Symbiodinium mitigate the combined effects of hypoxia and acidification on a noncalcifying cnidarian

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Title *Symbiodinium* mitigate the combined effects of hypoxia and acidification on a non-calcifying cnidarian

Running head CO_2 fuels a symbiotic cnidarian under hypoxia

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Abstract

Anthropogenic nutrient inputs enhance microbial respiration within many coastal ecosystems, driving concurrent hypoxia and acidification. During photosynthesis, *Symbiodinium* spp., the microalgal endosymbionts of cnidarians and other marine phyla, produce O_2 and assimilate CO_2, and thus potentially mitigate the exposure of the host to these stresses. However, such a role for *Symbiodinium* remains untested for non-calcifying cnidarians. We therefore contrasted the fitness of symbiotic and aposymbiotic polyps of a model host jellyfish (*Cassiopea* sp.) under reduced O_2 (~2.09mgL^{-1}) and pH (~pH 7.63) scenarios in a full factorial experiment. Host fitness was characterised as asexual reproduction and their ability to regulate internal pH and *Symbiodinium* performance characterised by maximum photochemical efficiency, chl*a* content, and cell density. Acidification alone resulted in 58% more asexual reproduction of symbiotic polyps than aposymbiotic polyps (and enhanced *Symbiodinium* cell density) suggesting *Cassiopea* sp. fitness was enhanced by CO_2-stimulated *Symbiodinium* photosynthetic activity. Indeed, greater CO_2 drawdown (elevated pH) was observed within host tissues of symbiotic polyps under acidification regardless of O_2 conditions. Hypoxia alone produced 22% fewer polyps than ambient conditions regardless of acidification and symbiont status, suggesting *Symbiodinium* photosynthetic activity did not mitigate its effects. Combined hypoxia and acidification, however, produced similar numbers of symbiotic polyps compared with aposymbiotic kept under ambient conditions, demonstrating that the presence of *Symbiodinium* was key for mitigating the combined effects of hypoxia and acidification on asexual reproduction. We hypothesise that this mitigation occurred because of reduced photorespiration under elevated CO_2 conditions where increased net O_2 production ameliorates oxygen debt. We show that *Symbiodinium* play an important role in facilitating enhanced fitness of *Cassiopea* sp. polyps, and perhaps also other non-calcifying cnidarian hosts, to the ubiquitous effects of ocean acidification. Importantly we

41 highlight that symbiotic, non-calcifying cnidarians may be particularly advantaged in
42 productive coastal waters that are subject to simultaneous hypoxia and acidification.

Introduction

Marine ecosystems are under increasing pressure from a suite of anthropogenic perturbations (Crain *et al.*, 2008). Environmental hypoxia (reduced oxygen) is a particular threat to coastal regions (Vaquer-Sunyer & Duarte, 2008, Chu & Tunnicliffe, 2015) and the ecosystem services they sustain, such as fishery production (Breitburg *et al.*, 2009) and nutrient cycling (Woulds *et al.*, 2007). Such hypoxic zones have been expanding since the mid-1900s via eutrophication associated with heavily populated coastlines, and by 2008 hypoxia was estimated to have affected more than 245,000 square kilometres of the Earth's surface (Diaz & Rosenberg, 2008). Hypoxic zones are expected to further expand as ocean waters warm (Deutsch *et al.*, 2015, Schmidtko *et al.*, 2017) and human populations become further concentrated along coastlines and catchments (Rabalais *et al.*, 2010) and thus, hypoxia is considered one of the most severe threats to coastal ecosystems.

Increased coastal hypoxia is primarily linked to accelerating rates of nutrient input along coastlines (Vaquer-Sunyer & Duarte, 2008, Altieri & Gedan, 2015), which stimulate excessive production of organic matter (OM) and microbial remineralisation processes to deplete oxygen (O_2) from the water column (hypoxia, typically defined as <2mg O_2 L^{-1}) (Cai *et al.*, 2011). Less well recognised, however, is that hypoxic waters become, in parallel, more acidic since carbon dioxide (CO_2) is simultaneously produced by respiration via microbial remineralisation, to reduce pH through the formation and dissociation of carbonic acid (Gobler & Baumann, 2016). Such hypoxia and associated acidification therefore become particularly amplified at night (Gobler & Baumann, 2016) when photosynthesis ceases and community respiration increases (Baumann *et al.*, 2015). Despite this inherent coupling of hypoxia and acidification, most experiments investigating the potential impacts of hypoxia have not accurately replicated the water chemistry associated with hypoxia. Instead, experiments commonly sparge seawater with N_2 gas (e.g. Wang & Widdows, 1991, Baker &

68 Mann, 1994, Gracey *et al.*, 2001, Eerkes-Medrano *et al.*, 2013), which simultaneously
69 displaces CO_2 and O_2, to create conditions that are hypoxic but less (not more) acidic.
70 Furthermore, this approach is unable to replicate the inherent variation to acidification and
71 hypoxia that occurs on a diel basis (e.g. Regnault & Aldrich, 1988, Landry *et al.*, 2007).
72 Hence most manipulative experiments of hypoxia create conditions that are inconsistent with
73 typical natural conditions (Gobler *et al.*, 2014).

74 Understanding the biological responses to hypoxic conditions is fundamentally
75 hindered by limited data on the potential interactive effects of hypoxia and acidification on
76 cnidarians (but see, Steckbauer *et al*., 2015). The few recent studies that have empirically
77 examined the interactive effects of hypoxia and acidification on marine invertebrates
78 generally demonstrate either additive (bivalves; Jakubowska & Normant, 2015, Jansson *et*
79 *al.*, 2015) or synergistic responses (gastropods, bivalves, anemones, respectively; Kim *et al.*,
80 2013, Gobler *et al.*, 2014, Steckbauer *et al.*, 2015) to the dual stressors (but see, bivalves; Sui
81 *et al.*, 2016). For example, metabolism of two non-symbiotic, non-calcifying anemones
82 (*Anemonia alicemartinae* and *Phymactis papillosa*) was depressed under the combined
83 effects of hypoxia and acidification but increased under acidification in isolation (Steckbauer
84 *et al.*, 2015). Taken together, these studies generally suggest that the combined effects of
85 hypoxia and acidification may be more severe than those of the individual stressors.

86 Whilst many marine organisms appear to be negatively impacted by acidification
87 (Kroeker *et al.*, 2010, Przeslawski *et al.*, 2015), most observations of marine algae and
88 seagrass suggest productivity and competitive fitness will be either enhanced (or at worst
89 generally unchanged, Roleda *et al.*, 2012, Asnaghi *et al.*, 2013, Young & Gobler, 2016) by
90 acidification. This poses an interesting conundrum for how the diverse range of invertebrates
91 that host symbiotic dinoflagellates (e.g. *Symbiodinium* spp.) may respond to microbial-driven
92 coastal hypoxia and associated acidification. Specifically, the presence of *Symbiodinium* spp.

93 may partially mitigate the combined effects of hypoxia and acidification, where
94 photosynthetic release of O_2 could potentially ameliorate oxygen debt induced during
95 hypoxic events (Malcolm & Brown, 1977) whilst the simultaneous acquisition of CO_2 may
96 reduce pCO_2 within their surrounding host cells (Laurent *et al.*, 2013, Gibbin *et al.*, 2014,
97 Laurent *et al.*, 2014). Indeed, photosynthetic activity of *Symbiodinium* spp. may mitigate
98 acidosis of host cells under high pCO_2 conditions (Gibbin *et al.*, 2014). Elevated pCO_2 of
99 surrounding seawater may also stimulate the photosynthetic activity of *Symbiodinium* spp.
100 where cells have become CO_2 limited (see Suggett *et al.*, 2012, Suggett *et al.*, 2013, Gibbin *et*
101 *al.*, 2014, Ventura *et al.*, 2016) and may act as a key condition needed to enhance
102 oxygenation of the host tissues. Whilst this potential mitigating role of endosymbiotic algae is
103 interesting, it may ultimately be restricted to certain *Symbiodinium* spp. genetic types with
104 inherently inefficient CO_2 acquisition modes under 'present day' seawater pCO_2 (see Brading
105 *et al.*, 2011, Brading *et al.*, 2013). Even so, the role of *in hospite Symbiodinium* spp. in
106 potentially mitigating the interactive effects of long-term hypoxia and acidification is
107 unexplored.

108 Here we contrast the fitness of symbiotic and aposymbiotic polyps for a model host of
109 *Symbiodinium* spp. (the jellyfish *Cassiopea* sp., Hofmann *et al.*, 1978) in a full factorial
110 design of reduced O_2 and pH for the first time. Absolute values and extent of diel variability
111 for pH and DO were selected to mimic current-day hypoxic ecosystems. We specifically
112 hypothesised that *Cassiopea* sp. polyps exposed to hypoxia and acidification (either in
113 isolation or combination) would exhibit negative physiological effects but that these effects
114 would be mitigated by the presence of *Symbiodinium*. Here we define host fitness as the rate
115 of asexual reproduction of polyps and their ability to regulate internal pH (during the night
116 and day), and the physiological response of *Symbiodinium* spp. was characterised via
117 measurements of maximum photochemical efficiency, chl*a* content, and *Symbiodinium* cell

118 density of symbiotic polyps. We also assessed whether different *Symbiodinium* spp.
119 genotypes were selected for under the different treatments. From this experiment we present
120 novel observations that demonstrate a potentially important role of *Symbiodinium* in
121 facilitating enhanced fitness of *Cassiopea* sp. polyps (and perhaps also other non-calcifying
122 cnidarians) to the ubiquitous effects of acidification and importantly that *Symbiodinium* also
123 appeared to sustain fitness of *Cassiopea* sp. polyps when acidification and hypoxia co-
124 occurred.

