Vol. 112: 149–159, 2014
doi: 10.3354/dao02802

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 2

Genetically divergent *Symbiodinium* sp. display distinct molecular responses to pathogenic *Vibrio* and thermal stress

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ABSTRACT: Global climate change and anthropogenic activities are threatening the future survival of coral reef ecosystems. The ability of reef-building zooxanthellate coral to survive these stressors may be determined through fundamental differences within their symbiotic dinoflagellates (*Symbiodinium* sp.). We define the *in vitro* apoptotic response of 2 evolutionarily distant *Symbiodinium* sp., subtypes B2 and C1, to determine the synergistic effects of disease and temperature on cell viability using flow cytometry. The putative yellow band disease (YBD) consortium of *Vibrio* spp. bacteria and temperature (33°C) had a positive synergistic effect on C1 apoptosis, while B2 displayed increased apoptosis to elevated temperature (29 and 33°C), the *Vibrio* consortium, and a lone virulent strain of *V. alginolyticus*, but no synergistic effects. Additionally, heat shock protein 60 expression revealed differential cell-mediated temperature sensitivity between subtypes via western blotting. This result marks the first evidence of *Symbiodinium* sp. apoptotic variations to YBD pathogens and emphasizes the potential impact of synergistic stress on globally distributed coral–*Symbiodinium* symbioses.

KEY WORDS: Coral · Apoptosis · Yellow band disease · YBD · Zooxanthellae · B2 · C1 · Heat shock protein 60 · HSP60

INTRODUCTION

Scleractinian corals are ecologically important and essential species in the structural and biological foundations of coral reef ecosystems. However, global coral abundance has declined dramatically over the past 40 yr. For example, coral cover of *Orbicella annularis* (previously classified as *Montastraea annularis*), an important framework building species in the Caribbean, experienced a 72% decline from 1988 to 1999 in some regions, with 50 yr projection models demonstrating a strong likelihood of species extinction (Edmunds & Elahi 2007). Disease and increasing global seawater temperatures are leading stressors threatening the immediate future of coral reefs (Harvell 2002). Remarkably, some corals are able to survive despite these stressors (Hughes et al. 2003). The key to tolerant coral assemblages may reside in fundamental differences between subtypes of corals' symbiotic algae, viz. *Symbiodinium* sp., also known as zooxanthellae (Buddemeier & Fautin 1993, Berkelmans & van Oppen, 2006, Sampayo et al. 2008). These differences may lead to localized, short-term preferential survival of some coral host–zooxanthellae assemblages via the presence of more robust subtypes, exemplified by

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the decrease in the release of zooxanthellae undergoing apoptosis, a form of programmed cell death, in *Sinularia* sp., as compared to *Xenia* sp. and *Sarcophyton ehrenbergi*, in the presence of elevated temperatures (Strychar et al. 2004). While the potential for this preferential survival has been rigorously studied (Berkelmans & van Oppen 2006, Sampayo et al. 2008, Bellantuono et al. 2012), little research has been conducted to evaluate differences in apoptotic cell death across multiple subtypes of zooxanthellae and multiple stressors.

As one of the most prevalent diseases affecting hermatypic corals in the Caribbean, yellow band disease (YBD) has resulted in high levels of infection and mortality, showing rates of up to 88% infection in *Montastraea* sp. in Bonaire (Donà et al. 2008). The putative YBD *Vibrio* pathogens were chosen here as they specifically target symbiotic zooxanthellae within coral gastrodermal tissues and result in the degeneration and deformation of organelles within zooxanthellae cells (Cervino et al. 2004a). In addition to the YBD consortium, a singular strain of *V. alginolyticus* was used in this study as it has been suggested as the sole cause of YBD. Previous laboratory inoculation of *O. faveolata* with the *V. alginolyticus* pathogen caused cellular damage similar to that seen in YBD (Cervino et al. 2008). In the same experiment, however, it did not form visible lesions when compared to the YBD consortium (Cervino et al. 2008).

The spread of YBD increases in the presence of elevated seawater temperatures (Cervino et al. 2004b). Elevated seawater temperatures, generally associated with climate change, are a common coral stressor that leads to a condition known as coral thermal bleaching (Yonge & Nicholls 1931, Goreau & Hayes 1994). Although not the only mechanism of coral bleaching, corals may expel their zooxanthellae at 1 to 2°C above their summer maximum temperatures, which may result in mortality depending on the duration and intensity of these bleaching events (Gates et al. 1992, Dunn et al. 2007, Lesser 2011). Further, zooxanthellae undergo apoptosis under bleaching conditions (Strychar et al. 2004).

Utilizing a balanced factorial design of temperature treatments (26, 29, and 33°C) and bacterial infection (YBD *Vibrio* consortium or *V. alginolyticus* alone), we examined the synergistic effects of increased temperature and YBD infection on the *in vitro* viability of 2 *Symbiodinium* strains (B2 and C1) with known physiological and genetic distance, to establish the plausibility of variable symbiont susceptibility to common stressors when experienced simultaneously. Subtype B2 is considered a temperate *Symbiodinium* sp. commonly reported in the Caribbean and mid-latitudes of North America (Thornhill et al. 2008), while C1 has been defined as an ecological generalist inhabiting a diverse array of hosts and locations (LaJeunesse et al. 2010). Additionally, these subtypes have been reported as symbiotic partners with hosts which are commonly affected with YBD. Zaragoza et al. (2014) report the experimental infection of *Aiptasia pallida*, commonly associated with B2 (LaJeunesse 2001) with YBD, while Correa et al. (2009) reported 2 instances of *O. annularis* fragments infected with YBD containing C1 symbionts.

