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Non-lethal effects of ocean acidification on two symbiont-bearing benthic foraminiferal species

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Abstract

We conducted experiments to assess the effect of elevated atmospheric carbon dioxide concentrations on survival, fitness, shell microfabric and growth of two species of symbiont-bearing coral-reef benthic foraminifera, using pCO_2 levels similar to those likely to occur in shallow marine pore waters in the decades ahead. Foraminifera were cultured at constant temperature and controlled pCO_2 (385 ppmv, 1000 ppmv, and 2000 ppmv) for six weeks, and total alkalinity and dissolved inorganic carbon were measured to characterize the carbonate chemistry of the incubations. Foraminiferal survival and cellular energy levels were assessed using Adenosine Triphosphate (ATP) analyses, and test microstructure and growth were evaluated using high resolution SEM and image analysis. Fitness and survival of *Amphistegina (A.) gibbosa* and *Archaias (A.) angulatus* were not directly affected by elevated pCO_2 and the concomitant decrease in pH and calcite saturation states (Ω_c values) of the seawater (pH and Ω_c values of 8.12, 7.86, and 7.50, and 5.4, 3.4, and 1.5, for control, 1000 ppmv, and 2000 ppmv, respectively). In *A. gibbosa*, a species precipitating low-Mg calcite, test growth was not affected by elevated pCO_2, but areas of dissolved calcium carbonate were observed even though Ω_c was >1 in all treatments; the fraction of test area dissolved increased with decreasing Ω_c. Similar dissolution was observed in offspring produced in the 2000 ppmv pCO_2 treatments. In *A. angulatus*, whose tests are more-solubile high-Mg calcite, growth was greatly diminished in the 2000 ppmv pCO_2 treatment compared to the control. These non-lethal effects of ocean acidification – reduced growth in *A. angulatus*, and enhanced dissolution in *A. gibbosa* – may reflect differences in test mineralogy for the two species; the long-term ecological consequences of these effects are not yet known.

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1 Introduction

Over the last two centuries, human activities have lead to an increase of almost 40 % in the concentration of atmospheric carbon dioxide (CO_2; Feely et al., 2004). Currently the atmospheric CO_2 concentration is above 385 ppmv and could be as high as 800 ppmv by the end of this century (Prentice et al., 2001; EPICA community members, 2004). As CO_2 dissolves into seawater, solution pH decreases (Feely et al., 2004; Orr et al., 2005) and thus, today's surface ocean pH is already 0.1 unit lower than pre-industrial values (Royal Society, 2005) and is predicted to be another 0.3 to 0.4 units lower by the end of this century (Prentice et al., 2001; Orr et al., 2005). This decrease in pH, commonly referred to as "ocean acidification", is likely to affect many marine organisms and, in particular, those that produce calcium carbonate shells or skeletons.

Taxa ranging from protists to metazoans have shown reduced calcification rates in ocean-acidification culture experiments (e.g., Riebesell et al., 2000; de Moel et al., 2009; Moy et al., 2009; Lombard et al., 2010; Kuffner et al., 2008; Martin and Gattuso, 2009; Semesi et al., 2009; Gattuso et al., 1998; Maier et al., 2009; Langdon et al., 2000, 2003; Leclercq et al., 2000; Andersson et al., 2009; Shirayama and Thorton, 2005; Gazeau et al., 2007; Comeau et al., 2009; Wood et al., 2008; Clark et al., 2009). On the other hand, some species have been shown to increase their calcification rates under experimentally induced pH decreases (Iglesias-Rodriguez et al., 2008; Ries et al., 2009; Checkley et al., 2009). Although the survival of many calcifying organisms seems not to be significantly affected by ocean acidification, many studies have found more subtle effects, such as reduced recruitment or growth rates, or changes in gene expression patterns, behavior or physiology (Kuffner et al., 2008; O'Donnell et al., 2008; Ellis et al., 2009; Parker et al., 2009; Arnold et al., 2009; Walther et al., 2009; Dixson et al., 2010).

To date, the majority of studies on the effects of ocean acidification have been conducted on metazoans and plants. Studies on protists have focused on two groups, coccolithophores, which exhibited species-specific calcification responses (e.g., Riebesell

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et al., 2000; Iglesias-Rodriquez et al., 2008), and planktonic foraminifera, in which calcification decreased in all species studied to date (e.g., Barker and Elderfield, 2002; de Moel et al., 2009; Moy et al., 2009; Lombard et al., 2010). Foraminifera are among the most ubiquitous marine calcifying organisms (e.g., Langer et al., 1997; Small and Adey, 2001; Harney and Fletcher, 2003), constitute an important link in marine food webs (e.g., Legendre and Le Févre, 1995; van Oevelen et al., 2006), and play important roles in the recycling of organic carbon (e.g., Moodley et al., 2000) and the production of carbonate (Langer et al., 1997).

Benthic foraminifera have been the subject of fewer ocean-acidification studies (Bernhard et al., 2009a, b; Kuroyanagi et al., 2009; Dissard et al., 2010, Haynert et al., 2011; Fujita et al., 2011). The stress-tolerant, shallow-water calcareous benthic species, *Ammonia tepida*, continued to calcify under elevated pCO_2 (2000 ppmv, $\Omega_c < 1$), although specimens grown at 2000 ppmv were generally lighter than those grown at 120 ppmv (Dissard et al., 2010). The neritic species, *Ammonia amoriensis*, exhibited reduced calcification and increased test dissolution at elevated pCO_2 levels beginning at 929 µatm and calcite saturation values below 1 (Haynert et al., 2011). Fujita et al. (2011) showed that net calcification of large symbiont-bearing foraminifera (*Baculogypsina sphaerulata and Calcarina gaudichaudii*), which secrete a hyaline shell, increased under intermediate levels of pCO_2 (580 and/or 770 µatm) and decreased at a higher pCO_2 level (970 µatm) whereas net calcification of the symbiont-bearing species *Amphisorus hemprichii*, which secretes a porcelaneous shell, tended to decrease at elevated pCO_2. Non-mineralizing benthic foraminifera have been shown to survive and sometimes even reproduce in extremely high pCO_2 (up to 200 000 ppmv) in short-term (10 to 14 days) laboratory experiments (Bernhard et al., 2009b). Moreover, in a field assessment testing the response to sequestration of liquid CO_2 on the deep-sea floor, Bernhard et al. (2009a) showed that survival of agglutinated and tectinous foraminifera was not significantly affected by a decrease in pH, while the survival of calcareous foraminifera was negatively impacted.

