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A gender bias in the calcification response to ocean acidification

M. Holcomb[1,*], **A. L. Cohen**[1], **and D. C. McCorkle**[1]

[1]Dept. Marine Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA
[*]current at: Centre Scientifique de Monaco, Avenue Saint Martin, 98000 Monaco-Ville, Monaco

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Correspondence to: M. Holcomb (mholcomb3051@gmail.com)

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Abstract

The effects of nutrients and pCO_2 on zooxanthellate and azooxanthellate colonies of the temperate scleractinian coral *Astrangia poculata* (Ellis and Solander, 1786) were investigated at two different temperatures (16 °C and 24 °C). Corals exposed to elevated pCO_2 tended to have lower relative calcification rates, as estimated from changes in buoyant weights. No nutrient effect was observed. At 16 °C, gamete release was not observed, and no gender differences in calcification rate were observed. However, corals grown at 24 °C spawned repeatedly and male and female corals exhibited two different growth rate patterns. Female corals grown at 24 °C and exposed to CO_2 had calcification rates 39 % lower than females grown at ambient CO_2, while males showed only a 5 % decline in calcification under elevated CO_2. At 16 °C, female and male corals showed similar reductions in calcification rates in response to elevated CO_2 (15 % and 19 % respectively). At 24 °C, corals spawned repeatedly, while no spawning was observed at 16 °C. The increased sensitivity of females to elevated pCO_2 may reflect a greater investment of energy in reproduction (egg production) relative to males (sperm production). These results suggest that both gender and spawning are important factors in determining the sensitivity of corals to ocean acidification and their inclusion in future research may be critical to predicting how the population structures of marine calcifiers will change in response to ocean acidification.

1 Introduction

Rising levels of CO_2 in the atmosphere are lowering the pH and carbonate ion concentration of the oceans, a process called "ocean acidification". These changes have fueled concern for the survival of many forms of marine life, in particular, marine calcifiers which incorporate carbonate ions into their skeletons (e.g. Feely et al., 2004; Orr et al., 2005; Hoegh-Guldberg et al., 2007). To date, many experimental investigations of the potential impacts of ocean acidification have focused on short-term calcification

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responses to decreased seawater pH and saturation state (e.g. Langdon and Atkinson, 2005; Orr et al., 2005). Experimental investigations have established that calcification by many marine organisms declines with decreasing seawater saturation state (Ω) associated with elevated pCO_2 (Langdon and Atkinson, 2005; Orr et al., 2005; Albright et al., 2008; Ries et al., 2009; Cohen et al., 2009).Nevertheless, the sensitivity to Ω differs substantially both within and between species (Ries et al., 2009; Holcomb et al., 2010), limiting our ability to predict how communities and ecosystems will respond to ocean acidification. Predicting the full impact of ocean acidification will require not just knowledge of short-term calcification responses, but an understanding of how multiple factors interact to affect the growth of an organism, how organism responses to these interacting factors change over seasonal cycles and life stages, and how ocean acidification affects interactions amongst organisms.

Here, the interactions of ocean acidification with a subset of potentially important factors are explored. This study investigated how nutrients and CO_2 interact to affect calcification in both zooxanthellate and azooxanthellate corals of the same species at different temperatures. However, previously unrecognized variables – gender and spawning – proved to be important factors which significantly affected the calcification response to ocean acidification.

2 Methods

2.1 Coral collection

Eight live colonies of the facultatively symbiotic, gonochoric coral *Astrangia poculata* were collected from the Woods Hole Oceanographic Institution pier (Great Harbor, Woods Hole, Massachusetts, USA). Four azooxanthellate colonies were collected at a depth of 2–3 m on 19 September 2008 at sites far from the edge of the pier (and thus never exposed to direct sunlight). Four zooxanthellate colonies were collected at a depth of 3–6 m on 25 September 2008 from pilings on the outer edge of the

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pier (exposed to sunlight). The temperature at the time of collection (recorded by a Hobo Pendant logger) was 20.4 °C for axoozanthellate corals, 19.2 °C for zooxanthellate colonies. Light levels (recorded by a Hobo Pendant logger) ranged from 1000–4000 lux (near mid-day) at the sites of collection of zooxanthellate colonies.

2.1.1 Initial processing

On the day of collection corals were returned to laboratory (in seawater), fragmented with wire cutters, and visibly bored regions, worms, crabs, sponges and other organisms were removed. Once cleaned and trimmed to size, each fragment was placed into a plastic cup suspended in seawater below a balance and weighed (see buoyant weight procedure below). Nine fragments from each colony were attached to individual acrylic slides with cyanoacrylate adhesive (EZ bond). Each acrylic slide measured ~2.5 cm by ~5 cm by 0.24 cm thick, with blue acrylic pieces attached (using acrylic solvent cement) near each corner to provide orientation reference points. Each slide had been engraved with a unique identifier, weighed, and soaked repeatedly in both seawater and freshwater prior to use. Once attached to slides, the cyanoacrylate was allowed to cure for one day before the fragments (with slide) were reweighed and assigned to individual aquariums. At least one fragment from each parent colony was assigned to each aquarium. All aquariums were initially at ambient temperature, nutrient, and pCO_2 conditions. To minimize stress to the coral, corals were handled underwater to the extent possible.

2.2 Aquarium set up

Coral fragments were placed in one of eight flow through aquaria. Each aquarium was housed in one of two temperature controlled baths (ultimately set at 16 and 24 °C), and each aquarium had its own water and air supply. Each aquarium in the 16 °C water bath contained 4 zooxanthellate corals, 4 azooxanthellate corals, and 2 slides without corals. Each aquarium in the 24 °C water bath contained 5 zooxanthellate corals, 5

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azooxanthellate corals, and 2 slides without corals. The experiment was carried out in four steps – an initial acclimation phase during which corals were gradually transitioned to experimental temperatures, a pre-treatment phase in which base line growth rates were established for each coral at the treatment temperature, a second acclimation phase during which corals were gradually transitioned to experimental nutrient and CO_2 levels, and a final treatment phase during which corals were maintained under the desired treatment conditions. The details of each component of the system and treatment conditions are detailed below.

