The Journal of Experimental Biology 216, 2813-2820

doi:10.1242/jeb.087858

RESEARCH ARTICLE

Stress and death of cnidarian host cells play a role in cnidarian bleaching

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SUMMARY

Coral bleaching occurs when there is a breakdown of the symbiosis between cnidarian hosts and resident *Symbiodinium* spp. Multiple mechanisms for the bleaching process have been identified, including apoptosis and autophagy, and most previous work has focused on the *Symbiodinium* cell as the initiator of the bleaching cascade. In this work we show that it is possible for host cells to initiate apoptosis that can contribute to death of the *Symbiodinium* cell. First we found that colchicine, which results in apoptosis in other animals, causes cell death in the model anemone *Aiptasia* sp. but not in cultured *Symbiodinium* CCMP-830 cells or in cells freshly isolated from host *Aiptasia* (at least within the time frame of our study). In contrast, when symbiotic *Aiptasia* were incubated in colchicine, cell death in the resident *Symbiodinium* cells was observed, suggesting a host effect on symbiont mortality. Using live-cell confocal imaging of macerated symbiotic host cell isolates, we identified a pattern where the initiation of host cell death was followed by mortality of the resident *Symbiodinium* cells. This same pattern was observed in symbiotic host cells that were subjected to temperature stress. This research suggests that mortality of symbionts during temperature-induced bleaching can be initiated in part by host cell apoptosis.

Key words: *Aiptasia*, apoptosis, Cnidaria, coral bleaching, innate immunity, *Symbiodinium*, symbiosis.

Received 6 March 2013; Accepted 27 March 2013

INTRODUCTION

Many cnidarians, including corals, form a mutualistic symbiosis with the photosynthetic dinoflagellate *Symbiodinium* spp., where the algae are housed in host gastrodermal cells, thereby giving corals their typical brown coloration. Host corals provide the resident symbionts with inorganic nutrients, a high-light environment and refuge from herbivory. In return, the symbionts supply the host coral with photosynthetically fixed carbon (Davy et al., 2012; Muscatine and Weis, 1992). Corals are the trophic and structural centerpiece of coral reefs, one of the most diverse biomes on the planet. Worldwide, the survival of coral reefs is threatened by a number of factors including elevated seawater temperature, ocean acidification, rising sea levels, overfishing and pollution (Richmond and Wolanski, 2011). One biological consequence of some of these stressors, especially elevated temperature, is the phenomenon of coral bleaching. Bleaching is the breakdown of the cnidarian–dinoflagellate symbiosis leading to the loss of *Symbiodinium* cells from host tissues, and thus loss of color. Coral bleaching results in reduced host fitness, as evidenced by reduced growth rates and fecundity, and increased susceptibility to disease (Coles and Brown, 2003; Rosenberg et al., 2007). The ecological and socio-economic implications of bleaching are therefore immense (Hoegh-Guldberg et al., 2007).

The process of coral bleaching is complex and involves several inter-related mechanisms that vary depending on the type, duration and intensity of environmental stress. While it is well known that environmental disturbances, chiefly elevated temperature, initiate the onset of bleaching, the cellular cascade of events that leads to symbiont loss is less clear (Lesser, 2011; Weis, 2008). There is a partial picture emerging of the multiple cellular pathways involved, including host cell death either in a sudden and uncontrolled manner, or *via* a controlled process of programmed cell death. In addition, *Symbiodinium* cells can be expelled from host cells or be degraded *in situ* (Brown et al., 1995; Dunn et al., 2002a; Dunn et al., 2007; Dunn et al., 2004). Teasing apart these mechanisms is difficult because of the potential dual effects of stress on the host cells and their resident symbionts individually, in addition to the interaction between the two partners.

To date, most studies on bleaching have contributed to a storyline where the cascade of events leading to bleaching is initiated by the symbiont (reviewed in Lesser, 2011; Weis, 2008). Many studies have shown that heat stress damages the symbiont's photosynthetic apparatus, which results in production of excess reactive oxygen species (ROS) that in turn overwhelm existing oxygen-handling mechanisms (Franklin and Berges, 2004; Lesser, 1997). ROS diffuse into the host cell, thereby activating its apoptotic pathway, a highly ordered program of self-destruction mediated by the caspase family of proteases; this leads to host cell death (Lesser, 2006; Suggett et al., 2008). The fate of *Symbiodinium* cells is less clear. There is evidence that released *Symbiodinium* cells can be in a healthy or damaged state depending on the severity of the heat stress and the strength of the *Symbiodinium* cell's antioxidant defenses, but that apparently healthy cells begin to die after a few days (Hill and Ralph, 2007). Furthermore, if the released *Symbiodinium* cells are dead, it is unknown whether their death is due to the stress itself, to host cell apoptosis or to some other cellular pathway, such as *in situ* degradation *via* host autophagic digestion.

Very few studies have examined the effect of stress on the host cell alone and whether host-specific effects could be contributing to the bleaching process. For example, different coral species

containing the same *Symbiodinium* clade have different responses to heat stress (Fitt et al., 2009; LaJeunesse, 2002). This could be, in part, a result of differences in host protective mechanisms between species. A variety of these protective mechanisms have been identified, such as the use of fluorescent proteins and mycosporine-like amino acids to manage high levels of ultraviolet and visible light, increased heat shock protein expression to mitigate a cellular stress response, and elevated production of antioxidants to manage high concentrations of ROS (reviewed in Baird et al., 2009). Differences in the susceptibility of corals to bleaching could also come from actions of the host as a result of stress directly impacting host cells. In studies on the model anemone *Aiptasia pulchella*, Nii and Muscatine (Nii and Muscatine, 1997) found that aposymbiotic anemones produced excess ROS under high heat and light conditions in the absence of symbionts. In other organisms, this is indicative of stress-induced mitochondrial dysfunction (Haak et al., 2009). Recently, Dunn et al. (Dunn et al., 2012) have shown that heat stress can induce mitochondrial damage in a host cell with healthy *Symbiodinium* cells. Together, this information raises the possibility that host stress and cell death alone could lead to stress and/or death of resident *Symbiodinium* cells.

