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Early cellular changes are indicators of pre-bleaching thermal stress in the coral host

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ABSTRACT

Thermal stress causes the coral-dinoflagellate symbiosis to disassociate and the coral tissues to whiten. The onset and occurrence of this coral bleaching is primarily defined via the dinoflagellate responses. Here we demonstrate that thermal stress responses occur in the coral host tissues in the days before the onset of coral bleaching. The observed sequence of thermal responses includes reductions in thickness of coral tissue layers and apoptosis of the cells prior to reductions in symbiont density. In the days before the onset of coral bleaching the outer coral tissue layer (epithelium) thickness reduces and apoptosis occurs within the gastrodermis. Two days following this, coinciding with an initial reduction of symbiont density (by approximately 25%), gastrodermal thickness decreased and apoptosis of host cells was identified in the epithelium. This was eventually followed by large reduction in symbiont density (by approximately 50%) consistent with coral bleaching. Both pro-apoptotic and anti-apoptotic genes are identified in the reef building coral *Acropora aspera*, demonstrating the necessary pathways are present for fine control of host apoptosis. Our study shows that defining periods of host stress based on the responses defined by dinoflagellate symbiont underestimates the importance of early cellular events and the cellular complexity of coral host.

1. Introduction

Mass bleaching of corals has been reported to be increasing in recent years and is directly correlated with increasing sea temperatures driven by global climate change (Hoegh-Guldberg, 1999). Bleaching is now considered one of the major threats to the sustainability of reefs worldwide (Hoegh-Guldberg, 1999; Hughes et al., 2003). Coral bleaching is broadly defined as the loss or reduction of endosymbiotic dinoflagellates or their associated pigments from the coral host cells; with severe or sustained bleaching resulting in mortality of the coral colony (Coles and Jokiel, 1977; Hoegh-Guldberg and Smith, 1989; Kleppel et al., 1989; Porter et al., 1989; Lesser, 1996; Szmant and Gassman, 1990; Glynn and D'Croz, 1990). This observable bleaching pattern in reef corals is a generalised response to stress and occurs as a reaction to many environment factors including high (Hoegh-Guldberg and Smith, 1989; Glynn, 1991; Glynn and D'Croz, 1990) and low water temperature (Hoegh-Guldberg and Fine, 2004), high light or UV (Lesser et al., 1990), aerial exposure (Leggat et al., 2006), cyanide (Jones and Hoegh-Guldberg, 1999), low salinity (Kerswell and Jones, 2003) and herbicides (Jones, 2005).

Increased seawater temperatures directly affect the photosynthetic apparatus of *Symbiodinium*, leading to an increase in production of reactive oxygen species and the disassociation of the coral-*Symbiodinium* endosymbiosis through a variety of possible cellular mechanisms (Gates et al., 1992). Proposed cellular mechanisms underlying coral bleaching include active host processes such as exocytosis and host cell apoptosis, and passive processes such as necrosis (Gates et al., 1992) (for reviews of apoptosis see Elmore, 2007 and apoptotic pathways of lower invertebrates see Zmasek et al., 2007). Despite intense study over the last 10 years, the exact point of thermal damage in *Symbiodinium* has not been comprehensively demonstrated. A variety of evidence has been presented supporting direct temperature effects on the photosystem II (PSII) (Iglesias-Prieto, 1997), in particular the protein D1 (Warner et al., 1999; Lesser and Farrell, 2004), the repair mechanism of PSII (Takahashi et al., 2004); the synthesis of accessory pigments (Takahashi et al., 2008),; the dark reactions of photosynthesis (Jones et al., 1998); degradation of *Symbiodinium* thylakoid membranes (Tchernov et al., 2004) and possibly a reduction of Rubisco activity (Leggat et al., 2004), all of which lead to a production of reactive oxygen species. All of these hypotheses are consistent with the observation that the onset of coral bleaching is often characterised by a reduction in the dark adapted photosynthetic yield of PSII associated with the reduction of endosymbiont density (Jones et al., 1998; Jones et al., 2000; Warner et al., 1999). This measurable reduction of photosynthetic yield and the ability to easily monitor photosystem II efficiency using fluorescence (Jones et al., 1999) has driven the pre-occupation of recent studies with the condition of the dinoflagellate symbiont during heat stress (Fitt et al., 2001). One consequence of this preoccupation has been a limited number of experimental studies that have examined the underlying cellular mechanisms occurring specifically within the coral animal host during

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the initial stages of thermal stress and subsequent bleaching (Edmunds and Gates, 2003; Lesser and Farrell, 2004; Dove et al., 2006). This is despite the proposal that a number of host mechanisms that may be involved in coral stress and bleaching (Gates et al., 1992).

The increasing incidence of disease prevalence among marine and terrestrial animals has been linked to climate change (Harvell et al., 2002; Sutherland et al., 2004; Bruno et al., 2007). Recent studies have proposed that this linkage is related to primary pathogens having an increased virulence during periods of increased sea temperatures (Bruno et al., 2007; Rosenberg et al., 2007). An alternative viewpoint suggests that primary environmental stressors such as extreme temperatures and thermal stress in summer months directly affect coral animal without the involvement of a primary pathogen (Lesser et al., 2007; Leggat et al., 2007b). Understanding the role of 'abiotic' disease in cases of coral disease has received little attention. Abiotic diseases and their impacts are being increasingly recognised in other systems (e.g. higher plants Ayres and Lombardero, 2000; Garrett et al., 2006; Boland et al., 2004) as an important threat related to climate change. In a review of abiotic disease and stress in plants, Ayres (1984) proposes a model for the integration of stress and various levels of sub-cellular, cellular and organism responses in governing disease outcomes and breakpoint (or point of irreversible change or damage) related to increasing stress impacts. An understanding of the links between stress and disease in coral reef systems has been hindered by a lack of understanding of coral cell biology and coral stress biology. In determining the role of stress, abiotic impacts, and increasing disease on coral reefs worldwide we require a greater understanding of the subcellular and cellular changes that occur not only during the onset of bleaching but also from the initial stress response and through the communication of stress across the holobiont.

