Calcification reduction and recovery in native and non-native Mediterranean corals in response to ocean acidification

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Abstract

In recent years, some of the ramifications of the ocean acidification problematic derived from the anthropogenic rising of atmospheric CO_2 have been widely studied. In particular, the potential effects of a lowering pH on tropical coral reefs have received special attention. However, only a few studies have focused on testing the effects of ocean acidification in corals from the Mediterranean Sea, despite the fact that this basin is especially sensitive to increasing atmospheric CO_2. In this context, we investigated the response to ocean acidification of the two zooxanthellate coral species capable of constituting the main framework of the community, the endemic *Cladocora caespitosa* and the non-native *Oculina patagonica*. To this end, we examined the response of both species to pCO_2 concentrations expected by the end of the century, 800 ppm, *vs* the present levels. Calcification rate measurements after 92 days of exposure to low pH conditions showed the same negative response in both species, a decrease of 32 - 35% compared to corals reared under control conditions. In addition, we detected in both species a correlation between the calcification rate of colonies in control conditions and the degree of impairment of the same colonies at low pH. Independent of species, faster growing colonies were more affected by decreased pH. After this period of decreased pH, we conducted a recovery experiment, in which corals reared in the acidic treatment were brought back to control conditions. In this case, normal calcification rates were reached in both species. Overall, our results suggest that *O. patagonica* and *C. caespitosa* will both be affected detrimentally by progressive ocean acidification in the near future. They do not display differences in response between native and non-native species but do manifest differential responses depending on calcification rate, pointing

40 to a role of the coral genetics in determining the response of corals to ocean
41 acidification.
42
43 **Keywords:** Ocean acidification; Coral calcification; Mediterranean coral; Resilience;
44 *Cladocora caespitosa*; *Oculina patagonica*

1. Introduction

A significant fraction of the anthropogenic CO_2 released to the atmosphere is being absorbed by the oceans (Canadell et al., 2007; Sabine et al., 2004), causing unprecedented changes in its chemical state and alterations in the physiology of a wide variety of marine organisms (Pelejero et al., 2010). Models predict that ocean pH will decrease by 0.3 to 0.4 pH units by the end of the century (Caldeira and Wickett, 2003).

In the Mediterranean Sea, the level of acidification is still poorly known, but certain characteristics of this semi-enclosed sea makes it especially sensitive to increasing atmospheric CO_2 (Calvo et al., 2011). On one hand, the high levels of total alkalinity (Schneider et al., 2007) increase its capacity to absorb large amounts of anthropogenic CO_2 compared with the open ocean (Goyet et al., 2009). On the other hand, the shorter residence time of deep waters (Bethoux et al., 2005) implies a more rapid penetration of anthropogenic CO_2. In agreement with the expected outcome of these characteristics, a first estimate indicates a pH decrease of up to 0.14 units since the pre-industrial era affecting the entire water column, with the largest effects observed in the western Mediterranean basin (Touratier and Goyet, 2011). This change is larger than the mean decrease of ~0.10 pH units for surface waters of the world's oceans over this period (Orr et al., 2005). Thus, the Mediterranean Sea seems to be among the world regions that are being most rapidly impacted by acidification. In this environmental framework, already under pressure by other anthropogenic stressors [e.g. (Calvo et al., 2011)], it is essential to evaluate the potential consequences of acidification on marine organisms.

Experimental studies examining the effects of ocean acidification on the growth of calcifying organisms have revealed a wide variety of sensitivity degrees within and among species [reviewed in (Doney et al., 2009; Guinotte and Fabry, 2008; Ries et al., 2009)]. Coral reef communities have been extensively studied because their calcifying organisms may be severely affected by the projected pH levels. The observed experimental effects of ocean acidification on most tropical shallow water corals have shown a reduction in calcification rate (Cooper et al., 2008 ; De'ath et al., 2009; Hoegh-Guldberg et al., 2007). Some species, however, have been shown to calcify even under very low saturation state conditions (Jury et al., 2010; Krief et al., 2010 ; Ries et al., 2010), pointing to the existence of different mechanisms controlling the carbonate chemistry at their sites of calcification [(Ries, 2011) and references therein]. Nevertheless, an overall reduction in calcification rate of the corals is expected as a result of ocean acidification. This decrease in calcification would increase the susceptibility of coral reefs to erosion which, together with the thermal stress caused by global warming, might lead to changes in their community structure and resilience (Fabricius et al., 2011). Moreover, acidification will interact with other factors affecting coral reef communities. In this sense, coral calcification has been shown to exhibit different responses upon the interaction between high pCO_2 and high temperature (Muehllehner, 2008; Reynaud et al., 2003; Rodolfo-Metalpa et al., 2010), nutrient enrichment (Ferrier-Pagès et al., 2000; Holcomb et al., 2010, 2012 ; Marubini and Atkinson, 1999 ; Renegar and Riegl, 2005), food supply (Edmunds, 2011), and light conditions (Marubini et al., 2001).

In contrast to the attention devoted to tropical coral species, thus far only three studies have been performed on the effects of ocean acidification on temperate

95 Mediterranean corals. These studies used different experimental approaches. First, Fine
96 and Tchernov (2007) exposed *Oculina patagonica* and *Madracis pharencis* to low pH
97 (7.4 units) in aquaria, which caused the complete dissolution of the skeleton. The
98 second study combined short (1 month) and long (1 year) exposure of *Cladocora*
99 *caespitosa* to low pH (7.8 units) in experimental aquaria and found no detrimental
100 effects on calcification rate of the colonies (Rodolfo-Metalpa et al., 2010). In the third
101 study, the transplantation of *C. caespitosa* corals to a gradient of naturally acidified
102 areas close to CO_2 vents (pH level range between 8.1 and 7.5 units) showed evidence of
103 dissolution (Rodolfo-Metalpa et al., 2011). This suggested that the effects of
104 acidification on these species may show up below a certain threshold of pH decrease.
105 The same study did not find evidence of dissolution in *Balanophyllia europaea* corals
106 exposed to the same gradient of natural acidification.
107
108 In the Mediterranean, *C. caespitosa* Linnaeus, 1767 and *O. patagonica* De
109 Angelis, 1908 are the only two zooxanthellate coral species that, under some
110 circumstances, can constitute the main framework of the shallow infralittoral
111 community (Kružić and Benković, 2008; Serrano et al., submitted a). *C. caespitosa* is a
112 widespread endemic species (Zibrowius, 1980) which has recently been severely
113 affected by mass mortality events (Garrabou et al., 2009; Lejeusne et al., 2010; Perez, T.
114 et al., 2000). In contrast, *O. patagonica* is an alien species (Zibrowius, 1974), which is
115 actually experiencing an increase in distribution and abundance throughout the
116 Mediterranean (Coma et al., 2011; Fine et al., 2001; Sartoretto, 2008; Serrano, et al.,
117 submitted b). In this study, we investigated the effects of, and recovery responses to,
118 ocean acidification in both coral species, by simulating the future pH conditions
119 projected by the end of the century. The results provide new information on biological

120 responses and resilience to low pH conditions for the native *C. caespitosa* and the alien
121 *O. patagonica* coral species in the Mediterranean Sea.
122
123 **2. Materials and methods**
124
125 *2.1 Specimen collection and preparation*
126
127 Ten widely separated colonies from each of the two coral species, *C. caespitosa*
128 and *O. patagonica*, were collected by scuba divers at 3-6 meters depth in L'Ampolla
129 (NE Spain, 40º48'N, 0º42'E) in April 2009. At the sampling area, located 10 km north
130 of the Ebro River Delta (northwestern Mediterranean Sea), both species are broadly
131 distributed in the shallow rocky infralittoral. Seawater temperature and light
132 measurements from the area were obtained using Onset Stow Away data-loggers set up
133 to register data at 1 h intervals over a full year cycle. The loggers were regularly either
134 cleaned or replaced by scuba divers to prevent bio-fouling and for data downloading.
135 The environmental conditions of the area are characterized by a marked seasonality,
136 with temperatures ranging from 12 ºC in winter to 27 ºC in summer, and often with low
137 irradiance due to the high turbidity of the water.
138
139 The collected specimens were placed immediately in large seawater containers
140 and transported to the Experimental Aquarium Zone (ZAE) at the Institute of Marine
141 Sciences (ICM) in Barcelona. Colonies were placed in a 225 L acclimation tank with 50
142 µm filtered running seawater (pumped from 300 m offshore, 10 m depth, in front of the
143 ICM). Temperature (14.5°C) and light conditions (~50 µmol photons m^{-2} s^{-1} on a 12:12
144 light:dark cycle) were chosen to simulate those at the collection site.

