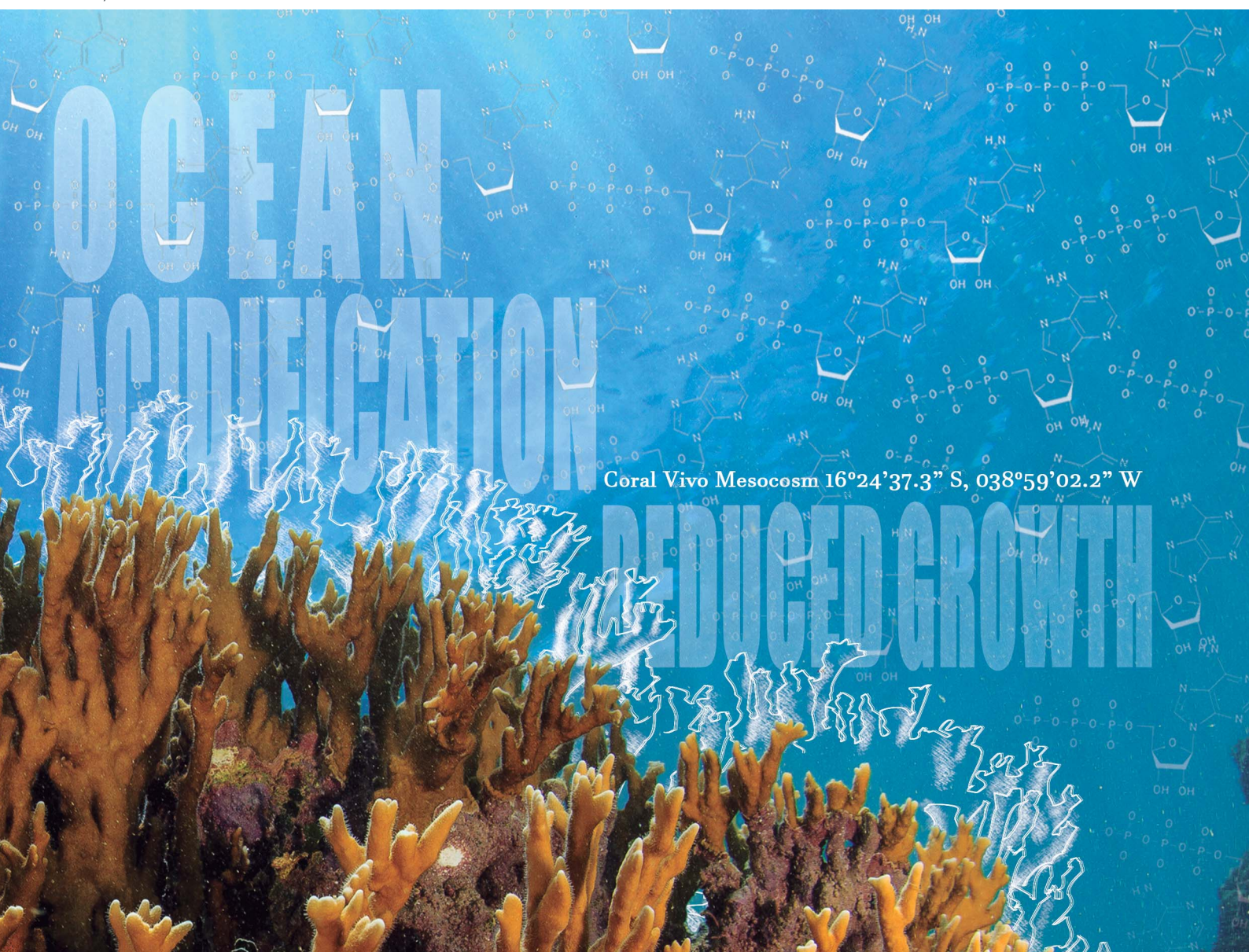


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Adalto Bianchini *et al.*

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Effect of seawater acidification on energy metabolism in the hydrocoral *Millepora alcicornis*: inhibition of citrate synthase activity indicates disruption in aerobic pathways†

Andrea Carlina Jesulich,^a Mariana Machado Lauer,^b Laura Fernandes de Barros Marangoni,^{ac} Joseane Aparecida Marques,^{ac} Yuri Dornelles Zebral,^b Cristiano Macedo Pereira,^c Gustavo Adolpho Santos Duarte,^d Débora de Oliveira Pires,^{cd} Clovis Barreira e Castro,^{cd} Emiliano Nicolas Calderon^c and Adalto Bianchini  ^{*bc}

