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Research Paper

Symbiophagy as a cellular mechanism for coral bleaching

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Coral bleaching is a major contributor to the global declines of coral reefs. This phenomenon is characterized by the loss of symbiotic algae, their pigments or both. Despite wide scientific interest, the mechanisms by which bleaching occurs are still poorly understood. Here we report that the removal of the symbiont during light and temperature stress is achieved using the host's cellular autophagic-associated machinery. Host cellular and subcellular morphologies showed increased vacuolization and appearance of autophagic membranes surrounding a variety of organelles and surrounding the symbiotic algae. Markers of autophagy (Rab 7 and LAS) corroborate these observations. Results showed that during stress the symbiont vacuolar membrane is transformed from a conduit of nutrient exchange to a digestive organelle resulting in the consumption of the symbiont, a process we term symbiophagy. We posit that during a stress event, the mechanism maintaining symbiosis is destabilized and symbiophagy is activated, ultimately resulting in the phenomenon of bleaching. Symbiophagy may have evolved from a more general primordial innate intracellular protective pathway termed xenophagy.

Bleaching in corals is marked by the loss of photosynthetic pigments or of the symbiotic dinoflagellate algae (zooxanthellae) from the coral host tissue, usually in association with environmental perturbations.[1] Bleaching can be induced by cold shock, bacterial infection, osmotic stress, exposure to chemical pollutants, high temperature, high-light intensity and persistent darkness.[1] Coral bleaching is thought to be the principal driver in recent mass coral mortality events that are occurring globally, especially in association with an increasing occurrence of El Niño events.[2]

How bleaching occurs in cnidaria, and whether there are multiple mechanisms that result in bleaching, has been contentiously debated for more than a century. Digestion of the symbiotic unicellular algae (zoochlorella and zooxanthellae) in cnidarians was documented as early as 1883,[3] confirmed and expanded by Boschma,[4] but challenged by Yonge, who argued that zooxanthellae were expelled, and not digested.[5] Work from Muscatine's lab in the 1980s provided the first evidence for phagosome-mediated digestion of unicellular algae (zoochlorella) in *Hydra viridis*.[6] Additional studies provided evidence that zooxanthella loss in nonscleractinian cnidarians was associated with a phagocytic-like process that resulted in their digestion.[7,8] Work on cold-induced bleaching in sea anemones and corals presented conflicting interpretations of whether the symbionts were expelled from the host through an exocytic-mediated pathway or via cellular detachment of the host cell containing the zooxanthellae.[9,10] Several investigations have demonstrated that bleaching in corals and anemones is positively correlated with oxidative damage and that the induction of the cellular-stress response is positively correlated with resistance to bleaching.[11,12] To date, no coherent cellular mechanism of bleaching has been proposed, particularly in regards to the order in which events occur during the bleaching process.

A recent study by Fang and coworkers gave the first clue as to the role vacuolar function may have in coral bleaching by establishing the role of the Rab family of proteins in mediating symbiosis in another cnidarian, *Aiptasia pulchella*.[13,14] They reported that the Aiptasia Rab11 is excluded from the vacuole body containing zooxanthellae under normal conditions. However, during a stress event that results in the degradation of the zooxanthellae, ApRab11 and especially ApRab7 were associated with endocytic phagosomes containing dead or dysfunctional zooxanthellae.[13,14] These findings provide key steps in developing an autophagic mechanism for cnidarian bleaching as both Rab11 (a small GTPase) and Rab7 are recognized markers of autophagic activity across taxa. Xenophagy, the digestion of potential intracellular pathogens, utilizes much of the same autophagic machinery, including the Rab proteins, for the digestion of these pathogens.[16] During xenophagy, the vesicle that envelopes the intracellular bacteria is transformed from a conduit of subsistence for the pathogen to a phagolysosome that degrades the bacteria.[16] In a similar fashion using the same autophagic machinery, we provide evidence that the zooxanthella is removed by the coral host in a xenophagic-like process we term *symbiophagy*. Symbiophagy of the zooxanthellae is achieved by transforming the symbiont vacuolar membrane from a conduit of nutrient exchange to a digestive organelle resulting in the consumption of the symbiont. We posit that during a stress event, the mechanism maintaining symbiosis is destabilized and symbiophagy is activated, ultimately resulting in the phenomenon of bleaching.

