

# Heat stress induces different forms of cell death in sea anemones and their endosymbiotic algae depending on temperature and duration

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## Abstract

**Bleaching of reef building corals and other symbiotic cnidarians due to the loss of their dinoflagellate algal symbionts (= zooxanthellae), and/or their photosynthetic pigments, is a common sign of environmental stress. Mass bleaching events are becoming an increasingly important cause of mortality and reef degradation on a global scale, linked by many to global climate change. However, the cellular mechanisms of stress-induced bleaching remain largely unresolved. In this study, the frequency of apoptosis-like and necrosis-like cell death was determined in the symbiotic sea anemone *Aiptasia* sp. using criteria that had previously been validated for this symbiosis as indicators of programmed cell death (PCD) and necrosis. Results indicate that PCD and necrosis occur simultaneously in both host tissues and zooxanthellae subject to environmentally relevant doses of heat stress. Frequency of PCD in the anemone endoderm increased within minutes of treatment. Peak rates of apoptosis-like cell death in the host were coincident with the timing of loss of zooxanthellae during bleaching. The proportion of apoptosis-like host cells subsequently declined while cell necrosis increased. In the zooxanthellae, both apoptosis-like and necrosis-like activity increased throughout the duration of the experiment (6 days), dependent on temperature dose. A stress-mediated PCD pathway is an important part of the thermal stress response in the sea anemone symbiosis and this study suggests that PCD may play different roles in different components of the symbiosis during bleaching.**

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**Keywords:** apoptosis; programmed cell death; necrosis; bleaching; cnidaria; coral; sea anemone

**Abbreviations:** PCD, programmed cell death; AI, apoptotic index; ROS, reactive oxygen species; ISEL, *in situ* end labelling;

ANCOVA, analysis of covariance; ATP, adenosine triphosphate; PAR, photosynthetic active radiation

## Introduction

Reef building corals and symbiotic sea anemones are cnidarians that host endodermal dinoflagellate algal symbionts. The symbionts, commonly known as zooxanthellae, promote calcium carbonate accretion by their coral hosts, and are important primary producers in coral reef ecosystems. The coral symbiosis is responsible for the deposition of the structural framework of these highly diverse ecosystems. The loss of the zooxanthellae and/or their photosynthetic pigments from the host results in a pale or whitened appearance that is called bleaching. Bleaching of corals leads to reduced carbonate accretion and ultimately in death of the coral. The increasing frequency and scale of mass bleaching and subsequent mortality events on coral reefs over the last two decades has been mainly attributed to environmental stress due to elevated temperature and solar irradiance,<sup>1,2</sup> mediated in some cases by bacterial pathogens.<sup>3</sup> Despite the global importance of coral reef ecosystems, the cellular mechanisms leading to bleaching remain largely unresolved and a recent review highlighted the need for a better understanding of the process at sublethal temperatures.<sup>4</sup>

Kültz<sup>5</sup> has recently argued that the cellular stress response is so well conserved because it represents a rapid, transient mechanism for promoting tolerance to temporary environmental extremes, thereby allowing for slower, stressor-specific mechanisms to be upregulated. Programmed cell death (PCD), or apoptosis, is recognised as an important part of the stress response. Once stabilisation and repair mechanisms have been exhausted or overwhelmed, PCD may mitigate further damage by preventing widespread inflammation and genetic instability.<sup>5</sup> Given this relationship, it is perhaps not surprising that heat shock (stress) proteins are implicated as regulators, usually inhibitors, of PCD and necrosis.<sup>6–8</sup> Conversely, reactive oxygen species (ROS), or the stress and cellular damage they cause, are known triggers of PCD. In plants, ROS may not only be toxic by-products of aerobic metabolism, but may be actively produced in response to stress and pathogens as a cell-death signalling mechanism.<sup>9,10</sup> In order to understand stress tolerance thresholds<sup>4</sup> and adaptive capacity of an organism to stress, it is clear that we must first determine whether different mechanisms are controlling cell death in response to different types and doses of stress.

The adaptive significance of different types of cell death in symbiosis has only recently been considered, but is critically important since PCD potentially provides a mechanism for host control of the intracellular symbiont populations by

deletion of the host cell containing the symbiont. PCD and necrosis of host animal cells were first proposed by Gates *et al.*<sup>11</sup> as possible mechanisms for release of the intracellular symbionts during bleaching. Later it was recognised that death and *in situ* degradation of the zooxanthellae may also be a significant pathway for loss of symbionts during bleaching events.<sup>12,13</sup> However, the importance of these processes relative to exocytosis of zooxanthellae or detachment of host cells<sup>11</sup> has not yet been resolved. Dunn *et al.*<sup>14–16</sup> recently showed evidence for the simultaneous occurrence of PCD and necrosis in both zooxanthellae and host cells in the symbiotic sea anemone *Aiptasia* sp during bleaching caused by heat stress. In the unicellular zooxanthellae, the operation of a ‘cell suicide’ mechanism may at first appear paradoxical but may indirectly benefit the population (which is cloned within the host by asexual division) by preventing an inflammatory response and further damage in the host. Furthermore, the relationship between the onset of bleaching, which may be relatively slow at a reef-scale, and the initiation of a rapid and possibly transient stress response to temperature elevation is unknown. Understanding this is key to predicting the future impact of global climate change on corals.