Ocean acidification is a major threat to coral reefs worldwide, with reduced growth already reported in the hydrocoral *Millepora alcicornis* (Linnaeus, 1758) under these conditions. Inhibition of enzymes related to energy metabolism is hypothesized as one of the mechanisms associated with the physiological impacts of ocean acidification. Therefore, a mesocosm experiment was conducted to investigate whether three levels of decreasing seawater pH could alter the activity of key enzymes involved in the energy metabolism in *M. alcicornis*. Hydrocorals were acclimated to marine mesocosm conditions for 20 days and then exposed to different seawater pH levels [ambient pH (8.1) and experimental pH (7.8, 7.5 and 7.2)] for 16 and 30 days. Endpoints analyzed included the activity of key enzymes involved in the regulation of the glycolytic pathway (hexokinase and pyruvate kinase), aerobic energy production via the Krebs cycle (citrate synthase) and anaerobic energy production via lactate formation (lactate dehydrogenase). The results obtained show that only citrate synthase was affected by seawater acidification, as a marked reduction in its activity was observed at all experimental pH levels tested (7.8, 7.5 and 7.2). This finding indicates that reduced growth previously reported for *M. alcicornis* under seawater acidification conditions can be explained, at least in part, by a negative impact on the Krebs cycle, a major pathway involved in aerobic energy production.

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Environmental significance

Ocean acidification is a key major threat to aquatic biota, including coral reefs. Despite the recognized negative effects of this global environmental stressor on marine biota, little is known about its mechanism of action in corals and hydrocorals. In the present study, we evaluated seawater acidification effects on the activity of energy metabolism-related enzymes in the hydrocoral *Millepora alcicornis*. Our findings indicate that reduced growth previously reported for this hydrocoral species under seawater acidification conditions can be explained by a negative impact on the Krebs cycle, a major pathway involved in aerobic energy production. Negative impacts on the growth of reef calcifying organisms result in reduced ecological complexity and biodiversity of the entire coral reef system.

Introduction

Ocean acidification refers to the decrease in seawater pH resulting from oceanic absorption of atmospheric CO₂ emissions associated with anthropogenic sources. It leads to changes in both the carbonate buffer system and the calcium carbonate (CaCO₃) saturation state of oceans. Indeed, the pH value of global seawater has remained stable at an average of 8.2 for millions of years. However, since industrialization began around the 1760s, ocean acidification has increased by 26% compared to the pre-industrial times and is expected to continue rising in the future. Indeed, the ambient pH of surface

^aPós-Graduação Em Oceanografia Biológica, Instituto de Oceanografia, Universidade Federal Do Rio Grande, Av. Itália km 8, Rio Grande, RS, 96203-900, Brazil

^bInstituto de Ciências Biológicas, Universidade Federal Do Rio Grande, Av. Itália km 8, Campus Carreiros, Rio Grande, RS, 96203-900, Brazil. E-mail: adaltobianchini@furg.br; Tel: +55 53 3293-5193

^cInstituto Coral Vivo, Rua Dos Coqueiros 87, Santa Cruz Cabralia, BA, 45807-000, Brazil

^dMuseu Nacional, Universidade Federal do Rio de Janeiro, Parque Quinta da Boa Vista, São Cristovão, Rio de Janeiro, RJ, 20940-040, Brazil

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After the 20 day acclimation period, six branches (three branches for each exposure time: 16 and 30 days) from each original colony were randomly distributed in each of the four experimental tanks used for the four different pH treatments. Therefore, one separate tank was used for each colony subjected

to each experimental condition tested (pH: 8.1 – control, 7.8, 7.5 and 7.2), thus totaling sixteen tanks. Therefore, four biological replicates were tested for each experimental condition, each one represented by branches from the same original colony exposed to the same experimental condition at each timepoint. In turn, technical replicates were represented by the three branches from each colony exposed to each experimental condition in the same tank at each timepoint.

The mesocosm structure and functioning were described in previous studies from our research group.^{16,18,20,28,29} Briefly, seawater, from the Mucugê reef located at 500 m from the research station, was used to mimic the abiotic characteristics and daily fluctuations of natural reefs in the marine mesocosm system. Reef seawater was continuously pumped into four 5000 L underground sumps. From there, seawater was again pumped to four 200 L elevated reservoirs, which were connected to the experimental tanks. The pH of the seawater from each of these reservoirs was continuously monitored and adjusted to reach the desired experimental conditions before reaching the experimental tanks through gravity. The experimental setup consisted of sixteen independent 100 L tanks. The seawater flux in each tank was adjusted to 8.3 L min⁻¹ using a flow meter (TM 050; GPI, Wichita, KS, USA).

The methodology for seawater acidification in the present study was the same as previously reported by Marangoni *et al.* (2019).¹⁶ Briefly, mesocosm conditions were maintained at three reduced pH levels (7.8, 7.5 and 7.2) with respect to the ambient pH (8.1). Experimental pH conditions were selected considering predictions based on models of atmospheric CO₂ emissions projected for years 2100 and 2300 under the IS92a scenario.² Seawater acidification was achieved by bubbling CO₂ into natural seawater. The seawater acidification process was controlled by means of a computerized system (Reef Angel® Controller, Dublin, CA, USA) coupled to pH electrodes (Gehaka 09RBCN, São Paulo, SP, Brazil). The pH sensors were calibrated every day, with pH 7.01 and pH 10.01 standard solutions (Hanna instruments, Woonsocket, RI, USA). In this case, the Reef Angel automatic calibration protocol was employed. Every week, the sensor's drift and response time were checked using a PG1800 Gehaka benchtop pH meter test protocol (Gehaka, São Paulo, SP, Brazil). In all cases, the free pH scale was employed.