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Table 1 **Each biomarker data set was tested for normality using the Kolmogorov-Smirnov test (with Lilliefors' correction) and for equal variance using the Levene Median test**

Cellular parameter	No light 25°C	No Light 32°C	Low Light 25°C	Low Light 32°C	High Light 25°C	High Light 32°C
DNA AP site (per 1 x 10^6 nucleotides)	22.5 ± 5.7[a]	15.2 ± 1.7[a]	33.2 ± 5.4[a]	73.7 ± 3.1[b]	165 ± 10.4[c]	690 ± 33.2[d]
Protein Carbonyl (nmol/mg TSP)	22.3 ± 2[a]	33.8 ± 2.7[a]	78.2 ± 5.1[b]	190.8 ± 6.2[c]	207.3 ± 11.3[c]	588 ± 12[d]
Hydroxynonenal (nanog/mg TSP)	24.8 ± 3.2[a]	81 ± 5.9[b]	36.8 ± 3.1[a]	250.3 ± 17.5[c]	342 ± 25.5[d]	763.8 ± 33.9[e]
Acid Phosphatase (relative units/ng TSP)	472 ± 33[a]	2086 ± 78.8[b]	428.3 ± 54.7[a]	2086.3 ± 103.6[b]	872.8 ± 47.8[c]	2099.25 ± 66.3[b]
Rab 7 (relative units/ng TSP)	950.3 ± 17.5[a]	547 ± 12.9[b]	1009.3 ± 34.2[c]	283.5 ± 3.9[d]	661 ± 18e	131 ± 15.2[f]

*Entries in the table give treatment means ± 1 SE. Treatment means with different superscripted letters differed significantly at $\alpha = 0.05$. If the data for that biomarker were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) was employed. When data for that biomarker did not meet the homogeneity of variances requirement for one-way ANOVA, a Kruskal-Wallis One-Way Analysis of Variance on Ranks was employed. When significant differences were found among treatment means, the Tukey-Kramer Honestly Significant Difference (HSD) method, the Student-Newman-Keuls Method post-hoc test, or the Holm-Sidak test was used as an exact alpha-level test to determine differences between each of the populations. Treatment means with different superscripted letters differed significantly at $\alpha = 0.05$. Entries in the table give treatment means ± 1 SE.

To resolve the mechanism of temperature and light dependent coral bleaching, we characterized the loss of dinoflagellates from the coral *Pocillopora damicornis* under different conditions, and provide evidence for a symbiophagic mechanism for this loss. We exposed coral nubbins previously acclimated to low light and 25°C to six environmental treatments. All experiments were begun at the same time in the morning immediately following a period of 8 hrs of darkness at 25°C. The treatments were carried out in triplicate for a period of ten hours each: (1) a reference treatment of low light (400 μmoles m^{-1} s^{-1} PAR peak-natural irradiance using a neutral density filter) at 25°C, (2) low light at 32°C, (3) high light (2007 μmoles m^{-1} s^{-1} PAR peak natural irradiance) at 25°C, (4) high light at 32°C, (5) darkness at 25°C and (6) darkness at 32°C. At the termination of the experiment, fragments were collected for morphological and biochemical assessment. Additional fragments maintained at the same experimental conditions for 48 hours and 5 days were then examined for changes in gross morphology. Corals exposed to treatments 2, 3 and 4 lost over 60 percent of their zooxanthellae after 48 hours, while corals exposed to the dark treatments showed no bleaching at 48 hours; after five days, however, significant bleaching was observed in dark exposed corals (Suppl. Fig. 1). Cellular and subcellular morphologies of samples from each treatment were characterized using transmission electron microscopy (TEM). Comparisons were carried out on sections from the same anatomical region of each polyp (Suppl. Fig. 2). Oxidative damage and endosome/phagosome maturation were measured using ELISA (Table 1). Tartrate-sensitive lysosomal acid phosphatase (LAS) protein levels in the host were measured because accumulation of this protein reflects upregulation of endosome/phagosome maturation.[17] Rab7 was measured since this protein regulates endosome/lysosome maturation, and is associated with organellar membranes of lysosomes, endosomes, phagosomes and autophagosomes via prenylation of the C-terminal double cysteines.[15] The activation of Rab7 acts to block the binding of an antibody generated against the prenylation site of Rab7 in an Inhibition ELISA. The decreased ELISA signal indicates increased activation of Rab7.[15,18]