To determine the role of PCD in heat-induced bleaching, we assessed the relationship between temperature elevation and cell death activity using cell death index<sup>17–24</sup> in the symbiotic anemone *Aiptasia* sp. This species was used as it has an identical symbiosis to reef corals, but is easier to culture and microscopy does not require decalcification. The frequency of cells *in situ* exhibiting healthy, apoptosis-like, or necrosis-like morphology was used as an index of PCD and necrosis activity. This index was based on criteria previously validated in *Aiptasia* sp<sup>14,15</sup> (Figures 1 and 2). Validation used a suite of established techniques, with colchicine treatment providing a positive control for PCD. These techniques included ISEL and agarose gel electrophoresis to determine fragmentation and endonuclease digestion of DNA. The morphological characteristics related to each of the cell death pathways were established using both light and electron microscopy in combination with stereological measurement and vital dye staining.<sup>14–16</sup> The index was determined for three cell types in *Aiptasia* sp subjected to a range of elevated temperatures over time. The cell types were: (1) the endosymbiotic zooxanthellae; (2) host anemone cells from the endoderm, the tissue layer where the zooxanthellae normally reside, and (3) host anemone cells from the ectoderm. Quantification of cell death activity in these three cell types allowed us to determine the relative importance of the PCD or necrotic pathways in bleaching for both host and endosymbiont.

## Results

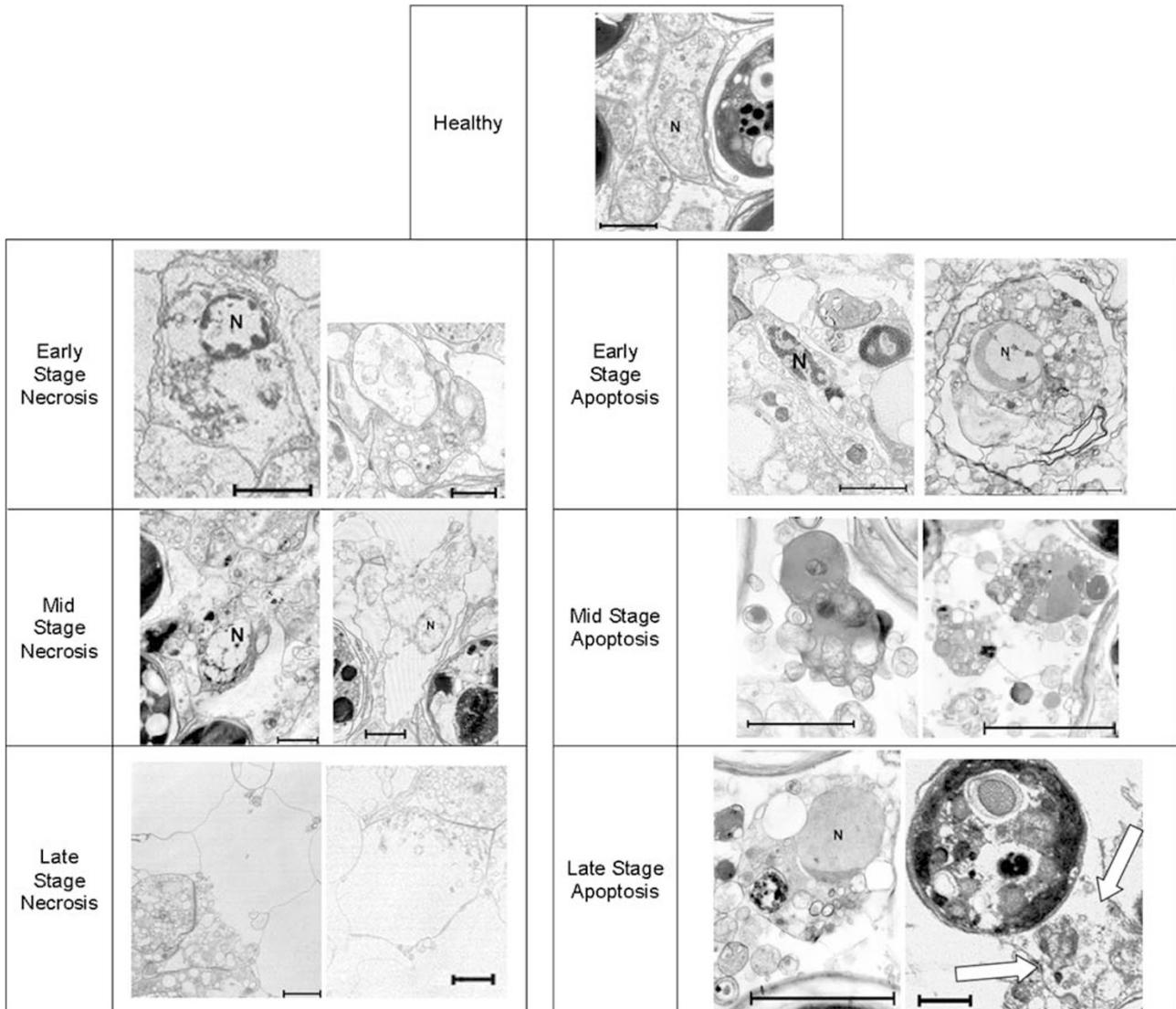
### Bleaching levels during experiments

Effect of temperature over time on zooxanthellae density and photosynthetic pigment content of anemones was assessed by ANCOVA using log-transformed data to meet statistical assumptions<sup>25</sup> (Table 1). There was a significant interaction between time and temperature for zooxanthellae density,

chlorophyll *a*, *c*<sub>2</sub> and total carotenoids, indicating that the rate of the response differed between treatment temperatures. In anemones subjected to temperatures of 31.5 and 33.5°C, there was a rapid initial loss of zooxanthellae and photosynthetic pigments within the first 18–48 h of treatment, with the rate of loss reaching an asymptote as zooxanthellae populations were depleted (Figure 3). There were no significant changes in the density of zooxanthellae or photosynthetic pigment concentrations between times in either the control (26.5°C) or 29.5°C temperature treatments (regression slope,  $t < 0.92$ ,  $P > 0.35$ , in all cases). There was also no significant difference in chlorophyll *a* per cell or chlorophyll *a*:*c*<sub>2</sub> ratio between temperatures or times (Table 1), indicating that reductions in photosynthetic pigment content of anemones were due to loss of zooxanthellae from the anemone, rather than changes in pigment concentration within the algae.