Five nubbins (12 ± 5 polyps) were harvested from each of the 10 collected colonies from each species, carefully cleaned of encrusting organisms and sediment and glued onto labeled methacrylate holders with an inert mastic compound. The buoyant weight of each nubbin was carefully measured before gluing to be able to subtract the holder and glue weight from the total weight measurements (see below). Temperature at the acclimation tank was increased gradually (0.4°C per day) up to 20°C (simulating mean summer conditions at the area of collection) over a two weeks period and maintained for one further week before the beginning of the experiment.

2.2 Experimental setup and carbonate system manipulation

We implemented a pH-manipulative experimental system following the experimental design described by Reynaud et al. (2003) (Fig. 1). Seawater was continuously supplied to two 150 L tanks and pH was adjusted to values of ~8.09 and ~7.83 units (total scale) simulating, respectively, current and future pH levels predicted for year 2100 following A2 IPCC SRES (Plattner et al., 2008). These pH levels correspond to Mediterranean seawater in equilibrium with an atmosphere of ~390 ppm CO_2 for the high pH condition, and ~800 ppm CO_2 for the low pH treatment (Table 1). In the two large tanks, we bubbled CO_2 (99.9% purity) or CO_2-free air (using a home-made filter filled with soda lime, Sigma Aldrich) to either reduce or increase pH, respectively. Seawater pH was monitored continuously by glass electrodes (LL Ecotrode plus - Metrohm) connected to a pH controller (Consort R305, Topac Inc., USA), which automatically opened and closed the solenoid valves of CO_2 or CO_2-free air when needed. To avoid drifts in the pH measurements, glass electrodes were

170 calibrated on a daily basis with a Tris buffer, following standard procedures (SOP6a of
171 Dickson et al., 2007). Water from every large tank was continuously transferred to two
172 replicate 25 L methacrylate experimental aquaria where the corals were maintained.
173 Seawater renewal rate in these aquaria was 10 times per day, and seawater was
174 continuously mixed with HYDOR Koralia pumps (4.5W, 1500 L h^{-1}). The aquaria were
175 covered with a methacrylate wrap to reduce evaporation and minimize surface-air gas
176 exchange. Two HQI-lamps (T5 ATI Aquablue Special 4x24W), running on a 12:12
177 light:dark cycle, were adjusted to the required irradiance with a plastic grey mesh.
178 Irradiance was measured using a Li-COR underwater spherical quantum sensor (Li-
179 1935B; Lincoln, NE; USA) and adjusted to 95 μmol photons m^{-2} s^{-1}, equivalent to the
180 mean daylight irradiance at 5 meters depth in June at the area of collection, when mean
181 water temperature is 20ºC (Onset Stow Away data-loggers). The pH-manipulative
182 experimental set-up was installed inside a thermostated room, ensuring constant values
183 (~20°C) during the whole experiment. Once a week, fresh *Artemia salina* nauplii (20
184 mg dry weight per coral fragment) were supplied by closing the seawater flow-through
185 for 4 hours to ensure a proper feeding.
186
187 Forty-eight out of the fifty initial nubbins for each species were distributed in the
188 four aquaria, such that at least one representative from each colony in each aquarium.
189 During the first week of the acidification experiment, the pH was gradually adjusted
190 (0.03 units per day) up to the final selected values and maintained there for 92 days.
191 After this period, we initiated the recovery experiment in which we gradually raised the
192 pH of the experimental acidified aquaria to match the conditions of the control aquaria
193 while maintaining the control aquaria as before (Fig. 2). The objective of this
194 manipulation was to determine whether any detrimental effects observed in the corals

195 exposed to acidic conditions could be reversed. The recovery experiment run from day
196 92 to day 216.
197
198 *2.3 Measured and derived parameters of the CO_2 system*
199
200 The temperature and pH of the large 150 L tanks were logged every 10 minutes
201 using Pt100 probes and glass electrodes, respectively. In addition, temperature and
202 salinity in the four experimental tanks were measured every 2-3 days, using an YSI-
203 30M/10FT probe. Small volumes of water were also taken from the tanks (once a week
204 during the first month and twice a month for the rest of the experiment) to analyze total
205 alkalinity (TA) by potentiometric titration (Perez, F.F. and Fraga, 1987; Perez, F.F., et
206 al., 2000) and pH using spectrophotometry (Clayton and Byrne, 1993) , which provides
207 better precision than with electrodes. TA and pH (always reported on total scale) were
208 used to calculate dissolved inorganic carbon (DIC), carbonate ion concentration $^{-}$,
209 bicarbonate ion concentration $^{-}$, dissolved CO_2, aragonite saturation state (Ω_A)
210 and atmospheric CO_2 concentration in equilibrium, using the CO2calc software
211 (Robbins et al., 2010), with dissociation constants for carbonate determined by
212 Mehrbach et al. (1973) and refit by Dickson and Millero (1987). Chemical and physical
213 conditions of both treatments during the experiment are shown in Table 1.
214
215 *2.4 Calcification rate*
216
217 Changes in coral calcification were assessed from measurements of buoyant
218 weight (Davies, 1989; Jokiel et al., 1978), using a 0.1 mg resolution balance (Mettler
219 Toledo AB204 SFACT). Measurements during the acidification experiment were

performed on days 15, 36, 49 and 92. A last weighing was performed at the end of the recovery experiment, on day 216. Before each measurement, epiphytes were carefully removed with a soft brush from all holders to avoid the presence of micro bubbles that could alter the weight of the organisms. Since the buoyant and dry weights are linearly correlated (with the regression passing through the origin), making percent-changes in both weights equivalent (Ries et al., 2009, 2010), in this work, we calculate the calcification rate directly from buoyant weight data. To this end, we normalize the net buoyant weight of the corals (total coral weight minus the coral holder and glue) to their initial mass. Growth rate (G) is expressed as mg of mass increase per gram of initial weight per day, taking as a reference the initial day of each period (day 0 in the acidification section of the experiment and day 92 in the recovery stage).

2.5 Density of symbionts

Coral tissue was removed from the skeleton of five nubbins from each species at the beginning of the acidification experiment and after the exposure to the control and low pH treatment conditions by means of a jet of re-circulated filtered seawater using an oral irrigator (WaterPik™). The resulting slurry was homogenized with a glass pestle and the volume of the homogenate was recorded (~10 mL). Density of symbiotic dinoflagellates was determined by using 5 replicate counts on a haemocytometer (Neubauer chamber) using a Zeiss standard microscope. Algal size was recorded for 15-20 cells in each sample. After correcting for homogenate volume, the density of symbiotic dinoflagellates was normalized to skeletal surface area, which was calculated by using the aluminium foil technique (Marsh, 1970).

245 *2.6 Scanning Electron Microscope (SEM) images of coral skeletons*

246

247 Only samples of *C. caespitosa* were analyzed by Scanning Electron Microscope.
248 Three nubbins were randomly selected from each treatment and covered with a thin
249 layer of gold-palladium (<200 Å) for morphology and microstructure SEM
250 observations. A SEM Hitachi S3500N, working at 5 kV, was used. Observations
251 focused on the amount and size of the septal flank spines.

252

253 *2.7 Statistical analyses*

254

255 The effect of both experiments (i.e., the acidification experiment - conducted
256 over the first 92 days-, and the recovery experiment - conducted from day 92 to day
257 216) was examined separately for each species. For the acidification experiment, a two-
258 way nested ANOVA was used for each species to examine whether calcification rate
259 varied between treatment (i.e., exposure to low pH conditions and exposure to current
260 pH conditions) and aquaria. Aquarium was considered as a random factor nested within
261 treatment. A two-way ANOVA was also used to examine whether calcification rate
262 varied between both species and aquaria in control conditions after 92 days of
263 experiment. For the recovery experiment, a two-way nested ANOVA was used for each
264 species to examine whether calcification rate varied between treatment (i.e., exposure to
265 current pH conditions of colonies previously exposed to low pH and exposure to current
266 pH conditions of colonies previously exposed to current pH conditions) and aquaria.
267 Aquarium was considered as a random factor nested within treatment. Normality
268 (Kolmogorov-Smirnov test) and heterocedasticity (Cochran's test) of both species
269 growth data were assessed after arctan-transformation. Calcification rate results are

expressed as mean ± standard error of the mean (SE). Non-parametric Kruskal-Wallis ANOVA was used to examine differences between both treatments from the acidification experiment in the abundance and size of symbionts. These statistical analyses were performed using the software package Statistica 6.0 (StatSoft, Inc. 2001).