Fragments maintained in the reference treatment (low-light, 25°C similar to the culture conditions these corals experienced over the previous two months) exhibited rapid regeneration of lesions from fragmenting the original colony indicating excellent tissue health.[25] Transmission electron microscopy of the reference samples showed cellular anatomical features of the zooxanthellae that were readily distinguishable and exhibited coherence and integrity. The coral's double vacuolar membrane was tightly laminated to the plasma membrane/thecal laminate of the zooxanthellae, and there was minuscule to nonperceptible volume of vacuolar matrix between the vacuolar membrane and dinoflagellate plasma membrane (Fig. 1A–C). In addition, the concentration of the oxidative damage markers and the endosome/lysosome maturation markers of the samples from this treatment reflect a reference range for the naturally occurring parent colony corals altogether indicating a relatively healthy physiological condition (Table 1).

Coral fragments exposed to low-light conditions at 32°C showed a marked change in cellular anatomy (Fig. 1D and E). There was no indication of exocytosis or detachment of gastrodermal cells. The gastrodermal cells showed perceptible vacuolization around the zooxanthellae, where the vacuolar matrix formed a considerable zone between the vacuolar membrane and the zooxanthellae's cellular boundary (Fig. 1D). The zooxanthellae's cell membrane and thecal laminate adjoining the vacuolar matrix were undetectable, suggesting that the vacuolar matrix was degrading (Fig. 1E). A significant increase in lysosomal acid phosphatase (LAS) levels indicated an increase in phagic activity (Table 1). Prenylation of Rab7 indicated an increase of phagic maturation activity (Table 1). Taken together, these data indicate that zooxanthellae are at the initial stages of lysis by the host employing a symbiophagic process.[16] Based on evidence that oxidative stress can initiate and regulate autophagy via redox-sensitive proteases,[19] the increased oxidative damage products resulting from the high-temperature treatment suggests that a similar mechanism may have initiated the symbiophagy (Table 1).

Exposure to intense irradiation is a significant factor in bleaching and can induce bleaching even in the absence of a temperature stress. Corals exposed to five times more photosynthetic active radiation at 25°C compared to the reference showed extensive signs of symbiophagy, though there were no indications of exocytosis or cell detachment. Vacuolar matrix surrounding the zooxanthellae was prominent, while zooxanthellae cell membrane and thecal plates were severely degraded (Fig. 2A and B). LAS levels significantly increased in response to the high light intensity, while Rab7 was activated—again indicating that a symbiophagic process had been initiated. LAS levels in the highlight/25°C treatment were significantly lower than the low light/32°C treatment, suggesting that heat stress can act inde-