In this study, we examined the role of host cell stress and death in the bleaching response. We performed our experiments with the symbiotic anemone *Aiptasia* sp., a useful laboratory model for the study of corals (Weis et al., 2008). First we found that colchicine, a pharmacological agent that is a known elicitor of apoptosis in metazoans, induces cell death in host cells, but not in cultured *Symbiodinium* CCMP-830 cells or those freshly isolated from hosts (Gorman et al., 1999). Treatment of symbiotic *Aiptasia* with colchicine resulted in death of *Symbiodinium in hospite*; an indication that host cell apoptosis can contribute to *Symbiodinium* cell death. A similar increase in death of *Symbiodinium in hospite* was observed when anemones were subjected to heat stress. Live-cell confocal microscopy of colchicine-treated symbiotic host cells revealed a pattern of host cell death followed by death of the resident *Symbiodinium* cells in the majority of cases. This same pattern was repeated when the symbiotic cell isolates where subjected to heat stress. These studies do not negate the crucial role of symbiont stress in bleaching, but they do suggest that cell death initiated in host cells can contribute to cnidarian bleaching.

MATERIALS AND METHODS

Symbiodinium and *Aiptasia* sp. cultures

Symbiodinium cells (CCMP-830, sub-clade B1) were maintained in f/2 medium at 25±1°C on a 12 h:12 h light:dark photoperiod at an irradiance of 70 μmol quanta m^{-2} s^{-1}, a light level at which there is no stress on the symbionts. For all experiments on cultured *Symbiodinium*, cells were washed and resuspended to a final concentration of 1×10^6 cells ml^{-1}. Individual anemones (*Aiptasia* sp.) containing clade B *Symbiodinium* (data not shown) were maintained in 0.1 μm ceramic filtered artificial seawater (FASW) under temperature and light conditions identical to those described above. Anemones were fed twice a week with freshly hatched brine shrimp nauplii. Anemones were transferred to 6- or 24-well culture plates and starved for 2 to 3 days prior to an experiment.

Incubation of symbiotic *Aiptasia* in colchicine to test for host cell death

The pharmacological agent colchicine was used to induce host cell death. Colchicine is known to induce apoptosis in animal cells including cnidarians (David et al., 2005; Dunn et al., 2004; Dunn and Weis, 2009; Pernice et al., 2011). Symbiotic individuals of *Aiptasia* were incubated in FASW alone or in 12.5 mmol l^{-1} colchicine dissolved in FASW for 3 h prior to addition of 50 μmol l^{-1} fluoroprobe Rhodamine 110 aspartic acid, a caspase substrate (Life Technologies, Grand Island, NY, USA); this concentration of colchicine has previously been shown to cause apoptosis in cnidarians (Dunn and Weis, 2009). Upon enzymatic cleavage *via* caspases, the non-fluorescent substrate is converted to the fluorescent Rhodamine 110. This method is described in detail in Detournay and Weis (Detournay and Weis, 2011). Images of anemone tissue were acquired with a Zeiss LSM 510 Meta microscope with a 40/0.8 water objective lens (Zeiss, Thornwood, NY, USA). Fluorescence excitation/emission for Rhodamine 110 was 488/505–530 nm. Relative caspase activity was quantified using ImageJ software (Schneider et al., 2012).

Incubation of cultured and freshly isolated *Symbiodinium* in colchicine to test for dinoflagellate cell death

Dinoflagellate cell survival has been found to be unaffected by incubation in colchicine (Cho et al., 2011; Wong and Kwok, 2005). We wanted to test this both in *Symbiodinium* CCMP-830 in culture and in symbiont cells freshly isolated from hosts. Cell death was measured using Sytox Green, a fluorescent probe that can only cross plasma membranes of dying cells, where it binds to nucleic acids. Sytox Green therefore labels an early stage of cell death prior to cell lysis.

Symbiodinium CCMP-830 cultures

Cultured *Symbiodinium* CCMP-830 cells were co-incubated in 5 μmol l^{-1} Sytox Green and 0, 6, 12.5 or 25 mmol l^{-1} colchicine. There was an additional treatment in Sytox Green and 10 mmol l^{-1} H_2O_2, an ROS and therefore a positive control for oxidative stress. A high concentration of H_2O_2 was used to ensure that cell death occurred rather than to mimic physiological conditions. Sytox Green fluorescence was measured every 30 min for 10 h. Total relative Sytox Green fluorescence was measured from triplicate experiments using a SPECTRAmax GEMINI Spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) set for high resolution and sensitivity, and an excitation/emission of 448/523 nm with a 515 nm cut-off.

Freshly isolated *Symbiodinium* from *Aiptasia*

To prepare freshly isolated *Symbiodinium* cells, two medium-sized *Aiptasia* (approximate oral disc diameter 4 mm) were homogenized for 30 s in 3 ml FASW using a glass tissue grinder. Homogenates were centrifuged for 1 min at 735 ***g***, the supernatant was removed and the algal pellet was resuspended in 1 ml FASW. This process was repeated three times to remove cellular debris. Between each centrifugation, the suspension was gently syringed 10 times with a 23-gauge needle to isolate the *Symbiodinium* cells from host cells. *Symbiodinium* cells were allowed to recover for 3 h at room temperature before experimental treatment and imaging.