Materials and methods

Species studied and response variables measured

We examined both symbiotic and aposymbiotic (without symbionts) polyps of the upside down jellyfish, *Cassiopea* sp. to test for the potential role of *Symbiodinium* in mitigating acidification/ hypoxia. *Cassiopea* sp. inhabit shallow tropical and sub-tropical coastal waters and lagoons (Hofmann *et al.*, 1996) that exhibit considerable fluctuations of DO and pH (e.g. Gray *et al.*, 2012, Tonetta *et al.*, 2014). *Cassiopea* sp. polyps were collected as larvae that had settled on a rock in a display tank containing at least 10 adult medusae at *Underwater World*, Sunshine Coast, Australia, in September 2013. Symbiotic polyps were sampled from the upper surface of the rock and aposymbiotic polyps from the underside under low light conditions. *Cassiopea* spp. larvae are aposymbiotic and metamorphose into aposymbiotic polyps (Sachs & Wilcox, 2006), which subsequently acquire *Symbiodinium* cells from the external environment ('horizontal transmission', Sachs & Wilcox, 2006, Thornhill *et al.*, 2006). Aposymbiotic and symbiotic *Cassiopea* sp. polyps thus serve as ideal study organisms to examine for the role of *Symbiodinium* (and hence host-*Symbiodinium* symbioses) in regulating host fitness.

Experimental Approach

Polyps were acclimated to laboratory conditions by maintaining them at 25ºC (±1SE, 0.02) under a 12:12 light: dark cycle of ~470 μmol photons m^{-2} s^{-1} (Aqua Zonic: Super Actinic Blue 26W, 400-500nm and Super Sun 30W, 400-700nm) in fresh 10 μm filtered seawater that was sourced from the Gold Coast Seaway, Queensland (27.56ºS, 153.25ºE). Seawater was stored in darkness for at least 10 weeks prior to the start of the experiment to minimise exposure of polyps to free-living *Symbiodinium* throughout experimentation. Polyps were fed newly hatched *Artemia* sp. nauplii every third day. All polyps were checked

149 for the presence or absence of symbionts using pulse amplitude modulated (PAM)
150 fluorometry (as used by: Steindler *et al.*, 2002, Lemloh *et al.*, 2009) and fluorescence
151 microscopy with UV Illumination prior to the start of the experiment and every second day
152 during the experiment.

153 The experimental design consisted of three orthogonal factors: pH (control (24h mean
154 ±1SE, 7.95 ± 0.01) versus reduced (7.63 ± 0.01)), dissolved O_2 (DO) concentration (control
155 (6.14 mgL^{-1} ± 0.03) versus hypoxic (2.09 mgL^{-1} ± 0.03)) and polyp type (symbiotic versus
156 aposymbiotic). Four replicate aquaria were randomly allocated to each combination of pH,
157 oxygen and polyp type (i.e. n=32). Six polyps were transferred using a toothpick into small
158 plastic petri dishes weighted with stainless steel weights. Three petri dishes were then
159 immersed in 1L glass aquaria (i.e. 18 polyps per aquarium); one petri dish was allocated for
160 asexual reproduction measurements and the other two were used for pH microelectrode and
161 chlorophyll fluorescence measurements. Three petri dishes were allocated to each replicate
162 aquarium to prevent overcrowding of polyps and to ensure that polyps used to measure one
163 response variable were not re-sampled for others during the experiment. The experiment ran
164 for 22 days and was performed in a controlled temperature laboratory with the ambient
165 temperature set at 25ºC.

166 Absolute values and extent of diel variability for pH and DO of control treatments
167 were replicated based on 24h field measurements taken in October, 2014 in Moreton Bay,
168 Australia (27.13ºS, 153.07ºE) (Fig. S1). In hypoxic systems, the magnitude of diel
169 fluctuations of pH and DO can vary depending on the ecosystem being tested. We, therefore,
170 selected moderate levels of DO and diel variation ($1.5mgL^{-1}$- $3.0mgL^{-1}$) from a range data
171 collected from current-day hypoxic ecosystems in coastal ecosystems (e.g. Park *et al.*, 2007,
172 Tyler *et al.*, 2009). Levels of DO and pH are stoichiometrically linked in marine ecosystems
173 (Cai *et al.*, 2011) and pH can vary between 6.9- 7.9 during hypoxic events (Gobler *et al.*,

2014, Gobler & Baumann, 2016). We, therefore, selected moderate pH levels (pH 7.5-7.75) for the low pH treatments from a range of pH data collected from coastal hypoxic systems (e.g. Cai *et al.*, 2011, Melzner *et al.*, 2012, Gobler *et al.*, 2014).

Manipulation of water chemistry

To achieve the desired water chemistry of each treatment, a series of gas mass controllers were used to deliver mixtures of CO_2, N_2 and O_2 gas to seawater (also see Bockmon *et al.*, 2013). The desired gas compositions (CO_2, N_2, O_2) were mixed from individual gas cylinders using four sets of three Omega® mass flow controllers (FMA-5400s, 0-20 mL/min (CO_2), 0-5 L/min (N_2), 0-2 L/min (O_2)), which allowed for four independent treatments. The mass flow controllers were operated and functions monitored by a desktop PC running NI LabVIEW™ software (32-bit version) with communication using a voltage generating Omega® Expandable Modular Data Acquisition System® (iNET-400) connected with three Omega® wiring boxes with screw terminals (iNET-510). The desired proportions of CO_2, N_2 and O_2 were mixed in a stainless steel manifold and the gas line that emerged from the manifold was split to provide identical gas mixtures to the replicate aquaria. Gas flow rates to replicate aquaria were manually adjusted using secondary stainless steel manifolds with control valves. For each treatment, two gas compositions (day and night) were used to closely mimic diel fluctuations in water chemistry in the field. NI LabVIEW™ was used to linearly transition between day and night gas mixtures but gas compositions were held constant at night and from 10am-2pm (Fig. S2).

Desired gas compositions for each treatment were continuously delivered to each aquarium using plastic air stones. Lids were placed loosely over each replicate aquarium with a head space of ~10mm to minimise evaporation and subsequent changes in water chemistry. 25% of the water for each aquarium was replaced every day using water of the same

chemistry. All aquaria were exposed to 12:12 light: dark cycle of ~470 μmol photons m^{-2} s^{-1} (Aqua Zonic: Super Actinic Blue 26W, 400-500nm and Super Sun 30W, 400-700nm) throughout the experiment to mimic diel patterns during summer.

Analysis of carbonate chemistry

Levels of pCO_2 were calculated based on measured levels of total alkalinity (TA), pH, DO, temperature and salinity using the program CO_2SYS (Lewis *et al.*, 1998) (see Table S1). Once per week, a 100mL water sample was collected for analysis of TA from one randomly selected replicate from each of the treatments. Samples were collected in clean glass amber bottles using a drawing tube. Bottles were filled from the bottom and water allowed to overflow for 10-15 seconds to minimise gas exchange with the atmosphere. All samples were fixed with 20μL of mercuric chloride to prevent biological activity and stored at 4 °C until analysed within 24 hours of collection. TA samples were analysed using an automatic 848 Titrino Plus Total Alkalinity Titrator (Metrohm©) calibrated every 3 days on the total scale using TRIS/HCl buffers in synthetic seawater. TA measurements on 50mL samples of certified reference material (provided by A. G. Dickson, batch #138) were used to verify TA values. Every third day, temperature, salinity, pH and DO were measured at 10am (Table S1). Temperature was recorded in each aquarium using a thermometer and salinity was measured using a conductivity-salinity metre (TPS salinity-conductivity metre, MC-84). The DO concentration in each aquarium was recorded using an optic DO sensor (Mettler Toledo OptiOPx, Mettler Toledo Ltd). The pH of each aquarium was measured using a FiveGo pH meter (Mettler Toledo Ltd) equipped with a TRIS-compatible electrode (Inlab Expert Pro Electrode, Mettler Toledo Ltd). Every 2-3 days, pH electrodes were calibrated using TRIS/HCl buffers in synthetic seawater. To accurately measure diel variation in O_2 and CO_2

during the experiment, pH and DO measurements were taken hourly (between 6am-6pm) from one randomly selected replicate from each of the treatments once per week (Fig. S2).

Asexual reproduction and internal pH measurements

Asexual reproduction and internal pH (i.e. pH micro-electrode profiles) of both symbiotic and aposymbiotic polyps were measured to investigate the extent to which *Symbiodinium* spp. metabolism within host tissues mitigate external DO and/ or pH exposure. Every third day, polyp dishes were removed from aquaria and checked for polyps undergoing strobilation (the production of young medusae via transversal fission). At the end of the experiment, all reproduction dishes were removed from aquaria and the number of individual polyps was recorded using a dissecting microscope. Only asexual buds that had metamorphosed into individual polyps from the planuloid stage were counted.