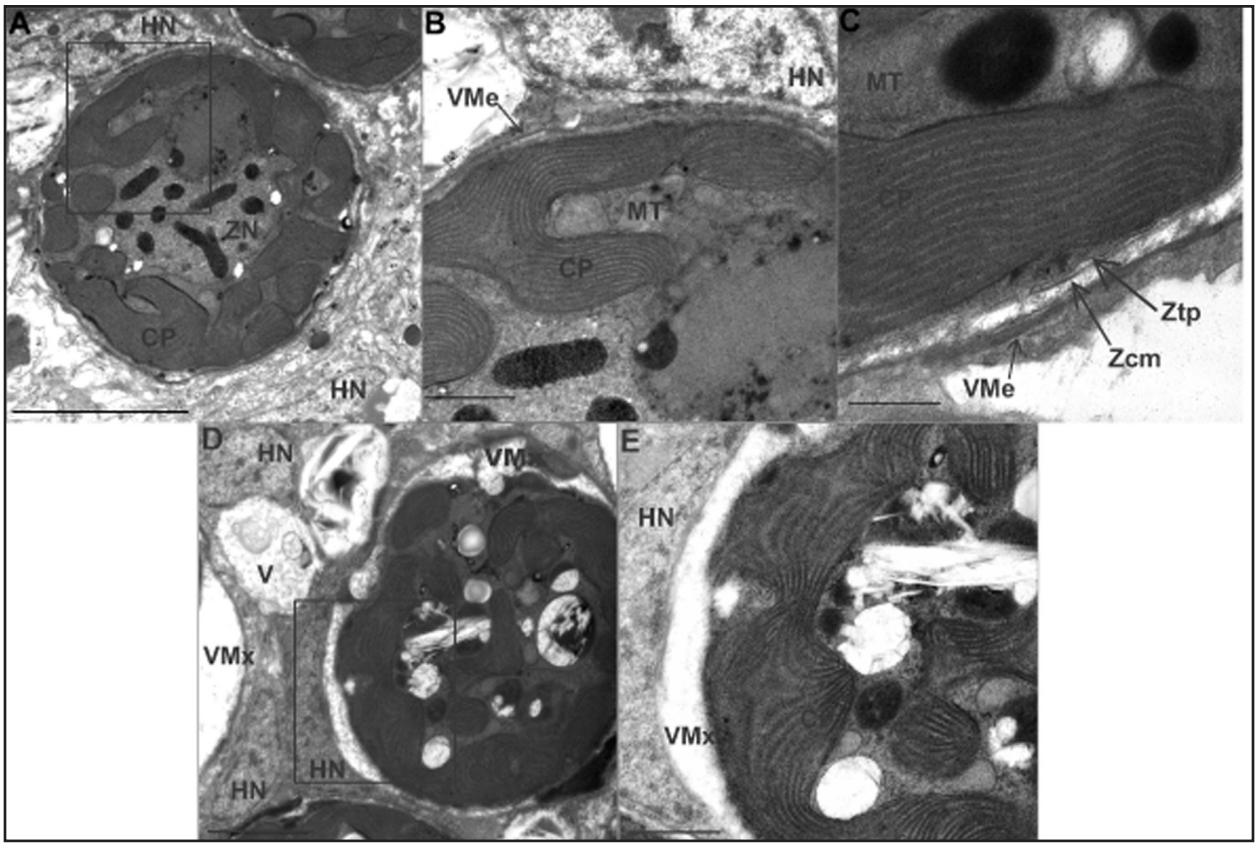

Figure 1. Transmission electron micrographs of a zooxanthella within the endodermal layer of the host coral *P. damicornis* exposed to 400 µmoles m^{-1} s^{-1} PAR peak irradiance (low light) at 25°C and 32°C. CP = chloroplast; HN = host nucleus; MT = mitochondria; P = pyrenoid body; VMe = host vacuolar membrane; VMx = Vacuolar matrix; Zcm = zooxanthellae cell membrane; Ztp = zooxanthellae thecal plate; Zn = Zooxanthella nucleus. (A) Low light at 25°C (reference treatment). Magnification 1500x, Scale bar = 5000 nm. (B) Magnified area from boxed area in (A). Magnification 4000x, scale bar = 1000 nm. (C) Low light at 25°C to illustrate the reference VMe, Zcm and Zcw. Magnification 8000x, scale bar = 500 nm. (D) Low light at 32°C. Magnification 2000x, scale bar = 2000 nm. (E) Magnified area from boxed area in (D). Magnification 4000x, scale bar = 1000 nm.

pendently to initiate symbiophagy, while oxidative damage under high light results in a quicker and more severe onset (Table 1).

Exposing corals to combined conditions of high temperature and high light resulted in extensive degradation of the zooxanthellae *in hospite*. Vacuolization around the zooxanthellae was prominent with extensive degradation of the zooxanthellae's cell membrane/thecal plates and darkening and thickening of the vacuolar double membranes (Fig. 2C and D). The zooxanthellae often exhibited subcellular structural deformations, including extensive thylakoid disorganization, dissolution of the nucleus, and blending of destabilized organellar structures (Fig. 2D). LAS levels were significantly elevated in comparison to the reference treatment and the high light/25°C treatment, but were not significantly different from the low light/32°C treatment (Table 1). This indicates that regulation of LAS accumulation may be influenced more by heat stress than oxidative stress. Rab7 activation was significantly higher in high light/high temperature than in any of the other treatments, reflecting the extensive vacuolization seen in the gastrodermal cells. Oxidative damage markers were also significantly higher in this treatment compared to any other treatment, corroborating other studies that associate oxidative stress with bleaching.[11,12]