2.2.1 Water supply

All experiments were carried out at the Environmental Systems Laboratory at WHOI. The laboratory is supplied with running seawater drawn at a depth of ~4 m from ~200 m off shore in Vineyard Sound. Seawater is pumped through one of two lines alternated periodically to limit the growth of fouling organisms within the lines, passed through a sand filter and pumped into a header tank. From the header tank, seawater is supplied to ambient, 10 °C, 14 °C, and 20 °C distribution lines which provide seawater throughout the facility. Water for the temperature controlled lines passes through titanium heat exchangers, and for the 14 °C, and 20 °C lines, water passes through gas exchange columns prior to entering the distribution system.

Coral fragments were maintained in a flow-through aquarium system. Two 450 l reservoirs were filled daily with seawater drawn from the 10 °C, 14 °C, 20 °C, or ambient seawater lines depending on the season – chilled water was used during the summer while heated water was used during the winter which helped to improve temperature stability in the aquarium system. Seawater passed through a 20 µm cartridge filter prior to entering the reservoirs, and water from each reservoir was continuously pumped to header tanks. From each header tank, water fed into gas mixing chambers to equilibrate the water with air or a CO_2/air mixture, and from there through valve manifolds used to deliver water to each aquarium. Water lines for each aquaria were coiled within the water bath holding the given aquarium to allow temperature equilibration at

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the experimental temperature; and just prior to entering each aquarium, water passed through a small gas mixing chamber held within the water bath to allow for partial CO_2 equilibration at the experimental temperature. Turnover rates were ~once per hour for all aquariums. Slightly lower flow rates were used for the 16 °C aquariums than the 24 °C aquariums to improve temperature stability.

2.2.2 Aquaria

Individual aquaria consisted of 43 × 5.5 cm polyethylene terephthalate copolyester (PETG) containers filled to ~8 cm depth. Half of each aquarium was exposed to light (for zooxanthellate corals), while the other half was kept dark (for azooxanthellate corals). The exterior of each aquarium was colored black to reduce stray light. Each aquarium was divided into two sections by a piece of opaque blue acrylic placed ~21.5 cm from the end and ~2 cm from the bottom and continuing to the surface. Aquariums were covered with PETG lids, one half of each lid was covered with blue acrylic to block light from entering the dark half of the aquarium. The darkened section in each aquarium was added following Holcomb et al. (2010) to ensure azooxanthellate colonies remained azooxanthellate over the course of the experiment. Each aquarium had two water inlets, three air inlets and two water outlets. Water inlets and outlets were placed at each end of the aquarium and the flow direction altered every ~10 d such that half the time water entered at the front of the aquarium and exited at the back, while the remainder of the time water entered at the back and exited at the front. Air inlets supplied air to aquarium air-stones placed at the front, middle, and back of each aquarium. A total of 8 aquaria were used, four in each of two temperature controlled water baths.

2.2.3 Temperature

Following a gradual (~4 °C month^{-1}) transition from ambient to experimental temperatures, one water bath was set at 16 °C, the other at 24 °C, near the seasonal

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maximum temperature in Great Harbor, MA. At this time, calcification and photosynthesis rates are expected to be at their highest values and photosynthesis in zooxanthellate colonies is expected to enhance growth relative to that of azooxanthellate colonies (e.g. Cummings, 1983; Jacques and Pilson, 1983). At 16 °C, the contribution of photosynthesis is expected to be considerably reduced, and growth rates of both symbiotic and asymbiotic colonies are expected to be similar (e.g. Cummings, 1983; Jacques and Pilson, 1983).

Temperature stability within each water bath was monitored using digital thermometers (VWR) to record daily minimum and maximum temperatures. Periodic manual checks were also made with a mercury thermometer (calibration checked against a Super-Thermometer II (Hart Scientific) and high precision temperature bath maintained by the WHOI CTD facility; a mercury thermometer was also used to check temperatures in individual aquaria). In addition temperature loggers recorded temperature every 12 min (U12 logger, TMC-HD probe, Onset Inc., data shown in Fig. S1). The total temperature variation was 0.8 °C about the set point (except for a brief period (<2 h) when the 24 °C bath was emptied for maintenance and the temperature dropped by ~1 °C) with typical daily variations of 0.15 °C for the 24 °C bath and 0.5 °C for the 16 °C bath. Each water bath was heated with aquarium heaters controlled by an Omega CN1504 controller with thermistor temperature probes, thermo-electric chillers (Ice-probe, Coolworks) were used to chill the 24 °C temperature bath, and a compressor based chiller (Prime, Current USA) used for the 16 °C bath. Aquarium power-heads were used to continually circulate water within each bath.

2.2.4 Lighting

Lighting was provided by two T5-HO bulbs (10 000 K bulbs, 48″ 54 w), on a 12 h light/dark cycle. All aquaria were positioned at the same distance from the bulbs, and the distance was chosen such that a Hobo pendant logger recorded ~1500 lux at the depth of the corals. However, light output varied along the length of the bulbs, so aquaria near the center of the bulbs received more light than those toward the end. The

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average irradiance (PAR, measured with the underwater quantum sensor of a diving-PAM (WALZ) at the depth of the corals at the end of the experiment) ranged from 15–24 µmol photons m^{-2} s^{-1}; within aquarium variability was ~ 7µmol photons m^{-2} s^{-1} based on measurements taken at the front, middle, and back of the lit section of each aquarium. Light levels were not measurable in the dark section of each aquarium.

2.2.5 Feeding

Each day freshly hatched brine shrimp (hatched from Ocean Star International brand eggs) were concentrated in a 150 µm sieve, mixed with filtered seawater and allowed to concentrate near a light source. Shrimp were added through two holes in the lid of each aquarium, one hole was on each side of the divider at the center of each aquarium. Half the aliquot of shrimp was added through each hole. A total of 6ml of shrimp suspension was added to the 16 °C aquaria, 9 ml to the 24 °C aquaria (more food was provided to the 24 °C corals to partially compensate for the higher metabolic rates (Jacques and Pilson, 1983) and greater coral densities). Periodically, 1 ml samples of shrimp suspension were taken, filtered through a pre combusted (10 h at 475 °C), pre-weighed 0.7 µm glass fiber filter (Fisher), rinsed briefly with distilled water, dried at 60 °C for 1 day and ashed at 450 °C for 4 h. The average ash free dry weight of the shrimp suspension was 5.8 mg ml^{-1} (standard deviation: 1.5 mg, $N = 14$).

