LIPID BIOMARKERS OF CORAL STRESS: CALIBRATION AND EXPLORATION

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THESIS ABSTRACT

Corals are increasingly threatened by warming sea surface temperatures and other anthropogenic changes. The delicate symbiosis between corals and their algal endosymbionts (zooxanthellae) is easily disrupted by thermal stress, leading to bleaching and eventual mortality. The use of lipid ratios as biomarkers of environmental conditions is well established. Coral biomass contains abundant lipids, and the potential of lipid parameters to diagnose thermal tolerance in zooxanthellae has been previously suggested. In this thesis, I explore the response of specific fatty acids, sterols, and thylakoid membrane lipids to thermal and disease stress in zooxanthellae grown in culture, as well as those isolated from living corals. I present the discovery of a bioactive thylakoid lipid within zooxanthellae cells, and show how this compound is selectively mobilized in thermally stressed cells. I present a plausible mechanism for the breakdown of this compound into products that may cause apoptosis and disrupt the coral-algal symbiosis, eventually causing bleaching. I present two new lipid biomarkers of thermal stress in zooxanthellae, the C18 fatty acid unsaturation ratio, and the fatty acid to sterol ratio. I calibrate the decline of these two parameters to levels of thermal stress comparable to those needed to cause bleaching. I further show that these parameters are sensitive to pathogen stress as well. In several case studies of diseased and thermally stressed corals, I demonstrate that these lipid biomarkers of coral stress may be applied to zooxanthellae isolated from environmental samples. I show that these same compounds are preserved within coral aragonite, which opens up the potential to retrieve lipid-based historical records of coral health from annual layers of coral skeleton. This work demonstrates the value of using lipid biomarkers to assess coral health and better understand the biochemical mechanisms of coral bleaching.

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CHAPTER 1:

AN INTRODUCTION TO CORAL BIOLOGY AND LIPID BIOMARKERS

The health of corals worldwide is seriously threatened by a variety of factors, including warming ocean temperatures, ocean acidification, increased nutrients and sediment from run-off, and overfishing (Wilkinson, 2008). Many coral species are also impacted by increasing incidence of disease (Sutherland et al., 2004). Environmental stress often disrupts the symbiosis between corals and their endosymbiotic dinoflagellates (zooxanthellae), causing the coral to bleach. Though coral reefs in many parts of the world are in poor condition, coral health has only been closely monitored in recent decades. According to a recent summary (Wilkinson, 2004), 20% of the world's reefs have been irreparably damaged, and a further 24% are threatened by anthropogenic pressures such as destructive fishing practices, increased sediment load, and eutrophication. Coral bleaching has increased in frequency and severity in recent decades, particularly in response to increased ocean temperatures exacerbated by El Niño events and global warming (Williams & Bunkley-Williams, 1990; Harvell et al., 1999; Hoegh-Guldberg et al., 2007). Marine diseases are becoming more devastating to reefs and may be exacerbated by abnormally warm temperatures (Cervino et al., 2004; Sutherland et al., 2004). The ability of corals to withstand disease can be compromised by long-term warming and other abiotic stresses (Harvell et al., 1999). Bleaching and disease outbreaks in recent years may only be the most severe symptoms of a longer-term decline in reef health (Pandolfi et al., 2003).

There are a few mechanisms by which corals might adapt to the changing environment. For example, in response to thermal stress, corals may acquire a more heat-tolerant phylotype of photosynthetic symbionts (LaJeunesse et al., 2010). There are drawbacks to harboring thermally tolerant zooxanthellae, however, since more sensitive clades of zooxanthellae confer a growth advantage on the host coral

(Little et al., 2004). Over much longer geologic timescales, coral species can migrate to a more suitable geographic range (Hughes et al., 2003). However, the magnitude, rate of change, and range of stresses now experienced by reefs due to global warming, coastal development, and agricultural land-use are much larger than natural environmental variability of the past (Wilkinson, 2008). Recent massive ecological shifts from coral to macroalgae dominance on Caribbean reefs appear unprecedented in the Holocene (Precht & Aronson, 2006). In order to better understand current trends in coral condition, it is critical that we identify specific environmental threats, quantify measures of declining coral health, and understand how natural stresses can be either exacerbated or mitigated by human activity.

Coral biology

Stony corals commonly exist as a colony containing many individual coral polyps that secrete a calcium carbonate "skeleton." Most reef-forming corals (Scleractinians) live in symbiosis between the coral animal and photosynthetic dinoflagellates (*Symbiodinium* spp.), known as zooxanthellae, living within the coral tissue. *Symbiodinium* cells are contained within a symbiosome in the coral host. A vast diversity of clade subtypes of *Symbiodinium* seems to be adapted to different conditions (Baker, 2003). Each individual coral colony commonly hosts only one or at most several individual varieties of *Symbiodinium* at a time, and the host-symbiont specificity can be stable to perturbations (Thornhill et al., 2006), though some clade subtypes ("phylotypes") are known to be particularly resilient. In particular, clade D is particularly tolerant of thermal stress and is known to either preferentially survive or more quickly recover from high temperature events (Baker, 2003). One phylotype each in clades A, B, C, and D are represented in this thesis, and the majority of the results focus on the ecologically common phylotypes C1 and D1.

Zooxanthellae provide a metabolic benefit to their coral hosts by translocating sugars, amino acids, and lipids to the host (Trench, 1971). However,

these metabolic products must cross several membrane layers, including the plasma membrane of endodermal cells and symbiosome membrane of the coral host and the plasma membrane and cell wall of the *Symbiodinium* (Yellowlees et al., 2008). Figure 1.1 illustrates these membranes and the close contact between the zooxanthellae cells and host tissue.

Coral bleaching

There are two separate physical changes that can cause zooxanthellate corals to visibly bleach. Under stressful conditions, the symbiotic dinoflagellates produce significantly less photosynthetic pigment, which causes them to pale (Kleppel et al., 1989). The other mechanism of bleaching is a decrease of number of dinoflagellate cells within the coral host tissue, which can occur from zooxanthellae death *in hospite*, host cell apoptosis or necrosis, or sloughing of host cells, among other mechanisms (Gates et al., 1992). Bleaching can be quite heterogeneous across a reef, and it is not well understood why some colonies are more susceptible to bleaching than others. After a major bleaching event, corals typically regain their zooxanthellae (and their color) within about a year. Those that do not regain their symbionts will not survive.

Coral stress and stress metrics

The most frequently recognized environmental cause of stress and subsequent bleaching in corals is exposure to abnormally high temperatures. The algae seem more tolerant to heat stress than the animal host, though Fitt et al. (2001) divide bleaching into "physiological" (e.g. seasonality), "algal-stress" and "animal-stress" causes. A decrease in the number of zooxanthellae cells can be a response to oxidative stress in the coral (Downs et al., 2002; Weis, 2008).

In addition to high temperature, other environmental stressors include pathogens such as *Vibrio*, intense light, turbidity (such as caused by increased river runoff or hurricanes), excess nutrients, or chemical pollutants (including herbicides

and petroleum compounds). Synergy between multiple stress agents can exacerbate their impact on corals (Fitt et al., 2001).

To assess the risk for bleaching caused by thermal stress, "degree heating weeks" (DHW) are typically used (Fitt et al., 2001). One DHW is equivalent to one sustained week of sea surface temperature 1°C above the expected summertime maximum for that location. Values around 10 DHWs are typically expected to produce significant bleaching. The use of degree heating weeks allows comparison between corals adapted to different climate zones, and is an attempt to combine the deleterious effects of abnormally high temperature and sustained exposure. Other coral stressors can only be concretely compared to thermal stress on the basis of the bleaching (measured with fluorometry (Fitt et al., 2001)) or mortality that they cause.

Potential of coral skeleton to preserve information

Coral skeletons provide an ideal archive of the environmental conditions the corals have experienced. Since the corals lay down aragonite layers of varying density each year, the age of individual layers can be determined by counting density bands from an x-ray image. Inorganic proxies, like oxygen isotopes and concentration of metals have been widely used to extract climatic and environmental data from coral skeletons (Cohen and McConnaughey, 2003; Cole et al., 2000; Goodkin et al., 2005; Fleitmann et al., 2007; Smith et al., 1979).

In contrast, coral organic proxies have not been widely explored, yet contain valuable additional information. For example, triterpenoid compounds in coral skeletons have been used to determine the source of petroleum pollution, with the suggestion that other organic compounds preserved in the skeleton could be used to identify changes in biogenic and external sources of organic matter (Readman et al., 1996). The preservation of lipids in coral aragonite skeletons at concentrations greater than 1 μmol C/g coral has previously been demonstrated, providing the potential for construction of paleoenvironmental records (Ingalls et al., 2003). Fatty

acids, which are abundant in coral tissue (Harland et al., 1993), and sterols, which are often highly specific (e.g. Robinson et al., 1984), have potential to be preserved as intra-crystalline lipids for centuries (Ingalls et al., 2003).

Lipid biomarkers

Several types of organic compounds have been successfully and widely used to indicate environmental conditions, such as the U_k^{37} alkenone unsaturation proxy (Prahl and Wakeham, 1987) and the TEX86 proxy (Schouten et al., 2003) from measurements of glycerol dialkyl glycerol tetraethers (GDGTs) to indicate temperature.

In this thesis, I set out to identify new lipid biomarkers of coral stress. Much of the work focused on several types of compounds that are both abundant and readily amenable to gas chromatographic analysis, specifically fatty acids and sterols. The other investigations for this thesis involved more complicated, but more highly specific lipids: the intact polar lipids that make up the thylakoid membranes in chloroplasts. Each molecule of intact polar lipid contains two fatty acid moieties.

Fatty acids come in a variety of chain lengths and numbers of double bonds (Figure 1.2). As free fatty acids, they are named by the number of carbons in the acyl chain and the number of double bonds they contain. For example, a C18:0 fatty acid consists of an 18-carbon chain with no double bonds and a carboxylic acid functional group at one end. A C18:4 fatty acid is the same compound, but with 4 double bonds somewhere along the chain. Typically, the double bonds are not conjugated (that is, they are spaced every 3 carbons and therefore do not readily share electrons in resonance). The compound is more specifically named by the position of the double bond closest to the methyl end of the carbon chain (n-x or ω-x nomenclature, such as the ω-3 fatty acids). In this thesis, the specific positions of the double bonds in fatty acids were not identified, and so compounds are given by their carbon chain length and number of double bonds only. Because acetyl-CoA is the precursor during fatty acid synthesis (e.g. Harwood ,1988), most common naturally

occurring fatty acids have even carbon numbers. Most natural unsaturated fatty acids have exclusively cis double bonds. Palmitic (C16:0) and stearic (C18:0) fatty acids are particularly abundant, and form the basis for further carbon chain extensions and desaturations (Harwood, 1988).

Fatty acids can be found in a number of different forms within the cell. They can be bound in fats (triglycerides), polar lipids including the phospholipids, sulfolipids, or glycolipids, or as wax esters. A small proportion of the total fatty acids are present as free fatty acids. The unsaturation of fatty acids within membrane lipids is known to regulate the fluidity of the membranes (e.g. Steim et al., 1969; Wada et al., 1990); increased unsaturation lowers the phase transition temperature and allows a membrane to maintain their liquid-crystalline phase even at low temperature.

The second major group of lipid biomarkers discussed in this thesis are sterols. The familiar compound cholesterol is illustrated in Figure 1.3. Sterols share the same fused 5-ring structure and differ from one another in the number and position of double bonds, as well as the presence and position of a variety of methyl and ethyl groups.

Sterols are important components of lipid membranes, and also play important roles are signaling molecules. The amount of sterols in lipid membranes may help regulate membrane fluidity, including the formation of lipid rafts (e.g. Brown and London, 1998). Some sterols, like cholesterol, are fairly ubiquitous amongst both animal and some plant cells. Other sterols are thought to be taxonomically specific, such as dinosterol in dinoflagellates. Bacteria do not produce sterols, but rather contain related triterpenoid compounds called hopanoids (Rohmer et al., 1984).

Polar lipids form lipid bilayer membranes. In photosynthetic cells, thylakoid membranes hold the photosynthetic machinery within chloroplasts. Thylakoid lipids contain four different polar head groups: sulfoquinovosyldiacylglyceride (SQDG), phosphatidylglyceride (PG), monogalactosyldiacylglyceride (MGDG) and

digalactosyldiacylglyderide (DGDG). Each polar head group is connected to two fatty acid chains, which can vary in carbon chain length and degree of unsaturation (Gounaris et al., 1986). In higher plants, the galactolipids MGDG and DGDG make up the majority of the total thylakoid lipids (Dörmann and Benning, 2002). The structure of one form of MGDG is shown in figure 1.4. MGDG in particular may serve as a substrate for C18 fatty acid desaturation within chloroplasts (reviewed in Harwood, 1988). In addition to its role a major chloroplast lipid and scaffolding for C18 fatty acid desaturation, several highly unsaturated forms of MGDG may have pronounced bioactivity as well. In particular, MGDG containing C18:4 and C18:5 fatty acids can induce apoptosis (cell death) in mammalian cells at micromolar concentrations (Andrianasolo et al., 2008).

Lipid biomarkers to indicate coral stress

Lipid biomarkers have not yet been exploited as indicators of coral condition, beyond the observation that coral lipids make up a lower proportion of total biomass when the corals are bleached (Grottoli et al., 2004). Heat shock proteins, commonly used as indicators of thermal stress in both coral animals and their algal symbionts, are more rapidly broken down than lipids and would likely not be preserved beyond the initial stress event. Other indications of coral stress, such as visual and fluorometry-based observations of coral bleaching (Fitt et al., 2001) are only possible prior to reef recovery.

A number of different investigators have characterized the lipid content, and particularly the fatty acid make-up, of natural coral and zooxanthellae samples. Grottoli et al. (2004) observed a decrease in total lipid abundance in bleached *Porites compressa* corals, but no change in *Montipora verrucosa*. Bachok et al. (2006) observed a marked decrease in lipid concentration and the total fatty acid component, particularly polyunsaturated fatty acids, in bleached *Pavona frondifera* corals compared to healthy nearby specimens.

Extracts of whole-coral tissue from a suite of reef-building corals in Japan show that the saturated stearic acid is generally in greater abundance than the C18 unsaturated acids, except in a few of their samples, most notably *Porites lutea* (Yamashiro et al., 1999). Comparison between coral host and algal symbiont fatty acid profiles has shown that zooxanthellae produce more poly-unsaturated fatty acids, and that the corals do assimilate some of the algae-derived acids (Papina et al., 2003). These results show a strong dominance of palmitic acid (30% of all fatty acids in zooxanthellae and 50% in coral tissue).

Zhukova and Titlyanov (1995) reported compound specific fatty acid profiles from symbiotic dinoflagellates isolated from several different reef-building corals; their natural samples show high amounts of highly unsaturated C18 acid (C18:4), in particular, which has been proposed as indicative of thermal stress tolerance in the zooxanthellae (Tchernov et al., 2004). However, they did not investigate whether particular lipid ratios changed in response to heat stress. Greater detail is needed to identify thresholds of thermal stress, in order to quantify zooxanthellae responses to high temperature and other environmental stress agents.

Objectives of this thesis

Common measures of coral stress disappear after a bleaching event, and have no opportunity for long-term preservation or are ambiguous. Lipids compose a large fraction of coral biomass, and individual lipid compounds can also be highly specific to certain types of organisms. Lipids are known to change in response to temperature in order to regulate membrane fluidity, and they have the potential to be preserved for up to millennia in coral skeletons (Ingalls et al., 2003). Saturation within fatty acids has been identified as a potential indicator of thermal sensitivity within a variety of *Symbiodinium* clades (Tchernov et al., 2004), which suggested that lipids may play a role in zooxanthellae stress tolerance.

In the course of this thesis work, I set out to identify lipid biomarkers of coral stress and calibrate the response of those parameters to levels of thermal stress. To

maximize the potential application of those biomarkers to historical coral health reconstructions based on compounds preserved in coral skeleton, I focused on abundant and readily measurable lipids. I additionally explored the response of those thermal stress biomarkers to disease stress.

My studies of lipids produced by the algal symbionts of reef-building corals indicate that lipid biomarkers are sensitive indicators of thermal stress in zooxanthellae. I discovered the presence of a bioactive polar lipid from the thylakoid membrane of zooxanthellae chloroplasts, which had previously been reported to induce apoptosis in mammalian cells (Andrianasolo et al., 2008). In Chapter 2, I show that the relative abundance of this bioactive compound declines drastically in 4 phylotypes of thermally stressed zooxanthellae cultures, and I present a possible mechanism for the involvement of this molecule in apoptosis and coral bleaching. In Chapter 3, I show that the degree of unsaturation within C18 fatty acids and the ratio of fatty acids to sterols both decrease in response to thermal stress. I present a calibration of these two new coral stress biomarkers to the degree of thermal stress experienced by phylotypes of *Symbiodinium* from heat-sensitive clade C and heat-tolerant clade D. Finally, in Chapter 4 I show that these stress biomarkers are also applicable to diseased zooxanthellae and diseased coral colonies collected from the field. The fifth chapter presents a summary of conclusions and some suggestions for future research.

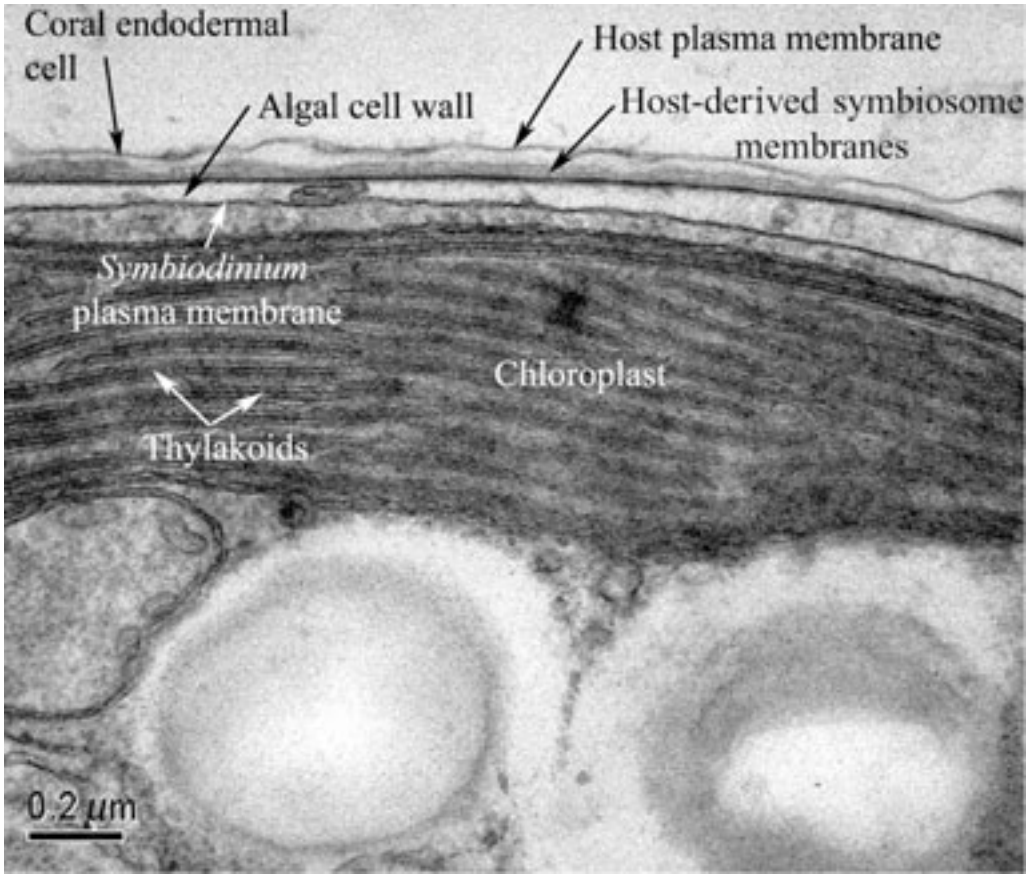

Figure 1.1 Coral-algal symbiosis. This close-up view depicts the interface between the endosymbiont (*Symbiodinium*) cell below and the coral animal cell above. Note that the coral plasma membrane and separate symbiosome membrane surround the algal cell wall and plasma membrane. Metabolic products such as sugars, amino acid, and lipids must cross these membranes in order to be transferred from the endosymbiont to the coral host. This figure also illustrates the neat stacking of thylakoid membranes within the *Symbiodinium* chloroplast. From Yellowlees et al., 2008.

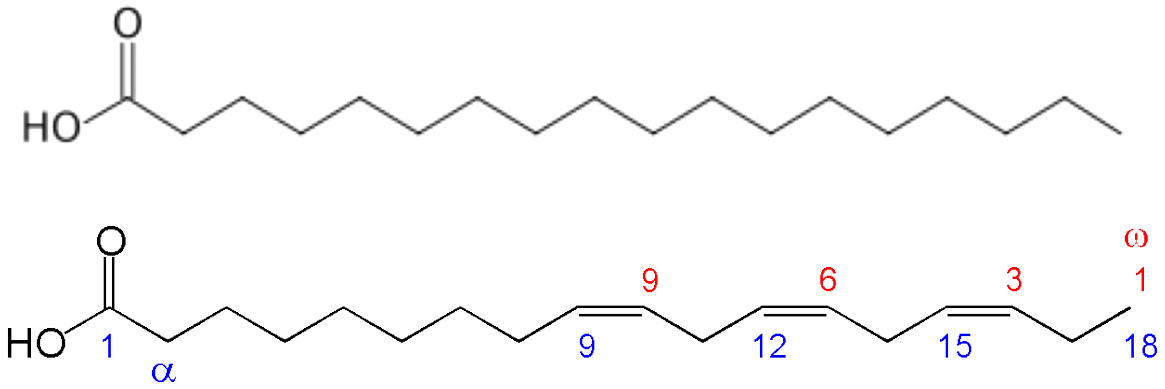

Figure 1.2 Chemical structure of saturated (top) and unsaturated (bottom) C18 fatty acids. The top compound is the fully saturated octadecanoic acid (stearic acid). The bottom compound is the tri-unsaturated 9Z, 12Z, 15Z-octadecatrienoic acid (α-linolenic acid). In the unsaturated molecule, blue numbers indicate IUPAC numbering starting with the carbonyl-carbon, and red numbers indicate the n-x or ω-x numbering convention beginning with the methyl terminal carbon. Note that the three double bonds are all in cis configuration and are not conjugated.

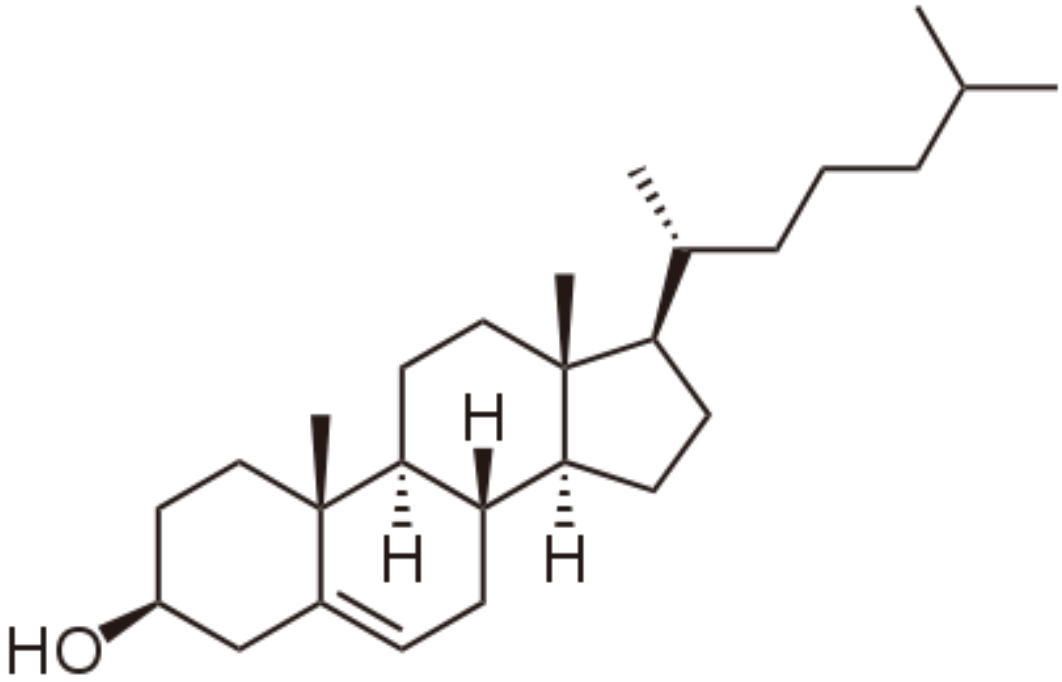

Figure 1.3 Chemical structure of cholesterol. Cholesterol is a common animal sterol, and is also produced in considerable abundance by Symbiodinium. It forms a structural part of lipid bilayer membranes, and may regulate membrane fluidity partly through the formation of lipid rafts. Other sterols differ from this structure in the number and position of double bonds and through the addition of methyl and ethyl groups in several locations in the ring structure or the side chain.

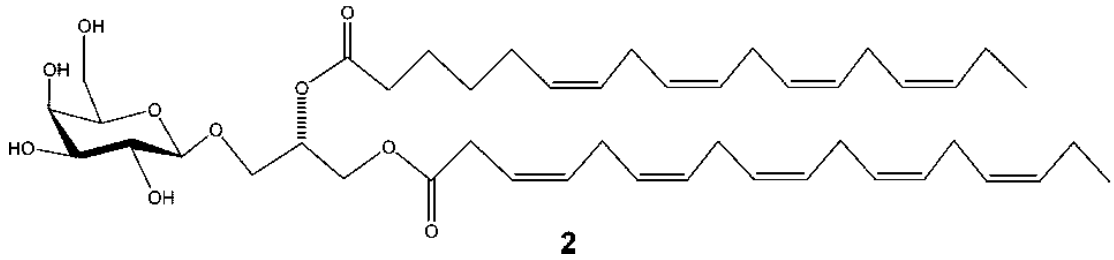

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Figure 1.4 Chemical structure of monogalactosyldiacylglycerol (MGDG). This MGDG molecule contains the galactose head group and C18:4 and C18:5 fatty acids. This compound has been shown to induce apoptosis in micromolar concentrations in mammalian cells (Andrianasolo et al., 2008.)