Cells were incubated in a seawater control, 10 mmol l^{-1} H_2O_2 (positive control) or 12.5 mmol l^{-1} colchicine for 3 or 24 h. Thirty minutes prior to imaging, Sytox Green was added to a final concentration of 5 μmol l^{-1} to cell suspensions in a glass-bottom dish (MatTek Corporation, Ashland, MA, USA). Images were captured with a Zeiss LSM 510 META confocal microscope through a Plan-APOCHROMAT 63×/1.4 oil DIC objective. Fluorescence excitation/emission was 488/505–530 nm for Sytox Green and 688/679–754 nm for *Symbiodinium* chlorophyll autofluorescence. Experiments were performed in triplicate and, for each sample, 10 random images were taken. Acquired images were then randomized

and a naïve counter quantified the total number of *Symbiodinium* cells and the fraction containing Sytox Green fluorescence, allowing for the calculation of a ratio of Sytox:control that accounted for any death induced by the isolation process.

Measurement of symbiont cell death when incubated in colchicine when *in hospite*

Specimens of symbiotic *Aiptasia* were incubated in 12.5 mmol l^{-1} colchicine for 3 or 24 h. *Symbiodinium* cells were isolated and cell mortality was quantified using confocal microscopy as described above.

Hyperthermic stress treatment of cultured *Symbiodinium* CCMP-830 and symbiotic *Aiptasia*

Symbiodinium CCMP-830 cell suspensions were incubated in a range of heat-stress conditions: 25 (ambient), 27, 30 or 33°C, for 3 or 24 h in glass-bottom dishes. Thirty minutes prior to imaging, Sytox Green was added to a final concentration of 5 μmol l^{-1}. The mortality of *Symbiodinium* cells at each temperature was quantified with confocal microscopy, as described above.

Specimens of symbiotic *Aiptasia* were incubated at the same set of temperatures and for the same duration as were the cultured *Symbiodinium* cells. *Symbiodinium* cells were subsequently isolated and incubated with 5 μmol l^{-1} Sytox Green, and their mortality was quantified using confocal microscopy as described above.

Maceration and culturing of symbiotic host cells

To isolate individual host gastrodermal cells from symbiotic *Aiptasia*, a method modified from Gates and Muscatine (Gates and Muscatine, 1992) was utilized. Animals were placed in relaxing solution (1:1, 370 mmol l^{-1} $MgCl_2$:FASW) 30 min prior to snipping off tentacles with small scissors and placing them into Ca^{2+}-free seawater. Tentacles were cut into 1 mm barrels with a scalpel and then gently pelleted by centrifugation for 1 min at 735 ***g***. Under aseptic conditions, the tentacle bits were resuspended in 0.05% collagenase (Sigma-Aldrich, St Louis, MO, USA) in 0.1 mol l^{-1} Na_3PO_4 buffer containing 3% NaCl and 0.004% CaCl (pH 7.4), and allowed to dissociate during a 1 h incubation at room temperature on a shaker table at 150 r.p.m. The cellular suspension was passed once through an 18-gauge needle and then centrifuged for 1 min at 735 ***g***. Cell macerates were resuspended in culture medium and plated onto poly-L-lysine-coated (Sigma-Aldrich) glass dishes. Several different culture media were tested, and a medium composed of 25% Dulbecco's modified Eagle medium (GIBCO, Life Technologies) in FASW resulted in the lowest level of mortality over time (data not shown). In all conditions, it was found that the rate of death decreased after a 3 h recovery period and thus cells were allowed to acclimatize for 3 h at room temperature before further experimental manipulation.

Live imaging of cell death in macerated symbiotic host cell isolates subjected to colchicine and heat treatments

After recovery, host cell macerates were placed in FASW at 25°C, 12.5 mmol l^{-1} colchicine in FASW at 25°C or FASW at 33°C, and followed for 8 h. For heat treatments, a CTI-controller 3700 humidified chamber (Zeiss) was utilized. Confocal imaging began 1–2 h after initiation of experimental treatments. For each of three replicates, images were acquired every 2 h from 16 different locations in the glass-bottomed dish. Each location contained a minimum of two symbiotic host cells housing multiple *Symbiodinium* cells. Confocal images were captured as described above. DIC-transmitted light images were captured simultaneously with fluorescence images, using the transmitted light detector. After acquisition, images were randomized and a naïve counter scored host cells and resident symbionts as either containing or missing Sytox Green labeling. Subsequent tabulation of all images for each host cell over the duration of the experiment resulted in four patterns of Sytox Green labeling, as shown in Fig. 1: (0) no label; (1) label in host cell only; (2) label in both partners; and (3) label in host cell first followed by appearance of label in symbiont. Because Sytox Green cannot penetrate the membrane of live host cells, we were unable to detect the presence of live host cells containing dead *Symbiodinium* cells. Therefore, healthy host cells containing both live and/or dying *Symbiodinium* cells showed no label. In addition, when label was detected in both host and *Symbiodinium* cells, we were unable to determine whether this occurred simultaneously or whether mortality of *Symbiodinium* cells occurred prior to host cell death.

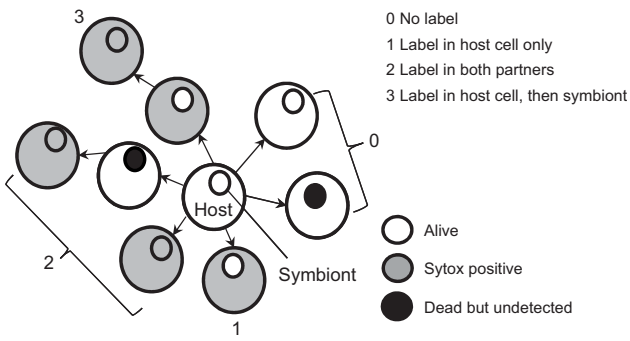

Fig. 1. Diagrammatic representation of the possible patterns of Sytox Green labeling in macerated symbiotic host cells visualized by confocal imaging. Four possible patterns emerged: 0, no label; 1, label in host cell only; 2, label in both partners; 3, label in host cell and then symbiont. Sytox Green cannot penetrate the membrane of healthy host cells, so this method cannot determine the status of symbionts when they are resident in a healthy host cell. Live cells are depicted with no shading, Sytox-positive cells with gray, and dead symbionts that are undetected by this method with black.