### Cell death indices

A total of 35 069 cells were scored, and the frequency of cells with apoptosis-like and necrosis-like morphology indicated that both PCD and necrosis increased in anemones as a function of temperature and time (Figure 4). At control temperatures, a low underlying rate of apoptosis-like cell death was seen throughout the experiment, similar to that reported for tissues of the sea anemone *Haliplanella lineata* prior to upregulation of PCD activity during longitudinal fission.<sup>26</sup> Multiple comparisons using a Monte-Carlo estimation of goodness-of-fit (two-sided log ratio, 10 000 re-samples, SPSS v11) showed a significant difference between control (26.5°C at time = 0) and the elevated temperature treatments immediately following exposure (<2 min) in the host endoderm cells (Figure 4a). A significant response was not detected in ectoderm cells until 20 min exposure at the two higher temperatures (Figure 4b). Zooxanthellae cell death distribution in the temperature treatments was not significantly different from controls until 1 h after exposure, and only at the highest temperature (33.5°C; Figure 4c). Moreover, significant effects in the zooxanthellae at the lowest treatment temperature (29.5°C) were not detected until 18 h exposure. Overall, it is clear that the ectoderm cells, which do not typically host zooxanthellae, showed less frequent cell death than the endoderm cells where the zooxanthellae normally reside. In both types of host animal cell, apoptosis-like morphology increased in frequency initially, but subsequently declined as necrosis-like morphology became more prevalent towards the end of the experiment (6 days). This effect was most pronounced at higher temperatures and overall severity (frequency of cell death) was higher in the endoderm (Figure 4a). The pattern of change in cell death type distribution was distinctly different in the zooxanthellae. Although significant changes were not detected until later, the severity (frequency of cell death) was greater in the zooxanthellae and both necrosis-like and apoptosis-like morphology increased in frequency throughout the experiment. At all stages, the frequency of apoptosis-like morphology of the zooxanthellae was greater than that of necrosis-like morphology.



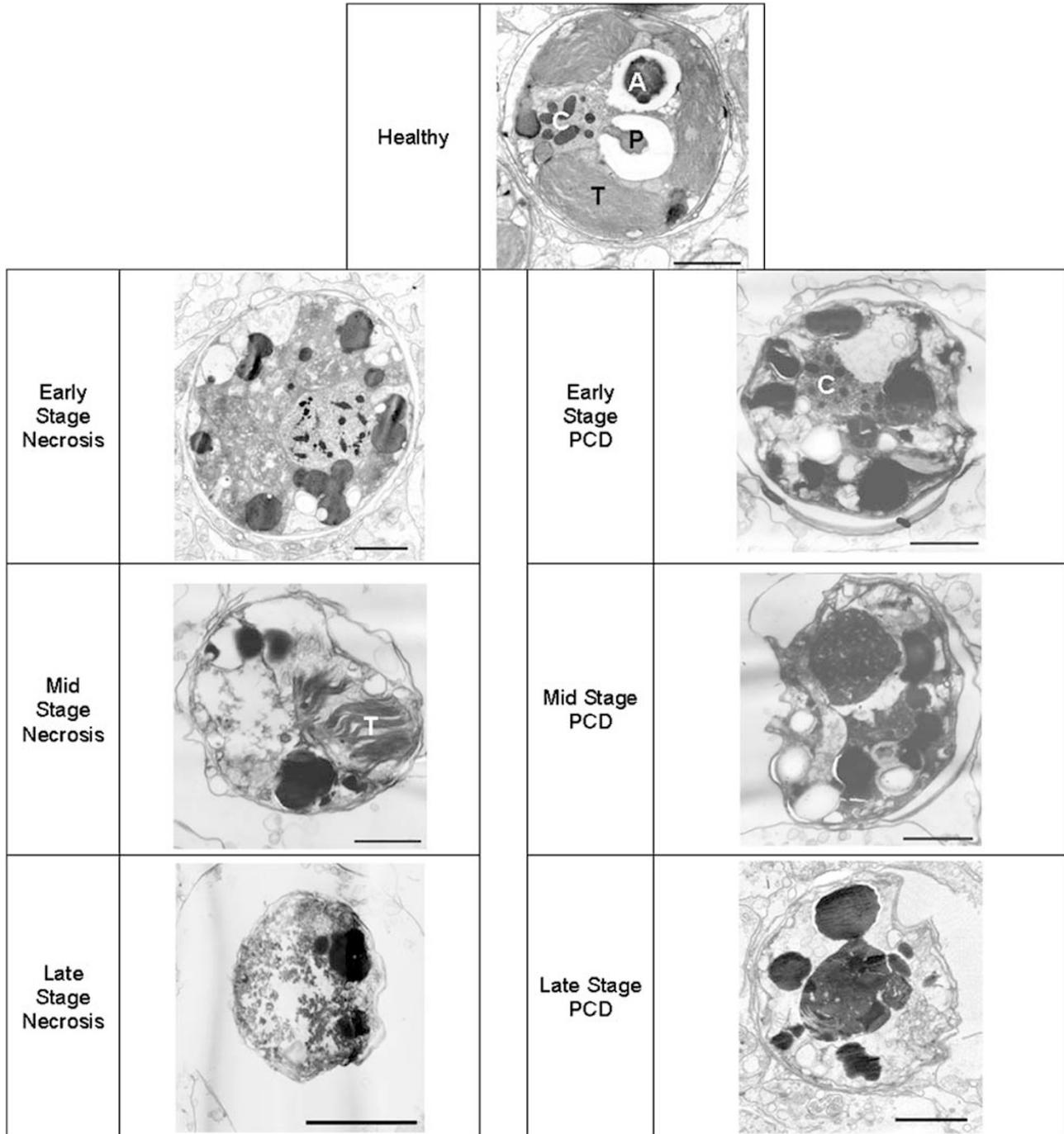
**Figure 1** Morphological criteria observed with transmission electron microscopy used to define healthy, necrosis-like and apoptosis-like condition of host cells of *Aiptasia* sp. These criteria have previously been validated against independent indicators of PCD and necrosis activity in this symbiosis.<sup>14,15</sup> Healthy host cells contain nuclei (N) with dispersed, lightly stained nuclear chromatin with organelle and cell membranes intact. During the early stage of necrosis, pyknotic chromatin condensation occurs, which forms into irregular clumps throughout the nucleus (N) and at the periphery of the nuclear envelope. Vacuolation of the cytoplasm occurs and organelles appear swollen and distended. Mid-stage necrosis involves rupture of organelle membranes including the nucleus with residual dispersed chromatin within the remains of the nucleus and throughout the highly vacuolated cytoplasm. Late stage necrosis is characterised by ruptured cell membranes with little or no evidence of cytoplasm or organelles remaining. Early stage PCD is characterised by condensation of organelles, cytoplasm and nuclear chromatin. Nuclear chromatin forms clear aggregations, often as crescentic caps at the periphery of the nucleus (N). Mid-stage PCD is associated with increased condensation of organelles and cytoplasm, blebbing and formation of membrane bound bodies (containing cell components) at the plasma membrane periphery. In the final stage of PCD there is dispersal and apparent phagocytosis (arrows) of membrane-bound 'apoptotic bodies' within the intercellular spaces. Scale bars = 2  $\mu$ m