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CHAPTER 2:

SELECTIVE MOBILIZATION OF A BIOACTIVE GLYCOLIPID IN THERMALLY STRESSED CORAL ZOOXANTHELLAE: THE POTENTIAL ROLE OF LIPIDS IN CORAL BLEACHING

ABSTRACT

Corals and their algal symbionts (zooxanthellae) are threatened by environmental stressors, particularly rising ocean temperatures. However, the cellular stress response in these sensitive organisms is still not fully understood. We cultured *Symbiodinium* dinoflagellates, known as zooxanthellae, to test whether their membranes change under thermally stressful conditions. Here we report the discovery of a known apoptosis-inducing galactolipid as a significant component of zooxanthellae membranes, and show how the membrane content of that poly-unsaturated fatty acid (PUFA)-rich lipid decreases in response to heat stress, in accordance with the expected thermal sensitivity of four different zooxanthellae (phylotypes A1, B1, C1, and D1). Together with observations of a simultaneous decrease in the relative amount of the PUFAs that would be the anticipated initial breakdown products of the bioactive galactolipid, we infer that the compounds are most likely degrading further to produce poly-unsaturated aldehydes (PUAs), which themselves have been implicated in apoptosis induction. We report here the first observation that an apoptosis-inducing galactolipid is a major component of zooxanthellae thylakoid membranes, which is selectively mobilized from the thylakoid during thermal stress.

INTRODUCTION

Corals are increasingly threatened by warming oceans and other anthropogenic impacts (Goreau et al., 2000; Hughes et al., 2003). Abnormally warm temperatures are known to cause coral bleaching and mortality (Williams and Bunkley-Williams, 1990). Bleaching represents a breakdown of coral-algal

symbiosis and occurs by several physical mechanisms (Fitt et al., 2001), but the chemical mechanism of this breakdown is not well understood. Scleractinian corals live in symbiosis with dinoflagellates of the genus *Symbiodinium*, referred to as zooxanthellae, which transfer organic products such as glycerol, glucose, amino acids, and fatty acids to the coral host (Trench, 1971; Papina et al., 2003). Considerable diversity within the *Symbiodinium* genus manifests in variable degrees of thermal tolerance between clades, such that the zooxanthellae population within a single coral colony may shift from thermally sensitive clade C phylotypes to more tolerant clade D symbionts in response to high temperature stress (Baker, 2003). Distantly related clade A (Pochon et al., 2006) appears restricted to light-rich shallow water environments, and phylotypes B1 and C1 are the most abundant of 28 *Symbiodinium* phylotypes studied in all depth ranges of the Caribbean (LaJeunesse, 2002). Though corals are also sensitive to heat stress, the zooxanthellae appear to respond more rapidly to high temperature conditions (Strychar and Sammarco, 2009).

Lipid changes have been observed in bleached corals and membrane lipid composition may indicate differences in thermal sensitivity between strains of zooxanthellae. Thermally stressed zooxanthellae cells exhibit physical disruption of the thylakoid membrane, and higher unsaturation in C18 fatty acids (C18:4 relative to C18:1) of that membrane may indicate sensitivity to heat stress (Tchernov et al., 2004). Increased unsaturation within the thylakoid membrane may improve the tolerance to hypothermal stress in cyanobacteria (Wada et al., 1994) but the specific connection between thylakoid unsaturation and high temperature stress remains untested.

Moderate thermal stress is known to cause photoinhibition and slow the repair of photosystem II in *Symbiodinium* (Takahashi et al., 2009; Warner et al., 1999). It has been suggested that overproduction of reactive oxygen species (ROS) interfere with photosynthesis yield and lead to thylakoid membrane breakdown and disorganization during oxidative stress (Lesser, 1996; Tchernov et al., 2004).

Reactive oxygen species have also been implicated in the breakdown of zooxanthellae-coral symbiosis (Lesser, 1997; Weis, 2008) and more specifically bleaching may be a mechanism by which the coral mitigates oxidative damage from zooxanthellae (Downs et al., 2002).

Several galactolipids produced by marine diatoms have been shown to induce apoptosis at micromolar concentrations in mammalian cells (Andrianasolo, 2008), which suggests a possible role for membrane lipids in regulating the coral-zooxanthellae symbiosis. A careful investigation of membrane lipid composition is needed to test the effect of thermal stress on thylakoid membranes, identify the source of highly unsaturated C18 fatty acids that appear to confer a short term thermal tolerance advantage on select strains of zooxanthellae, and better understand the biochemical mechanism of thermal tolerance and species survival within the zooxanthelle of hermatypic reef-building corals.

RESULTS AND DISCUSSION

To assess the effect of temperature stress on lipid membranes, we cultured four *Symbiodinium* phylotypes under ambient and elevated temperature for up to 4 weeks. Phylotype A1 was particularly sensitive to the high temperature treatment and only survived to 2 weeks at elevated temperature. We extracted and measured intact polar lipids and free fatty acids to assess the connection between membrane lipids and thermal tolerance. Microscopy of cultured zooxanthellae shows thylakoid membrane disruption (Figure 2.1). At high temperature, the grana of the thylakoids become disorganized and are no longer contained within the thylakoid membranes. Some of the cells display localization of phosphatidylserine (determined by Annexin V stain) to the outer edge of the zooxanthellae plasma membrane at high temperature; this phosphatidyl serine migration is used to assess apoptosis (Vermes et al., 1995). The percentage of cells undergoing apoptosis increases in response to elevated temperature (Figure 2.2). Apoptosis increases from less than 5% of cells to between 13% (phylotype D1) and 49% (phylotype C1) after the first

24 hours of heat exposure. In the more thermally sensitive phylotypes A1, B1, and C1, the proportion of cells undergoing apoptosis decreases again at 48 hours. Heat tolerant phylotype D1 shows increasing apoptosis through 48 hours, though the proportion of cells undergoing apoptosis in phylotype D1 is less than in the more thermally sensitive phylotypes (Figure 2.2). We have no apoptosis data for our cultures beyond 48 hours, but sufficient cells were still growing to sample the biomass for lipid assays after two weeks in phylotype A1 and up to four weeks in phylotypes B1, C1, and D1.

We have demonstrated that thermal stress causes an increase in apoptosis within several days of exposure to elevated temperature, particularly in thermally sensitive phylotypes of zooxanthellae. However, weeks of sustained high temperatures are necessary to cause coral bleaching, and we observed that sufficient zooxanthellae cells survive the initial die-off and continue living in culture to enable additional sampling after one and four weeks in phylotypes B1, C1, and D1, and one and two weeks in clade A1. We further investigated thylakoid membrane lipids to identify longer-term biochemical changes over week-long timescales in response to thermal stress in the surviving cells.

Thylakoid membranes, which house the photosynthetic apparatus within chloroplasts, are composed of lipids with 4 different polar head groups (sulfoquinovosyldiacylglyceride [SQDG], phosphatidylglyceride [PG], monogalactosyldiacylglyceride [MGDG] and digalactosyldiacylglyderide [DGDG]). Each polar head group is attached to two fatty acids, which can be saturated (no double bonds), monounsaturated (one double bond) or polyunsaturated (multiple double bonds). A variety of saturated and unsaturated fatty acids make up the two acyl moieties attached to each polar head group (Gounaris et al., 1986). In higher plants, the galactolipids MGDG and DGDG make up the majority of the total thylakoid lipids (Dörmann and Benning, 2002). The relative amount of phospholipids in some organisms may decrease in response to phosphorus limitation (Van Mooy et al., 2009). In our nutrient-replete *Symbiodinium* cultures,

the sulfolipid SQDG makes up the majority of the thylakoid lipids (70-95 mol%) but the relative amounts of the galactolipids MGDG and DGDG decrease in the thermally stressed samples (Figure 2.3, DGDG data not shown).

A closer, molecular-level characterization of the major MGDG species in these cultures revealed that two MGDG compounds make up the majority of MGDG in unstressed cultures. The most striking discovery within our zooxanthellae cultures is that one of these two abundant MGDG compounds is the bioactive MGDG molecule containing C18:4 and C18:5 fatty acids. This compound is known to induce apoptosis in mammalian cells at micromolar concentration (Andrianasolo et al., 2008). The second highly-abundant MGDG compound contains two C18:4 fatty acids, so it differs from the apoptosis-inducing compound by only one double bond in one of the two fatty acid moieties. In unstressed zooxanthellae cultures, these two MGDG compounds containing C18:4/C18:4 and C18:4/C18:5 polyunsaturated fatty acids (PUFAs) are the two most abundant MGDG compounds (Figure 2.4). The C18:4/C18:5 MGDG has been previously observed in cultured dinoflagellates and is a substantial component of thylakoid membranes (Gray et al., 2009) in dinoflagellates closely related to the *Symbiodinium* strains cultured for this study. In our unstressed cultures, the C18:4/C18:5 MGDG represents a similar proportion of total MGDG and DGDG (7-14%, data not shown) to the 3 strains of *Symbiodinium microadriaticum* explored by Gray et al. (2009) (12-15%).

The second important observation from our *Symbiodinium* thermal stress experiment is that the relative amount of these PUFA-rich compounds within the MGDG pool is highly sensitive to thermal stress (Table 2.1). In unstressed cultures at 26°C, the two PUFA-rich MGDG compounds make up an average of 71% (±10%, 1 standard deviation) of the total MGDG. Over several weeks of thermal stress, the relative amount of PUFA-rich MGDG declines, particularly in the thermally sensitive zooxanthellae phylotypes (Figure 2.4). After one to four weeks of exposure to elevated temperature at 31°C, these two PUFA-rich MGDG compounds become significantly less dominant or disappear entirely in thermally sensitive

zooxanthellae phylotypes A1 (13% of MGDG after 1 week, $p < 0.005$ by one-tailed t-test), B1 (24% of MGDG after 4 weeks, $p < 0.005$) and C1 (0% of MGDG after 4 weeks, $p < 0.005$). The relative concentration of these compounds is apparently not affected in the high temperature treatment of thermally tolerant phylotype D1. The clear decrease in these MGDG species under high temperature conditions indicates a fundamental change in thylakoid membrane composition. The comparable PUFA-rich species of DGDG, which differ from the PUFA-rich MGDGs by only one sugar group, do not decline in response to thermal stress (Figure 2.S1). This indicates that the PUFA-rich MGDGs are being selectively targeted.

The relative decreased inventory of PUFA-rich MGDG compounds in thermally stressed cultures could be due to either a decrease in production or an increase in breakdown. Since PUFA-rich MGDGs are produced by progressive desaturation of more saturated C18-MGDG compounds (Gounaris et al., 1986), a decrease in PUFA-rich MGDG production could either result from a decrease in MGDG overall or a decrease in desaturation activity within the existing pool of MGDG. In phylotype B1, the proportion of MGDG of the total thylakoid lipids does not decrease in the high temperature treatment (Figure 2.3), though the proportion of PUFA-rich compounds within that MGDG pool does decrease (Figure 2.4 and Table 2.1). This would point to a possible decrease in desaturation activity in this phylotype. In phylotypes A1 and C1, the total amount of MGDG decreases in the thermally stressed cells (Figure 2.3) while the proportion of highly unsaturated MGDG also decreases (Figure 2.4). While a simultaneous decrease in both total MGDG production and desaturation activity in these two phylotypes cannot be ruled out, a simpler explanation is that the highly unsaturated compounds are preferentially being degraded. Subsequent discussion will consider the implications of this interpretation.

The breakdown process of MGDG is well characterized. Initially, hydrolysis of MGDG releases free PUFAs, which then further degrade to polyunsaturated aldehydes (PUAs) (D'Ippolito et al., 2004) via lipid peroxidation of lipid

hydroperoxides (Fontana et al., 2007). The PUFA-rich MGDGs are preferentially disappearing from the thylakoids of thermally stressed zooxanthellae, so we next looked for a possible buildup of the C18 PUFAs that are the initial breakdown product. Rather than an increase in C18 PUFAs, the free fatty acid pool shows a decrease in C18:4 (and all C18 PUFAs) under heat stress conditions, relative to saturated fatty acids (Figure 5) and relative to sterols (data not shown). Again, we cannot exclude the possibility of decreased production of C18:4 fatty acids, but some amount of C18:4 PUFAs would necessarily be released by the breakdown of PUFA-rich MGDG compounds. We therefore surmise that these PUFAs are breaking down further to release poly-unsaturated aldehydes (PUAs) and other degradation products into the cell. PUAs and subsequent degradation products are known to repel grazers and may play a role in apoptosis induction (Jüttner 2005; Romano et al., 2010).

Apoptosis, an organized form of cell death, has been reported in *Symbiodinium* as a result of thermal stress (Strychar et al., 2004). To reconcile the observed presence of a known apoptosis-inducing lipid, which then declines in abundance in heat-stressed zooxanthellae, we propose the following mechanism. In thermally stressed cells, the PUFA-rich MGDG is readily broken down to release PUFAs, which themselves have may repel potential grazers (Romano et al., 2010). Those PUFAs can be further degraded to PUAs, which also induce apoptosis and have known anti-grazing properties (Romano et al., 2010). In diatom biofilms that produce the PUFA eicosapentaenoic acid (EPA), the EPA is rapidly broken down to PUAs, which repel and impair reproduction in grazers (Miralto et al., 1999) and are toxic to some zooplankton (Jüttner, 2005). The conversion from PUFA to PUA occurs via lipid peroxidation, which may allow formation of a transient lipid radical (Catalá, 2009). Lipid radicals are more stable than superoxide or hydrogen peroxide radicals, so they can spread the damage of the additional reactive oxygen species produced during thermally-induced oxidative stress. The lipid radicals can then trigger a protease cascade and induce apoptosis.

If the PUFA-rich MGDG species were breaking down to release their fatty acid moieties into the cell, we would expect an increase in C18 PUFAs, assuming other sources and sinks are unchanging. However, C18:4 decreases under the same conditions where PUFA-rich MGDG compounds are preferentially disappearing. There are several possible explanations we must consider for the relative decrease in highly unsaturated C18 fatty acids. A decline in production due to down-regulation of fatty acid desaturase enzymes could explain the C18:4 decrease, but a metagenomic analysis of the coral holobiont determined that genes involved in fatty acid catabolism and anabolism increased upon high temperature treatment (Vega Thurber et al., 2009). There could also be a preferential increase in the more saturated C18 fatty acids due to release from other polar lipids, including other membrane lipids, or triglycerides. Since the total amount of C18 free fatty acids declines relative to sterols, however, a preferential release of more saturated compounds is unlikely. Instead, the most consistent scenario for the decreases in both PUFA-rich MGDG and free C18 PUFAs under thermally stressed conditions is that the bio-active MGDG is breaking down further through interaction with ROS species to produce lipid peroxides and poly-unsaturated aldehydes (PUAs).

The possible influence of zooxanthellae on host cell apoptosis has been previously suggested (Lesser and Farrell, 2004). Both animal and algal cells in the symbiosis are sensitive to thermal stress, but there is some debate about which organism responds first. In a heat-stressed anemone-algal symbiosis, both the animal and algal cells show features of both necrosis and programmed cell death (Dunn et al., 2002), but the anemone endoderm cells are affected on shorter time scales (2 minutes) compared to zooxanthellae (1 hour), and endodermal cells (which typically harbor zooxanthellae) showed a higher incidence of apoptosis-like cells than ectodermal cells (which do not typically harbor zooxanthellae) (Dunn et al., 2004). In two species of scleractinian corals, the zooxanthellae showed signs of both types of cell death within hours of comparable high temperature exposure, but apoptosis in host cells was negligible for the course of the 48-hour experiment

(Strychar and Sammarco, 2009). The C18:4/18:5 MGDG has known apoptosis-inducing and anti-grazing properties (Andrianasolo et al., 2008) and thus has the potential to disrupt the coral-zooxanthellae symbiosis if this compound and its degradation products are released into the coral cells. Mobilization of this compound from zooxanthellae thylakoid membranes may then induce apoptosis in the coral host that could lead to the destabilization of symbiosis. Several layers of membrane, including both host-derived and zooxanthellae-derived lipids, physically contain the zooxanthellae cells *in hospite* (Wakefield and Kempf, 2001), and chemical signals released by the zooxanthellae would need to cross this barrier before affecting coral endoderm cells; PUFAs, PUAs, and ROS would more readily cross into coral endoderm cells than the intact PUFA-rich MGDG.

The breakdown of the bioactive 18:4/18:5 MGDG into free fatty acid components (C18 PUFAs) is not seen, in fact C18 PUFAs (as free fatty acids) decrease relative to other fatty acids at higher temperature. Thus it is likely that the C18 PUFAs are further degrading into PUAs, which may be the agents that induce apoptosis in the zooxanthellae or lead to a breakdown of the symbiosis *in hospite*. The highest concentration of the PUFA-rich MGDG compounds occurs in phylotype A1, which also shows the highest thermal sensitivity and fastest depletion of those MGDGs. Thermally tolerant phylotype D1 shows almost no MGDG loss, which may explain its known hardiness (Baker, 2003). The participation of PUFA-rich thylakoid lipids in apoptosis induction may explain the observed correlation between high levels of C18:4 fatty acids and thermal sensitivity in different zooxanthellae clades (Tchernov et al., 2004).

Apoptosis induction, as indicated by phosphatidylserine in cell membranes, begins on a timescale of 24 hours or less, but the large initial amount of bioactive MGDG available in the cells dwarfs the micromolar concentrations needed for apoptosis induction (Andrianasolo et al., 2008), and the observed dramatic decrease in these bioactive thylakoid lipids occurs only after several weeks of heat treatment. Lipid changes are on a consistent timescale with coral bleaching (Fitt et al., 2001),

therefore we suggest that in an intact coral-zooxanthellae symbiosis the bioactive thylakoid lipids or their breakdown products can transfer to the coral host and induce apoptosis in the coral animal as well. This would explain the observed apoptosis in corals and anemone with symbiotic zooxanthellae (Dunn et al., 2004; Lesser and Farrell, 2004). Reports of apoptosis induction in mammalian cells by galacto-lipids (Andrianasolo et al. 2008) hinted at the role membrane lipids may play in cellular signaling, and in particular we suggest that the presence of such galacto-lipids in zooxanthellae cells may help regulate the coral-algal symbiosis by ensuring the coral host cells do not degrade their photosymbionts, in an intra-symbiotic anti-grazing capacity. Thermal stress in the zooxanthellae could release these compounds and disrupt the symbiosis, contributing to coral bleaching. This work highlights a potential role of a bioactive thylakoid lipid in determining sensitivity to thermal stress and suggests a mechanism for chemical ecology in maintaining the coral-zooxanthellae symbiosis.

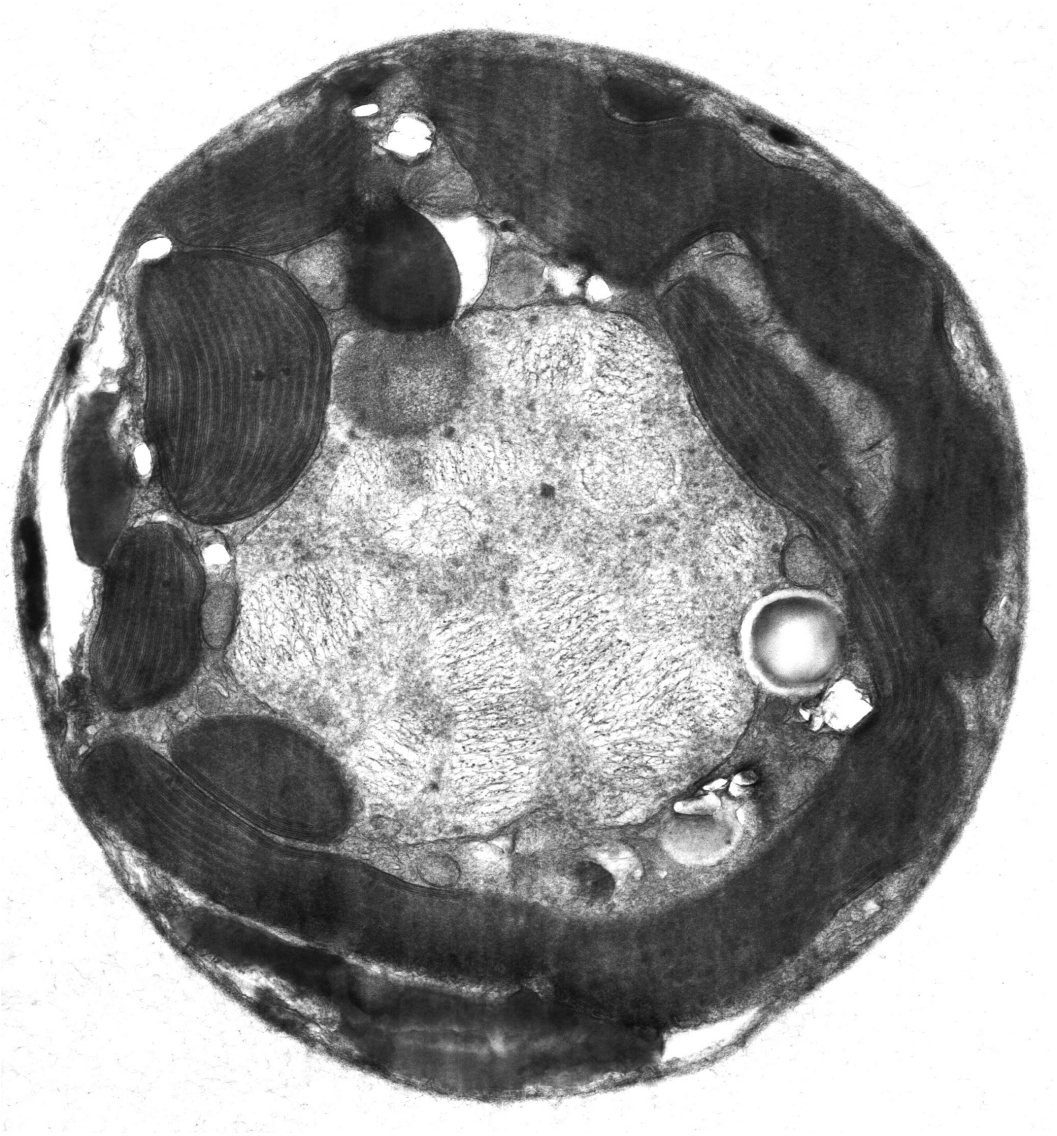

Figure 2.1a: Healthy *Symbiodinium* cell showing well-organized, stacked thylakoid membranes fully contained within chloroplast membranes.

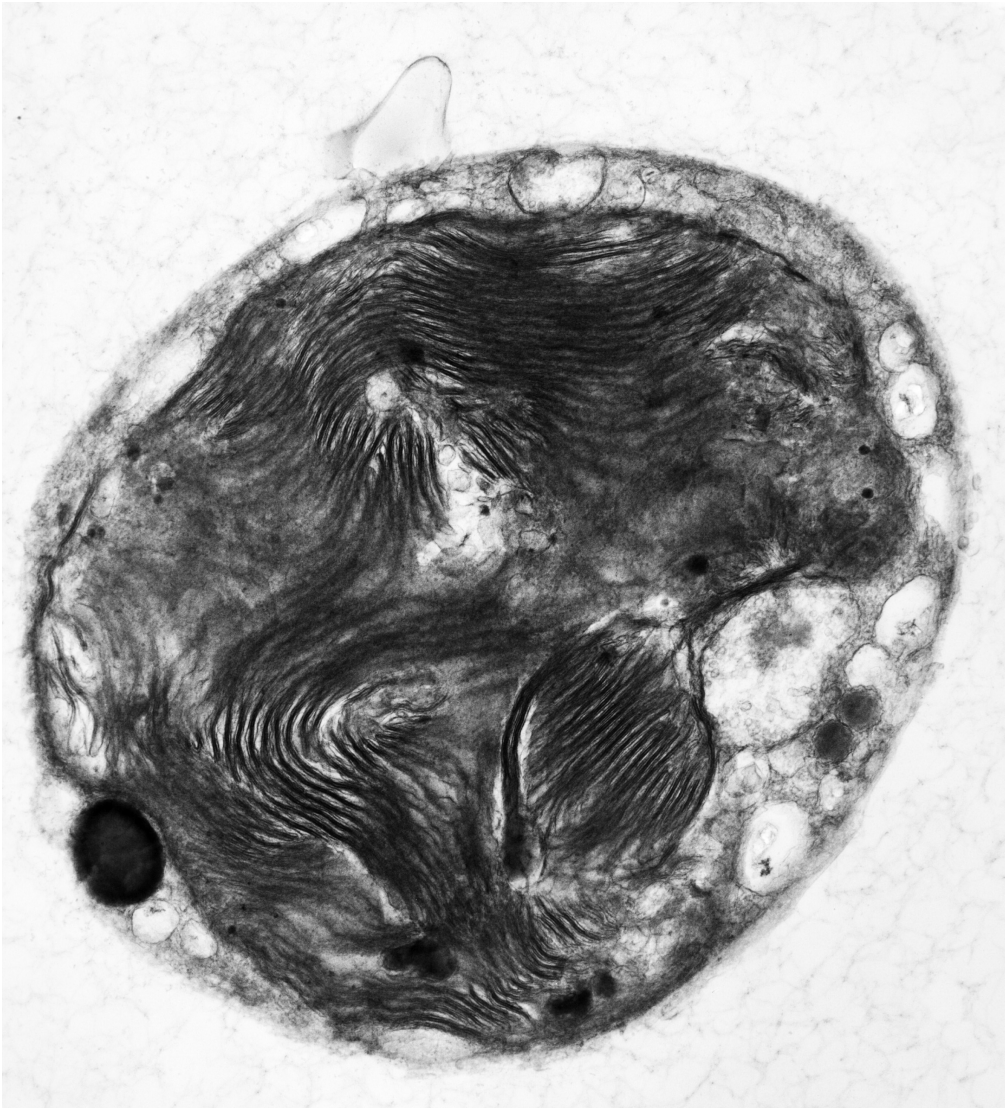

Figure 2.1b: Thermally stressed *Symbiodinium* cell undergoing apoptosis, with disorganized thylakoid membranes no longer contained by chloroplast membranes.

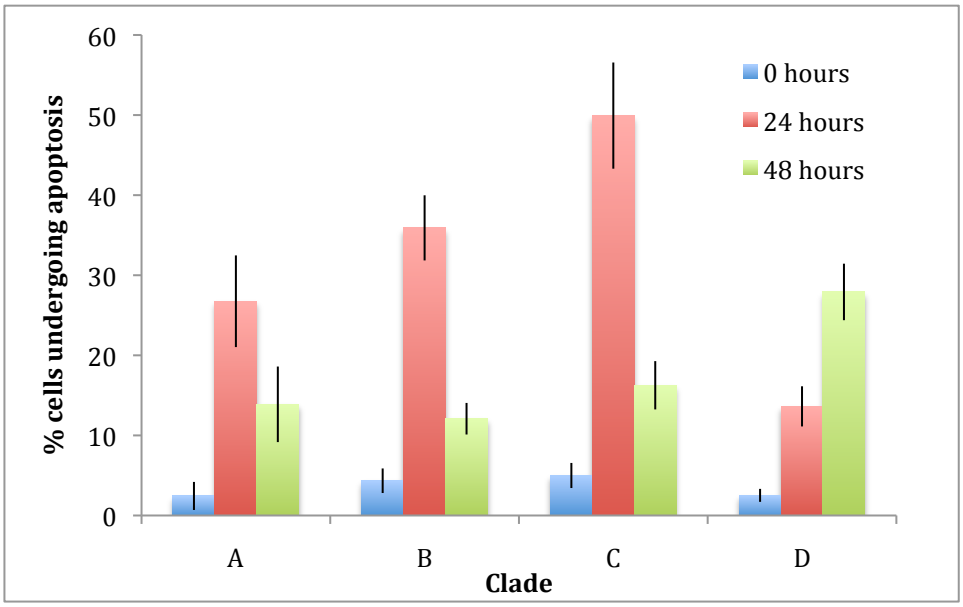

Figure 2.2: Percentage of heat-stressed cells (33°C) showing phosphatidylserine localization to the outer membrane indicated by Annexin V staining, which is symptomatic of apoptosis. Error bars represent one standard deviation (n=24 for 1 and 24 hours, n=8 for 48 hours).