In the present study, we explore the cellular and tissue changes that occur in the coral host during the early stages of thermal stress. In doing this, we describe a scenario where the host cellular biology is affected by early thermal stress prior to the bleaching response being observed in the *Symbiodinium*.

2. Materials and Methods

2.1. Experimental design

Acropora aspera colonies (3 large distinct patch colonies) on the reef flat of Heron Island (23.44°S, 151.91°E) were selected as the experimental organism and a total of 108 branches of at least 7 cm in length were collected and transported immediately to holding tanks in a flow-through seawater system. The coral branches were randomly assigned to one of 4 experimental 60 l plastic aquaria with flow rates of approximately 10 l min^{-1}, and stored in polypropylene racks at least 10 cm above the base of the tank to ensure mixing and flow of seawater around the entire branches. Two of the four aquaria were used as heat treatment tanks, and two as control tanks. Control and treatment tanks were fed from separate 1000 l sumps; this design was chosen to allow for effective heating of water in the experimental tanks. The tanks were maintained for 4 days prior to coral collection to allow the microbial surface layers to stabilise. After collection of the coral colonies, the system was left at ambient flow through conditions for 5 days to acclimate before initiation of the experiment. The control tanks remained at ambient sea surface temperatures throughout the experiment, while heat treatments involved a daily 1 °C increase in water temperature at 0800 hours on each experimental day throughout the experimental period (Fig. 1). Temperatures increased above the control 28 °C in experimental tanks on the 9th of December to 29 °C (Day 3), the 10th of December to 30 °C (day 4), the 11th of December to 31 °C (day 5), and over the bleaching threshold of 32 °C on the 12th (day 6) continuing until the 15th of December (Day 9). To replicate natural reef conditions, normal temperature fluctuations over the 24 hour day/night periods were maintained (Fig. 1). Temperature in each of the four tanks was monitored using Odyssey data loggers (Christchurch, New Zealand) every two minutes through the experimental and acclimation periods.

Two entire branches were arbitrarily sampled from each tank each day, from one day before the experimental period and daily throughout the experimental period for tissue processing. Five branches in each experimental aquaria were assigned solely for measurement of dark adapted Fv/Fm using an Imaging-PAM Fluorometer (Walz, Germany) to avoid handling effects on tissues and cell responses. These five branches were used to record the onset of coral bleaching as measured by a sustainable downturn in photosynthetic yield (Jones et al., 1998). Branches were also sampled from *A. aspera* coral colonies during a natural bleaching event in the field during December 2005 and January 2006. The branches were collected at midday on the low tide and were transported, immediately fixed, handled, processes and measured as per the experimental coral samples.

2.2. Sample preservation and tissue processing

Coral braches were randomly selected and sampled at precisely mid-day, daily, from both experimental and control aquaria. Branches were fixed individually in 50 ml of 4% (w/v) paraformaldehyde in sterile phosphate buffered saline (PBS, pH 7.4) (Ainsworth et al., 2006; Ainsworth et al., 2007) for 12 h at 4 °C, and then stored in PBS at 4 °C. Prior to decalcification with 20% (w/v) EDTA (pH 8), the coral branches were dissected into 3 regions lengthwise, to allow cross-sectioning of

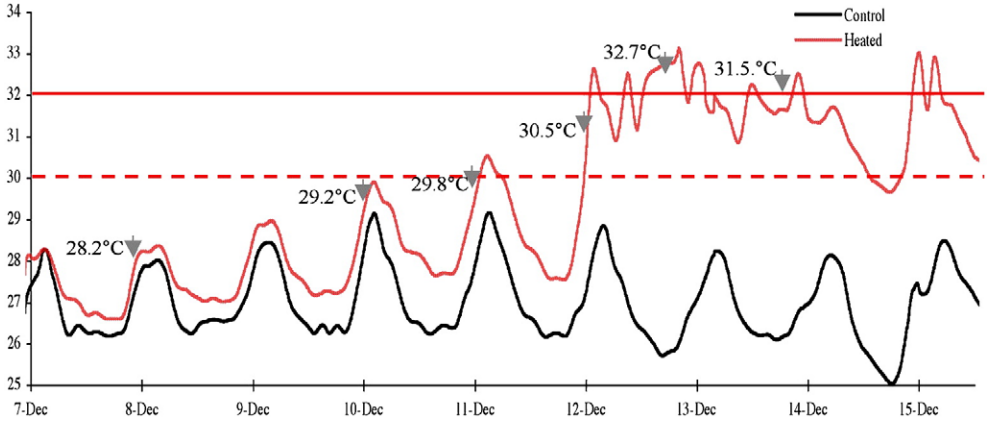

Fig. 1. Thermal log of heated and control flow through aquaria for the duration of the experimental period. Grey arrowheads indicate sampling points. Dashed line indicates 30 °C thermal impact, solid line indicates 32 °C thermal threshold.

the coral braches and measurements of the coral tissue layers to be taken from all regions along the length of the coral branch (base, centre and tip) to account for variability within the coral branch. Decalcified coral samples were then processed for standard paraffin embedding, this included washes of 70%, 80%, two of 95% and three of 100% ethanol for 40 min each, three xylene washes for 40 min and then 3 paraffin washes under vacuum for 40 min each prior to embedding in paraffin. Serial tissue sections (4 μm) were taken from samples each day of the experimental period and prior to experimental manipulation, and were collected onto Superfrost Plus slides (Menzel, Brauschweig, Germany). The tissue sections were then stained using Harris's haematoxylin and eosin (with Phyloxine B) (Sigma-Aldrich Pty Ldt, # HHS32 and HT110-1-32).