2.2.6 Nutrients

After an initial phase of eight months (during which base line growth rates were established at each temperature), nutrient and CO_2 levels were gradually elevated in a subset of aquariums to reach treatment values. During the treatment phase of the experiment, nutrient levels were elevated by adding $NaNO_3$, K_2HPO_4, and $FeCl_2$ stock solutions at a ratio of 2 µl stock solution l^{-1} seawater to one of the reservoirs to increase concentrations of NO_3^-, PO_4^{3-}, and Fe^{2+} by 5 µmol l^{-1}, 0.3 µmol l^{-1} and 2 nmol l^{-1} (per Holcomb et al., 2010). Measured values were: 0.31 ± 0.04 µmol l^{-1} NH_4,

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$1.6 \pm 0.5\,\mu mol\,l^{-1}$ NO_3, $0.15 \pm 0.24\,\mu mol\,l^{-1}$ PO_4 in ambient seawater prior to the start of treatments, $0.6 \pm 0.5\,\mu mol\,l^{-1}$ NH_4, $3.3 \pm 0.8\,\mu mol\,l^{-1}$ NO_3, $0.6 \pm 0.1\,\mu mol\,l^{-1}$ PO_4 for ambient seawater during treatment, and $0.3 \pm 0.2\,\mu mol\,l^{-1}$ NH_4, $8.7 \pm 1.2\,\mu mol\,l^{-1}$ NO_3, $0.9 \pm 0.1\,\mu mol\,l^{-1}$ PO_4 for nutrient treated seawater. Nutrient levels were monitored both within reservoirs and within individual aquariums by taking water samples every ~10 days throughout the experiment (see Table 1 for average values and Fig. S1 for plots). Each sample was filtered through a syringe filter (0.45 µm) into high density polyethylene (HDPE) scintillation vials with foamed polyethylene lid liners (Wheaton) and stored frozen until measured. Vials used for nutrient sampling were soaked for over 12 h in distilled water, rinsed repeatedly and air dried prior to use; ~15 ml of sample was used to rinse the syringe filter prior to sample collection. Samples were analyzed for ammonium, silicate, phosphate, and nitrate/nitrite (expressed as NO_3) by the WHOI Nutrient Analytical Facility using a Lachat Instruments QuickChem 8000 flow injection system.

2.2.7 CO_2

To establish experimental CO_2 enrichments CO_2 levels were increased gradually over the course of one month for one set of gas mixing chambers and their corresponding aquaria using mixtures of ambient air and CO_2. Ambient air was provided by a Porter Cable oil free air-compressor, and passed through mechanical air filters and activated carbon prior to use. Flow rates for CO_2 and air were controlled by rotameters (Alborg Instruments) to provide an air/CO_2 mixture with CO_2 levels ~400 ppm above ambient during the treatment phase. A Qubit s151 CO_2 analyzer and a commercially prepared CO_2 standard (Corp Brothers, certified 1036 ppm CO_2) were used daily to assess the stability of CO_2 levels (Fig. S1). A dial barometer (Fisher) was used to measure atmospheric pressure at the time of each CO_2 measurement. Gas flow rates to all aquaria were ~1 $l\,min^{-1}$.

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2.3 Chemistry

Water samples for nutrients, total alkalinity (A_T), pH, total inorganic carbon (C_T; see Supplement), and salinity were collected every ~10 days near the end of the day. Oxygen was measured occasionally as well using a Hach LDO probe (both during the day and at night, values were always near saturation). Following sampling, aquariums were cleaned of algal growth using a plastic card to scrape algae from the sides of the aquariums and a pipet to remove algae and detritus from the aquarium. Flow directions were then switched and rates adjusted if needed. Temperature, salinity, nutrient, A_T and pH on the total scale (pH_T) values (see Fig. S1) were used to calculate carbonate chemistry for each aquarium before and after the start of treatments (Table 1).

2.3.1 Total alkalinity

Total alkalinity samples were taken in scintillation vials (Wheaton). Samples were stored refrigerated for no more than 1 month prior to measurement (see Supplement for assessment of sample stability). Total alkalinity was measured via titration with 0.01 N HCl containing ~40.7 g NaCl l^{-1} using a Metrohm Titrando 808 dosimat and 730 Sample Changer controlled by Tiamo software to perform automated normalized Gran titrations of 1 ml samples. Certified seawater reference material supplied by the lab of Andrew Dickson (Scripps Institution of Oceanography) was run each time samples were run to determine acid normality. See Supplement for additional details.

2.3.2 pH

Prior to the treatment phase, 14 ml pH samples were collected in 15 ml polypropylene conical tubes (Corning) which had previously been washed repeatedly with fresh water and soaked repeatedly with seawater (to leach out acidic compounds in the plastic). Thymol blue (Fisher lot#990191) was added to each sample (160 or 320 µl of a 2.05 mmol l^{-1} solution). Samples were allowed to temperature equilibrate in a

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water bath prior to transfer to a 1 cm cuvette for absorbance measurements. Spectra were collected using an Ocean Optics USB4000 spectrophotometer with a blue filtered (SCHOTT BG-34 glass) tungsten light source (Ocean Optics LS-1-LL). For each sample, 150 spectra were averaged, seawater from one of the sampled aquariums was used for establishing reference absorbance. pH_T was calculated following Zhang and Byrne (1996) including the correction of Delvalls and Dickson (1998).

During the treatment phase, pH values in the CO_2 treatments declined and m-cresol purple appeared to give more consistent results than thymol blue (TB) for the lower pH values, thus m-cresol purple (mCP Acros 199250050, lot A014923001) was used instead. Initial m-cresol purple measurements were made as described above except dye additions were 50 or 100 µl of a 9.4 mmol l^{-1} solution, but most were made using a 10 cm path length flow cell instead of a cuvette. For measurements with the flow cell, samples were collected in pre-cleaned 10 ml polypropylene syringes (Becton Dickson), and 50 or 100 µl of a 0.946 mmol l^{-1} solution of mCP added. Syringes were capped and placed in a constant temperature bath prior to injection into the flow cell. pH_T calculations followed DOE (1994), code used for processing spectra and calculations is available online (https://sites.google.com/site/coralcalcify/home/matlab-code). pH_T values measured with TB and mCP usually agreed within measurement error, typical reproducibility was 0.015, measurements on standards (tris buffer and seawater (CRM) supplied by the laboratory of Andrew Dickson) were generally within 0.02 of the expected value, expected values for CRM were calculated from A_T and C_T using CO2Sys (van Heuven et al., 2009) using constants from Mehrbach et al. (1973) as refit by Dickson and Millero (1987).