The experiment was carried out under natural lighting. Light intensity (LI-COR, LI 250A Light Meter, LI-193 Underwater Spherical Quantum Sensor, Lincoln, NE, USA), pH (Gehaka 09RBCN pH electrodes, São Paulo, SP, Brazil), temperature and salinity (Instrutemp ITREF 10 optical refractometer, São Paulo, SP, Brazil) were monitored twice a day (08:00 a.m. and 08:00 p.m.). Every day (08:00 a.m.), seawater samples from each experimental tank were collected for measurements of total alkalinity and dissolved organic carbon concentration. Total alkalinity and dissolved organic carbon concentration were measured immediately after seawater sample collection. Total alkalinity was analyzed following Dickson *et al.* (2007)³⁰ using an alkalinity titrator (AS-ALK2, Apollo SciTech Inc., Bogart, GA, USA) and certified reference material (ID: 132; Scripps Institute of Oceanography, La Jolla, CA, USA). In turn, dissolved organic carbon concentration was measured after seawater sample

filtration (0.45 µm mesh filter) using a total organic carbon analyzer (TOC analyzer 5050A, Shimadzu, Japan). Levels of *p*CO₂ and aragonite saturation state (Ω_{ar}) values were calculated for each treatment. Calculations were performed based on the total alkalinity and mean pH data using CO₂calc. version 1.2.9 software. The *p*CO₂ values were 351.8 µatm, 939.0 µatm, 1683.4 µatm, and 3494.3 µatm for pH 8.1, pH 7.8, pH 7.5 and pH 7.2, respectively. In turn, the Ω_{ar} values corresponded to 3.89, 1.96, 1.23, and 0.64, respectively.

After the 20 day period of hydrocoral acclimation to the mesocosm condition, twelve branches of *M. alcinornis* (three branches of each of the four hydrocoral colonies) were collected, individually placed into a cryovial and immediately frozen in liquid nitrogen. Therefore, three technical replicates (three branches of each colony) were collected per tank, while four biological replicates (branches of the four different colonies) were collected and frozen as described above. Samples collected were further maintained in an ultra-freezer (−80 °C) until laboratory analysis. The remaining branches were exposed to the control condition or to the three different seawater acidification treatments, for 16 and 30 days. Three branches of each of the four colonies were tested per tank, totaling twelve hydrocoral branches for each replicate at each timepoint. Four independent replicates (four raceway tanks) were tested for each experimental condition (control or acidification treatment). During the experiment, the hydrocorals fed only on natural foods present in the seawater. A qualitative color gradient ranging from six (darkest tone) to zero (white color), adapted from a Coral Health Chart (<https://coralwatch.org/>), was used to monitor variation in hydrocoral tissue color. Under all experimental conditions, no clear sign of bleaching was observed over the whole experimental time. After the exposure period, hydrocoral samples were collected, frozen and stored as described above for acclimated hydrocorals.

In the laboratory, samples (hydrocoral branches) were macerated in liquid nitrogen, using a mortar and pestle. Macerated samples were divided into aliquots, conditioned in cryovials and immediately frozen in liquid nitrogen. Frozen samples were stored in an ultra-freezer (−80 °C) until enzyme activity analysis.

Enzyme assays

The activities of glycolytic and Krebs cycle enzymes were analyzed according to Lallier and Walsh (1991),³¹ and Lauer *et al.* (2012),³² with slight modifications. Each sample aliquot (~0.5 g) was homogenized in ice-cold buffer (50 mM imidazole; 0.1 mM PMSF; pH 7.8), using a sonicator (Qsonica Model CL-188, Newtown, CT, USA). The homogenate was then centrifuged (10 000 × *g*; 20 min; 4 °C) and the supernatant was used as the enzyme source.

Enzyme activities were measured spectrophotometrically using a microplate reader (ELx808IU, BioTek Instruments, Winooski, VT, USA). Glycolytic enzyme (hexokinase, pyruvate kinase and lactate dehydrogenase) assays were performed using an imidazole buffer solution (75 mM; pH 7.4). Enzyme activity was measured based on NAD⁺/NADH oxidation/reduction by