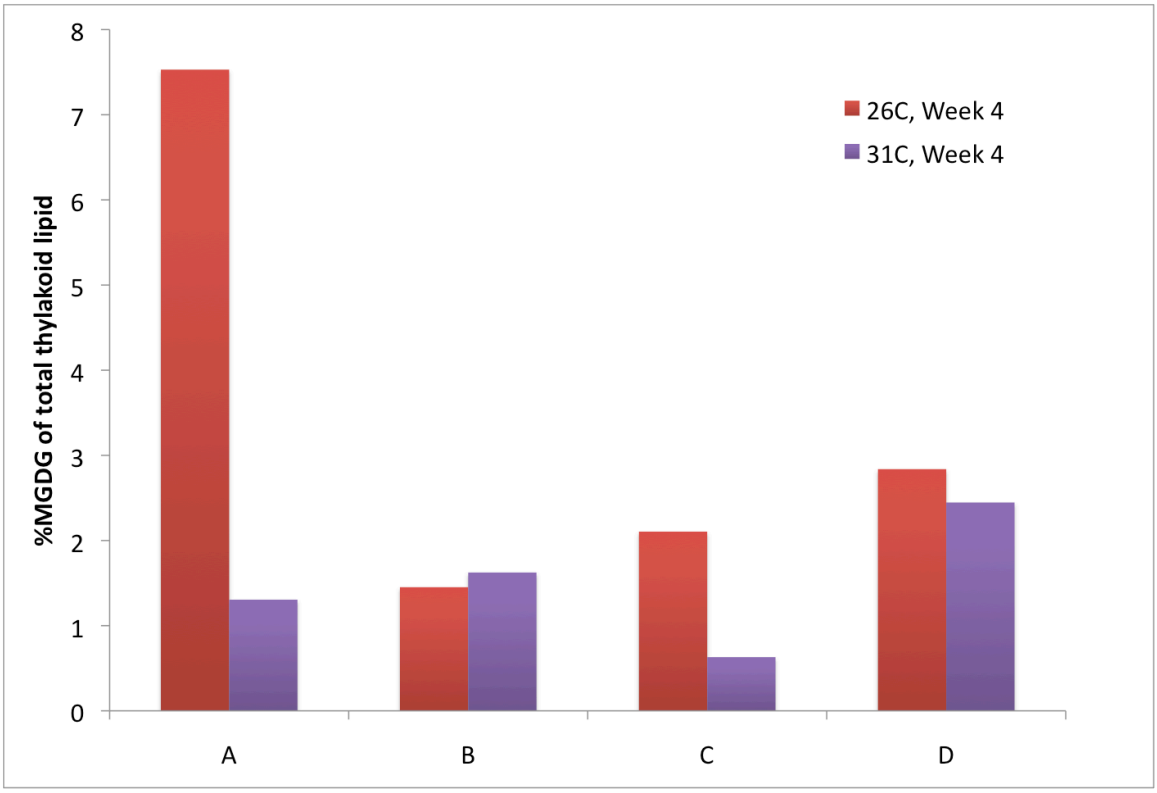

Figure 2.3: Percentage of MGDG in total thylakoid lipids in unstressed (red) and thermally stressed (purple) cultured zooxanthellae in clades A1, B1, C1, and D1.

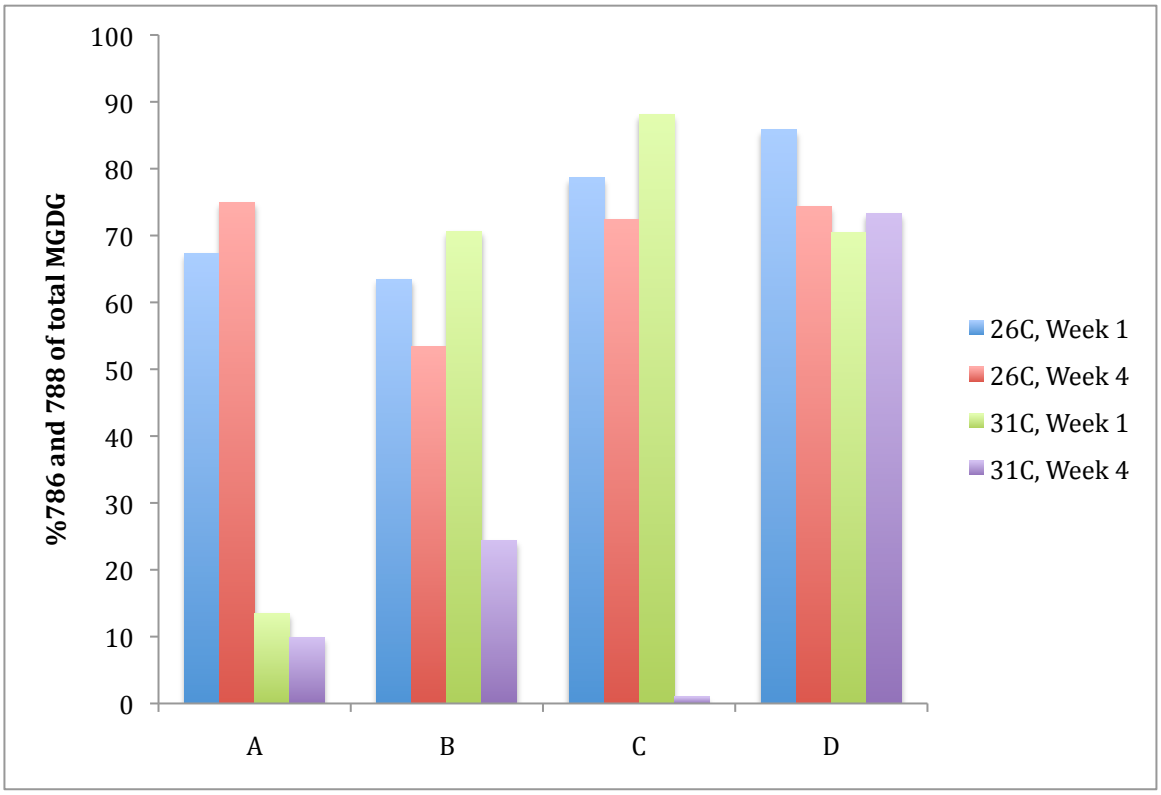

Figure 2.4: Proportion of bio-active MGDG (C18:4/C18:4 and C18:4/C18:5) relative to total MGDG pool in cultured zooxanthellae. In unstressed cultures (26°C for 1 and 4 weeks) these two compounds make up 50-80% of the thylakoid MGDG. In thermally stressed cultures (31°C for 4 weeks in clades B1 and C1, and 31°C at both 1 and 4 weeks in the most sensitive clade A1) these bio-active MGDG compounds are significantly lower ($p < 0.005$, one-tailed t-test).

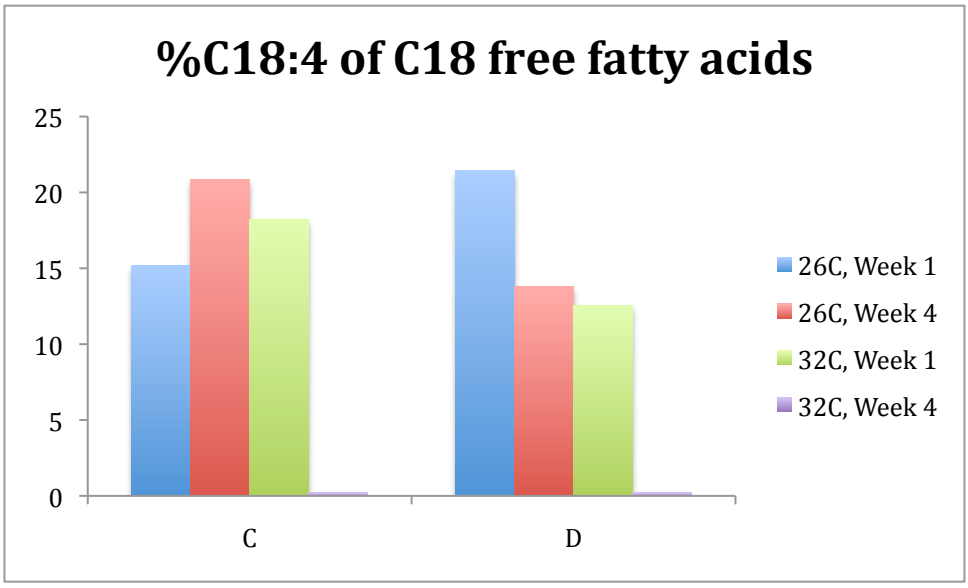

Figure 2.5: Percent C18:4 free fatty acid, relative to all C18 fatty acids in thermally sensitive phylotype C1 and thermally tolerant phylotype D1. C18:4 free fatty acids were undetectable after four weeks of exposure to 32°C in either strain. This value is significantly different from the low temperature (26°C) conditions (one-tailed t-test, $p < 0.05$)

Supplemental Figure

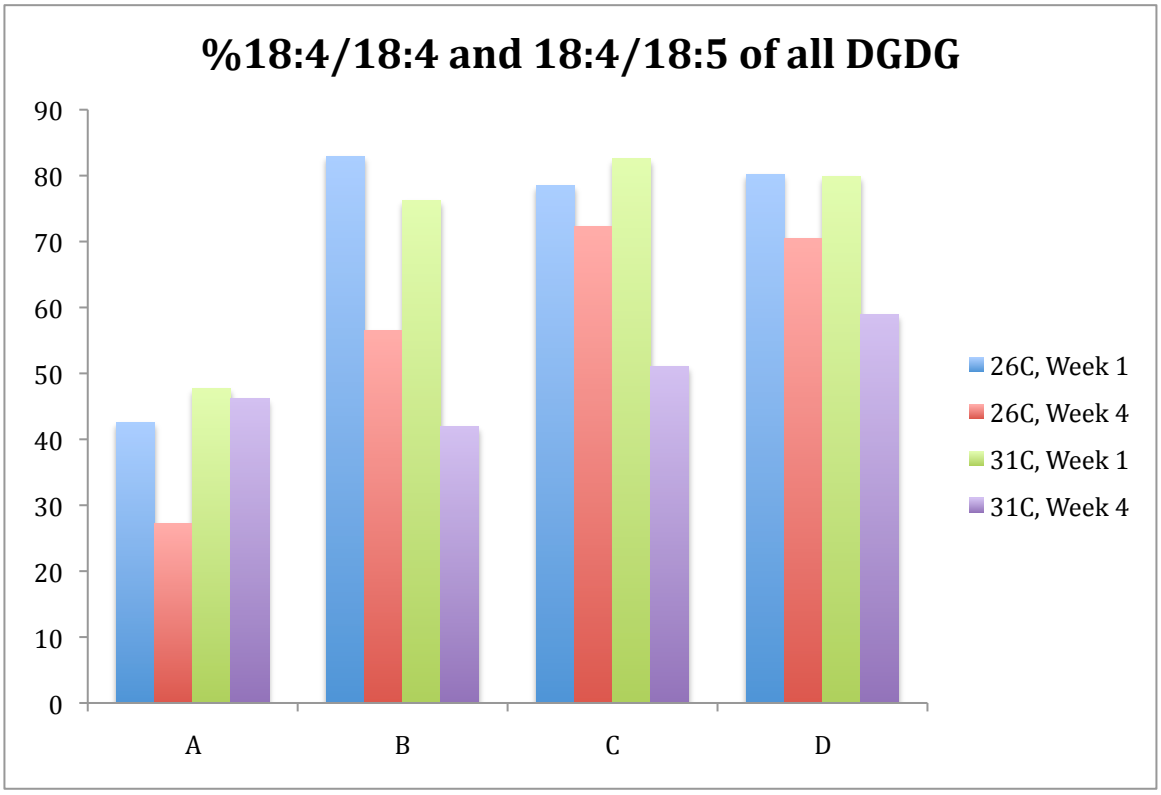

Figure 2.S1. Relative proportion of PUFA-rich DGDG of all cellular DGDG in cultured zooxanthellae. MGDG and DGDG differ by only one extra galactose moiety in the DGDG. As with MGDG, DGDG also contains a high proportion of C18:4 and C18:5 polyunsaturated fatty acids, particularly in clades B, C, and D. Though the PUFA-rich bioactive MGDG species decrease markedly in heat stressed cultures, a similar pattern is not observed in the analogous DGDG molecules. This suggests that the mobilization of PUFA-rich MGDG molecules in thermally stressed cells is highly specific.

Phylotype	Temperature (°C)	Time (weeks)	% PUFA-rich MGDG
A1	26	1	67
A1	26	2	75
B1	26	1	63
B1	26	4	53
C1	26	1	79
C1	26	4	72
D1	26	1	86
D1	26	4	74
***A1**	**31**	**1**	**13**
***A1**	**31**	**4**	**10**
B1	31	1	71
***B1**	**31**	**4**	**24**
C1	31	1	88
***C1**	**31**	**4**	**ND**
D1	31	1	70
D1	31	4	73

Table 2.1 Proportion of MGDG containing C18:4 and C18:5 PUFAs. The percentage of PUFA-rich MGDG is calculated as the sum of C18:4/C18:4 and C18:4/C18:5 MGDG divided by the sum of the six most abundant MGDG compounds in cultured *Symbiodinium* cells. Values denoted by an asterisk and bold type are significantly different from the low temperature control values (one-tailed t-test, $p < 0.005$).

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CHAPTER 3:

LIPID BIOMARKERS IN SYMBIODINIUM DINOFLAGELLATES: POTENTIAL FOR A NEW INDEX OF CORAL HEAT STRESS

ABSTRACT

Lipid content and fatty acid profiles of corals and their dinoflagellate symbionts are known to vary in response to high temperature stress. To develop lipid biomarkers as indicators of heat stress in reef-building corals, we investigated cultures of *Symbiodinium* dinoflagellates of clade subtypes C1 and D1 grown under a range of temperatures and durations. The predominant lipids produced by *Symbiodinium* are palmitic (C16) and stearic (C18) saturated fatty acids and their unsaturated analogs and a variety of sterols. The relative fatty acids compared to sterols in zooxanthellae tissue decreases in response to thermal stress. Prolonged exposure to high temperature also causes the relative amount of unsaturated acids within the C18 fatty acids to decrease. This shift in fatty acids and sterols is common to both clade subtypes C1 and D1, but the apparent threshold of heat stress is lower for clade subtype C1. This work indicates that fatty acids and sterols in zooxanthellae can be used as sensitive indicators of thermal stress in corals.

1. INTRODUCTION

Tropical reef-building corals around the world are threatened by a variety of biotic and abiotic factors (Wilkinson, 2008), but understanding the detailed causes and

effects of coral decline remains incomplete. Increasing sea surface temperature is one of the most severe threats to coral health. In recent years, high temperatures have been commonly observed to correlate with coral bleaching (e.g. Harvell et al., 1999), and coral diseases may be exacerbated by abnormally warm temperatures (Sutherland et al., 2004; Cervino et al., 2004). The ability of corals to withstand disease can be compromised by long-term warming and other abiotic stresses (Harvell et al., 1999). Bleaching and disease outbreaks in recent years are only the most severe symptoms of a longer-term decline in reef health (Pandolfi et al., 2003).

Bleaching occurs when corals are exposed to unusually high temperature for periods of weeks or longer (Goreau and Hayes, 1994). Coral heat stress is typically measured in degree heating weeks, defined as the product of duration (in weeks) and temperature above the maximum expected summer temperature (in degrees Celsius). Mechanisms of bleaching include expulsion of the photosynthetic symbionts (zooxanthellae) and loss of pigment within remaining zooxanthellae cells (Douglas, 2003). In response to repeated thermal stress, some corals may acquire a more heat-tolerant clade of zooxanthellae (Jones et al., 2008). Over much longer timescales, corals may eventually migrate to a more appropriate geographic range (Hughes et al., 2003). However, the magnitude and rate of increased stress now experienced by corals due to global warming, coastal land-use, and runoff are historically unprecedented. In order to better understand current trends in coral condition, it is critical that we quantify both specific environmental threats and

measures of declining coral health, and understand how natural stresses can be either exacerbated or mitigated by human activity.

We present here an investigation of the potential for lipid biomarkers in two *Symbiodinium* clades to serve as proxies for heat stress. We have examined the lipid composition of the dinoflagellate zooxanthellae that typically live symbiotically within hermatypic corals and have characterized how those lipids vary in response to heat stress. We saponified a subset of samples to determine whether a measurement of free fatty acids is representative of the total fatty acids in *Symbiodinium* cells. In order to identify potential biomarkers that may be applied to reconstructions back through time, we focus on the ratios between abundant compounds. We then examined how these lipid ratios evolved over time in thermally stressed cells, and compared the lipid ratios to a degree-heat-week index to assess their value as biomarkers of thermal stress.

2. METHODS

2.1 *Symbiodinium* cultures

Clones of *Symbiodinium* spp. dinoflagellates of clade subtypes C1 (*S. goreauii* strain CCMP 2466) and D1 (CCMP 2469) were cultured under 12/12 hour light/dark cycle in f/2 growth medium at 26°C, 28°C, 30°C, and 32°C for up to 4 weeks. 500 μl of *Symbiodinium* stock culture was inititally placed in 15mL glass test tubes and supplemented with f/2 medium during incubation. Fresh f/2 medium was replenished during sampling times to compensate for evaporation. Triplicate

samples at each temperature were removed for analysis each week. Control cultures grown at the initial temperature of 24°C were analyzed for comparison. Samples were frozen at -4°C prior to extraction for lipid analysis.

2.2 Cytological analyses

At each sampling interval, aliquots for mitotic index, the percentage of cells actively undergoing mitosis (Wilkerson *et al*., 1988; Jones and Yellowlees, 1997), were preserved in 10% gluteraldehyde seawater solution and immediately counted. The liquid supernatant was discarded and the pellet was resuspended in a solution of filtered sea water with 10% gluteraldehyde and re-centrifuged for zooxanthellae cell counts. Zooxanthellae abundance and mitotic index were determined by direct examination under a phase contrast microscope at 400x and 1000x magnification, and counted using a Neubauer ruling hemocytometer.

2.3 Lipid characterization

Lipids were extracted from the cultures with a modified Bligh and Dyer (1959) procedure using methylene chloride, methanol, and phosphate buffer. Extracts were derivatized with pyridine and bis-(trimethylsilyl)trifluoroacetamide (BSTFA). Compound concentrations were quantified on an Agilent 6850 series gas chromatograph with flame ionization detection (GC-FID) and compound identification was determined with time of flight mass spectrometry (GC-TOF-MS) and comparison to spectra and retention times of known compounds. Ratios of

major compounds were compared to temperature and degree-week stress indices to identify potential heat stress biomarkers. Heat stress in degree-weeks (McClanahan et al., 2007) is defined here for application to lab cultures as the product of duration in weeks and temperature anomaly in degrees above 27°C.

2.4 Saponification

Total lipid extracts were saponified at 70°C for 2 hours in 0.5N potassium hydroxide in methanol to liberate bound fatty acids. Basic and acidic fractions were extracted with hexane (3x each) and recombined prior to derivatization with pyridine and BSTFA. Saponified fatty acids and sterols were then analyzed by GC-FID.

3. RESULTS

3.1 Cellular evidence of stress

After several weeks of exposure to elevated temperature, the zooxanthellae cells appeared stressed (Figure 3.1), as evidenced by visibly decreased pigmentation, abundant cell lysis and fragmentation, and vacuolated cells. The visible evidence of cellular stress is corroborated by the more quantitative mitotic index, which initially increases under moderate thermal stress and then decreases at higher stress in both clades (Figure 3.2). Clade C shows a consistent but not significant elevation in mitotic index at 28°C relative to the 26°C control condition, and these two temperature conditions are treated as the control group in statistical analyses. Clade C, known to be more heat sensitive (Baker, 2003), shows decreased mitotic index

relative to controls after one week of exposure to 32°C ($p < 0.0001$, one-tailed t-test). At 30°C, mitosis in clade C is initially elevated relative to controls at 1 week ($p < 0.0001$, one-tailed t-test) and decreases with each subsequent week of 30°C treatment, becoming significant lower than controls after 4 weeks of exposure ($p < 0.0001$, one-tailed t-test). The strong response of mitotic index to heat stress in clade C is consistent with its expected thermal sensitivity. In more thermally tolerant clade D, mitotic index is elevated in the first two weeks ($P < 0.0001$) and then declines to values lower than control after 3 weeks of exposure to 32°C. At the intermediate heat stress of 30°C, clade D shows an initial mitosis increase in the first two weeks ($p < 0.0001$) and subsequent decrease in mitotic index relative to controls at 3 and 4 weeks of exposure.

3.2 Lipid ratios

The most abundant lipids produced by zooxanthellae are saturated and unsaturated fatty acids. Several sterols each account for a small amount of the lipid analyzed as well. Other compound classes were not characterized due to low abundance. Within the fatty acids and sterols, several ratios stood out for their sensitive response to thermal stress.

3.2.1 Fatty acid unsaturation

The most abundant saturated fatty acids in zooxanthellae tissue are palmitic (C16) and stearic (C18) acids. Unsaturated fatty acids included palmitoleic (C16:1), oleic

(C18:1), linolenic (C18:3) and stearidonic (C18:4) acids, as well as a poly-unsaturated C22 fatty acid. Linoleic (C18:2) acid coelutes with oleic acid, and mass spectrometry showed it to be a minor contributor to the oleic acid peak in gas chromatographic data.

To characterize the relative change in C18 fatty acid unsaturation, we define a C18 unsaturation ratio as the sum of unsaturated C18 fatty acids (C18:4, C18:3, and C18:1 with coelution of C18:2 included) divided by the sum of all saturated and unsaturated C18 fatty acids (C18:4, C18:3, C18:1 with coelution of C18:2, and C18:0).

At low temperatures (26°C and 28°C), both clades show high C18 fatty acid unsaturation ratios (88 ± 3% in clade C and 86 ± 2% in clade D), which does not change over the 4-week experiment. The C18 unsaturation index shows a strong decrease in response to high temperature stress (Figure 3.3). In sensitive clade C, the decrease in C18 unsaturation becomes significant after 1 week of exposure to 32°C ($p < 0.001$, one-tailed t-test) and continues declining to a low value around 30% in weeks 3 and 4. At 30°C, the C18 unsaturation in clade C first declines significantly below control values after 2 weeks of high temperature exposure ($p < 0.0001$, one-tailed t-test), and is statistically indistinguishable from the highest temperature values in weeks 3 and 4. In more tolerant clade D, 32°C causes a decline in C18 unsaturation after one week of exposure ($p < 0.05$, one-tailed t-test), and values decline below 30% in weeks 3 and 4 as with clade C. At the intermediate 30°C thermal treatment, clade D shows no decrease in C18 unsaturation until week

3 ($p < 0.0005$, one-tailed t-test). After 4 weeks exposure to 30°C, C18 unsaturation in clade D drops to a value around 30%, consistent with the highest temperature treatment. Clade D shows a higher threshold for heat stress response compared to clade C, and at the highest thermal stress conditions both clades converge to the same low C18 unsaturation value. The apparent time and temperature thresholds above which a stress response is seen are consistent between the C18 unsaturation ratio and mitotic index.

3.2.2 Fatty acid to sterol ratios

In addition to changes within the unsaturation of fatty acids, we observe a decrease in the concentrations of fatty acids compared to sterols in heat-stressed cultures. This is evident through several different indices of fatty acid to sterol ratios. Figure 3.4 shows the ratio of the sum of fatty acids (C16:0, C16:1, C18:0, C18:1, C18:3, C18:4, and C22 PUFA) to the sum of the three most abundant sterols (cholesterol, dinosterol and 4α,24-dimethyl-5α-cholesta-3β-ol). Fatty acid to sterol ratios show more variability within the control cultures than was observed in C18 unsaturation ratios. Like the mitotic index, the fatty acid to sterol ratios typically increase slightly in response to intermediate levels of heat stress, and then rapidly decrease at the highest heat stress.

Both clades show a somewhat elevated fatty acid to sterol ratio at 28°C relative to 26°C that appears consistent, but these two temperatures will continue to be treated as control conditions in light of the scatter within the datasets. In

response to 32°C, clade C shows a slight but insignificant decrease in fatty acid to sterol ratio after 1 week, and a significant decrease after 2 weeks ($p > 0.001$, one-tailed t-test). At 30°C, the fatty acid to sterol ratio is elevated in the first week ($p < 0.01$, one-tailed t-test), and then declines to significantly lower values after two weeks of exposure ($p < 0.005$, one-tailed t-test). In clade D, there is a slight but barely significant elevation ($p < 0.05$, one-tailed t-test) in fatty acid to sterol ratio after 1 week at 32°C. An initial increase in fatty acid to sterol ratio at one week ($p < 0.05$) and two weeks ($p < 0.005$) at 30°C is followed by a decrease that first becomes significant after 4 weeks of exposure ($p < 0.005$). In the ratio of summed fatty acids to sterols, there again appears to be a threshold type response, where for sensitive clade C, a response is earlier and more drastic at intermediate thermal stress compared to clade D.

The pattern of decrease in fatty acid to sterol ratios at high heat stress can be illustrated by a variety of different individual compound ratios as well. In order to develop an index that can be used in whole coral samples, which will include lipid signatures from coral host tissue, we investigated ratios of compounds specific to dinoflagellate algae. Figure 3.5 shows the ratio between a C22 poly unsaturated fatty acid and 4α,24-dimethyl-5α-cholesta-3β-ol as a function of time and temperature. This ratio of algae-specific compounds, while not as robust to variability between samples as the summed fatty acid to sterol parameter, nonetheless shows a similar pattern of initial increase at moderate temperatures and durations followed by a large decrease in response to heat stress. Clade C shows a significantly lower ratio

after 2 weeks at 30°C ($p < 0.005$) and after two weeks at 32°C also ($p < 0.005$). For clade D, the C22 PUFA to dinoflagellate sterol ratio is higher than controls after 2 weeks at 30°C ($p < 0.001$) and first becomes significantly lower than control values after 4 weeks at 30°C ($p < 0.01$) and after 2 weeks at 32°C ($p < 0.005$). The apparent thresholds are comparable, though not identical, for this ratio compared to the summed fatty acid to sterol ratio.