One polyp was selected randomly from each replicate aquarium to measure internal pH every three days during the day and night to account for the lack of photosynthesis in symbiotic polyps at night when acidification and hypoxia are amplified. All polyps were gently detached from the polyp dishes allocated for pH profile measurements using stainless steel manicure scissors and collected with ~30mL of their respective treatment water. If polyps were observed to retract their oral arms, they were considered as 'stressed' and not used for microelectrode measurements. Polyps were placed in a 30mm plastic petri dish under a dissecting microscope. The pH microelectrode (pH-25 Unisense, Denmark, 20-30µm tip diameter) was mounted on, and controlled by, a micromanipulator. *Cassiopea* sp. polyps are 'sticky' in texture and each polyp was gently placed flush against the side of the petri dish to ensure the polyp would remain in a fixed position. The pH microelectrode was introduced into the treatment water and external pH measurements were taken ~5mm from the polyp. All microelectrode measurements were performed horizontally through the polyp wall when

245 polyps were alive and upright (i.e. perpendicular to the microelectrode). The microelectrode
246 was inserted halfway between the base of the polyp and the beginning of the oral arms, to
247 ensure consistency of microelectrode profiles among all polyps. The epidermis formed a seal
248 around the electrode and prevented fluid exchange between the external seawater and the
249 polyp tissue (*sensu* Revsbech *et al.*, 1995, Köhler-Rink & Kühl, 2000). Polyps varied slightly
250 in shape and size and so to ensure consistency, pH was recorded continuously through one
251 side of the polyp until the gastrovascular cavity was reached. Four increments through the
252 polyp were then determined relative to the thickness of the body wall (i.e. the number of
253 measurements taken through the polyp wall) to account for differences in shape and size,
254 yielding 6 measurements per polyp (i.e. external pH, increments 1-4 through the polyp wall,
255 and gastrovascular cavity). Prior to pH measurements, the pH micro-electrodes were
256 calibrated with pH 7 and 10 buffer solutions. Fluorescent pH sensitive dye, 5(6)-
257 Carboxynaphthofluorescein, (sourced from Sigma-Aldrich, CAS no: 128724-35-6) was used
258 to confirm relative pH changes of microelectrode profiles using a fluorescence microscope.
259 Polyps were noninvasively incubated at 25ºC in their aquarium water supplemented with dye
260 to a final concentration of 50 μmol L^{-1} 5(6)-Carboxynaphthofluorescein for 30 minutes to
261 allow sufficient uptake. Polyps were placed in 3mL vials and the pH-sensitive dye solution
262 was allowed to overflow the vials into water baths at a rate of ~2mL min^{-1} to ensure the
263 solution was gently mixed. Following staining, polyps were gently placed on depression
264 microscope slides using a pipette and photographed horizontally using a Nikon Eclipse 80*i*
265 fluorescence microscope with UV Illumination. The pH-sensitive dye solution was excited at
266 a rate of 0.2Hz at A_{598nm}. To confirm the response of the fluorescent dye, phosphate buffers
267 of known concentrations were supplemented with the pH-sensitive dye to a final
268 concentration of 50 μmol L^{-1} to construct a two-point calibration curve. All polyps used for
269 internal pH measurements were sacrificed.

Maximum photochemical efficiency (F_v/F_m), chla content and Symbiodinium cell density

We measured another three response variables on symbiotic polyps (maximum photochemical efficiency, F_v/F_m), Chl*a* content and *Symbiodinium* density) to assess potential changes in *Symbiodinium* growth and photophysiological status in response to the various treatments tested. Prior to the commencement of the experiment, and at weekly intervals throughout the experiment, symbiotic polyps were sampled to measure maximum photochemical efficiency of *Symbiodinium* using a Maxi-Imaging pulse amplitude modulator (Maxi-PAM, Walz GmbH, Germany). Within each replicate aquarium, one polyp was transferred from the allocated petri-dish for fluorescence measurements into an individual well of a black, non-binding 96-well plate (Greiner Bio-One GmbH, cat no. 655090). All polyps were dark acclimated for 20 minutes prior to PAM measurements. Repeated (n= 10, separated by 0.1s) chl*a* fluorescence inductions were made to return values of the minimum (F_0) and maximum fluorescence yield (F_m), and hence the maximum PSII photochemical efficiency (F_v/F_m, = $[F_m-F_0]/F_m$).

At the end of the experiment, four polyps of similar shape and size were sampled from each replicate aquarium containing symbiotic polyps and stored in 1mL of 0.22µm filtered seawater. Polyps were macerated with a tissue homogeniser for 30s and a 100µL aliquot was taken from each sample for *Symbiodinium* counts. 100µL of glycerol was added to each 100µL aliquot and samples were frozen at -80ºC until analysed. Defrosted samples were mixed and ten 0.10µL drops from each sample were counted using a Neubauer haemacytometer. The remaining 900µL samples were used for estimates of chl*a* content. All chl*a* samples were centrifuged at 3000 × g at 4ºC for 10 minutes and the supernatant discarded. The pelleted *Symbiodinium* were resuspended in 95% ethanol and extracted overnight in the dark at 4ºC before centrifugation. Absorption of the supernatant was then determined at 647nm and 664nm using a UV-1800 Shimadzu© spectrophotometer. All

samples were analysed in 1cm quartz cuvettes and the instrument was calibrated using 95% ethanol blanks. Chl*a* content was determined using coefficients from a spectrophotometric equation ($-2.6094 \times A_{629} + 12.4380 \times A_{665}$) for dinoflagellates in ethanol (Ritchie, 2006). Chl*a* (units) concentrations were normalised to corresponding measures of cell content (units) to also yield the chl*a* $[cell]^{-1}$ (units). *Symbiodinium* cell density and chl*a* concentrations were also normalised to the number of polyps analysed to yield the *Symbiodinium* and chl*a* $[polyp]^{-1}$ (units), respectively.

Identification of Symbiodinium genotype

Symbiodinium genetic type identity was further determined across all (symbiotic) treatments to confirm whether any changes associated with *Symbiodinium* reflected alterations in physiology of the same type versus a switch in dominate type (e.g. Suggett *et al.*, 2012). At the end of the experiment, ten polyps from each replicate aquarium containing *Symbiodinium* were sampled and stored in DMSO preservation buffer (Seutin *et al.*, 1991). Samples were washed twice with phosphate buffered saline (PBS) and the total DNA was extracted using the MO BIO PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, CA, USA, cat no. 13400-50) following the manufacturer's bead-beating protocol with an extra phenolic separation step. The *Symbiodinium* partial 5.8S, ITS2, and partial 28S region was amplified by PCR using the forward *ITS-dino* (5′GTGAATTGCAGAACTCCGTG 3′) and reverse *ITS2-rev2* (5′CCTCCGCTTACTTATATGCTT 3′) primers (Stat *et al.*, 2011). ITS2 amplicons were purified through gel electrophoresis, sequenced by the Australian Genome Research Facility (AGRF), and compared to *Symbiodinium* entries in NCBI using the Basic Local Alignment Search Tool (BLAST), yielding a % match. Sequences retrieved by this study were deposited in NCBI under the accession numbers KX533944 through KX533954 (Table S3).

Statistical analyses

Dependent variables of number of polyps, Chl*a*, *Symbiodinium* density and *Symbiodinium* Chl*a* per cell, were analysed using linear mixed models (LMMs) in SPSS (SPSS, Released 2013). Prior to analyses, data were checked for normality and homoscedasticity using standardised residual plots and Q-Q plots and, if required, data were either ln or ln(x+1) transformed. All factors were fixed and the number of factors for each dependent variable differed according to how the data were collected. The factors for number of polyps were pH, oxygen concentration, and polyp type, and for *Symbiodinium* Chl*a*, cell density and chl*a* [cell]$^{-1}$ were pH and oxygen concentration. The dependent variables of internal pH and F_v/F_m were analysed using repeated measures LMMs. The factors for internal pH were pH, oxygen concentration, polyp type, day/ night, and distance (through polyp), which was the repeated measure, whereas for F_v/F_m were pH, oxygen concentration, and time, which was the repeated measure. In all repeated measures LMMs, various models (e.g. AR(1), AR(1) heterogeneous, CS) were investigated to assess the model of best fit by comparing several goodness-of-fit statistics (e.g. -2 Restricted Log Likelihood, Akaike's Information Criteria (AIC) and Bayesian Information Criterion (BIC)). Preliminary analysis for the dependent variable internal pH revealed no significant effect of the factor oxygen for all terms, and thus this was removed and the analysis re-run. If significant differences were found, estimated marginal means were used to determine which means differed.

Results

Survival and asexual reproduction

All polyps of *Cassiopea* sp. survived experimentation, with polyp numbers increasing via asexual production in all treatments. At the end of the experiment, polyp numbers differed among pH treatments but their response depended on symbiont status, resulting in a significant pH × symbiont interaction (Table 1, Fig. 1); specifically, greatest numbers were produced by symbiotic polyps under low pH conditions, with symbiotic polyps producing 58% more than aposymbiotic polyps. Symbiotic polyps still produced 17% more than aposymbiotic polyps under ambient pH. Thus host fitness (asexual reproduction) was consistently higher when *Symbiodinium* was present but the magnitude of the difference was greater in low pH conditions. Polyp numbers also differed among oxygen treatments, with 22% fewer polyps occurring in hypoxic (mean ± 1SE: 9.63 ± 0.53) compared to ambient treatments (12.25 ± 0.67), regardless of pH conditions or symbiont status (Table 1). Overall, symbiotic polyps exposed to both low pH and low oxygen produced a similar number of polyps (12.25 ± 0.41) as for symbiotic and aposymbiotic polyps exposed to ambient conditions (13.25 ± 0.54, 11.00 ± 0.70, respectively) (Fig. S3). Thus the presence of *Symbiodinium* appeared to enhance host fitness under acidification conditions that was otherwise hindered under hypoxia alone. However, presence of *Symbiodinium* appeared to sustain host fitness when hypoxia and acidification coincided. Strobilation was not observed during the experiment.