We assessed cell death (apoptosis and necrosis) as a measure of zooxanthellae viability using flow cytometry. Apoptosis and necrosis, while both forms of cell death, are achieved via different mechanisms. Apoptosis is a highly organized and predictable process where cells are fragmented and disposed of through the regulation of specific cell signaling pathways. Necrosis, on the other hand, is traditionally described as a passive form of cell death that results from external trauma, such as physical damage (Edinger & Thompson 2004). The physiological differences of these processes may be targeted using flow cytometry to differentiate between cell populations. Additionally, we used western blotting to identify changes in the protective heat shock protein 60 (HSP60).

MATERIALS AND METHODS

Supply of cultured zooxanthellae

Strains of 2 axenic *Symbiodinium* cell cultures, B2 and C1 (CCMP nos. 2459 and 2466, respectively), were obtained from The National Center for Marine Algae and Microbiota (formerly the Center for the Culture of Marine Phytoplankton [CCMP]) at the Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA. Sub-clade B2 was originally isolated from the scleractinian coral *Oculina diffusa* in the Sargasso Sea, while C1 was isolated from the anemone *Discosoma sanctithomae* in the Caribbean Sea. Although subclade C1 in this study was originally isolated from an anemone, this subclade has also been routinely isolated from scleractinian corals (van Oppen et al. 2001). Specific subclades were chosen as representatives of the 2 broader clades B and C, which are characterized by lower and higher stress tolerance levels, respectively (DeSalvo et al. 2010).

Cultures were grown, shipped, and stored in sterile F/2 media purchased from CCMP. Once received, algae were grown and stored in individual 500 ml flasks in 350 ml of F/2 media. Flasks were stored within a water bath at 26°C (±0.5°C). Soft white lights were used as a light source on a 13/11 h light/dark schedule with a constant irradiance of 100 µmol quanta s^{-1} m^{-2}. Fifty percent of the media was changed on a weekly basis to maintain healthy cultures. Sub-culturing was performed monthly by adding 5 ml of homogeneous algae to 345 ml of fresh F/2 media.

Experimental protocol

A factorial fixed factor analysis of variance (ANOVA) design was used to determine cell viability of zooxanthellae following treatment at 1 of 3 temperatures (26, 29, and 33°C; temperature-alone treatments labeled as '*n*') and exposure to either an inoculum of the 5 *Vibrio* pathogens associated with YBD (R-370, R-1207, R-1216, R-1257, R-1249; Cervino et al. 2008), labeled here as '*v*,' or an inoculum of *Vibrio alginolyticus* alone (R-370), labeled here as '*k*,' over the course of 24 h. Temperature treatments were treated with a control inoculum, which lacked bacteria. The 3 temperatures used were chosen to represent ambient temperatures, summer maximum temperatures, and projected temperatures in the Caribbean Sea, the general region in which both strains were originally isolated. Temperature treatments were maintained with electronically controlled water baths and monitored with calibrated alcohol thermometers (± 0.5°C).

Treatments of inoculum with non-pathogenic bacteria, to test for stress responses due solely to high concentrations of bacteria, were not used here, as previous studies with 4 strains of *Bacilli* sp. found on healthy corals did not show any pathogenic conditions during culture experiments and whole coral experiments. *Escherichia coli* strains also showed no negative effects *in vivo* during early experiments on the host and symbiont (Cervino et al. 2004a,b).

Bacterial strains were cultured from −80°C archived cultures as a full lawn at 32°C on glycerol artificial seawater media (Smith & Hayasaka 1982) overnight. Inoculum was prepared by adding a 10 ml aliquot of sterile seawater (SSW; 32 ppt) to each plate and dissociating the bacterial lawn from the agar using a sterile glass stir-rod bent at a 90° angle. Inocula from each of the 5 individual cultures YBM23, YB36, YBFLG2A, YB-K, and YBFL3122 were combined and vortexed in a 50 ml conical falcon tube to a final density of ca. 10^8 CFU ml^{-1} as outlined by Cervino et al. (2008). *V. alginolyticus* inoculum was prepared in the same way using only the YB-K strain, again diluted to a final density of ca. 10^8 CFU ml^{-1}.

For experimentation, each replicate was contained in 9 ml borosilicate culture tubes. Three replicates of each treatment (temperature and *Vibrio* inocula) were used for each of 5 sample periods (0, 3, 8, 12, and 24 h; n = 270). Two ml of zooxanthellae, at 10^6 cells ml^{-1} within phylotypes, were added to each tube and allowed to acclimate overnight at each experimental temperature. The average doubling time for zooxanthellae in culture is between 2 and 20 d (as summarized by Wilkerson et al. 1988). Therefore, any potential changes in zooxanthellae population concentrations across treatments were deemed dismissible. After the 24 h acclimation period, an equal volume of bacterial inoculum (200 µl at ca. 10^8 CFU ml^{-1}; i.e. treatments *v* or *k*) was added to each tube, except the temperature-alone treatments. To ensure equal volume, controls and temperature treatments were inoculated with SSW. All tubes, including temperature treatments and controls, were inverted 3 times to ensure equal exposure of bacteria.

Flow cytometry

Zooxanthellae viability was analyzed using a flow cytometry apoptosis assay in which viable, dead, and apoptotic cells may be differentiated based upon the exclusion and binding of fluorescent dyes (Vybrant Apoptosis Kit no. 6; Invitrogen). Membrane permeability is a key characteristic of dead or dying cells and, as such, the membrane-impermeable DNA-binding dye propidium iodide (PI) is able to bind to exposed nuclear material and fluoresce red (FL3-H; 630 ± 22 nm). Apoptotic cells may be differentiated from necrotic (dying) and viable (live) cells through the identification of phosphatidylserine exposure on the outer leaflet of the cell membrane, a well-known distinguishing characteristic of apoptotic cells. The protein Annexin V binds to this particular membrane component and when conjugated to the fluorescent dye Alexa *fluor*488®, may be excited at 488 nm to produce green fluorescence (FL1-H; 530 ± 30 nm). Live cells fluoresce considerably less with respect to this wavelength. Samples were prepared as described by Strychar et al. (2004).