In addition to spectrophotometric measurements, pH (NBS scale) measurements were made on day/night cycles using an Orion Ross 8165BNWP electrode calibrated with NBS buffers (Ricca). Measurements were carried out in a flow cell with water supplied via siphon from a given aquarium. The siphon tube was inserted through the feeding hole, thus the aquarium lids were not opened for electrode based measurements. Daily cycles in pH were ~0.1, with lower values at night (Fig. S1).

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2.3.3 Salinity

Salinity was monitored using a dip type conductivity probe (Hach). Conductivity was converted to salinity values using equations given by Fofonoff (1985). The reliability of the conductivity probe was periodically checked by simultaneously taking samples for measurement with a Guildline Autosal model 8400B salinometer (Figs. S1 and S7). The conductivity probe values were typically within 1 of the Autosal value.

2.4 Buoyant weights

Buoyant weight measurements (e.g. Davies, 1989) were made every 1-2 months. A Sartorius GC803S balance (resolution 0.2 mg, reproducibility of the calculated dry weight for a standard weighing ~7 g was better than 7 mg) with weigh-below hook was used to weigh corals, coral growth rates averaged $3.3 \pm 2\,\mathrm{mg\,day^{-1}}$. Glass and aluminum weights were used to check reproducibility. Temperature was recorded at the time of every measurement and controlled using an Omega cn1504 temperature controller and aquarium heater, chilled seawater (~10 °C) was added as needed to reduce the temperature. Seawater was at ambient conditions for all buoyant weight measurements, only temperature was changed to match the treatment temperature. Salinity was recorded once to twice over the course of each weighing session and used to calculate expected seawater density (Fofonoff, 1985). Mass changes are assumed to reflect changes in the mass of aragonite, an aragonite density of $2.9\,\mathrm{g\,cm^{-3}}$ was used to calculate the dry weight of calcium carbonate deposited. Buoyant weight measurements were corrected for the contribution of the acrylic slide and cyanoacrylate as outlined in the Supplement. The mass of the slide with adhesive was estimated from the buoyant weight change between the initial weighing of the unmounted coral fragment and the mounted weight the following day, coral growth was assumed to be negligible during this interval and thus required only a correction for changes in seawater density.

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2.5 Gender identification

Genders were established either by direct observation of gamete release, or by verifying the presence of sperm in incubations of individual specimens in which the water had become cloudy. Direct observation of spawning was made when corals were weighed. During most weighing sessions, some corals spawned allowing for the gamete type released by individual polyps to be identified. Spawning males were readily identified as specimens tended to release sperm in several pulses providing several opportunities to observe gamete release. Female identification was more difficult as fewer eggs were released, generally in one or two pulses. Only those specimens observed as they were releasing eggs were identified as female. In separate individual incubations carried out for alkalinity depletion and oxygen consumption measurements (not discussed), spawning males often released sperm in sufficient quantity to cloud the water and microscopic examination of water samples from individual incubations which had become cloudy allowed for the identification of sperm and establishment of gender. At 24 °C, the corals spawned repeatedly, allowing identification of colony gender: females released eggs, males released sperm. All fragments from a given parent colony spawned as the same gender and gender did not change over time. No spawning was observed by either male or female corals in the 16 °C aquaria. In these aquariums, gender assignments are based on the gamete type released by fragments from the same parent colony which had been assigned to the 24 °C aquaria.

2.6 Statistics

Pre-treatment growth rates were determined using the weight increase from shortly after treatment temperatures were reached to just prior to the start of treatments (November 2008–June 2009). Treatment growth rates were based on the weight increase from the first set of weights after treatment conditions had been reached to the final set of measurements for the study (July–October 2009). Linear regression (Systat v.9) was used to determine pre-treatment and treatment calcification rates (regression slopes).

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Differences between pre-treatment and treatment rates for each fragment were normalized to pre-treatment rates for that fragment by calculating a relative change in growth rate:

$$\Delta\text{relative rate} = \frac{(\text{growth rate during treatment}) - (\text{pretreatment growth rate})}{(\text{pretreatment growth rate})}$$

See Supplement for discussion of normalization approaches and alternative presentations of the data. Percentage differences between treatments were calculated as (with 1 being the "control" condition (ambient seawater)):

$$\text{percentage difference} = 100 \times \frac{\frac{\text{treatment rate}_1}{\text{pretreatment rate}_1} - \frac{\text{treatment rate}_2}{\text{pretreatment rate}_2}}{\frac{\text{treatment rate}_1}{\text{pretreatment rate}_1}}$$

Data were initially analyzed using a blocked factorial ANOVA model per the experimental design (factors nutrients and CO_2, blocked by parent colony, different temperatures and symbiont statuses analyzed separately) using SAS version 7.2 (Table 2). However, inspection of the raw data (Figs. 2, S9, S10) revealed the presence of two distinct growth patterns which corresponded to gender (at 24 °C), and thus suggested that the initial blocked factorial ANOVA analysis might not be the most appropriate. Unfortunately, insufficient replicates of each gender precluded testing the multi-way interactions with gender in the context of the original experimental design. To increase the number of replicates for testing the effect of gender, data were pooled. Inspection of the raw data suggested males and females behaved differently at 24 °C regardless of symbiont status, and, due to the use of relative growth rate changes, differences between zooxanthellate and azooxanthellate growth rates were not expected (a lack of a significant difference was confirmed using a t-test, $t_{30} = 1.41$, $p = 0.168$ at 16 °C, $t_{38} = -0.98$, $p = 0.33$ at 24 °C), thus data for zooxanthellate and azooxanthellate corals were pooled. Corals grown at 16 °C were analyzed in the same manner for comparison purposes, though there was no obvious gender difference. The effects of gender were tested using the pooled data sets in a nested block factorial ANOVA model (Table 3),

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followed by a blocked factorial model on each gender separately for 24 °C data due to a significant CO_2 gender interaction. In the case of significant treatment effects, Dunnetts procedure was used to determine which treatments significantly ($p < 0.05$) differed from ambient. Residuals from ANOVA analysis were examined using normal probability plots and plotted against predicted values to assess violations of ANOVA assumptions. All data are presented as mean ± standard deviation.

