Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals

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Abbreviations footnote: Reactive Oxygen Species (ROS).

Data deposition footnote: The new sequences reported herein have been deposited in GenBank (accession # XXXXX to XXXXX).
Abstract

Over the past three decades, massive bleaching events of zooxanthellate corals have been documented across the range of global distribution. Although the phenomenon is correlated with relatively small increases in surface sea temperature (SST) and enhanced light intensity, the underlying physiological mechanism remains unknown. In this paper we demonstrate that thylakoid membrane lipid composition is a key determinate of thermal-stress sensitivity in symbiotic algae of Cnidarians. Microscopic analyses of thylakoid membranes reveals that the critical threshold temperature separating thermally tolerant from sensitive species of zooxanthellae is determined by the saturation in the membrane lipids. The lipid composition is potentially diagnostic of the differential nature of thermally induced bleaching found in scleractinian coral. Using fast repetition rate fluorescence measurements, we found that thermally damaged membranes become energetically uncoupled, but still are capable of splitting water. A fraction of the photosynthetically produced oxygen appears to reduced by photosystem I via the Mehler reaction to form reactive oxygen species (ROS), which rapidly accumulates at high irradiance levels and triggers death and expulsion of the endosymbioic algae. Differential sensitivity to thermal stress among the various species of *Symbiodinium* is not predicted based on large subunit ribosomal sequence analyses, but rather appears to be related to the genes in the lipid biosynthetic pathways. We suggest that the evolutionary history of symbiotic algae in Cnidarians reduced the tolerance to elevated temperatures in the latter portion of the Cenozoic.
Coral bleaching on a global scale is a growing concern both because of the reduction in essential ecological services provided by zooxanthellate corals within reef communities (1, 2), and the potentially devastating economic impacts accompanying the phenomenon (3). Small, positive deviations in temperature of <2º C can trigger massive losses of symbiotic algae, *Symbiodinium* spp., from their cnidarian host cells (4). However, not all corals within a reef are equally susceptible to elevated temperature stress (5, 6). Although elevated temperatures often lead to a reduction in the quantum yield of photochemistry, a concomitant increase in the rate of protein turnover in oxygen generating reaction center, photosystem II (PSII)(7-9), and an increase in the production of ROS (10-12), no mechanism has been elucidated. Here we show that thermal sensitivity in isolated clones of zooxanthellae and in symbiotic animal hosts is correlated with the degree of saturation of the lipids in the thylakoid membranes in the algal plastids. Our results provide a mechanistic basis for understanding and diagnosing coral bleaching patterns in nature.

**Materials and Methods**

**Cultures and Corals.** Cultures of *Symbiodinium* spp, obtained from culture collections or isolated from hosts, were grown in F/2 medium under a 10:14 light dark cycle and illuminated with 100 µmol quanta m⁻² s⁻¹. Corals were grown at 26º C 800L aquaria with running artificial seawater (Instant Ocean Sea Salt) as described (13). For thermal stress experiments, aquaria were heated to 32º C. Light, with a fluence of 200 µmol quanta m⁻² s⁻¹ on a 12:12 light:dark cycle was provided by 400W metal halide bulbs
(Iwasaki). Nutrients (NO$_3^-$, NO$_2^-$, NH$_4^+$ and PO$_4^{3-}$) were kept at submicromolar concentrations by foam fractioning and biological filtration (e.g. live sand).

**Variable fluorescence.** Variable chlorophyll fluorescence transient kinetics were measured with custom built fasat repetition rate fluorometer using protocols described by Kolber et al. (14).

**Lipid Analysis.** Lipids were saponified, methylated and extracted into hexane/methyl tertiary butyl ether as described (15). Fatty acid methyl esters were analysed by GC-mass spectrometry using an Agilent Series 6890 GC System and 5973 Mass Selective Detector, equipped with a HP5MS capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm) with helium as the carrier gas.

