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

Running head CO₂ 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, chla 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 cnidian hosts, to the ubiquitous effects of ocean acidification. Importantly we
highlight that symbiotic, non-calcifying cnidarians may be particularly advantaged in
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 &
Mann, 1994, Gracey et al., 2001, Eerkes-Medrano et al., 2013), which simultaneously displaces CO$_2$ and O$_2$, to create conditions that are hypoxic but less (not more) acidic. Furthermore, this approach is unable to replicate the inherent variation to acidification and hypoxia that occurs on a diel basis (e.g. Regnault & Aldrich, 1988, Landry et al., 2007). Hence most manipulative experiments of hypoxia create conditions that are inconsistent with typical natural conditions (Gobler et al., 2014).

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

Whilst many marine organisms appear to be negatively impacted by acidification (Kroeker et al., 2010, Przeslawski et al., 2015), most observations of marine algae and seagrass suggest productivity and competitive fitness will be either enhanced (or at worst generally unchanged, Roleda et al., 2012, Asnaghi et al., 2013, Young & Gobler, 2016) by acidification. This poses an interesting conundrum for how the diverse range of invertebrates that host symbiotic dinoflagellates (e.g. Symbiodinium spp.) may respond to microbial-driven coastal hypoxia and associated acidification. Specifically, the presence of Symbiodinium spp.
may partially mitigate the combined effects of hypoxia and acidification, where photosynthetic release of O$_2$ could potentially ameliorate oxygen debt induced during hypoxic events (Malcolm & Brown, 1977) whilst the simultaneous acquisition of CO$_2$ may reduce pCO$_2$ within their surrounding host cells (Laurent et al., 2013, Gibbin et al., 2014, Laurent et al., 2014). Indeed, photosynthetic activity of Symbiodinium spp. may mitigate acidosis of host cells under high pCO$_2$ conditions (Gibbin et al., 2014). Elevated pCO$_2$ of surrounding seawater may also stimulate the photosynthetic activity of Symbiodinium spp. where cells have become CO$_2$ limited (see Suggett et al., 2012, Suggett et al., 2013, Gibbin et al., 2014, Ventura et al., 2016) and may act as a key condition needed to enhance oxygenation of the host tissues. Whilst this potential mitigating role of endosymbiotic algae is interesting, it may ultimately be restricted to certain Symbiodinium spp. genetic types with inherently inefficient CO$_2$ acquisition modes under ’present day’ seawater pCO$_2$ (see Brading et al., 2011, Brading et al., 2013). Even so, the role of in hospite Symbiodinium spp. in potentially mitigating the interactive effects of long-term hypoxia and acidification is unexplored.

Here we contrast the fitness of symbiotic and aposymbiotic polyps for a model host of Symbiodinium spp. (the jellyfish Cassiopea sp., Hofmann et al., 1978) in a full factorial design of reduced O$_2$ and pH for the first time. Absolute values and extent of diel variability for pH and DO were selected to mimic current-day hypoxic ecosystems. We specifically hypothesised that Cassiopea sp. polyps exposed to hypoxia and acidification (either in isolation or combination) would exhibit negative physiological effects but that these effects would be mitigated by the presence of Symbiodinium. Here we define host fitness as the rate of asexual reproduction of polyps and their ability to regulate internal pH (during the night and day), and the physiological response of Symbiodinium spp. was characterised via measurements of maximum photochemical efficiency, chla content, and Symbiodinium cell
density of symbiotic polyps. We also assessed whether different *Symbiodinium* spp. genotypes were selected for under the different treatments. From this experiment we present novel observations that demonstrate a potentially important role of *Symbiodinium* in facilitating enhanced fitness of *Cassiopea* sp. polyps (and perhaps also other non-calcifying cnidarians) to the ubiquitous effects of acidification and importantly that *Symbiodinium* also appeared to sustain fitness of *Cassiopea* sp. polyps when acidification and hypoxia co-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 25°C (±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
for the presence or absence of symbionts using pulse amplitude modulated (PAM) fluorometry (as used by: Steindler et al., 2002, Lemloh et al., 2009) and fluorescence microscopy with UV Illumination prior to the start of the experiment and every second day during the experiment.