Previous studies showed that prolonged darkness (>4 days) resulted in bleaching, with the expulsion of the zooxanthellae as the dominant observation.[20] In the present study, corals were subjected to 18 hours of darkness at 25°C as a control for high temperature and light exposures. Although no expulsion of zooxanthellae was observed after 18 hrs of darkness, an extensive change in cellular morphology of the gastrodermal cells (Fig. 3A and B) was detected. There was significant vacuolization surrounding the zooxanthellae, but in contrast to the other treatments exhibiting vacuolization, a majority of zooxanthellae had cell membrane/thecal plates that were distinct with little indication of degradation (Fig. 3A and B). In the dark treatment, the host tissue showed significant morphological differences from the four light treatments, exhibiting an abundance of microvacuolization bodies in the gastrodermal cells (Fig. 3A). Nuclear morphologies of the gastrodermal cells did not exhibit any of the classic signs of either apoptosis or autophagic cell death. Darkness-induced bleaching is a relatively long process, and the sampling occurred very early in the bleaching process, which may explain why LAS levels were not significantly different from the reference, though Rab7 activation was increased (Table 1). Oxidative damage markers were either not significantly different, or were lower compared to the reference, indicating that a different regulatory mechanism may have activated symbiophagy. In the dark, zooxanthellae are unable to produce photosynthate, thereby starving the host cell of expected calories. Since it is known that starvation induces autophagy,[21] it is possible that the same regulating pathway may activate the initiation of symbiophagy in dark-treated corals.

Corals exposed to 18 hours of darkness with 10 of those hours at 32°C showed an exacerbated pathomorphology compared with corals exposed to extended darkness at 25°C. There was widespread vacuolization surrounding the zooxanthellae, and the cell membrane/thecal plate exhibited signs of extensive degradation (Fig. 3C and E). Sub-cellular morphology of the zooxanthellae was largely intact, though there were indications of disorganization of the thylakoid membrane in some of the chloroplasts (Fig. 3D and E). In this treatment, as in the high temperatures in the light treatment, LAS levels were significantly higher than the reference, but not significantly different from the other two 32°C treatments; concurring that LAS expression and lysosomal maturation is influenced by heat stress. Rab7 activation was significantly greater than in the reference or the darkness/25°C treatment, corroborating its involvement in the activation of symbiophagy. This result also suggests that its activation may be triggered by cellular signals other than temperature stress. DNA and protein damage markers showed a similar pattern as the dark/25°C pattern, while the lipid damage marker was significantly higher in this treatment compared to the reference and the dark/25°C. This is not surprising, since lipid autooxidation is generally more sensitive to changes in reactive oxygen species content from its microenvironment.[22] Signs of gastrodermal tissue distress and even necrosis were prominent, indicating that the role of heat stress may be additive to the induction of phagic processes (Fig. 3C and E; Suppl. Fig. 2). Autophagosome bodies and whorls were pronounced in gastrodermal cells not yet necrotic (Fig. 3C). Many gastrodermal cells were devoid of most organellar structures, except for vacuolated zooxanthellae, nuclei, and the littering of cystoplasmic remnants that adhered to vacuolar and plasma membranes (Fig. 3E). There were no morphological signs of apoptosis, and with respect to the activation of symbiophagy and autophagy, gastrodermal nuclei also lacked many of the classic signs of autophagic programmed cell death. For example, in Figure 3E, the nucleus lacks blebbing, or vacuolization, instead the chromatin material is seen adhering to the nuclear membrane, and there is initial shearing of the nuclear membrane. Micrographs of other cnidarian species, similar to these shown here, abound in the literature, and were interpreted as cell detachment or exocytosis.[9,10] These results indicate that dark exposure may result in a form of autophagic cell death similar to that recently reported in other organisms.[23-25] Thus, similar to what occurs during Kazal1 silencing in Hydra endoderm, the autophagic necrosis of the gastrodermal tissue in dark exposed *P. damicornis* (at 25° or 32°C) may be the result of a failed process to safe guard the tight control of the tissue from extensive cellular autophagy.[26]