Internal pH profiles

The pH profiles of polyp walls differed among pH treatments, symbiotic versus aposymbiotic polyp, and between day and night, resulting in a significant day/night × symbiont × pH × distance interaction (Table 2, Fig. 2). During the night, pH profiles throughout the polyps matched the external experimental conditions, whereby pH remained consistent across the external to internal body wall and did not differ between symbiotic and aposymbiotic polyps (Fig. 2a). In contrast, during the day and for the symbiotic polyps, pH slowly increased from Increment 1 to Increment 4 through the polyp tissues and then decreased in the gastrovascular cavity to levels similar to that of the surrounding water (Fig. 2b). Under ambient conditions, the internal pH of symbiotic polyps was greater than aposymbiotic polyps and the internal pH of symbiotic polyps increased by 0.19 units at Increment 4 relative to aposymbiotic polyps (Fig. 2b). As expected, this pattern reflected the drawdown of inorganic carbon by *Symbiodinium* photosynthetic activity and was also observed for the low pH treatments, but the magnitude of difference between symbiotic and aposymbiotic polyps was greater under low pH conditions. The internal pH of symbiotic polyps in the low pH treatment was highest at Increment 4 and increased by 0.27 units relative to aposymbiotic polyps (Fig. 2b). At Increment 4, the internal pH of symbiotic polyps matched the internal pH of aposymbiotic polyps under ambient conditions (Fig. 2b). There was no significant difference between the pH of the gastrovascular cavities of symbiotic and aposymbiotic polyps, but the pH levels of the gastrovascular cavity of polyps exposed to low pH conditions were reduced (Fig. 2b).

Maximum photochemical efficiency

F_v/F_m remained generally consistent over time for all treatments (range: 0.143-0.325, mean ±1SE: 0.258 ±0.003) and were similar to F_v/F_m values measured prior to the commencement of the experiment (range: 0.265-0.338, mean ±1SE: 0.265 ±0.009). Although a significant oxygen × pH × time interaction was detected (Table S2, Fig. S4), the magnitude of difference between treatments was small and no consistent patterns were observed.

Symbiodinium identification, Chla content and cell density

Symbiodinium ITS2 sequences from all replicate aquaria, except for one replicate sample in the control treatment that could not be sequenced, were confirmed to match *Symbiodinium* ITS2 subtype C1 (see NCBI Genbank accession KX533944 through KX533954 and Table S3 for ITS2 sequences generated in this study). Consequently, any change in fitness of symbiotic *Cassiopea* sp. polyps reflects a change of the physiology (and/or cell density) of the existing *Symbiodinium* type rather than a change towards alternate types with differing physiologies.

We subsequently examined chl*a* content and *Symbiodinium* cell density to evaluate how the presence of symbionts potentially benefitted the host (as per Fig. 1). Specifically, at the end of the experiment, *Symbiodinium* cell density polyp^{-1} and the *Symbiodinium* specific chl*a* content (units cell^{-1}) varied among pH treatments, but patterns were not consistent among oxygen treatments (Table 3). Consistent with observations that symbiotic polyps produced more polyps under low pH, *Symbiodinium* cell density in polyps in the low pH treatment was 39% higher than those under ambient conditions and was higher than that of all other treatments (Fig. 3a). However, this response was lost when low DO coincided with low pH, where polyps in the hypoxic and low pH treatment had similar *Symbiodinium* cell densities to those in the control treatment (Fig. 3a). Contrary to observations that low DO reduced host fitness (asexual reproduction), polyps exposed to low DO had similar

404 *Symbiodinium* cell densities to those in the control treatment regardless of the pH conditions
405 they were exposed to (Fig. 3a). Intriguingly, observations of *Symbiodinium* chl*a* $cell^{-1}$ were
406 not consistent with those of *Symbiodinium* cell densities. *Symbiodinium* chl*a* $cell^{-1}$ was
407 highest in the low pH and low DO treatment and exceeded that of all other treatments (Fig.
408 3b). Consistent with reduced asexual reproduction of polyps under low DO conditions,
409 hypoxia in isolation resulted in a 52% lower chl*a* $cell^{-1}$ concentration relative to the control
410 treatment (Fig. 3b). This response was not consistent when symbiotic polyps were exposed to
411 hypoxia and low pH in combination, where chl*a* $cell^{-1}$ was 52% higher under hypoxic and
412 low pH conditions than under low pH conditions alone (Fig. 3b).

Discussion

Elevated CO_2 (reduced pH) can benefit *Symbiodinium* both as free living cells (Brading *et al.*, 2011) and *in hospite* of cnidarians (e.g. anemones, Suggett *et al.*, 2012, Towanda & Thuesen, 2012; and corals, Crawley *et al.*, 2010, Suggett *et al.*, 2013). Our data are highly consistent with these observations; specifically, that *Symbiodinium* spp. (ITS2 type C1) facilitated enhanced fitness of *Cassiopea* sp. polyps under acidification conditions, whereby acidification alone resulted in 58% more symbiotic polyps than aposymbiotic polyps and enhanced numbers of *Symbiodinium* cells per polyp. Some studies, however, have reported no change (e.g. Brading *et al.*, 2011) or even decreases (e.g. Anthony *et al.*, 2008) in rates of photosynthesis of *Symbiodinium* under elevated CO_2 and thus, this effect may ultimately be restricted to certain *Symbiodinium* spp. genetic types. Hypoxia alone reduced host fitness (asexual reproduction) regardless of acidification and symbiont status, suggesting *Symbiodinium* photosynthetic activity did not mitigate the negative effects of hypoxia. Most importantly, however, we observed that hypoxia and acidification in combination produced as many symbiotic polyps as the aposymbiotic polyps kept under ambient conditions. Hence, by enhancing photosynthetic activity, exposure to elevated CO_2 appears to offset the negative effects of hypoxia in taxa that host *Symbiodinium*. Our observations suggest that *Cassiopea* sp., and perhaps other symbiotic non-calcifying cnidarians, may still thrive when hypoxia and acidification co-occur but non-symbiotic cnidarians may be negatively impacted by the dual stressors. Species that host *Symbiodinium* only as juveniles or adults, however, may still face challenges if their populations rely on recruitment of aposymbiotic larvae.

Photosynthesis and photorespiration depend on the relative availability of O_2 and dissolved inorganic carbon (DIC) (Larkum *et al.*, 2003, Crawley *et al.*, 2010) but no studies have investigated the concurrent effects of hypoxia and acidification on these processes in symbionts. Whilst our data cannot pinpoint the mechanism that appears to mitigate the

438 observed negative effects of hypoxia under acidification conditions, we hypothesise that our
439 various observations potentially indicate an important role for photorespiration upon
440 exposure to the dual stressors. The apparent mitigation of hypoxia only when acidification
441 co-occurred in the presence of *Symbiodinium in hospite* is consistent with reduced
442 photorespiration under elevated CO_2 conditions (see Crawley *et al.*, 2010). A reduction in
443 photorespiration would then increase net O_2 production and thus increase O_2 concentrations
444 within host tissues thereby ameliorating oxygen debt. Although data are limited on the effects
445 of acidification on rates of photorespiration in symbiotic cnidarians, some studies
446 demonstrate increases in net oxygen production of non-calcifying, symbiotic cnidarians
447 (anemones, Suggett *et al.* 2012, Jarrold *et al.* 2013, Gibbin & Davy 2014) under elevated
448 pCO_2 conditions despite increased respiration rates at elevated pCO_2 concentrations (in some
449 cases, Suggett *et al.* 2012, Gibbin & Davy 2014). Indeed, any increase in net oxygen
450 production must equate to a net increase in CO_2 fixation during photosynthesis (Reece *et al.*,
451 2015). In addition to reduced costs of carbon acquisition under high pCO_2 (Ventura *et al.*,
452 2016), we hypothesise that inhibition of photorespiration may also partially explain the
453 higher rate of asexual reproduction of symbiotic polyps under acidification alone due to
454 increased efficiency of carbon fixation and increased availability of organic carbon for
455 growth. Clearly, better understanding processes such as photorespiration is needed to assess
456 why only some *Symbiodinium* types, i.e. perhaps those more susceptible to Rubisco
457 oxygenation under relatively low CO_2 conditions via differences in Rubisco pool sizes and/or
458 turnover, appear to benefit from elevated CO_2 availability (e.g. Brading *et al.*, 2013).

459 Survival of marine organisms in hypoxic systems may partly depend on their ability
460 to regulate their internal pH under the lowered pH conditions that result from elevated DIC
461 concentrations. During the day, under low pH conditions, the internal pH of symbiotic polyps
462 matched the pH levels of aposymbiotic polyps exposed to ambient conditions, which suggests

463 that photosynthesis of *Symbiodinium* regulated the internal pH of the polyps. Aposymbiotic
464 polyps, however, conformed to the pH of their respective treatments, indicating that
465 *Cassiopea* sp. polyps, as hosts, may have limited ability to regulate their internal pH. Our
466 results are consistent with the few other studies that have investigated the diel regulation of
467 internal pH of cnidarian host cells (Venn *et al.,* 2009, Laurent *et al.*, 2013, Laurent *et al.*,
468 2014, Gibbin *et al.,* 2014. For example, the only other study that has compared the internal
469 pH of symbiotic and non-symbiotic cells (isolated from a coral) under elevated CO_2
470 conditions reported that the internal pH of non-symbiotic cells decreased by 0.3-0.4 when
471 exposed to decreasing pH (from 7.8 to 6.8) but the internal pH of symbiotic cells recovered to
472 control levels (Gibbin *et al.*, 2014). Combined, these studies highlight the significant control
473 that *Symbiodinium* exert over the internal pH of their host tissues and suggest that symbiotic
474 biota will typically be more robust to hypoxic environments than non-symbiotic biota.