3 Results

3.1 Seawater chemistry

Our experimental set-up allowed a precise adjustment of the selected pH conditions, which were maintained during the first three months at 8.09 ± 0.01 and 7.83 ± 0.02 pH units for the control and acidified pH treatment, respectively (Table 1; Fig. 2). Total alkalinity values remained constant in both treatments (2534 ± 11 and 2539 ± 9 μmol kg^{-1} for the control and acidified pH treatment, respectively) throughout the entire experiment. The average calculated Ω_A and χCO_2 (mole fraction of CO_2 in dry air) for the control pH treatment were 3.6 and 390 ppm, respectively. In the acidified treatment, these values changed to 2.1 and 800 ppm, respectively. Average values of other parameters of the carbonate system are summarized in Table 1. Temperature and salinity were constant throughout the whole experiment (20.0 ± 0.6 ºC and 37.4 ± 0.2, respectively).

3.2 Effects of low pH on coral calcification rates

Significant differences were observed between the calcification rates of the two species reared under control conditions after 92 days of experiment (Table 2), being

295 37% higher in *O. patagonica* than in *C. caespitosa* (Fig. 3a). The effect of the low pH
296 treatment was similar; both species showed a significant decrease in average
297 calcification rate compared to control conditions: 32% lower for *O. patagonica* and
298 35% lower for *C. caespitosa* (Table 2, Fig. 3a). At the end of the acidification period the
299 decrease in skeletal growth rate exhibited by both species was similar and no significant
300 differences were observed between them (Table 2). The survivorship in each treatment
301 was 100% and no tank effect was detected between aquaria replicates of the same
302 treatment in any analysis.
303
304 On the basis of the absence of differences between duplicate aquaria, we
305 calculated the effect of low pH on the skeletal growth (difference in growth between the
306 control and the low pH treatments) of each of the 10 distinct coral colonies used in the
307 experiments. As previously described, each colony was split in five and distributed
308 among the 2 treatments (4 aquaria). A one-way ANOVA was performed to examine
309 whether the effect of exposure to low pH differed between the two species. We
310 observed that the decrease in calcification rates at the end of the acidification
311 experiment was similar for both species (Table 2). However, when the mean growth
312 reduction of each colony from both species in the acidified experiment (after 92 days)
313 was compared with the average growth rate of the same colony in control conditions
314 (Fig. 4), an interesting pattern arose: colonies exhibiting faster growth rates were more
315 affected by the decreased pH.
316
317 The difference in *O. patagonica* calcification between the treatment and control
318 exposure exhibited a large spread of responses among the colonies during the first
319 month, which later progressively diminished (i.e., there was a reduction of the variance

along the length of the experiment, Fig. 5). The previously observed pattern of a larger detrimental effect of acidification on coral colonies that grew faster (Fig. 4) is contributing to this trend, because it causes an attenuation of the differences between the growth rate of the colonies over time. Although the same effect is observed in *C. caespitosa* (Fig. 4), the slow growth rate of this coral species prevents measurement of a clear reduction of the variance over time (Fig. 5). This observation highlights the importance of running experiments long enough to assess more realistically the effect of these environmental perturbations.

During the recovery experiment, the nubbins of *O. patagonica* and *C. caespitosa* grown under low pH conditions gradually recovered after being returned to the current pH conditions of the control treatment. At the end of the recovery experiment (216 days), no significant differences in overall mean calcification rate were detected between the nubbins in control and those in recovered conditions for both species (Table 2, Fig. 3b). The recovery potential showed by both species was similar and no significant differences were detected between them (Table 2). Encouraged by the absence of aquaria effect, we then calculated the difference in skeletal growth between the control and recovery treatments for each of the 10 distinct coral colonies used in the experiment. At the end of the recovery experiment (216 days), we did not observe a significant variation in skeletal growth rate of the treatment colonies with respect to control between both species. In addition, the recovery experiment did not exhibit any significant relationship between the average calcification rate of each colony exposed to the recovery treatment and that of the same colony exposed to the control conditions (R^2 = 0. 29, p = 0.11 in *O. patagonica* and R^2 = 0.34, p = 0.08 in *C. caespitosa*; n = 10), that

344 is, growth rate under control conditions did not predict growth rate under recovery
345 conditions for a given colony.
346
347 *3.3 Density of symbionts*
348
349 The initial abundance of zooxanthellae was about 2-fold higher in *O. patagonica* (8.39
350 ± 6.23 x10^6 cells cm^{-2}; mean ± SD) than in *C. caespitosa* (4.85 ± 2.46 x10^6 cells cm^{-2};
351 mean ± SD; Fig. 6a; Kruskal-Wallis, p = 0.03). In contrast, zooxanthellae were on
352 average half a micron smaller in *O. patagonica* (6.8 ± 0.4 μm; mean ± SD) than in *C.*
353 *caespitosa* (7.5 ± 0.6 μm; mean ± SD; Fig. 6b; Kruskal-Wallis, p = 0.05). Zooxanthellae
354 density (*O. patagonica*, Kruskal-Wallis, p = 0.518; *C. caespitosa*, Kruskal-Wallis, p =
355 0.304), and cell size (*O. patagonica*, Kruskal-Wallis, p = 0.338; *C. caespitosa*, Kruskal-
356 Wallis, p = 0.395) did not differ between treatments (control and acidified) for either
357 species, indicating that zooxanthellae in the two Mediterranean coral species were not
358 affected by the level of acidification to which they were subjected.
359
360 *3.4 Microimaging of coral skeleton*
361
362 SEM observations of *C. caespitosa* nubbins revealed no clear differences in the
363 skeletal morphology and microstructure between treatments (Fig. 7). At a gross
364 morphological level, slight differences were found in the appearance of the distal tips of
365 the septa (thinner in nubbins grown under lower pH conditions) as well as on the
366 number and size of the septal flank spines. However, at a much higher magnification, no
367 differences were apparent in the size and arrangement of the microcrystalline units, and

368 different fiber crystallization patterns were observed within the same corallite regardless
369 of the treatment.
370
371 **4 Discussion**
372
373 *4.1 Effects of acidification on corals from the Mediterranean Sea*
374
375 During the first stage of the experiment, colonies of *O. patagonica* and *C.*
376 *caespitosa* reared in the low pH treatment (pH 7.83), suffered a decrease in calcification
377 rates of 32 to 35% compared with colonies maintained in control conditions (pH 8.09;
378 Fig. 3). Our experiment thus exhibited the expected result of detrimental effects on coral
379 calcification. Previous experiments with Mediterranean corals testing the effect of
380 acidification on calcification rates have revealed different responses, pointing to some
381 complexity in the effect of low pH on temperate corals. Our results are consistent with
382 those of the only experiment reported to date with *O. patagonica* and *Madracis*
383 *pharensis* (Fine and Tchernov, 2007). In that study, *O. patagonica* reared in aquaria
384 under different pH treatments, showed a dissociation of the colony form and complete
385 skeleton dissolution when exposed to seawater pH of 7.3 – 7.6. However, differences in
386 the low pH treatment between Fine and Tchernov's (2007) study and ours are
387 substantial, preventing an exact comparison of both works. First, the pH in the acidified
388 treatment was lower in the earlier study (pH ~7.4) than in our experiment (pH ~7.83), in
389 which our goal was to mimic realistic projections for the year 2100. In addition, the pH
390 adjustment in Fine and Tchernov's (2007) experiment was performed by adding HCl
391 (reducing alkalinity) whereas, in our case, we bubbled CO_2 (maintaining alkalinity
392 constant), a method that provides a more realistic approach. In any case, despite the