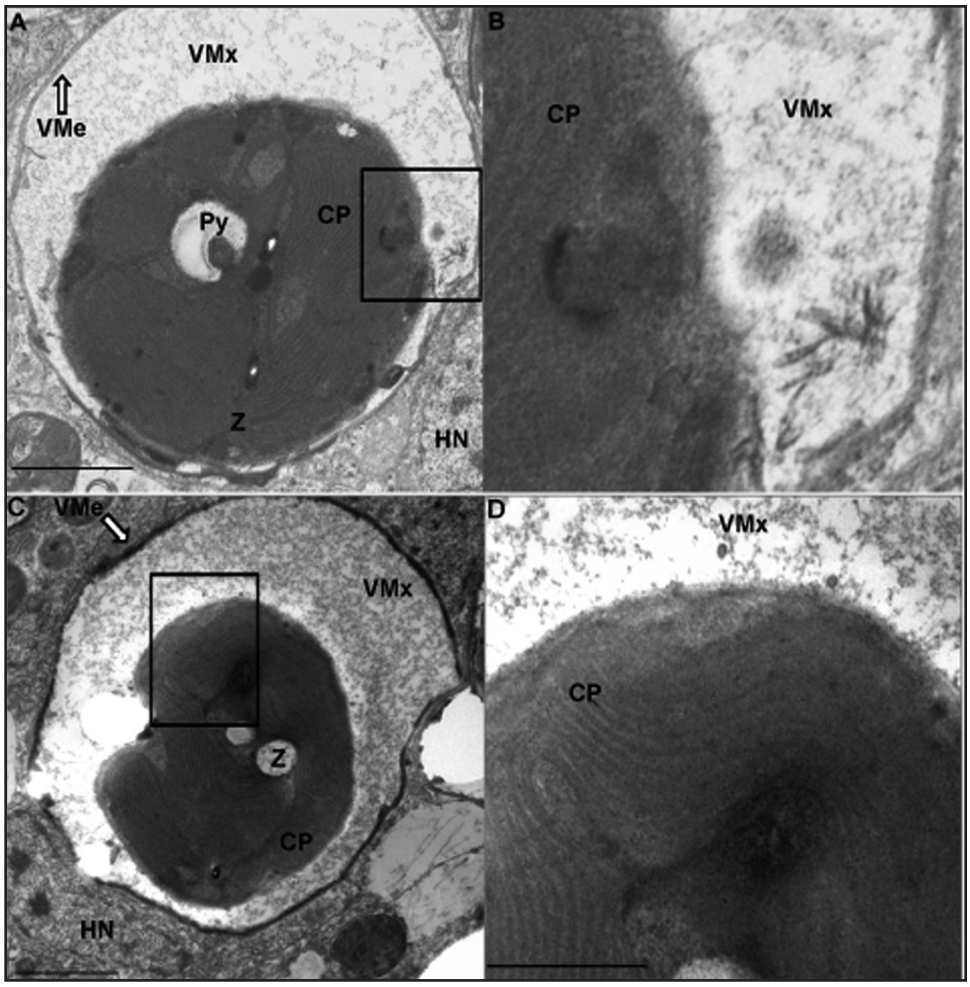

Figure 2. Transmission electron micrographs of a zooxanthella within the endodermal layer of the host coral *P. damicornis* exposed to 2007 µmoles m^{-1} s^{-1} PAR peak irradiance (high light) at 25°C and 32°C. CP = chloroplast; HN = host nucleus; MT = mitochondria; P = pyrenoid body; VMe = host vacuolar membrane; VMx = Vacuolar matrix; Z = zooxanthellae. (A) High light at 25°C. Magnification 2000x, scale bar = 2000 nm. (B) Magnified area from boxed area in (A). (C) High light at 25°C. Magnification 2000x, scale bar = 2000 nm. (D) Magnified area from boxed area in (C). Magnification 6000x, scale bar = 1000 nm.

Symbiophagy of the zooxanthellae is a dominant coral survival response. Understanding symbiophagy in cnidaria is important for advancing our knowledge of the role of autophagic processes as a primordial intracellular protective pathway. Maintenance of symbiosis requires the suppression of phagolysosomal activation and maturation of the zooxanthellae's vacuolar membrane. Other xenophagic models, such as those involving pathogens like *Mycobacterium tuberculosis*, Vibrios and Shigella, were shown to block phagolysosomal activation and maturation, and that starvation or other stress events were found to overcome this regulation, thereby inducing autophagy and xenophagy of intracellular bacteria as part of host innate immunity.[16,27,28] Corals infected with the intracellular, pathogenic *Vibrio coralliilyticus* often become bleached.[29] This suggests that the innate immune response of the coral towards the Vibrio may become nonspecific and expand its xenophagic reaction towards the zooxanthellae.[30] If this is true, then many of the cytokines and