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Rising pCO_2 has been speculated to be a cause of aberrant test morphologies, dissolution, and bleaching (i.e., loss of photosynthetic endosymbionts) in calcareous reef foraminifera (Tsimilli-Michael et al., 1998; Crevison and Hallock, 2007), though Souder et al. (2010) found no increase in either aberrant test morphologies or dissolution in a study comparing tests collected in 1982–83 by Hallock et al. (1986a) and 2006–08 from the same site.

In this study we conducted experiments to assess the effect of elevated atmospheric carbon dioxide concentrations on two species of benthic, symbiont-bearing coral-reef foraminifera using pCO_2 levels (1000 and 2000 ppmv) similar to those likely to occur in shallow marine pore waters in the decades ahead. The parameters assessed in this study were survival and fitness, using ATP analysis, and shell microfabric, using scanning electron microscopy (SEM). The two species used differ in the chemical composition of their tests. *Amphistegina (A.) gibbosa* precipitate low-Mg calcite while the tests of *Archaias (A.) angulatus* consist of high-Mg calcite, which, at 10–15 mole % (Toler et al., 2001) is more soluble than either aragonite or calcite (Andersson et al., 2008). The pCO_2 levels (1000 and 2000 ppmv) used were similar to those likely to occur in shallow reef waters in the decades ahead.

2 Material and methods

2.1 Specimen collection and preparation

Specimens of *Amphistegina gibbosa* (Amphisteginiidae, Rotaliida) and *Archaias angulatus* (Soritidae, Miliolida) were collected in the Florida Keys, USA, in April and June 2009. The collection site for *A. gibbosa* was Tennessee Reef lighthouse vicinity, 6–8 m depth, which is a site studied extensively over the past 20 yr (e.g., Hallock et al., 1995; Williams et al., 1997; Baker et al., 2009). The basic sample collection and processing methods also are well established and previously described in those papers. SCUBA divers collected palm-sized pieces of reef rubble into resealable plastic bags at depth, scrubbing each piece of rubble with a small brush to remove algae and microorganisms

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from the rock surface, discarding the rubble pieces, and "rinsing" the resultant slurry to remove finer sediment and algal debris. The resultant sediment-organism slurry was brought to the surface in sealed plastic bags that were placed into a covered bucket containing ambient seawater to protect the specimens from exposure to high light intensities and temperature fluctuations. At Keys Marine Laobratory, bulk samples were decanted into several one-liter wide-mouth jars, each containing approximately half centimeter depth of sediment and ~600 ml of seawater. Jars were tightly sealed for transport.

The collection site for *A. angulatus* was adjacent to the Keys Marine Laboratory in Florida Bay, a site described by Fujita and Hallock (1998). At roughly 1 m depth, depending upon the tide, this site is easily accessible by snorkeling. Handfuls of benthic macroalgae were placed into resealable plastic bags and manually agitated to dislodge the abundant *A. angulatus* specimens. The bulk organic matter was decanted and the samples were rinsed repeatedly to minimize presence of finer particulate organic matter. The resultant sediment-ogranism slurry was dispersed into one-liter wide-mouth jars as described above.

All jars were tightly sealed and placed in an insulated box for transport to the laboratory at the University of Florida. There they were decanted into petri dishes and placed into an incubator on a 12-h light/dark cycle and maintained at 25 °C. The morning of shipment, healthy-appearing specimens were picked into insulated jars, packed in an insulated box, and shipped using overnight delivery to WHOI.

After arrival at WHOI, specimens were allowed to acclimatize for 7–10 d before experiments commenced. During this period and throughout the experiments, the foraminifera were fed a freeze-dried mixture of equal proportions of the algae *Isochrysis galbana* and *Dunaliella tertiolecta* once per week. On the day of experimental initiation, specimens were transferred into Petri dishes containing 0.22 µm-filtered seawater (FSW). After about 15 min, specimens were examined under a Nikon SMZ-2B stereo dissecting microscope and those with visible pseudopodia were deemed to be alive and distributed randomly among the experimental treatments.

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2.2 Experimental overview

Experiments were run at two elevated CO_2-concentrations, 1000 ppmv and 2000 ppmv, with ambient pCO_2 (~385 ppmv) as the control concentration. Experiments lasted for 6 weeks with time points after 1 and 6 weeks.

2.3 Treatments

To assess survival, each experiment consisted of three treatments: one ambient pCO_2 (Control) and two elevated pCO_2 treatments performed at either 1000 or 2000 ppmv. Specimens from one of the elevated treatments were extracted for adenosine triphosphate (ATP) directly from the elevated pCO_2incubator (the "Direct" treatment). The second elevated pCO_2 treatment was placed in the control chamber after the enriched CO_2 incubation, and allowed to re-equilibrate to atmospheric conditions for ~24 h prior to subsequent analyses (the"Rebound" treatment, Bernhard et al., 2009b). Only the Control and Direct treatments were used for test microstructure analysis. One 24-well plate was used per treatment and per time point for Control, Direct and Rebound and one plate each for SEM Control and SEM Direct, with one individual per well. Prior to the beginning of the experiments, photographs of all specimens from the SEM, Control and Rebound plates, as well as 6 specimens from the Direct plates, were taken with an Olympus DP 70 digital camera attached to a Leica MZ FLIII stereo dissecting microscope.

2.4 Incubators

Control plates were kept in an acrylic desiccator cabinet (Nalgene) within a larger diurnal incubator (Thermo low temperature illuminated incubator 818) maintained at 25 °C (±0.5) under a 12 h light-dark cycle. The cabinet was aerated daily by opening and closing the door repeatedly for 40 s. Direct and Rebound plates were kept in a DigiTherm® 38-l Heating/Cooling Incubator with an integrated circadian lighting

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system (Tritech Research) at 25 °C (±1.8) under a 12 h light-dark cycle. Since the standard light source of the incubator was insufficient for our purposes, the intensity of PAR (photosynthetic active radiation) was enhanced by adding a 14 watt 6700 K full-spectrum aquarium light and a combination of mirrors to focus the light toward specimens. Elevated atmospheric pCO_2 was maintained using a feedback controlled infrared CO_2-sensor (Biospherix ProCO_2 system; ±2 % accuracy) that, together with a CO_2 inlet tube, was inserted through a hole in the incubator's side wall. The inlet tube was placed just below an integrated fan to ensure fast mixing of gases. Calibration was performed and checked using a QuBit infrared CO_2 analyzer (QuBit Systems), calibrated with a N_2 gas blank and a standard gas of 1036 ppmv CO_2 in N_2. Pilot experiments, using spectrophotometric pH measurements, (data not shown) established that the pCO_2 of the seawater media equilibrated with the incubator atmosphere within 40 h. A 10 min exposure to ambient conditions, as during the weekly feeding, resulted to an increase in pH of 0.02–0.03 units.