393 differences between the studies, our results, together with those from Fine and Tchernov
394 (2007), show decreased calcification rate of *O. patagonica* at lower pH and Ω_A in
395 seawater. In contrast, results for a temperate coral species from the same genera
396 (*Oculina arbuscula*, not present in the Mediterranean), showed a nonlinear response of
397 calcification rates to CO_2-induced ocean acidification (Ries et al., 2010) by exhibiting
398 no changes until Ω_A was reduced to 0.8 (equivalent to a pH of 7.48 units). These results
399 suggest a greater resistance to low pH of *O. arbuscula* in comparison to *O. patagonica*
400 as reported by Fine and Tchernov (2007) and our study.
401
402 Regarding the endemic Mediterranean species, a previous study by Rodolfo-
403 Metalpa et al. (2010) exhibited no significant difference in calcification rate of *C.*
404 *caespitosa* when reared in aquaria under elevated pCO_2 (700 ppm equivalent to ~7.88
405 pH units), pointing to a lower sensitivity of this temperate coral species to ocean
406 acidification. However, further work with *C. caespitosa* and *Balanophyllia europaea*
407 grown under the influence of natural high pCO_2 vents (Rodolfo-Metalpa et al., 2011),
408 showed that, whereas net calcification in *B. europaea* remained positive even at pH 7.3,
409 net calcification rates of *C. caespitosa* became negative at pH 7.5. According to these
410 authors, the presence of a protective external organic layer, a condition that has also
411 been documented to modulate the effects of ocean acidification in corals and other
412 organisms (Ries et al., 2009), could explain the observed differences between the two
413 species. In those corals under the influence of CO_2 vents, *C. caespitosa* presented large
414 parts of the skeleton exposed with evident marks of dissolution, while *B. europaea*
415 skeletons remained completely covered by tissue. In our experiment, the skeletons of *C.*
416 *caespitosa* corals maintained a full organic coverage of the polyps and the coenosarc,
417 thus preventing the direct exposure of the skeleton to the surrounding seawater.

418

419 Concerning possible skeletal microstructural differences between treatments, our
420 SEM images of *C. caespitosa* reared under low pH conditions revealed no obvious
421 evidence of localized dissolution, in line with of the results reported by Ries *et al*.
422 (2010) with *O. arbuscula*. No clear differences in skeletal microstructure between
423 treatments were detected at high magnification (Fig. 7e, f). Only slight differences were
424 observed at a lower magnification; distal ends of some of the septa displayed a thinner
425 and sharper appearance in the colonies reared under acidic conditions, while the size of
426 the septal dentation was apparently smaller (Fig. 7a-d). The lack of evidences for
427 dissolution (e.g. disordered aragonite crystals) is probably related to the fact that, over
428 the course of the study, the skeleton of *C. caespitosa* was never exposed to the corrosive
429 effects of aragonite-undersaturated waters. This suggests that the decrease in net
430 calcification observed in our experiment was more related to decreased calcification rate
431 than to dissolution.

432

433 Temperature conditions represent an important difference between our study and
434 that of Rodolfo-Metalpa *et al*. (2010), in which no decrease in calcification was
435 observed in *C. caespitosa* under acidified conditions (7.88 pH units). Our experiment
436 was conducted at a constant temperature (~20ºC), whereas Rodolfo-Metalpa *et al*.
437 (2010) explored possible responses following an annual natural temperature cycle. In
438 our case, we chose to focus our experiment in the warming season, the time of the year
439 when *C. caespitosa* exhibits most of its growth, while in winter its metabolism is
440 reduced to a minimum (Montagna et al., 2007; Rodolfo-Metalpa et al., 2010). The
441 increase in atmospheric CO_2 is also causing global seawater warming, which in the
442 Mediterranean Sea has been shown to be responsible of a significant lengthening of

443 summer conditions (Coma et al., 2009). Up to a certain threshold, this trend should
444 favor calcification of the studied species. Nevertheless, as shown by our experiment,
445 acidification may counterbalance the positive effects on calcification of sea water
446 warming.
447
448 *4.2 Acidification and energetically costly calcification*
449
450 The ability to elevate pH and $^{-}$ at the site of calcification is an energy
451 demanding process (Al-Horani et al., 2003; Cohen and Holcomb, 2009). Under
452 lowered-pH conditions, this process needs more energy, which could otherwise be
453 devoted to other activities, such as locomotion, reproduction, tissue growth or to
454 counteract other environmental stresses (Brewer and Peltzer, 2009; Hoegh-Guldberg et
455 al., 2007). This is consistent with the fact that feeding rate and nutrient availability are
456 also variables that has been shown to modulate the effects of acidification on coral
457 growth and calcification. Several studies with tropical species have shown that
458 enhanced heterotrophic feeding and inorganic nutrient enrichment help to counteract the
459 negative impacts of acidification on calcification and photosynthesis [e.g. (Chauvin et
460 al., 2011; Edmunds, 2011; Houlbrèque, 2004 ; Langdon and Atkinson, 2005)].
461 Regarding temperate corals, Holcomb et al. (2010, 2012) observed that *Astrangia*
462 *poculata* exhibited a sharp decline in calcification under low pH and normal nutrient
463 concentration; whereas no significant differences were found under enhanced nutrient
464 concentrations or high feeding rates, indicating that a supplementary diet might partly
465 compensate the energy demand of calcifiers growing in a more corrosive ambient.
466

In our experiment, food supply consisted of *Artemia* nauplii once a week. By comparison, feeding rates in previous works with *O. patagonica* and *C. caespitosa* were higher. In the case of Ries et al. (2010), *Artemia sp*. was added every other day and, in the other studies, corals were naturally fed with unfiltered seawater in the aquaria (Rodolfo-Metalpa et al., 2010), or kept at natural sea input conditions (Rodolfo-Metalpa et al., 2011). Therefore, the greater sensitivity observed in our study to acidic conditions might also be related to a lower energy availability caused by less frequent feeding in comparison to previous experiments. The design of our experiment may be relevant, as global warming will very likely induce an increase in water stratification with a consequent diminishment of nutrient and plankton availability in surface waters (Coma et al., 2009; Doney et al., 2009), which could lend coral communities to be more vulnerable than previously thought to global environmental pressures.

Overall, our study focused on the time period and conditions during which acidification may exert its larger effect (warm temperatures and low food supply characteristic of summer conditions). Our results also agree with the fact that *C. caespitosa* (but also *B. europaea*), were not found in nature below pH 7.8 (Rodolfo-Metalpa et al., 2011) which suggests that long term exposure to these conditions is detrimental to the development of the species and could be related to the high metabolic cost of maintaining a high pH at the site of calcification (Cohen and Holcomb, 2009) in an oligotrophic environment such as the Mediterranean.

4.3 Effect of nubbin parent colony on response to low pH

High natural variability in coral growth has been documented as a result of several factors such as size, seasonal cycle, health status, gender and/or genetic variation (Buddemeier and Kinzie, 1976). Since all colonies had approximately the same size, were simultaneously collected and temperature was kept constant during the whole experiment, the size and possible seasonal factors can be discarded. The absence of dead nubbins and the healthy appearance of the tissue (always covering the entire skeleton and with a zooxanthellae density and size comparable between colonies from both treatments, Fig. 6a, b) also suggest that differences in health did not modulate natural variability in growth. A recent study has reported a similar correlation in the temperate coral *Astrangia poculata* (Holcomb et al., 2012), with fast growers being the more affected by a lowering in pH. In that case, intercolonial growth variability was related to gender, with a larger effect of acidification in females during the spawning season, and little or no effect on non-breeding males and females. In our experiment, the gender of the colonies was not determined, so we cannot discount such an effect in modulating coral growth. However, as mentioned above, gender is just one among the many factors contributing to the high natural variability commonly observed (Buddemeier and Kinzie, 1976).

Given the unlikeliness of strong influences from most of the factors discussed above, we suggest that intraspecific genetic variability could be key in explaining the wide variety of responses observed in our studied coral colonies. Thus, coral colonies that have a genetically-determined ability to grow rapidly will be more sensitive to a lowering in pH. The corals that grow faster should also have greater energetic requirements for modulating pH at the site of calcification and, when subjected to more corrosive conditions, may be the first to exhibit a negative response to this pressure.

Similar responses have been pointed out by other studies comparing several species. Rodolfo-Metalpa et al. (2010), for instance, discussed on this possibility when comparing tropical *vs* temperate corals, highlighting that the faster growing tropical species may have more requirements in terms of enhanced saturation state and concentration of carbonate ion to maintain their high rates of calcification. More recently, Jokiel (2011) and Edmunds et al. (2012) also noted a stronger reduction in calcification in the more rapidly growing tropical coral species. In the framework of the *proton flux hypothesis* proposed in the Jokiel's (2011) study, this was interpreted as reflecting the need to dissipate a larger flux of protons through the boundary layer in the fast growing species. Our experimental results suggest that analogous energy consuming constraints may also modulate the effects of ocean acidification on different colonies from a single coral species.

