MS. SHANNON GRACE KLEIN (Orcid ID : 0000-0001-8190-3188)

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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

Authors [1]Shannon G. Klein, [1]Kylie A. Pitt, [2]Matthew R. Nitschke, [2]Samantha Goyen, [3]David T. Welsh, [2]David J. Suggett, [3]Anthony R. Carroll

Institute of Origin [1]Australian Rivers Institute – Coasts and Estuaries, Griffith School of Environment, Gold Coast, Griffith University, QLD 4222, Australia. [2] Climate Change Cluster (C3), University of Technology Sydney, NSW 2007, Australia. [3]Environmental Futures Research Institute, Griffith School of Environment, Gold Coast, Griffith University, QLD 4222, Australia.

Corresponding Author Shannon G. Klein, Ph: +61-7- 5552-8463, shannon.klein@griffithuni.edu.au

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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 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, chl*a* 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 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 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

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

164 Absolute values and extent of diel variability for pH and DO of control treatments were
165 replicated based on 24h field measurements taken in October, 2014 in Moreton Bay, Australia
166 (27.13ºS, 153.07ºE) (Fig. S1). In hypoxic systems, the magnitude of diel fluctuations of pH and
167 DO can vary depending on the ecosystem being tested. We, therefore, selected moderate levels of
168 DO and diel variation ($1.5mgL^{-1}$- $3.0mgL^{-1}$) from a range data collected from current-day hypoxic
169 ecosystems in coastal ecosystems (e.g. Park *et al.*, 2007, Tyler *et al.*, 2009). Levels of DO and pH
170 are stoichiometrically linked in marine ecosystems (Cai *et al.*, 2011) and pH can vary between
171 6.9- 7.9 during hypoxic events (Gobler *et al.*, 2014, Gobler & Baumann, 2016). We, therefore,
172 selected moderate pH levels (pH 7.5-7.75) for the low pH treatments from a range of pH data
173 collected from coastal hypoxic systems (e.g. Cai *et al.*, 2011, Melzner *et al.*, 2012, Gobler *et al.*,
174 2014).

175 *Manipulation of water chemistry*

176 To achieve the desired water chemistry of each treatment, a series of gas mass controllers
177 were used to deliver mixtures of CO_2, N_2 and O_2 gas to seawater (also see Bockmon *et al.*, 2013).
178 The desired gas compositions (CO_2, N_2, O_2) were mixed from individual gas cylinders using four
179 sets of three Omega® mass flow controllers (FMA-5400s, 0-20 mL/min (CO_2), 0-5 L/min (N_2),
180 0-2 L/min (O_2)), which allowed for four independent treatments. The mass flow controllers were
181 operated and functions monitored by a desktop PC running NI LabVIEW™ software (32-bit
182 version) with communication using a voltage generating Omega® Expandable Modular Data
183 Acquisition System® (iNET-400) connected with three Omega® wiring boxes with screw
184 terminals (iNET-510). The desired proportions of CO_2, N_2 and O_2 were mixed in a stainless steel
185 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 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 25ºC 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 80*i* fluorescence microscope with UV Illumination. The pH-sensitive dye solution was excited at a rate of 0.2Hz at A_{598nm}. 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_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* $[\text{cell}]^{-1}$ (units). *Symbiodinium* cell density and chl*a* concentrations were also normalised to the number of polyps analysed to yield the *Symbiodinium* and chl*a* $[\text{polyp}]^{-1}$ (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 *Symbiodinium* cell densities to those in the control treatment regardless of the pH conditions they were exposed to (Fig. 3a). Intriguingly, observations of *Symbiodinium* chl*a* cell^{-1} were not consistent with those of *Symbiodinium* cell densities. *Symbiodinium* chl*a* 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 chl*a* 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 chl*a* cell^{-1} was 52% higher under hypoxic and 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 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 co-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, Chl*a* $cell^{-1}$) 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, 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 '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 N_2 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 O_2 levels with N_2 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.

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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

Source	Numerator df	Denominator df	F	Sig.
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	

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 ($P < 0.05$).

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**

Table 3 Summary of results for three LMMs comparing *Symbiodinium* density (cell polyp^{-1}) and chl*a* 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.

Variable	***Symbiodinium* polyp^{-1}**	**Chl*a* cell^{-1}**
Transformation	Ln	None
Information Criterion	BIC= 10.359 AIC= 9.874	BIC= 59.840 AIC= 59.355
Source of variation	*P*	*P*
pH	0.131 *F*=2.633	**0.035** *F*=5.623
Oxygen	0.124 *F*=2.720	0.419 *F*=0.709
pH × Oxygen	**0.047** *F*=5.095	**0.002** *F*=16.215