Statistical analyses

All data were tested for normality and heteroscedasticity before being analyzed by two-way ANOVA, which applied treatment (chemical or elevated temperature *versus* control) and time as effects. *T*-tests with a Bonferroni correction were employed when multiple comparisons were made (GraphPad, La Jolla, CA, USA). Data are presented as means ± s.e.m. All observations are derived from at least three independent experiments. Significance was set at $P<0.05$.

RESULTS

Colchicine induces cell death in *Aiptasia* tissue but not in cultured or freshly isolated *Symbiodinium* cells

To determine the fate of the *Symbiodinium* cells *in hospite* after host cell death, the pharmacological agent colchicine was used to induce host cell but not *Symbiodinium* cell mortality. Incubation of symbiotic *Aiptasia* in 12.5 mmol l^{-1} colchicine for 3 h elicited apoptotic host cell death, as evidenced by the presence of Rhodamine 110 fluorescence, a measure of caspase activity, in whole-mount *Aiptasia* (Fig. 2). Levels of fluorescence increased in both the epidermis and gastrodermis from relative fluorescence of 957 and 307 in control animals to 4012 and 1814 in colchicine-incubated

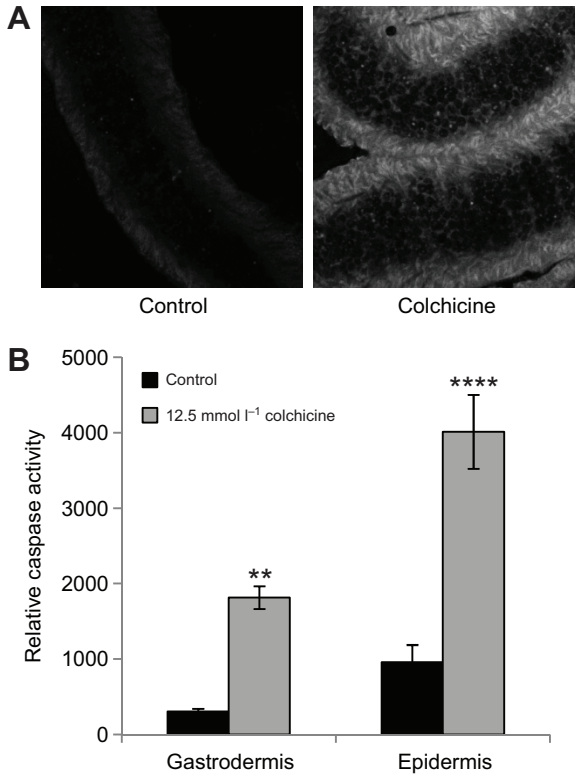

Fig. 2. Cell death in *Aiptasia* incubated in colchicine as measured by caspase activity. Caspase activity was measured using a Rhodamine 100 aspartic acid fluoroprobe after a 3 h incubation in 0 (control) or 12.5 mmol l^{-1} colchicine. (A) Confocal images of live tentacles from whole-mount *Aiptasia*. (B) Relative caspase activity in the gastrodermis and epidermis of *Aiptasia* measured by the fluorescence intensity of the Rhodamine 100 aspartic acid fluoroprobe and quantified using ImageJ software. Bars represent means ± s.e.m. (*N*=3; two-way ANOVA with Bonferroni correction, ****P<0.0001, **P<0.01).

animals, an increase of fourfold (P<0.0001) and sixfold (P<0.01), respectively (Fig. 2B).

Other investigators have found that colchicine does not affect dinoflagellate cell survival (Cho et al., 2011; Wong and Kwok, 2005). We wanted to test this both in *Symbiodinium* CCMP-830 cells in culture and in cells freshly isolated from their host. Control CCMP-830 cells displayed no increase in fluorescence throughout the duration of the experiment (Fig. 3A). In contrast, cells incubated in H_2O_2 displayed a steady increase in fluorescence throughout the experiment to a final treatment:control ratio of 5 (Fig. 3A). However, there was no significant difference in Sytox Green fluorescence in either the 6 or 12.5 mmol l^{-1} colchicine treatments compared with controls (P>0.05). Between 1 and 2.5 h, cells incubated in 25 mmol l^{-1} colchicine exhibited a slight increase in fluorescence to ~1.6 times the control value (P<0.0001) and subsequently remained at this level for the duration of the experiment.

Because *Symbiodinium* cells *in hospite* display a different phenotype than those in culture, and could potentially be differentially susceptible to colchicine, we tested the effect of colchicine on *Symbiodinium* cells freshly isolated from *Aiptasia* and incubated in a FASW control, 10 mmol l^{-1} H_2O_2 (positive control) or 12.5 mmol l^{-1} colchicine for 3 or 24 h (Fig. 3B). Whereas *Symbiodinium* cells incubated in H_2O_2 exhibited six (P<0.0001) and 12 times (P<0.0001) greater mortality than controls at 3 and 24 h, respectively, the level of mortality in *Symbiodinium* cells incubated in colchicine was not significantly different from that of control incubations, regardless of incubation time (P>0.05). Together, these results suggest that colchicine does not induce *Symbiodinium* cell mortality under experimental conditions.