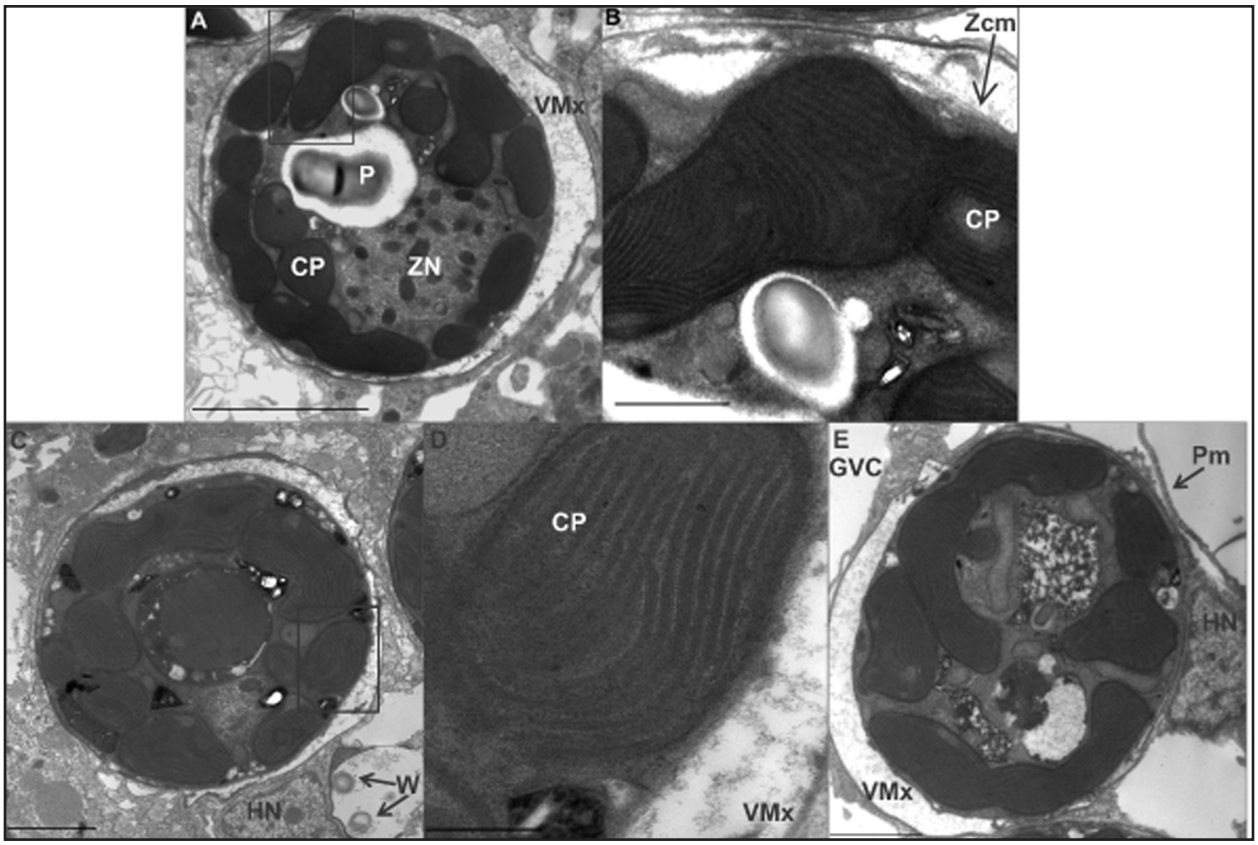

Figure 3. Transmission electron micrographs of a zooxanthella within the endodermal layer of the host coral *P. damicornis* exposed to darkness at 25°C and 32°C. CP = chloroplast; GVC = gastrovascular cavity; HN = host nucleus; MT = mitochondria; P = pyrenoid body; Pm = plasma membrane; VMe = host vacuolar membrane; VMx = Vacuolar matrix; Z = zooxanthellae; Zcw = zooxanthellae cell membrane. (A) Darkness at 25°C. Magnification 1500x, scale bar = 5000 nm. (B) Magnified area from boxed area in (A). Magnification 5000x, scale bar = 1500 nm. (C) Darkness at 32°C. Magnification 2000x, scale bar = 2000 nm. "W" indicates autophagic whorls. (D) Magnified area from boxed area in (C). Magnification 10,000x, scale bar = 500 nm. (E) Darkness at 32°C. Magnification 2000x, scale bar = 2000 nm.

stressors associated with the induction of autophagy and bacterial/viral xenophagy may also activate symbiophagy. It is therefore imperative to explore this phenomenon in the context of primordial innate immunity as an intracellular protective pathway, particularly since this pathway is likely to be a part of a more general and evolutionarily conserved defense mechanism in cnidaria and other phyla.

Methods Summary

Collection of coral. *Pocillopora damicornis* were collected from the eastern side of Coconut Island at the Hawaii Institute of Marine Biology, Oahu, Hawaii, and maintained in raceways.