Other individual saturated and unsaturated acids, when compared to cholesterol, 4α,24-dimethyl-5α-cholesta-3β-ol, or a sum of the most abundant sterols, show a similar pattern. While moderate exposure to high temperature initially causes an increase in the fatty acid to sterol ratio, after 1 to 3 weeks at high temperature the fatty acid to sterol ratio decreases dramatically, with some unsaturated fatty acids falling below the detection limit at the highest thermal stress levels. This drop in fatty acid to sterol ratio occurs more rapidly at the highest temperature, and occurs at a lower temperature or after shorter duration for clade C1 than for clade D1.

When the C18 unsaturation and fatty acid to sterol ratios are examined in relation to degree heating weeks (DHW, defined relative to 27°C), the importance of combining temperature and time into a single heat stress parameter is clear. The C18 unsaturation ratio decreases after a certain threshold of heat stress is exceeded. For sensitive clade C1, all C18 unsaturation values are significantly lower than control at 6 DHW and higher. In the more tolerant clade D1, C18 unsaturation values are lower for all data at or above 9 DHW (Figure 3.6). Initial increase and

subsequent decrease in fatty acid to sterol ratios demonstrate higher apparent thermal threshold values due to greater scatter at low temperatures; the decline in fatty acid to sterol ratios becomes significant above 10 DHW in clade C1 and 12 DHW in clade D1 for both fatty acid to sterol ratios (Figures 3.7 and 3.8).

3.2.3 Saponified (total) fatty acids

To assess whether the heat stress indices measured in free fatty acids are representative of the total lipid pool, we saponified a subset of the lipid extracts. When lipid extracts are saponified to release fatty acids bound in diglycerides, triglycerides, and wax esters, the total C18 fatty acid pool still shows a decrease in unsaturation at high thermal stress (Figure 3.9). The unstressed (low temperature) C18 unsaturation value for the total fatty acids is very slightly lower than observed in the free fatty acids alone (80-85% vs. 86-88% in free fatty acids). In total (saponified) fatty acids, the C18 fatty acid unsaturation shows a significant decrease due to 4 weeks of thermal stress, although the magnitude of the decrease is not as drastic as for free fatty acids alone. From this we conclude that bound fatty acids pools contain relatively more stearic acid (C18:0). Clade C again shows a more severe response to high temperature stress than clade D.

As with the free fatty acids, the ratio of summed fatty acids to cholesterol in saponified samples shows a decline at high temperature (Figure 3.10). Though there is some variability among the low temperature samples, already after 1 week at higher temperature (32°C) fatty acids have declined relative to the control cultures

at 26°C. In contrast to the fatty acid to sterol ratios measured with free fatty acids only, the total fatty acid to cholesterol ratio does not show clear clade-specific differences. Overall, these saponified samples indicate that the lipid stress signatures we observe are a robust feature of the cell's total lipid pool.

4. DISCUSSION

Lipids make up a considerable portion of coral biomass, ranging from 15-37% of dry tissue weight, of which approximately 10% is free fatty acid (Yamashiro et al., 1999; Harland et al., 1992; Harland et al., 1993). Variability of the lipid content in coral tissue may indicate changes in energy storage molecules or membrane compounds, which all contain fatty acid moieties. The most abundant lipids extracted from our *Symbiodinium* clones were the fatty acids. Palmitic acid (C16:0) was the most abundant of these, and unsaturated C16 and C18 fatty acids were also prevalent.

The lipid profiles of our cultured zooxanthellae show that under heat stress, sustained for a period of weeks, the relative amount of fatty acids decreases compared to sterols. Poly-unsaturated fatty acids (PUFAs) account for the majority of the C18 fatty acids, but decline relative to stearic acid (C18:0) in heat-stressed cultures in a temperature, time, and clade-dependent manner. PUFAs may be broken down to poly-unsaturated aldehydes (PUAs; Catalá, 2009), which could help explain the relative decline in PUFAs in thermally stressed *Symbiodinium*. Particularly, reactive oxygen species produced more abundantly in thermally stressed cells (Lesser, 2006) could help catalyze the PUFA to PUA reaction through a

lipid peroxidation intermediate (Catalá, 2009). Other work on these same cultured zooxanthellae (Chapter 2, this thesis) suggests that apoptosis-inducing thylakoid membrane lipids (Andrianasolo et al., 2008) may break down during thermal stress, releasing PUFAs that can further degrade to PUAs and interact with reactive oxygen species to induce apoptosis in thermally stressed cells. A culture study of *Synechocystis* cells genetically unable to produce PUFAs showed that this strain was more sensitive to thermal stress than the PUFA-producing wild type (Gombos et al., 1994). The earlier decline in fatty acid unsaturation observed in our clade C *Symbiodinium* cultures compared to clade D may likewise account for the increased sensitivity of clade C.

In whole coral analyses, total lipid content may decrease in response to thermal stress. Grottoli et al. (2004) observed a decrease in total lipid abundance in bleached *Porites compressa* corals, but no change in *Montipora verrucosa*. Bachok et al. (2006) observed a marked decrease in both total lipid concentration and the relative fatty acid component in bleached *Pavona frondifera* corals compared to healthy nearby specimens. Taken together, these results suggest that the low relative abundance of fatty acids in our heat stressed *Symbiodinium* is a result of decreased total production of lipid, and particularly those rich in fatty acids. Sterols are commonly membrane lipids and may be biologically more indispensable, such that they are still produced at relatively constant concentrations under stressed conditions. Since the bulk of the lipids in cultured zooxanthellae under minimal heat stress are fatty acids, the decline in fatty acid in coral bulk tissue could be a result of

the expulsion of these fatty acid-rich symbionts during bleaching, in addition to the proportional decline of fatty acids within any remaining zooxanthellae.

The sharp decrease in unsaturated fatty acids relative to their saturated analogs is striking, but less easily understood in the context of coral field samples. Extracts of whole-coral tissue from a suite of reef-building corals in Japan show that stearic acid is generally in greater abundance than the C18 unsaturated acids, except in a few samples, notably *Porites lutea* (Yamashiro et al., 1999). If the dominance of unsaturated C18 acids we observed in the zooxanthellae clones is characteristic of all zooxanthellae, this could indicate that the *P. lutea* coral specimen examined in that study received a greater portion of its fatty acids from algal symbionts, rather than synthesizing the acids *de novo*. Alternatively, the low proportion of unsaturated C18 fatty acids in Japanese corals other than *P. lutea* could indicate that zooxanthellae in those corals were experiencing thermal stress that was nonetheless below the threshold for visible bleaching. Comparison between fatty acid profiles from coral host *Montipora digitata* and its symbionts has shown that zooxanthellae produce more poly-unsaturated fatty acids, particularly C18:3(n-3) (Papina et al., 2003). However, in another study, zooxanthellae isolated from 5 different coral species showed almost no contribution of C18:3, but rather much higher C18:4 (Zhukova and Titlyanov, 2003). Papina and coworkers (2003) examined saponified (total) fatty acids, whereas Zhukova and Titlyanov (2003) analyzed free fatty acids, which may account for some of the differences observed. Although our study focused on free fatty acids, we found a result more strongly in

agreement with Papina et al. (2003). Both studies found high amounts of PUFAs in coral zooxanthellae from the field, which is consistent with our high C18 unsaturation index in unstressed cultures.

It has been suggested that high amounts of C18:3 may be indicative of rapid growth (Piorreck and Pohl, 1984; Napolitano et al., 1997), as decreasing nutrient availability may cause a shift from predominantly polar lipids to neutral lipids (and thus a change in associated fatty acids). While we cannot rule out changes in fatty acid composition related to nutrient effects, our clade-specific heat stress thresholds more strongly support a thermal stress interpretation. Additionally, the decrease in fatty acids relative to sterols we observe is inconsistent with a build-up over time of neutral lipids, primarily triacylglycerols, which are rich in fatty acids.

Certain ω-3 and ω-6 fatty acids are not produced by animals (Dewick, 1997), and the 18:4(ω-3) acid is considered characteristic of dinoflagellates (Zhukova and Aizdacher, 1995). Some of these polyunsaturated fatty acids, initially produced by the algal symbionts, are then incorporated into coral tissue (Papina et al., 2003). In order to detect zooxanthellae heat stress from whole coral tissue extracts, the ideal marker would be both abundant and specific to zooxanthellae. While corals may produce saturated fatty acids, thus affecting the C18 unsaturation ratio, the C22 polyunsaturated fatty acid and 4α,24-dimethyl-5α-cholesta-3β-ol are particular to dinoflagellates (Volkman et al., 1998). The ratio of the C22 PUFA to 4α,24-dimethyl-5α-cholesta-3β-ol makes a particularly good marker of zooxanthellae stress, which would not suffer from interference from coral tissue in the more complicated host-

symbiont system. The absence of C22 PUFA in the presence of measurable 4α,24-dimethyl-5α-cholesta-3β-ol indicates heat stress in excess of 4-6 degree heating weeks, above which some bleaching of the coral would be expected.

5. CONCLUSIONS AND FUTURE WORK

We have demonstrated that lipids extracted from cultured *Symbiodinium* clones show a high proportion of C16 and C18 fatty acids, but the saturated C18 acid is generally less abundant than the unsaturated C18 acids. As heat stress increases, the relative amounts of unsaturated C18 fatty acids decrease and overall fatty acid to sterol ratio decrease. This stress response occurs in both clades C1 and D1, but clade C1 responds earlier or at lower temperature, in agreement with the higher thermal tolerance reported for clade D (Baker, 2003).

Additionally, from this work we anticipate that these stress signals from the zooxanthellae should be measurable in stressed corals, and careful culturing of corals under stressful conditions will help test these biomarkers for eventual field use. There is some indication that fatty acids and sterols can be preserved within coral skeleton (Ingalls et al., 2003). A historical record of coral stress reconstructed from skeletal lipids would allow a better characterization of the anthropogenic impact on coral health.

Further investigation of intact polar lipids will help to identify the cellular source of these stress signals within specific lipid classes (e.g. different phospholipids and glycolipids). In depth analysis of lipids from separated coral

animal tissue and *Symbiodinium* growing *in hospite* will help determine whether lipid profiles produced by the symbiotic system as a whole can be used as markers of heat stress.

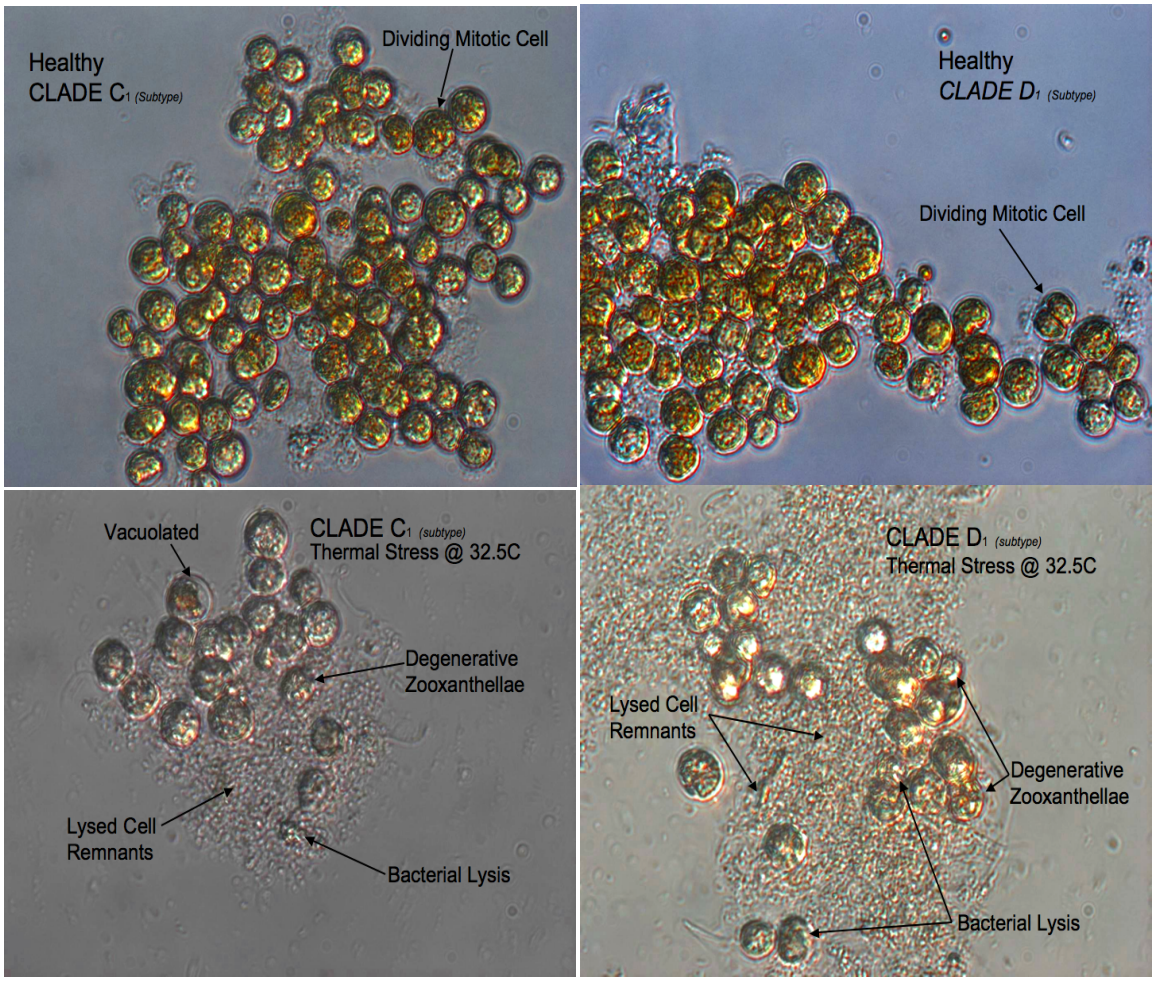

Figure 3.1. Microscope images of healthy and thermally stressed *Symbiodinium* cells. There is a decrease in pigmentation, degenerative cell morphology, and abundant cell lysis in heat stressed cells (32.5°C for 1 week) relative to lower temperature controls (26°C).

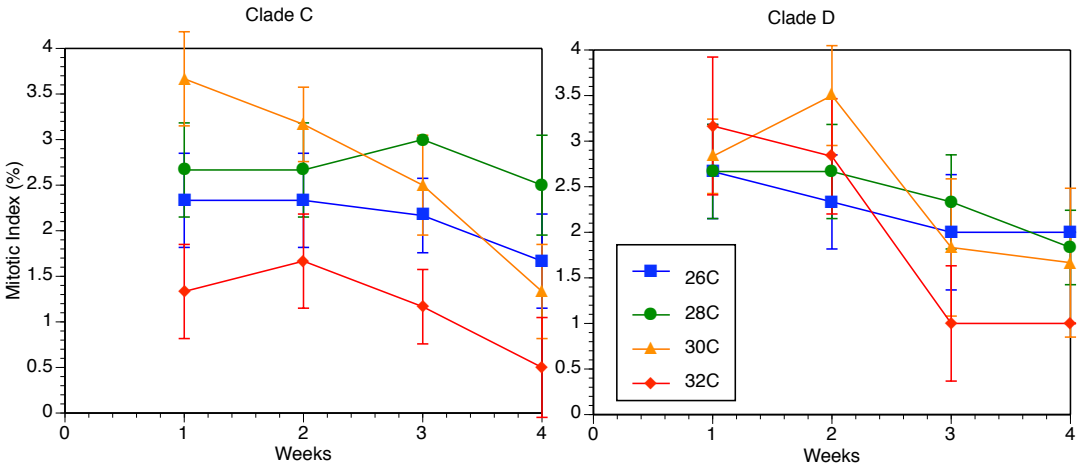

Figure 3.2. Mitotic index of cultured *Symbiodinium* cells.

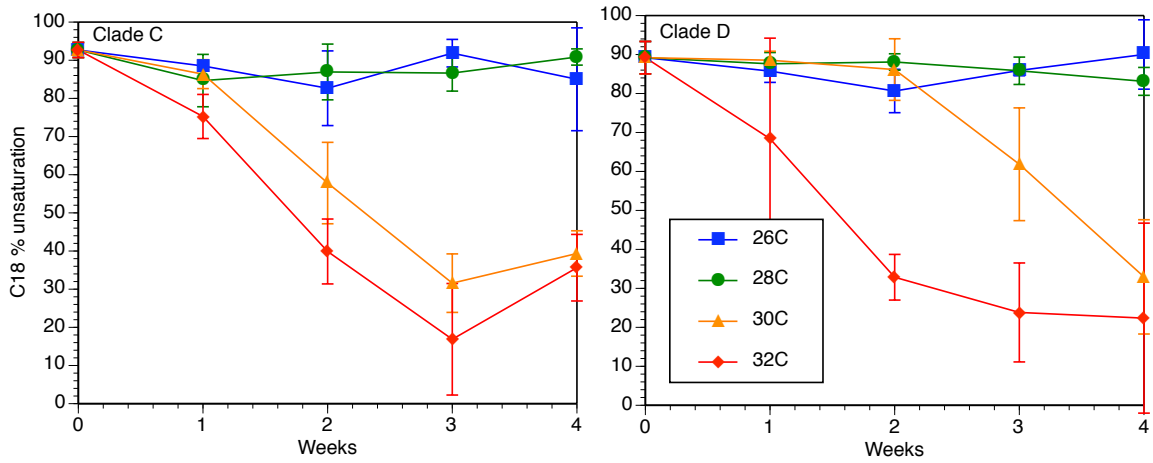

Figure 3.3. Unsaturation ratio of C18 free fatty acids in thermally stressed *Symbiodinium*. The C18 % unsaturation is defined as the ratio of the summed C18 fatty acids (C18:1, C18:2, C18:3, and C18:4) over the sum of all C18 fatty acids.

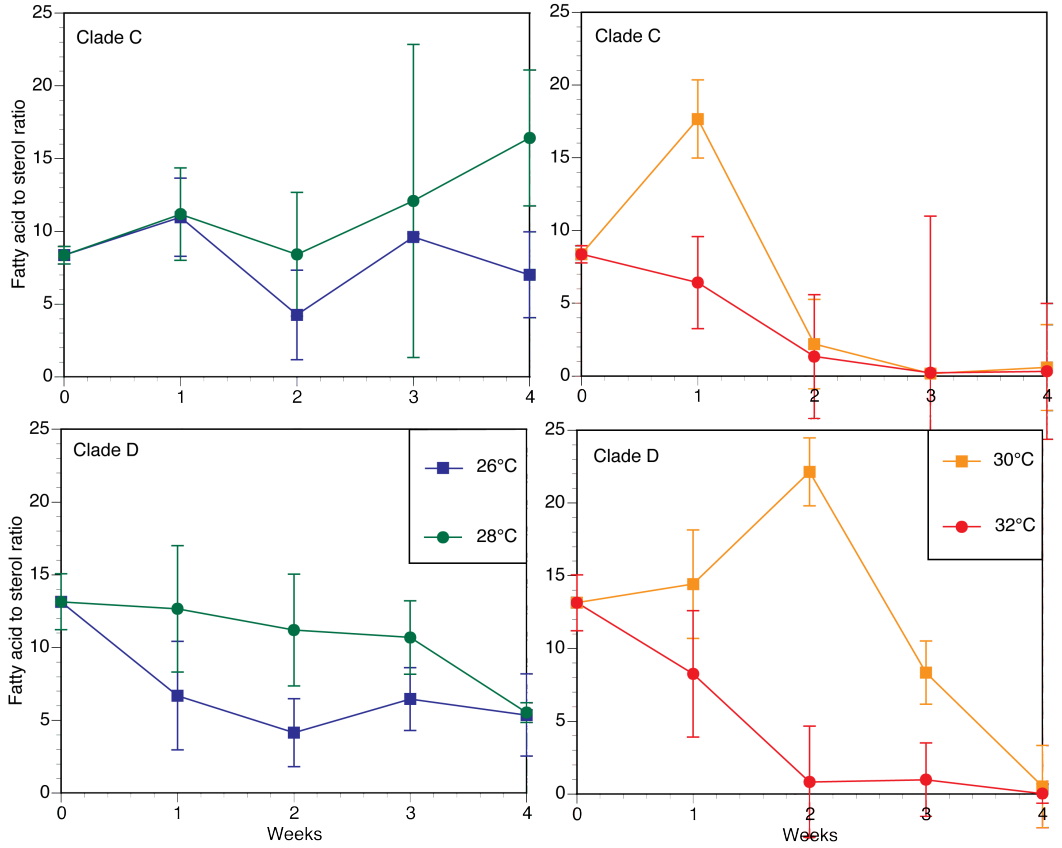

Figure 3.4. Ratio of fatty acids to sterols in thermally stressed *Symbiodinium*. The fatty acid to sterol ratio is calculated as the sum of fatty acids (C16:0, C16:1, C18:0, C18:1, C18:3, C18:4, and C22 PUFA) to the sum of sterols (cholesterol, dinosterol and 4α,24-dimethyl-5α-cholesta-3β-ol) in unstressed (left-hand panels) and thermally stressed (right-hand panels) *Symbiodinium.*

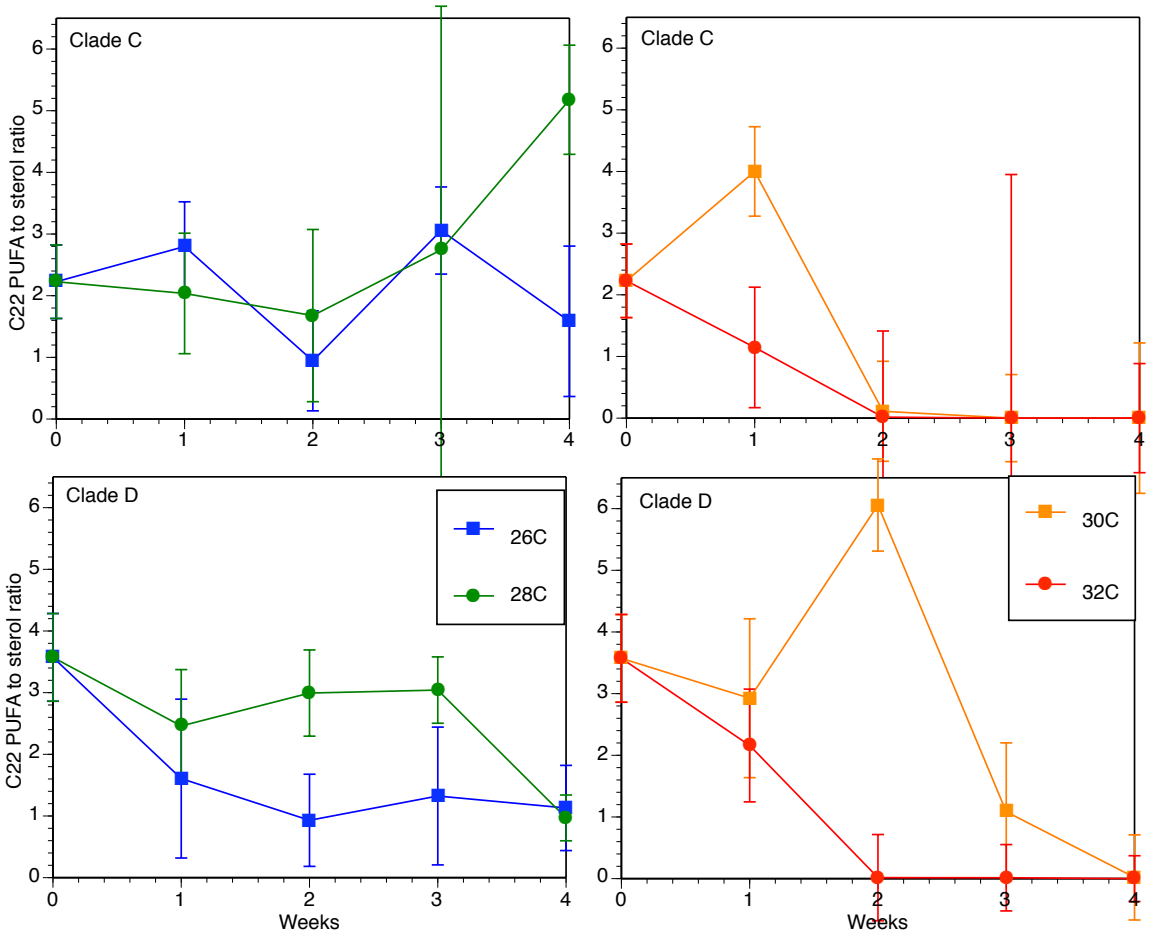

Figure 3.5. Ratios of C22 polyunsaturated fatty acid to 4α,24-dimethyl-5α-cholesta-3β-ol in thermally stressed *Symbiodinium*. Unstressed (left-hand panels) and thermally stressed (right-hand panels) values are plotted separately for clarity..

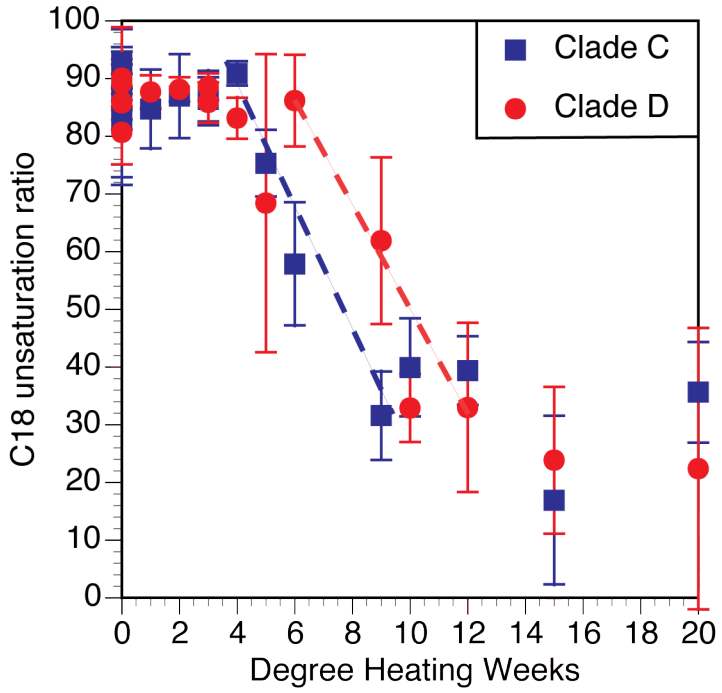

Figure 3.6. C18 fatty acid unsaturation ratio as a function of degree heating weeks. For the purposes of this culture study, degree heating weeks are defined as the product of temperature above 27°C and the duration in weeks.