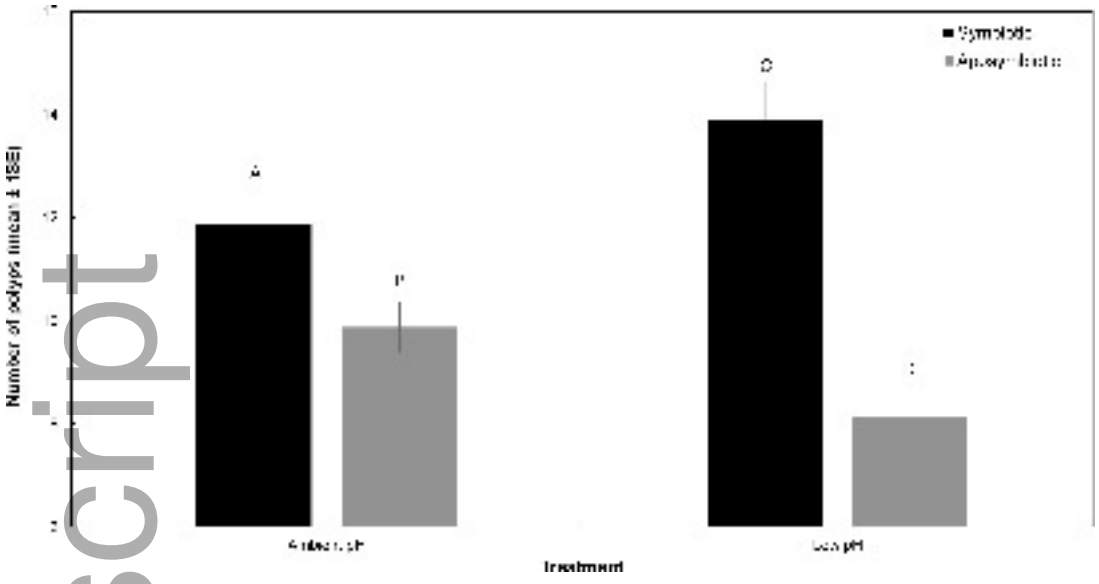
Figure captions

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.

846

847 **Figure 3** Mean (±1SE) *Symbiodinium* cells polyp^{-1} (a) Chl*a* *Symbiodinium* cell^{-1} (pg) (b) at Day
848 22 of the experiment. Letters above error bars indicate similarities (e.g. AA) or differences (e.g.
849 AB) between treatments, as determined by estimated marginal means.



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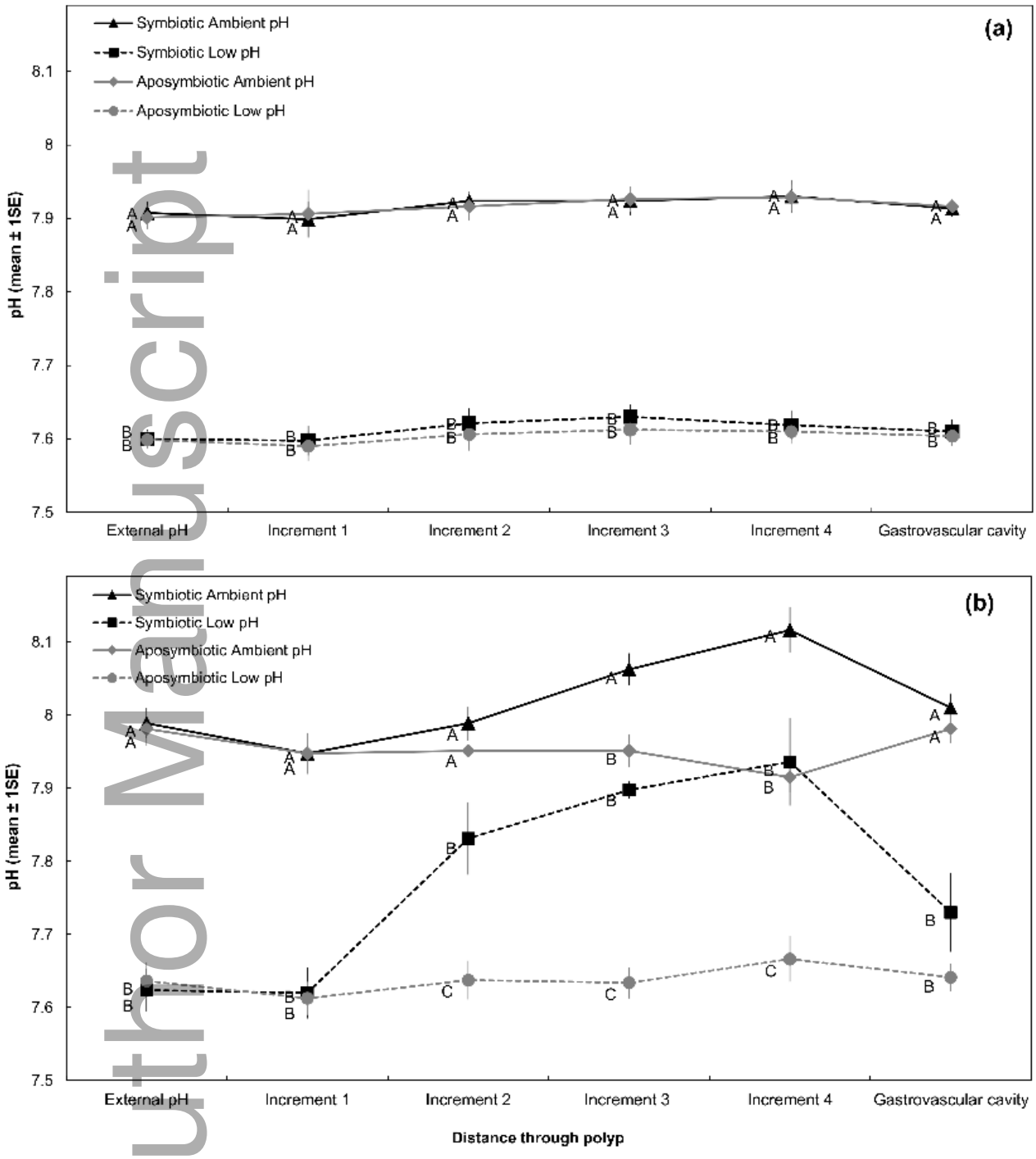