Prior to the start of the experiment, irradiance (PAR) in both incubators were measured using an underwater fluorometer (Diving PAM, Walz Mess-und Regeltechnik). Since the two species require different light intensities, plates containing *A. gibbosa* were covered with nearly opaque white tape on all sides to reduce light availability. Irradiance in the 1000 ppmv experiment was 11 µmol m^{-2} s^{-1} for *A. gibbosa*, which is in the range of optimal light conditions for this species (Walker et al., 2011), and 24 µmol m^{-2} s^{-1} for *A. angulatus*, slightly below the optimal irradiance range for this species (Walker et al., 2011). For the 2000 ppmv experiment, however, irradiance for *A. angulatus* was reduced by also covering the top of the plates with nearly opaque white tape since specimens in the 1000 ppmv (earlier) experiment were overgrown by algae. Thus, *A. angulatus* was kept under less than optimal light conditions (15–18 µmol m^{-2} s^{-1}) to minimize co-occurring algal growth in the 2000 ppmv experiment. All plates were relocated randomly in both incubators throughout the course of each experiment to ensure that no "location effect" took place.

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2.5 Carbonate system parameters

Surface seawater for these experiments was collected in 2008 and 2009 from ~5 m water depth using the uncontaminated seawater intake of an underway research vessel. Due to the small volume of the treatment containers (24-well slides, 1.7 ml per well), seawater for carbonate parameter measurements were kept in 22-ml glass scintillation vials in the control and elevated CO_2 incubator. Samples for total alkalinity (A_T) and dissolved inorganic carbon (C_T) were taken at four time points throughout the course of each experiment (at the end of weeks 1, 2, 4, and 6). Samples were filtered (0.45 µm) and poisoned with 10 µl $HgCl_2$ at the time of collection. Alkalinity samples were stored in glass vials, and C_T samples were sealed in glass ampoules. Alkalinity values were determined by automated Gran titrations of 1 ml samples, standardized using certified reference materials obtained from A. Dickson (Scripps Institution of Oceanography). The relative standard deviation of alkalinity analyses of replicate samples through all experiments averaged ±0.9 % ($n = 39$). Dissolved inorganic carbon concentrations were determined manometrically on 3 to 5 ml samples, using an automated vacuum extraction system. The relative standard deviation of C_T analyses of replicate samples through the experiments averaged ±1.1 % ($n = 47$). Calculations of carbonate system parameters were performed using a spreadsheet version of the CO2SYS program (Lewis and Wallace, 1998) using the total pH scale and the dissociation constants of Hansson and Mehrbach as refit by Dickson and Millero (1987). The impact of evaporation on the 24-well slides was minimized by distilled water additions, but unfortunately this was not done for the vials used for chemical analyses, and the salinity in the vials increased through each experiment. Because we do not think that the foraminifera experienced this increase in salinity, we report only the pH and $\Omega_{c/a}$ values from week 1, because we think that these values reflect most accurately the conditions experienced by the foraminifera. The estimated uncertainty in these values is based on the observed water chemistry variability between timepoints in a separate six-week experiment with temperate benthic foraminifera at 7 °C (data not shown).

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2.6 Survival assessment

To assess survival, specimens were extracted and assayed for ATP content. The nucleotide ATP is an indicator of cellular energy that has been used to quantify benthic foraminiferal populations (e.g., DeLaca, 1986; Bernhard and Reimers, 1991; Bernhard, 1992; Bernhard et al., 2008). Just before ATP extraction, photographs were taken of all individuals from the Control and Rebound plates and about six specimens from the Direct treatment. Only a subset from the Direct treatment was imaged due to the time-dependent nature of the (ATP) response. Within 10–15 min after removal from the incubator, individuals were measured for their diameter (rounded to the nearest 20 µm) using a Nikon SMZ-2B stereo dissecting microscope, and extracted for ATP in 1.0 ml boiling phosphate-citrate buffer (DeLaca, 1986) for 5 min, after which extracts were frozen for later analysis. Specimens of *A. gibbosa* that had reproduced during the experiment were not extracted for ATP because reproduction in foraminifera results in death of the dividing individual. Instead, two offspring per "parent" were measured and extracted. Frozen ATP extracts were thawed and subsequently analyzed using a Berthold Lumat LB 9507 luminometer and luciferin–luciferase reaction per standard protocol (e.g., Bernhard, 1992).

To estimate each individual's test volume, both species were assumed to have the idealized geometric shape of an oblate spheroid. The diameter of each, along with a calculated height to diameter ratio (*h/d*) which was derived from *h/d* estimates from a subset of specimens, was used in the calculations. To account for differences between calculated volume and actual volume, a set of empty tests of both species was photographed and the test areas in two focal planes were calculated using the mathematical equation for an ellipse. Subsequently, areas were also measured with imaging analysis software (Image J; US National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). A comparison of the two sets of measurements revealed that the ellipse equation overestimated the actual volume by 15 % in *A. gibbosa* and 3 % in *A. angulatus*. Final-volume calculations of all specimens were adjusted accordingly.

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Tests of dead foraminifera can house bacteria and microeukaryotes such as flagellates, which can occur in decaying cytoplasm (Bernhard et al., 2010) or living on surfaces of empty tests. These microbes contain ATP and thus provide the rationale for using a live-dead threshold for foraminifera. The live/death threshold of ATP concentration used for deep-sea foraminifera (415 ng mm^{-3}; Bernhard and Reimers, 1991) was adjusted to determine survival in our samples because the ratio of cytoplasm to calcium carbonate in the two reef species is lower than in the deep-sea foraminiferal species for which the threshold was originally developed. This adjustment (to 58 ng mm^{-3}) was established by determining the contribution of calcium carbonate to the overall specimen volume by rehydrating dried specimens of *A. gibbosa* ($n = 7$) and *A. angulatus* ($n = 9$) in DI water. These specimens were soaked in 5 % NaOCl solution for 30 min to remove any cytoplasmic remnants and then rinsed three times in deionized water. Specimens were stored in a solution of sodium borate until analysis, when each wet specimen was weighed with a micro balance (Mettler Toledo MX5; accuracy 0.8 µg). Care was taken to weigh specimens while all chambers were filled with water. Individuals were then dried overnight at 50 °C and reweighed. The mean difference between dry weight and wet weight was 20 % for both species and was considered to be the contribution of cytoplasm to the overall volume.

2.7 Image analysis

The imaging software ImageJ was also used for assessing growth after experiment termination. Cross-sectional areas were measured on images taken before the start of the experiment and at the timepoints and percent increase (i.e., growth) was calculated as (final area – initial area)/(initial area).