475 Survival of symbiotic organisms ultimately depends on the physiological limitations
476 of both the host and their symbionts. Maximum photochemical efficiency values of
477 *Symbiodinium* were low relative to those previously observed for *Symbiodinium in hospite* of
478 cnidarians (e.g. Enochs *et al.*, 2014, Hoadley *et al.*, 2015, including polyps of *Cassiopea* sp.
479 Klein *et al.*, 2016) and could reflect a number of biological (e.g. high light fields,
480 chlororespiration), or measurement artefacts (e.g. lower values expected with imaging PAM,
481 Levin *et al.*, 2017) that cannot presently be ascertained. Low maximum photochemical
482 efficiency values were unlikely induced by stress since all other response variables (i.e.
483 asexual reproduction, *Symbiodinium* densities, Chl*a* $cell^{-1}$) indicate that ambient experimental
484 conditions were optimal for *Symbiodinium*. Even so, maximum photochemical efficiency
485 appeared to be unaffected by the various treatments tested, suggesting that any artefact was
486 constant. *Symbiodinium* densities (and host asexual reproduction), however, were highest in
487 the low pH treatment, suggesting that low pH conditions are favourable for *Symbiodinium*.

Such a pattern is consistent with studies on anemones where elevated CO_2 (reduced pH) not only enhanced *Symbiodinium* (ITS2 type A19) numbers and productivity, but natural population sizes were also substantially increased in proximity to a natural CO_2 vent (Suggett *et al.,* 2012). As with this previous observation, ours similarly suggests that *Symbiodinium* of *Cassiopea* sp. polyps may be limited by the availability of dissolved inorganic carbon (DIC) under present day pCO_2 conditions. Exposure to low pH and low DO simultaneously did not increase densities of *Symbiodinium*; however, chl*a* cell^{-1} increased in response to the dual stressors, consistent with observations that *Symbiodinium* resource investment into pigment synthesis and/ or division rate appears carbon limited under ambient conditions (e.g. ITS2 type A13, Brading *et al.*, 2011). No studies have examined the response of *Symbiodinium* to the combined effects of hypoxia and acidification but observations of other non-calcifying symbiotic cnidarians exposed to elevated CO_2 in isolation demonstrate inconsistent responses of *Symbiodinium* cell densities and Chl*a* content (e.g. Suggett *et al.*, 2012, Towanda & Thuesen, 2012, Gibbin & Davy, 2014, Horwitz *et al.*, 2015). For example, *Symbiodinium* cell densities and chlorophyll content of the anemone *A. elegantissima* were unaffected by high CO_2 conditions despite increased rates of photosynthesis (Horwitz *et al.*, 2015). However, consistent with the current study, acidification increased symbiont densities in two anemones, *Anthopleura elegantissima and Aiptasia* sp. (Suggett *et al.*, 2012, Gibbin & Davy, 2014). Together these results further highlight the complexity of responses of cnidarian associations and demonstrate differential effects of high CO_2 conditions among *Symbiodinium* genotypes.

Some cnidarians host multiple, genetically distinct variants of *Symbiodinium* that tolerate different types or levels of environmental stress (Baker, 2003, Thornhill *et al.*, 2006, Putnam *et al.*, 2012). Hosting multiple variants of *Symbiodinium* may confer a fitness benefit to the host, particularly if the composition of the symbiont community varies (e.g. via competitive displacement) in response to changing environmental conditions (so called

513 'symbiont shuffling') (Little *et al.*, 2004, Berkelmans & van Oppen, 2006). However, in our
514 study we only detected *Symbiodinium* ITS2 type C1, which is a generalist symbiont
515 (LaJeunesse, 2005) that has been observed in >100 host species, including *Cassiopea*
516 (Franklin *et al.*, 2012, Tonk *et al.*, 2013, Mellas *et al.*, 2014). Since we detected only one
517 variant of *Symbiodinium*, there was no evidence of major symbiont shuffling of the dominant
518 symbiont populations, although we cannot rule out that the techniques used in this study and
519 the ITS2 marker may not fully capture changes in dominance of population heterogeneity,
520 notably for type C1 (see Howells *et al.*, 2016, Wham & LaJeunesse, 2016). It is also possible
521 that cryptic *Symbiodinium* variants may have occurred at levels below the detection
522 thresholds of the techniques we used (e.g. Boulotte *et al.*, 2016), although the short duration
523 of our experiment (but see Lewis & Coffroth, 2004) probably precluded the potential for
524 shuffling to occur. Even if some *Cassiopea* sp. polyps harbour only one *Symbiodinium* type,
525 they may still change their symbionts via uptake of new symbiont types from the
526 environment (termed symbiont 'switching', *sensu* Baker, 2003). Whilst the typical symbionts
527 that are *in hospite* of *Cassiopea* spp. populations in Australia are at present unknown, it is
528 well demonstrated that *Cassiopea* spp. polyps can host multiple variants of *Symbiodinium*
529 and acquire additional symbiont types at the polyp stage (including clades A, B, C and D,
530 Mellas *et al.*, 2014). However, polyps in our experiment were not exposed to exogenous
531 *Symbiodinium* cells and thus could not have acquired new symbiont types unless symbionts
532 were shared horizontally between polyps (see Sachs & Wilcox, 2006). To more accurately
533 predict how symbiotic cnidarians, as a group, may respond to hypoxia and acidification we
534 must also consider that hosts may acquire more resistant symbionts, including types that are
535 physiologically adapted to extreme environmental conditions in the longer term (Brading *et*
536 *al.*, 2011).

Various combinations of host and symbiont type may provide physiological advantages under changing ocean conditions. Our observations suggest that *Cassiopea* sp. harbouring *Symbiodinium* subclade C1 responded positively to acidification conditions. Although no studies have investigated the combined effects of hypoxia and acidification on *in hospite Symbiodinium*, our results are consistent with studies that investigated the future effects of ocean acidification on other cnidarians that host *Symbiodinium* types. Indeed, high CO_2 conditions stimulated the productivity of two anemones, *Anthopleura elegantissima and Anemonia viridis,* harbouring *Symbiodinium* clade B and A19, respectively (Suggett *et al.*, 2012, Towanda & Thuesen, 2012). To better determine how biota may respond to hypoxia and acidification, we must now determine whether our results for *Symbiodinium* C1 and *Cassiopea* sp. polyps are consistent with other symbiont types and hosts, and investigate possible interactions between other environmental stressors. Indeed, *Cassiopea* spp. harbour other clades of *Symbiodinium* including A, B and D (Santos *et al.*, 2002, Thornhill *et al.*, 2006, Mellas *et al.*, 2014), and whether our observations here scale to other *Cassiopea* species, life history stages, and/or symbiont types remains to be tested.

Our understanding of the responses of marine biota to hypoxic conditions is hindered because the majority of hypoxia studies manipulate O_2 levels with N_2 gas (Gobler & Baumann, 2016), thereby increasing (up to pH 8.6, see Gobler *et al.*, 2014) and not decreasing pH. In the current study, the fitness of symbiotic polyps appeared to be enhanced by acidification under hypoxic conditions, suggesting that studies that do not account for concurrent changes in O_2 and CO_2 may produce results that do not accurately reflect the response of symbiotic biota to hypoxic environments. Aposymbiotic polyps, however, were negatively affected by hypoxia regardless of the pH conditions they were exposed to, suggesting that acidification did not exacerbate the effects of low oxygen availability. Our observations of aposymbiotic polyps are inconsistent with the only other study to examine the

562 interactive effects of low DO and pH on non-symbiotic, non-calcifying cnidarians (two
563 species of anemones (*Anemonia alicemartinae* and *Phymactis papillosa*) (Steckbauer *et al.*,
564 2015). Although both anemones are naturally non-symbiotic (unlike *Cassiopea* sp.), the study
565 demonstrated that exposure to acidification alone increased the metabolism of *A.*
566 *alicemartinae* and *P. papillosa* but exposure to acidification and hypoxia in combination
567 depressed metabolism of both species. However, we cannot determine whether our results are
568 consistent with other studies that mimic hypoxia using N_2 gas because we did not expose
569 aposymbiotic polyps to low DO and high pH in combination; for this, studies will need to
570 further compare the response of biota to low DO and high pH in combination to those
571 exposed to hypoxia in isolation to assess the reliability of results obtained by studies of
572 hypoxia that manipulate O_2 levels with N_2 gas.

573 *Symbiodinium* are clearly important in mitigating the combined effects of hypoxia and
574 acidification on *Cassiopea* sp. polyps. Our data suggest that symbiotic *Cassiopea* sp. may
575 still thrive in hypoxic environments and although aposymbiotic *Cassiopea* sp. may persist,
576 they are unlikely to proliferate when exposed to the dual stressors. Symbiotic (non-
577 calcifying) cnidarians, such as jellyfish (tested here) but perhaps also other non-calcifying
578 cnidarians may therefore have a greater competitive advantage in current-day hypoxic zones.
579 Our observations that *Symbiodinium* mitigated the negative effects of hypoxia when
580 acidification co-occurred highlights the importance of investigating the concurrent effects of
581 hypoxia and acidification on symbiotic biota. Although the response of aposymbiotic polyps
582 to hypoxia was unaffected by acidification, manipulative experiments of hypoxia need to
583 consider concurrent changes in pH to accurately reflect field observations of hypoxic zones.
584 We therefore advocate for a prompt re-alignment of future studies of hypoxia and suggest
585 that future experiments consider concurrent changes in DO and pH. Whilst *Symbiodinium*
586 may benefit non-calcifying cnidarians in current-day hypoxic zones (tested here),

587 *Symbiodinium* are sensitive to transient heat stress and thus to understand whether this
588 potentially important role of *Symbiodinium* holds under future additional warming scenarios
589 we now need to investigate the effects of additional warming in combination with hypoxia
590 and acidification.