**Membrane Inlet Mass Spectrometry.** Light dependent production and consumption of oxygen was measured using a membrane inlet system attached to a Prisma QMS-200 (Pfeiffer) quadrapole mass spectrometer with closed ion source recording at mass/charge ($m/z$) ratios of 32 ($^{16}$O$_2$), 36 ($^{18}$O$_2$), and 40 (Ar). The membrane inlet system was modified from a water-jacketed DW/2 oxygen electrode chamber (Hansatech Instruments Ltd.) in which the electrode base plate was replaced by a stainless steel base plate with a gas port drilled through the center. The standard Teflon membrane (thickness 12.5 µm) supplied with the DW/2 oxygen electrode system was used. Illumination was provided by a high pressure halogen arc source at 300 µmol quanta m$^{-2}$ s$^{-1}$. Temperature was maintained at 26°C. Oxygen signals were calibrated with O$_2$ saturated water and zero (+ sodium dithionite) O$_2$ water and normalized to Ar. Oxygen production and consumption rates were calculated by linear regression analysis.
**Reactive Oxygen Species.** Cultures were harvested by centrifugation and resuspended in culture medium which had been stripped of O₂ by bubbling with N₂ gas. Subsamples were incubated for 3 h at 150 µmol quanta m⁻² s⁻¹ in 96 well plates in the presence of 15 µM dihydrorhodamine 123, a reactive oxygen specie sensitive dye (10). Fluorescence (i.e., ROS production) was measured kinetically with a plate reader (Molecular Devices) at excitation λ = 488nm and emission λ = 525 nm.

**Transmission Electron Microscopy.** Cells were harvested by centrifugation (15 min at 7000 x g) and fixed in cacodylate buffer containing 4% glutaraldehyde and 8.6% sucrose. Pellets were washed in a series of cacodylate buffers with descending sucrose concentration, and post-fixed in osmium tetroxide for 2h. After dehydration in an ascending series of ethanol (70% to 100%), samples were embedded in agar and Epon, sectioned (50 nm thickness) with a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and observed under a JEOL 100 CX transmission electron microscope.

**LSU rDNA Sequencing and Phylogenetic Analyses.** Genomic DNA was extracted from zooxanthellae isolated from cultures and corals using the DNeasy Plant Minikit (Qiagen). Standard PCR amplification of nuclear ribosomal DNA was performed using two sets of primers: (1) S-DINO (cgctctacgcgattgagtga) and L-DIN-1 (aagcgtgtgcacgtcagta), which are *Symbiodinium*-specific and cover the ITS-1/5.8S/ITS-2/partial LSU rDNA, and (2) D1R (acccgctgaatttaagcatat) and D2C (ccttggtccggtt), which are dinoflagellate-specific and target a 5’ fragment of the LSU rDNA. PCR products were purified using shrimp alkaline phosphatase and exonuclease I, and directly sequenced using an ABI 3100-Avant automatic sequencer. The new
sequences reported herein have been deposited in GenBank (accession # XXXXX to XXXX).

The D1 and D2 sequences of the LSU rDNA were manually aligned to the 294 homologous gene fragments from *Symbiodinium* spp. available in GenBank. All redundant, identical sequences were removed from the alignment, which resulted into a final DNA matrix containing 84 sequences and 556 nucleotidic sites (297 parsimony informative characters). Hierarchical likelihood ratio tests were applied to our data-set in order to select the most appropriate DNA substitution model: a general time reversible model considering the proportion of invariant sites as well as rate heterogeneity among sites (\(\gamma\)-shaped distribution, \(\gamma=1.2581\)) (16). Phylogenetic trees were inferred using Bayesian (1 million MCMC generations, substitution model parameters =GTR+G+I), Maximum Likelihood (substitution model parameters=TIM+G+I), and Neighbor Joining (substitution model parameters=Tamura and Nei+G) statistics, with the softwares MrBayes, PAUP*, and Lintree, respectively (17-19). In order to give a time dimension to our tree, the 13 consensus, highly resolved clades (thick branches in the tree of Fig. 5) were tested for molecular clock deviation using relative rate tests (20), with the Clade A used as an outgroup. None of the LSU rDNA *Symbiodinium* clades are evolving significantly faster than others (threshold risk for 12 clades and 66 tests: \(P<0.08\%\)). Consequently, we used Lintree\(^5\) to infer a clock-enforced, linearized tree (Fig. 5), which was calibrated in time via a “dinoflagellate” rate of LSU rDNA substitution based on previously published DNA-fossil comparative data-set\(^1\).