## Discussion

We have shown that heat stress initiates cell death pathways that differ in rate and magnitude between the three cell types of the anemone/zooxanthellae symbiosis. The degree-minute onset of cell death activity was within 6, 180 and 3240°C-min for the endoderm, ectoderm and zooxanthellae, respectively, for a 3°C heat stress. This rapid response contrasts with the effect of elevated sea-surface temperatures for reef ecosystems, which is currently determined in terms of degree heating weeks.<sup>27</sup> In bleaching events in the field, relatively small

diurnal fluctuations in temperature may be far more important than previously recognised,<sup>4</sup> and may selectively promote the rapid PCD response over slower necrotic cell death.

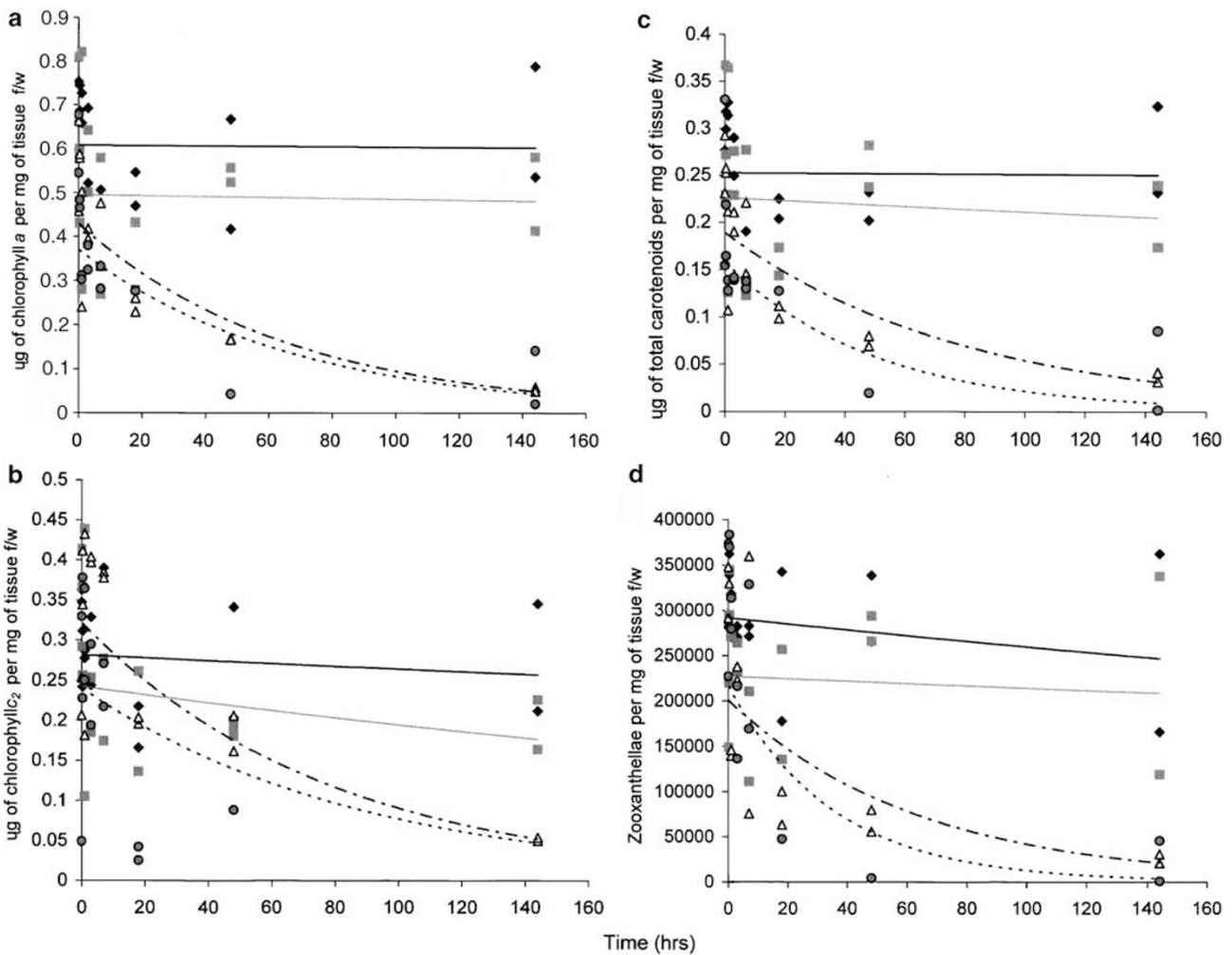
The rapid initial burst of apoptosis-like cell death of host cells in the anemone was not sustained and gave way to necrosis-like cell death over time. The timing of the peak of apoptosis-like cell death, at around 3 h exposure, was similar in ectoderm and endoderm cells and at all temperatures, but was more pronounced (higher frequency) at higher temperatures and in the endoderm, the tissue layer that hosts the zooxanthellae. One explanation for this pattern is that PCD is