Colchicine treatment of *Aiptasia* induces *Symbiodinium* cell death *in hospite*

Next, we were interested in determining whether induction of cell death in hosts could lead to cell mortality of *Symbiodinium* cells *in hospite*. Treatment of *Aiptasia* for 3 h in colchicine led to a 2.3-fold increase (P<0.05) in *Symbiodinium* cell mortality compared with controls, and further increased to 4.7-fold (P<0.001) after 24 h of colchicine treatment (Fig. 4). Because colchicine does not induce *Symbiodinium* cell mortality, as demonstrated in Fig. 3, these data suggest that apoptosis of host gastrodermal cells may play a role in mortality of *Symbiodinium* cells *in hospite*.

Heat stress causes an increase in *Symbiodinium* cell death in a dose- and time-dependent manner

With an understanding that host cell death could be contributing to symbiont cell mortality, the next step was to test the hypothesis that heat stress causes host cell death, which in turn can contribute to symbiont cell mortality. First, to assess the mortality levels in *Symbiodinium* CCMP-830 cells due to elevated temperature, the cultured dinoflagellates were incubated under a range of heat stress conditions [25 (ambient), 27, 30 or 33°C for 3 or 24 h] and mortality was quantified with Sytox Green fluorescence and confocal

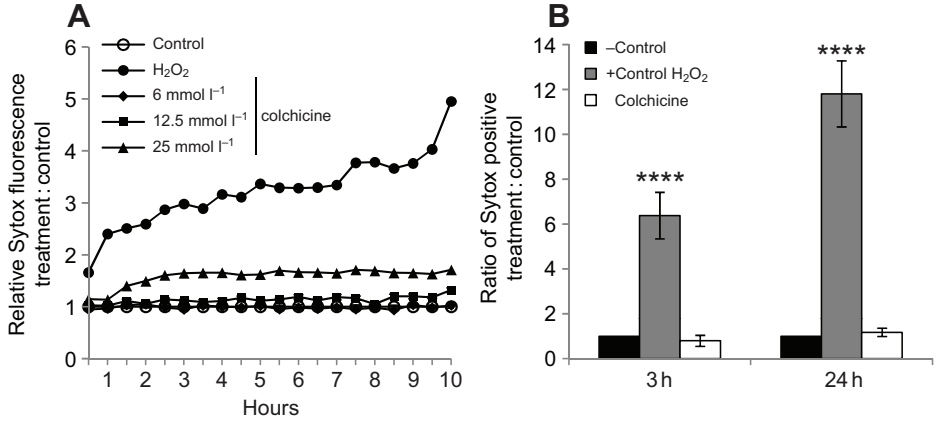

Fig. 3. Effect of colchicine on *Symbiodinium* cell health. (A) Health of cultured *Symbiodinium* CCMP-830 cells measured by quantification of relative Sytox Green fluorescence every 30 min for 10 h. Cells were incubated in control conditions, 10 mmol l^{-1} H_2O_2 (a positive control for ROS), or varying concentrations of colchicine. (B) Health of freshly isolated *Symbiodinium* cells quantified by counting Sytox-Green-labeled cells *via* confocal microscopy, in cells incubated in seawater only, 10 mmol l^{-1} H_2O_2 or 12.5 mmol l^{-1} colchicine for 3 or 24 h. Bars represent means ± s.e.m. (*N*=4; two-way ANOVA with Bonferroni correction, ****P<0.0001).

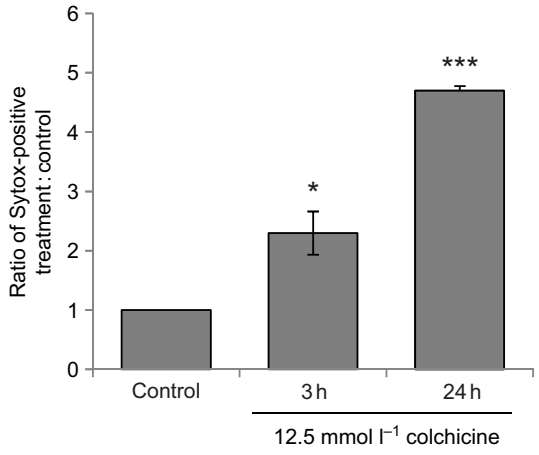

Fig. 4. Effects of colchicine treatment on the health of *Symbiodinium in hospite*. Anemones were incubated for 3 h in 0 (control) or 12.5 mmol l^{-1} colchicine, before *Symbiodinium* cells were isolated from host tissues. Health of the isolated *Symbiodinium* cells was then determined using confocal microcopy to calculate the ratio (treatment:control) of Sytox-positive *Symbiodinium* cells. Bars represent means ± s.e.m. (*N*=3; two-way ANOVA with Bonferroni correction, **P*<0.05, ****P*<0.001).

microscopy (Fig. 5A). Cells sampled at 3 h increased from treatment:control ratios of 1 at 25°C to a high of 2.5 at 33°C, and those sampled at 24 h increased from ratios of 1 at 25°C to a high of 5.2 at 33°C. Cells incubated at 30°C (3 h *P*<0.01, 24 h *P*<0.0001) and 33°C (3 and 24 h both *P*<0.0001) displayed significantly increased rates of mortality compared with controls. The level of mortality in *Symbiodinium* cells incubated at 33°C for 24 h was 2.5-fold higher than the level in *Symbiodinium* cells incubated for 3 h (*P*<0.0001). These findings reflect an increase in mortality that is both dose and time dependent.

A similar experiment was carried out to determine whether levels of mortality due to heat stress were different in *Symbiodinium* cells *in hospite* compared with those in culture. As in treatments of cultured *Symbiodinium* cells, mortality of algae *in hospite* increased with rising temperature and prolonged duration of treatment (Fig. 5B). Cells sampled at 3 h increased from treatment:control ratios of 1 at 25°C to a high of 4.6 at 33°C, and those sampled at 24 h increased from ratios of 1 at 25°C to a high of 7.7 at 33°C. Values at 30°C (3 h, *P*<0.05; 24 h, *P*<0.0001) and 33°C (3 h, *P*<0.0001; 24 h, *P*<0.0001) were significantly higher than those at ambient temperatures, with the rate of symbiont cell mortality significantly elevated between 3 and 24 h (30°C, *P*<0.01; 33°C, *P*<0.0001).