following the sample absorbance at 340 nm. Krebs cycle enzyme (citrate synthase) assay was performed using a HEPES buffer solution (50 mM; pH 8.1). Enzyme activity was measured based on 5-5'-dithio-2-nitrobenzoic (DTNB) reduction by following the sample absorbance at 412 nm. Specific conditions were used for each enzyme assay, as follows (final concentrations): hexokinase (150 mM MgCl_2 , 10 mM D-glucose, 1.2 mM NAD^+ , 120 U mL^{-1} glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and 60 mM ATP); pyruvate kinase (150 mM MgCl_2 , 750 mM KCl, 37.5 mM ADP, 3 mM NADH, 1500 U mL^{-1} lactate dehydrogenase and 5 mM phosphoenolpyruvate); lactate dehydrogenase (7.7 mM pyruvate and 3 mM NADH); and citrate synthase (2.3 mM acetyl coenzyme A, 3.5 mM DTNB and 3.5 mM oxaloacetate). All assays were performed at 25 °C. For each experimental treatment, three branches of each hydrocoral colony were analyzed, thus totaling twelve branches per pH condition (pH 8.1, pH 7.8, pH 7.5, and pH 7.2) at each experimental time (0, 16 and 30 days). Protein content in the sample homogenate was determined based on the absorbance at 550 nm, using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). Data were expressed as enzyme units (EU) per mg protein (UE per mg protein).

Data presentation and statistical analysis

Data on seawater physicochemical parameters (pH, temperature and salinity: $n = 60$; alkalinity and dissolved organic carbon: $n = 30$) and enzyme activity ($n = 4$, i.e., four coral colonies; three branches of each coral colony were measured at each exposure time) were expressed as mean $\pm$ standard deviation. Data on seawater physicochemical parameters were compared by one-way analysis of variance (ANOVA). Enzyme activity data after hydrocoral acclimation to the mesocosm conditions and after exposure to the control pH condition (ambient seawater pH: 8.1) for 16 and 30 days were compared by one-way ANOVA. In turn, enzyme activity data after exposure to different experimental conditions for 16 and 30 days were subjected to two-way (exposure time and pH treatment) ANOVA. Post-hoc significant differences among the different experimental treatments were evaluated using Fisher's Least Significant Difference (LSD) test. ANOVA assumptions, i.e., data normality and homogeneity of variances, were previously

verified using the normal probability plot for raw residuals and the Cochran C test, respectively. In all cases, the significance level adopted was 95% ($\alpha = 0.05$). All statistical analyses were performed using Statistica 7.0 software (StatSoft, Tulsa, OK, USA).

Results

Data on seawater chemistry are presented in Table 1. The results obtained showed that pH treatments were successfully achieved and maintained throughout the experimental period. No significant difference was observed among replicates for the same experimental condition ($p > 0.05$; one-way ANOVA). However, a significant difference was observed among the different experimental conditions tested ($p < 0.05$; one-way ANOVA, followed by the LSD test). General mean values ($\pm$ standard deviation) were 8.10 ± 0.096 (nominally pH 8.1; pH range: 7.71–8.34), 7.74 ± 0.185 (nominally pH 7.8; pH range: 6.60–8.41), 7.51 ± 0.160 (nominally pH 7.5; pH range: 6.22–8.36), and 7.21 ± 0.190 (nominally pH 7.2; pH range: 6.15–8.35). No significant difference in light intensity, temperature, salinity, alkalinity and dissolved organic carbon concentration was observed among the different replicates for the same experimental condition, as well as among the different experimental conditions ($p > 0.05$; one-way ANOVA). General mean values for the whole experimental period corresponded to 285.95 ± 39.13 $\mu\text{mol photons per m}^2$ per s, 25.12 ± 0.30 °C, 35.04 ± 0.38 , 117.00 ± 1.77 mg CaCO_3 per L, and 3.35 ± 0.35 mg C per L, respectively.

Data on enzyme activities in hydrocoral collected after the 20 day period of acclimation are presented in Table S1 (ESI).† For each enzyme, no significant difference was observed among mean values for the four hydrocoral colonies ($p > 0.05$; one-way ANOVA). Data on enzyme activities in hydrocoral collected after the 16 day and 30 day periods of exposure are presented in Tables S2 and S3, respectively (ESI).† For each enzyme, no significant difference was observed ($p > 0.05$; one-way ANOVA) among the mean values for the four hydrocoral colonies for each pH condition (pH 8.1, 7.8, 7.5, and 7.3) at each exposure time (16 and 30 days). Therefore, mean values for each pH condition and exposure time were calculated based on the

Table 1 Seawater chemistry under the different experimental conditions over the 30 day experimental period. Data on pH, temperature (°C), salinity, alkalinity (mg CO_3 per L), dissolved organic carbon (DOC; mg C per L) and light intensity ($\mu\text{mol photons per m}^2$ per s) are expressed as mean $\pm$ standard deviation. Different capital letters indicate significantly different mean values among the different experimental conditions ($p < 0.05$; one-way ANOVA, followed by the LSD test) for each parameter analyzed