**Figure 2** Morphological criteria observed with transmission electron microscopy used to define healthy, necrosis-like and apoptosis-like condition of zooxanthellae. These criteria have previously been validated against independent indicators of PCD and necrosis activity in this symbiosis.<sup>14,15</sup> Healthy zooxanthellae are associated with well-defined organelle structure such as thylakoids (T), pyrenoid body (P) and accumulation body (A), as well as chromosomes (C). Within the early stage of necrosis, pyknotic chromatin condensation occurs and chromosomes form irregular clumps throughout the nucleus. Vacuolation of the cytoplasm is evident and organelles appear swollen and distended. Electron-dense aggregate bodies appear in the cytoplasm. At mid-stage necrosis, dilation and rupture of organelles and membranes (e.g. thylakoids; T) occurs and the cytoplasm becomes highly vacuolated. At late stage necrosis cell membranes and walls become ruptured and degraded with little or no evidence of cell contents. The early stages of PCD are characterised by condensation of the cytoplasm and organelles. The nucleus becomes condensed but chromosomes (C) remain intact. As with necrosis, there is an increase in electron-dense aggregate bodies but with little or no vacuolation of the cytoplasm. The periphery of the plasma membrane appears crenated. Mid-stage PCD is characterised by increased crenation of the cell wall and plasma membranes, further condensation of organelles and cytoplasm, larger aggregate bodies and the disappearance of defined chromosomes within the condensed nucleus. At late stage PCD the cell wall becomes 'halo-like' and the aggregate bodies remain the only well-defined cellular components. There is very little or no evidence of membrane rupture during PCD. Scale bars = 2  $\mu$ m

**Table 1** ANCOVA analysis of the effect of temperature on zooxanthellae density and photosynthetic pigment content of anemones with time as the covariate. Main effects of temperature and time are not shown where there was a significant interaction

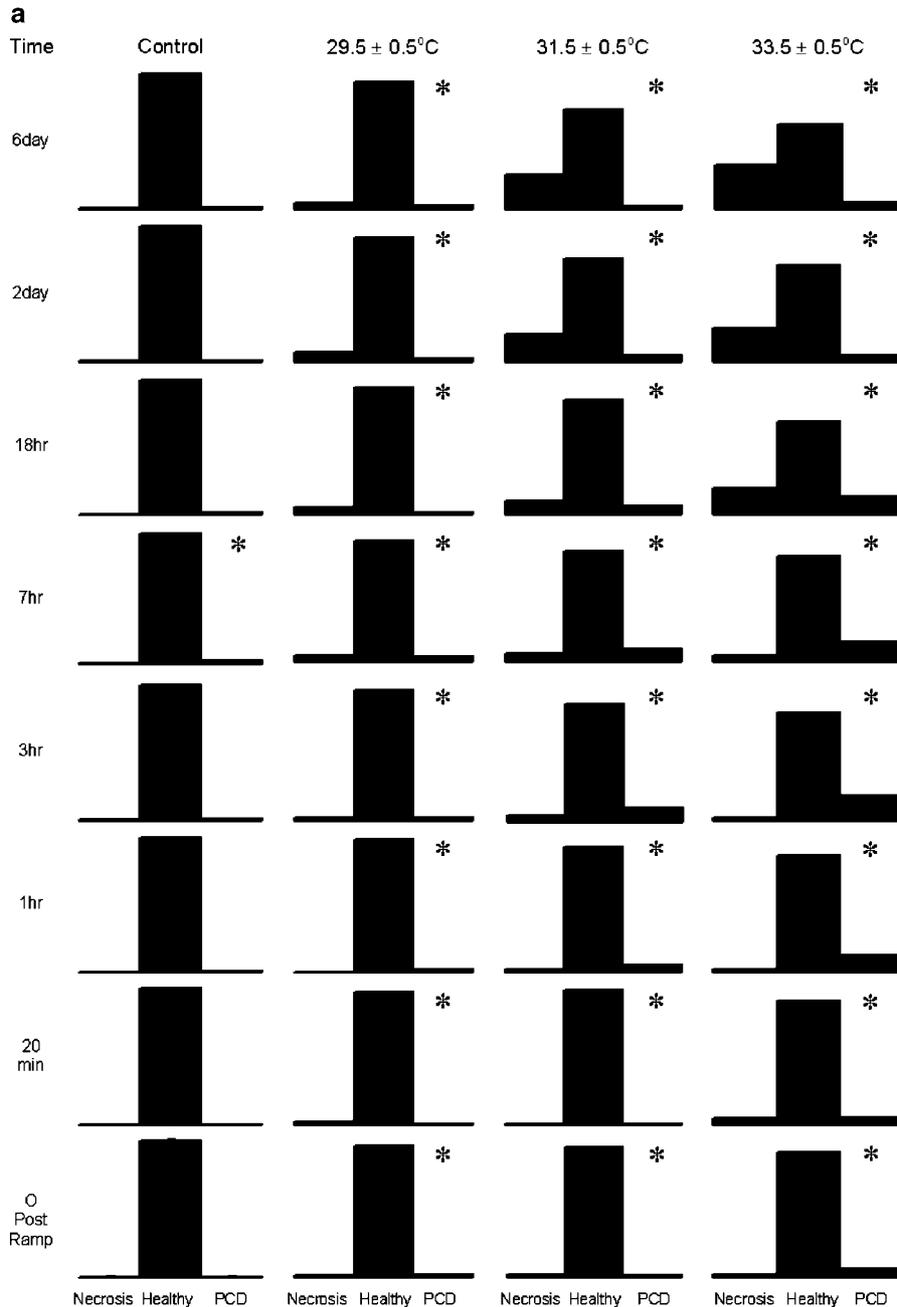
Dependent variable	Source of effect	F	P
Chlorophyll <i>a</i> ( $\mu\text{g}/\text{mg}$ fresh wt)	Temperature $\times$ time	16.8	< 0.0001
Chlorophyll <i>c</i> <sub>2</sub> ( $\mu\text{g}/\text{mg}$ fresh wt)	Temperature $\times$ time	6.0	< 0.001
Total carotenoids ( $\mu\text{g}/\text{mg}$ fresh wt)	Temperature $\times$ time	11.7	< 0.0001
Zooxanthellae density (cells/mg fresh wt)	Temperature $\times$ time	13.1	< 0.0001
Chlorophyll <i>a</i> : <i>c</i> <sub>2</sub> ratio	Time	0.03	> 0.05
	Temperature	1.6	> 0.05
	Temperature $\times$ time	0.5	> 0.05
Chlorophyll <i>a</i> per zooxanthella (mg)	Time	1.5	> 0.05
	Temperature	0.5	> 0.05
	Temperature $\times$ time	0.4	> 0.05