Increases in *Symbiodinium* cell mortality appeared more pronounced in cells that had been *in hospite* than in culture: the dose- and time-dependent responses were more dramatic in symbiont cells resident in the host. The level of mortality in *Symbiodinium* cells *in hospite* incubated for 24 h at 33°C was nearly eightfold higher (*P*<0.0001) than controls, compared with 5.5-fold higher (*P*<0.0001) than controls in *Symbiodinium* cells in culture. These data suggest that the health of the host cell has a direct impact on the health of the endosymbiont during stress.

Host cell death can contribute to mortality of resident *Symbiodinium* cells

Finally, to further test the hypothesis that host cell stress and death can contribute to symbiont mortality, the dynamics of cell death in individual host cells with resident symbionts was temporally monitored. The health of individual symbiotic host cells obtained by maceration was monitored by live-cell imaging over an 8 h period. The progression of host cell death and *Symbiodinium* mortality was then classified as one of three patterns or modes of cell death based on the presence of Sytox Green fluorescence labeling (Fig. 6A): (1) death of host cell only with healthy *Symbiodinium* cells; (2) death of host cell and *Symbiodinium* cell mortality observed simultaneously; and (3) death of host cell observed first followed by *Symbiodinium* cell mortality. As mentioned in the Materials and methods (Fig. 1), data from pattern 2 are difficult to interpret because the technique is unable to detect dying symbiont cells in live host cells. Pattern 2 could include live host cells where *Symbiodinium* mortality preceded host cell death. This approach, therefore, cannot quantify a pattern of symbiont cell mortality preceding host cell death.

Under control conditions, 28% of symbiotic host cells died during the 8 h incubation (Fig. 6B). Of these cells, 92% died according to the first pattern (*P*<0.0001), with 2 and 6% displaying patterns 2 and 3, respectively. In contrast, incubation of symbiotic host cell macerates in 12.5 mmol l^{-1} colchicine led to 98% host cell death (*P*<0.0001). Of these dead cells, the majority (67%; *P*<0.0001) displayed death according to pattern 3, with 38% (*P*<0.05) and 5% (*P*>0.05) exhibiting patterns 1 and 2, respectively. Taken together, these results suggest that induction of host cell apoptosis *via* colchicine incubation leads primarily to a pattern of host cell death followed by *Symbiodinium* mortality.

Finally, to determine whether a similar pattern of death is observed under heat stress conditions, symbiotic host cells were subjected to 33°C temperature stress for 8 h. All host cells subjected

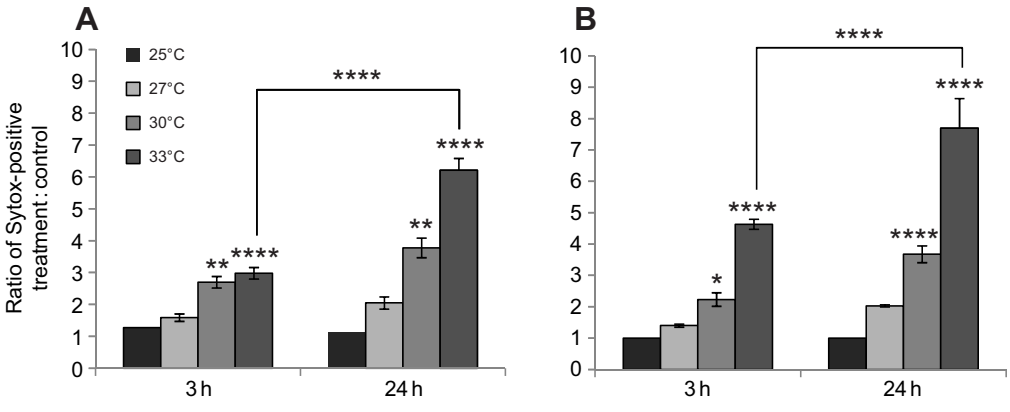

Fig. 5. Effects of elevated temperature on the health of *Symbiodinium* cells. Health of (A) cultured *Symbiodinium* CCMP-830 cells and (B) *Symbiodinium* cells freshly isolated from *Aiptasia* after being subjected to a range of temperature conditions (25, 27, 30 or 33°C). Health was determined by quantifying the ratio (treatment:control) of Sytox-Green-labeled cells using confocal microscopy. Bars represent means ± s.e.m. (*N*=3; two-way ANOVA with Bonferroni correction, **P*<0.05, ***P*<0.01, *****P*<0.0001).