4.4 Potential for recovery from ocean acidification

Based on data from the recovery experiment, when more acidic conditions were brought progressively back to current pH, our study provides insight on the potential for recovery of both coral species following events of acidification. Given the existence of natural oscillations in seawater pH at different timescales both in the open ocean and around coral calcifying communities [(Pelejero et al., 2010) and references therein], our recovery experiment could also be taken as an analog for these naturally occurring transitions. A similar study performed on *O. patagonica* showed rapid recovery after increasing pH to control conditions (Fine and Tchernov, 2007), but this is the first attempt to assess such recovery potential in an endemic Mediterranean species such as *C. caespitosa*. Interestingly, this endemic species showed a recovery very similar to that

541 of the alien species *O. patagonica*. Furthermore, and in contrast to the acidification
542 experiment, in which the colonies that grew faster were most affected by acidification
543 (Fig. 4), no trend was detected through the recovery stage, so the recovery took place
544 independent of colony growth.
545
546 The recovery potential shown by *O. patagonica* in this experiment is in
547 agreement with the only study published so far evaluating the resilience of this species
548 after an acidification event (Fine and Tchernov, 2007). These authors observed that
549 despite the complete skeleton dissolution of the *O. patagonica* polyps, they maintained
550 their symbionts and normal gametogenesis during the low pH treatment. When
551 transferred back to ambient pH conditions, colonies reformed, showing high plasticity
552 and acclimation capacity. A decrease in the growth rate under acidic conditions and a
553 subsequent recovery after return to normal conditions has also been observed in tropical
554 corals (Marubini and Atkinson, 1999). This supports the existence of certain degrees of
555 reversibility in the effects of ocean acidification in corals, if levels of pH rise back to
556 normal conditions. There is the possibility that this capacity to recover is a result of
557 evolutionary adaptation to the natural oscillations in seawater chemistry that are
558 ubiquitous in the oceans.
559
560 As mentioned, natural pH fluctuations are commonly experienced by upper
561 oceanic waters, particularly within coral reefs or shallow areas dominated by marine
562 calcifiers [(Pelejero et al., 2010) and references therein]. It is thus conceivable that,
563 during the early stages of anthropogenic ocean acidification, similar natural cycles will
564 be superimposed on the general trend of decreasing pH. In this sense, the observed
565 capacity of these species to recover in the framework of natural cycles would probably

566 mitigate the effects of acidification in corals, which will benefit intermittently from
567 periods when pH will be brought back towards the more basic conditions that they
568 prefer. However, as global ocean acidification evolves, these reinvigorating periods for
569 corals will become shorter and shorter, eventually disappearing as the full range of pH
570 changes shifts away from preindustrial values [e.g. (Friedrich et al., 2012)]. Even
571 though the resilience showed by certain species of corals could improve their survival
572 capacity for the coming years, this could be counteracted by the combined effect of
573 ocean acidification and other global and regional pressures such as warming,
574 overfishing, high sedimentation rates (due to land use changes) and nutrient enrichment
575 in coastal areas, all leading to a future that will likely be bleak for corals [e.g. (Hoegh-
576 Guldberg, 2012) and references therein].
577
578 **5 Conclusions**
579
580 Our data from pH-manipulative experiments show a high sensitivity of *O.*
581 *patagonica* and *C. caespitosa* to near future acidification in the Mediterranean Sea. A
582 low pH-driven decrease of up to 35% was detected in the calcification rates of both
583 species compared to control conditions. In contrast, acidification caused no apparent
584 effect on coral-associated zooxanthellae or in the coral skeleton microstructure. We also
585 found a high intraspecific variability in the response to acidification among different
586 colonies, with the fastest growing organisms displaying the greatest sensitivity. This
587 suggests an important role for energy consuming constraints in modulating the effects
588 of ocean acidification in corals. In addition, these results highlight the importance of
589 future studies targeting genetic variability to better understand the observed differential
590 intercolonial response.

591

592 This study also assesses, for the first time, the recovery potential of an endemic
593 Mediterranean coral species after an acidification event, with results that suggest a
594 gradual recovery similar to that of the alien species used as comparison. This points
595 towards a certain degree of acclimation capacity of these species to periodic pH
596 oscillations, although the energy demands could eventually be detrimental to the
597 organism's ability to fulfill other important physiological or reproductive processes.
598 Considering that the projected progressive warming of seawater might lead to higher
599 metabolism rates and lower prey availability due to longer stratification periods, the
600 survival threshold of these coral species could be exceeded sooner than expected under
601 the influence of single stressors.

602

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604

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Table 1. Parameters of the seawater carbonate system in each treatment. Total alkalinity, pH_T, salinity and temperature were used to calculate all the other parameters using the CO2calc software (USGS). For the four measured parameters we report the values as mean ± standard deviation (SD) and range (in brackets). All other calculated parameters are expressed as mean ± SD. n = 12 and 6 for the control and high-CO_2 treatment, respectively.

Measured parameters

Treatment	**pH_T**	**TA**	**Sal**	**T**
Control	8.092 ± 0.008 (8.070 - 8.100)	2534 ± 11 (2520 - 2550)	37.4 ± 0.2 (37.0 - 37.7)	20.0 ± 0.6 (19.1 - 21.1)
High-CO_2	7.830 ± 0.021 (7.807 - 7.862)	2539 ± 9 (2525 - 2550)	37.3 ± 0.2 (37.0 - 37.5)	19.8 ± 0.3 (19.5 - 20.2)

Calculated parameters

Treatment	**pCO_2**	**χCO_2**	**DIC**	**$[CO_2]_{aq}$**	**$[HCO_3^-]$**	**$[CO_3^{2-}]$**	**Ω_A**
Control	381 ± 9	390 ± 9	2211 ± 15	12.1 ± 0.5	1966 ± 20	232 ± 6	3.6 ± 0.1
High-CO_2	780 ± 40	800 ± 40	2362 ± 14	25 ± 1.4	2197 ± 18	140 ± 7	2.1 ± 0.1

"pH_T" = pH in total scale; "TA" = total alkalinity (µmol/kg-SW); "Sal" = salinity; "T" = temperature (°C); "pCO_2" = partial pressure of CO_2 of air in equilibrium with seawater (ppm); "$_{\chi}CO_2$" = mole fraction of CO_2 in dry air (ppm); "DIC" = dissolved inorganic carbon (µmol/kg-SW); "$[CO_2]aq$" = CO_2 concentration in seawater (µmol/kg-SW); "$[HCO_3^-]$" = bicarbonate ion concentration (µmol/kg-SW); "$[CO_3^{2-}]$" = carbonate ion concentration (µmol/kg-SW); "Ω_A "= saturation state of seawater with respect to aragonite.

Table 2. Summary of the outputs ANOVA tests evaluating the effect of two levels of pH (8.09 and 7.83 units) on the growth of *Cladocora caespitosa* and *Oculina patagonica* and the subsequent recovery. Bold face numbers indicates $P < 0.05$.

Source of variation	df	MS	F-ratio	P-value
Comparison of growth in control conditions				
Species	1	0.09	7.65	**0.0083**
Aquarium	1	0.00	0.06	0.80
Error	44	0.01		
Effect of low pH in *O. patagonica*				
Treatment	1	0.05	5.91	**0.0192**
Aquarium (treatment)	2	0.01	1.32	0.28
Error	44	0.01		
Effect of low pH in *C. caespitosa*				
Treatment	1	0.06	7.00	**0.0113**
Aquarium (treatment)	2	0.00	0.34	0.71
Error	44	0.01		
Comparison of low pH effect on both species				
Species	1	0.00	0.01	0.94
Error	18	0.42		
Recovery in *O. patagonica*				
Treatment	1	0.01	2.17	0.15
Aquarium (treatment)	2	0.00	0.62	0.54
Error	44	0.01		
Recovery in *C. caespitosa*				
Treatment	1	0.02	1.74	0.19
Aquarium (treatment)	2	0.00	0.44	0.64
Error	44	0.01		
Comparison of recovery potential on both species				
Species	1	0.00	0.69	0.42
Error	18	0.00		

Figure captions

Fig. 1. Experimental setup used to control and modify the seawater pH in each aquarium. **A)** and **B)** large 150 L tanks for seawater conditioning at pH ~7.83 and 8.09, respectively; **C)** glass electrodes for pH and PT100 probes for temperature measurements; **D)** pH controller and data logger; **E)** solenoid valves; **F)** soda lime filter; **G)** 50 kg CO_2 bottle; **H)** seawater filtering system (sand, sediment trap and 50µm cartridge filter); **I)** and **J)** control and low pH experimental aquaria, respectively (two replicates per treatment); **K)** HQI-lamps; **L)** Microbubble diffusers.