Parameter	Experimental condition			
	pH 8.1	pH 7.8	pH 7.5	pH 7.2
pH	8.10 ± 0.096^A	7.74 ± 0.185^B	7.51 ± 0.160^C	7.20 ± 0.190^D
Temperature	25.19 ± 0.03^A	25.00 ± 0.03^A	25.16 ± 0.03^A	25.13 ± 0.04^A
Salinity	35.11 ± 0.39^A	35.02 ± 0.41^A	35.13 ± 0.37^A	35.07 ± 0.34^A
Alkalinity	119.2 ± 1.7^A	117.1 ± 1.5^A	115.8 ± 2.6^A	115.9 ± 1.5^A
DOC	3.43 ± 0.37^A	3.56 ± 0.36^A	3.20 ± 0.31^A	3.19 ± 0.34^A
Light intensity	285.0 ± 41.3^A	296.4 ± 41.5^A	285.5 ± 33.9^A	277.0 ± 39.8^A



twelve branches analyzed (three branches of each of the four hydrocoral colonies).

In hydrocorals kept at ambient seawater pH (8.1), no significant change ($p > 0.05$; one-way ANOVA) was observed in enzyme activity [hexokinase (Fig. 1), pyruvate kinase (Fig. 2), lactate dehydrogenase (Fig. 3) and citrate synthase (Fig. 4)] over the experimental period (0, 16 and 30 days). In hydrocorals exposed to seawater acidification for 16 days, no significant change ($p > 0.05$; two-way ANOVA) was observed in hexokinase (Fig. 1), pyruvate kinase (Fig. 2), lactate dehydrogenase (Fig. 3) and citrate synthase (Fig. 4) activity. In hydrocorals exposed to seawater acidification for 30 days, no significant change ($p > 0.05$; two-way ANOVA) was observed in hexokinase (Fig. 1), pyruvate kinase (Fig. 2) and lactate dehydrogenase (Fig. 3) activity. However, citrate synthase activity was markedly inhibited ($p < 0.05$; two-way ANOVA, followed by the LSD test) in hydrocorals exposed to seawater acidification treatments (Fig. 4).

Discussion

Based on the hypothesis outlined for the present study, the reduced growth rate previously observed in the hydrocoral *M. alcyonensis* could be associated with lower energy production related to the reduced activities of key enzymes linked to glycolysis and the Krebs cycle. Therefore, we would expect the activities of the tested enzymes to decrease under an ocean acidification scenario. However, our data reported show that CO₂-driven seawater acidification, as low as pH 7.1, did not

affect hexokinase and pyruvate kinase activities, even after 30 days of exposure to this environmental stressor. This finding indicates that the pyruvate formation rate through the glycolytic pathway was likely not affected after *M. alcyonensis* exposure to the different levels of seawater acidification tested.

Regarding the lack of response of the lactate dehydrogenase, it is important to note that this enzyme plays a crucial role, especially under anaerobic conditions. In fact, pyruvate formed in the glycolytic pathway is converted into lactate, following a reaction catalyzed by this enzyme.²⁴ Therefore, the lack of the seawater acidification effect on lactate dehydrogenase activity observed in the present study is evidence that hydrocorals maintained aerobic metabolism throughout the whole experimental period.

Differently from the other enzymes analyzed, the activity of the citrate synthase was markedly reduced in the hydrocoral *M. alcyonensis* exposed to any of the seawater acidification conditions (pH 7.8, 7.5 and 7.2) for 30 days. Therefore, aerobic energy production was certainly compromised in *M. alcyonensis* chronically exposed to seawater acidification. The reduced citrate synthase activity together with the lack of change in lactate dehydrogenase activity observed in hydrocoral exposed to seawater acidification in the 30 day exposure indicates that *M. alcyonensis* was likely not able to sustain an energy production steady state. This finding can thus explain, at least in part, the reduced growth previously reported for this hydrocoral species.¹⁸ This statement is in line with other biochemical and molecular studies on corals subjected to seawater acidification. In fact, Edmunds and Wall (2014)³³ reported that protein