2.8 Test microfabric assessment

Specimens used for SEM analysis were rinsed in DI water and air dried on micropaleontology slides. Mounted specimens were sputter coated (Leica EM MED020; 10 nm

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palladium) and imaged using a Zeiss Supra 40VP scanning electron microscope. Initially, all specimens were imaged at various magnifications to determine if any changes in test microstructure could be detected. To quantify the extent of test dissolution in *A. gibbosa*, the penultimate and antepenultimate chamber of each adult individual was imaged at a consistent magnification. The percentage surface area with changed microfabric was determined using ImageJ. Between 4 and 6 specimens were analyzed for each treatment and timepoint. Several whole specimens of *A. gibbosa* offspring were also imaged and analyzed. None of the *A. angulatus* specimens from the 2000 ppmv treatment dedicated for SEM analysis precipitated calcium carbonate and, thus, an analysis of test microfabric of newly precipitated calcium carbonate was not possible for this species.

2.9 Loss of symbiont color

Symbiont color loss was assessed by comparing the proportion of olive-green color (representing symbionts), compared with white areas devoid of color (e.g., Talge and Hallock, 2003), at the experiment start to that percent at time points 1 and 6. Images were taken before the start of the experiment and just before ATP extraction or SEM processing (see above). Images were only used when specimens were oriented in the same way (i.e., umbilical or spiral side up) at the start of the experiment and at the time point. Degree of color loss was assessed using the following index: 4 = 75–100 % of test olive-green in color; 3 = 50–74 %; 2 = 25–49 % and 1 = 0–24 %.

3 Results

3.1 Carbonate system parameters

Culture pH and calcite and aragonite saturation states decreased with increasing pCO_2, but the seawater remained saturated with respect to calcite and aragonite in all three pCO_2 conditions (Fig. 1; Table 1).

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3.2 Survivorship and ATP concentrations

Survival rates for *A. gibbosa* were high in all three treatments in both experiments. There was no obvious or consistent pattern to survivorship (Fig. 2) and differences in survival after one week (χ^2 test, $p = 0.077$) and six weeks were not significant (χ^2 test, $p = 0.067$). ATP concentrations were comparable in both experiments, ranging from 100 to 239 ng mm^{-3} in the 1000 ppmv experiment and 133–239 ng mm^{-3} in the 2000 ppmv experiment, with no pattern related to time or treatment (Fig. 2). While no true replication of treatments was performed due to logistical constraints, the lack of a pattern when comparing results from the 1000 ppmv and 2000 ppmv experiment suggest that survival was not significantly affected by elevated pCO_2 .

For *A. angulatus*, only results from the 2000 ppmv experiment are presented because specimens in the 1000 ppmv experiment were overgrown with algae after only one week. In the 2000 ppmv experiment, survivorship was very low after Week 1 in all three treatments (4 % in Control, 8 % in both Direct and Rebound). However, while survivorship remained at 4 % in the Control treatment after Week 6, survivorship rates were significantly higher in the Direct (42 %) and Rebound (43 %) (χ^2 test, $p = 0.036$; Fig. 3) treatments. ATP concentrations of survivors in Direct and Rebound ranged from 66 to 109 ng mm^{-3} (Fig. 3). The ATP content of the sole survivor in the Control plates after Week 1 and Week 6 was 163 ng mm^{-3} and 59 ng mm^{-3}, respectively. The high survivorship in Direct and Rebound treatments at Week 6 may be an artifact of small sample sizes, or of location within the incubator, resulting in different light and/or temperature conditions for the Week 1 plates.

3.3 Growth

Growth in *A. gibbosa* was comparable in all three treatments. Mean growth in *A. angulatus* in the Control treatment was 55 % (±9.2 %) after 6 weeks and only 3 % (±1.1) in the 2000 ppmv after the same period. No statistical tests were performed on any of these results due to the varied survivorship (n) in individual plates.

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3.4 Reproduction

Asexual reproduction occurred only in *A. gibbosa*. In the 2000 ppmv experiment, mean yield (number of offspring per parent) within each of three Control plates (Control Week 1 and Week 6, Control SEM) in which reproduction occurred, were 138.6 (±32.4), 37.3 (±16.5) and 48.5 (±21.5) (Table 2). Mean yield within each of the three Direct and Rebound plates (Direct Week 6, Direct SEM week 6, Rebound week 6) in which reproduction occurred were 52.5 (±7), 24.5 (±0.5) and 41.3 (±13.7). Although the grand-mean yield of the three Control plates was higher than that of the 6 Direct and Rebound plates (74.8 vs. 39.7), the difference was not significant (Two-sample Kolmogorov Smirnoff test; Quinn and Keough, 2006).

3.5 Test microfabric

In *A. gibbosa*, alterations in test microfabric were visible as areas of dissolved calcium carbonate (Fig. 4). All imaged specimens contained cytoplasm and symbionts and thus were considered to be alive at the time of processing. Patterns of test dissolution in these specimens were distinctly different than patterns of test disintegration in specimens which had reproduced during the experiment (Fig. 5). In live specimens, the test surface was dissolved only in small, well defined patches that appeared to be distributed randomly over the whole test surface. In contrast, in dead specimens, the entire test surface appeared to dissolve, resulting in delamination of layers of calcium carbonate. The percent of dissolved area increased with decreasing Ω_c (increasing pCO_2; Fig. 6). No alterations were observed in any of the adult Control specimens or in adult specimens from elevated pCO_2 after Week 1. After 6 weeks, however, adult specimens from both elevated pCO_2 levels exhibited test dissolution, with larger areas affected in specimens exposed to 2000 ppmv CO_2 (11.5 %, ±4.8, $\Omega_c = 1.7$) than those exposed to 1000 ppmv CO_2 (1.0 %, ±0.7, $\Omega_c = 2.8$). In offspring, test microfabric alterations were only observed in specimens incubated in 2000 ppmv CO_2 after 6 weeks (Fig. 7).

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3.6 Loss of symbiont color

Patterns of color loss in *A. gibbosa* were similar in both experiments. At the start of the experiments the mean color index was 4 (no visible symbiont loss). In the Control treatments, the color index remained at 4 after one week and declined to 3 after six weeks. In Direct and Rebound treatments the index decreased to 3 after week 1 and to 2 in week 6. Due to the lack of suitable images no index was assigned to any specimens from the 2000 ppmv direct treatment after 6 weeks.

4 Discussion

The impacts of elevated pCO_2 on *A. gibbosa* and *A. angulatus* were varied and few negative effects were observed (Table 3).