2.3. Tissue structure and physiology

Measurements of the thickness of coral tissue layers were conducted on an Olympus BX4 microscope at 100 times magnification. Tissue regions between polyps comprising the coenosarc were selected for measurement due to the consistency and evenness of the tissues. Two tissue sections, separated by at least 150 μm from each of the three branch regions (tip, centre and base) were investigated; from each tissue section, 9 measurements were made of the epithelial and gastrodermal tissues, resulting in 54 measurements taken from each coral on each experimental day, which were averaged for each region, with two samples taken per aquarium per day, 216 measurements of each parameter were taken each day from both treated and control coral branches. The population density of *Symbiodinium sp.* was determined via cell counts within each of the tissue sections. The density of endosymbiotic algae within each tissue section is normalised to the total area of the coral tissue (symbionts per cm^2 of coral tissue) as opposed to the traditional measure of symbiont density as per cm^2 of coral skeleton.

2.4. Histopathology

The extent of mass tissue necrosis (swelling and lysis of cells, disruption of cell structure) was determined via visual assessment using light microscopy of H&E stained tissue sections. *In situ* labelling of 3′ end of DNA fragments was used to investigate the presence and extent of programmed cell death (Ainsworth et al., 2007; Ainsworth et al., 2006) using the ApopTag *in situ* apoptosis detection kit as per manufacturers recommendations (S7101, Chemicon International, Inc. USA). This has been shown to distinguish apoptosis from necrosis by specifically detecting DNA cleavage and chromatin condensation associated with apoptosis and confirmed by the lack of necrotic morphology. Cells were defined as apoptotic if the nuclear area of the cell was positively labelled as indicated by red stain as opposed to the blue haematoxylin counter-stained non-apoptotic nuclei (Ainsworth et al., 2007).

2.5. Statistical analysis

All parameters were analysed using a nested ANOVA design using Statistic V7 (Statsoft Oklahoma, USA). Post-hoc analysis was performed with a Tukey's post hoc test.

2.5.1. PAM flourometry

An imaging Pulsed Amplitude Modulated (iPAM) fluorometer (imaging-PAM, Waltz Gmbh, Germany) was used to examine the photosynthetic efficiency of the algal endosymbionts. At 1800 h each day coral branches were dark adapted for 30 min and the dark adapted quantum yield of photosystem II determined using the Genty equation $Y=(F_m-F_o)/Fm$ (Genty et al., 1989).

2.5.2. Apoptotic gene detection, sequencing and phylogenetic analysis

In order to support the assumption that apoptotic cellular processes occur in Acroporid corals, apoptotic pathway genes were identified in *A. aspera*. An Expressed Sequence Tag (EST), which was later confirmed as originating from the coral host, was identified as Bcl-2 like from an existing EST sequence library (Leggat et al., 2007a), derived from the *Symbiodinium* isolated from this coral. This library was known to contain approximately 10% coral host contamination (Leggat et al., 2007a). Subsequently primers were designed for this sequence and used to amplify the full length transcript from the cDNA library. In addition, the corresponding sequence was obtained from a library constructed from the coral holobiont *Acropora millepora*. Total RNA was isolated from *A. millepora* by grinding an entire branch under liquid nitrogen, RNA was then purified using the RNAspin kit (GE Healthcare, Uppsala Sweden). mRNA was subsequently purified using the Poly(A)Purist kit (Ambion, Texas, USA) and a directional phage library was constructed in λ-ZAP II (Stratagene, La Jolla, California, USA) as per the manufacturer's instructions, this library was used as a template to amplify the complete cDNA sequences using vector and gene specific primers (Leggat et al., 2007a). Searches of the NCBI database revealed a partial Bax-like sequence (accession number DY581529) from *A. millepora*, this was used to design primers and amplify the complete cDNA sequence from both the *A. aspera* and *A. millepora* holobiont libraries. The cDNA sequence for both the Bcl-2-like and Bax-like proteins were identical for both *A. aspera* and *A. millepora*. The origin of these genes (coral vs *Symbiodinium*) was confirmed by amplifying a 450 bp genomic DNA fragment from *A. millepora* sperm, which is *Symbiodinium* free, and genomic DNA isolated from long-term *Symbiodinium* cultures. Products were only amplified from the coral genomic DNA, when this product was sequenced it exactly matched the obtained cDNA sequence. Phylogenetic analyses were conducted using the program Clustal W (Thompson et al., 1994) from BioManager by ANGIS (http://www.angis.org.au), alignments were bootstrapped 1000 times and the trees constructed using maximum likelihood (Felsenstein, 1989) with Tree Puzzle (Version 5.2).

2.5.3. Satellite tracking of localised thermal anomalies

Weekly averages of sea-surface temperature (SST) were constructed for the Great Barrier Reef using only high quality-control values (Pathfinder Version 5.0 dataset, NOAA 2007, http://www.nodc.noaa.gov/sog/pathfinder4km/). This provides daily global SST data at approximately 4-km resolution (Global Area Coverage) for the period during 1985–2006. Missing data pixels and data gaps were temporally and spatially filled using standard techniques of climatology-comparisons. The resulting dataset comprises 52 one-week periods for each year, where the weekly-mean (WM) temperature is the average of each week's SST values throughout 1985–2006. The NOAA Coral Reef Watch maximum monthly means (MMM) climatology covers the globe at 0.5-degree (50-km) resolution http://coralreefwatch.noaa.gov.