The experimental design consisted of three orthogonal factors: pH (control (24h mean ±1SE, 7.95 ± 0.01) versus reduced (7.63 ± 0.01)), dissolved O$_2$ (DO) concentration (control (6.14 mgL$^{-1}$ ± 0.03) versus hypoxic (2.09 mgL$^{-1}$ ± 0.03)) and polyp type (symbiotic versus aposymbiotic). Four replicate aquaria were randomly allocated to each combination of pH, oxygen and polyp type (i.e. n=32). Six polyps were transferred using a toothpick into small plastic petri dishes weighted with stainless steel weights. Three petri dishes were then immersed in 1L glass aquaria (i.e. 18 polyps per aquarium); one petri dish was allocated for asexual reproduction measurements and the other two were used for pH microelectrode and chlorophyll fluorescence measurements. Three petri dishes were allocated to each replicate aquarium to prevent overcrowding of polyps and to ensure that polyps used to measure one response variable were not re-sampled for others during the experiment. The experiment ran for 22 days and was performed in a controlled temperature laboratory with the ambient temperature set at 25ºC.

Absolute values and extent of diel variability for pH and DO of control treatments were replicated based on 24h field measurements taken in October, 2014 in Moreton Bay, Australia (27.13ºS, 153.07ºE) (Fig. S1). In hypoxic systems, the magnitude of diel fluctuations of pH and DO can vary depending on the ecosystem being tested. We, therefore, selected moderate levels of DO and diel variation (1.5mgL$^{-1}$ - 3.0mgL$^{-1}$) from a range data collected from current-day hypoxic ecosystems in coastal ecosystems (e.g. Park et al., 2007, Tyler et al., 2009). Levels of DO and pH are stoichiometrically linked in marine ecosystems (Cai et al., 2011) and pH can vary between 6.9- 7.9 during hypoxic events (Gobler et al.,
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$_2$SYS (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 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
polyps were alive and upright (i.e. perpendicular to the microelectrode). The microelectrode was inserted halfway between the base of the polyp and the beginning of the oral arms, to ensure consistency of microelectrode profiles among all polyps. The epidermis formed a seal around the electrode and prevented fluid exchange between the external seawater and the polyp tissue (sensu Revsbech et al., 1995, Köhler-Rink & Kühl, 2000). Polyps varied slightly in shape and size and so to ensure consistency, pH was recorded continuously through one side of the polyp until the gastrovascular cavity was reached. Four increments through the polyp were then determined relative to the thickness of the body wall (i.e. the number of measurements taken through the polyp wall) to account for differences in shape and size, yielding 6 measurements per polyp (i.e. external pH, increments 1-4 through the polyp wall, and gastrovascular cavity). Prior to pH measurements, the pH micro-electrodes were calibrated with pH 7 and 10 buffer solutions. Fluorescent pH sensitive dye, 5(6)-Carboxynaphthofluorescein, (sourced from Sigma-Aldrich, CAS no: 128724-35-6) was used to confirm relative pH changes of microelectrode profiles using a fluorescence microscope.

Polyps were noninvasively incubated at 25°C in their aquarium water supplemented with dye to a final concentration of 50 µmol L⁻¹ 5(6)-Carboxynaphthofluorescein for 30 minutes to allow sufficient uptake. Polyps were placed in 3mL vials and the pH-sensitive dye solution was allowed to overflow the vials into water baths at a rate of ~2mL min⁻¹ to ensure the solution was gently mixed. Following staining, polyps were gently placed on depression microscope slides using a pipette and photographed horizontally using a Nikon Eclipse 80i fluorescence microscope with UV Illumination. The pH-sensitive dye solution was excited at a rate of 0.2Hz at A598nm. To confirm the response of the fluorescent dye, phosphate buffers of known concentrations were supplemented with the pH-sensitive dye to a final concentration of 50 µmol L⁻¹ to construct a two-point calibration curve. All polyps used for internal pH measurements were sacrificed.
Maximum photochemical efficiency (F\textsubscript{v}/F\textsubscript{m}), chla content and Symbiodinium cell density