**Results and Discussion**
Representative transmission electron micrographs, selected from thousands of zooxanthellae cells, revealed that when thermally tolerant clones of *Symbiodinium* spp., grown at 26°C, were transferred to 32°C (a thermal stress that induces bleaching), the stacking properties and ultrastructural integrity of thylakoid membranes remained unaffected (Fig. 1 A, B, C, E; Supplemental online Table 1). In contrast, thylakoid membranes of thermally sensitive clones subjected to the higher temperature were significantly disrupted, and the organized stacking pattern, which is essential for efficient photochemical energy transduction, was compromised (Fig. 1 D, F). This process is not reversible, and was further observed in zooxanthellae *in hospite* in heat sensitive corals cultivated in the laboratory prior to bleaching.

The effect of thermal stress on the photochemical energy conversion efficiency was confirmed by fast repetition rate fluorescence (FRRF) measurements (14) on a variety of isolated, cultured clones of zooxanthellae (Figure 2). Thermally induced changes in membrane integrity were initially accompanied by both an increase in the rate of electron transport on the acceptor side of PSII and a simultaneous decrease in the maximum quantum yield of photochemistry within the reaction center (Supplemental online Table 2). In an energetically coupled thylakoids the fastest component of fluorescence decay corresponds to a single electron transfer from the primary quinone acceptor, QA⁻ to QB or QB⁻ (21), and occurs with a time constant ranging from 300 to 500 μs (22). In temperature sensitive clones of zooxanthellae, the measured time constant fell from an average of 304 ± 54 to 200 ± 46 μs, while in thermally tolerant clones the time constant remained statistically unchanged, averaging 318 ± 24 μs at 26°C and 341 ± 9 μs at 32°C. The marked change in electron transfer times in thermally sensitive clones was
accompanied by a 40% decrease in (but not loss of) photochemical energy conversion efficiency in PSII reaction centres. These two phenomena are diagnostic of an energetically uncoupled system where the trans-membrane proton gradient, established by the photochemical reactions in the functional reaction centres, is dissipated without generating ATP (23). This fluorescence kinetic pattern, uniquely found in thermally sensitive zooxanthellae, qualitatively differs from photoinhibition (24-26), where the time constant for electron transfer increases as the reaction centres become increasingly impaired (27). Moreover, in thermally sensitive clones of zooxanthellae, the pattern of change in photochemical energy conversion occurs over a very narrow thermal window of < 2°C. These results not only demonstrate that high resolution, kinetic measurements of variable chlorophyll fluorescence can be used to rapidly assess the sensitivity of zooxanthellae to thermal stress, but moreover suggest that thylakoid membrane integrity is potentially a critical determinant of thermal tolerance.

We further examined the patterns of thermal sensitivity and bleaching in colonies of the zooxanthellate corals, *Stylophora pistillata, Montipora samarensis*, and the symbiotic anemone *Aptasia* sp. cultivated *ex situ*. *S. pistillata* and *Aptasia* sp. both lost over 50% of their symbiotic algae within 72 h after exposure waters of 32°C. In contrast *M. samarensis* retained zooxanthellae at the elevated temperature for over a month. In the thermally sensitive species, not only was there a change in membrane integrity (e.g., Fig. 1 F) and loss of photochemical competence, but production of ROS in isolated zooxanthellae, detected using dihydrorhodamine 123, also increased by more than two-fold at high irradiances levels. The production of ROS corresponded to a light-dependent increase in O₂ consumption, as measured by membrane inlet mass spectrometry with
using 10% $^{18}$O$_2$ as a tracer (data not shown) (28). These results strongly suggest that the production of ROS is due to the Mehler reaction, i.e., the photochemical reduction of O$_2$ in photosystem I (29). Moreover, the dye tracer measurements clearly indicate that ROS produced in the algae leaks out of the cells. If this phenomenon happens in hospite, ROS would be transferred directly to the animal host, inducing a physiological stress.