A fluorescent activated cell sorter (Becton Dickson Vantage SE) was used to detect relative fluorescence corresponding to each cell's physiological state, i.e. viable, dead, or apoptotic. Instrument calibration was

performed using chicken red blood cells (BioSure®) to manufacturer specifications prior to sample analysis. Ten thousand cells were analyzed for each replicate. Bacterial background fluorescence was excluded from analysis based upon forward scatter (threshold = 120). Compensation was required to differentiate algal autofluorescence (Sammarco & Strychar 2013). All flow cytometry data were analyzed using FlowJo V. 8.8.6 software.

Western blotting

Western blot analysis for HSP60 expression, a protein chaperone commonly linked with apoptosis, was performed on samples of B2 and C1 exposed to temperature treatments only. Heat shock proteins are highly conserved between organisms and, as such, antibody-based detection systems poorly differentiate between the epitopes of bacterial, metazoan, and plant HSPs. Therefore, we only determined changes in HSP60 within B2 and C1 subtypes exposed to thermal stress (26, 29, and 33°C). Samples for western blotting were prepared for treatment as discussed above. After removal from water baths (at time treatments 0, 3, and 8 h), the samples were collected and transferred to 1.5 ml tubes. Each sample was then spun down at 13000 × *g* (2 min), and the supernatant was removed and snap frozen at −80 ± 0.5°C.

As *Symbiodinium* contain chlorophyll pigments, the standard technique of protein equalization on a spectrophotometer was not used. Instead, cell counts on a hemocytometer were utilized to obtain equal cell concentrations between samples. Samples were suspended in 1 ml FSW. Ten µl of this homogeneous mixture were removed and placed onto a Neubauer-improved bright-line hemocytometer (Hausser Scientific). Counts were performed and equalized based on the sample with the lowest concentration. Equal protein concentrations were double-checked using the modified Ghosh method post lysis (Ghosh et al. 1988). Western blotting was performed as described by Krucher et al. (2006) using 1 µg ml^{-1} of anti-human HSP60 antibody (LK-2; StressGen).

Statistical analysis

The distribution of the responses at the lowest treatment level, incubation time, with regards to all other treatment levels was first examined with box plots. Those treatments with interquartile ranges 10% greater than or less than the mean were re-analyzed using FlowJo for quality control. Comparing to internal replicates, instances of irregular bi-plot patterns, indicative of clogs in the flow cytometer fluidics system, were removed from the data set (n = 2 points). In both cases, large shifts in the bi-plot were observed resulting in uncharacteristically low-viability cell populations. Although the initial concentration of cells remained unchanged across all treatments, the initial percentage of viable, dead, and apoptotic *Symbiodinium* varied significantly between clades B2 and C1 at all treatment levels ($p < 0.001$; *t*-test). Starting viable percentages for clades B2 and C1 were 85.7 and 84.3, respectively. Although not a large difference, we corrected for this bias in the data set by taking the difference of the mean of control samples (time 0 h) and each value of those treated for 24 h ($p > 0.05$; *t*-test). For statistical analyses, negative values were corrected for by adding 1 greater than the absolute value of the minimum percentage to all response variable data so that the lowest value in the dataset was 1. For example, 80.82 was added to −79.82 to give a lowest value of 1. This technique was done to all data points in order to maintain both the variance within each response and the relative magnitude of difference between each value, without affecting the statistical outcomes.

Correlations between response variables (i.e. viable, necrotic, or apoptotic) were determined using linear regression. A general linear model with a Poisson distribution was used to analyze the data for significance between treatment levels. The Poisson distribution best simulates data obtained relating to counts. The error distribution of data displaying over- or underdispersion was corrected utilizing a quasi-likelihood approach to estimate ø rather than defining it using a particular distribution (McCullagh & Nelder 1989). Model simplification was carried out using the drop1 function with an *F*-test. Variables and/or interactions displaying insignificance were removed from the model, in order from the most interactions to the least. All statistical analysis was performed using R v. 2.10.1 (R Foundation for Statistical Computing).

RESULTS

Cell-death pathways of *Symbiodinium* subtypes to synergistic stress

A strong inverse relationship existed between frequency of apoptotic (Fig. 1, Table 1) and viable (Fig. 2, Table 1) *Symbiodinium* irrespective of subtype at all treatment levels ($R^2 = 0.997$). Necrotic cells