We measured another three response variables on symbiotic polyps (maximum photochemical efficiency, F\textsubscript{v}/F\textsubscript{m}), Chla 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) chla fluorescence inductions were made to return values of the minimum (F\textsubscript{0}) and maximum fluorescence yield (F\textsubscript{m}), and hence the maximum PSII photochemical efficiency (F\textsubscript{v}/F\textsubscript{m} = (F\textsubscript{m} - F\textsubscript{0})/F\textsubscript{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 -80°C 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 chla content. All chla samples were centrifuged at 3000 × g at 4°C for 10 minutes and the supernatant discarded. The pelleted Symbiodinium were resuspended in 95% ethanol and extracted overnight in the dark at 4°C 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. Chla content was determined using coefficients from a spectrophotometric equation (-2.6094 × A629 + 12.4380 × A665) for dinoflagellates in ethanol (Ritchie, 2006). Chla (units) concentrations were normalised to corresponding measures of cell content (units) to also yield the chla [cell]⁻¹ (units). Symbiodinium cell density and chla concentrations were also normalised to the number of polyps analysed to yield the Symbiodinium and chla [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, Chla, Symbiodinium density and Symbiodinium Chla 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 Chla, cell density and chla [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 (asesexual 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 chla 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 chla 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
Symbiodinium cell densities to those in the control treatment regardless of the pH conditions they were exposed to (Fig. 3a). Intriguingly, observations of Symbiodinium chla cell\(^{-1}\) were not consistent with those of Symbiodinium cell densities. Symbiodinium chla cell\(^{-1}\) was highest in the low pH and low DO treatment and exceeded that of all other treatments (Fig. 3b). Consistent with reduced asexual reproduction of polyps under low DO conditions, hypoxia in isolation resulted in a 52% lower chla cell\(^{-1}\) concentration relative to the control treatment (Fig. 3b). This response was not consistent when symbiotic polyps were exposed to hypoxia and low pH in combination, where chla cell\(^{-1}\) was 52% higher under hypoxic and low pH conditions than under low pH conditions alone (Fig. 3b).
Discussion

Elevated CO₂ (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 CO₂ 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 CO₂ 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 O₂ 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
observed negative effects of hypoxia under acidification conditions, we hypothesise that our
various observations potentially indicate an important role for photorespiration upon
exposure to the dual stressors. The apparent mitigation of hypoxia only when acidification
coco-occurred in the presence of *Symbiodinium in hospite* is consistent with reduced
photorespiration under elevated CO$_2$ conditions (see Crawley *et al.*, 2010). A reduction in
photorespiration would then increase net O$_2$ production and thus increase O$_2$ concentrations
within host tissues thereby ameliorating oxygen debt. Although data are limited on the effects
of acidification on rates of photorespiration in symbiotic cnidarians, some studies
demonstrate increases in net oxygen production of non-calcifying, symbiotic cnidarians
(anemones, Suggett *et al.*, 2012, Jarrold *et al.*, 2013, Gibbin & Davy 2014) under elevated
pCO$_2$ conditions despite increased respiration rates at elevated pCO$_2$ concentrations (in some
cases, Suggett *et al.*, 2012, Gibbin & Davy 2014). Indeed, any increase in net oxygen
production must equate to a net increase in CO$_2$ fixation during photosynthesis (Reece *et al.*, 2015). In addition to reduced costs of carbon acquisition under high pCO$_2$ (Ventura *et al.*, 2016), we hypothesise that inhibition of photorespiration may also partially explain the
higher rate of asexual reproduction of symbiotic polyps under acidification alone due to
increased efficiency of carbon fixation and increased availability of organic carbon for
growth. Clearly, better understanding processes such as photorespiration is needed to assess
why only some *Symbiodinium* types, i.e. perhaps those more susceptible to Rubisco
oxygenation under relatively low CO$_2$ conditions via differences in Rubisco pool sizes and/or
turnover, appear to benefit from elevated CO$_2$ availability (e.g. Brading *et al.*, 2013).

Survival of marine organisms in hypoxic systems may partly depend on their ability
to regulate their internal pH under the lowered pH conditions that result from elevated DIC
concentrations. During the day, under low pH conditions, the internal pH of symbiotic polyps
matched the pH levels of aposymbiotic polyps exposed to ambient conditions, which suggests
that photosynthesis of *Symbiodinium* regulated the internal pH of the polyps. Aposymbiotic polyps, however, conformed to the pH of their respective treatments, indicating that *Cassiopea* sp. polyps, as hosts, may have limited ability to regulate their internal pH. Our results are consistent with the few other studies that have investigated the diel regulation of internal pH of cnidarian host cells (Venn et al., 2009, Laurent et al., 2013, Laurent et al., 2014, Gibbin et al., 2014. For example, the only other study that has compared the internal pH of symbiotic and non-symbiotic cells (isolated from a coral) under elevated CO$_2$ conditions reported that the internal pH of non-symbiotic cells decreased by 0.3-0.4 when exposed to decreasing pH (from 7.8 to 6.8) but the internal pH of symbiotic cells recovered to control levels (Gibbin et al., 2014). Combined, these studies highlight the significant control that *Symbiodinium* exert over the internal pH of their host tissues and suggest that symbiotic biota will typically be more robust to hypoxic environments than non-symbiotic biota.