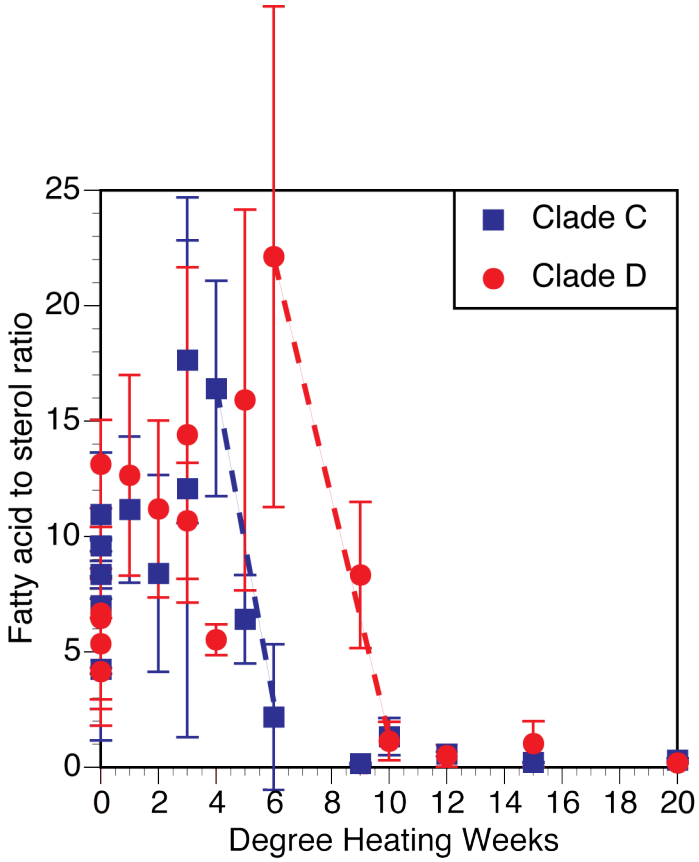

Figure 3.7. Fatty acid to sterol ratio as a function of degree heating weeks. For the purposes of this culture study, degree heating weeks are defined as the product of temperature above 27°C and the duration in weeks.

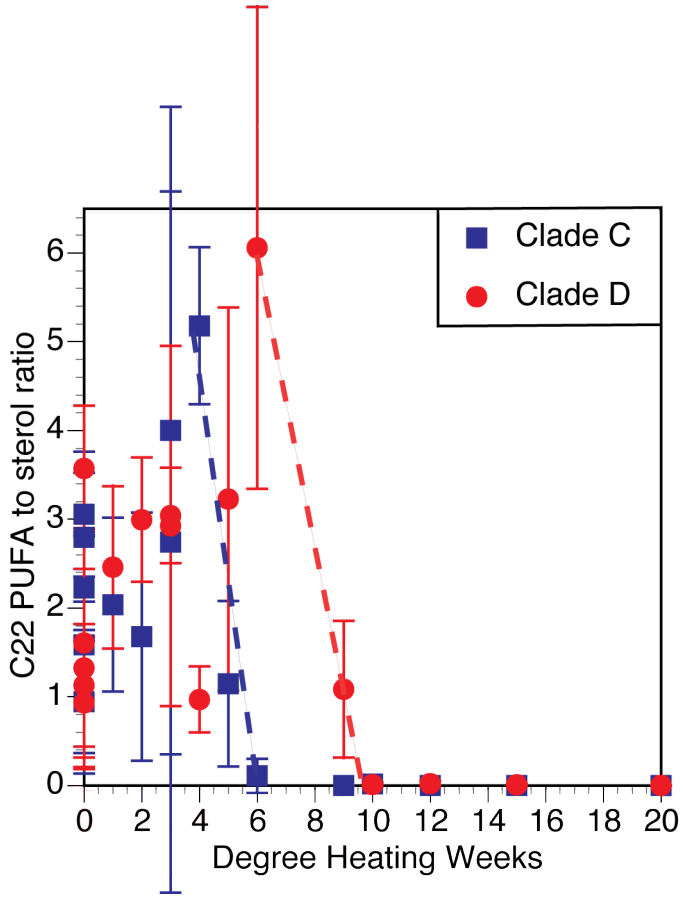

Figure 3.8. C22 PUFA to sterol ratio as a function of degree heating weeks. For the purposes of this culture study, degree heating weeks are defined as the product of temperature above 27°C and the duration in weeks.

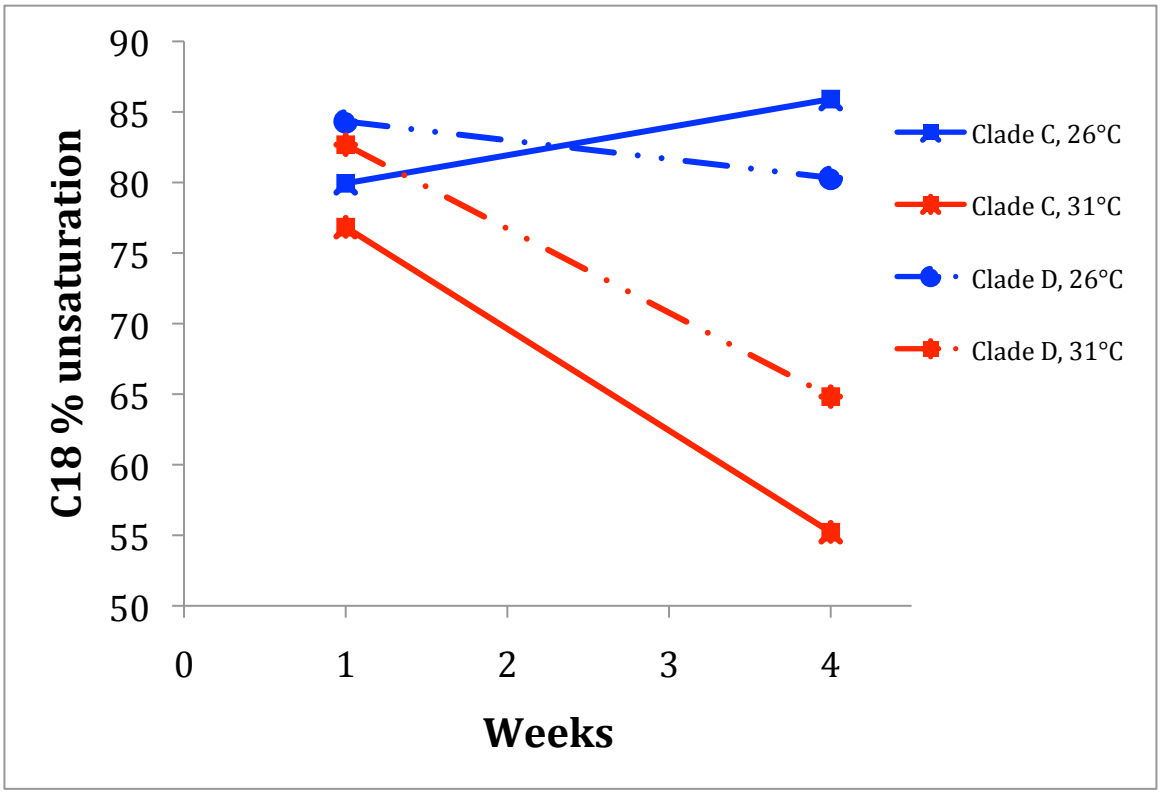

Figure 3.9. C18 % unsaturation in saponified (total) fatty acids.When lipid extracts are saponified to release fatty acids bound in diglycerides, triglycerides, and wax esters, the total C18 fatty acid pool still shows a decrease in unsaturation at high thermal stress, here 31°C for 4 weeks.

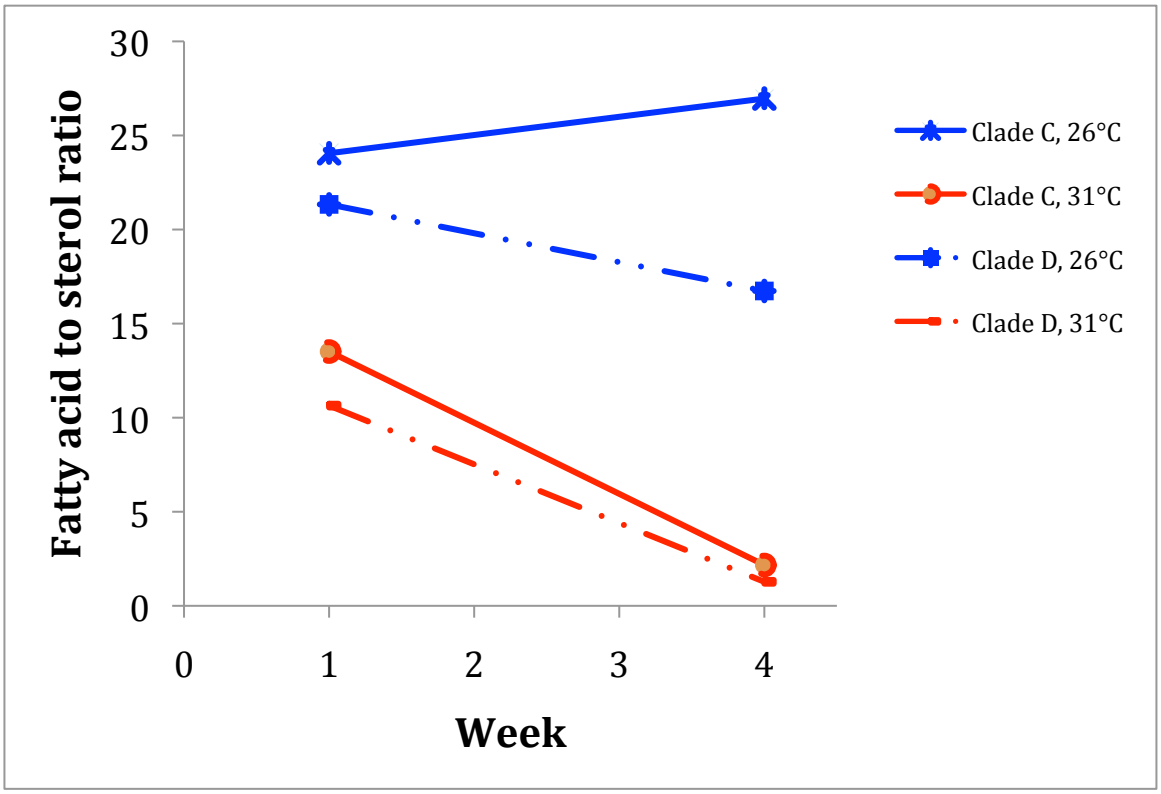

Figure 3.10. Fatty acid to sterol ratio from saponified (total) fatty acids. The ratio of summed fatty acids to cholesterol in saponified samples shows a decline in fatty acids at high temperature.

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CHAPTER 4:

LIPID BIOMARKERS OF DISEASE STRESS IN CORALS AND THEIR ZOOXANTHELLAE

ABSTRACT

Corals worldwide are increasingly threatened by a variety of stressors, including the increasing prevalence of coral disease. To better understand the biochemical effects of disease on corals and their algal symbionts (zooxanthellae), we investigated the impact of bacterial infection on several lipid biomarkers previously shown to respond to thermal stress in zooxanthellae. We examined a hierarchy of biological samples: infected zooxanthellae, coral fragments infected in controlled aquarium experiments, and diseased coral samples collected from the field. We find that lipid biomarkers of coral stress can be detected in infected corals and zooxanthellae, confirming the potential to use lipid biomarkers to assess coral health and better understand the cellular mechanisms of coral disease.

1. INTRODUCTION

Corals are threatened by many factors, including rising ocean temperatures, increasing ocean acidification, anthropogenic pollution, overfishing, and spreading coral diseases. Coral disease is a particular concern because disease prevalence is increasing. Infection rates and pathogen virulence may both be exacerbated by increasing ocean temperatures. Only recently have large-scale attempts been made to monitor the occurrence of coral disease. The association between several species of *Vibrio* bacteria and diseased corals is well established, such as association between *Vibrio harveyi* and diseased *Pocillopora damicornis* (Luna et al., 2007). The etiology of many coral diseases is still poorly understood, as disease may affect the coral host, its *Symbiodinium spp*. endosymbionts (zooxanthellae), or both.

Yellow band/blotch disease (YBD) is known to be a particular threat to corals in the Caribbean, as well as in the Indo-Pacific (Cervino et al, 2008). In the

Caribbean, it affects a variety of coral species (Santavy and Peters, 1997; Garzón-Ferreira et al., 2001), most notably the *Montastraea* species complex. YBD may be exacerbated by high temperatures; higher bleaching prevalence was correlated with an increase in disease incidence after a major thermal bleaching event in the Caribbean (Cróquer and Weil, 2009). *Montastraea faveolata* tissues with visible YBD lesions contain a microbial community with a notably different functional potential from the microbial community in healthy coral tissue (Kimes et al., 2010). A consortium of 4 strains of *Vibrio* spp. bacteria has been shown to cause YBD in *Montastraea spp*. corals (Cervino et al., 2004). It is thought that the disease primarily impacts the *Symbiodinium* spp. photosymbionts harbored by zooxanthellate corals. Disease or thermal bleaching stress may cause the endosymbiont population to shift towards more tolerant or resilient *Symbiodinium* clades (Toller et al., 2001). The specific biochemical effects of *Vibrio* infection on *Symbiodinium* remains poorly characterized, however.

In this study, we infected several strains of cultured Symbiodinium with either the 4 *Vibrio* strains associated with YBD or with a single Vibrio strain, with and without additional thermal stress. We measured the effect of Vibrio infection on established lipid biomarkers of coral stress (this thesis, Chapter 3). We additionally collected field samples of *M. faveolata* and *Fungia spp.* with apparent YBD lesions to test whether our stress biomarkers were applicable to diseased corals in the field. We present here the first applicability test of lipid biomarkers of coral stress.

2. METHODS

2.1 Cultured zooxanthellae

Symbiodinium dinoflagellates of strains C1 (S. goreaui strain CCMP 2466) were cultured in f/2 media under a 12/12 hour light/dark regime. Cultures were maintained at 26˚C, 28˚C, 30˚C, and 32˚C, and subsamples were collected at 8 hours, 24 hours, 96 hours, 1 week, and 2 weeks, 3 weeks, and 4 weeks. Samples were immediately frozen and maintained at -20˚C prior to further analysis.

Under each temperature condition, a full set of cultures was subjected to each of two different disease treatments. The first disease treatment was inoculated with 4 strains of *Vibrio* bacteria that had been previously shown to induce yellow band disease in Montastraea spp. corals (Cervino et al., 2004), identified as *Vibrio rotiferianus*, *Vibrio harveyi*, *Vibrio alginolyticus*, and *Vibrio proteolyticus*. The second disease treatment was inoculated with a single strain of *Vibrio alginolyticus*. Cultures were inoculated at time t=0 and were maintained at 26˚C, 28˚C and 33˚C. Subsamples were collected at 8 hours, 24 hours, 96 hours, 1 week, and 2 weeks. Samples were immediately frozen and stored at -20˚C prior to further analysis.

2.2 Diseased *Montastraea faveolata* and *Fungia spp.*

2.2.1 *M. faveolata* aquarium experiments

During April 2009, fragments of approximately 4cm x 6cm each were broken off from a single colony of *M. faveolata* available at Mote Tropical Research Lab, Summerland Key, FL and dispersed to 6 different covered 5-gallon aquaria in a common raceway. 2 tanks each were heated to temperatures of approximately 29˚C and 33˚C (measured 29.6±1.0˚C and 31.8±0.7˚C respectively), while the remaining 2 tanks were allowed to stay at the ambient temperature of approximately 26˚C (measured 26.6±0.8˚C). One tank of each temperature was designated a "diseased" tank. Fragments in the diseased tanks were inoculated by immersing each fragment in a prepared bacterial slurry containing the 4 *Vibrio* strains in the YBD core group prior to returning it to the aquarium. Approximately 10% of the water in each tank was exchanged daily, with sufficient freshwater addition to correct for evaporation effects and maintain a salinity of approximately 37. One fragment was sacrificed from each tank for sampling at t=0, 22 hours, 46 hours, 70 hours, 96 hours, 120 hours, 1 week, 2 weeks, 3 weeks, and 4 weeks of treatment. Immediately after sampling, tissue was separated from each fragment using a WaterPik filled with saline solution of salinity 15. Tissue slurries were centrifuged at 3000 rpm and 10˚C for approximately 30 minutes to separate and concentrate the zooxanthellae and

coral host tissue fractions. Mitotic index was measured in a subsample of zooxanthellae using a Neubauer ruling hemocytometer. For the culturing experiment, three vials of each experimental condition were counted for mitotic index, which was determined by the average of six separate counts from each culture vial. The remaining tissue fractions were then carefully pipetted into cryogenic storage vials and frozen in liquid nitrogen (LN_2) prior to transport in an LN_2 dry shipper. Following shipment, samples were stored at - 80˚C until further analysis.

2.2.2 *M. faveolata* field sample collection

Colonies of *Montastraea faveolata* displaying lesions of yellow band disease (YBD) were visually identified near Looe Key, FL. 3 separate lesioned colonies and 3 nearby healthy colonies were sampled with a hole saw. From each lesioned colony, 2 samples were collected near the edges of the lesion and one visibly healthy sample was taken away from the lesion. All samples were collected by SCUBA in less than 5 meters water depth, and samples were preserved in liquid nitrogen at the conclusion of the dive. After transport in an LN_2 dry shipper, samples remained frozen at -80˚C until further processing. Genetic identifications of zooxanthellae clade were determined by cloning and sequencing a portion of the chloroplast 23S gene. For each sample, 16 clones were sequenced.

2.2.3 *Fungia spp.* field sample collection

Individual mushroom corals in the *Fungia* genus were collected in June 2009 from the southern Red Sea off the coast of Saudi Arabia. At each of five off-shore reef sites, individuals were identified as either healthy (normal pigmentation) or diseased (pale, yellow spots indicative of Yellow Band Disease). Samples were collected by SCUBA in approximately 10 meters water depth, and preserved in liquid nitrogen at the conclusion of the dive. After transport in an LN_2 dry shipper, samples remained frozen at -80˚C until further processing.

2.2.3 *M. faveolata* and *Fungia spp*. tissue separation and zooxanthellae isolation

An airbrush was used to separate tissue from the coral skeleton. For the diseased *M. faveolata* samples, care was taken to sample exclusively lesioned tissue. Diseased *Fungia* samples were too spatially heterogeneous to allow exclusive sampling of the yellow disease spots. Samples were kept on ice during processing to keep them as cool as possible. After airbrushing, blastate was centrifuged at 1300g and 10˚C for approximately 20 minutes to pre-concentrate coral and zooxanthellae tissue. The concentrated blastate was then suspended in a solution of sorbitol Percoll saline (SPS) and ultracentrifuged for 30 minutes at 70,000x and 10˚C (Tytler and Spencer Davies, 1983). A thin layer containing zooxanthellae was carefully pipetted into a new vial and again centrifuged if necessary at 1300g and 10˚C for 10-20 minutes to further concentrate the zooxanthellae cells.

2.3 Lipid extraction and analysis

2.3.1 Bligh and Dyer extraction

Cultured or isolated zooxanthellae were extracted using a modified Bligh and Dyer procedure (Bligh and Dyer, 1959). Briefly, the concentrated whole cells were dissolved in methanol, phosphate buffered saline, and dichloromethane. After resting in a -20˚C freezer for approximately 10 minutes, the aqueous and organic phases separated, and the organic phase was carefully pipetted into a separate vial. The extract was then concentrated under a gentle nitrogen stream and split into two fractions for analysis of free fatty acids and sterols in the first fraction and intact polar lipids in the second fraction.

2.3.2 Free fatty acid and sterol analysis

Total lipid extracts (TLE) were run over a sodium sulfate column to remove any water from the extract. They were then concentrated under nitrogen and derivatized with bis-trimethylsilyltrifluoroacetamide (BSTFA) and pyridine in

dichloromethane. Fatty acids and sterols were quantified by Agilent gas chromatograph with flame ionization detection (GC-FID). Quantification was achieved by comparison with external standard curves.

2.3.3 Intact Polar Lipid (IPL) Analysis

TLE fractions for IPL measurements were augmented with a phosphatidylethanolamine (DNP-PE) standard. IPL concentrations were measured by HPLC-ESI-MS (reversed phase high performance liquid chromatography connected to a triple stage quadrupole mass spectrometer with electrospray ionization). External standard curves were used for quantification. Samples were capped with argon and stored at -80˚C prior to analysis.

3. RESULTS

3.1 Cultured and inoculated *Symbiodinium*

3.1.1 Cellular response to disease stress

Thermal and disease stress cause visible changes in the cultured *Symbiodinium* cells (Figure 4.1). Mitotic index (MI, the percentage of cells dividing) is an indicator of cell cycle responses to stress, and is expected to initially increase in response to moderate temperature increase, and then decrease in thermally stressed cells (Suharsono and Brown, 1992). In our cultures, MI is impacted by both thermal and disease stress (Figure 4.2). Increased temperature alone causes an initial increase in mitosis after 24 hours of exposure ($p < 0.0001$, one-tailed t-test) and then begins to decline at high temperature (33˚C). The mitotic index at high temperature first becomes significantly lower than control after 72 hours of exposure ($p < 0.0001$, one-tailed t-test). The addition of YBD *Vibrios* causes an additional decrease in mitotic index relative to high temperature alone, which becomes significant after 48 hours in the low temperature treatment ($p < 0.0001$, one-tailed t-test) and after only 24 hours in the high temperature treatment ($p < 0.0001$). The largest effect is seen in cultures inoculated with only *V. alginolyticus*, which causes a large suppression of

mitosis relative to control conditions, even after only 24 hours at low temperature ($p < 0.0001$, one-tailed t-test). The addition of thermal stress to cultures inoculated with *V. alginolyticus* causes a small additional decrease in mitosis, which becomes significantly different from *V. alginolyticus* exposure alone after 48 hours of treatment ($p < 0.0001$).

3.1.2 C18 fatty acid unsaturation

The amount of unsaturation within C18 free fatty acids has been established as a sensitive indicator of heat stress, declining from ~90% unsaturation in unstressed cells to around 20-30% unsaturation in thermally stressed cells (Chapter 3, this thesis). Inoculation with *Vibrio* bacteria also causes a decrease in C18 fatty acid unsaturation, but on a somewhat different timescale (Figure 4.3). As a direct result of bacterial inoculation, the introduction of bacterial biomass to the cultures initially decreases the C18 unsaturation, because bacterial cells produce mainly saturated fatty acids (Urdaci et al., 1990). For the purpose of statistical tests, the initial measurements at 8 hours in all three temperatures of the disease-treated cultures are considered the control group. Treatment with the 4 strains of YBD *Vibrio* causes no clear trend in C18 unsaturation over the 2-week course of our experiment at 26˚C, though the values were significantly lower at 1 day, 4 days, and 2 weeks, and higher at 1 week ($p < 0.0001$, one-tailed t-test). At the slightly elevated temperature of 28˚C , again no clear trend is observed, though differences from control are significant ($p < 0.0001$, one-tailed t-test). Even at the highest temperature of 33˚C, the decrease of in C18 unsaturation relative control is statistically significant ($p < 0.0001$) but small, with no trend over exposure time. In general, no C18 unsaturation response is seen in YBD-inoculated cultures even when combined with thermal stress (Figure 4.3, upper panel)).

In contrast, inoculation with *V. alginolyticus* causes a strong decrease in C18 fatty acid unsaturation (Figure 4.3, lower panel). The C18 unsaturation decrease occurs on a much shorter timescale than thermal stress alone. As soon as 4 days

after *V. alginolyticus* exposure, the C18 unsaturation has declined below unstressed values at all temperatures ($p < 0.0001$, one-tailed t-test), and continues to decline over the 2-week course of the experiment. The decrease in C18 unsaturation is comparable at all 3 experimental temperatures (26˚C, 28˚C and 33˚C); no additional effect of combined thermal and disease stress is observed except perhaps after a mere 24 hours of exposure, where 32˚C causes an early decrease in C18 unsaturation relative to the 8 hour control conditions ($p < 0.0001$, one-tailed t-test).

3.1.3 Fatty acid to sterol ratios

Free fatty acid acid to sterol ratios in zooxanthellae have been shown to initially increase, and then subsequently decrease in response to thermal stress (Chapter 3, this thesis). To test the effect of disease stress on fatty acid abundance, we calculated the ratio of C16 and C18 free fatty acids to cholesterol, a common and abundant sterol in zooxanthellae cells. In zooxanthellae cultures inoculated with Vibrio bacteria, we observed a relative decrease in fatty acids (Figure 4.4). The addition of bacterial biomass initially affects the fatty acid to sterol ratio by increasing the amount fatty acids, since bacterial cells contribute fatty acids, but do not produce cholesterol. To correct for this effect, we calculated the fatty acid to cholesterol ratio, normalized to the initial, unstressed value. As with the C18 unsaturation data, the 8-hour inoculation cultures at all temperature are used as the control condition for statistical tests.

Both thermal stress and disease stress cause a relative decrease in free fatty acids. Inoculation with the 4 YBD Vibrio strains causes fatty acids to decline. The fatty acid to sterol ratios in the low temperature treatments (26˚C and 28˚C) become less than control values after 4 days of exposure ($p < 0.0001$, one-tailed t-test). In the highest temperature treatment (33˚C), the fatty acid to sterol initially decreases after one day of exposure ($p < 0.0001$) but then climbs above control values at 4 days and 1 week of thermal stress ($p < 0.0001$). *V. alginolyticus* infection causes a more monotonic decrease in fatty acids. The decline in fatty acid content of

zooxanthellae infected with *V. alginolyticus* occurs after shorter exposure than for thermal stress alone. The fatty acid decline becomes significant 4 days after inoculation at 28˚C and after only 24 hours at 26˚C and 33˚C. The decline and continues through the 2-week experiment at all temperatures, with the exception of the 26˚C, 2 week sample, which may be a spurious data point. The rate of decline in the fatty acid to sterol ratio is not sensitive to temperature. The timescale of lipid changes and the difference between the two disease treatments are consistent between both biomarkers of stress.

3.2 Inoculated *Montastraea faveolata* fragments

3.2.1. Mitotic index

Zooxanthellae isolated from the stony coral *M. faveolata* show an initial increases and subsequent decrease in mitotic index (MI) in response to thermal stress (Figure 4.5). Zooxanthellae from fragments kept at the ambient temperature of 26˚C show a constant MI of approximately 3% over the 5-day period. A moderate temperature elevation to 29˚C causes an initial increase in MI over a period of 3 days to a maximum value around 4.5%, which then decreases again to the 3% after 5 days. Temperatures of 31˚C and 33˚C, high enough to cause thermal stress in zooxanthellae cultured in isolation (Chapter 3, this thesis), cause MI in the *in hospite Symbiodinium* to increase to around 5% after 1 day of exposure. The MI in these thermally stressed zooxanthellae then decreases to values below control conditions by the fifth day of the experiment.