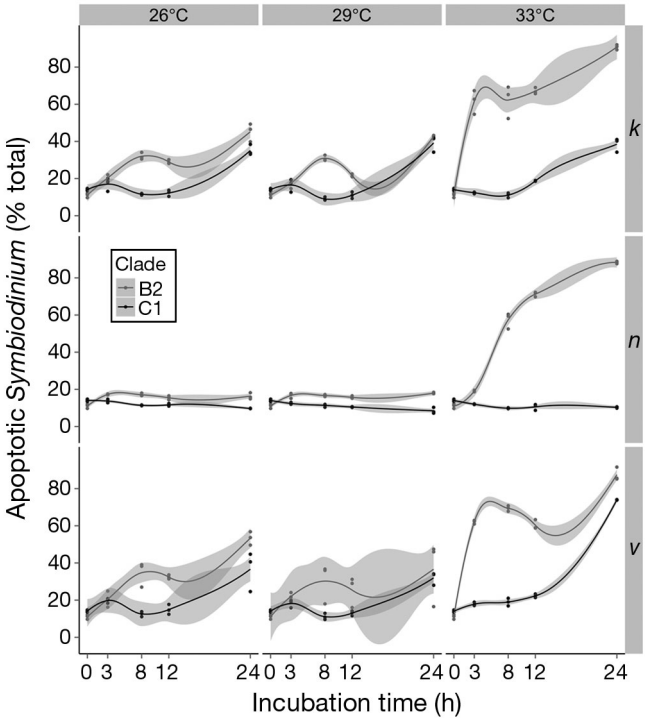

Fig. 1. Percent apoptotic cells at 3 tempertures in treated axenic cultures of *Symbiodinium* subtypes B2 and C1 after 0, 3, 8, 12, and 24 h. Horizontal rows labeled '*k*,' '*n*,' and '*v*' represent samples exposed to *Vibrio alginolyticus*, temperature alone, and the yellow band disease *Vibrio* consortium, respectively. A loess regression smoothing function was fit to the data; shaded area represents 95% confidence intervals for the fit of this function

(Fig. 3, Table 1) did not share a significant relationship with the other response variables (necrotic:viable R^2 = 0.011; necrotic:apoptotic R^2 = 0.017). Therefore, *Symbiodinium* cells studied *in vitro* entered a death phase via an apoptotic pathway, indicating a cell-directed response occurring at the symbiont level, rather than as a direct effect of the stressor, which is typical of necrosis. As changes in necrotic cells were negligible and changes in viable cells mirrored apoptotic cells (Figs. 1–3), we will report results via variation in apoptotic cells.

Cell-directed responses of *Symbiodinium* subtypes to thermal stress

The thermal susceptibility of B2 and C1 was established by incubation at each of 3 temperatures (26, 29, and 33°C) over 24 h (0, 3, 8, 12, and 24 h sampling times). Significant response variation between B2 and C1 was evident after 24 h of exposure ($p < 0.0001$, Fig. 1, Table 1). As no significant changes in apoptotic cells were seen in either subtype at 26 or 29°C, the majority of variation was driven by susceptibility at 33°C. Apoptotic cells increased by 70% in subtype B2 while decreasing by 5% in subtype C1 (Fig. 4).

Table 1. Simplified statistical models obtained from a balanced 3-way model describing apoptosis, viability, and necrosis in *Symbiodinium* clades B2 and C1, including clade, temperature (temp), and bacterial inoculation treatments (Vib *n*: temperature alone, no *Vibrio*; Vib *v*: inoculum of the 5 *Vibrio* pathogens associated with yellow band disease) as predictors. **Bold** type indicates significance ($p < 0.05$)

Predictor	Estimate	SE	*t*	p(>|*t*|)
Apoptosis				
(Intercept)	3.3179	0.2738	12.12	**0.0000**
Clade C1	1.2048	0.4074	2.96	**0.0052**
Temp	0.0523	0.0092	5.71	**0.0000**
Vib *n*	−1.4704	0.4080	−3.60	**0.0009**
Vib *v*	0.4692	0.3840	1.22	0.2289
Clade C1:Temp	−0.0480	0.0137	−3.50	**0.0012**
Clade C1:Vib *n*	1.2398	0.6180	2.01	0.0516
Clade C1:Vib *v*	−1.6059	0.5853	−2.74	**0.0090**
Temp:Vib *n*	0.0439	0.0136	3.24	**0.0024**
Temp:Vib *v*	−0.0146	0.0128	−1.13	0.2633
Clade C1: Temp:Vib *n*	−0.0467	0.0208	−2.25	**0.0301**
Clade C1: Temp:Vib *v*	0.0564	0.0196	2.88	**0.0064**
Viability				
(Intercept)	10.0979	0.8987	11.24	**0.0000**
Clade C1	−5.2450	1.0849	−4.83	**0.0000**
Temp	−0.2301	0.0322	−7.14	**0.0000**
Vib *n*	0.3897	0.1079	3.61	**0.0007**
Vib *v*	−0.2126	0.1290	−1.65	0.1063
Clade C1:Temp	0.2033	0.0384	5.29	**0.0000**
Necrosis				
(Intercept)	4.3500	0.0109	398.22	**0.0000**
Clade C1	0.0366	0.0111	3.30	**0.0020**
Temp	0.0010	0.0004	2.79	**0.0079**
Vib *n*	0.0177	0.0133	1.33	0.1910
Vib *v*	0.0354	0.0137	2.59	**0.0132**
Clade C1:Temp	−0.0010	0.0004	−2.80	**0.0077**
Clade C1:Vib *n*	−0.0184	0.0026	−7.13	**0.0000**
Clade C1:Vib *v*	−0.0001	0.0027	−0.02	0.9824
Temp:Vib *n*	0.0000	0.0004	0.07	0.9467
Temp:Vib *v*	−0.0012	0.0005	−2.54	**0.0148**

Cell-directed responses of *Symbiodinium* subtypes to *Vibrio alginolyticus*

Next, we measured the response of subtypes B2 and C1 exposed to *V. alginolyticus* (treatment *k*) at all 3 temperatures (26, 29, and 33°C). Responses of B2 and C1 varied with respect to the 24 h change in the percentage of apoptotic cells (Fig. 4). When