4.1 *Amphistegina gibbosa*

In our 6-week experiments, the survival of *A. gibbosa* was not significantly affected by elevated pCO_2 and concomitant decreases in pH and Ω_c. The similarity in ATP concentration of surviving specimens across treatments, pCO_2, and time points further indicate that pCO_2 did not affect the level of cellular energy as a measure of health of *A. gibbosa* (Fig. 2). Since specimens were maintained in static conditions over a relatively long time period, ammonia from excretion and mineralization of organic detritus like unconsumed food could have accumulated, causing a general decrease in survivorship (e.g., Lin and Chen, 2003), but this potential impact should have affected all treatments similarly. Also, the reproductive yield was not significantly affected by increased pCO_2. Although elevated pCO_2 did not affect *A. gibbosa* survival directly in our experiments, it may have indirect effects on survival over longer time periods, or in the field, by affecting both the extent of symbiont loss and the test microfabric.

Symbiont loss was evident in all experimental conditions, with the most loss occurring in the elevated pCO_2 treatments. When the symbionts of *A. gibbosa* are damaged,

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the host may digest their symbionts (Talge and Hallock, 1995). Digestion resulting in breakdown of symbionts, together with subsequent breakdown of foraminiferal cytoplasm, resulting in partial to nearly complete loss of golden-brown to olive-green color, has been termed bleaching (Talge and Hallock, 1995, 2003). Bleaching due to photic stress has been common among *A. gibbosa* field populations since 1991 (e.g., Hallock et al., 1995; Hallock, 2000) and can be induced in experimental treatments by above optimal light intensities (Hallock et al., 1986b; Talge and Hallock, 2003; Williams and Hallock, 2004). Bleaching in field populations can result in reproductive failure (Hallock et al., 1995), increased susceptibility to predation (Hallock and Talge, 1994), and shell breakage (Toler and Hallock, 1998). Ocean acidification contributes to photo-oxidative stress, which is the primary cause of bleaching (Tsimilli-Michael et al., 1998; Wooldrige, 2009), thereby increasing pressure on already stressed *A. gibbosa* populations.

The patterns of test degradation in live specimens differed from those of empty tests of specimens that reproduced in culture. In the dead specimens, dissolution caused exfoliation of the surface and exposure of the underlying crystalline structure, a typical taphonomic mechanism of test degradation (e.g., Cottey and Hallock, 1988; Berkeley et al., 2009). In contrast, in live specimens, patches of dissolution were relatively small and well defined, and, although the extent of dissolution was only estimated on the antepenultimate chamber of each specimen, the dissolution appeared to be distributed over the whole test surface without any discernible pattern. The inverse relationship between the extent of dissolution and the calcite saturation state of the seawater suggests that increased pCO_2 was the cause of dissolution, even though water in all three pCO_2 conditions was supersaturated with respect to calcite.

Nienhuis et al. (2010) found that the calcitic shells of intertidal snails dissolved in experiments with calcite saturation states greater than 1 and that the shell dissolution rate increased roughly linearly with increasing CO_2 levels, while shell deposition rates did not change. The fact that dissolution was not observed on the entire test surface of live *A. gibbosa* may reflect partial protection of the test surface by the foraminifer's reticulopodial network, which extend over the foraminiferan test surface (Travis and Bowser,

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1991) and may isolate the test surface from direct contact with the surrounding seawater. Test surface dissolution did not affect the survival and fitness (as measured) of *A. gibbosa*, but the longer-term ecological consequences of partial test dissolution have yet to be established. Dissolution of non-growing shell regions can result in structural weakness (e.g., McDonald et al., 2009) and, thus, could make *A. gibbosa* more susceptible to predation. Tests of offspring produced in 2000 ppmv pCO_2 were affected by dissolution in a similar manner as adults. Enhanced susceptibility to predation of juvenile *A. gibbosa* could lead to a decline in population sizes because fewer specimens reach reproductive maturity.

Asexual reproduction by *A. gibbosa* in all three pCO_2 treatments could mean one of two things: that experimental conditions were favorable for the foraminifera, or that reproduction was a stress response. In general, *A. gibbosa* are amenable to culture conditions and reproduce regularly in culture (Hallock et al., 1986b, 1995). In our experiments reproductive yield did not differ significantly between specimens exposed to ambient and elevated pCO_2, but the lack of statistical significance might have been due to the low number of replicates available for analysis. Whether or not reproduction was a stress response is difficult to assess. For *A. gibbosa,* coiling ratio in the asexual brood has been suggested to be an indicator for stress in the parent (Hallock and Larsen, 1979; Hallock, 1988). Here, the coiling ratios in the broods were similar in ambient and elevated pCO_2 treatments (data not shown). Elevated incidences of juvenile deformity have previously been observed in offspring of severely stressed *A. gibbosa* field populations (Hallock et al., 1995). Here, the ratio of "normal" to "abnormal" offspring did not differ between treatments. Although twinning was only observed in offspring from 2000 ppmv CO_2, there were too few twins overall to influence the ratios of abnormal to normal morphologies (data not shown). Experiments with more specimens, solely focused on determining the reproductive yield of *A. gibbosa* , are necessary to determine if reproduction was a stress response and to establish if offspring in seawater exposed to elevated pCO_2 are able to complete their life cycle successfully.

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4.2 *Archaias angulatus*

Survival of *A. angulatus* after 6 weeks was significantly higher in the elevated pCO_2 treatments than in the ambient control conditions, suggesting that *A. angulatus* can tolerate elevated pCO_2, although our sample sizes were small. Regardless, our experiment suggests that survival by this species was not negatively affected by elevated pCO_2. Due to the small sample size and lack of true replicate dishes, this possible adaptation requires further study. However, *A. angulatus* thrives in areas of relatively high organic productivity, including the mixed macroalgae-seagrass beds from which they were collected for this study, and the site studied by Hallock et al. (1986a) and Souder et al. (2010). In such environments the diurnal fluctuation of pCO_2 can be substantial, with dissolution occurring at night (Yates and Halley, 2006a, b). Moreover, although *A. angulatus* and its algal symbionts can survive elevated pCO_2, this benefit may be offset by the apparent reduction in calcium carbonate production, as indicated by lack of growth in the treatment specimens. In the elevated 2000 ppmv pCO_2, very little calcium carbonate was deposited. This is not surprising since the aragonite saturation state in those treatments was below 1 and *A. angulatus* produces tests consisting of high-Mg calcite, which is more soluble than either aragonite or calcite (Andersson et al., 2008). Reduced growth rates have been observed in the high-Mg test producing, symbiont-bearing benthic foraminifera, *Marginopora kujakajimensis* (pH 7.9 and 7.7; Kuroyanagi et al., 2009) and *A. hemprichii*, *B. sphaerulata*, and *C. gaudichaudii* (pCO_2 = 970 µatm; Fujita et al., 2011).