Survival of symbiotic organisms ultimately depends on the physiological limitations of both the host and their symbionts. Maximum photochemical efficiency values of *Symbiodinium* were low relative to those previously observed for *Symbiodinium in hospite* of cnidarians (e.g. Enochs et al., 2014, Hoadley et al., 2015, including polyps of *Cassiopea* sp. Klein et al., 2016) and could reflect a number of biological (e.g. high light fields, chlororespiration), or measurement artefacts (e.g. lower values expected with imaging PAM, Levin et al., 2017) that cannot presently be ascertained. Low maximum photochemical efficiency values were unlikely induced by stress since all other response variables (i.e. asexual reproduction, *Symbiodinium* densities, Chla cell$^{-1}$) indicate that ambient experimental conditions were optimal for *Symbiodinium*. Even so, maximum photochemical efficiency appeared to be unaffected by the various treatments tested, suggesting that any artefact was constant. *Symbiodinium* densities (and host asexual reproduction), however, were highest in 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, chla 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 Chla 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
‘symbiont shuffling’) (Little et al., 2004, Berkelmans & van Oppen, 2006). However, in our study we only detected *Symbiodinium* ITS2 type C1, which is a generalist symbiont (LaJeunesse, 2005) that has been observed in >100 host species, including *Cassiopea* (Franklin et al., 2012, Tonk et al., 2013, Mellas et al., 2014). Since we detected only one variant of *Symbiodinium*, there was no evidence of major symbiont shuffling of the dominant symbiont populations, although we cannot rule out that the techniques used in this study and the ITS2 marker may not fully capture changes in dominance of population heterogeneity, notably for type C1 (see Howells et al., 2016, Wham & LaJeunesse, 2016). It is also possible that cryptic *Symbiodinium* variants may have occurred at levels below the detection thresholds of the techniques we used (e.g. Boulotte et al., 2016), although the short duration of our experiment (but see Lewis & Coffroth, 2004) probably precluded the potential for shuffling to occur. Even if some *Cassiopea* sp. polyps harbour only one *Symbiodinium* type, they may still change their symbionts via uptake of new symbiont types from the environment (termed symbiont ‘switching’, *sensu* Baker, 2003). Whilst the typical symbionts that are *in hospite* of *Cassiopea* spp. populations in Australia are at present unknown, it is well demonstrated that *Cassiopea* spp. polyps can host multiple variants of *Symbiodinium* and acquire additional symbiont types at the polyp stage (including clades A, B, C and D, Mellas et al., 2014). However, polyps in our experiment were not exposed to exogenous *Symbiodinium* cells and thus could not have acquired new symbiont types unless symbionts were shared horizontally between polyps (see Sachs & Wilcox, 2006). To more accurately predict how symbiotic cnidarians, as a group, may respond to hypoxia and acidification we must also consider that hosts may acquire more resistant symbionts, including types that are physiologically adapted to extreme environmental conditions in the longer term (Brading et 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
interactive effects of low DO and pH on non-symbiotic, non-calcifying cnidarians (two species of anemones (Anemonia alicemartinae and Phymactis papillosa) (Steckbauer et al., 2015). Although both anemones are naturally non-symbiotic (unlike Cassiopea sp.), the study demonstrated that exposure to acidification alone increased the metabolism of A. alicemartinae and P. papillosa but exposure to acidification and hypoxia in combination depressed metabolism of both species. However, we cannot determine whether our results are consistent with other studies that mimic hypoxia using N₂ gas because we did not expose aposymbiotic polyps to low DO and high pH in combination; for this, studies will need to further compare the response of biota to low DO and high pH in combination to those exposed to hypoxia in isolation to assess the reliability of results obtained by studies of hypoxia that manipulate O₂ levels with N₂ gas.