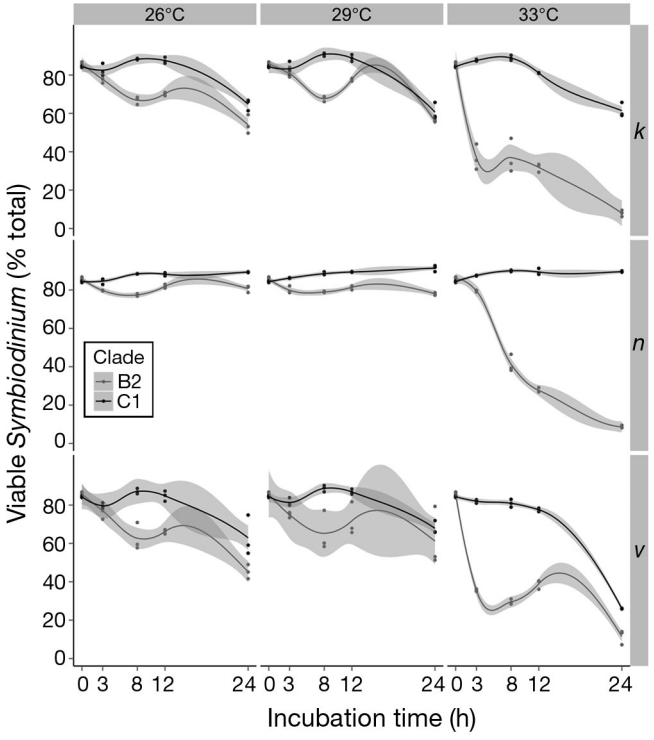

Fig. 2. Percent viable cells in treated *Symbiodinium* sub types B2 and C1. Other details as in Fig. 1

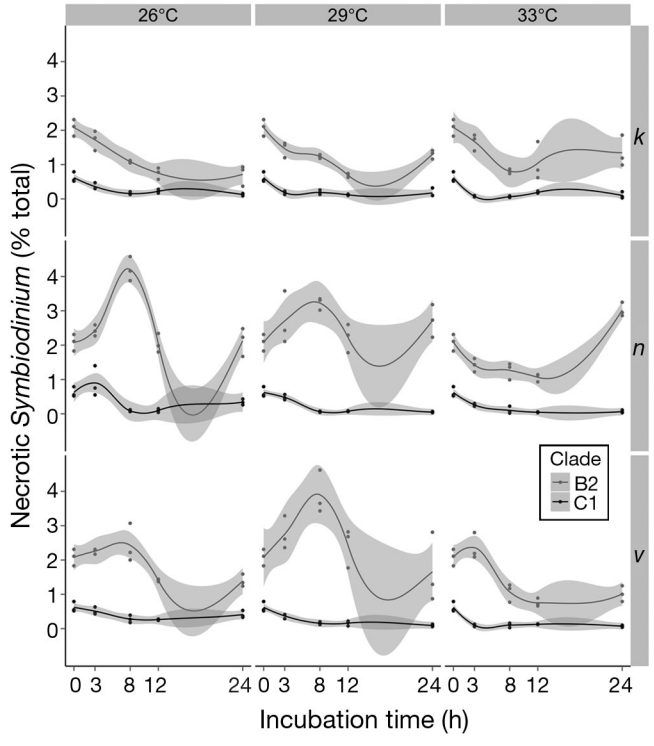

Fig. 3. Percent necrotic cells in treated *Symbiodinium* sub types B2 and C1. Other details as in Fig. 1

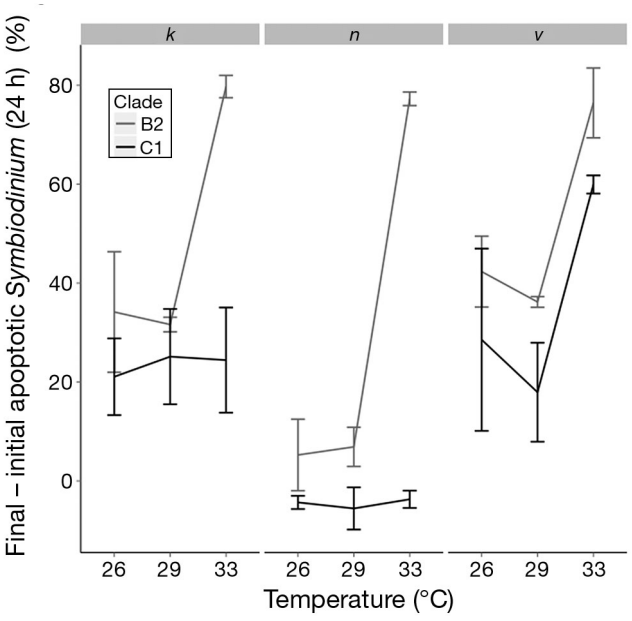

Fig. 4. Percent change of apoptotic cells in treated axenic cultures of *Symbiodinium* subtypes B2 and C1 over 24 h calculated as means from time 0, in response to varying degrees of thermal (26, 29, and 33°C) and bacterial stress. Physiological state was determined simultaneously by flow cytometry. Vertical columns labeled '*k*,' '*n*,' and '*v*' represent samples exposed to *Vibrio alginolyticus*, temperature alone, and the yellow band disease *Vibrio* consortium, respectively. Error bars indicate 95% confidence intervals

exposed to *V. alginolyticus* at 26 and 29°C, each subtype responded with similar trends over 24 h (Fig. 1). Within these trends, B2 had ~10 to 15% more apoptotic cells than C1; however, these differences were not significant (Fig. 4). When incubated at 33°C in the presence of *V. alginolyticus*, trends differed between the 2 subtypes. The mean percentage of apoptotic cells increased for subtype B2 from 30 to 80% ($p < 0.0001$). This change was significantly different when compared to that of subtype C1 ($p < 0.0001$), which did not display a temperature-dependent response (Table 1). The percent changes of apoptotic cells from both subtypes at all treatment levels were significantly different from temperature treatments devoid of bacteria ($p < 0.0001$; Fig. 4).