5 Summary and conclusions

Our results suggest that fitness, as measured by ATP content, and survival of *A. gibbosa* and *A. angulatus* in culture are not directly affected by elevated pCO_2 and the concomitant decrease in pH and calcite/aragonite saturation states of the seawater. A range of non-lethal effects of ocean acidification was observed, including partial

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dissolution and symbiont loss, that differed between the two species. The ecological consequences of these non-lethal effects have yet to be established on the scale of generations.

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Table 1. Calculated pH, Ω_c, and Ω_a and standard errors in seawater exposed to 385 ppmv, 1000 ppmv, and 2000 ppmv pCO_2, respectively. Since only single values were used for each parameter in the present experiment, standard errors (SE) were calculated as an estimate of uncertainty, using percent standard error obtained in a different set of experiments ("temperate experiments") with a similar experimental design.

		Present experiments value (±SE)
pH_T	385 ppmv pCO_2	8.14 (0.06)
	1000 ppmv pCO_2	7.88 (0.02)
	2000 ppmv pCO_2	7.52 (0.05)
Ω_c	385 ppmv pCO_2	5.25 (0.57)
	1000 ppmv pCO_2	3.30 (0.15)
	2000 ppmv pCO_2	1.46 (0.09)
Ω_a	385 ppmv pCO_2	3.47 (0.36)
	1000 ppmv pCO_2	2.18 (0.10)
	2000 ppmv pCO_2	0.96 (0.06)

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Table 2. Number of *A. gibbosa* offspring per parent (yield) in each of the 7 plates exposed to 385 ppmv pCO_2 and 2000 ppmv pCO_2 for up to six weeks in which reproduction occurred.

385 ppmv pCO_2			2000 ppmv pCO_2			
Wk 1	Wk 6	SEM	Wk 1	Wk 6	SEM	Rebound Wk 6
27	153	17	34	73	67	200
70	109	70		49	25	75
	41	25		56		25
	240			24		26
	150			45		34
	29					35
						25
						75

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Table 3. Summary of effects of elevated atmospheric pCO_2 on *A. gibbosa* and *A. angulatus*. nc = no change; neg = negativ effect; pos = positiv effect; na = not available (see text).

	A. gibbosa		*A. angulatus*	
	1000 ppmv pCO_2	2000 ppmv pCO_2	1000 ppmv pCO_2	2000 ppmv pCO_2
Fitness	nc	nc	na	nc
Survival	nc	nc	na	nc[1], pos[2]
Growth	nc	nc	na	neg
Dissolution	neg	neg	na	na

[1] no change after 1 week; [2] increased survival could be an artifact of small sample size, as noted in the text.

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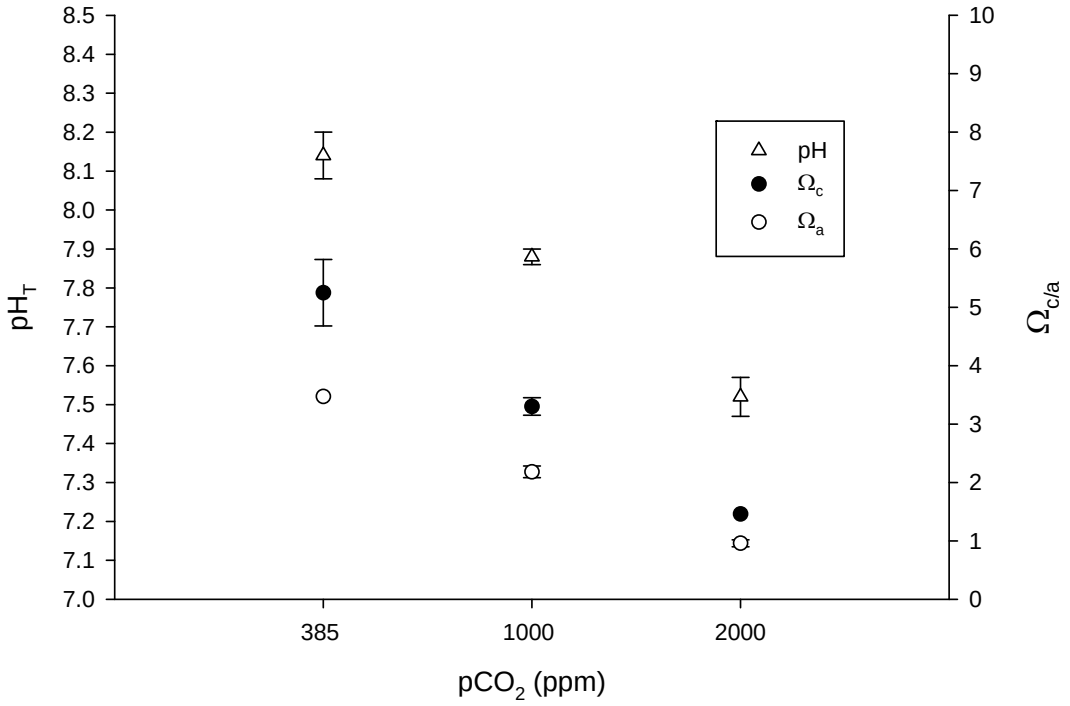

Fig. 1. Calculated pH_T, Ω_c, and Ω_a in seawater exposed to 385 ppmv, 1000 ppmv, and 2000 ppmv pCO_2, respectively. Error bars were calculated using percent standard error estimates of four sampling points over a 6-week period in a related experiment.