Fig. 2. pH fluctuations during the two stages of the experiment. The solid gray line and the black dotted line represent the pH logged using glass electrodes in the acidified and control large conditioning tanks, respectively. Stars indicate corresponding discrete pH measurements performed by spectrophotometry in the aquaria.

Fig. 3. Skeletal growth rates of *O. patagonica* and *C. caespitosa* after the first three months under the two pH treatments (A) and during the following four months when the acidified treatment was progressively basified to match the control pH (B). Black and grey bars represent the growth data corresponding to control (~8.09 pH units) and treatment (~7.83 pH units during the first three months, brought to ~8.09 pH units during the following four months), respectively (n = 24, mean ± SE). Growth rates are expressed as mg $CaCO_3$ per gram of initial weight per day, taking as a reference the initial day of each stage (day 0 in (A) and day 92 in (B).

Fig. 4. Correlation between the average growth rate of colonies in the control pH and the difference in growth between these colonies in the acidified and the control conditions, corresponding to *C. caespitosa* (solid line, grey dots) and *O. patagonica* (dashed line, white dots). Linear regressions are statistically significant in both cases (*O. patagonica* b = -0.57; R^2 = 0.78; p = 0.0007; n = 10 and *C. caespitosa* b = -0.48; R^2 = 0.75; p = 0.0011; n = 10).

Fig. 5. Variability in the differences in growth between colonies in the acidified treatment and the corresponding counterparts in the control conditions during all the time intervals when corals were weighted. White and grey boxes represent *O.*

patagonica and *C. caespitosa*, respectively. Horizontal dotted line represents identical growth of the colonies in each treatment. The corresponding box-and-whisker diagrams depict the median (solid line) and the 10^{th}, 25^{th}, 75^{th} and 90^{th} percentiles (n = 10). Skeletal growth data during each time point were calculated in relation to the initial weight (day 0).

Fig. 6. Zooxanthellae density (A) and symbiotic dinoflagellate size (B) measured at the beginning and after the exposure to the control and acidified conditions. White and grey bars represent *O. patagonica* and *C. caespitosa*, respectively. Data are mean ± SD (n = 5).

Fig. 7. Examples of Scanning Electron Microscope (SEM) images of *C. caespitosa* polyps at the end of the experiment, that were exposed to control (A, C, E) and acidified (B, D, F) conditions. Polyp distal view (A, B, scale bar represents 3mm); morphology of distal septal edge from enlarged view (C, D, scale bar represents 500 μm) and septal flank spine from at greater magnification (E, F, scale bar represents 5μm).

Figure 1

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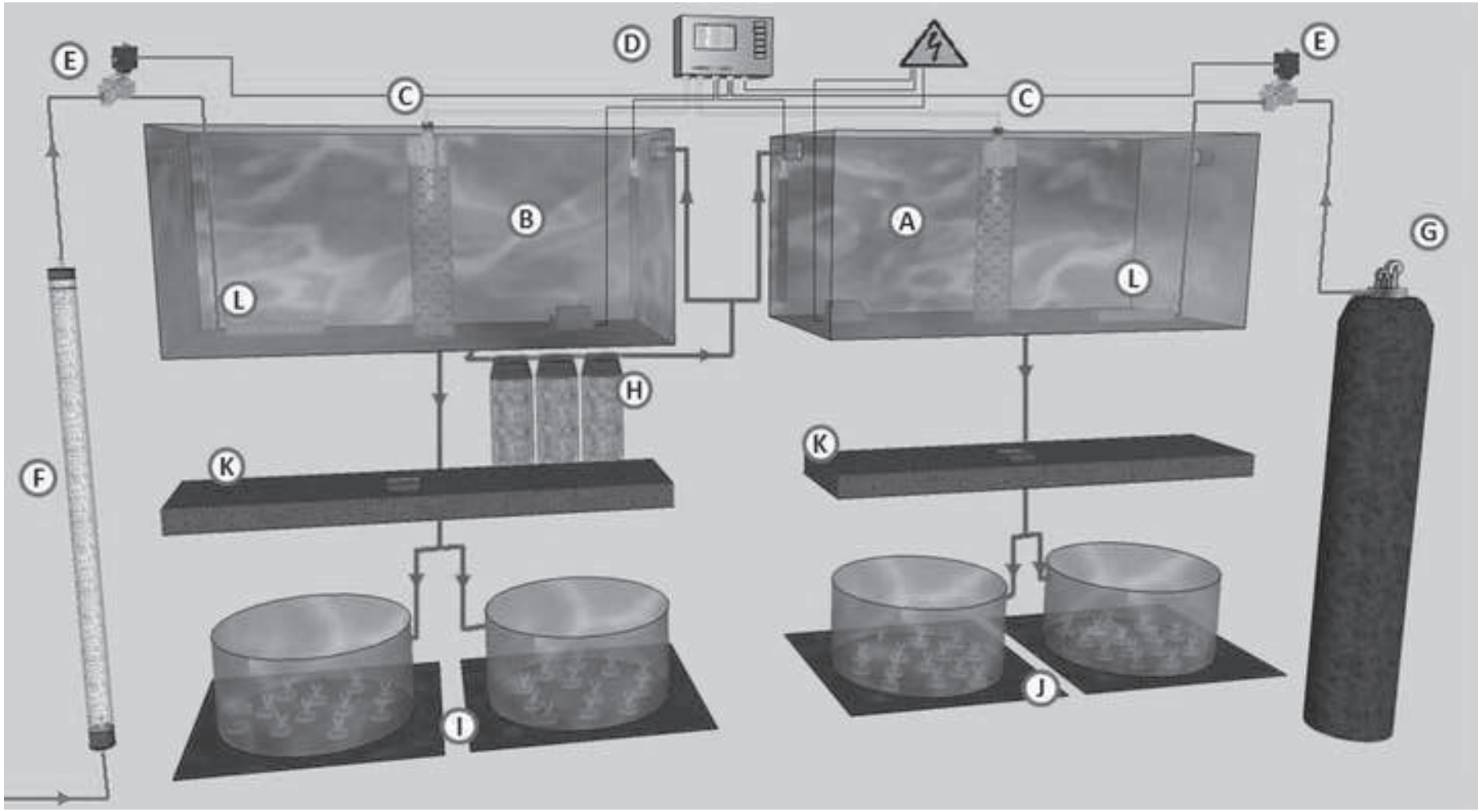

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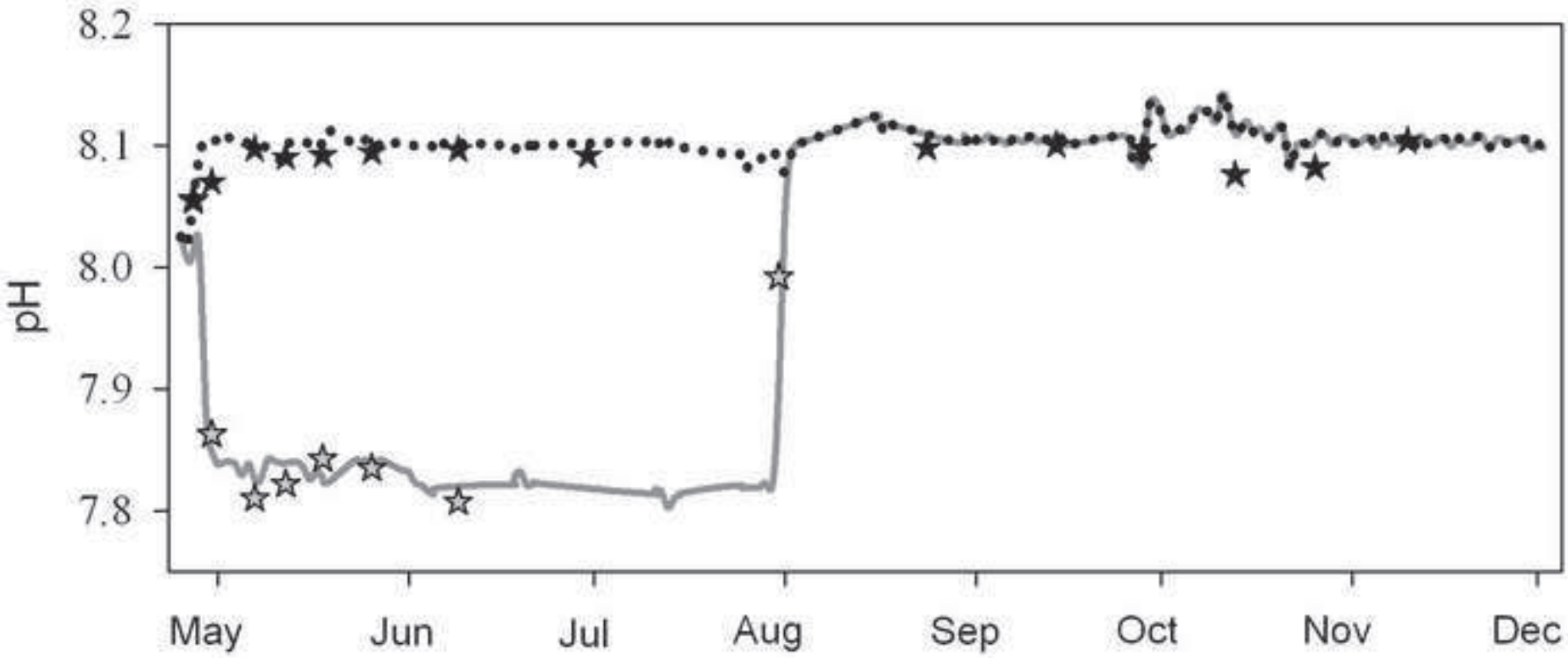