When fragments of this same coral are inoculated with 4 strains of *Vibrio* bacteria from the Yellow Band Disease core group (Cervino et al., 2008), the zooxanthellae MI is drastically impacted. In the lower temperature treatments (26˚C and 29˚C), MI slowly decreases over the 5-day experiment, with final values around 1% or below after 5 days. The effect of combined thermal and disease stress on MI at 31˚C and 33˚C is clear. In the inoculated and thermally stressed zooxanthellae, MI has decreased to below 1% after only 1 day of exposure. No dividing zooxanthellae

cells are observed after 4 days exposure to thermal and disease stress in the highest temperature (33˚C) treatment.

3.2.2 Fatty acids and sterols

Fatty acid and sterol abundances were too low in the *M. faveolata* fragments to be characterized.

3.2.3 Intact polar lipids

Molecules of mologalactosyldiacylglycerol (MGDG) rich in poly-unsaturated fatty acids (PUFAs) have been shown to induce apoptosis in mammalian cells (Andrianasolo et al., 2008) and decrease markedly in thermally stressed zooxanthellae (Chapter 2, this thesis). In zooxanthellae isolated from *M. faveolata* fragments, we again were able to observe the presence MGDG compounds containing C18:4/C18:5 and C18:4/C18:4 fatty acids. These compounds make up between 8-17% of the total MGDG in zooxanthellae from corals maintained at 26˚C (no thermal stress). This is considerably less than the 60-80% of PUFA-rich MGDG found in unstressed cultures of 4 different phylotypes of *Symbiodinium* (Chapter 2, this thesis). In the low-temperature treatments, inoculation with 4 strains of YBD *Vibrio* has no detectable effect on the amount of PUFA-rich MGDG in zooxanthellae (Figure 4.6).

In thermally stressed *M. faveolata* fragments, a different response is seen. After 2 weeks of high temperature exposure, no PUFA-rich MGDG compounds can be detected in zooxanthellae from either untreated or inoculated fragments. However, the relative amount of PUFA-rich MGDG appears to recover to control conditions after 4 weeks of high temperature exposure, regardless of disease exposure.

The amount of MGDG in the total thylakoid lipids (MGDG, DGDG, PG, and SQDG) ranges from 3-12%. The %MGDG in the thylakoid roughly triples from 4% to 12% between 2 and 4 weeks in the low temperature control condition. However, the

thylakoid MGDG content does not change from 2 to 4 weeks in response to thermal stress, disease stress, or combined thermal stress and disease exposure. Relative to the control condition, then, thylakoid MGDG content decreases in response to thermal and disease stress. Disease stress has a slightly larger effect than thermal stress, and the combined effect of disease and thermal stress on thylakoid MGDG content is slightly larger than thermal or disease stress alone. The relative amounts of MGDG in the zooxanthellae thylakoids are comparable in magnitude to those observed in zooxanthellae cultured outside of the coral host (Chapter 2, this thesis).

3.3 Field samples of *Montastraea faveolata* with Yellow Band Disease from Looe Key

3.3.1 Visual appearance of *M. faveolata* colonies

Colonies of *Montastraea faveolata* identified as affected by Yellow Band Disease (YBD) show yellowish lesions that form a ring around dead tissue and exposed skeleton (Figure 4.8). The yellow lesions themselves are typically a few centimeters wide. The entire affected area in the 3 diseased colonies sampled for this study had diameters of at least 10s of centimeters. Tissue away from the lesion did not appear abnormal.

3.3.2 Zooxanthellae C18 fatty acid unsaturation

We compared C18 free fatty acid unsaturation ratios between zooxanthellae isolated from healthy and diseased portions of 3 different diseased *M. faveolata* colonies, as well as 4 nearby healthy colonies that served as controls. C18 unsaturation is lower in zooxanthellae isolated from lesioned tissue than in healthy tissue on the same colony. There is no statistical difference between C18 unsaturation from healthy tissue on diseased colonies and nearby healthy colonies. Though not as stark as for healthy and diseased zooxanthellae in culture, lipid differences between healthy and diseased zooxanthellae from *M. faveolata* are still detectable.

3.3.3 Zooxanthellae fatty acid to sterol ratios

Fatty acid to cholesterol ratios from zooxanthellae from *M. faveolata* did not respond as strongly to disease stress. Given the variability between individual samples, two out of three diseased corals showed lower fatty acid to sterol ratios in the diseased tissue than in the healthy portion of the same colony. However, the average fatty acid to sterol ratio in the nearby healthy colonies is consistent with the values from the lesioned tissue. Thus fatty acid to cholesterol ratios cannot be used in these samples to discriminate between healthy and diseased tissue.

3.3.4 Differences in zooxanthellae clade among *M. faveolata* colonies

At the clade level, we detect differences in the zooxanthellae population between healthy and diseased *M. faveolata* tissue. Clade B is abundant in all samples, composing over 60% on average in diseased samples (n=6), healthy tissue from diseased colonies (n=3) and healthy colonies (n=4). Clade B made up 12.5 - 100% of zooxanthellae DNA from individual samples. The contribution of clade C varies considerably, from 0 – 88% in individual healthy samples. No diseased samples contained detectable contributions of DNA from clade C. Clade A was only observed in diseased samples, and contributed 0 – 63% of the DNA in any particular sample. The average contribution from clade A in all diseased samples was 35%. Given the variability between individual samples, it is not possible to distinguish between healthy tissue on a diseased colony and a healthy colony on the basis of zooxanthellae ecology. Diseased samples, on the other hand, could be distinguished from healthy samples by the presence of clade A, with the exception of one sample out of six.

3.4 Field samples of *Fungia spp*. from the Saudi Arabian Red Sea

3.4.1 C18 unsaturation ratio in diseased Red Sea *Fungia spp*.

In general, values of the C18 unsaturation ratio in zooxanthellae isolated from both healthy and diseased *Fungia* are somewhat lower than observed in zooxanthellae

cultured alone. This is consistent with a greater contribution from bacterial or coral animal tissue, which would tend to contribute primarily saturated C18 fatty acids. Comparison between zooxanthellae from healthy and diseased samples from the same sites (Figure 4.12) shows a small increase in diseased C18 fatty acid unsaturation compared to healthy samples (55% vs. 57% unsaturated, $p < 0.0001$, two-tailed t-test). The data is strongly influenced by the complete absence of saturated C18:0 fatty acid in the single diseased sample from site 4. Excluding site 4 from the analysis results in a lower average C18 unsaturation value in diseased samples compared to healthy controls (47% vs. 57% unsaturated, $p < 0.0001$, two-tailed t-test). This result agrees with expectations of a lower unsaturation ratio in diseased zooxanthellae cultures (Figure 4.3) and in thermally stressed zooxanthellae (Chapter 3, this thesis).

3.4.2 Fatty acid to sterol ratio in diseased Red Sea *Fungia spp*.

The fatty acid to cholesterol ratio is lower on average in diseased samples compared to healthy *Fungia* at the same sites (Figure 4.13, $p < 0.0001$, one-tailed t-test) and considerably lower at 4 of the 5 sites. This result agrees with our expectation of a lower fatty acid to cholesterol ratio in diseased zooxanthellae based on inoculated cultures (Figure 4.4).

4. DISCUSSION

To test the use of lipid biomarkers as indicators of disease stress in corals and zooxanthellae, we examined several previously established lipid ratios in three types of samples: cultured diseased zooxanthellae, a well-controlled set of inoculated *Montastraea faveolata* fragments, and a suite of healthy and diseased *M. faveolata* samples from the field. Cell cycle measurements indicate that diseased zooxanthellae are experiencing stress. In both cultured Symbiodinium and zooxanthellae isolated from coral fragments, we find a temporary increase and subsequent decline of mitotic index (MI) in thermally stressed zooxanthellae.

Mitotic index decreases in all diseased samples. The magnitude of the decrease depends on which pathogens are introduced.

Three lipid parameters have been previously established as thermal stress indicators in cultured zooxanthellae: C18 free fatty acid unsaturation (Chapter 3), fatty acid to sterol ratios (Chapter 3), and PUFA-rich MGDG content in thylakoids (Chapter 2). All three of these parameters decrease in response to thermal stress in cultured zooxanthellae, and here we present evidence that they decrease in response to thermal and disease stress in zooxanthellae from whole corals as well. Our lipid biomarkers respond more rapidly to disease stress than to thermal stress alone. In general, the addition of thermal stress to diseased cultures or coral fragments causes no additional change in the lipid biomarkers.

4.1 Mitotic index decreases in diseased zooxanthellae

In the cultured zooxanthellae, a disease treatment with 4 YBD-associated *Vibrio* strains caused no significant lipid biomarker signal, even though microscopy and MI evidence indicates these cells are stressed. A treatment with *V. alginolyticus* caused significant decreases in C18 unsaturation ratio and fatty acid to sterol ratio, regardless of the temperature at which the cultures were maintained.

4.2 Fatty acid unsaturation decreases in response in disease stress

Vibrio bacteria do not produce PUFAs. Bacterial lipids could still contribute to the C18 fatty acid unsaturation through contribution of saturated (C18:0) and mono-unsaturated (C18:1) fatty acids. To some degree, additional bacterial lipids, relative to the amount of zooxanthellae lipids in a sample, would tend to decrease the fatty acid unsaturation. However, bacterial fatty acid unsaturation may also respond to stress. In particular, the proportion of mono-unsaturated C18 fatty acid in several *Vibrio* species decreases in response to high temperature and greater salinity (Urdaci et al., 1990; Danevčič et al., 2005). *V. alginolyticus*, the strain of *Vibrio* that had the biggest impact on zooxanthellae lipids in our study, produces less C18:1

fatty acid at higher temperature. A greater contribution of *Vibrio* biomass would thus have the effect of adding the saturated stearic acid and decreasing average C18 fatty acid unsaturation. That effect should be even stronger at high temperature when the bacteria contribute mostly stearic acid. We do not observe such a temperature-dependent response of C18 unsaturation in the Vibrio-inoculated zooxanthellae cultures. Furthermore, the unsaturation decrease is pathogen-specific; *V. alginolyticus* causes a more pronounced stress response than the 4-*Vibrio* YBD consortium. We can thus conclude that the changes in C18 unsaturation are due to changes induced in the zooxanthellae fatty acids, rather than merely a direct contribution of *Vibrio*-sourced lipids.

In the diseased *M. faveolata* sampled from the field, we also observed a lower C18 unsaturation ratio in the zooxanthellae isolated from within the YBD lesion compared to healthy tissue on the same colonies. C18 unsaturation in healthy tissue from diseased colonies was not distinguishable from that in healthy colonies. Diseased *Fungia* from the Red Sea also showed a slightly lower C18 unsaturation ratio than healthy individuals from the same sites, after one sample with questionable data was excluded from the analysis. In sites or colonies where the difference between healthy and diseased values does not agree with expectations based on cultured zooxanthellae, it is important to consider that contributions of saturated C18 fatty acid from bacteria and coral biomass could mask the zooxanthellae signal. Given the complications of applying a simple chemical marker to a complicated biological system, it is already surprising that a significant difference between healthy and diseased tissues is observed. This confirms the general usefulness of the C18 unsaturation ratio as a marker of stress in environmental samples.

Our analysis of the zooxanthellae clades within the healthy and diseased tissue of *M. faveolata* indicated that clade A was only present in lesioned tissue, and clade C was only present in healthy tissue. Though we have no data for the C18 unsaturation ratio in free fatty acids of clade A, the PUFA-rich MGDGs show the most

drastic decline in thermally stressed clade A (Chapter 2, this thesis). We might therefore expect that clade A contributes a higher relative amount of saturated fatty acids when it is thermally stressed. If, like clade C, clade A also responds to disease stress similarly to thermal stress, we would also expect the C18 unsaturation ratio to decrease in diseased clade A *Symbiodinium*. This shift of clades could provide the explanation for the lower C18 unsaturation ratio in diseased coral tissue.

4.3 Fatty acid to sterol ratios decrease in diseased zooxanthellae

The amount of free fatty acids relative to sterols has been shown to decline in thermally stressed zooxanthellae (Chapter 3, this thesis.) Much like the C18 unsaturation ratio, we find that the fatty acid to sterol ratio also decreases in diseased zooxanthellae. Since bacteria produce hopanoids rather than steroids, bacterial biomass contributes fatty acids and not sterols. Bacterial inoculation thus causes an initial increase in the fatty acid to sterol ratio. However, over time this ratio declines in cultures inoculated with *V. alginolyticus*. The decline is substantial, and insensitive to temperature. The decline in fatty acids cannot be attributed to a decrease in bacterial biomass, however, because in that case we would expect to see a substantial decline in both disease treatments. Additionally, we would expect that the contribution from bacterial biomass would be sensitive to temperature, with higher bacterial growth rate at higher temperature. The relative amount of fatty acids in the 4-*Vibrio* YBD treatment is not sensitive to disease stress, so we conclude that the signal is not due to the presence or absence of bacterial biomass, but rather is a signal of zooxanthellae lipids changing in response to pathogenic stress.

In diseased *M. faveolata* collected from the field, the fatty acid to sterol ratios show considerable variability and no clear difference from healthy tissues from the same or nearby colonies. This likely reflects variable contributions of fatty acids and cholesterol from coral animal tissue. Bacteria may also contribute fatty acids, though not cholesterol. *Fungia* from the Red Sea, in contrast, showed a clear difference in fatty acid to sterol ratio between healthy and diseased samples. In four

out of five sites, the diseased sample showed considerably lower fatty acid to sterol ratio than the healthy individual from the same site. This result agrees with the expectation set by *in vitro* cultured zooxanthellae, and is surprising in its consistency, given the complications of applying this biomarker to zooxanthellae grown within the more biologically complicated holobiont. Differences between zooxanthellae from *M. faveolata* and *Fungia* might be attributable to variations in zooxanthellae clade within these two species. We have clear evidence for a shift in zooxanthellae population within diseased *M. faveolata* (Figure 4.11). *Fungia scutaria* in contrast is known to show considerable specificity in zooxanthellae association (Rodriguez-Lanetty et al., 2004). Lipid biomarkers of zooxanthellae stress may thus be considerably more useful when applied to coral species that have highly specific zooxanthellae associations.

4.4 PUFA-rich MGDG decreases in zooxanthellae from thermally stressed corals

The relative amount of two PUFA-rich MGDG compounds within the MGDG of zooxanthellae thylakoids responds sensitively to thermal stress in cultured zooxanthellae (Chapter 2, this thesis). One of these compounds (C18:4/C18:5) has been shown to induce apoptosis in mammalian cells (Andrianasolo et al., 2008) and the other (C18:4/C18:4) is chemically similar enough to have similar breakdown products and thus a similar putative mechanism of biochemical action (Chapter 2, this thesis). In zooxanthellae from our M. faveolata fragments, we find that the proportion of PUFA-rich MGDG responds sensitively to thermal stress. These compounds are not detectable in the 2 high temperature samples (high temperature control and YBD-inoculated) after only 2 weeks exposure. This shows a much faster response than would be expected from zooxanthellae cultured without a coral host (Chapter 2).

Intriguingly, the PUFA-rich MGDG compounds then come back in the thermally stressed *M. faveolata* fragments after 4 weeks of exposure. This is true for both the high temperature control and the YBD-inoculated treatments. There are a

few possible explanations for this apparent return to control conditions. The high temperature fragments looked visibly less pigmented and appeared to have a lower density of zooxanthellae per coral polyp, so we can be reasonably confident that they were in fact still stressed. The most reasonable explanations for the return of PUFA-rich MGDG compounds after 4 weeks in the high temperature fragments involve either a shift in the zooxanthellae population towards more tolerant clades, or a greater relative contribution from other organisms within the coral holobiont.

Contributions from other photosynthetic organisms within the coral holobiont are a likely source of MGDG. A variety of photosynthetic organisms are known to produce MGDG. Diatoms produce PUFA-containing MGDGs, though C18 unsaturated fatty acids were not associated with MGDG in one common marine diatom (Arao et al., 1994). Some species of red and brown algae produce significant amounts of C18-PUFA-rich MGDG (Sanina et al., 2004). Cyanobacteria have the ability to make MGDG, though through a different pathway than higher plants (Awai et al., 2006) and it is unclear whether those MGDG compounds contain C18 PUFAs. Because thermally stressed corals show a lower density of zooxanthellae cells, the relative contribution of biomass from other organisms would be higher in these samples. Though we used an ultracentrifuge technique for isolating zooxanthellae cells, we cannot rule out some small contribution of lipids from other organisms, particularly if those compounds are adhering to zooxanthellae cell aggregates.

An ecological shift towards more thermally tolerant zooxanthellae phylotypes provides a more likely explanation for the return of PUFA-rich MGDG compounds after 4 weeks of thermal stress. In this ecological shift scenario, MGDG from the relatively small proportion of clade D that might be present in initially unstressed coral fragments would be swamped by the MGDG produced by thermally sensitive clades B and C. After 4 weeks of exposure to high temperature, clades B and C would preferentially die and the remaining zooxanthellae would be primarily the tolerant clade D, which continues to produce abundant PUFA-rich MGDG after 4 weeks at high temperature (Chapter 2, this thesis). Our samples of *M. faveolata* from

the field indicate that healthy corals contain primarily clades B and C, and diseased (but not heat-stressed) corals harbor clade A. Clade D was not detectable based on our techniques.

Overall, our lipid biomarkers of coral stress do show some response to disease in both cultured zooxanthellae and zooxanthellae isolated from whole coral samples. The responses are pathogen-specific and not sensitive to temperature for zooxanthellae inoculated in isolation. Within the more complicated system of the coral holobiont, zooxanthellae still show some lipid changes in response to thermal and disease stress. Shifts in predominant zooxanthellae clade can help the coral survive stress events, but may confound the lipid biomarker signals. Given the complications of the whole coral system, the success of our lipid biomarkers in responding to coral stress is surprising. Further exploration of lipid biomarkers within other species and in response to additional stressors will be needed to diagnose the utility of lipid biomarkers of coral stress in environmental samples. Lipid biomarkers of coral stress will be most readily applicable to coral species with highly clade-specific zooxanthellae associations.

Acknowledgment

James Cervino and his students provided mitotic index data. Briana Hauff assisted with the coral culturing experiment at Looe Key. Amy Apprill and Cosette Bardawil provided genetic data for zooxanthellae from the Looe Key samples.

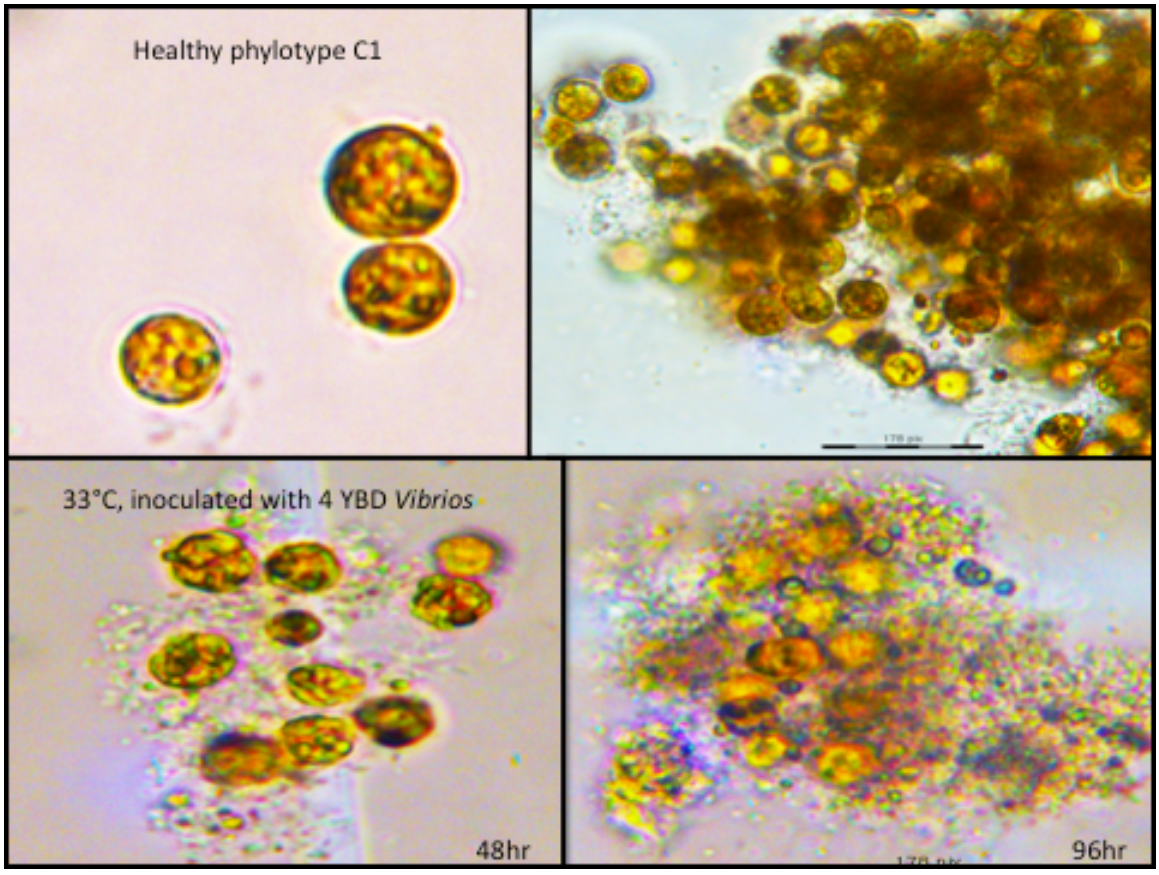

Figure 4.1. Microscopy of healthy and diseased zooxanthellae. High temperature and disease causes cells to fragment and become deformed.

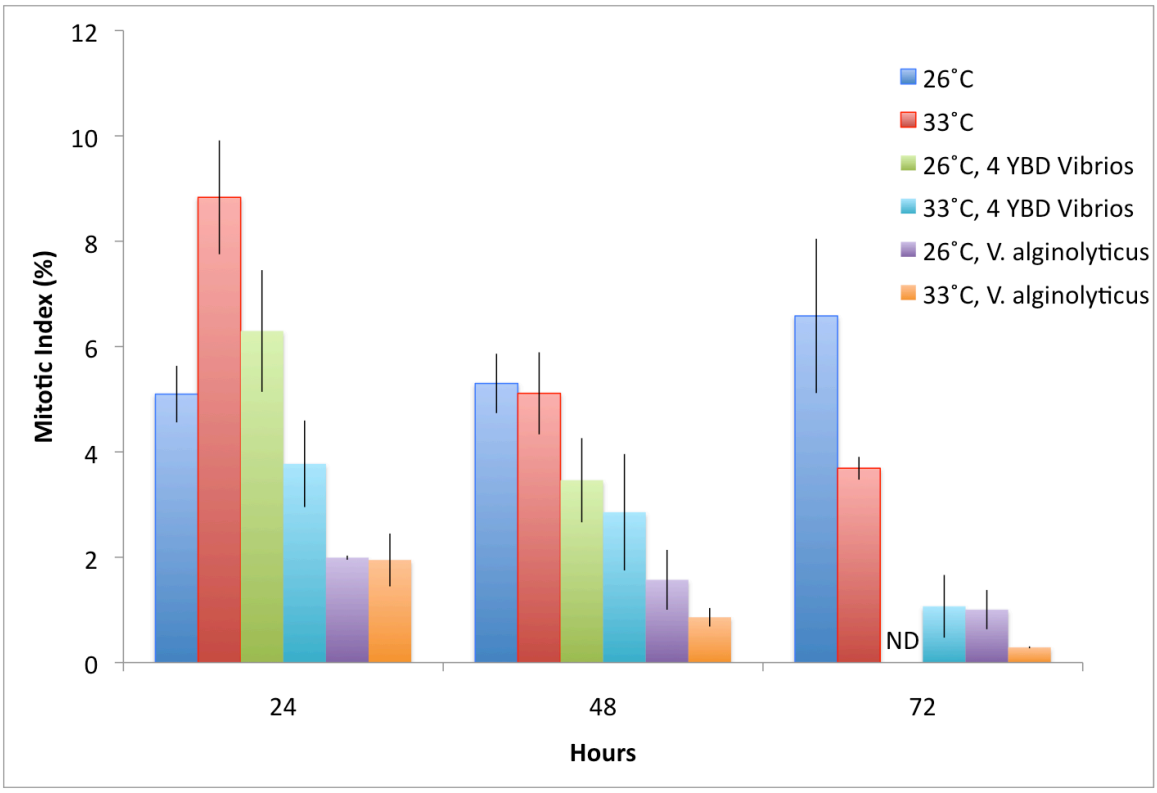

Figure 4.2. Mitotic index in cultured *Symbiodinium* inoculated with 4 *Vibrio* strains in the YBD core group and *V. alginolyticus*. Increased temperature alone causes an initial increase in mitosis which drops below control values after 72 hours of exposure ($p < 0.0001$, one-tailed t-test). The addition of YBD *Vibrios* causes an additional decrease in mitotic index relative to high temperature alone, which is significant after 48 hours in the low temperature YBD treatment, 24 hours in the high temperature YBD treatment ($p < 0.0001$). The largest effect is seen in cultures inoculated with *V. alginolyticus*, which causes a large suppression of mitosis relative to thermal stress alone, significant after only 24 hours at both low and high temperature ($p < 0.0001$). The addition of thermal stress to cultures innoculated with *V. alginolyticus* causes a small additional decrease in mitosis, which becomes significantly lower than *V. alginolyticus* exposure alone after 48 hours of exposure ($p < 0.0001$).