Cell-directed responses of *Symbiodinium* subtypes to the YBD consortium

To establish subtype sensitivity to the synergistic effect of temperature and infection with the putative YBD *Vibrio* pathogens, we measured the rate of cell death of subtypes B2 and C1 following exposure to

the consortium at all 3 temperatures (26, 29, and 33°C). As with the previous treatments, the most significant change in the apoptotic cells occurred at 33°C following 24 h exposure. The percentage of apoptotic B2 cells changed from ~10% to ~80%, while C1 increased from ~10% to ~70%, with a significant difference within subtypes at different temperatures ($p < 0.0001$), as well as between subtypes at 33°C ($p < 0.01$; Fig. 1).

We observed similar trends between the bacterial treatments at 26 and 29°C regardless of single or multiple bacterial species when time interval changes were taken into account. In each case, the variance surrounding the mean of each response was greater for B2 when a greater number of *Vibrio* sp. were present, indicating a more detrimental environment due to the presence of the YBD consortium, but implicating *V. alginolyticus* as a key player in the disease.

When subtypes were incubated at 33°C with the YBD consortium, we observed variation between the responses of each cell line (Figs. 1 & 4). B2 responded similarly to previous treatments at 33°C with and without *V. alginolyticus*. The apoptotic cells for C1, however, increased exponentially upon bacterial infection at 33°C. Increase in apoptotic cells at 33°C with the YBD consortium is significantly different than other temperature treatments with these bacteria, 33°C in the presence of *V. alginolyticus*, and 33°C devoid of bacteria ($p < 0.000$). These data show a synergistic response to high temperatures and the presence of YBD consortium pathogens after 24 h of exposure in zooxanthellae subtype C1 (Table 1).

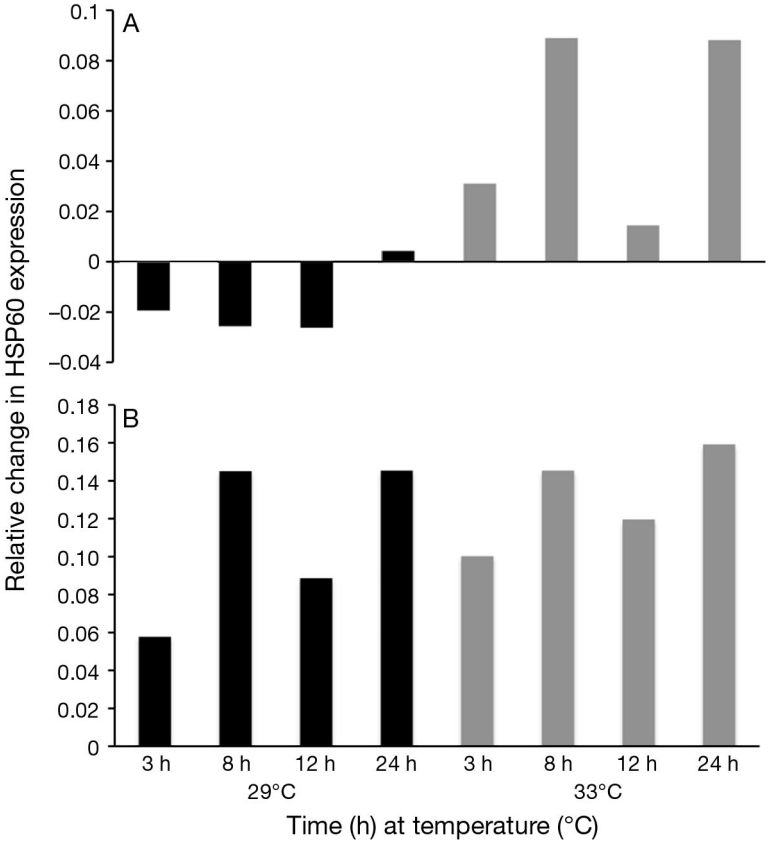

Fig. 5. Realtive changes in heat shock protein 60 (HSP60) expression compared to constitutive levels in *Symbiodinium* clades (A) B2 and (B) C1 exposed to increased temperatures. All experimental samples were standardized relative to HeLa cell expression for cross-comparison and constitutive expression at 'normal' 26°C temperatures (black bars: samples exposed to 29°C; grey bars: 33°C). All samples were run in duplicate

HSP60 responses of *Symbiodinium* subtypes to thermal stress

As evidenced by Fig. 5, relative to constitutive expression, HSP60 was down-regulated at 3, 8, and 12 h in B2 cells exposed to 29°C. At 24 h, expression was slightly higher than constitutive levels, signaling the initiation of cellular stress. All time treatments of B2 at 33°C experienced an increase in HSP60 expression relative to constitutive amounts, with maxima at 8 and 24 h. C1 cell lines expressed higher amounts of HSP60 in response to increased temperatures (Fig. 2C,D). Trends for 29 and 33°C were similar, with bimodal peaks in expression at 8 and 24 h, similar to B2 at 33°C.

DISCUSSION

To our knowledge, no studies have compared thermal stress viability in cultured coral zooxanthellae subtypes of clades B and C specifically. Individually, most subtypes of both clades have been regarded as thermally intolerant, specifically clade B, when compared to representatives of the thermally tolerant clade D (Garren et al. 2006). Our results show that subtype B2 is more susceptible to stress at all experimental levels tested here than subtype C1 (Fig. 1). The lower susceptibility of C1 reported here may be explained by our controlled light conditions (i.e. 100 µmol quanta s^{-1} m^{-2}), as much of the previously

reported intolerance of clade C to elevated temperatures has been linked to increases in irradiance that may accompany thermal stress (Rowan et al. 1997). In contrast to Rowan et al. (1997) however, DeSalvo et al. (2010) reported a significant decrease in maximum quantum efficiency, or photosynthetic yield, in subtype B1 in response to thermal stress when compared to C7 in a single coral host species (*Orbicella faveolata*), corroborating our findings of increased cell death in response to thermal stress in B2 compared to C1.