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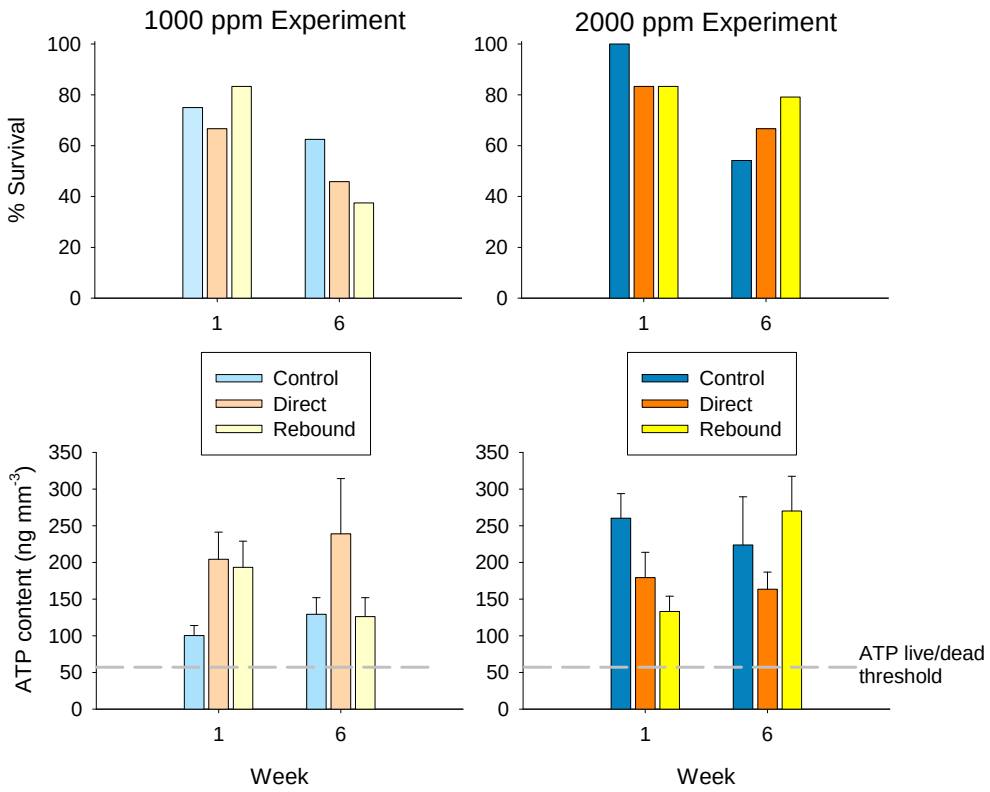

Fig. 2. Percent survival and ATP concentrations of surviving *A. gibbosa* in experiments using 385 ppmv and 1000 ppmv, and 385 ppmv and 2000 ppmv pCO_2.

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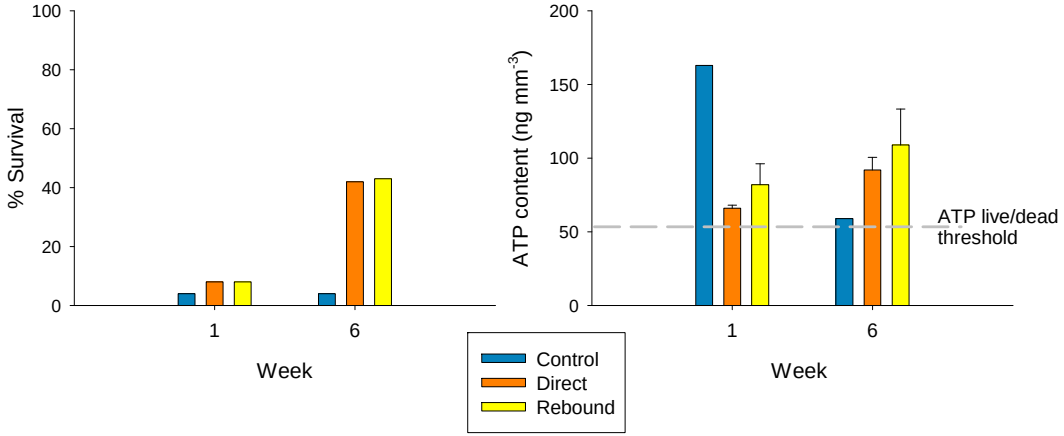

Fig. 3. Survival and ATP concentrations of surviving *A. angulatus* in seawater exposed to 385 ppmv and 2000 ppmv pCO_2.

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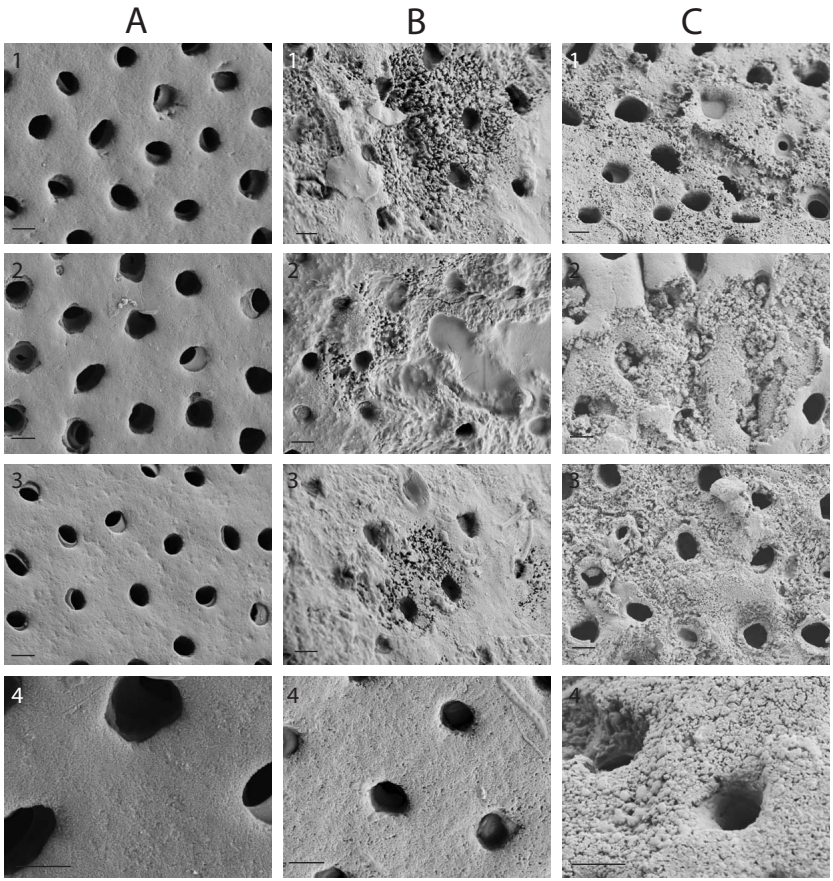

Fig. 4. SEM micrographs of adult *A. gibbosa* kept at 385 ppmv pCO_2 **(A)**, 1000 ppmv pCO_2 **(B)** and 2000 ppmv pCO_2 **(C)** for six weeks. Note the pristine texture in **(A)** versus the degraded texture in **(B)** and **(C)**. Scale bars: 2 µm.