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Tables

Table 1 Summary of results for a LMMs analysis comparing the number of polyps between treatments at day 22 of the experiment. Df= Degrees of freedom. *P* values in bold are statistically significant ($P < 0.05$). BIC (Bayesian Information Criterion) =85.204, and AIC (Akaike's Information Criterion) =84.026. For all sources of variation numerator df =1 and denominator df =24.

Source	F	Sig.
Symbiont	106.778	**<0.001**
Oxygen	49.00	**<0.001**
pH	0.111	0.742
Symbiont × Oxygen	1.000	0.327
Symbiont × pH	25.000	**<0.001**
Oxygen × pH	0.111	0.742
Symbiont × Oxygen × pH	0.111	0.742

837 **Table 2** Summary of results for a LMMs analysis comparing day and night pH
838 microelectrode profiles between treatments at Day 22 of the experiment. The model-of-best-
839 fit was AR(1), BIC (Bayesian Information Criterion) = -1268.564, AIC (Akaike's
840 Information Criterion) = -1276.198. Note, the factor Oxygen was included in preliminary
841 analysis but removed due to non-significance for all terms (P>0.05) and LMMs analysis was
842 re-run. Df= Degrees of freedom (numerator, denominator). P values in bold are statistically
843 significant ($P < 0.05$).

844

Source	Numerator df	Denominator df	F	Sig.
Day/ Night	1	89.181	309.977	**<0.001**
Symbiont	1	89.181	120.335	**<0.001**
pH	1	89.181	3810.007	**<0.001**
Day/ Night × Symbiont	1	89.181	98.877	**<0.001**
Day/ Night × pH	1	89.181	7.747	**0.007**
Symbiont × pH	1	89.181	17.477	**<0.001**
Day/ Night × Symbiont × pH	1	89.181	10.509	**0.002**
Distance	5	239.171	45.393	**<0.001**
Day/ Night × Distance	5	239.171	19.691	**<0.001**
Symbiont × Distance	5	239.171	36.126	**<0.001**
pH × Distance	5	239.171	12.215	**<0.001**
Day/ Night × Symbiont × Distance	5	239.171	32.763	**<0.001**
Day/ Night × pH × Distance	5	239.171	13.975	**<0.001**
Symbiont × pH × Distance	5	239.171	5.520	**<0.001**
Day/ Night × Symbiont × pH × Distance	5	239.171	4.628	**<0.001**

845 **Table 3** Summary of results for three LMMs comparing *Symbiodinium* density (cell polyp^{-1})
846 and chl*a* cell^{-1} (pg) between treatments of symbiotic polyps at Day 22 of the experiment. Df =
847 degrees of freedom. BIC= Bayesian Information Criterion and AIC = Akaike's Information
848 Criterion. *P* values in bold are statistically significant ($P < 0.05$). For all sources of variation
849 numerator df =1 and denominator df =24.

Variable	***Symbiodinium* polyp^{-1}**	**Chl*a* cell^{-1}**
Transformation	Ln	None
Information Criterion	BIC= 10.359	BIC= 59.840
	AIC= 9.874	AIC= 59.355
Source of variation	*P*	*P*
pH	0.131	**0.035**
	F=2.633	*F*=5.623
Oxygen	0.124	0.419
	F=2.730	*F*=0.700
pH × Oxygen	**0.047**	**0.002**
	F=5.095	*F*=16.215

851 **Figure captions**

852

853 **Figure 1** Mean ±1SE number of polyps recorded at Day 22 of the experiment. Letters above
854 error bars indicate similarities (e.g. AA) or differences (e.g. AB) between treatments, as
855 determined by estimated marginal means.

856

857 **Figure 2** Mean (±1SE) pH measurements taken through polyp walls taken during the night
858 (a) and day (b). Letters next to data points indicate similarities (e.g. AA) or differences (e.g.
859 AB) between treatments, as determined by estimated marginal means.

860

861 **Figure 3** Mean (±1SE) *Symbiodinium* cells polyp^{-1} (a) Chl*a* *Symbiodinium* cell^{-1} (pg) (b) at
862 Day 22 of the experiment. Letters above error bars indicate similarities (e.g. AA) or
863 differences (e.g. AB) between treatments, as determined by estimated marginal means.

Supporting information

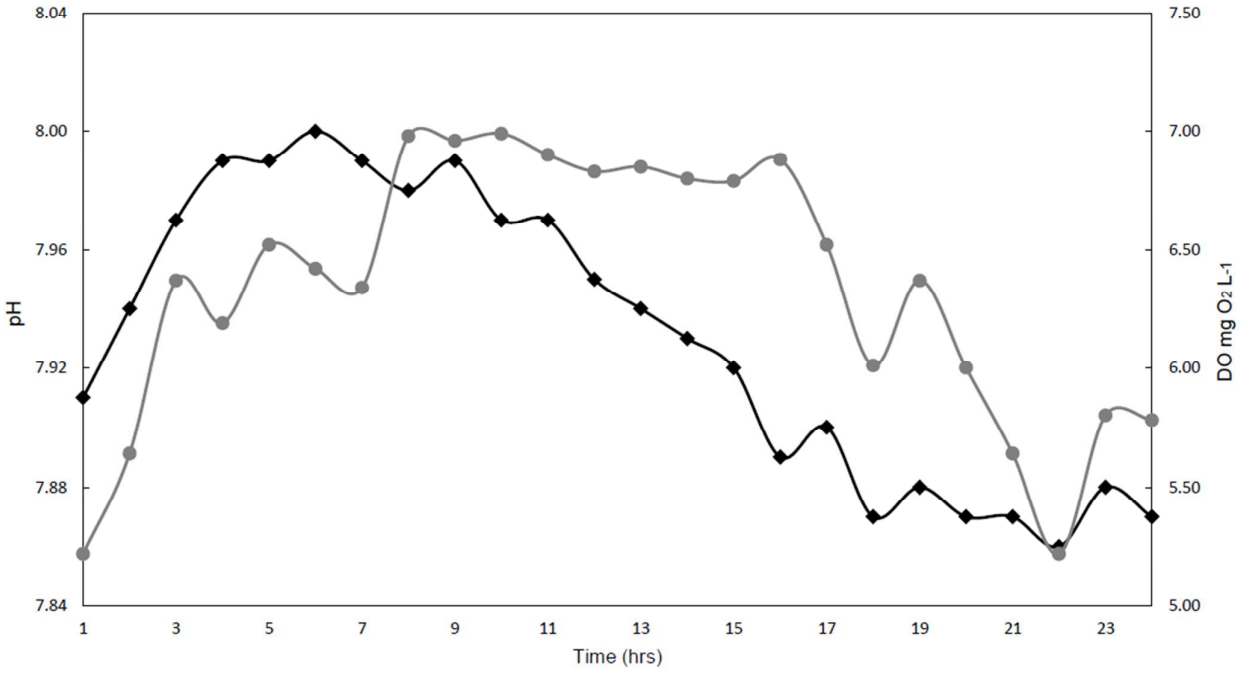
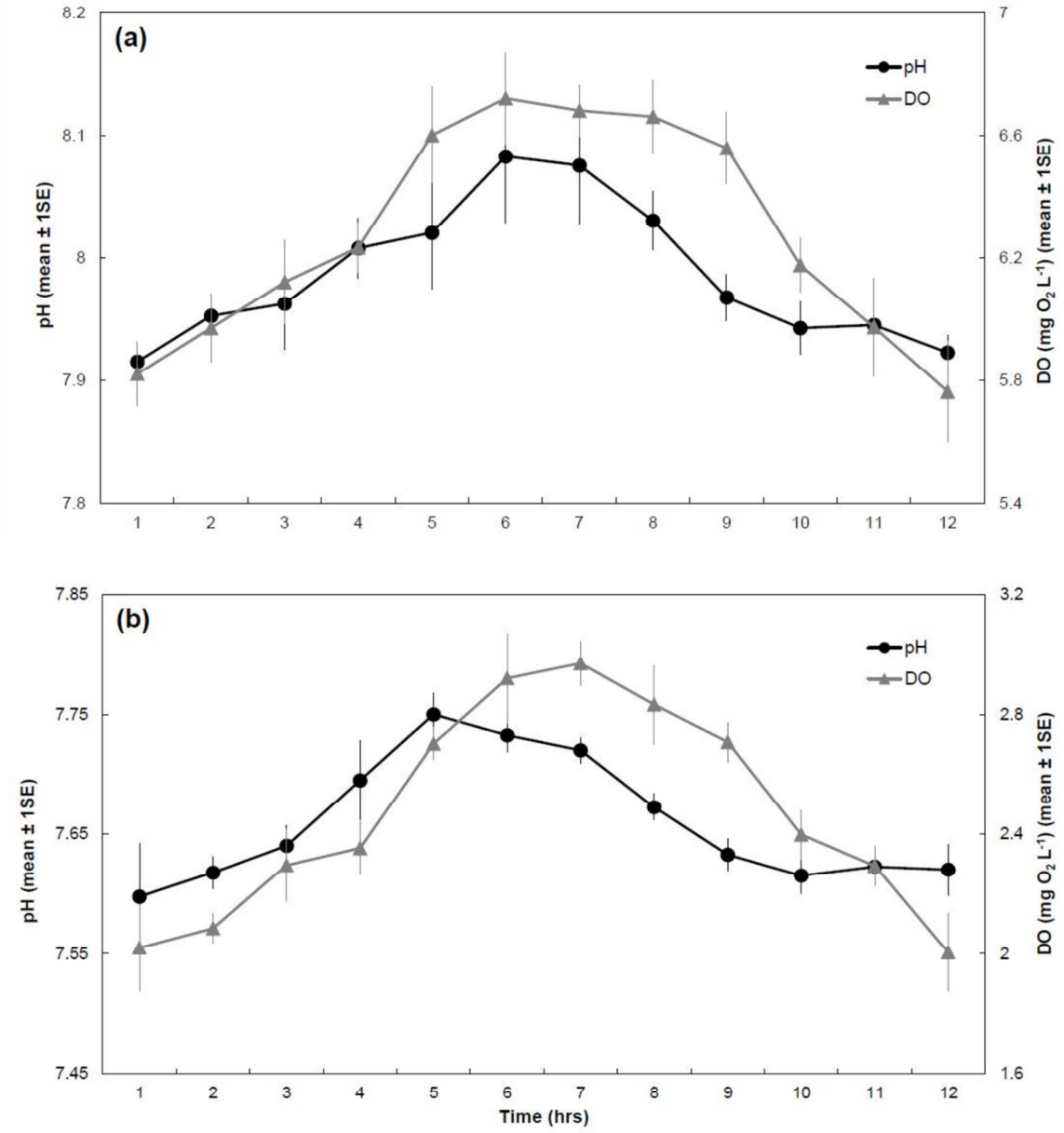