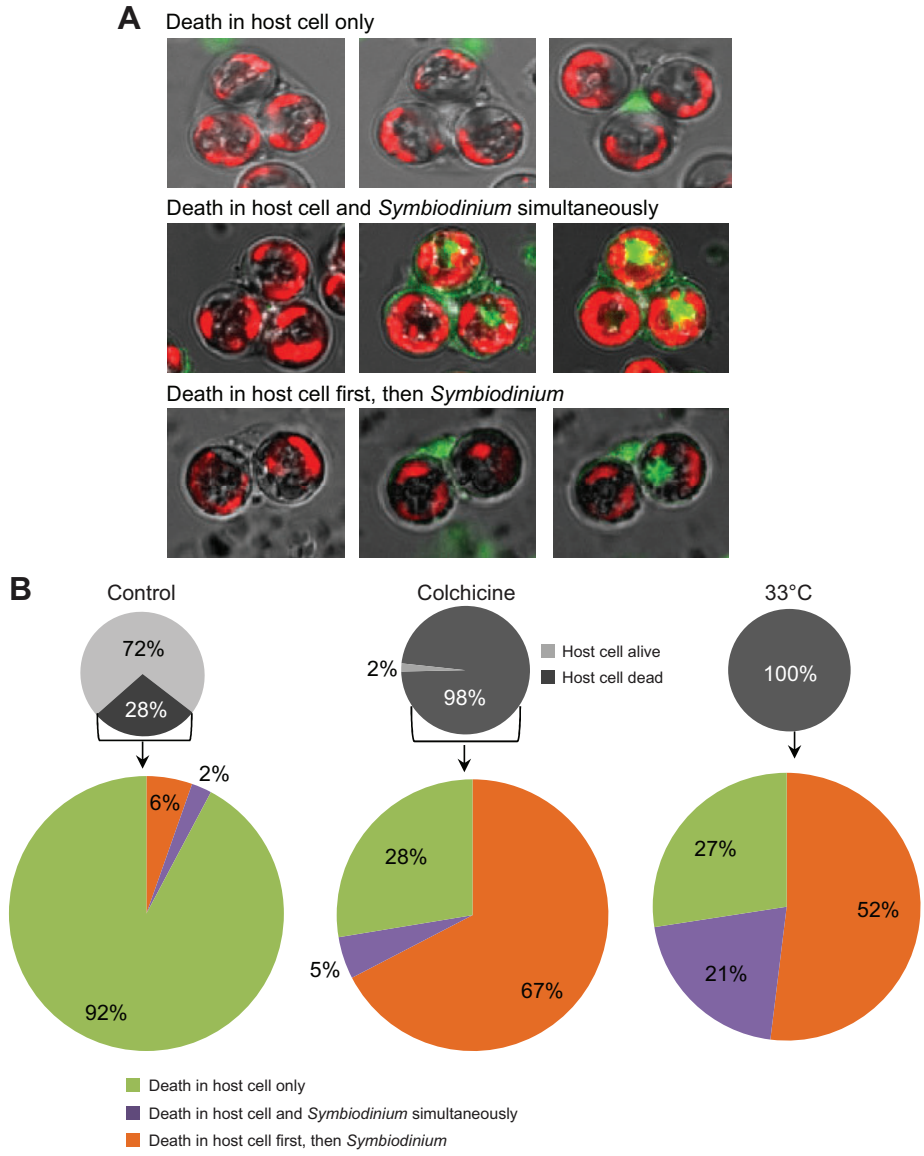

Fig. 6. Mode and timing of cell death of macerated host and resident *Symbiodinium* cells subjected to colchicine and elevated temperature. (A) Sample images of macerates dying over time. The mode of cell death was determined for each cell macerate containing two or more *Symbiodinium* cells over an 8 h time course. Each cell was viewed temporally to determine whether the host cell macerates remained alive (no Sytox Green labeling) or died (Sytox Green positive). For the host cells that were classified as dead, the pattern of host cell death in relation to the health status of the resident *Symbiodinium* cell(s) was observed and classified as belonging to one of three patterns: (1) death of host cell only; (2) death of host cell and *Symbiodinium* cell(s) simultaneously; and (3) death of host cell first then *Symbiodinium* cell(s). (B) Patterns of cell death in symbiotic host cell macerates incubated for an 8 h time course at 25°C (control), or in 12.5 mmol l^{-1} colchicine at 25°C or at an elevated temperature of 33°C. Each percentage is a mean of the replicates.

to heat stress died. A slight majority of macerates (52%; $P<0.0001$) exhibited death according to pattern 3. A further 27% ($P<0.001$) and 21% ($P<0.001$) died by patterns 1 and 2, respectively. These data suggest that, in many cells, heat stress induces apoptosis in host cells that in turn could be contributing to death of the symbionts.

DISCUSSION

Multiple studies have shown that programmed cell death in the host and possibly in the symbiont plays an important role in high-temperature-induced cnidarian bleaching (Dunn et al., 2002a; Franklin et al., 2004; Pernice et al., 2011; Strychar and Sammarco, 2009). However, details such as the order in which each process occurs, in which partner, and the effect that one partner's stress has on the other remain difficult to dissect out from the complexity of the endosymbiosis. To aid our attempts to tease apart the effects of stress on each partner individually, in addition to the interaction between the two partners, we wanted to identify a pharmacological agent that would cause programmed cell death of the host cell but not the symbiont cell within the time frame of our experiments. Building on previous studies in *Aiptasia* and other animals, and in dinoflagellates, we focused on colchicine. Colchicine, a commonly used anti-cancer drug, is a microtubule-disrupting agent. In mammals, it also induces apoptosis through poorly understood mechanisms that cause release of cytochrome *c* from mitochondria and subsequent caspase activation (Gorman et al., 1999). Researchers studying apoptosis and/or bleaching in cnidarians have shown that colchicine causes apoptosis in a variety of cnidarians including *Hydra*, the corals *Acropora millepora* and *Fungia scutaria*, and in *Aiptasia* sp. (David et al., 2005; Dunn et al., 2004; Dunn and Weis, 2009; Pernice et al., 2011). In contrast, studies to date on dinoflagellates have shown that colchicine causes cell cycle arrest in G1 but no programmed cell death (Cho et al., 2011). Our experiments confirm these previous findings (Figs 2, 3). Incubation of aposymbiotic anemones in colchicine caused apoptosis, whereas no cell death was observed in cultured or freshly isolated *Symbiodinium* cells incubated in colchicine.

With the tool of a host-only apoptosis-inducer in hand, we found that induction of apoptosis in hosts when incubated in colchicine also results in increased cell death of symbionts *in hospite* (Fig. 4). In addition, we provide evidence from colchicine-incubated host cell macerates that, in the majority of dying cells, host cell apoptosis occurs first, followed by death of the resident symbionts (Fig. 6A,B).