3. Results

3.1. Experimental bleaching

Dark-adapted photosynthetic yield was significantly different between treatments ($F_{1,\ 128}=347.96$, $p<0.00005$) with a significant treatment×day effect ($F_{14,128}=185.10$, $p<0.00005$) while there was no significant tank effect. Post-hoc analysis found significant differences between control and heated coral photosynthetic yields on days 7 and 8 (Fig. 2b). *Symbiodinium* cell densities were also significantly different between treatments and controls with post hoc analysis showing significant differences between population densities on day 7 and 8 (Fig. 2a). These decreases in dark-adapted yield and cell density occurred when temperatures exceeded 32 °C for 24 h (Figs. 1 and 2 a, b). An identical thermal threshold (32 °C) was identified in previous studies on the same species at Heron Island (Dove, 2004). As temperatures of 32 °C and above continued, PSII yield and *Symbiodinium* cell densities

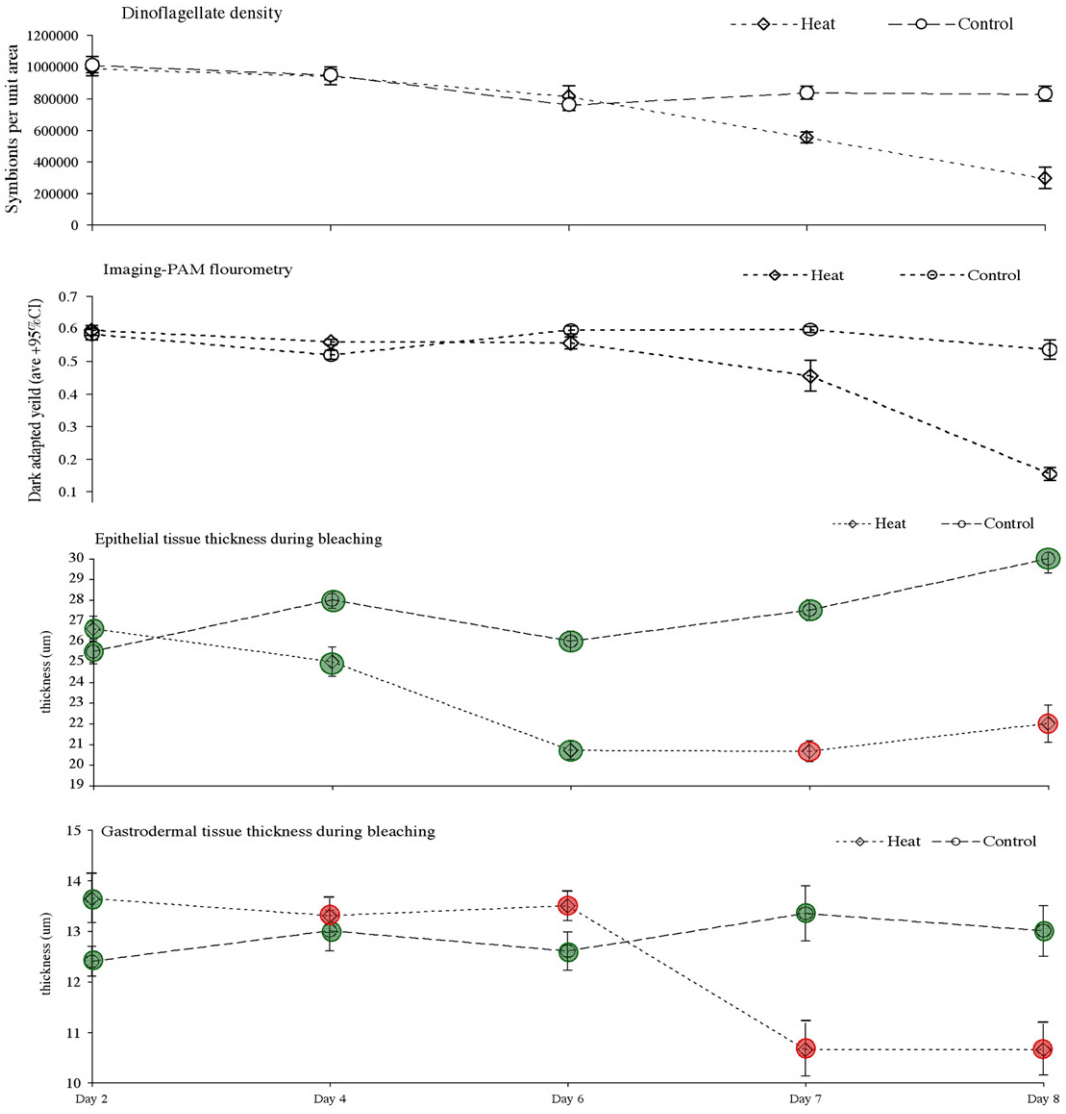

Fig. 2. Endosymbiotic algal densities (a) and reductions of PSII, as determined by imaging PAM (b), occur after prolonged exposure to 32 °C. Reduction of epithelial (c) and gastrodermal (d) tissues layers associated with increasing thermal stress and coral bleaching, and detection of apoptosis (as indicated by red dots) occur at temperatures as low as 30 °C, and several days prior to the onset of bleaching. Red dots indicate detection of apoptosis in cells, green dots indicate no detection of apoptosis. Symbionts per unit area, equates to symbionts per cm^2 of coral tissue. Where no stated error bars represent the standard error.

continued to decline until conditions of severe bleaching were reached (PSII yield <0.3, cell density reduction >50%).

Coral branches were sampled for histological analysis throughout the experimental period. Significant changes in the tissue pathology of *A. aspera* were detectable long before any reductions in the population densities or dark-adapted photosynthetic yields of *Symbiodinium* were observed. The thickness of the coral epithelial layer was significantly smaller in heated corals than in controls ($F_{1, 20}=47.694$, $p<0.00005$) with a significant treatment by day effect ($F_{8, 20}=6.5616$, $p=0.00031$). Post hoc analysis showed that the treatment thickness was significant smaller after exposure to 29 °C (day 4) with a reduction of approximately 30% after exposure to 31 °C (day 6) (Fig. 2c). In contrast, significant reductions in the coral gastrodermal layer did not occur until the reduction of photosynthetic yield and loss of the endosymbiotic algae on experimental days 7 and 8 (Fig. 2d).

Apoptotic cell death was detectable in tissue layers of *A. aspera* following exposure to 30 °C (Fig. 2). This was first found only in the gastrodermal cell layers (Fig. 2d) and was limited primarily to small regions associated with the mesenterial tissues (Fig. 3). It was also more pronounced following exposure to 32 °C (Figs. 2 and 3). Apoptotic cell death was evident within all tissue layers after the first period of 32 °C occurring on experimental day 7 at which point initial bleaching effects could be observed in the endosymbiont (Fig. 2c, d). Evidence for mass necrotic cell death or a loss of tissue integrity occurred following the reduction of endosymbiotic algal density on experimental day 8 (Fig. 4), as would be expected with the severe bleaching observed. The corals remaining within the experimental conditions following day 8 showed no signs of photosynthetic activity or tissue integrity and macro-algal overgrowth was observed to occur rapidly.