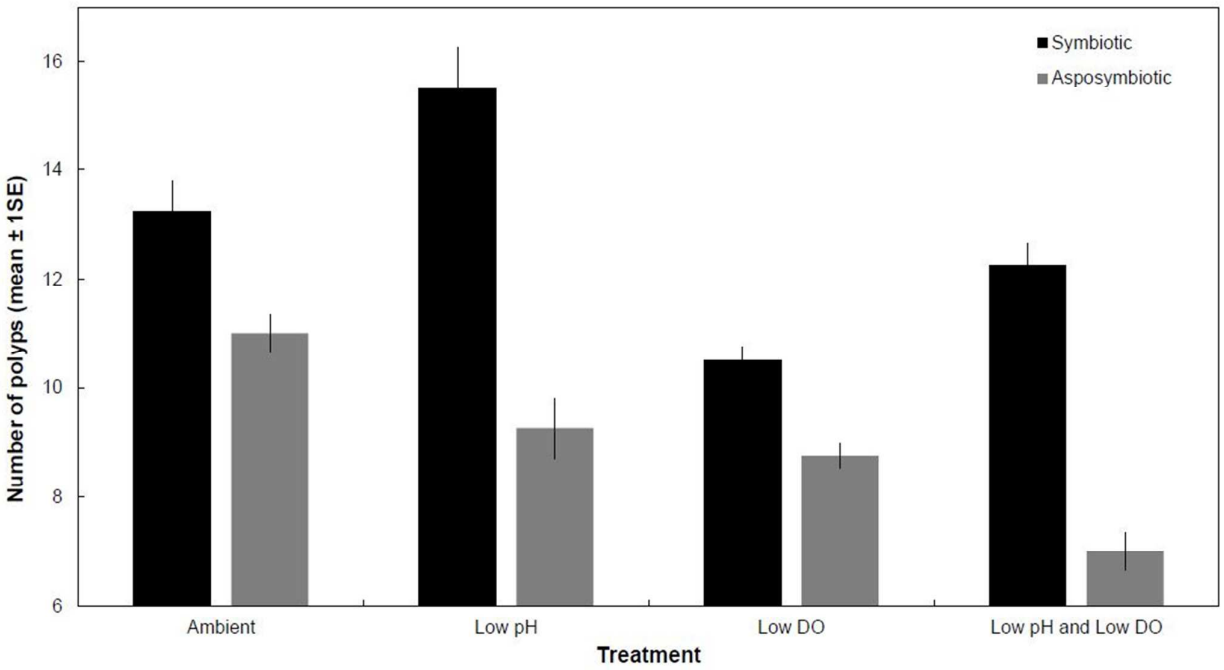
Figure S1 Absolute values and extent of diel variability for pH and DO taken at hourly intervals in October, 2014 in Moreton Bay, Australia (27.13ºS, 153.07ºE).



867

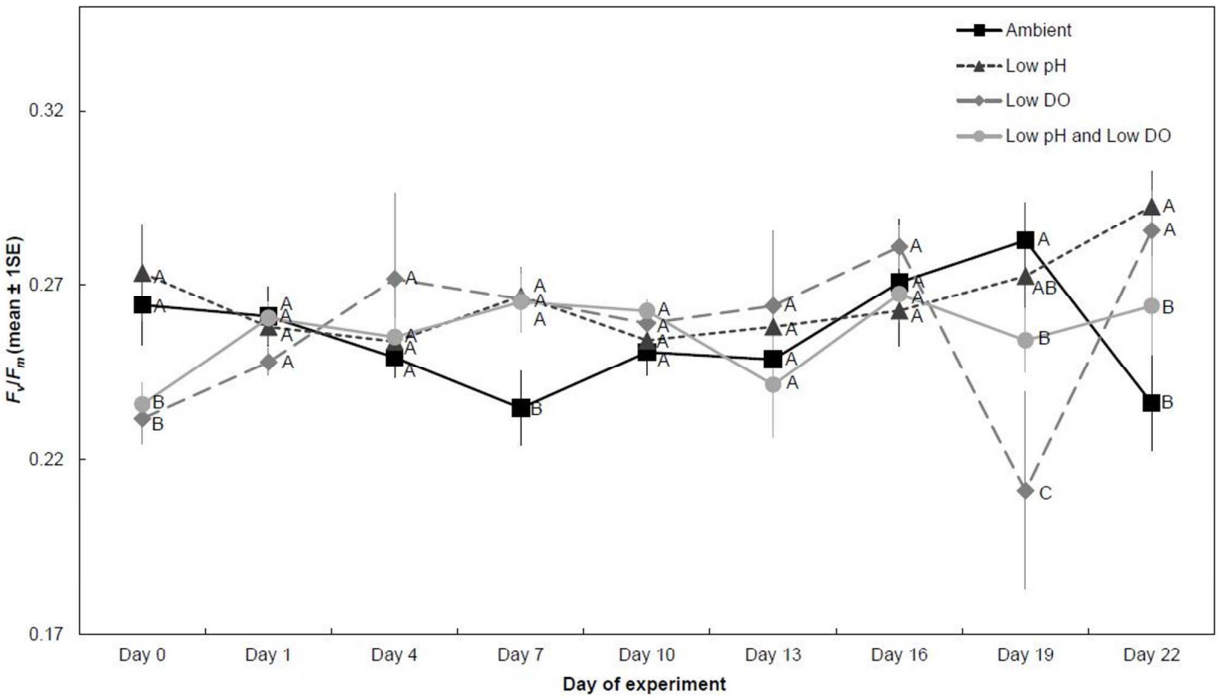
868 **Figure S2** Mean (±1SE) pH and DO measurements taken in (a) ambient treatments (b) low

869 pH and DO treatments taken at hourly intervals throughout the day.



870 **Figure S3** Mean ±1SE number of polyps in all treatments recorded at Day 22 of the

871 experiment.



872 **Figure S4** Mean (±1SE) maximum photochemical efficiency (F_v/F_m) of symbiotic polyps
873 during the 22 day experiment. Letters next to data points indicate similarities (e.g. AA) or
874 differences (e.g. AB) between treatments, as determined by estimated marginal means.

875 **Table S1** Mean ± 1SE water chemistry measurements taken during the 22 day experiment. Temperature, salinity and pH measurements were
876 taken at 10am every 3 days during the experiment and TA and pCO_2 measurements were taken weekly (Weeks 1, 2, 3, and 4).

Treatment	Temp (ºC)	Salinity (ppt)	Alkalinity (µeq kg $^{-1}$)	pH	Oxygen (mg O_2 L^{-1})	Calculated pCO_2 (µatm)
Symbiotic						
Control	25.1±0.02	36.3±0.09	2299.06± 9.3	8.00±0.02	6.3±0.14	421.07±27.7
Oxygen	25.1±0.03	36.2±0.07	2276.20±19.3	8.01±0.03	2.0±0.13	405.32±25.1
pH	25.1±0.02	36.2±0.07	2281.67±20.9	7.62±0.01	6.3±0.13	1151.36±48.3
Oxygen, pH	25.0±0.05	36.1±0.08	2267.18±30.6	7.63±0.02	2.3±0.20	1173.34±32.3
Aposymbiotic						
Control	25.1±0.02	36.3±0.08	2336.06± 7.3	8.01±0.03	6.2±0.19	410.01±28.6
Oxygen	25.1±0.04	36.2±0.07	2249.87±22.6	8.02±0.02	2.2±0.26	402.03±32.3
pH	25.2±0.03	36.4±0.08	2303.82±10.6	7.61±0.01	6.5±0.18	1189.02±43.9
Oxygen, pH	25.0±0.06	36.3±0.08	2260.85±32.5	7.63±0.01	2.1±0.18	1186.25±41.9

877

878 **Table S2** Summary of results for a LMMs analysis comparing effective quantum yield (F_v/F_m)
879 values between treatments during the 22 day experiment (Days 0, 1, 4, 7, 10, 13, 16, 19, 22). The
880 model-of-best-fit was CS, BIC (Bayesian Information Criterion) = -501.960, AIC (Akaike's
881 Information Criterion) = -507.325. Df= Degrees of freedom (numerator, denominator). *P* values in
882 bold are statistically significant ($P < 0.05$).