Gas chromatography/mass spectroscopy analysis of seven zooxanthellae isolates revealed a striking contrast in the relative composition of lipids associated with thylakoid membranes between thermally sensitive and resilient clones (supplemental online Table 3). Specifically, thermally tolerant, cultured *Symbiodinium* clones and zooxanthellae freshly isolated from corals that did not bleach after experimental thermal stress (see supplemental data), have a markedly lower content of the major polyunsaturated fatty acid (PUFA), $\Delta$6,9,12,15-cis-octadecatetraenoic acid (18:4) in relation to $\Delta$9-cis-octadecatetraenoic (18:1) acid, independent of the experimental temperature (Fig 3). The differences in this lipid profile are statistically significant at the 0.001 level (ANOVA). The higher relative concentration of the saturated PUFA enhances thermal stability in eukaryotic thylakoid membranes and simultaneously reduces the susceptibility of the membrane lipids to attack by ROS(30-32). These experimental results strongly suggest that the wide variety of *Symbiodinium spp.* we analysed are not able to significantly modify their thylakoid lipid composition in response to changes in temperature, and hence, unlike most eukaryotic algae, are confined to relatively narrow thermal regimes. The absence of qualitative differences in thylakoid lipid composition between the heat sensitive and tolerant species suggests that differential susceptibility to elevated temperature results from changes in lipid biosynthetic pathways not associated with lipid
desaturases per se, but rather with regulatory elements of the enzyme(s) that control(s)
the relative amount of desaturation in specific pools of fatty acids.

Phylogenetic analyses of the zooxanthellae isolates used in this study clearly show that thermal tolerance is not associated with a single, monophyletic clade. Heat sensitive *Symbiodinium* spp. are found in totally different subdivisions of the LSU rDNA-based tree (A, B, C, and E), where thermally tolerant phylotypes systematically branch as closely related sister species (Fig. 4). This evolutionary pattern suggests that the reduced physiological ability to acclimate to elevated temperatures by enhancing thylakoid lipid saturation levels was either acquired in the common ancestor of all modern *Symbiodinium* clades and was subsequently lost independently in individual taxa within each clade, or was selected multiple times in independent lineages belonging to different clades.

The application of a molecular clock to the *Symbiodinium* spp. phylogenetic tree suggests that the ancestor of the species complex appeared at the K/T boundary, which corresponds to a major transition time from the Mesozoic rudist-based reefs to the modern scleractinian dominated reefs. Juxtaposition of the clocked *Symbiodinium* spp. phylogenetic tree with a sea surface temperature (SST) curve derived from oxygen isotope analysis of tropical planktonic foraminifera for the last 65 Myr (33) suggests that the first several million years in the Cenozoic Era, zooxanthellate-based symbioses evolved in warm tropical waters. We hypothesize that extensive cooling periods, starting in the Eocene, selected for cold-tolerant, heat-sensitive, *Symbiodinium* species, which may have been subject to negative selection (bleaching) later in the Pleistocene, and even more strongly in the contemporary Anthropocene period.
Our combined physiological, biochemical, and molecular data confirm that the widely accepted, but rather arbitrarily defined, *Symbiodinium* taxonomic “clades” (34), often referred to as genetic or functional units, are in fact multi-million year old groups containing a broad diversity of modern species that are physiologically differentiated. Phylotypes belonging to different “clades” can present similar pattern of sensitivity to elevated temperatures, but differ from their closely related, sister phylotypes. This analysis clearly indicates that rDNA genotyping is not a useful diagnostic tool for *a priori* detection of thermal sensitivity in zooxanthellate symbiotic associations.