3.2. Characterisation of Bcl-2 like and Bax-like genes

Apoptosis is controlled by a variety of regulatory proteins of the Bcl-2 family, which includes anti-apoptotic *Bcl-2-like* proteins and pro-apoptotic *Bax-like* proteins. In higher organisms, the expression of these "*life and death proteins*" governs apoptosis of the cells (Spierings et al., 2005; Lanave et al., 2004). To take the first steps to understand the capability of corals to express *Bcl-2-like* and *Bax-like* proteins, existing cDNA libraries were screened to detect the

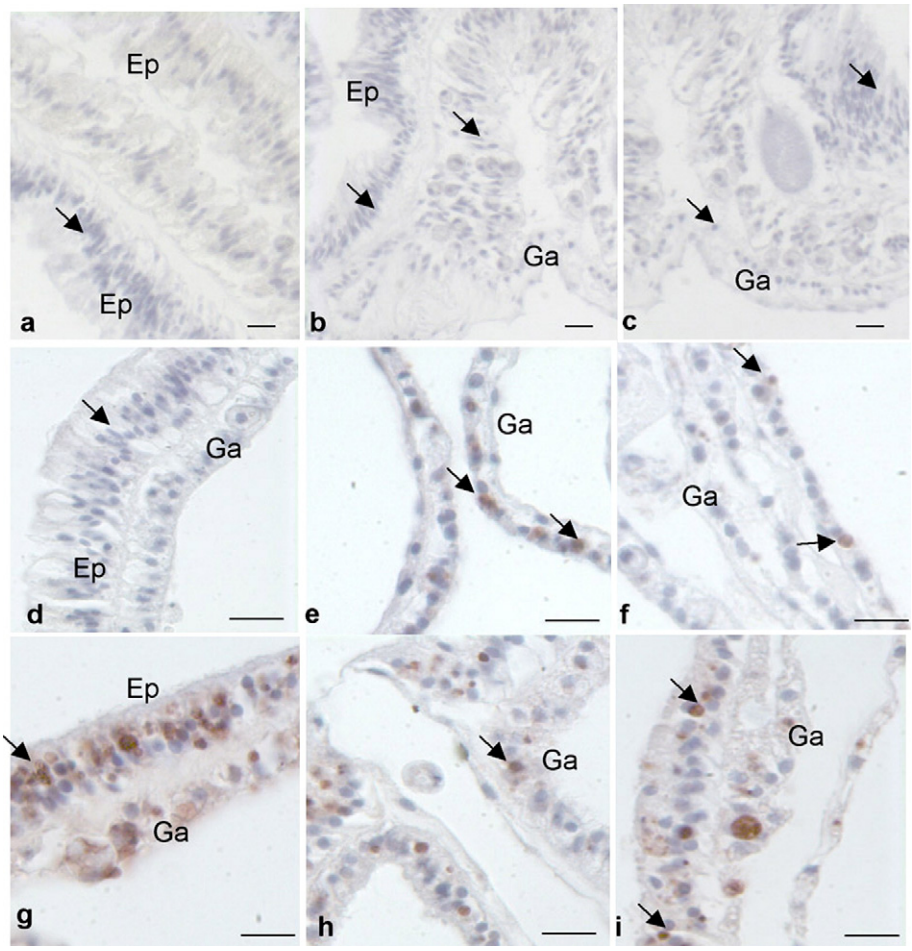

Fig. 3. Prior to thermal stress (a,b,c) no apoptotic cell death is detected within any of the tissue layers. The occurrence of apoptotic cell death associated with thermal stress of 30 °C at day 4 is detected in the gastrodermis only (d,e,f) and subsequently then in the epithelial layer during defined coral bleaching at prolonged exposure to 32 °C (g,h,i) in *Acropora aspera*. Arrowheads indicate nuclear stain. Apoptotic nuclei red, counterstain nuclei blue. Scale bar, 20 μm, Ep, epithelium, Ga, Gastroderm.

presence of Bcl-2-like and Bax-like genes. Transcripts encoding for both a Bcl-2-like (EU161957) and Bax-like (EU161958) protein were found (Fig. 5a), indicating that pathways similar to those in other organisms operate within Acroporid corals of the GBR. Exploration of the sequence information revealed that both transcripts contained putative BH1, BH2, and BH3 domains (Fig. 5a), while the Bcl-2 like transcript also contained a putative BH4 domain, which is characteristic of the anti-apoptotic members of this protein family. Phylogenetic comparison of the BH3 domain of coral Bcl-2 and Bax homologs cluster with the anemone (*Aipatsia sp.*) (Dunn et al., 2006) homolog and representative of both vertebrate and invertebrate proteins found that the coral and anemone sequences form a distinct cluster (Fig. 5b). Within the Cnidarian cluster the *Aiptasia sp.* and coral pro-apoptotic genes were found on the same branch with high bootstrap support (Fig. 5b). The BH3 domain has previously been identified as a suitable region for phylogenetic analysis of the Bcl-2 family of proteins (Lanave et al., 2004).