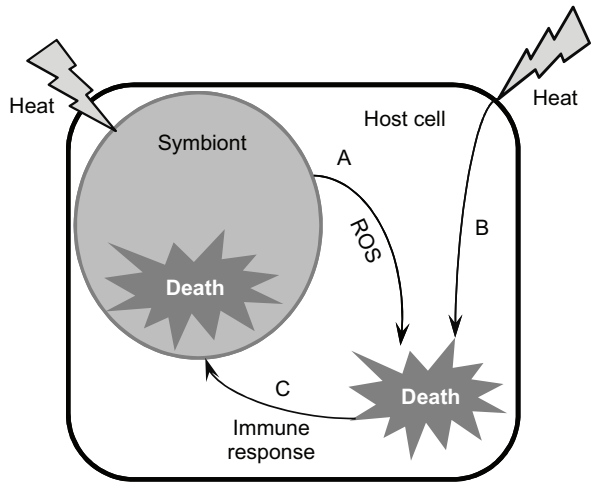

Fig. 7. Schematic diagram of proposed stress and cell death pathways due to heat stress in a symbiotic cnidarian cell. In pathway A, heat stress affects the *Symbiodinium* cell, which then releases ROS into the host cell, ultimately leading to host cell death. In pathway B, heat stress can cause direct damage and death of the host cell. In pathway C, death of the host cell can cause an immune response contributing to the death of the *Symbiodinium* cell.

These results suggest that host cell apoptosis can contribute to cell death of symbionts without prior signaling from stressed or dying symbionts. This response exhibits characteristics of a classic innate immune response, such as the removal and/or destruction of foreign bodies, so well characterized in other animals (Postigo and Ferrer, 2009; Wilson et al., 2009). A similar phenomenon has also been described in aposymbiotic larvae of the coral *F. scutaria* challenged with *Symbiodinium* sub-clade C31, which is unable to colonize this coral species (Dunn and Weis, 2009). Larvae challenged with C31 cells exhibited high numbers of apoptotic cells in the gastroderm, suggesting that hosts use apoptosis to eliminate inappropriate symbionts from their tissues.

Moving from simple studies using a single pharmacological agent to those that use heat stress, which causes a myriad of effects, could we show evidence that temperature stress on host cells contributes to cell death of symbionts *in hospite*? Results shown in Fig. 5 indicate that *Symbiodinium* cells subjected to elevated temperature die at a higher rate *in hospite* than they do in culture, suggesting that there is a negative host effect on *Symbiodinium* cell survival during heat stress. Furthermore, in the majority of cases, the host cell dies first, followed by mortality of the symbiont (Fig. 6B). However, the percentage of cases exhibiting simultaneous death of host and symbiont cells was much higher in heat-stress treatments compared with the colchicine treatment, suggesting that multiple effects are at play in the observed cell death patterns. Previous studies in *Aiptasia* sp. using quantitative ultrastructural techniques (Dunn et al., 2002; Dunn et al., 2000) have described a variety of host and symbiont cell death scenarios [cf. fig. 11 in Dunn et al. (Dunn et al., 2002)] depending on the duration and degree of the stress. Included in this were death profiles similar to what we describe in this study: death of both the host and the symbionts. Other studies have found evidence of elevated temperature stress in host tissues. ROS are produced in aposymbiotic anemones subjected to elevated temperature (Nii and Muscatine, 1997) or light (Dykens et al., 1992). Host cells from *Aiptasia* containing apparently healthy symbionts show mitochondrial damage (and presumably subsequent ROS generation) after heat stress (Dunn et al., 2012). Together with our results from colchicine treatment, these findings suggest that host stress plays a role in the bleaching response.

The present study has several limitations that constrain our ability to fully interpret the results. First, we were not able to observe *Symbiodinium* mortality in live host cells (see Fig. 1, pattern 0) and therefore we were unable to assess a potential pattern of symbionts dying in healthy host cells. However, if this pattern were observable, it would decrease the percentage of live host cells bearing healthy symbionts observed, but would not affect patterns 1–3 described in Fig. 6. Second, we were not able to assess a potential pattern of symbiont cells dying first followed by death of the host cells (see Fig. 1, pattern 2). If this pattern were observable, it would decrease the percentage of pattern 2. Finally, in the heat stress experiments, it is possible that initiation of host cell death was not caused directly by the host cell response to heat stress, but instead was a result of stress signals, such as ROS, from the *Symbiodinium* cell that are released prior to symbiont cell mortality; these in turn would launch a host innate immune response. Despite these limitations, the results provided in this study allow us to add to existing cellular models for cnidarian bleaching proposed in other studies and reviews (Fig. 7) (Lesser, 2006; Lesser, 2011; Weis, 2008). Specifically, with an elevated temperature stress, ROS generated by symbionts leak from the symbiont into host tissues and contribute to a host cell death response (pathway A). Based on data from this study and others (Dunn et al., 2012; Dykens et al., 1992; Nii and Muscatine, 1997), elevated temperature will stress host cells, causing host cell death (pathway B). Finally, our study suggests that host cell death can contribute to subsequent symbiont cell mortality (pathway C), a process that appears similar to a host innate immune response.

ACKNOWLEDGEMENTS

We thank Sheila Kitchen for help setting up the maceration protocol, Jessica Flesher and Kelsey Lance for their work counting and classifying cells, Riana Wernick for spell-checking and editing early drafts of the manuscript, and members of the Weis Laboratory for comments on the manuscript. We also wish to acknowledge the Confocal Microscopy Facility of the Center for Genomic Research and Biocomputing at Oregon State University.

AUTHOR CONTRIBUTIONS

C.W.P., S.K.D. and V.M.W. designed the experiments, C.W.P. conducted the research, and C.W.P., S.K.D. and V.M.W. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This research was supported by National Science Foundation grants to V.M.W. [IOB09190730] and to C.W.P. [1002947].

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