Additionally, with regard to bleaching susceptibility, Rowan et al. (1997) described a fitness gradient between members of clades A, B, and C, viz. A > B >> C, with clade A having the highest fitness/lowest susceptibility. Results from Rowan et al. (1997), as compared to those of DeSalvo et al. (2010), likely elucidate the importance of stress tolerance variation within different coral–symbiont assemblages. DeSalvo et al. (2010) attempted to remove the effect of host coral on zooxanthellae tolerance by keeping the host species constant. Rowan et al. (1997), on the other hand, generalized large-scale clade fitness amongst a multitude of coral species. This aspect of zooxanthellae–host assemblage specific interactions likely explains the differences between our findings and those of Rowan et al. (1997).

Although large-scale cladal generalizations are important in determining overall reef health, fine-scale differences in the susceptibilities of subclades have been reported. Sampayo et al. (2008) reported bleaching susceptibilities in *Stylophora pistillata* to be dependent on the presence of subclades C78, C79, C35/a or C8/a, with C79 and C35/a being significantly more susceptible to thermal stress. Although our results for subclades B2 and C1 corroborate findings for B1 and C7 from DeSalvo et al. (2010), there may be other subtypes within clades B and C that behave differently under similar conditions. Therefore, researchers should be mindful of specific coral–zooxanthellae assemblages when reporting bleaching susceptibilities.

In addition to differences in thermal tolerance, we report differences between B2 and C1 in terms of susceptibility to bacterial pathogens. As indicated in Fig. 1, apoptotic cells were more frequently identified for subtype B2 at all temperature levels in the presence of *Vibrio alginolyticus* when compared to C1 cells of the same treatments. This provides further support that B2 is more susceptible to these stressors than C1. The significance of *V. alginolyticus* to algal cell physiology is demonstrated, as both subtypes experienced significantly more apoptosis with the addition of *V. alginolyticus* compared to treatments without bacterial inoculum. B2 displays a temperature-dependent response as significantly more apoptosis was observed at 33°C compared to 26°C and 29°C, but no synergism, as this level was not significantly different from *n* treatments at 33°C. C1 subtype did not display a temperature-dependent response, as levels of apoptosis were not significantly different from one another across all temperatures within the *k* treatment, but did display susceptibility to *V. alginolyticus*, as all C1 treatments in *k* significantly differed from *n*. It has been shown that some coral disease-related *Vibrio* species increase virulence in the presence of higher temperatures (Ben-Haim et al. 2003). Here, however, it is more likely that susceptibility of our B2 cells decreased with increasing temperature, rather than bacterial virulence increasing at higher temperatures, as C1 cells did not display higher apoptosis at 33°C.

In treatments containing the YBD-consortium, B2 again displayed a temperature sensitivity at 33°C and greater susceptibility than C1 (Fig. 1). Interestingly, C1 also displayed a temperature-dependent response with significantly more apoptotic cells at 33°C than at 26 and 29°C (Fig. 4). The amount of apoptotic C1 cells at 33°C in *v* treatments was significantly different from those at 26°C-*v* (YBD consortium alone) and 33°C-*n* (elevated temperature alone). This demonstrates a synergistic effect of the YBD consortium and temperature. Significant increases in apoptotic cells of C1 exposed to the YBD consortium at 33°C compared to *V. alginolyticus* at the same temperature suggest the importance of the YBD consortium. It is likely that bacterial virulence has increased, as opposed to cell tolerance decreasing, thus having a more negative impact on the survival potential of a widely distributed, generally stress-resistant *Symbiodinium* subclade.

The synergistic response of C1 to temperature and YBD pathogens is significant as subtypes of clade C are routinely isolated from both Caribbean and Indo-Pacific reefs (LaJeunesse et al. 2010). Additionally, YBD has recently been identified in the Indo-Pacific, although it was once thought to be a Caribbean-specific disease (Cervino et al. 2008). Our results suggest a possible explanation for increases in coral disease outbreaks during warm summer months (Cervino et al. 2004b) as well as the importance of diseases in the long-term survival of coral reefs if average sea surface temperatures increase in the near future due to climate change. Our results may also help explain the prevalence of YBD in the Caribbean due to B2's widespread presence (LaJeunesse

2001) and high susceptibility to YBD-associated pathogens as shown in this study.

We have demonstrated that apoptosis is the cell-death mechanism displayed by *Symbiodinium* sp. C1 and B2 under elevated temperature and YBD stress. Our results also show that C1 subtypes are better able to down-regulate or avoid apoptosis in the presence of elevated temperatures and YBD than B2. Strychar et al. (2004) showed that various coral–algal assemblages yield a higher chance of survival under bleaching conditions as an effect of bleaching-mediated zooxanthellae apoptosis. Rogers (2013) suggested that the coming decades will see a shift in coral species abundances and distributions, rather than complete extinction. As proposed by Strychar et al. (2004), less susceptible *Symbiodinium* subtypes, such as C1, may represent genetic pools to aid the continued survival of scleractinian corals in the face of environmental changes associated with global climate change and anthropogenic effects.

In addition to changes in the prevalence of apoptotic cells, we analyzed changes in constitutive expression of HSP60, a well-known protein chaperone that is commonly linked with the stress response and in particular, apoptosis (Samali & Cotter 1996). The association of HSP60 with rubisco activase in heat-stressed D43 plants suggests that HSP60 could function in plants as a mechanism for stress tolerance (Holland et al. 1998). Expressed at both healthy states and at times of stress with varying concentrations (Tsan & Gao 2004), disparity from constitutive concentrations of this protein may be used as a proxy to help identify the stress response of *Symbiodinium* as a cell-directed response (Downs et al. 2000, 2012).