3.3. Host changes during field bleaching at Heron Island

Almost coincidental with the experimental bleaching of *Acropora aspera* a thermal anomaly (20th Dec 2004 – 4th Jan 2005) resulted in bleaching on the reef flat of Heron Island (151°54′40″E, 23°46′22″S). The progression of weekly-averaged sea-surface temperature (SST)

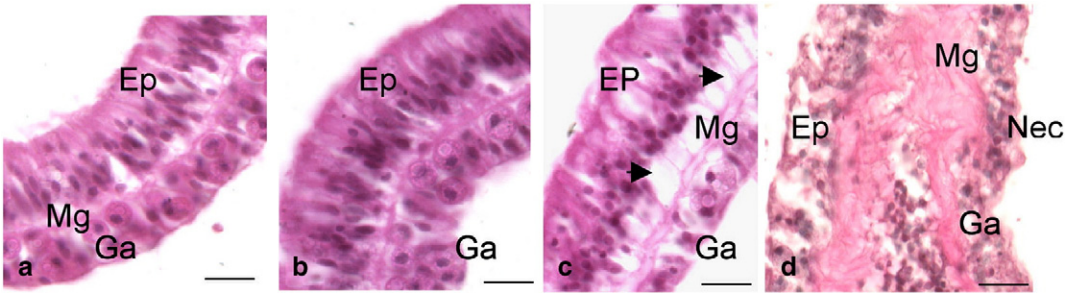

Fig. 4. Tissue structure of *Acropora aspera* remains intact through initial temperature stress of day 4 (a) and day 6 (b), an apparent early loss of structure occurs simultaneous to bleaching and symbiont loss (Day 7) (c), the onset of necrotic cell death occurs after the gross impact of bleaching and endosymbiont loss (day 8) (d).

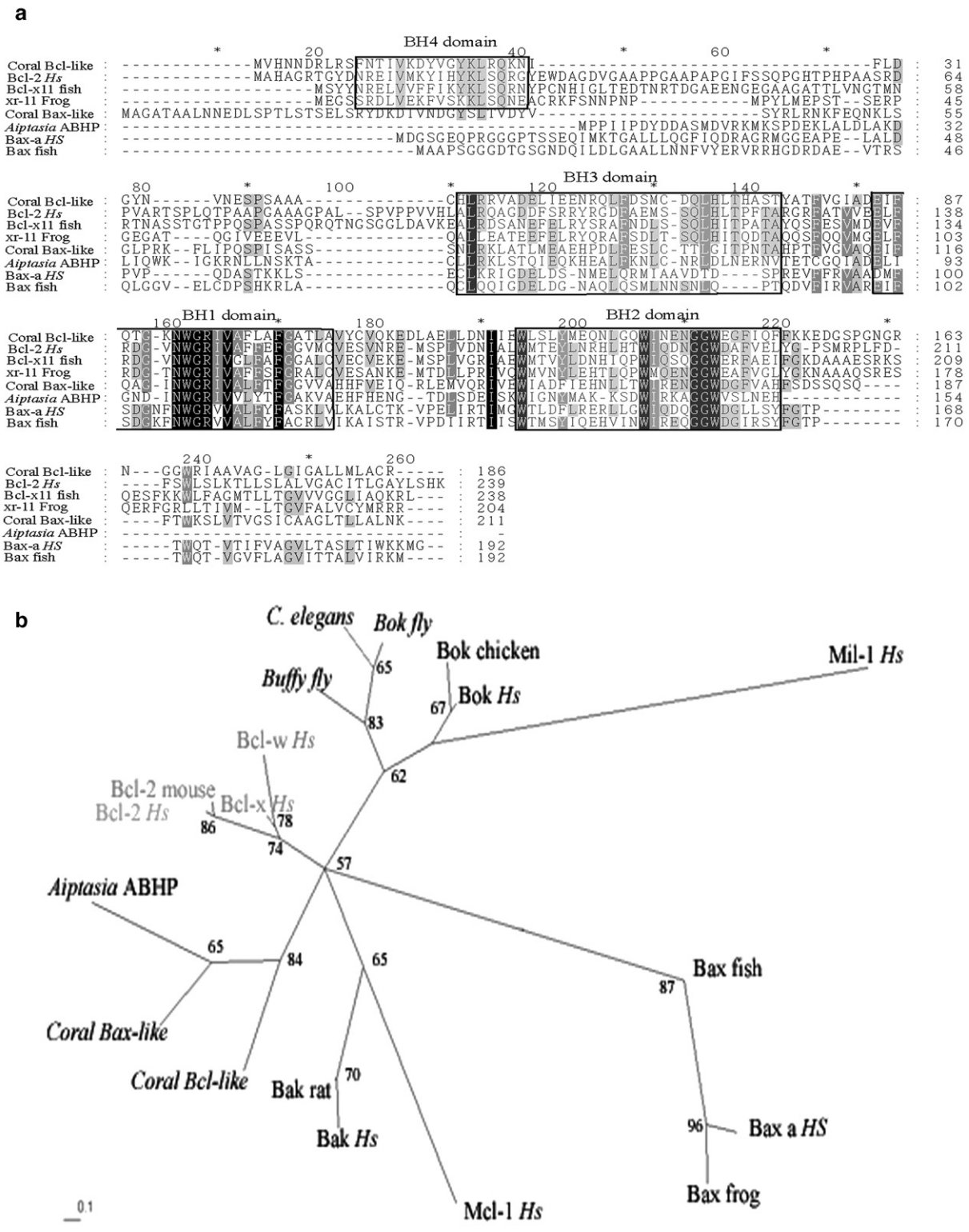

Fig. 5. Gene alignment and domain identification of coral Bcl-2 and Bax like genes based on multi-domainal analysis and identification of BH4 domain governing pro-apoptotic status (a) and Phylogenetic tree (b) of coral Bcl2-like and coral Bax-like genes, showing delineation with *Aiptasia sp.* ABHP. Invertebrate sequences are in italics. Bcl-2 family members are in grey. Bootstrap values are indicated at branches.

measurements at two-week intervals through Dec 2005 indicates the rapid increase in thermal stress across the Capricorn and Bunker groups (Fig. 6). Significant temperature increases occurred during the one-week periods ending 16 Dec 2005 and 30 Dec 2005 for Heron Island (4 km-weekly satellite SST) (Fig. 6). The long-term temperature average (maximum monthly means (MMM) and the 4 km weekly-mean (WM)) demonstrate that temperatures were above the usual weekly average for that time of year throughout the month of December and exceeded the average summertime maximum (MMM) (Fig. 7a). While the observed temperature anomaly was not as sustained as that used in the experimental treatments it provided a unique opportunity to determine if similar processes those under experimental conditions, would be observed in within an ecological setting.