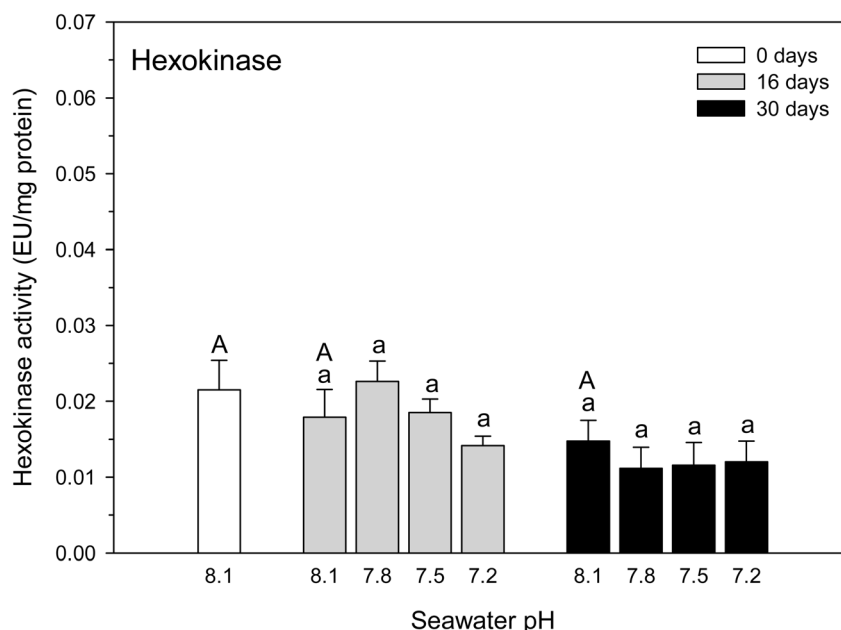


Fig. 1 Hexokinase activity (UE per mg protein) in the hydrocoral *Millepora alcyonensis* kept under the control condition (ambient seawater: pH 8.1) or exposed to different levels of CO₂-driven seawater acidification (pH 7.8, 7.5 and 7.2) for 16 and 30 days in a marine mesocosm system. Data are expressed as mean $\pm$ standard deviation ($n = 12$). Different capital letters indicate significantly different mean values ($p < 0.05$; one-way ANOVA, followed by the LSD test) under the control condition (pH 8.1) at the different experimental times (0, 16 and 30 days). Different lowercase letters indicate significantly different mean values ($p < 0.05$; two-way ANOVA, followed by the LSD test) among the experimental conditions (exposure time and pH treatment).



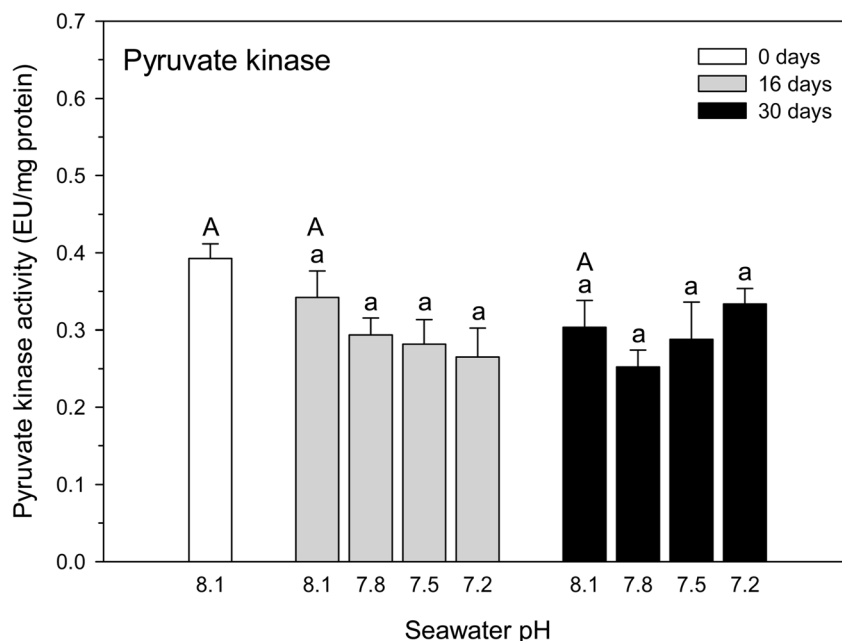


Fig. 2 Pyruvate kinase activity (UE per mg protein) in the hydrocoral *Millepora alcicornis* kept under the control condition (ambient seawater: pH 8.1) or exposed to different levels of CO₂-driven seawater acidification (pH 7.8, 7.5 and 7.2) for 16 and 30 days in a marine mesocosm system. Data are expressed as mean $\pm$ standard deviation ($n = 12$). Different capital letters indicate significantly different mean values ($p < 0.05$; one-way ANOVA, followed by the LSD test) under the control condition (pH 8.1) at the different experimental times (0, 16 and 30 days). Different lowercase letters indicate significantly different mean values ($p < 0.05$; two-way ANOVA, followed by the LSD test) among the experimental conditions (exposure time and pH treatment).