**Figure 3** Bleaching of anemones measured as (a) chlorophyll *a*, (b) chlorophyll *c*<sub>2</sub>, (c) total carotenoids, and (d) zooxanthellae density. Least-squares regression lines are shown based on  $\log_{10}$ -transformed data. Key =  $\blacklozenge$  (—) Control,  $\blacksquare$  29.5°C,  $\blacktriangle$  (---) 31.5°C and  $\circ$  (.....) 33.5°C

initiated as a stress response, but the cells are unable to sustain PCD during prolonged heat stress due to progressive protein degradation,<sup>28</sup> membrane disruption<sup>29</sup> and/or ATP depletion.<sup>30,31</sup> Damaged cells subsequently undergo necrosis rather than PCD. However, this effect would likely be dose-

dependent, resulting in the earlier cessation of PCD at higher temperatures. Alternatively, PCD activity may have been reduced by the stress-mediated upregulation of heat shock proteins that are known inhibitors of PCD.<sup>6–8</sup> A shift from PCD to necrosis occurs in several vertebrate systems in response



**Figure 4** Frequency histograms of cell death morphologies observed in (a) endoderm tissue, (b) ectoderm and (c) zooxanthellae in response to different temperatures over a logarithmic set of time intervals. N = necrosis-like, H = healthy and PCD = apoptosis-like cell death. Error Bars (95% CI) are shown for the control temperature at time = 0. A Monte-Carlo estimation of goodness-of-fit (two-sided log ratio, 10 000 re-samples, SPSS v11) was used to test for differences between the frequency distribution of cell morphologies between the control ( $26.5^\circ\text{C}$  at time = 0) and that of anemones from each temperature at each time interval. \*Denotes distributions that were significantly different from the control using a Bonferroni correction to account for multiple comparisons ( $\alpha = 0.0016$ )

to temperature,<sup>29,32</sup> other stressors and disease.<sup>33–35</sup> A similar pattern in the anemone, a lower invertebrate, suggests that the mechanisms controlling the shift between different forms of cell death in response to stress may be highly conserved.

The higher frequency and earlier onset of cell death in endoderm *versus* ectoderm tissues may indicate that the endoderm cells are more susceptible to heat stress, or that the

extrinsic stress has a greater intrinsic impact. This is plausible, as oxidative stress contributes to temperature-induced coral bleaching<sup>36</sup> and it is likely that damage to photosystems of the zooxanthellae<sup>37</sup> results in increased production of ROS, so the endodermal cells, which host the zooxanthellae, may well be subjected to greater levels of stress. Another explanation is that host cell PCD may act as a mechanism to remove dysfunctional symbionts.<sup>11,14–15</sup> The release of zooxanthellae