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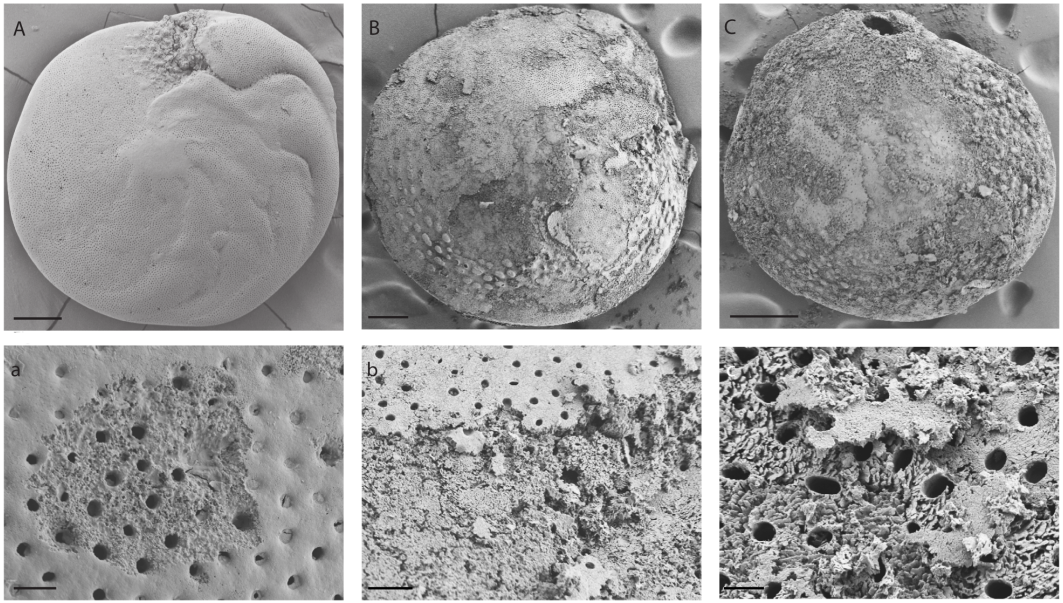

Fig. 5. SEM micrographs of *A. gibbosa* specimens that reproduced (and thus "died") under ambient pCO_2 (385 ppmv; **A**, **a**) and elevated pCO_2 (2000 ppmv; **B**, **b**) conditions. Specimens were dead for several weeks (385 ppmv) and one week (2000 ppmv pCO_2) before being harvested. Scale bars: **A–C** = 100 µm, **a**, **c** = 4 µm, **b** = 10 µm.

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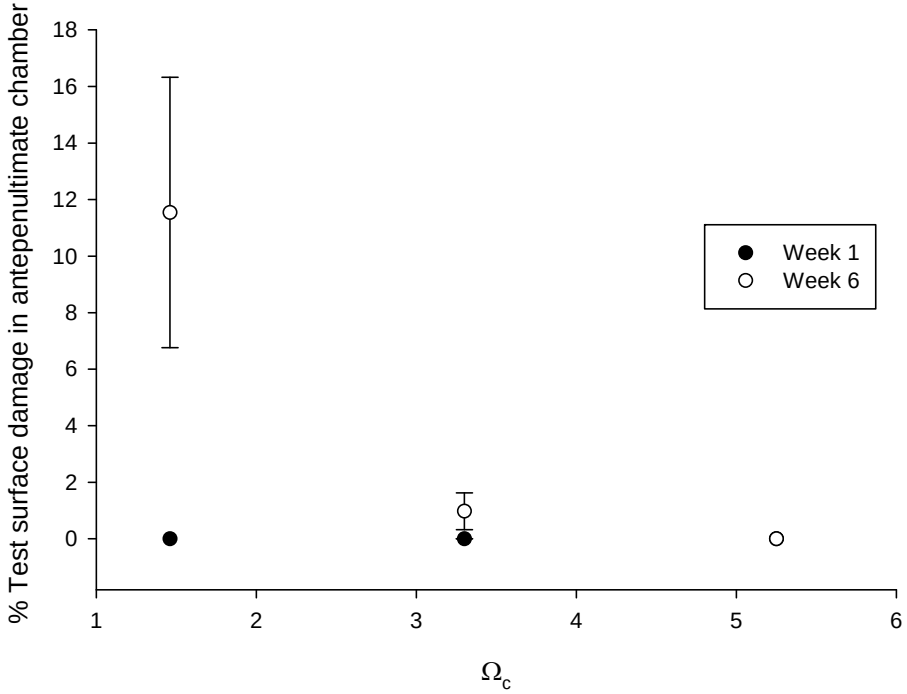

Fig. 6. Percent damage to tests of adult *A. gibbosa* tests exposed to one of three different calcite saturation states and harvested after 1 and 6 weeks. The $\Omega_c = 5.25$ data for week 1 and week 6 superimpose.

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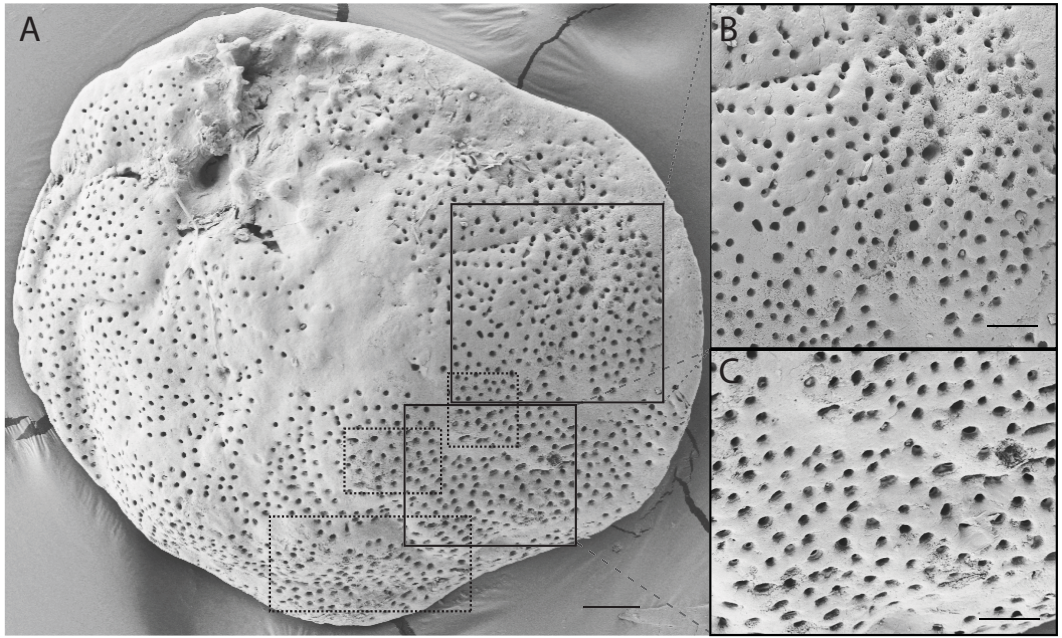

Fig. 7. *A. gibbosa* offspring produced in week 5, grown in 2000 ppmv pCO_2 and harvested after 6 weeks. Boxes indicate approximate areas of mild test dissolution; two are shown as enlargements **(B, C)**. Arrows point to test aperture. Scale bars: **A** = 30 µm, **B**, **C** = 10 µm.

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