Experimental treatments. Coral nubbins were subjected, in triplicate, to six environmental treatments using 10-gallon aquarium tanks. The treatments were carried out for a period of ten hours each beginning at 6:00 am: (1) a reference treatment of low light (400 µmoles m^{-1} s^{-1} PAR peak-natural irradiance using a neutral density filter) at 25°C, (2) low light at 32°C, (3) high light (2007 µmoles m^{-1} s^{-1} PAR peak natural irradiance) at 25°C, (4) high light at 32°C, (5) darkness for 10 hours (18 hr total) at 25°C, and (6) darkness for a total of 18 hours with the first 8 hours at 25°C and the next 10 of those hours at 32°C.

Transmission electron microscopy. A single polyp was biopsied, fixed in a series of different fixative buffers and osmium tetroxide, dehydrated and embedded in Araldite (502). The block was sectioned using an ultramicrotome and mounted on 300 mesh copper grids. The ultrathin sections were stained with lead citrate. Sections through the same approximate mid polyp body area were examined using a JEM-1230 at 80 kV. Images were taken using TVIPS TemCam-F214.

DNA abasic lesions. Tissues from frozen coral nubbins were prepared under ultracold conditions. DNA was isolated from each sample using the Dojindo pureDNA kit-Cell, tissue (GK03-20) with one slight modification to address Maillard chemistry artifact. DNA concentration was determined using an Invitrogen/Molecular Probes Quant-iT^{TM} DNA Assay Kit, Broad Range (cat# Q33130) using a Bio-Tek FL800 fluorescent microplate reader. DNA AP concentration was determined using the Dojindo DNA Damage Quantification Kit-AP Site Counting (cat# DK-02-10) and was conducted according to manufacturer's instructions.

Protein carbonyl. Protein carbonyl groups were measured with a method that uses 2,4-dinitrophenylhydrazine as a carbonyl derivatizing reagent. A quantitative Enzyme-linked Immuno-sorbent Assay (ELISA) that consisted of a primary antibody against dinitrophenyl, a horseradish peroxidase-conjugated secondary antibody, and a luminol/luminescent detection system was used for the detection of the derivatized carbonyl.

Hydroxynonenal adducted to protein. Antibody was generated against a HNE-bovine serum antigen. Coral samples were homogenized

in lysis/redox buffer and subjected to a quantitative ELISA using the anti-HNE/protein antibody as the primary antibody, a horseradish peroxidase-conjugated secondary antibody, and a luminol/luminescent detection system was used for signal detection.

Rab7 and lysosomal acid phosphatase (LAS) antibody production and ELISA. Protein sequences from invertebrates and vertebrates were aligned, conserved and unique domains of each protein determined, and an antigen region for each protein was determined. A synthetic peptide of each antigen domain was synthesized, conjugated to a carrier protein, and immunized into a host. Antibody for each protein was validated to detect the antigen, as well as a protein of the correct migration rate in a SDS-PAGE/western blot assay. Rab7 and LAS were measured in coral samples with a quantitative ELISA using the respective primary antibodies. Synthetic antigens were used as the calibrant standard curves.

Statistics. Data were tested for normality using the Kolmogorov-Smirnov test (with Lilliefors' correction) and for equal variance using the Levene Median test. If the data were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) was employed. When data did not meet the homogeneity of variances requirement for one-way ANOVA, we instead used a Kruskal-Wallis One-Way Analysis of Variance on Ranks. When significant differences were found among treatment means, we used the Tukey-Kramer Honestly Significant Difference (HSD) method, the Dunn's posthoc test or the Holm-Sidak test as an exact alpha-level test to determine differences between each of the populations.

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C.A.D. designed the study, processed all samples for analysis, conducted all biomarker assays, designed the antigens, oversaw antibody production, validated the antibodies, statistically analyzed the data, rendered all figures, and was primary author of the paper. E.K.-W. and C.A.D. acquired the transmission electron microscopy images. E.K.-W. generated the semithin histological images. C.A.D., J.M. and G.K.O. collected the coral, conducted the coral culturing, and conducted the environmental experimental treatments. E.K-W, A.K., C.M.W., Y.L. and G.K.O. were responsible for editing of the manuscript. C.M.W. acquired protein sequences of Rab7 and LAS, and conducted the protein alignments. All authors discussed results and participated in the writing of the manuscript.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/DownsAUTO5-2-Sup.pdf

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