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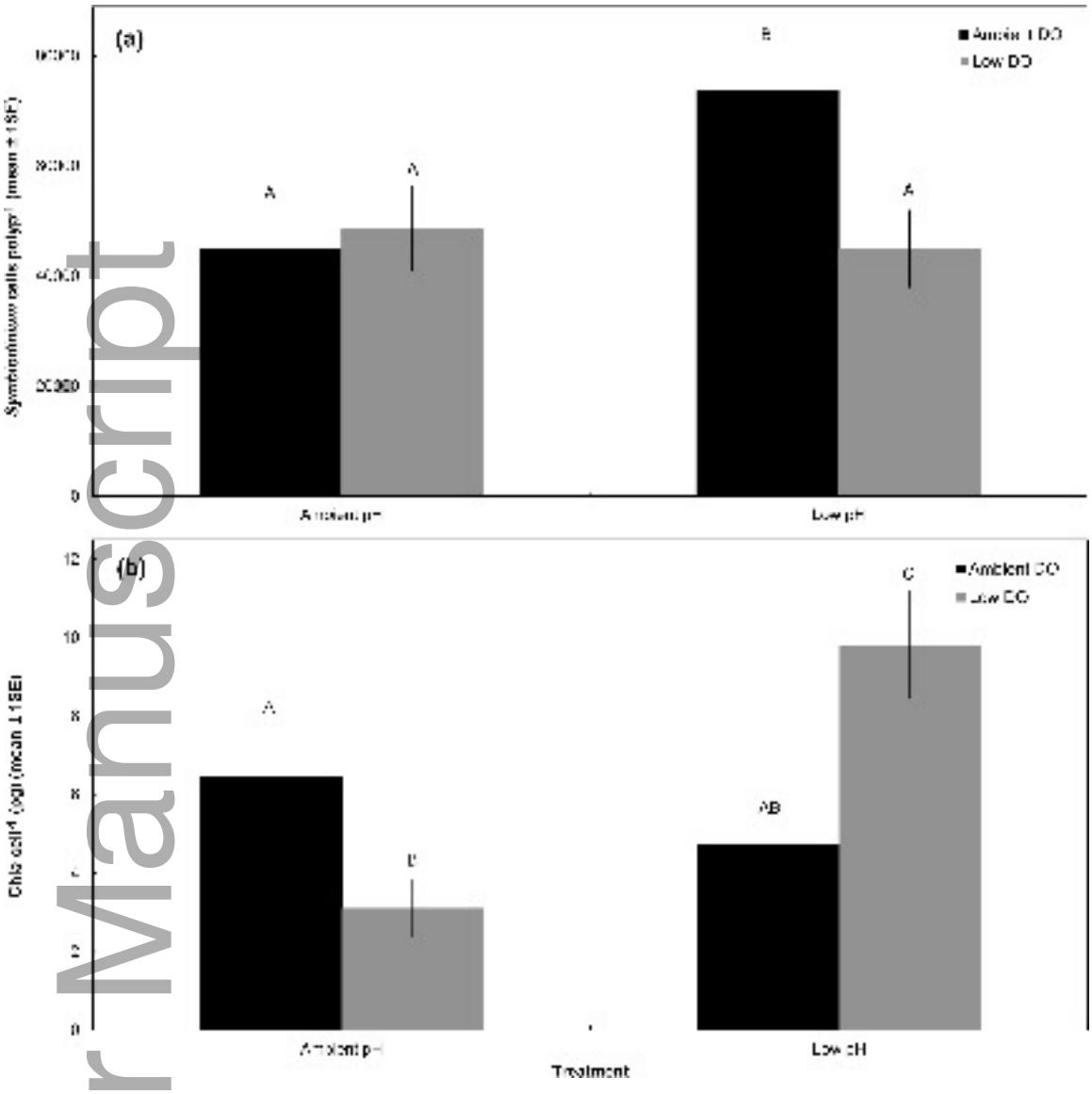

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