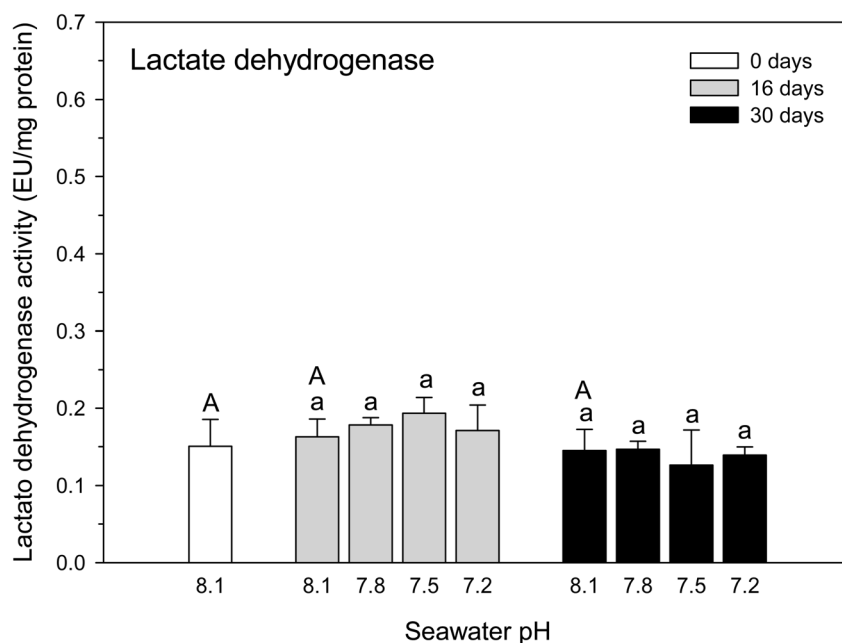


Fig. 3 Lactate dehydrogenase activity (UE per mg protein) in the hydrocoral *Millepora alcicornis* kept under the control condition (ambient seawater: pH 8.1) or exposed to different levels of CO₂-driven seawater acidification (pH 7.8, 7.5 and 7.2) for 16 and 30 days in a marine mesocosm system. Data are expressed as mean $\pm$ standard deviation ($n = 12$). Different capital letters indicate significantly different mean values ($p < 0.05$; one-way ANOVA, followed by the LSD test) under the control condition (pH 8.1) at the different experimental times (0, 16 and 30 days). Different lowercase letters indicate significantly different mean values ($p < 0.05$; two-way ANOVA, followed by the LSD test) among the experimental conditions (exposure time and pH treatment).



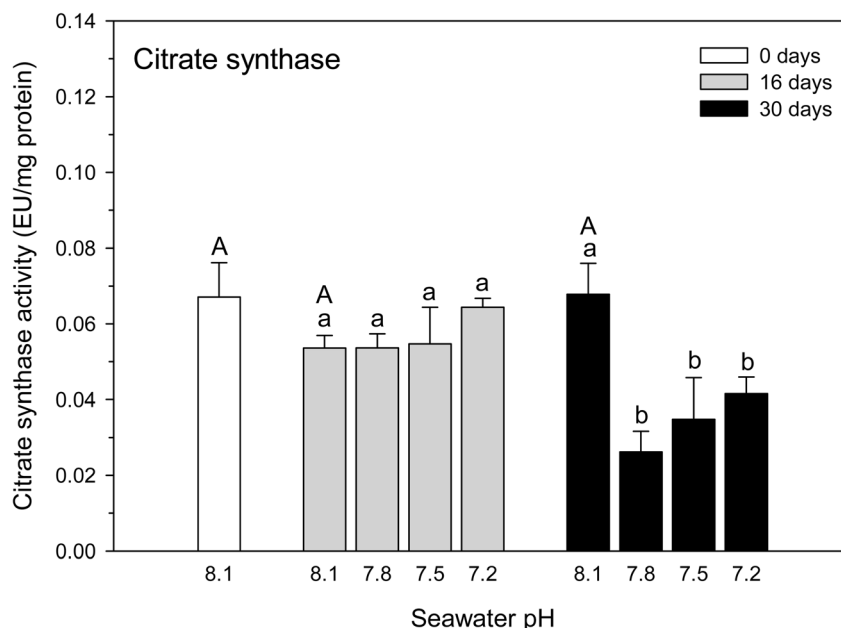


Fig. 4 Citrate synthase activity (UE per mg protein) in the hydrocoral *Millepora alcicornis* kept under the control condition (ambient seawater: pH 8.1) or exposed to different levels of CO₂-driven seawater acidification (pH 7.8, 7.5 and 7.2) for 16 and 30 days in a marine mesocosm system. Data are expressed as mean $\pm$ standard deviation ($n = 12$). Different capital letters indicate significantly different mean values ($p < 0.05$; one-way ANOVA, followed by the LSD test) under the control condition (pH 8.1) at the different experimental times (0, 16 and 30 days). Different lowercase letters indicate significantly different mean values ($p < 0.05$; two-way ANOVA, followed by the LSD test) among the experimental conditions (exposure time and pH treatment).

synthesis was negatively affected by high $p\text{CO}_2$ in recruits of the scleractinian coral *Seriatopora caliendrum*, with consequences on aerobic respiration. In turn, Kaniewska *et al.* (2012)³⁴ reported that genes involved in the Krebs cycle were down-regulated in the scleractinian coral *Acropora millepora* exposed to pH 7.8–7.9 and 7.6–7.7 for 28 days. Also, Vidal-Dupiol *et al.* (2013)²¹ reported that exposure to pH ≤ 7.4 for 21 days induced a downregulation of genes coding for proteins involved in coral calcification, as well as heterotrophic and autotrophic related proteins in the scleractinian coral *Pocillopora damicornis*. More recently, Glazier *et al.* (2020)¹⁰ reported a lowered expression of genes involved in the energy metabolism of the scleractinian coral *Lophelia pertusa* exposed to pH 7.6 for six months in comparison to those kept at ambient pH (7.9).