3 Results

3.1 Aquarium conditions

Conditions in all aquariums were similar during the initial phase, and consistent differences between treatment conditions were maintained throughout the treatment period (Table 1, Fig. S1). However, the composition of the source water, pumped from Vineyard Sound, changed between pre-treatment and treatment periods – alkalinity was lower and nutrient levels higher during the treatment period relative to the pre-treatment phase. The rise of ambient nutrient levels during the treatment phase (by ~0.4 µmol l^{-1} for PO_4^{3-}, 1.7 µmol l^{-1} NO_3^- relative to pre-treatment conditions) exceeded intended treatment elevations for PO_4^{3-}, and represented over 1/4 of the intended treatment elevation for NO_3^-. Individual aquariums experienced daily pH variations of ~0.1, with lower values at night, however CO_2 treatments consistently had lower pH values than aquariums not treated with CO_2, and the difference between treatments was greater than the daily cycle (Fig. S1). Values reported in Table 1 are likely to be biased toward lower pCO_2, A_T, and nutrient values, as all water samples were collected near the end of the day shortly before aquariums were cleaned of algal growth, airstones replaced, and water flows adjusted (water flow tended to drop between adjustments). Water flow rates were usually sufficient to maintain water chemistries in each aquarium similar to that of the incoming source water. However valves occasionally became blocked leading to reduced water flow, such time points are characterized by low A_T values (Fig. S1).

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Saturation state variations associated with water flow induced alkalinity variations were generally small relative to treatment differences (Fig. S1).

3.2 Coral growth

Regardless of treatment, all corals showed a net increase in mass over the course of the experiment and mass increased between each weighing for all corals (Figs. S9, S10). Elevated CO_2 levels invariably reduced relative calcification rates compared to non-CO_2 treatments (Fig. 1). However, only for zooxanthellate corals at 24 °C was the effect of CO_2 significant ($p = 0.006$, Table 2). Nutrient enrichment had no significant effect, nor was there a significant interaction between nutrients and CO_2. The source coral colony was significant for both zooxanthellate and azooxanthellate corals at 24 °C ($p = 0.02$), suggesting fragments derived from different colonies had different patterns of growth at 24 °C. Examination of changes in skeletal mass over time suggested two distinct growth rate patterns, one nearly linear with a relatively shallow slope and the other increasing with time (see Fig. 2 for an example, Figs. S9 and S10 for all fragments). Regardless of treatment or zooxanthellate status, the 24 °C experiment yielded two different growth patterns that were linked to the parent colony; fragments from a given colony showed similar growth patterns. Once genders had been established for all coral colonies, the different growth patterns were found to correspond to gender. Females showed slower, more linear growth rates and males showed increasing growth rates with time (Fig. S9). No such pattern was observed at 16 °C; as noted above, corals were not observed to spawn at this temperature.

The correspondence between growth patterns and coral gender indicated that gender should be included as a factor in the data analysis. Since only three female parent colonies in total were present, and these were in-turn divided between the zooxanthellate and azooxanthellate groups, the zooxanthellate and azooxanthellate colonies were pooled. Consistent with visual assessment of the data, gender was highly significant at 24 °C ($p < 0.0001$ Table 3) in the pooled data set, and there was a significant effect of CO_2 ($p = 0.006$) in the pooled dataset. However, there was also a significant

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CO_2 *x* gender interaction ($p = 0.031$). To interpret the effects of CO_2 and nutrients, each gender was analyzed separately. For female corals at 24 °C, relative calcification rates were significantly reduced by elevated pCO_2 ($p = 0.002$), while males were not significantly affected (Fig. 3). Nutrients had no significant effect. At 16 °C, gender had no significant effect, nor did nutrients, but CO_2 was associated with a reduction in calcification ($p = 0.024$).

4 Discussion

Consistent with many studies of the effects of ocean acidification on corals (e.g. Langdon and Atkinson, 2005; Cohen et al., 2009), relative calcification rates were lower in corals exposed to elevated pCO_2 than in corals grown under ambient conditions, regardless of symbiont status or temperature (Fig. 1). However only for the zooxanthellate corals grown at 24 °C was the decline significant. In contrast to previous work with *Astrangia poculata* (Holcomb et al., 2010), there was no indication that elevated nutrient levels offset the effects of ocean acidification on calcification. However, high nutrient levels in the source water during the treatment period (relative to the pre-treatment period) precluded the existence of a true low nutrient control, which could have reduced the potential for further nutrient enrichment to alter the response to CO_2. In addition, feeding rates were higher in the current study than in Holcomb et al. (2010), which may have reduced the relative importance of photosynthesis to the corals energy needs, or reduced the need for inorganic nutrients.

Complicating the interpretation of the CO_2 and nutrient results is the presence of both male and female corals. At 16 °C, gender appears to have no effect on how coral calcification responded to treatments and is not statistically significant (Table 3, Fig. 3). The absence of a gender difference at 16 °C suggests that the gender difference observed at 24 °C is not due to an inherent difference between the corals, but may reflect an interaction of gender with temperature. One potential source of such an

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interaction is gamete production. At 16 °C, no gender difference was observed and corals were not observed to release gametes. This observation is consistent with that of Szmant-Froelich et al. (1980) who suggested that gametogenesis is not completed in *A. poculata* at low temperatures. At 24 °C, however, gamete release was observed on a regular basis and two different growth patterns were observed which appear linked to gender. One way that gamete production could influence calcification rates is by altering resource allocation within the coral, and the degree of alteration may depend on the gamete type (egg vs. sperm) produced.

The metabolic cost of calcification, and more specifically, differences amongst individuals and species in the resources devoted to calcification, has been implicated in the observed variability amongst corals in their response to ocean acidification (Cohen and Holcomb, 2009; Holcomb et al., 2010). If true, this implies that processes or events which deplete an organism's resources, such as reproduction, could increase its sensitivity to the effects of ocean acidification.