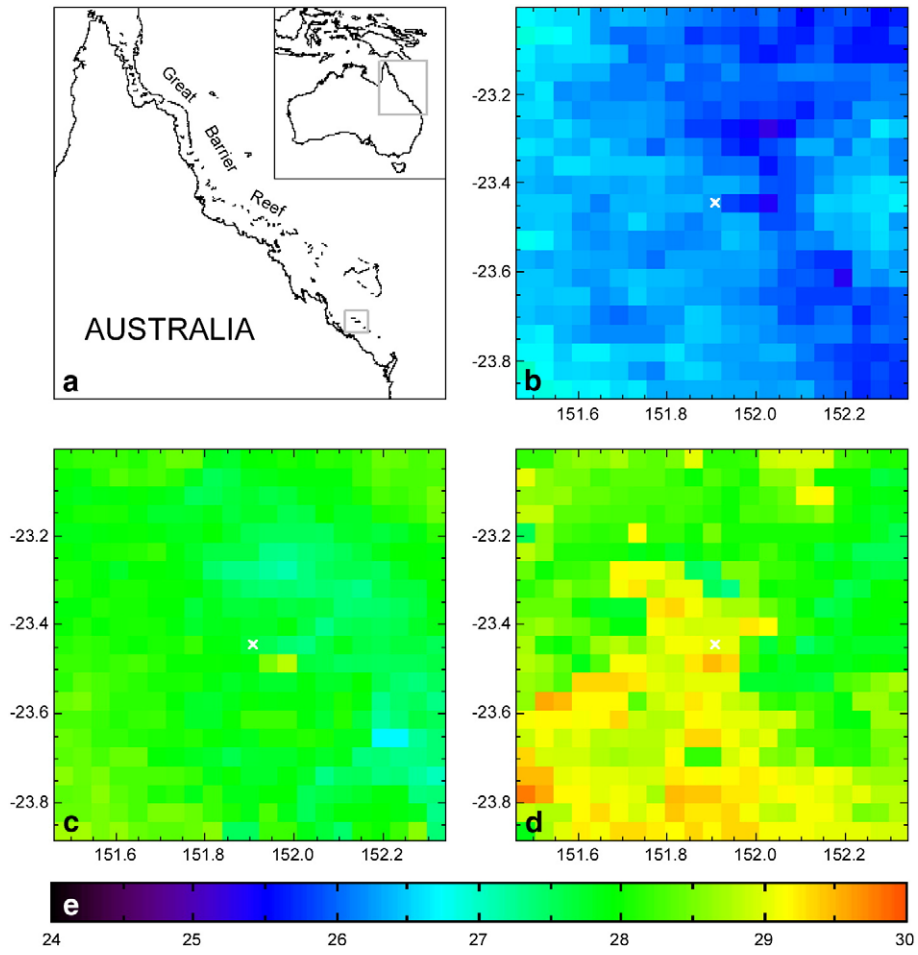

Fig. 6. The field-site location (grey box) in the southern Great Barrier Reef (a), and regional sea-surface temperature evolution during the study period of 02 Dec (b), 16 Dec (c) and 30 Dec (d) 2005. The location of Heron Island is indicated by symbol 'x'. Sea-surface changes are represented by the colour scale (°C) (e).

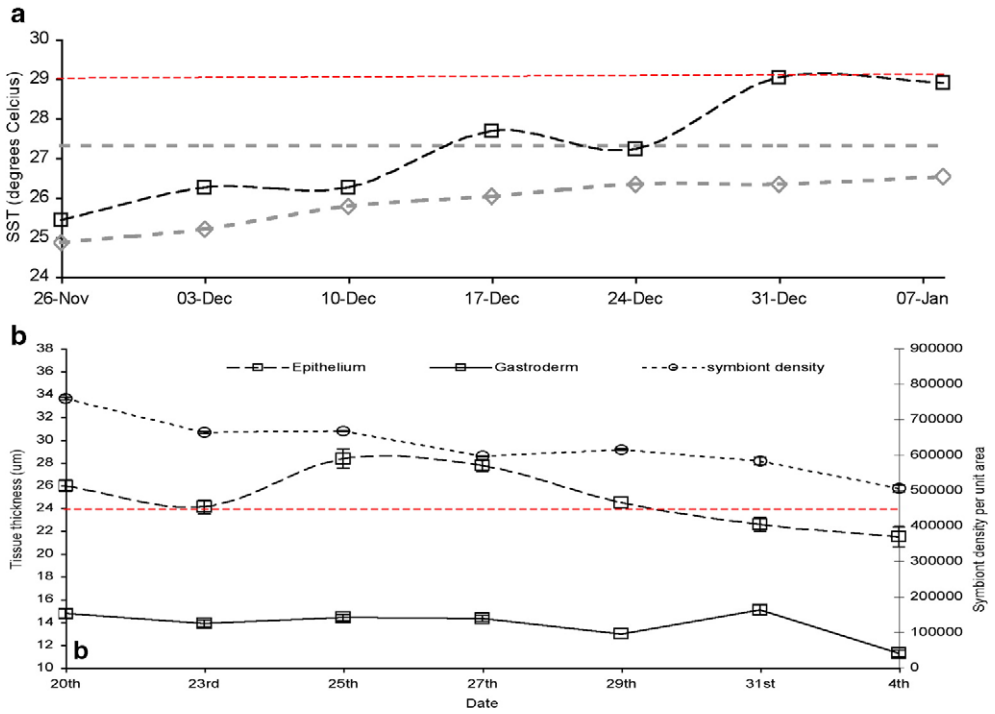

Fig. 7. The regional sea-surface temperature time-series, mean monthly maxima, and weekly maxima for Heron Island during December 2005 display the local temperature anomalies (a) and responding cellular and tissue changes of *Acropora aspera* to thermal anomalies on the reef flat of Heron Island for December 2005. Symbionts per unit area, equates to symbionts per cm^2 of coral tissue. Red dashed line indicates 29 degrees Celsius (a) and 24um tissue thickness (b).