Symbiodinium are clearly important in mitigating the combined effects of hypoxia and acidification on Cassiopea sp. polyps. Our data suggest that symbiotic Cassiopea sp. may still thrive in hypoxic environments and although aposymbiotic Cassiopea sp. may persist, they are unlikely to proliferate when exposed to the dual stressors. Symbiotic (non-calcifying) cnidarians, such as jellyfish (tested here) but perhaps also other non-calcifying cnidarians may therefore have a greater competitive advantage in current-day hypoxic zones. Our observations that Symbiodinium mitigated the negative effects of hypoxia when acidification co-occurred highlights the importance of investigating the concurrent effects of hypoxia and acidification on symbiotic biota. Although the response of aposymbiotic polyps to hypoxia was unaffected by acidification, manipulative experiments of hypoxia need to consider concurrent changes in pH to accurately reflect field observations of hypoxic zones. We therefore advocate for a prompt re-alignment of future studies of hypoxia and suggest that future experiments consider concurrent changes in DO and pH. Whilst Symbiodinium may benefit non-calcifying cnidarians in current-day hypoxic zones (tested here),
Symbiodinium are sensitive to transient heat stress and thus to understand whether this potentially important role of Symbiodinium holds under future additional warming scenarios, we now need to investigate the effects of additional warming in combination with hypoxia and acidification.
Acknowledgements

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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.

<table>
<thead>
<tr>
<th>Source</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symbiont</td>
<td>106.778</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oxygen</td>
<td>49.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH</td>
<td>0.111</td>
<td>0.742</td>
</tr>
<tr>
<td>Symbiont × Oxygen</td>
<td>1.000</td>
<td>0.327</td>
</tr>
<tr>
<td>Symbiont × pH</td>
<td>25.000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oxygen × pH</td>
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<td>0.742</td>
</tr>
<tr>
<td>Symbiont × Oxygen × pH</td>
<td>0.111</td>
<td>0.742</td>
</tr>
</tbody>
</table>
Table 2 Summary of results for a LMMs analysis comparing day and night pH microelectrode profiles between treatments at Day 22 of the experiment. The model-of-best-fit was AR(1), BIC (Bayesian Information Criterion) = -1268.564, AIC (Akaike’s Information Criterion) = -1276.198. Note, the factor Oxygen was included in preliminary analysis but removed due to non-significance for all terms (P>0.05) and LMMs analysis was re-run. Df= Degrees of freedom (numerator, denominator). P values in bold are statistically significant (P < 0.05).

<table>
<thead>
<tr>
<th>Source</th>
<th>Numerator df</th>
<th>Denominator df</th>
<th>F</th>
<th>Sig.</th>
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</thead>
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<tr>
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<td>89.181</td>
<td>309.977</td>
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<td>Symbiont</td>
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<td>89.181</td>
<td>120.335</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH</td>
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<td>89.181</td>
<td>3810.007</td>
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<td>Day/Night × Symbiont</td>
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<td>89.181</td>
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<tr>
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<tr>
<td>Day/Night × Symbiont × pH</td>
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<td>10.509</td>
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<tr>
<td>Distance</td>
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<td>239.171</td>
<td>19.691</td>
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<td>32.763</td>
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<td>239.171</td>
<td>4.628</td>
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Table 3 Summary of results for three LMMs comparing *Symbiodinium* density (cell polyp\(^{-1}\)) and chla cell\(^{-1}\) (pg) between treatments of symbiotic polyps at Day 22 of the experiment. Df = degrees of freedom. BIC = Bayesian Information Criterion and AIC = Akaike’s Information Criterion. *P* values in bold are statistically significant (*P* < 0.05). For all sources of variation numerator df =1 and denominator df =24.

<table>
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<tr>
<th>Variable</th>
<th><em>Symbiodinium</em> polyp(^{-1})</th>
<th>Chla cell(^{-1})</th>
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<td>Transformation</td>
<td>Ln</td>
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<tr>
<td>Information Criterion</td>
<td>BIC = 10.359</td>
<td>BIC = 59.840</td>
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<tr>
<td></td>
<td>AIC = 9.874</td>
<td>AIC = 59.355</td>
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<td>Source of variation</td>
<td><em>P</em></td>
<td><em>P</em></td>
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<td></td>
<td><em>F</em>=2.633</td>
<td><em>F</em>=5.623</td>
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<tr>
<td>Oxygen</td>
<td>0.124</td>
<td>0.419</td>
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<td></td>
<td><em>F</em>=2.730</td>
<td><em>F</em>=0.700</td>
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<tr>
<td>pH × Oxygen</td>
<td>0.047</td>
<td>0.002</td>
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<tr>
<td></td>
<td><em>F</em>=5.095</td>
<td><em>F</em>=16.215</td>
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Figure captions