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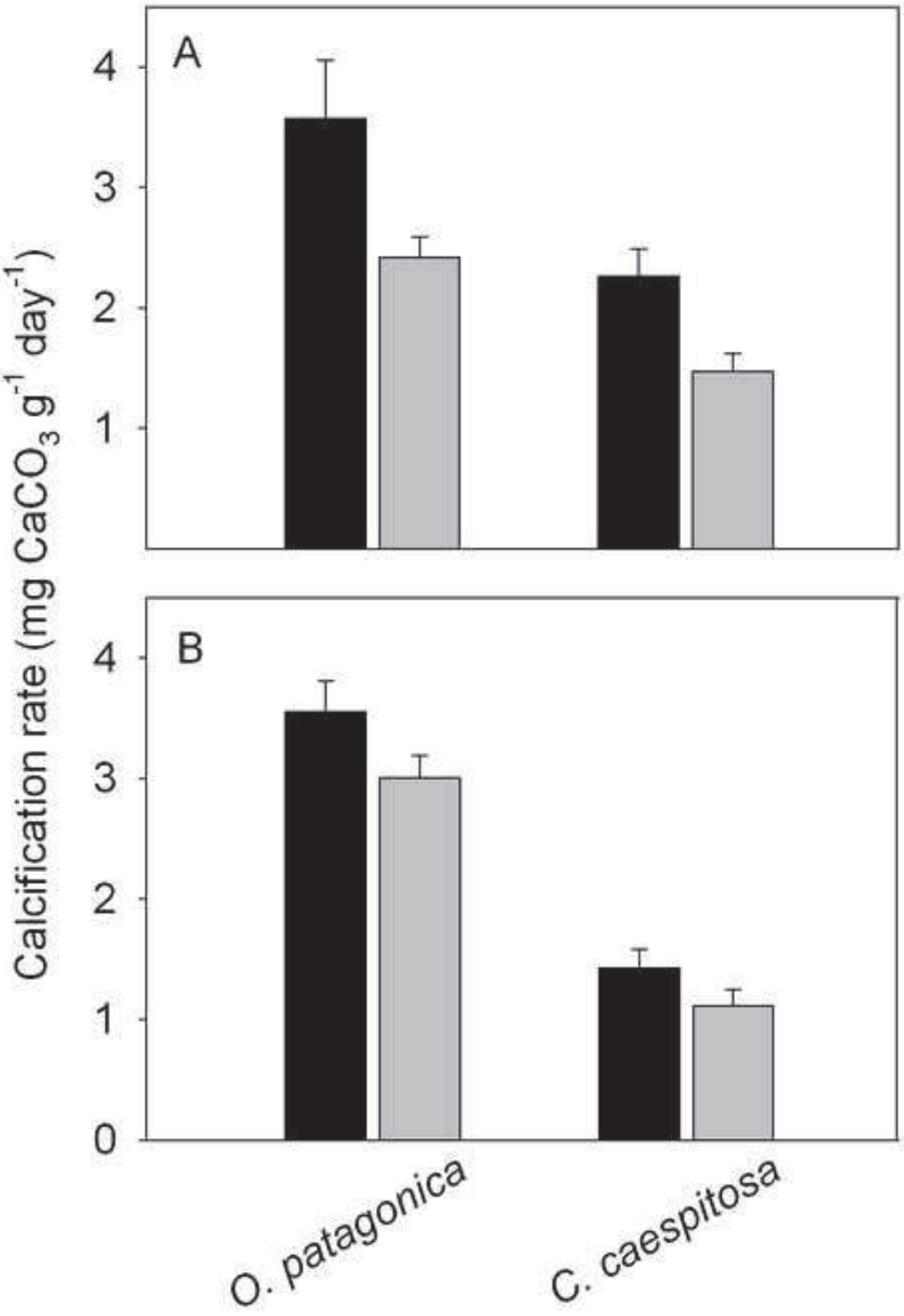

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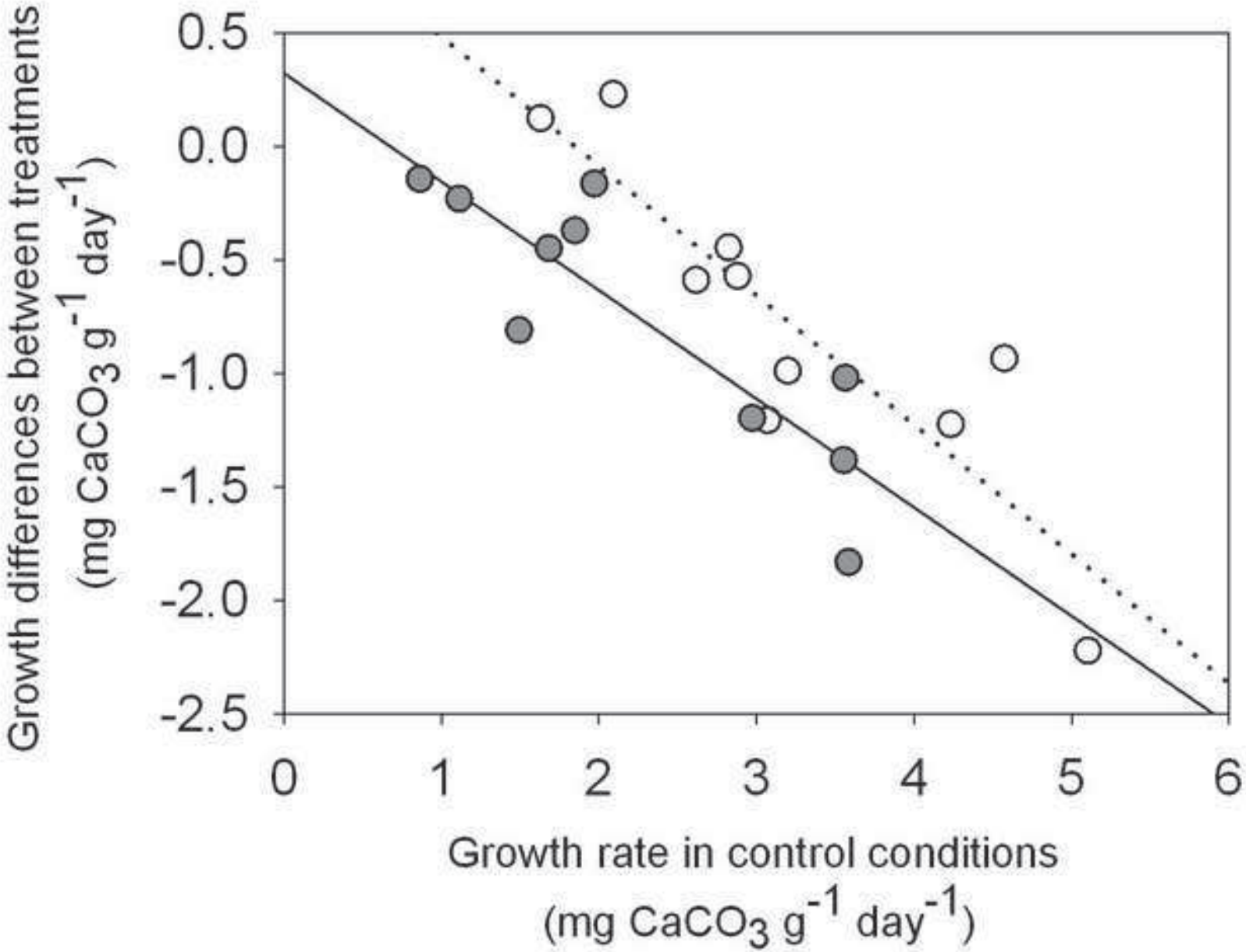