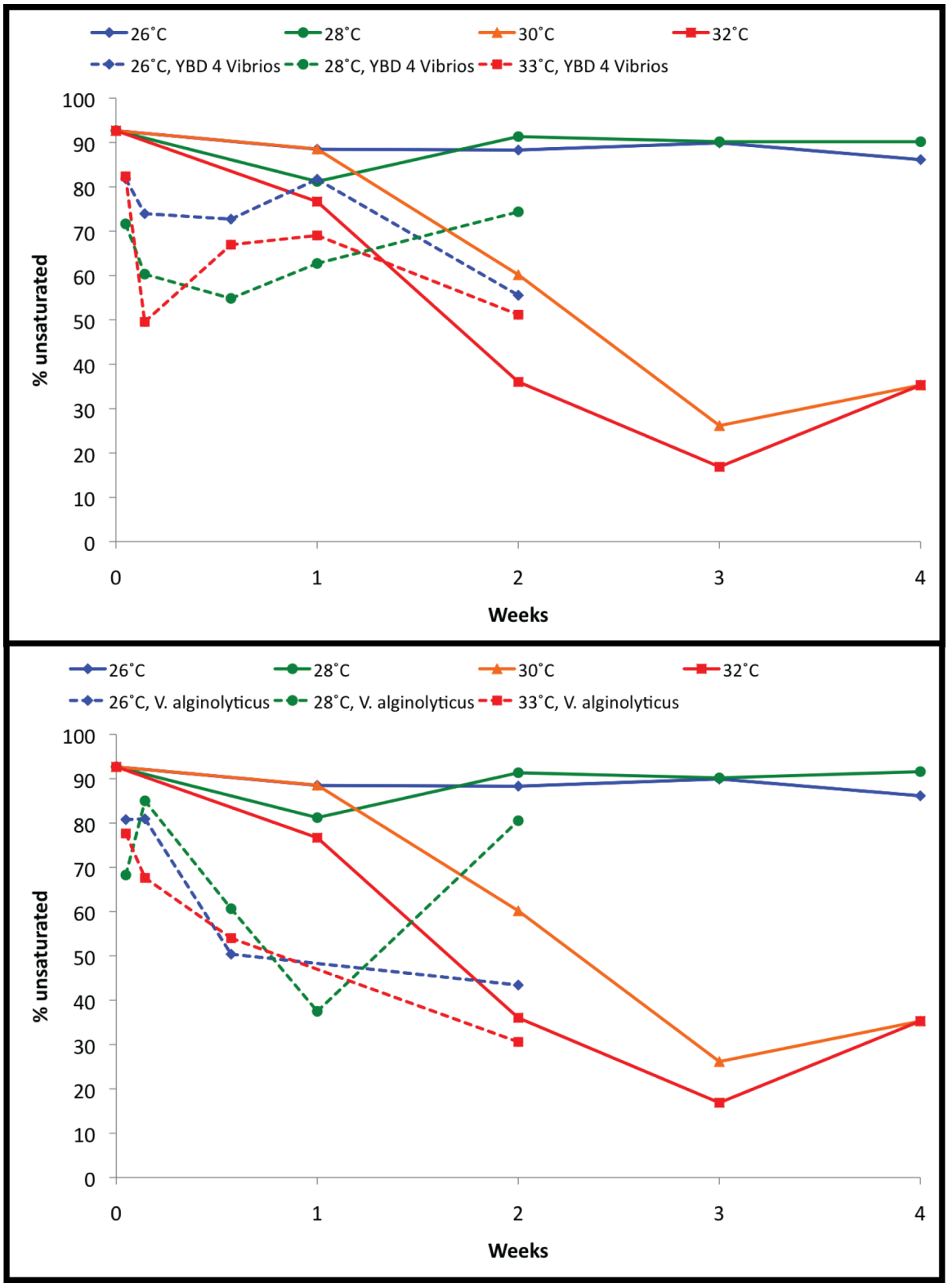
26˚C
28˚C
30˚C
32˚C
26˚C, YBD 4 Vibrios
28˚C, YBD 4 Vibrios
33˚C, YBD 4 Vibrios
% unsaturated
Weeks
26˚C
28˚C
30˚C
32˚C
26˚C, V. alginolyticus
28˚C, V. alginolyticus
33˚C, V. alginolyticus
% unsaturated
Weeks

Figure 4.3. C18 fatty acid unsaturation index in cultured *Symbiodinium* inoculated with 4 *Vibrio* strains in the YBD core group (top panel) and V. alginolyticus (bottom panel). Cultures in the disease treatment were inoculated with bacteria from the 4-strain consortium of the *Vibrio* core group, shown to cause Yellow Band Disease (Cervino et al., 2008) or a single strain of *V. alginolyticus*. The solid lines show data from thermal stress only experiments (Chapter 3, this thesis). The initial fatty acid unsaturation decrease in diseased cultures results from the addition of bacterial biomass, which is enriched in saturated fatty acids, particularly stearic acid. The decrease in fatty acid unsaturation over time occurs earlier for disease exposure than for heat treatment, and appears insensitive to temperature. There is no significant temperature effect in either of the *Vibrio* treatments.

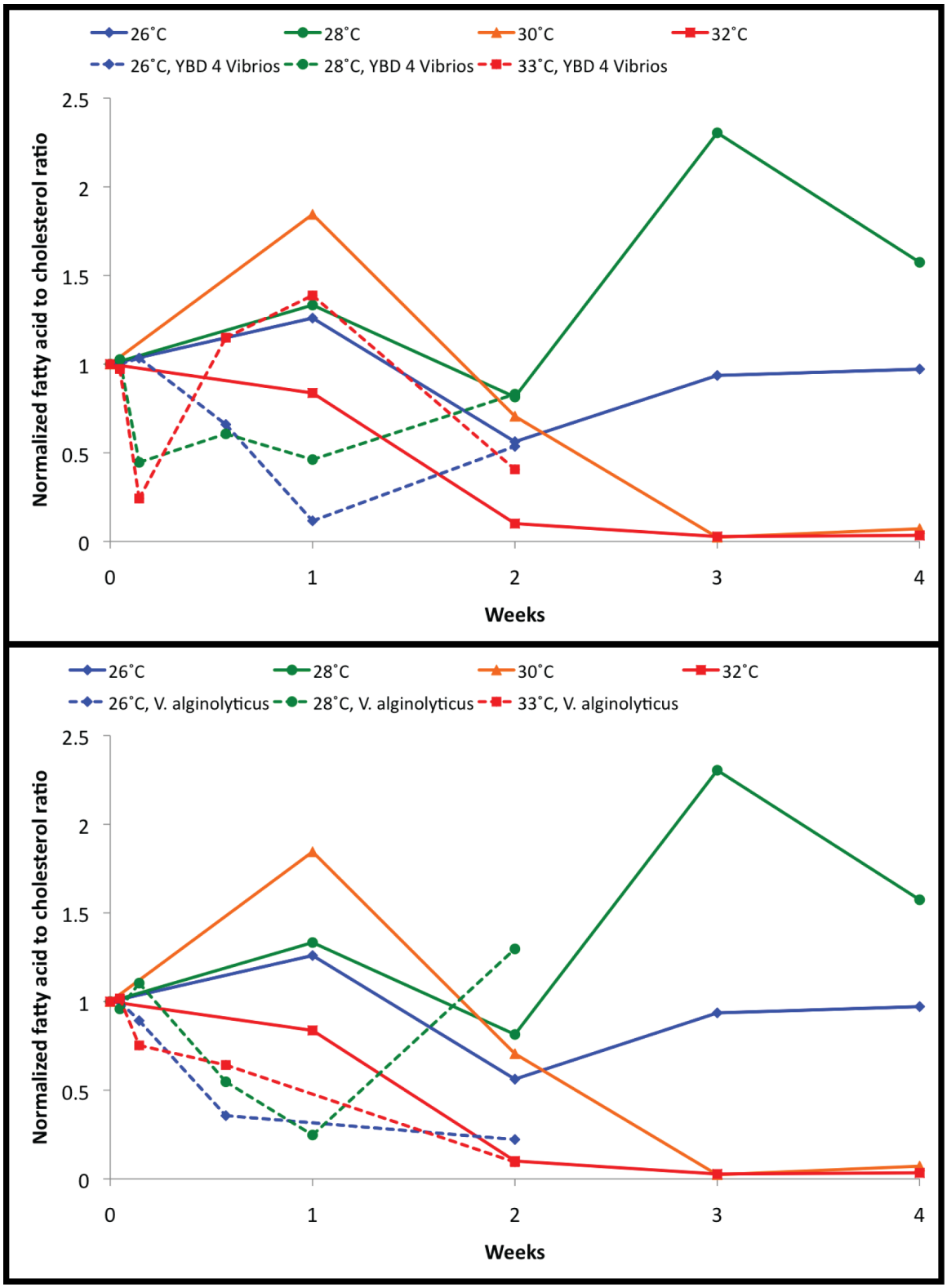
26˚C
28˚C
30˚C
32˚C
26˚C, YBD 4 Vibrios
28˚C, YBD 4 Vibrios
33˚C, YBD 4 Vibrios
Normalized fatty acid to cholesterol ratio
2.5
2
1.5
1
0.5
0
0
1
2
3
4
Weeks
26˚C
28˚C
30˚C
32˚C
26˚C, V. alginolyticus
28˚C, V. alginolyticus
33˚C, V. alginolyticus
Normalized fatty acid to cholesterol ratio
2.5
2
1.5
1
0.5
0
0
1
2
3
4
Weeks

Figure 4.4. Fatty acid to cholesterol ratio in cultured *Symbiodinium* inoculated with 4 *Vibrio* strains in the YBD core group (top panel) and *V. alginolyticus* (bottom panel). All values were normalized to the initial fatty acid to cholesterol ratio at the start of each experiment, to account for the additional contribution of bacterial fatty acids in the inoculated treatments. *Vibrio* bacteria do not produce cholesterol.

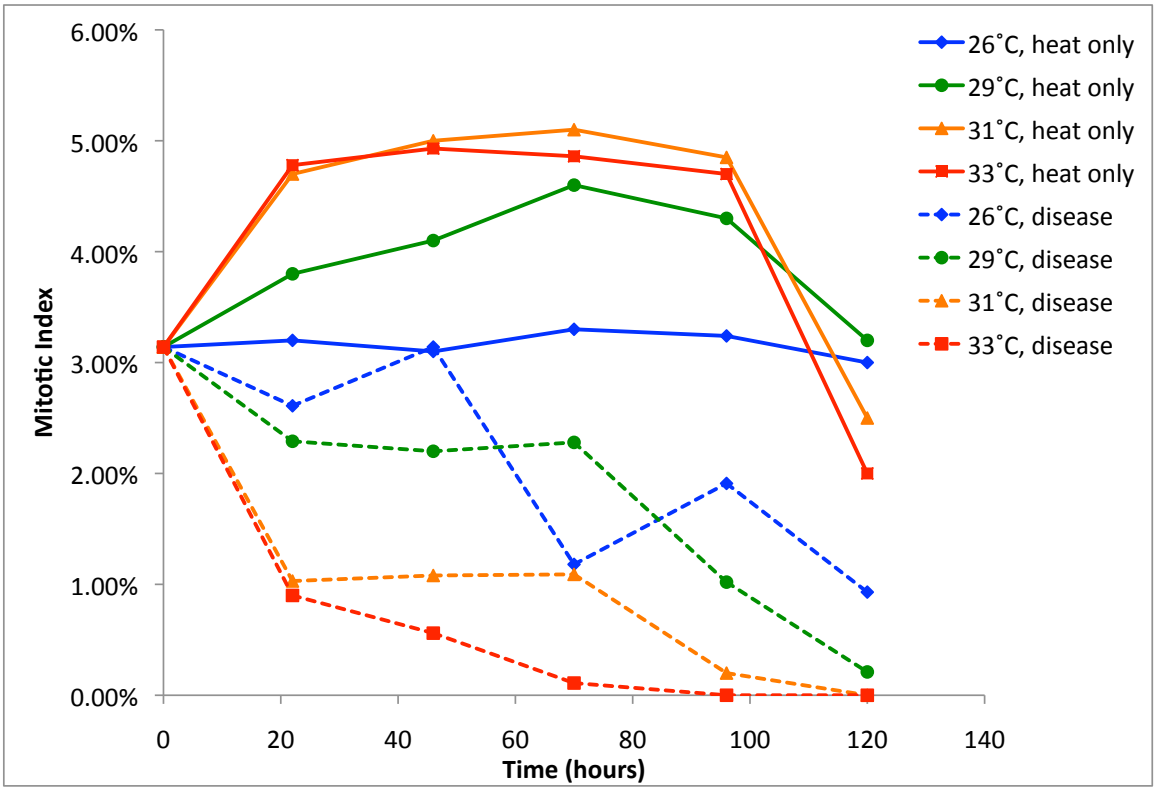

Figure 4.5. Mitotic index in zooxanthellae isolated from *Montastraea faveolata* inoculated with 4 *Vibrio* strains in the YBD core group. *Montastraea faveolata* fragments were grown under different temperature conditions. Cooler colors (blue and green) indicate cooler, unstressed temperatures of 26˚C and 28˚C, whereas orange and red indicate 31˚C and 33˚C, temperatures expected to cause thermal stress in corals. Dashed lines indicate coral fragments that were exposed to an inoculum containing 4 strains of *Vibrio* bacteria previously shown to cause Yellow Band Disease (Cervino et al., 2008). Mitotic index is initially elevated in thermally stressed cultures, and then declines after 5 days of high temperature exposure. Inoculation with *Vibrio* bacteria causes the mitotic index to decline in all four temperature treatments, and the sharpest decline is seen when thermal stress and pathogen stress are combined.

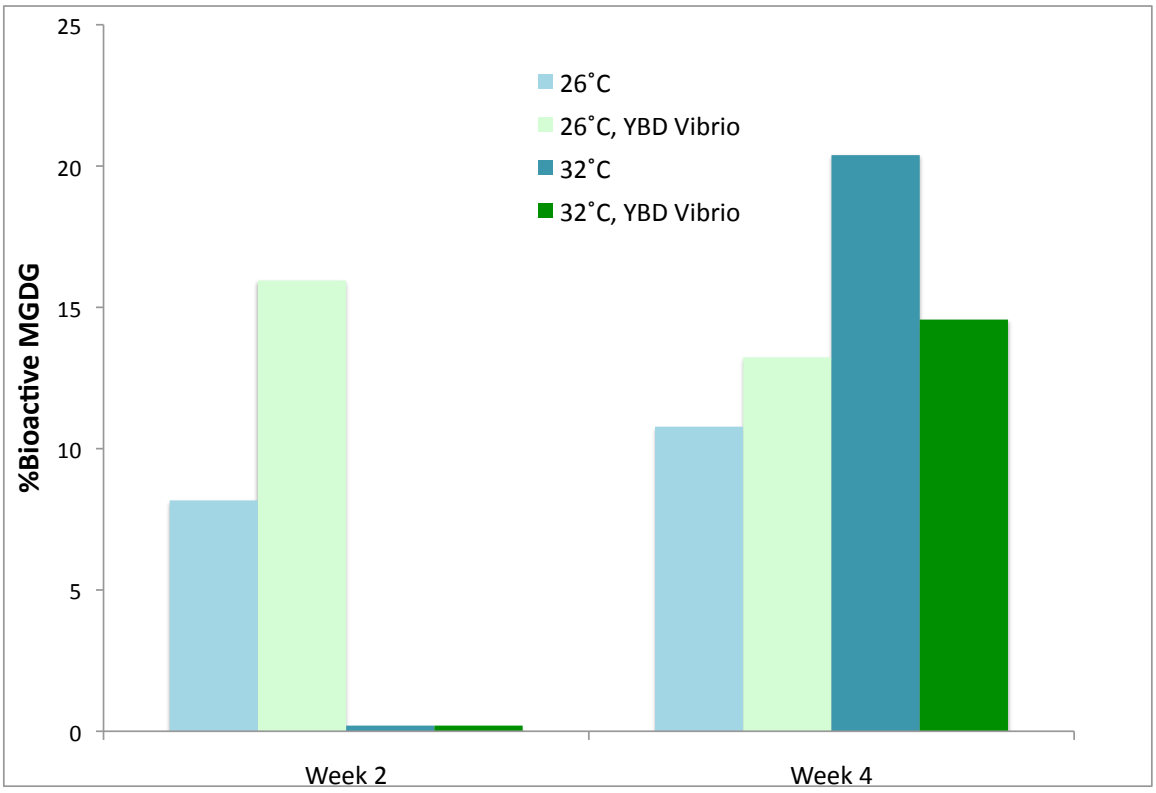

Figure 4.6. Proportion of PUFA-rich MGDG in zooxanthellae isolated from healthy and inoculated *Montastraea faveolata*. PUFA-rich MGDG include C18:4/C18:4 and C18:4/C18:5 fatty acids, and are shown as the proportion of total MGDG from zooxanthellae isolated from cultured *Montastraea faveolata* fragments. This index of highly-unsaturated thylakoid membrane compounds has been previously shown to decline in response to thermal stress (Chapter 2, this thesis). The lowest temperature fragments (26˚C, light blue bars) show a slight increase in the relative proportion of PUFA-rich MGDG from 2 weeks to 4 weeks in aquarium conditions. The low temperature fragments that were inoculated with the 4-*Vibrio* YBD core group pathogens so a slight decrease over time of PUFA-rich MGDG. Striking changes are seen over time in the high temperature (32˚C) fragments, where after 2 weeks of exposure no PUFA-rich MGDG compounds are detected in either diseased or uninoculated heat treatments. These compounds return after 4 weeks, however. This could be due to an ecological shift in the symbiont community towards more resilient phylotypes of zooxanthellae. All treatment conditions (thermal stress, disease stress, and combined) are statistically different from the two low-temperature controls ($p < 0.0001$, two-tailed t-test).

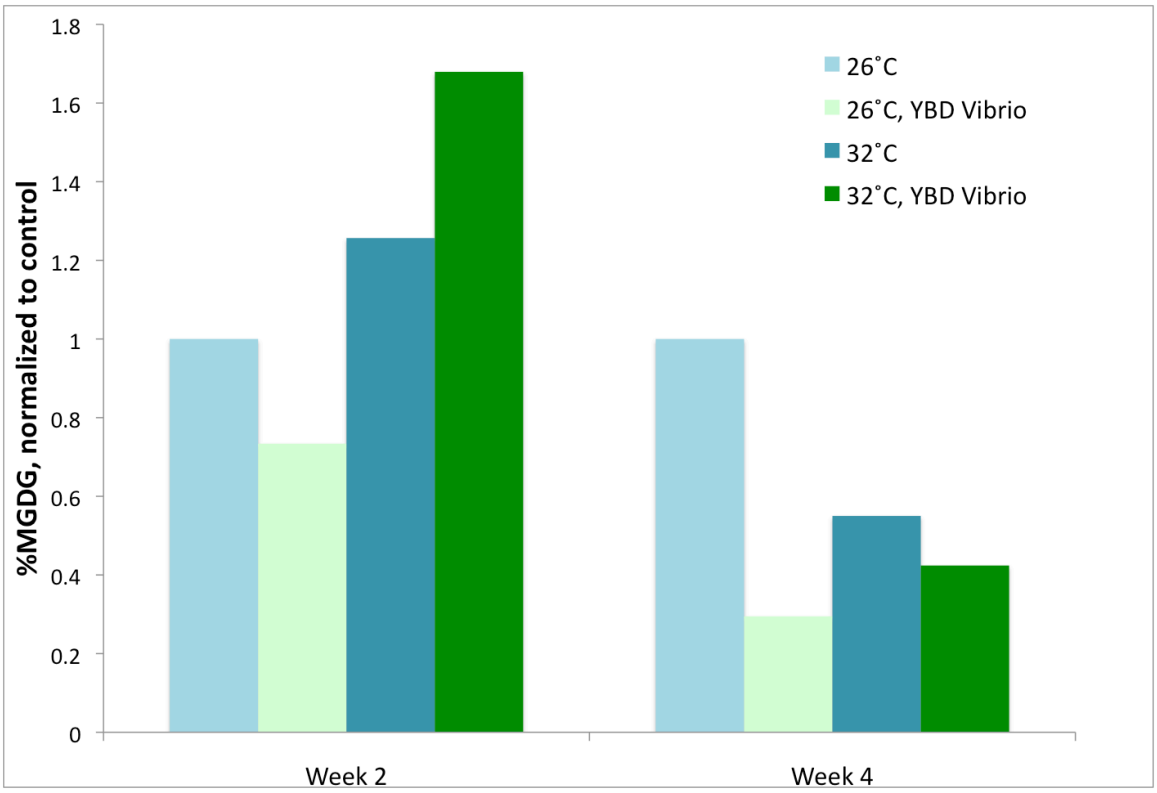

Figure 4.7. Proportion of MGDG in thylakoid lipids of zooxanthellae isolated from healthy and inoculated *Montastraea faveolata*. Total thylakoid lipids include MGDG, DGDG, PG, and SQDG. The proportion of MGDG (normalized to the control tank at 26˚C) decreases somewhat over time in all stressed conditions (thermal stress, disease stress, and combined). Though the relative amount of PUFA-rich MGDG compounds was significantly lower in the higher temperature conditions at 2 weeks (previous figure) the total amount of MGDG is static. This demonstrates that preferential changes in PUFA-rich MGDGs represent a compound-specific response to thermally stressful conditions, rather than an overall shift in thylakoid composition.

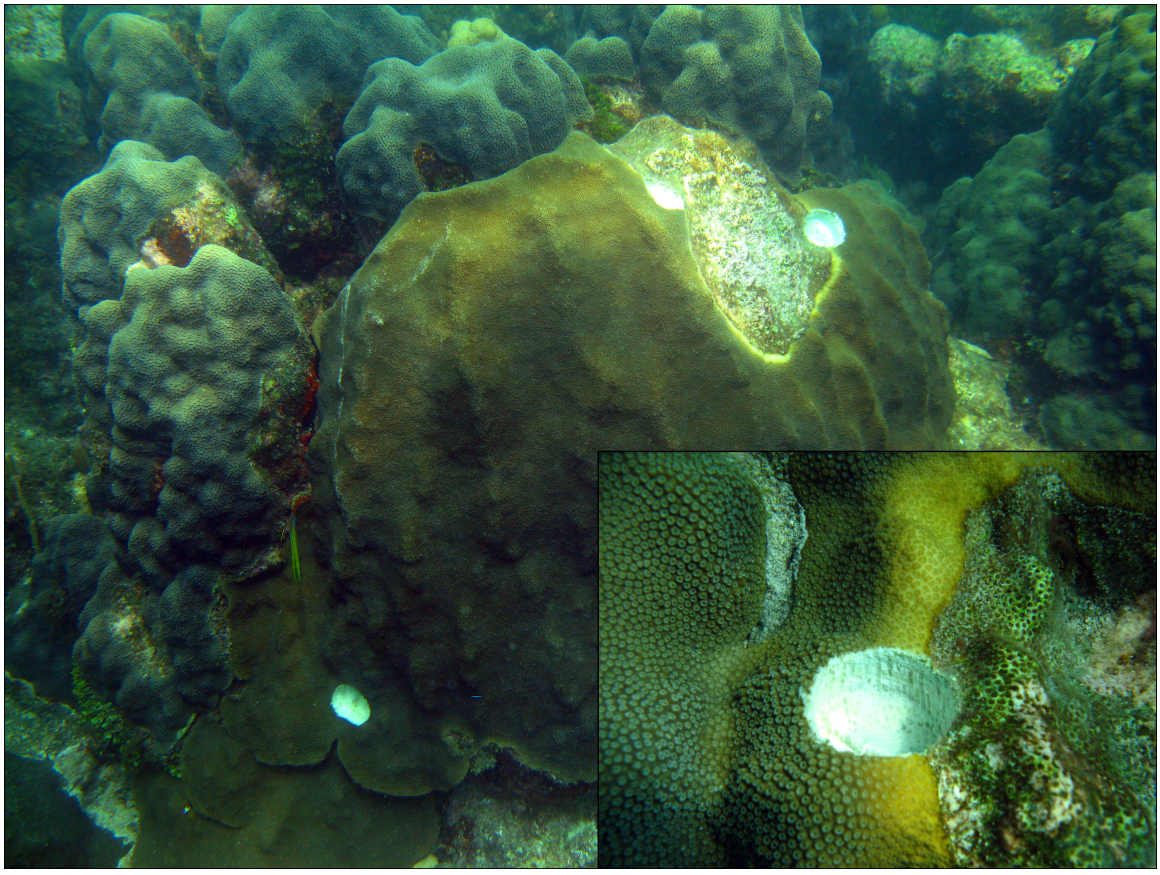

Figure 4.8. Underwater photographs of *Montastraea faveolata* infected with Yellow Band Disease in Looe Key, FL. This *Montastraea faveolata* colony is typical of the three diseased colonies sampled for this study. Note the replicate diseased samples taken from the Yellow Band Disease lesion and the control sample taken from a healthy portion of this diseased colony. Inset photo shows a closer view of an individual sample hole. When airbrushing to remove the diseased tissue from the skeleton, care was taken to sample only the yellowish, diseased tissue.

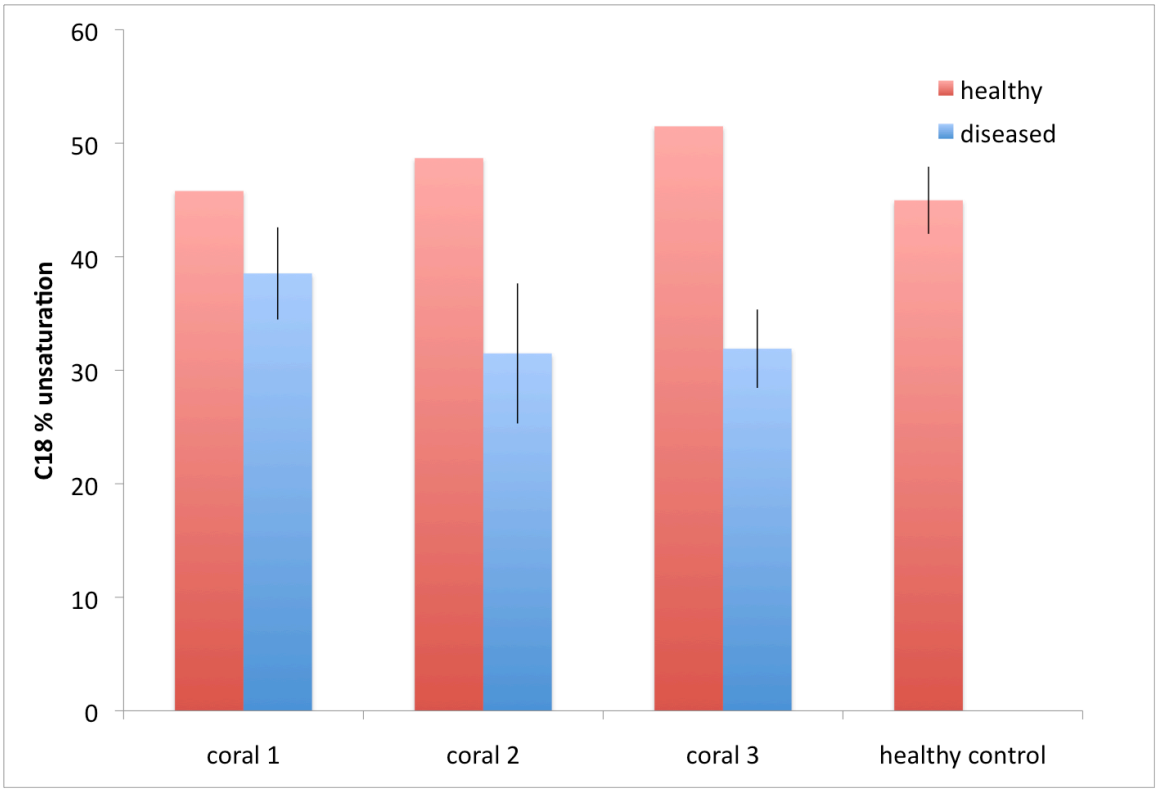

Figure 4.9. C18 fatty acid unsaturation index in zooxanthellae from *M. faveolata* with Yellow Band Disease. Each diseased value represents the average of two samples from a single colony, and the healthy value for each coral is from a sample of visibly healthy tissue from the same colony. The healthy control represents an average of 1 sample each from 3 different healthy colonies from the same reef. The healthy portions of diseased colonies show a very slightly higher C18 unsaturation ratio compared to the healthy controls (<4% average elevation, $p < 0.0001$, two-tailed t-test). Diseased tissue has lower C18 unsaturation relative to either group of healthy samples ($p < 0.0001$, two-tailed t-test).