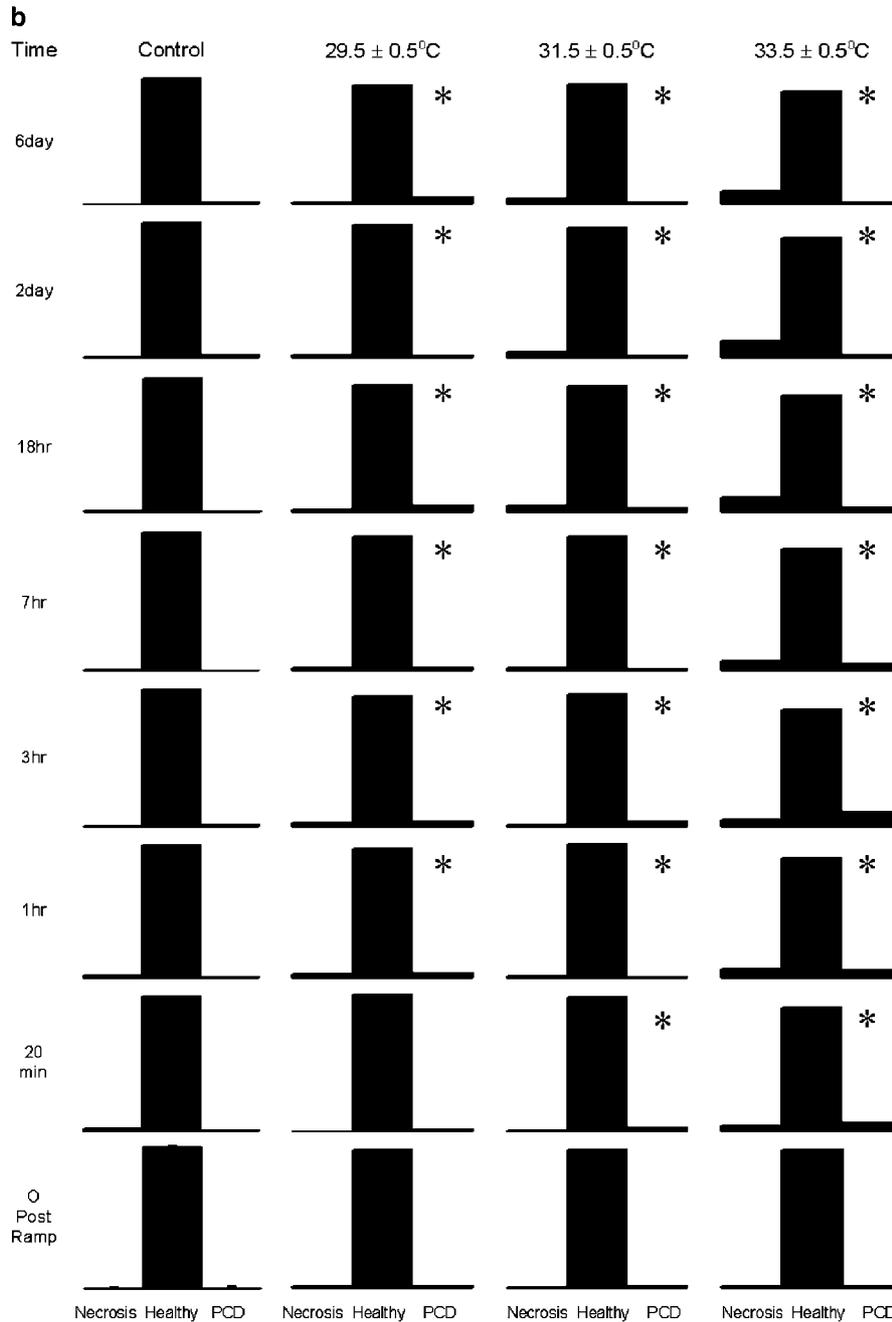
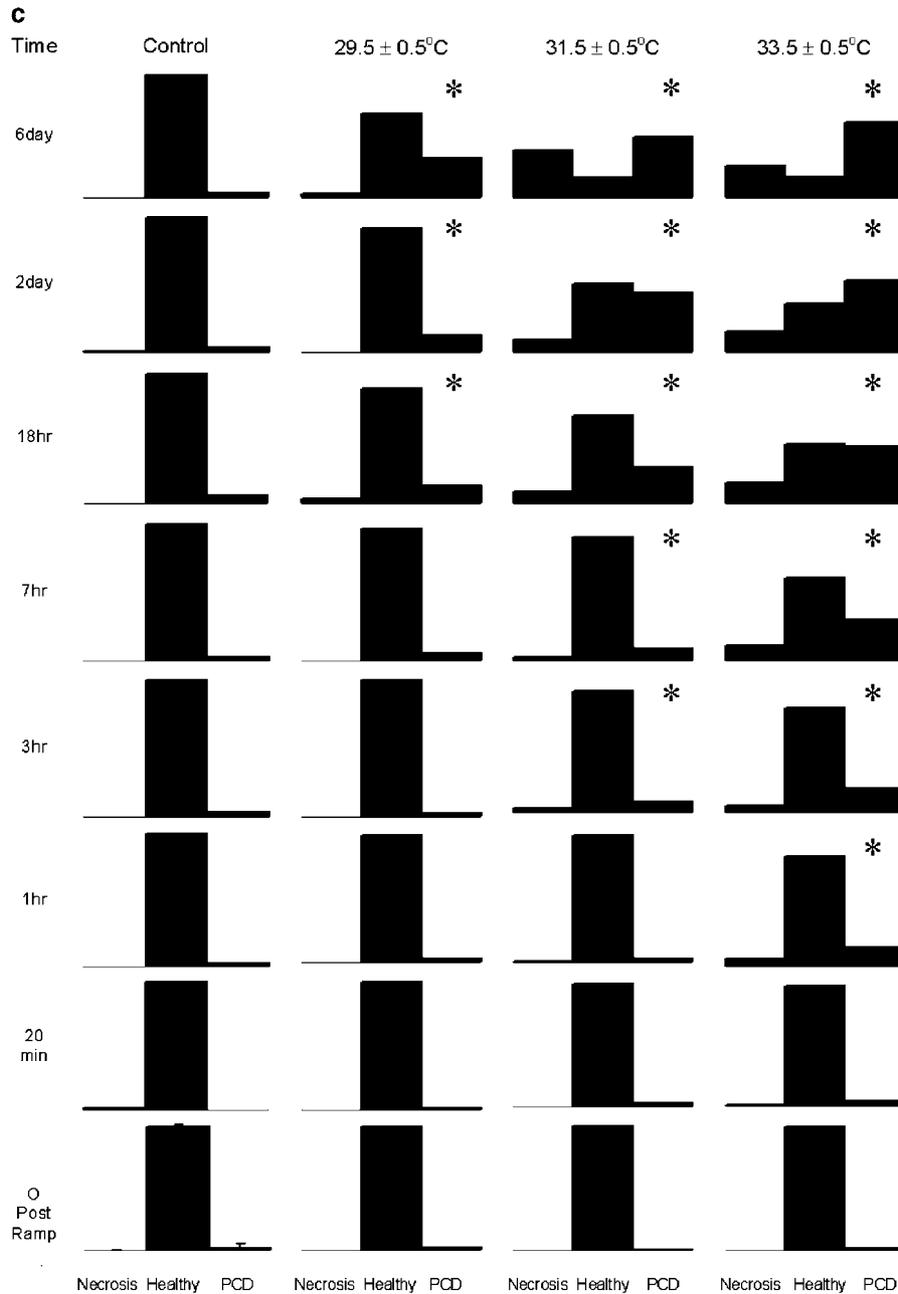