Figure 5

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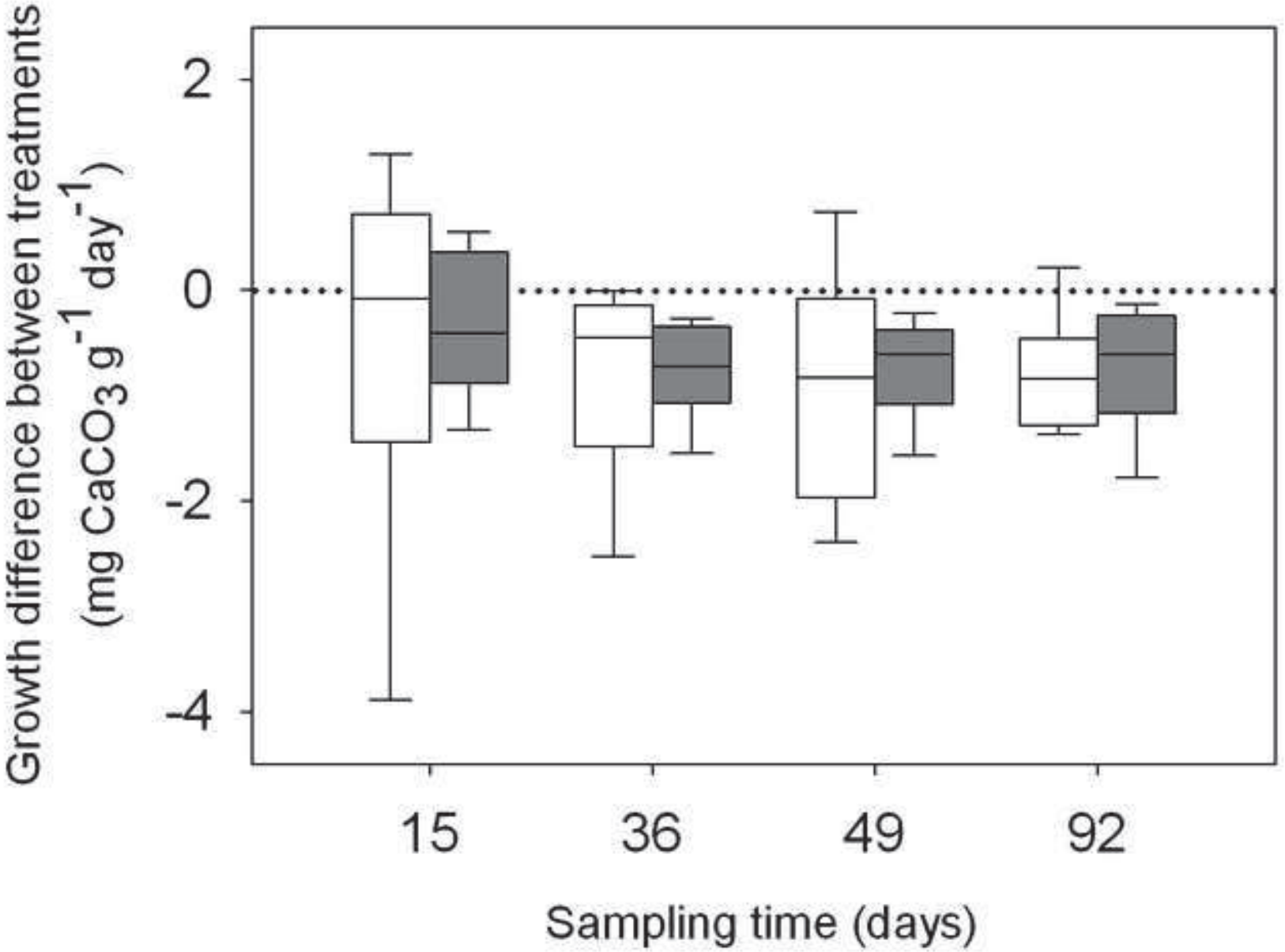

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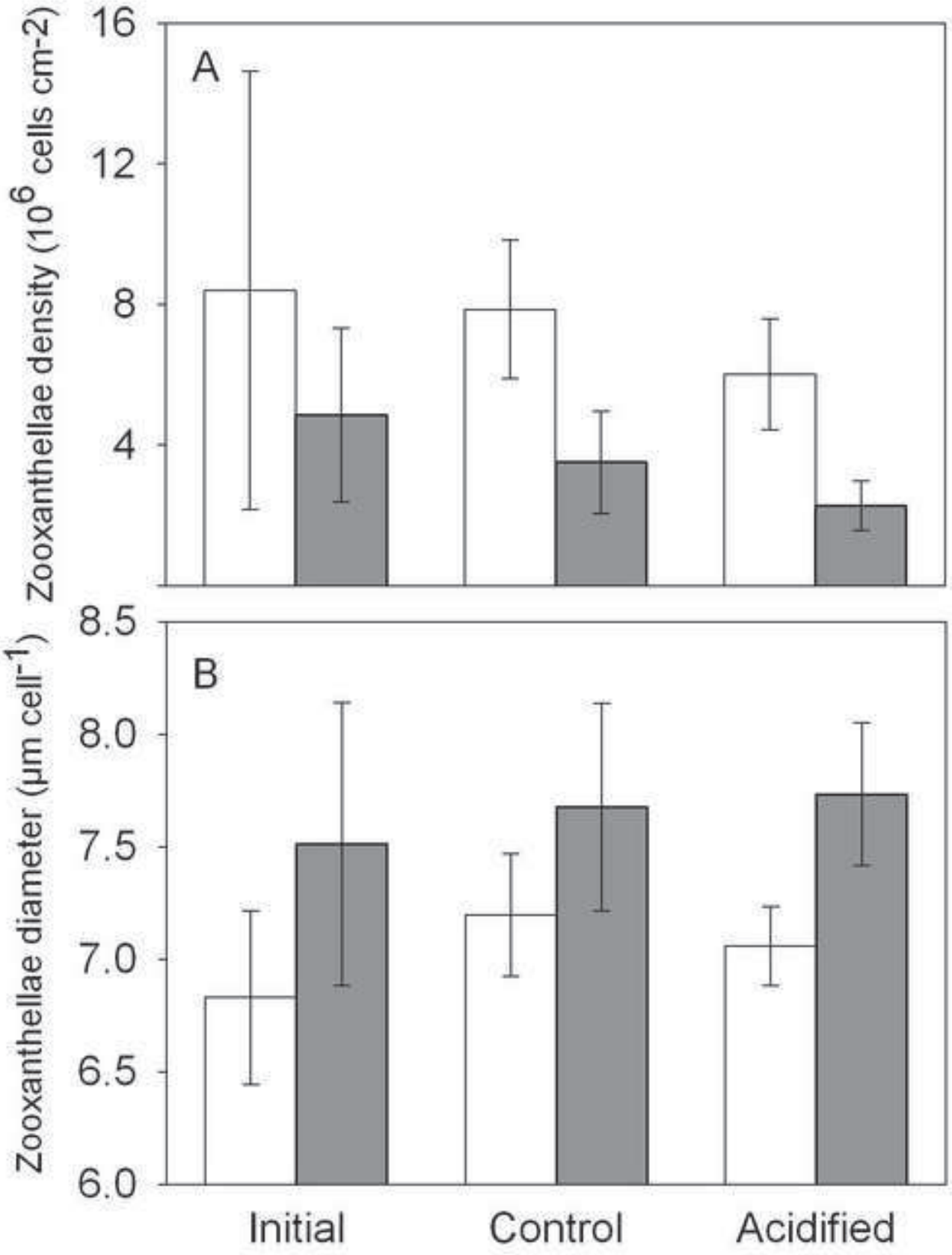

Figure 7
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