Source	Df	F	Sig.
Oxygen	1, 45.2	1.2	0.269
pH	1, 45.2	1.9	0.172
Time	8, 60.4	1.3	0.255
Oxygen × pH	1, 45.2	2.8	0.102
Oxygen × Time	8, 60.4	3.7	**0.002**
pH × Time	8, 60.4	0.9	0.514
Oxygen × pH × Time	8, 60.4	2.4	**0.026**

883

Table S3: Internal transcribed spacer 2 (ITS2) sequences used to genotype *Symbiodinium* cells in *Cassiopea sp.* polyps in this study. Note: only the sequences retrieved by this study with 100% query coverage to previously described *Symbiodinium* genotypes have been deposited in NCBI genbank.

pH treatment	Oxygen treatment	Sequence length (bp)	Accession (Genbank)	Type
Low pH	Control	291	KX533944	C1
Low pH	Control	291	KX533945	C1
Low pH	Control	291	KX533946	C1
Low pH	Control	301	KX533947	C1
Control	Hypoxic	302	KX533948	C1
Control	Hypoxic	291	KX533949	C1
Control	Hypoxic	291	KX533950	C1
Control	Hypoxic	302	KX533951	C1
Low pH	Hypoxic	291	KX533952	C1
Low pH	Hypoxic	291	KX533953	C1
Low pH	Hypoxic	291	KX533954	C1

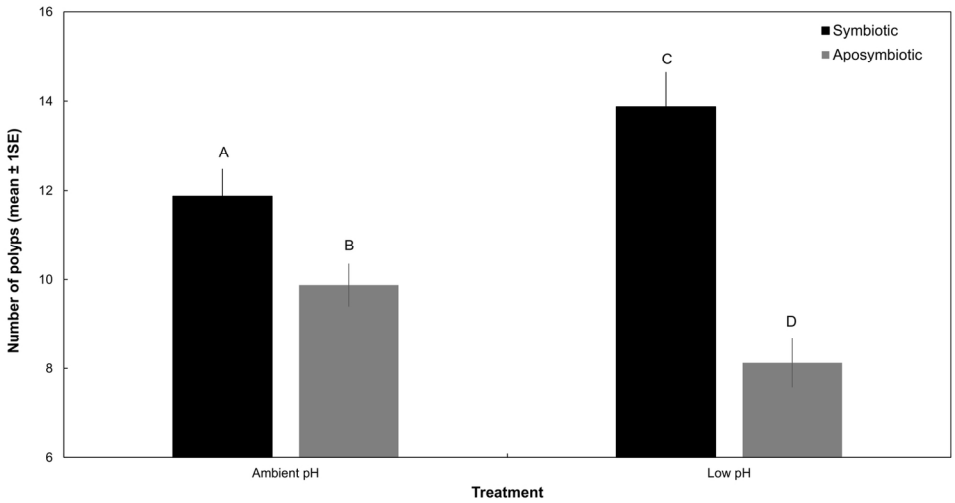


Figure 1 Mean ±1SE number of polyps recorded at Day 22 of the experiment. Letters above error bars indicate similarities (e.g. AA) or differences (e.g. AB) between treatments, as determined by estimated marginal means.
Figure 1
140x77mm (300 x 300 DPI)

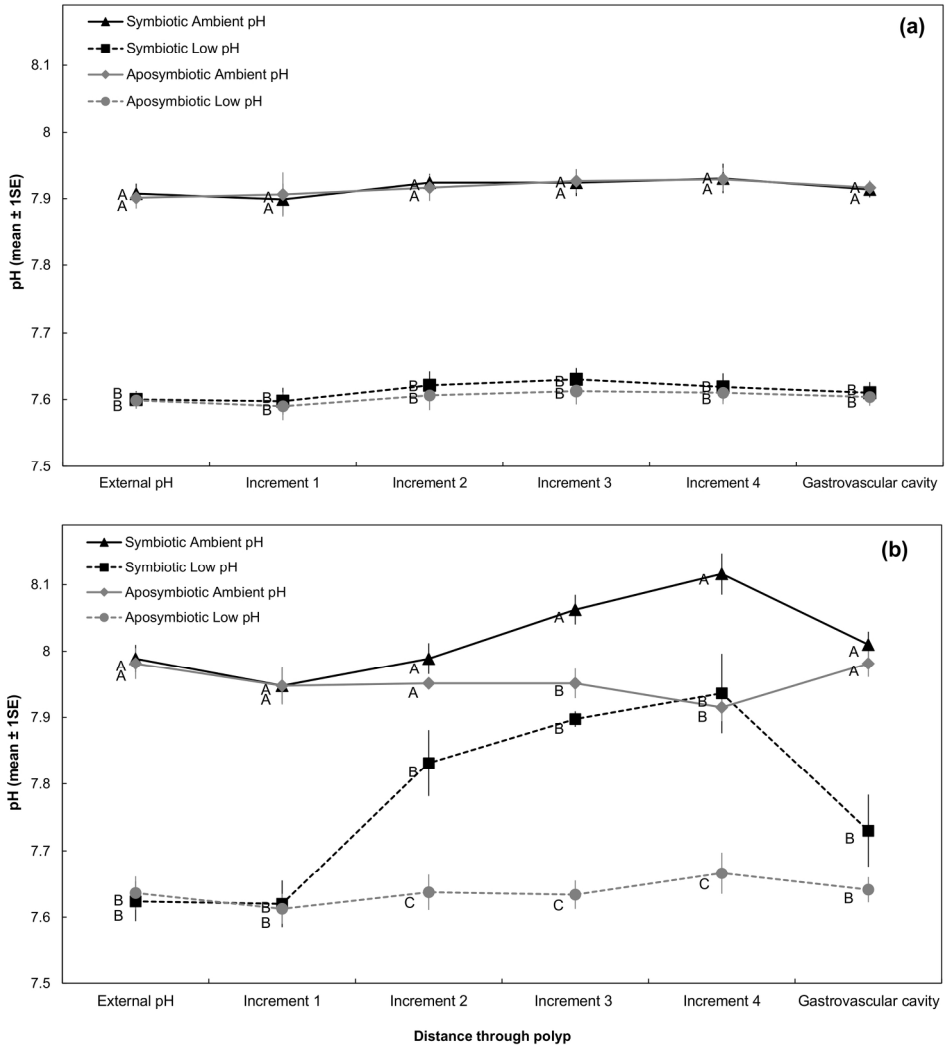


Figure 2 Mean (±1SE) pH measurements taken through polyp walls taken during the night (a) and day (b). Letters next to data points indicate similarities (e.g. AA) or differences (e.g. AB) between treatments, as determined by estimated marginal means.

Figure 2
199x223mm (300 x 300 DPI)

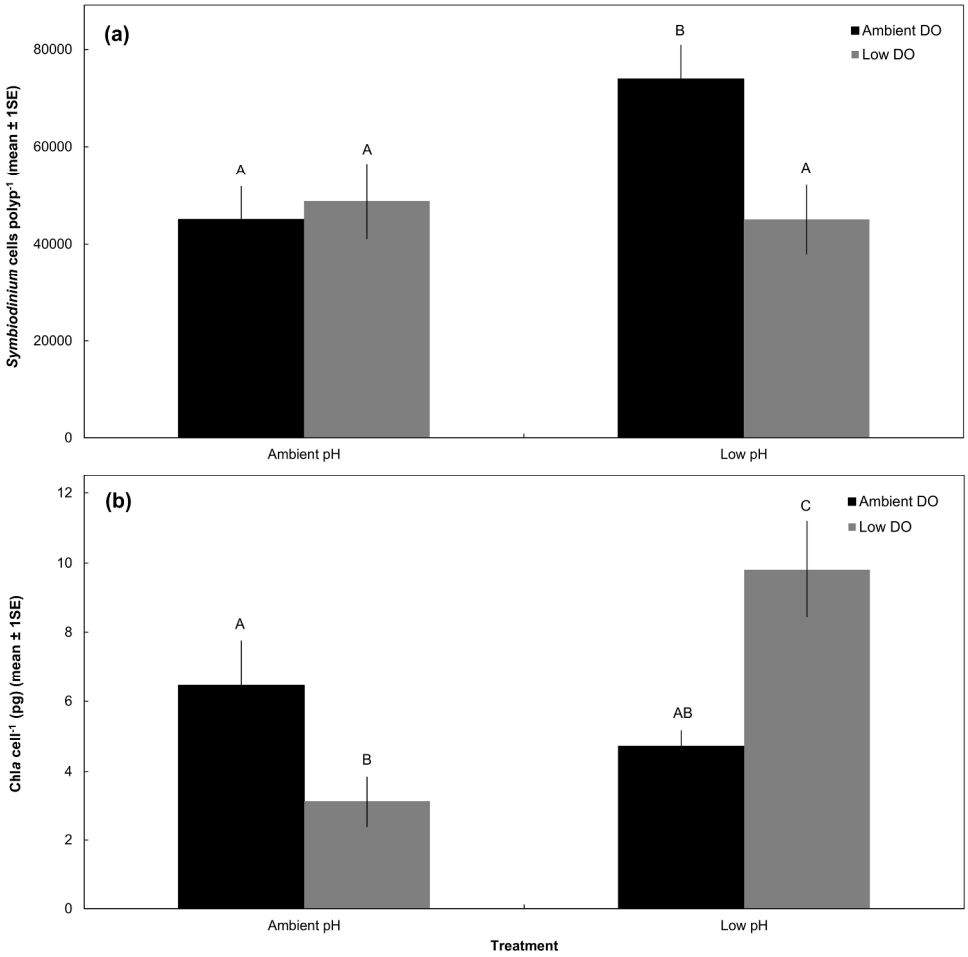


Figure 3 Mean (±1SE) Symbiodinium cells polyp-1 (a) Chla Symbiodinium cell-1 (pg) (b) at Day 22 of the experiment. Letters above error bars indicate similarities (e.g. AA) or differences (e.g. AB) between treatments, as determined by estimated marginal means.

Figure 3
266x268mm (300 x 300 DPI)