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 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 3 Mean (±1SE) *Symbiodinium* cells polyp$^{-1}$ (a) Chl *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.
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 (27.13°S, 153.07°E).
Figure S2 Mean (±1SE) pH and DO measurements taken in (a) ambient treatments (b) low pH and DO treatments taken at hourly intervals throughout the day.
Figure S3 Mean ±1SE number of polyps in all treatments recorded at Day 22 of the experiment.
Figure S4 Mean (±1SE) maximum photochemical efficiency ($F_v/F_m$) of symbiotic polyps during the 22 day experiment. Letters next to data points indicate similarities (e.g. AA) or differences (e.g. AB) between treatments, as determined by estimated marginal means.
**Table S1** Mean ± 1SE water chemistry measurements taken during the 22 day experiment. Temperature, salinity and pH measurements were taken at 10am every 3 days during the experiment and TA and pCO₂ measurements were taken weekly (Weeks 1, 2, 3, and 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp (ºC)</th>
<th>Salinity (ppt)</th>
<th>Alkalinity (µeq kg⁻¹)</th>
<th>pH</th>
<th>Oxygen (mg O₂ L⁻¹)</th>
<th>Calculated pCO₂ (µatm)</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.1±0.02</td>
<td>36.3±0.09</td>
<td>2299.06±9.3</td>
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<td>Oxygen</td>
<td>25.1±0.03</td>
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<td>2.3±0.20</td>
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<td><strong>Aposymbiotic</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.1±0.02</td>
<td>36.3±0.08</td>
<td>2336.06±7.3</td>
<td>8.01±0.03</td>
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<td>pH</td>
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<td>25.0±0.06</td>
<td>36.3±0.08</td>
<td>2260.85±32.5</td>
<td>7.63±0.01</td>
<td>2.1±0.18</td>
<td>1186.25±41.9</td>
</tr>
</tbody>
</table>
Table S2 Summary of results for a LMMs analysis comparing effective quantum yield ($F_v/F_m$) values between treatments during the 22 day experiment (Days 0, 1, 4, 7, 10, 13, 16, 19, 22). The model-of-best-fit was CS, BIC (Bayesian Information Criterion) = -501.960, AIC (Akaike’s Information Criterion) = -507.325. Df= Degrees of freedom (numerator, denominator). $P$ values in bold are statistically significant ($P < 0.05$).

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>1, 45.2</td>
<td>1.2</td>
<td>0.269</td>
</tr>
<tr>
<td>pH</td>
<td>1, 45.2</td>
<td>1.9</td>
<td>0.172</td>
</tr>
<tr>
<td>Time</td>
<td>8, 60.4</td>
<td>1.3</td>
<td>0.255</td>
</tr>
<tr>
<td>Oxygen $\times$ pH</td>
<td>1, 45.2</td>
<td>2.8</td>
<td>0.102</td>
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<tr>
<td>Oxygen $\times$ Time</td>
<td>8, 60.4</td>
<td>3.7</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>pH $\times$ Time</td>
<td>8, 60.4</td>
<td>0.9</td>
<td>0.514</td>
</tr>
<tr>
<td>Oxygen $\times$ pH $\times$ Time</td>
<td>8, 60.4</td>
<td>2.4</td>
<td><strong>0.026</strong></td>
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</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>pH treatment</th>
<th>Oxygen treatment</th>
<th>Sequence length (bp)</th>
<th>Accession (Genbank)</th>
<th>Type</th>
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<tbody>
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<td>Low pH</td>
<td>Control</td>
<td>291</td>
<td>KX533944</td>
<td>C1</td>
</tr>
<tr>
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<td>Control</td>
<td>291</td>
<td>KX533945</td>
<td>C1</td>
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<tr>
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<td>Control</td>
<td>291</td>
<td>KX533946</td>
<td>C1</td>
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<td>Low pH</td>
<td>Control</td>
<td>301</td>
<td>KX533947</td>
<td>C1</td>
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<tr>
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<td>Hypoxic</td>
<td>302</td>
<td>KX533948</td>
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</tr>
<tr>
<td>Control</td>
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<td>291</td>
<td>KX533949</td>
<td>C1</td>
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<td>291</td>
<td>KX533950</td>
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<td>291</td>
<td>KX533954</td>
<td>C1</td>
</tr>
</tbody>
</table>
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)