Our results suggest that the physiological basis of bleaching is initiated when thylakoid membrane integrity is compromised at elevated temperatures, leading to an uncoupling of photosynthetic energy transduction. The accompanying proton leak and loss of ATP restricts photosynthetic carbon assimilation, however O₂ generated by PSII can react with the photochemically generated electrons in PSI to form ROS, which, in turn oxidizes membrane lipids. The oxidized lipids initiate a positive feedback of ROS that is accelerated by high light. Ultimately the ROS kills the intracellular algal symbionts and damages the host cells. The symbiotic algae literally are bleached, and/or expelled from their hosts. These results provide the first experimental demonstration of a biochemical adaptation associated with thermal tolerance in zooxanthellae and suggest that lipid analysis, could potentially provide a rapid, sensitive tool to diagnose the susceptibility of corals to thermally induced bleaching.
Acknowledgements

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Figure Legends

Figure 1. Transmission electron micrographs of thin sections of *Symbiodinium* spp. isolated from: **A & B:** *Tridacna* spp (CCMP 828); **C & D:** the sea anemone *Aptasia* sp. (CCMP-831); **E:** the coral, *Montipora samarensis*; **F:** the coral, *Stylophora pistillata*. Samples were incubated at 26°C (A,C) and 32°C (B, D, E, F). All cultures were grown in F/2 medium(35), under 12:12 light dark cycle. The corals were grown in a closed system, supported by a biological filtration system under 10:14 light dark cycle. Note the degradation of the thylakoid membranes within the plastids of the heat sensitive strains.

Figure 2. Maximum quantum yields of fluorescence (Fv/Fm, dimensionless) and electron transfer rates (τ, µs) from the primary electron acceptor in photosystem II, QA, to the secondary quinone, QB, for all clones of zooxanthellae. Fluorescence parameters were derived from measurements with a custom-built fast repetition rate fluorometers (14, 24). All cultures were grown in F/2 medium; cultures were incubated for up to 224 hours (to verify resilience and non-reversibility of thermally damaged cultures) under 10:14 light dark cycle, at 26°C and 32°C for each species tested. Maximum quantum yields of photochemistry (Fv/Fm) of the thermally tolerant clones averaged 0.57 ± 0.05 at 26 ºC and 0.55 ± .01 at 32 ºC; the corresponding electron transfer rates (τ) were 318 ± 24 and 341 ± 9 µs. In heat sensitive clones, the maximum quantum yields averaged 0.50 ± .07 at 26 ºC and 0.31 ± .03 at 32 ºC; the corresponding electron transfer rates were 304 ± 54 and 200 ± 46 µs.

Figure 3. Ratios of Δ9-*cis*-octadecatetraenoic (18:1) acid to Δ6,9,12,15-*cis* -octadecatetraenoic acid (18:4) for seven clones of *Symbiodinim* spp. ANOVA of the log
transformed data indicates a statistically significant difference between heat sensitive and heat tolerant clones.

Figure 4. LSU rDNA based evolution of the *Symbiodinium* species complex (SSC) and phylogenetic position of the zooxanthellae isolates analysed in Figures 1 to 4. Heat sensitive and resilient phylotypes are in red and blue, respectively. Clades A to G are the 7 recognized *Symbiodinium* phylogenetic groups (34), with A and B (yellow shaded) being typically considered as bleaching resistant, shallow-water types, and C (pink shaded) as bleaching sensitive, deeper living types. Our analysis suggests that at least 13 clades can be recognized based on genetic distances (thick branches in the tree), and that thermal sensitivity is not clade specific. The ultrametric, linearized tree shown here allowed us to apply a crude clock and calibrate the evolution of the SSC in time. The sea surface temperature curve, based on tropical planktonic foraminifera $\delta^{18}O$, serves as an approximate time-scale for SSC evolution. Note that 2 to 3 DNA substitutions in the LSU rDNA correspond to 1 My of evolution, so that speciation events in the last 500 000 y may not be detectable using this genetic marker. Neighbor Joining (1000 replicates) and Bayesian statistical values (1,000,000 generations) are indicated on the main internal branches.
References


mean DNA substitution rate = 0.29 % per My

Heat sensitive

Heat tolerant

SST

Age: Million years before present