As a protein chaperone, HSP60 expression increases in the presence of cellular signals that indicate denatured housekeeping proteins, threatening the status of cellular integrity. Here, C1 cells showed a strong response to both a moderate (29°C) and severe (33°C) stress via an up-regulation of HSP60. Comparably, B2 cells displayed a delayed response to moderate stress exemplified by the down-regulation of HSP60 upon initial exposure to 29°C, followed by up-regulation during a more severe stress. Barshis et al. (2013) showed that more resilient corals expressed genes relating to a stress response at higher constitutive levels. They determined that this 'front-loading' allowed less-susceptible corals to respond to stress more quickly than those which did not front-load. Here, the ability for C1 to mount a stress response more quickly than B2, and therefore avoid apoptosis, may be due to an ability to front-load stress-related genes.

Previous research on the role of HSP60 in apoptosis activation has produced confounding results. Xanthoudakis et al. (1999) found that under stressed conditions, HSP60 was able to substantially accelerate the maturation of pro-caspase-3, and, as an important regulatory molecule in apoptosis, lead to downstream activation of apoptosis. Conversely, Cappello et al. (2006) found positive HSP60 expression to be linked with inhibition of apoptotic cell death. As such, links between changes in constitutive HSP60 expression and apoptosis seem to be species- and stress-specific. The small changes in HSP60 expression in B2 cells at 29°C correlate with insignificant changes in apoptosis relative to controls. Higher levels of expression in B2 cells experienced at 33°C correlated with a significant increase in apoptosis over 24 h. However, the relatively large change in expression levels of HSP60 experienced by C1 cells over 24 h correlate with insignificant changes of cellular apoptosis. As such, these data further suggest that *Symbiodinium* sp. sub-types B2 and C1 experience distinct changes in response to thermal stress, but call into question the reliability of HSP60 as a lone stress biomarker in this system. Although there are many steps between HSP60 expression and apoptosis activation, this research marks the first attempt to correlate HSP60 expression in *Symbiodinium* sp. symbionts to downstream apoptosis effects during increased temperature and bacterial stress.

Zooxanthellae used in this study were obtained from a subset of cultured cells. The ability to generalize findings to all members of this sub-clade may be dependent on zooxanthellae symbiotic status. Santos et al. (2001) found that cultured zooxanthellae do not accurately represent density ratios of the original population isolated from the host. They found that *in hospite* samples taken from the sea anemone *Aiptasia pallida* contained mainly representatives from clade A, with smaller concentrations of clade B. After 1 yr in culture, *Symbiodinium* from clade B dominated 4 of 8 cultures. Axenic growth of a heterogeneous community of microorganisms is inherently selective due to specific techniques used in culturing and the interactions between organisms within the culture. For example, some species within a community may reproduce more rapidly than others depending on the media used or may be preferentially selected based on motility or sub-culturing techniques (Santos et al. 2001). Because we used monoculture samples in this study, the logistical issues associated with heterogeneous communities are not present. As a consequence, the critical question in our study is not whether these particular symbionts

are present as a community member or axenic group. The important factor to consider is whether different *Symbiodinium* spp. are experiencing different levels of cell death. This goal was accomplished using these methods, as we present different cell-directed responses of these organisms when in adverse situations due to thermal and bacterial stress. Further, a study by Rosic et al. (2011) found that symbiotic status of *Symbiodinium* sp. did not affect expression of HSP70 and HSP90 in response to temperature stress.

As the ability of some corals to multi-associate with monophyletic clades of zooxanthellae likely evolved as a mechanism to cope with changing environments, it may be assumed that those corals possessing zooxanthellae with higher plasticity in their stress response will yield higher survival to the combined effects of temperature and disease (Douglas 1998). We have shown that 2 subclades of *Symbiodinium* sp., B2 and C1, display differential apoptotic responses to the separate and combined effects of elevated temperatures and inoculation with YBD pathogens. Our results have also demonstrated a synergistic effect of elevated temperature and YBD infection in C1. This synergism may have a significant impact on reefs given current global climate projections, due to the global distribution of C1 and its association with many host–zooxanthellae assemblages. It is important to note, however, that symbiotic zooxanthellae genotyping may be insufficient in determining stress-tolerant host–zooxanthellae assemblages, as the individual bio-physiology of localized *Symbiodinium* subtypes may determine their immune response, sensu stricto, and therefore the tolerance of certain host–algal assemblages to thermal stress (Warner et al. 1996, Iglesias-Prieto & Trench 1997, LaJeunesse 2001, Cervino et al. 2004a). Previous works hint at the possibility of these resilient assemblages (Rowan & Powers 1991, Buddemeier & Fautin 1993, Baker 2001), but existence of these holobionts may be obscured by our insufficient knowledge of zooxanthellae biophysiology, diversity, and host-specific interactions.

Acknowledgements. We thank our sponsors for funding this project: (1) Coastal Preservation Network LLC, College Point, NY, and (2) National Institutes of Health (NIH Award No. CA120019) awarded to N.K. We also thank Angela Richards-Dona, Pamela Lawther, and Gabriel DeLeon for laboratory assistance and Samantha Kerath for many editorial comments on this manuscript. We are grateful to the 3 anonymous reviewers, whose comments and critiques greatly enhanced the clarity and quality of the manuscript. Lastly, we acknowledge the use of scientific equipment offered by Texas A&M University-Corpus Christi and Pace University.

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Editorial responsibility: Garriet Smith, Aiken, South Carolina, USA

Submitted: March 25, 2014; Accepted: September 1, 2014
Proofs received from author(s): November 14, 2014