A significant portion of a coral's energy budget is devoted to reproduction (Richmond, 1987; Rinkevich, 1989; Leuzinger et al., 2003) and reallocation of resources from skeletal growth to sexual reproduction is thought to lead to a decline in skeletal extension in some coral species (Richmond, 1987; Mendes, 2004). Thus reproduction may change calcification rates in *A. poculata*. In females of some coral species, reproduction is delayed until a larger colony size is reached (Benayahu and Loya, 1986; Chornesky and Peters, 1987), suggesting a trade off between reproduction and growth which is more pronounced for females. Investment in skeletal growth versus tissue growth may be hormonally regulated, as exposure to estrone has been found to reduce skeletal growth rates, and is associated with thicker tissue (Tarrant et al., 2004). Thus, spawning (and associated hormonal changes) may be expected to alter growth rates, and if egg production has a greater cost than sperm production, then females would be expected to have more pronounced declines in their calcification rates. Further, spawning female corals were much more sensitive to CO_2 than their male counterparts (Fig. 3), suggesting that spawning females were not able to compensate for

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reduced saturation states, while males were able to compensate for the less favorable calcification environment.

Our findings highlight the less visible long-term impacts of ocean acidification on population structure, reproduction, and ultimately, species and ecosystem survival. With reduced rates of skeletal growth, females could become more vulnerable than males to the effects of boring organisms, and less able to repair damage caused by parrotfish bites, disease, or storms. Over time, such damage could lead to a shift in gender ratios, as has been found on polluted reefs (Harrison and Wallace, 1990). If this occurred, the decline in the proportion of females within a population could compromise sexual reproduction. These findings are not limited to corals but likely apply to a range of calcifying organisms which make a substantial investment in gamete production, and thus may reduce resource allocation to calcification for some portion of the year. Studies which have not taken gender and spawning into account may have underestimated the long-term sensitivity of calcifying organisms to ocean acidification caused by rising pCO_2.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/8/8485/2011/bgd-8-8485-2011-supplement.zip.

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Table 1. Day-time water chemistry during initial and treatment phases for each aquarium (A), and source water chemistry (samples taken from the reservoirs supplying the aquariums) (B). Average A_T (μmol kg^{-1}), pH_T (value at experimental temperature), aragonite saturation state (Ω_{Arag}), C_T (μmol kg^{-1}), CO_3^{2-} (μmol kg^{-1}), HCO_3^- (μmol kg^{-1}), pCO_2 (ppmv, water saturated air), salinity, NH_4^+, H_2SiO_4, PO_4^{3-}, and NO_3^- (μmol l^{-1}). Measured A_T, temperature, salinity, nutrient, and pH_T values were used to calculate aragonite saturation state and carbon speciation using a Matlab implementation of CO2SYS (van Heuven et al., 2009; using constants from Mehrbach et al., 1973; Dickson and Millero 1987; Dickson 1990) which incorporated the contribution of ammonia (Clegg and Whitfield, 1995). Nutrient values were not measured for all aquariums for all sampling points, average values for the respective period/aquarium were inserted for missing values for carbonate chemistry calculations. Values are means, standard deviation in parenthesis, n.d. = not determined.

A. Initial	A_T	pH_T	Ω_{Arag}	C_T	CO_3^{2-}	HCO_3^-	pCO_2	S	NH_4^+	H_2SiO_4	PO_4^{3-}	NO_3^-
24 °C ambient	2116	7.97	2.53	1897	158	1726	462	32.1	0.97	3.0	0.28	2.55
	(23)	(0.03)	(0.13)	(26)	(8)	(29)	(35)	(0.4)	(0.26)	(2.0)	(0.41)	(2.92)
24 °C nutrient	2112	7.98	2.56	1891	159	1719	452	32.0	0.76	2.9	0.14	0.75
	(14)	(0.03)	(0.14)	(20)	(9)	(26)	(38)	(0.3)	(0.29)	(1.6)	(0.09)	(0.41)
24 °C CO_2	2123	7.96	2.5	1908	156	1739	474	32.0	0.81	3.2	0.14	0.75
	(16)	(0.03)	(0.14)	(19)	(9)	(24)	(38)	(0.3)	(0.19)	(1.4)	(0.07)	(0.35)
24 °C nut & CO_2	2124	7.97	2.52	1908	157	1737	468	32.0	0.68	3.1	0.11	0.98
	(12)	(0.03)	(0.13)	(15)	(8)	(19)	(33)	(0.3)	(0.24)	(1.4)	(0.07)	(0.52)
16 °C ambient	2124	8.03	2.10	1940	135	1790	402	31.1	0.99	3.0	0.18	0.83
	(17)	(0.03)	(0.13)	(16)	(8)	(20)	(31)	(0.4)	(0.45)	(1.0)	(0.13)	(0.24)
16 °C nutrient	2118	8.01	2.01	1943	129	1799	422	31.0	0.85	3.5	0.17	0.67
	(18)	(0.02)	(0.08)	(17)	(5)	(18)	(21)	(0.4)	(0.24)	(1.6)	(0.09)	(0.34)
16 °C CO_2	2122	8.02	2.09	1940	134	1791	405	31.1	0.70	3.3	0.16	0.56
	(16)	(0.02)	(0.10)	(15)	(6)	(17)	(25)	(0.4)	(0.17)	(2.0)	(0.10)	(0.20)
16 °C nut & CO_2	2120	8.00	2.00	1946	128	1802	425	30.9	1.08	3.2	0.23	1.18
	(16)	(0.02)	(0.06)	(16)	(4)	(16)	(18)	(0.4)	(0.31)	(1.9)	(0.15)	(0.43)
Treatment												
24 °C ambient	2037	8.01	2.57	1812	159	1641	407	31.2	1.0	4.8	0.61	4.04
	(29)	(0.04)	(0.21)	(33)	(13)	(39)	(44)	(0.6)	(0.4)	(1.2)	(0.13)	(0.49)
24 °C nutrient	2035	8.00	2.54	1813	158	1643	410	31.1	0.90	5.0	0.92	8.19
	(28)	(0.03)	(0.16)	(28)	(10)	(31)	(32)	(0.6)	(0.4)	(0.8)	(0.18)	(0.81)
24 °C CO_2	2045	7.81	1.77	1903	110	1773	688	31.1	1.0	5.5	0.67	3.0
	(33)	(0.04)	(0.16)	(26)	(10)	(25)	(67)	(0.5)	(0.3)	(0.4)	(0.18)	(0.6)
24 °C nut & CO_2	2043	7.78	1.67	1911	104	1785	741	31.1	0.94	5.1	0.93	8.39
	(32)	(0.04)	(0.17)	(28)	(11)	(29)	(79)	(0.5)	(0.26)	(0.9)	(0.13)	(0.51)
16 °C ambient	2037	8.05	2.09	1851	134	1704	363	30.7	1.13	3.8	0.61	2.86
	(37)	(0.02)	(0.11)	(36)	(7)	(35)	(24)	(0.2)	(0.19)	(0.9)	(0.20)	(0.70)
16 °C nutrient	2012	8.01	1.93	1840	123	1702	394	30.7	2.40	3.0	1.25	9.67
	(42)	(0.04)	(0.16)	(37)	(10)	(36)	(38)	(0.2)	(1.75)	(0.9)	(0.35)	(1.45)
16 °C CO_2	2039	7.78	1.22	1949	78	1843	732	30.7	0.92	4.1	0.66	3.17
	(37)	(0.04)	(0.11)	(30)	(7)	(27)	(62)	(0.2)	(0.56)	(1.4)	(0.21)	(0.94)
16 °C nut & CO_2	2033	7.75	1.14	1951	73	1849	783	30.7	1.52	3.8	1.12	8.71
	(35)	(0.03)	(0.08)	(52)	(5)	(31)	(52)	(0.2)	(0.66)	(0.9)	(0.19)	(0.69)