Figure 4 (Continued)

due to apoptosis-like cell death of the host cell was observed in this and previous studies.<sup>14,15</sup> In addition, the timing of the peak and subsequent decline in apoptosis-like cell death (ca. 3 h) corresponds well with the pattern of loss of zooxanthellae, which was rapid within the first 18 h but declined afterwards during the period that necrosis-like cell death of host cells became dominant. This mechanism of symbiont removal may be analogous to pathogen-induced PCD activation seen in both animal<sup>38</sup> and plant systems.<sup>39</sup>

The pattern of cell death in the zooxanthellae was different to that of the host animal and showed a strong dose-dependent response, with both necrosis and apoptosis-like

cell death commencing earlier at higher temperatures. From this study it is not possible to determine the relative contribution of loss of zooxanthellae via host cell death *versus* death of zooxanthellae within the host cell, because the durations of the two mechanisms are unknown. However, the relative frequency of apoptosis-like cell death of the zooxanthellae was higher than that of necrosis at all stages of the experiment. These findings are consistent with the hypothesis that oxidative stress brought about by damage to photosystems triggers PCD in the zooxanthellae.<sup>9,10</sup> It is possible therefore that the burst in PCD seen in host endodermal cells represents an intrinsic stress response aimed at expelling



**Figure 4** (Continued)

damaged, and hence potentially damaging, symbionts. Conversely, PCD in the zooxanthellae may be an extrinsic response which is triggered by the overproduction of ROS, which are normally used in plants as cell death signalling molecules in cell homeostasis.<sup>9</sup> Alternatively, zooxanthellae PCD may be a 'pseudo-altruistic' mechanism by which dysfunctional symbionts initiate cell suicide in order to protect the host and thereby promote survival of the zooxanthellae clone population.<sup>40,41</sup>

Clearly, the operation of different cell death pathways in symbiosis in response to stress warrants further study and it is important that we understand the triggers of PCD and

necrosis in the two components of the symbiosis during bleaching. The adaptive and evolutionary significance of the cell death process is critical to understanding of coral bleaching, a serious ecological phenomenon that is threatening the existence of coral reef ecosystems.<sup>1,2</sup>

## Materials and Methods

### Culture conditions and treatments

*Aiptasia* sp. of unknown geographical origin were obtained from various suppliers and set up as mixed cultures in aquaria containing 80 l of artificial

seawater (Tropic Marin™ salinity 34–35, pH 8.2) at  $26.5 \pm 0.5^\circ\text{C}$ . Aquaria were illuminated by a 150 W (5200 K) metal halide lamp (Hitlite HIT-DE 150 dw) providing  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR at the water surface with a 12:12 h light/dark regime. Anemones were fed 5 ml liquid invertebrate food (Waterscene Enterprises) and two frozen *Artemia* shrimp once a week. Experimental temperature treatments were  $26.5 \pm 0.5$  (control),  $29.5 \pm 0.5$ ,  $31.5 \pm 0.5$  and  $33.5 \pm 0.5^\circ\text{C}$ . A ramp of 30 min from control to treatment temperature was used prior to sampling. Anemones were removed from each treatment over a logarithmic time series at time 0, 20 min, 1, 3, 7, and 18 h, 2 and 6 days (= 0, 0.33, 1, 3, 7, 18, 48, 144 h) to enable the quantification of rapid and longer term cell death activity.

### Photosynthetic pigment and zooxanthellae counts

Three anemones were removed from each treatment and control at each sampling time and were bisected and weighed. One half of each anemone was processed for photosynthetic pigment analysis in accordance with Dunn *et al.*<sup>14,15</sup> Zooxanthellae density (per mg wet weight) in the remaining half was determined from  $3 \times 50 \mu\text{l}$  subsamples of homogenised tissues using an Improved Neubauer haemocytometer (Weber, England).

### Cell death activity

Anemones were processed for ultra-thin sectioning for transmission electron microscopy according to Le Tissier.<sup>42</sup> Only tissues from the tentacles were examined. Between 268–1030 ectoderm cells, 247–595 endoderm cells and 100–232 zooxanthellae were assessed using the cell death index<sup>14–16</sup> (Figures 1 and 2). Frequencies of cell death activity were assessed in tentacle samples from five anemones from the control temperature ( $26.5 \pm 0.5^\circ\text{C}$ ) at time = 0.

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