Haphazard sampling was only possible during the field bleaching, however it was determined that *Symbiodinium* densities had decreased by approximately 20% during the period of 20th–29th December 2005 and 33% by 4th January 2006, consistent with the period when the SST was greater than 1 °C above the MMM. While changes were smaller than those seen in the experiments, there was a decrease from the endosymbiont densities observed on the 20th December 2005. Unfortunately it was not possible to obtain field measurements of PSII dark adapted yield during this time. Host tissue and cellular changes were similar to those seen in the experiments, and tracked changes in sea temperature observed in the Capricorn bunker group of Islands (Fig. 7a) during the period 20th–29th December 2005 (Fig. 7a). Increases in seawater temperature corresponded to a reduction in epithelial tissue thickness, prior to bleaching associated reductions in *Symbiodinium* densities. This pattern of tissues changes within an ecological setting reflects that of the experimental trials (Fig. 7b). The changes seen in the field were however muted by comparison to the changes seen in the experiments, reflecting the milder time course of changes occurring in the field (Fig. 2 versus Fig. 7).

4. Discussion

Here we see that sub-cellular and cellular responses occur in the coral host at lower temperatures not previously considered detrimental. Coral host cellular responses occur at temperatures below 30 °C, with early changes within the host tissues including apoptotic cell death in the gastrodermis and a reduction in the epithelial cell layer thickness. These cellular changes continue as thermal stress increases, with extensive cellular changes evident before the onset of symbiont loss or reduction in maximum photosynthetic yields of the dinoflagellate. This demonstrates that important changes to the host are induced during periods of extreme, but not bleaching level climate conditions. This has the potential to have implications to our understanding of coral cell stress biology and physiology that have previously been overlooked.

Gates et al. (1992) outlined 5 cellular mechanisms that may underpin the loss of symbionts from the coral host during coral bleaching events. Our study reveals that different cellular mechanisms are involved in the sequence of events associated with thermal stress within the host prior to and during the loss of symbionts. Apoptotic cell death and reduction of tissue layers occurred early in the stress response, whereas mass necrosis was is evidence for gross injury to the organism related to the breakdown of the symbiosis. The loss of dinoflagellates and/or their pigments from symbiosis during bleaching is a relatively late event in the cellular sequence of thermal stress by comparison to the mechanisms occurring within the host. The presence of putative pro- and anti-apoptotic genes also confirms that coral host cells have the potential to trigger host controlled cellular processes such as apoptosis and this represents the first time that both putative pro- and anti-apoptotic genes have been described from Cnidarians and shows similarity to that described across lower invertebrates (for reviews of identified lower invertebrate apoptotic genes see David et al., 2005 and Zmasek et al., 2007). Given that apoptosis probably incurs a cost to overall organism fitness, for example through the expression of new protein sets, sea surface temperatures below 30 °C may have major implications to holobiont physiology which have previously been overlooked. Sea surface temperatures of only 1 °C above long term summer maxima have been shown to cause coral bleaching (Lui et al., 2003; Gleeson and Strong, 1995). The impact of thermal stress on coral cell physiology maybe more complicated than those linked to observable mass bleaching events. As seen here sub-cellular and cellular responses in the host occur at lower temperatures, the role of these host thermal stress responses in potentially driving disease susceptibility or colony mortality in times of further stress needs to be investigated.

Our understanding of the long-term effects of these sub-cellular and cellular stressors in corals is extremely limited. For example, some coral species are suggested to exhibit lower capability for thermal adaptation and higher susceptibility to bleaching and disease, however there is little understanding of what is driving this at a physiological and cellular level. Understanding the thermal stress impacts to the host may help to understand if different mechanisms are driving long-term consequences to coral colonies. For example, the epithelial layer of corals is largely comprised of mucus producing cells, and mucus plays an important role in waste removal, antimicrobial defences and coral stasis (Wild et al., 2004; Brown and Bythell, 2005; Ritchie, 2006). It is estimated that the dominant reef building coral genus *Acropora* may exude up to 4.8 l of mucus per square metre of reef per day, equating approximately 10–21 mmol of particulate organic carbon (Wild et al., 2004). This release is thought to increase during periods of environmental stress and the impacts of pre-bleaching temperatures on the host cell biology may have significant impacts to the long-term energy reserves of the holobiont. Reduced epithelial thickness as seen in this study may have resulted from mucus release and depletion of the mucus reserves within the epithelial layer. This has the potential to contribute further demands to the symbiont (the primary source of energy) during the onset of bleaching temperature thermal stress and further impact the coral physiology, mucus production. Given that Acroporid corals are considered among the most susceptible to both bleaching (Marshall and Baird, 2000) and disease (Sutherland et al., 2004), the short and long-term impact of epithelial reductions and mucus loss during early environmental stressors needs to be determined especially considering the role of mucus in waste removal and energy reserves. Furthermore the 'pre-bleaching' temperatures and subsequent impacts to the host as shown in this study should also be included in models investigating links between thermal stress and later disease events.

5. Conclusion

In conclusion, Miller et al. (2007) have shown the coral host is much more complex than previously thought, having a compliment of molecular pathways through which the coral can illicit complex immune and stress responses. This study demonstrates that pre-bleaching thermal stressors are important to investigate and that the molecular, sub-cellular and cellular stress responses of the host, which have the potential to impact the holobiont physiology, need to be determined especially if we are to understand the role of environmental stressors in long-term outcomes such as disease. Understanding how stress affects both partners in the symbiosis and in combination (holobiont) is necessary if we are to understand the ecosystem changes that are already occurring in the environment in response to environmental stress. Given the early onset of sub-cellular and cellular responses as demonstrated within this study, it must be noted that impacts of environmental stress to coral colonies involve more than those occurring during observable "bleaching events". The role of these sub-cellular and cellular impacts in governing short and long-term survival of the individual coral colonies and consequently coral reefs must also be considered.

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