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Fig. 1. Continued.

B. Initial	A_T	pH_T	Ω_{Arag}	C_T	CO_3^{2-}	HCO_3^-	pCO_2	S	NH_4^+	H_2SiO_4	PO_4^{3-}	NO_3^-
ambient seawater	2140 (10)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.31 (0.04)	3.7 (1.6)	0.15 (0.24)	1.6 (0.5)
nutrient enriched (pre-enrichment)	2130 (11)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.35 (0.08)	3.3 (0.3)	0.08 (0.03)	1.9 (0.6)
Treatment												
ambient seawater	2050 (30)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.5 (0.5)	7.5 (2.0)	0.62 (0.15)	3.5 (0.6)
nutrient enriched	2050 (30)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.32 (0.17)	7.1 (2.0)	0.91 (0.11)	8.7 (1.2)

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Fig. 2. ANOVA results for the effects of CO_2, nutrients (nut), and interaction there of on the change in relative calcification rates, using the source coral colony (coral) as a blocking variable.

Source	DF	SS	MS	F	*p*
24 °C zooxanthellate					
Model	6	2.422	0.404	4.890	0.008
Error	13	1.072	0.082		
CO_2	1	0.866	0.866	10.500	0.006
nut	1	0.097	0.097	1.170	0.298
coral	3	1.153	0.384	4.660	0.020
CO_2^*nut	1	0.015	0.015	0.180	0.679
24 °C azooxanthellate					
Model	6	2.370	0.395	2.950	0.048
Error	13	1.738	0.134		
CO_2	1	0.046	0.046	0.340	0.569
nut	1	0.280	0.280	2.100	0.171
coral	3	1.820	0.607	4.540	0.022
CO_2^*nut	1	0.088	0.088	0.660	0.432
16 °C zooxanthellate					
Model	6	0.972	0.162	2.070	0.1576
Error	9	0.706	0.078		
CO_2	1	0.273	0.273	3.490	0.095
nut	1	0.022	0.022	0.280	0.607
coral	3	0.674	0.225	2.870	0.096
CO_2^*nut	1	0.002	0.002	0.020	0.878
16 °C azooxanthellate					
Model	6	1.446	0.241	2.510	0.104
Error	9	0.865	0.096		
CO_2	1	0.318	0.318	3.310	0.102
nut	1	0.003	0.003	0.030	0.868
coral	3	1.042	0.347	3.610	0.058
CO_2^*nut	1	0.083	0.083	0.860	0.378

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Fig. 3. ANOVA results for the effects of CO_2, nutrients (nut), gender and interaction there of on the change in relative calcification rates, with the source coral colony (coral) nested within gender. Due to a significant CO_2 * gender interaction at 24 °C, 24 °C data were then tested for the effects of CO_2, nutrients, and the interaction there of on each gender separately. Since there was a significant effect of CO_2 for female corals at 24 °C, Dunnetts HSU test was used determine which treatments differed from ambient – CO_2 only was significant $p = 0.025$. Data for zooxanthellate and azooxanthellate corals have been pooled.

Source	DF	SS	MS	F	p
24 °C					
Model	12	4.914	0.410	3.840	0.002
Error	27	2.880	0.107		
co2	1	0.939	0.939	8.800	0.006
nut	1	0.014	0.014	0.130	0.723
gender	1	2.616	2.616	24.520	< .0001
co2*nut	1	0.068	0.068	0.640	0.430
gender*co2	1	0.556	0.556	5.210	0.031
gender*nut	1	0.000	0.000	0.000	0.989
coral(gender)	6	0.140	0.023	0.220	0.967
16 °C					
Model	12	2.649	0.221	2.610	0.030
Error	19	1.605	0.084		
co2	1	0.509	0.509	6.030	0.024
nut	1	0.006	0.006	0.070	0.795
gender	1	0.028	0.028	0.330	0.575
co2*nut	1	0.055	0.055	0.650	0.429
gender*co2	1	0.015	0.015	0.180	0.678
gender*nut	1	0.002	0.002	0.020	0.884
coral(gender)	6	1.954	0.326	3.860	0.011
24 °C male					
Model	7	0.245	0.035	0.260	0.960
Error	17	2.258	0.133		
co2	1	0.034	0.034	0.260	0.620
nut	1	0.011	0.011	0.080	0.778
co2*nut	1	0.070	0.070	0.530	0.478
coral	4	0.122	0.031	0.230	0.918
24 °C female					
Model	5	1.233	0.247	3.620	0.045
Error	9	0.614	0.068		
co2	1	1.181	1.181	17.320	0.002
nut	1	0.007	0.007	0.100	0.760
co2*nut	1	0.007	0.007	0.110	0.751
coral	2	0.013	0.007	0.100	0.910