From an ecological perspective, our findings suggest the possibility of ocean acidification impacts on reef-builder corals. Indeed, it was recently demonstrated that two natural coral reef communities on the back reef of Mo'orea (French Polynesia) showed a decreased net calcification of the natural coral reef community subjected to a high $p\text{CO}_2$ condition (949 μatm) compared to that exposed to the ambient $p\text{CO}_2$ condition (393 μatm).³⁵ In the context of global changes, it is interesting to note that citrate synthase activity was also shown to be inhibited in the hydrocoral *M. alcicornis* and the scleractinian corals *Mussismilia hispida* and *Porites branneri* subjected when exposed to combined seawater acidification and increased temperature (pH 7.8 and 28.5 $^{\circ}\text{C}$) compared to the control conditions (pH 8.1 and 26.0 $^{\circ}\text{C}$).³⁶ Therefore, the inhibition of citrate synthase activity induced by reduced seawater pH, as demonstrated in

the present study with the hydrocoral *M. alcicornis* and scleractinian corals,³⁶ can be a good biomarker of the reduced growth observed in hydrocorals and scleractinian corals after chronic exposure to ocean acidification.^{16,18} Indeed, it was demonstrated that the hydrocoral *M. alcicornis* is capable of coping with acidic conditions only for a short period of time (16 days). However, it shows clear negative responses (oxidative stress) after chronic exposure (30 days) to seawater acidification, which could compromise hydrocoral health.¹⁷ Interestingly, we also found a significant and marked reduction in the citrate synthase activity only after 30 days of *M. alcicornis* exposure to seawater acidification. Based on these findings, it is expected that both corals and hydrocorals would negatively respond to ocean acidification over the long term, showing compromised health, a lower growth rate and gross production.

Conclusion

The observed lack of changes in the activity of hexokinase and pyruvate kinase provides some evidence that the glycolytic pathway in the hydrocoral *M. alcicornis* is functioning normally even after 30 days of exposure to seawater acidification (pH 7.8, 7.5 and 7.2). In turn, the observed inhibition of the citrate synthase activity indicates that the first step of the Krebs cycle for energy production is compromised, leading to a reduced activity of citrate synthase in the hydrocoral *M. alcicornis*. This would be implicated in a reduced energy availability to sustain an adequate metabolic rate. Such a condition could thus explain, at least in part, the reduced growth previously reported



in this hydrocoral species subjected to seawater acidification for 30 days. Therefore, future work would be focused on the establishment of a direct connection between lower energy production through the Krebs cycle and the reduced growth in hydrocorals and corals long-term exposed to an ocean acidification scenario.

Data availability

The data that support the findings of this study are available on request from the corresponding author, [AB].

Author contributions

Conceptualization: Andrea Carlina Jesulich, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, Cristiano Macedo Pereira, Gustavo Adolpho Santos Duarte, Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano Nicolas Calderon, and Adalto Bianchini. Data curation: Andrea Carlina Jesulich, Mariana Machado Lauer, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, and Yuri Dornelles Zebral Adalto Bianchini. Formal analysis: Andrea Carlina Jesulich, Mariana Machado Lauer, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, Yuri Dornelles Zebral, and Adalto Bianchini. Funding acquisition: Débora de Oliveira Pires, Clovis Barreira e Castro, and Adalto Bianchini. Investigation: Andrea Carlina Jesulich, Mariana Machado Lauer, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, Cristiano Macedo Pereira, Gustavo Adolpho Santos Duarte, Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano Nicolas Calderon, and Adalto Bianchini. Methodology: Andrea Carlina Jesulich, Mariana Machado Lauer, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, Yuri Dornelles Zebral, Cristiano Macedo Pereira, Gustavo Adolpho Santos Duarte, Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano Nicolas Calderon, and Adalto Bianchini. Project administration: Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano Nicolas Calderon, and Adalto Bianchini. Resources: Andrea Carlina Jesulich, Mariana Machado Lauer, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, Yuri Dornelles Zebral, Cristiano Macedo Pereira, Gustavo Adolpho Santos Duarte, Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano Nicolas Calderon, and Adalto Bianchini. Supervision: Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano, and Adalto Bianchini. Writing – original draft: Andrea Carlina Jesulich, Mariana Machado Lauer, Laura Fernandes de Barros Marangoni, Joseane Aparecida Marques, Yuri Dornelles Zebral, Cristiano Macedo Pereira, Gustavo Adolpho Santos Duarte, Débora de Oliveira Pires, Clovis Barreira e Castro, Emiliano Nicolas Calderon, and Adalto Bianchini. Writing – review & editing: Yuri Dornelles Zebral and Adalto Bianchini.

Conflicts of interest

There are no conflicts of interest to declare.

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