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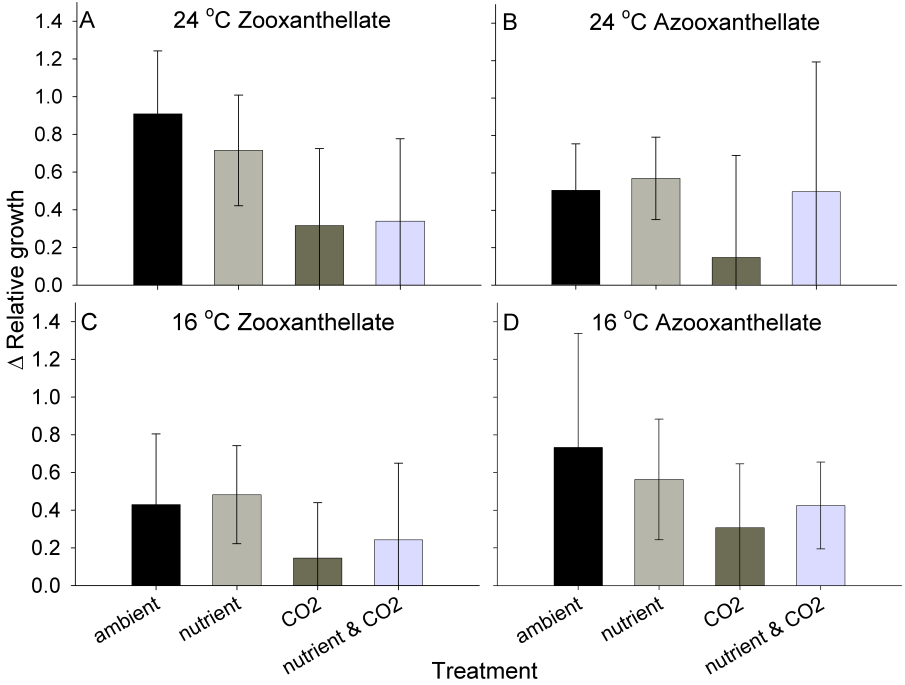

Fig. 1. Relative change in growth rate versus treatment. Zooxanthellate corals grown at 24 °C **(A)**, azooxanthellate corals grown at 24 °C **(B)** zooxanthellate corals grown at 16 °C **(C)**, azooxanthellate corals grown at 16 °C **(D)** plotted versus treatment. Bars represent means ($N = 5$ for 24 °C, 4 for 16 °C), error bars are standard deviation.

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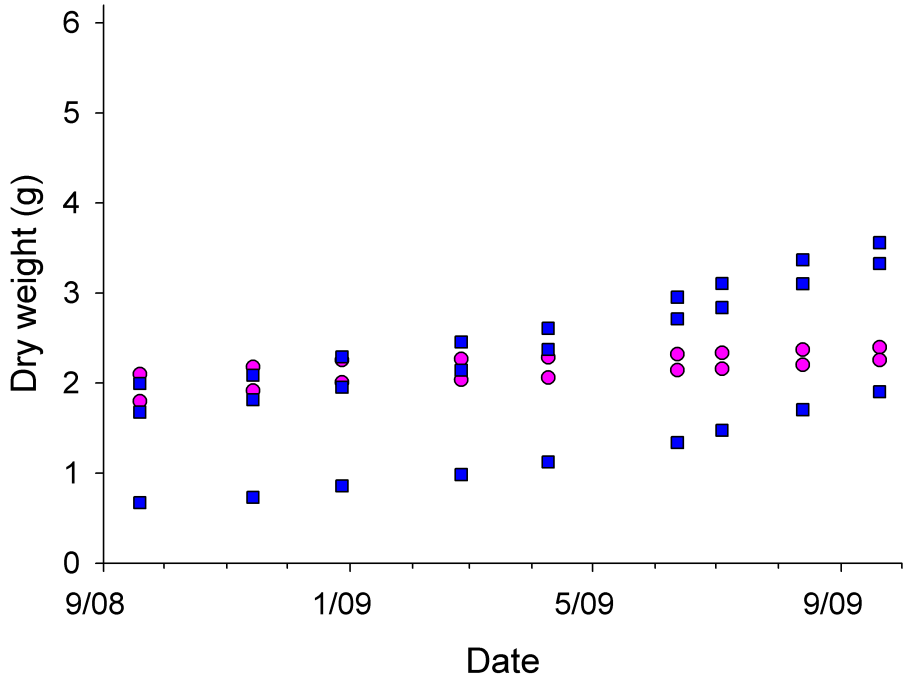

Fig. 2. Example plot of coral dry weights (calculated from buoyant weights) versus time. Data come from 24 °C azooxanthellate corals under ambient conditions. Values for females are indicated by pink circles, males by blue squares.

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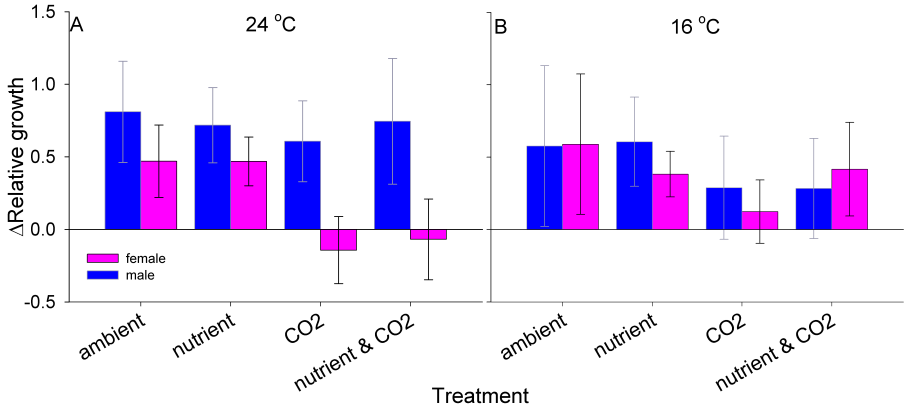

Fig. 3. Relative change in growth rate versus treatment by gender. Corals were grown at 24 °C **(A)** or 16 °C **(B)**. Males are represented by blue bars, females by pink. Data for zooxanthellate and azooxanthellate corals have been pooled. Bars represent means ($N = 5$ or 6 for males, 3 or 4 for females), error bars are standard deviation.

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