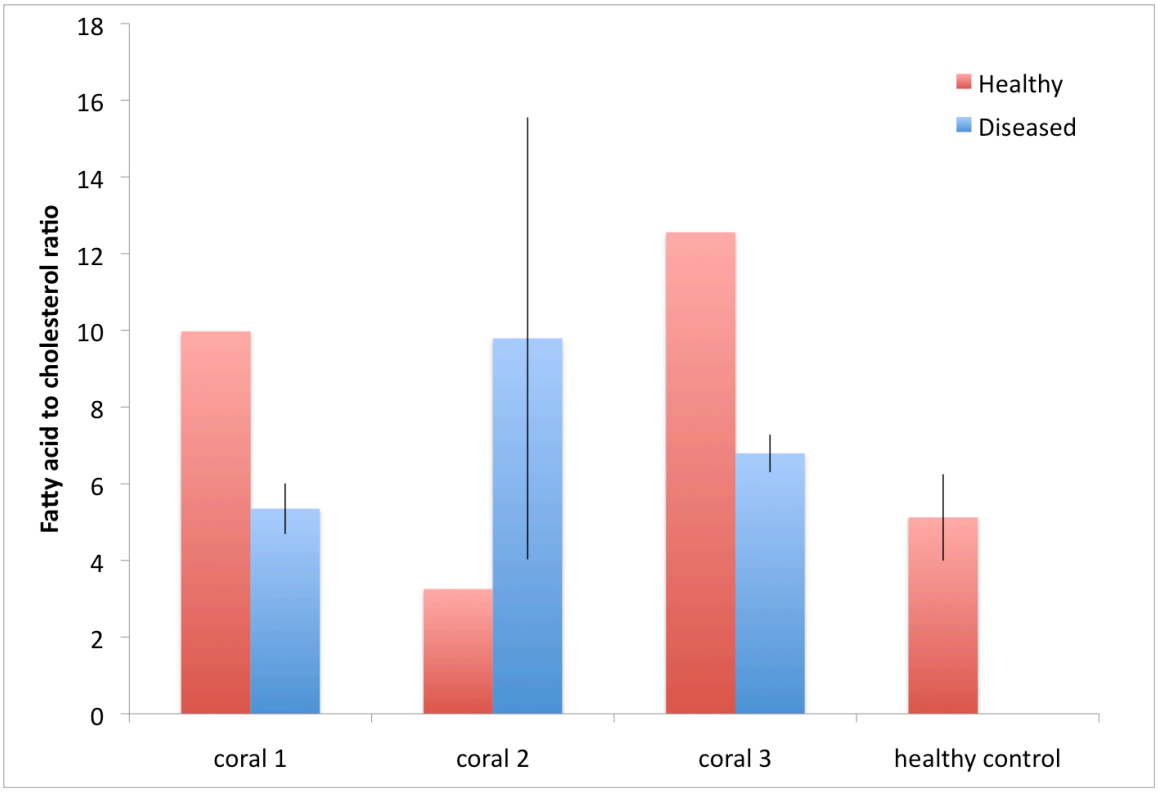

Figure 4.10. Fatty acid to sterol ratio in zooxanthellae from *M. faveolata* with Yellow Band Disease and healthy controls. The healthy control represents an average of 1 sample each from 3 different healthy colonies from the same reef. The visibly healthy tissue of diseased colonies shows a very slightly higher fatty acid to sterol ratio compared to the healthy controls ($p < 0.0001$, two-tailed t-test). Diseased tissue also has an elevated fatty acid to cholesterol ratio than healthy tissue ($p < 0.0001$, two-tailed t-test), which disagrees with our expectation for a lower fatty acid to sterol ratio in stressed zooxanthellae (Chapter 3, this thesis). There is, however, considerable variability between samples, including between adjacent samples from the same disease lesion. Values for diseased tissue are slightly lower than for healthy tissue from the same colonies ($p < 0.0001$, two-tailed t-test).

Figure 4.11. Zooxanthellae clade distribution in *M. faveolata* with Yellow Band Disease and healthy controls. Diseased colonies showed visible Yellow Band Disease lesions, and data represents an average of 2 samples from each of 3 diseased colonies. Healthy samples from the same 3 diseased colonies are shown for comparison, as well as an average from 4 healthy colonies from the same reef.

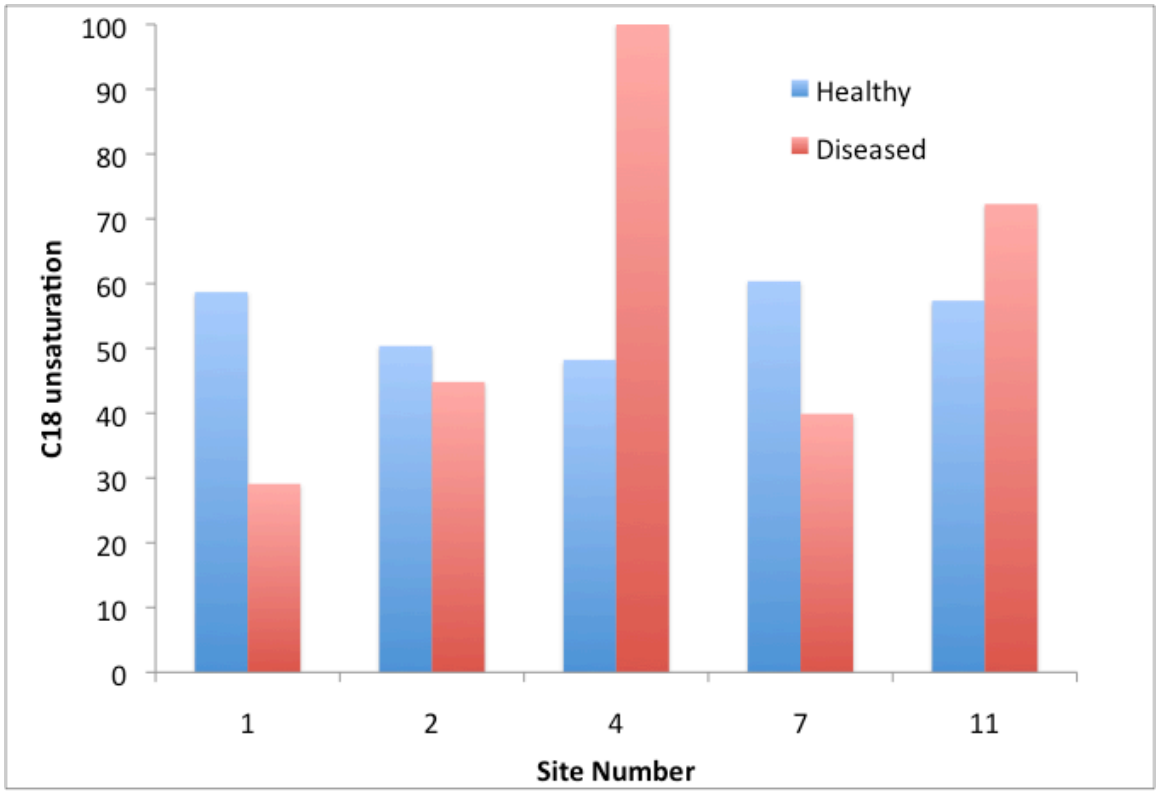

Figure 4.12. C18 unsaturation ratio in zooxanthellae from healthy and diseased *Fungia spp*. Diseased zooxanthellae show very slightly higher fatty acid unsaturation compared to healthy samples from the same site (55% vs. 57%, $p < 0.0001$, two-tailed t-test). However, the data is strongly influenced by the complete absence of saturated C18:0 fatty acid in the diseased sample from site 4. If site 4 is excluded from the analysis, the diseased samples then show a lower C18 unsaturation value compared to healthy controls (57% vs. 47%, $p < 0.0001$, two-tailed t-test). This result agrees with expectations of a lower unsaturation ratio in diseased zooxanthellae cultures (Figure 4.3).

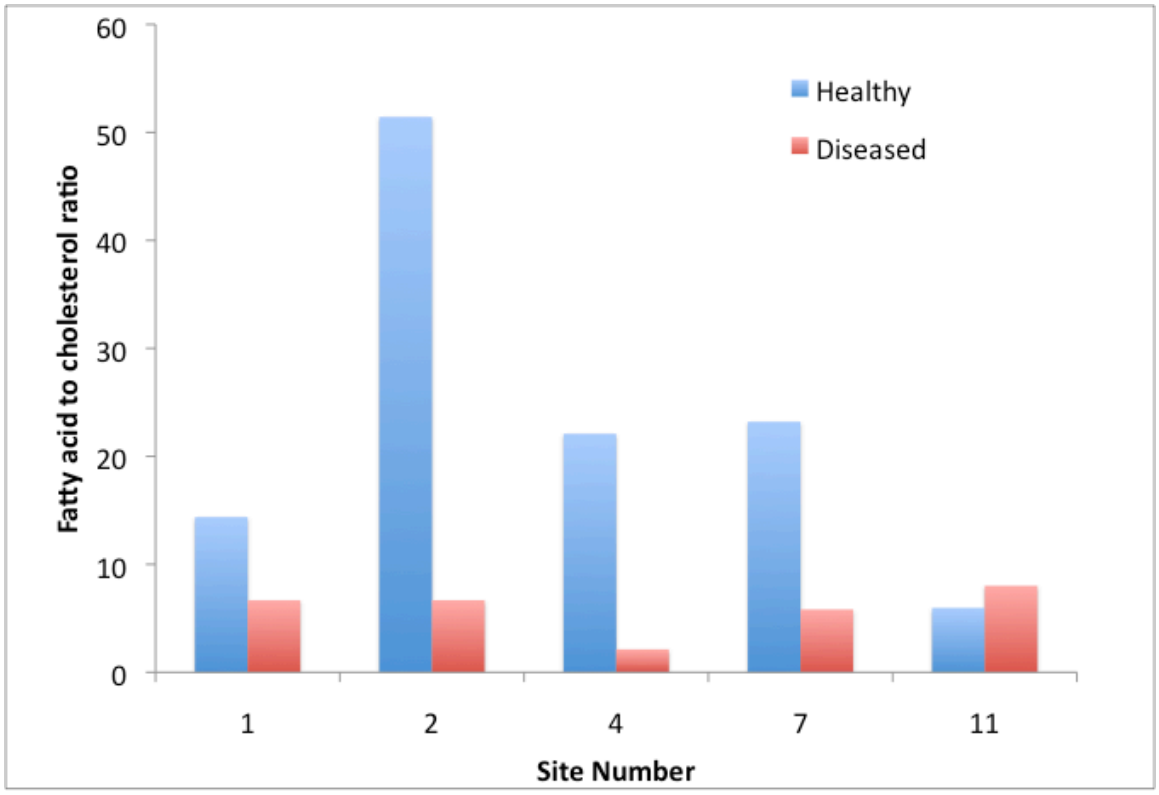

Figure 4.13. Fatty acid to cholesterol ratio in zooxanthellae from healthy and diseased *Fungia spp*. The fatty acid to cholesterol ratio is lower on average in diseased samples ($p < 0.0001$, one-tailed t-test) and considerably lower at 4 of the 5 sites. This result agrees with our expectation of a lower fatty acid to cholesterol ratio in diseased zooxanthellae based on inoculated cultures (Figure 4.4).

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CHAPTER 5:

CONCLUSIONS

In this thesis, I presented results from the first well-controlled study of the response of lipid biomarkers to thermal and disease stress in coral zooxanthellae. We measured a variety of parameters in zooxanthellae cultured under controlled thermal stress conditions, outside of their usual coral hosts. After establishing several lipid parameters that respond sensitively to thermal stress (Chapters 2 and 3), we looked for these same parameters in cultured zooxanthellae exposed to disease-causing bacteria (Chapter 4). We further tested the usefulness of our thermal and disease stress biomarkers in zooxanthellae isolated from diseased and heat-stressed corals (Chapter 4).

Chapter 2 highlighted the discovery of a particular bioactive molecule that forms part of the thylakoid membrane in zooxanthellae chloroplasts. This form of monogalactosyldiacylglycerol (MGDG) has been previously shown to induce apoptosis (organized cell death) in mammalian cells (Andrianasolo et al., 2008). The mere presence of this molecule in zooxanthellae cells is already exciting, and furthermore we discovered that the relative amount of this compound declines in thermally stressed zooxanthellae cells. In that chapter I presented a plausible mechanism by which the bioactive MGDG preferentially degrades in thermally stressed zooxanthellae, releasing degradation products that may contribute to apoptosis in the zooxanthellae cells, as well as potentially in the coral host. Ultimately, these hazardous degradation products may contribute to coral bleaching. Clearly, further research into these breakdown products (PUFAs, PUAs, and the reactive oxygen species they interact with) could help determine whether PUFA-rich MGDG compounds contribute to apoptosis and bleaching in corals.

In the third chapter, I presented evidence that ratios within abundant fatty acids and sterols can be used as sensitive indicators of the level of thermal stress

experienced by zooxanthellae. I introduced two new biomarkers of coral stress: the C18 free fatty acid unsaturation ratio, and the fatty acid to sterol ratio. Both of these parameters decline in thermally stressed cells. Furthermore, they do so in a manner that is consistent with the degree-heating-weeks measure for the extent of thermal stress. The amount of thermal stress needed to produce large changes in C18 unsaturation and fatty acid to sterol ratios is similar to the degree heating weeks after which coral bleaching would be expected. Additionally, the more thermally sensitive clade C zooxanthellae show lipid ratio changes after reaching a lower threshold of thermal stress than the more tolerant clade D symbionts. The threshold-type, clade-specific response in the lipid biomarkers is similar to the observed bleaching behavior of corals harboring these symbionts. If these lipids are not directly involved with the mechanism of bleaching as was hinted at by the results of Chapter 2, they are at least quantitatively indicative of the broader biochemical changes experienced by zooxanthellae undergoing thermal stress.

Finally, the fourth chapter presented results from an experiment in which our cultured zooxanthellae were exposed to bacterial pathogens as well as thermal stress. I tied those results together with several tests of the use of coral stress biomarkers in whole coral samples exposed to thermal stress and disease pathogens. I found that our lipid biomarkers of thermal stress do in fact respond to disease stress as well, though not for all pathogens. Where disease causes a lipid response, the decline in our C18 unsaturation and fatty acid to sterol ratios occurs on a much shorter timescale than with thermal stress alone. The addition of thermal stress to diseased cultures produces no additional change; the effect of disease stress appears to overwhelm any thermal stress effects.

Moving from controlled zooxanthellae cultures to the whole coral system, complications are to be expected. I helped develop the ultracentrifugation method to separate intact zooxanthellae cells from coral host tissue, as well as from biomass contributed to the holobiont by bacteria or other organisms. Nonetheless, it is expected that zooxanthellae grown *in hospite* might show different lipid changes

from those cultured outside of a coral host. Additionally, from the whole coral samples we have only sparse data to indicate which clade phylotypes of zooxanthellae are present. I can only speculate about how the ecological composition of a presumably heterogeneous zooxanthellae population changes in response to experimental manipulations. Given the potential complications of looking at zooxanthellae from a whole coral system, then, it is surprising that I was able to observe changes in the lipid biomarkers that are broadly consistent with the expectations set by the better controlled zooxanthellae cultures. The bioactive MGDG was again observed in zooxanthellae isolated from whole coral fragments. The abundance of this apoptosis-inducing compound within *in hospite* zooxanthellae appears sensitive to thermal, but not disease stress. Shifts in the zooxanthellae community or contributions of MGDG from other organisms may explain the otherwise puzzling reappearance of bioactive MGDG after several more weeks of thermal stress. C18 fatty acid unsaturation is lower in zooxanthellae from Yellow Band Disease lesions than in healthy tissue in natural coral field samples. The difference is not as large as in our diseased zooxanthellae cultures. Given the difference in predominant *Symbiodinium* clade between healthy and diseased coral tissue, however, it is remarkable that we are still able to detect a difference in the stress biomarker. Results from the fatty acid to sterol ratios in these samples were more ambiguous, displaying a large variability between samples that overwhelmed any difference between healthy and diseased conditions. Variable contributions from bacterial biomass (which contains fatty acids, but not sterols) could explain variability in the fatty acid to sterol ratio.

Coral tissue is a complex biological environment. Coral polyps harbor algal symbionts, a variety of beneficial or potentially harmful bacteria, and other organisms. All of these components of the coral holobiont can exchange metabolites, including lipids, in complex pathways. This thesis presents the first calibration of lipid biomarkers produced by *Symbiodinium* cells as sensitive indicators of thermal and disease stress in zooxanthellae. I have additionally suggested a mechanism by

which some of these lipid biomarkers may induce apoptosis in zooxanthellae or coral cells and contribute to regulation of the symbiosis. I have confirmed that these lipid biomarkers are present in zooxanthellae growing within corals collected in the field. Though the biology of those intact corals is more complicated, I was able to observe significant changes in some of the lipid biomarkers in response to thermal and disease stress within the whole coral. These results contribute several new parameters by which to assess coral health, and they strongly suggest new avenues for exploration into the biochemical mechanisms of coral stress and bleaching. The threats to corals from global warming and the increased spread of marine diseases are unlikely to abate in the near future. The new lipid biomarkers of coral stress that I presented here can help contribute to better predictions of the particular threats faced by corals. Ultimately, specific management actions that take into account the biochemical mechanisms of bleaching will be needed to give threatened coral species the best possible chance at survival in a rapidly changing environment.

APPENDIX I:

LIPID BIOMARKERS PRESERVED IN CORAL ARAGONITE

INTRODUCTION

Corals are threatened by rapidly warming oceans and other anthropogenic threats, but no long-term records of past coral health exist (Pandolfi et al., 2003). Coral skeletons provide a potential archive of the environmental conditions the corals have experienced. Scleractinian corals produce aragonite layers of different density each year, so the age of individual layers can be determined by counting density bands from an x-ray (Figure A.1). Isotopic and elemental ratios within the coral aragonite have been successfully used as recorders of the environmental conditions the corals experienced, such as temperature and salinity (e.g. Cohen and McConnaughey, 2003; Cole et al., 2000; Goodkin et al., 2005; Fleitmann et al., 2007; Smith et al., 1979). Newly developed lipid biomarkers of coral stress could provide additional information about the health history of an individual coral colony (Chapters 2-4, this thesis). Lipids have been previously extracted from coral aragonite (Ingalls et al., 2003), but have not yet been used to extract information about coral condition. We have extracted lipids from coral aragonite and identified some of the same compounds that are used to calculate our lipid biomarkers of thermal stress (Chapters 3 and 4). Lipids preserved in coral aragonite show great promise for reconstructing histories of coral health.

METHODS

Two drill core samples of living *Montastraea faveolata* colonies were collected in 2005 at Sapodilla Caye, Belize. Samples of the coral aragonite representing approximately annual bands were ground to a fine powder with a solvent-cleaned mortar and pestle, and then sonicated first with methanol and then dichloromethane to remove any lipids that may have been protected in pore spaces.

The cleaned powder was dissolved with hydrochloric acid, then neutralized with potassium hydroxide. Lipids were extracted using liquid/liquid extraction between the aqueous potassium chloride solution and a 9:1 hexane:MTBE organic phase. Total lipid extracts were dried over sodium sulfate columns and derivatized with BSTFA. Compounds were quantified by GC-FID and identified by GC-TOF-MS.

RESULTS AND DISCUSSION

We were able to successfully recover intracrystalline fatty acids and sterols from milligram quantities of coral aragonite. The most abundant compounds were C16 and C18 saturated free fatty acids (Figure A.2). Sterols were present, though the sterols isolated from coral aragonite typically differed from those found in zooxanthellae cultures, with the exception of cholesterol. Given the complicated biological system within the coral holobiont, we would expect lipid contributions from animal, algae, bacteria, and other organisms.

We measured the coral stress biomarkers C18 unsaturation and fatty acid to cholesterol ratio in a 10-year record of coral aragonite from 2 different *Montastraea faveolata* colonies from Sapodilla Caye, Belize. Though lipid abundances were low, the ratios could be successfully measured in the majority of samples. C18 unsaturation (Figure A.3) and fatty acid to sterol ratio (Figure A.4) do show variability over time in both records. Unfortunately, there is considerable disagreement between records from the two colonies. Part of this may be attributable to slight differences in handling, as we were just developing new methods to handle coral aragonite samples. SMF 1 had a lower relative abundance of unsaturated C18 fatty acids, which causes both stress biomarkers to be lower on average than in the core. Smaller sample sizes from SMF 1 meant that the unsaturated fatty acids were often below detection limit.

In general, we would have expected values for both stress biomarkers to drop significantly during 1998-1999, which was a year of strong bleaching in the Caribbean, and indeed worldwide (Wilkinson, 2006). This expected stress signal is

not observed, and there are several reasons why that may be the case. Coral skeletal extension rate declines after a bleaching event, though corals may continue to increase the density in the most recently deposited aragonite (Figure A.1). It is therefore possible that lipid signatures of the bleaching event itself are not well recorded, as no new porous skeleton is being produced during the stress event. Additionally, during bleaching the density of zooxanthellae cells within each polyp declines, resulting in a decreased relative contribution of zooxanthellae to the lipids present. Finally, it is also possible that other organisms such as endolithic algae, fungi, or bacteria may modify the lipid signal after deposition. Post-depositional modification would be specific to each colony and may explain differences between the two cores.

This research provides tantalizing evidence that coral stress biomarkers may be preserved within the coral skeleton. We have successfully extracted intracrystalline lipids from within coral aragonite and show results of two short down-core records of our stress biomarkers. Corals represent a complex biological environment, and lipids within the aragonite may undergo post-depositional alteration that could mask the signal of coral stress. Further work will be necessary to quantify lipid contributions from various components of the coral holobiont and their individual stress responses before these stress biomarkers can be successfully applied to reconstruct longer records of coral health.

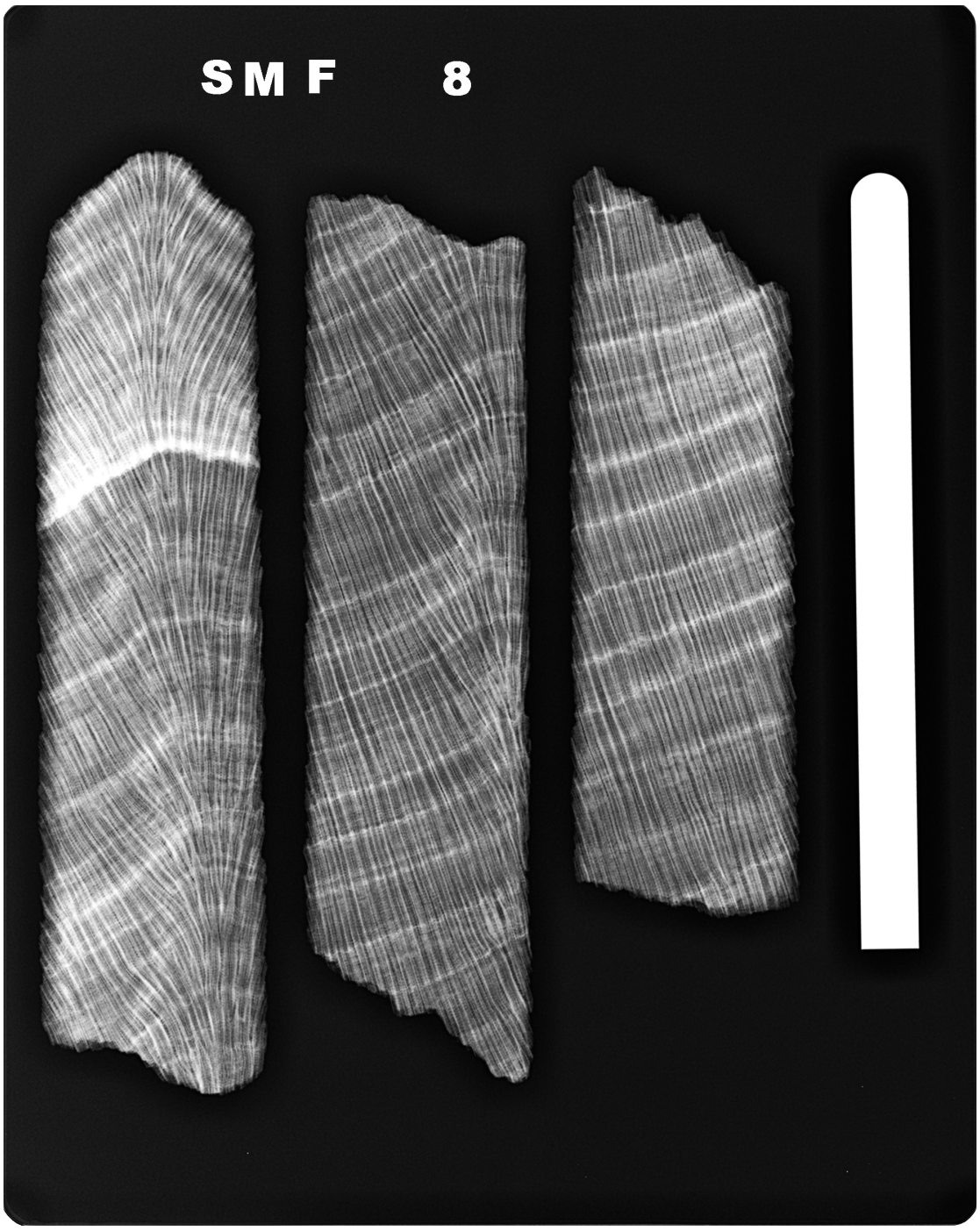

Figure A.1. X-ray image of a sectioned core of a *Montastraea faveolata* coral skeleton, collected at Sapodilla Caye, Belize in 2005. Light and dark density bands reflect seasonal variations in growth rate. The bright layer in the far-left, core-top section represents the 1998 bleaching event. That year, corals worldwide experienced extensive bleaching.

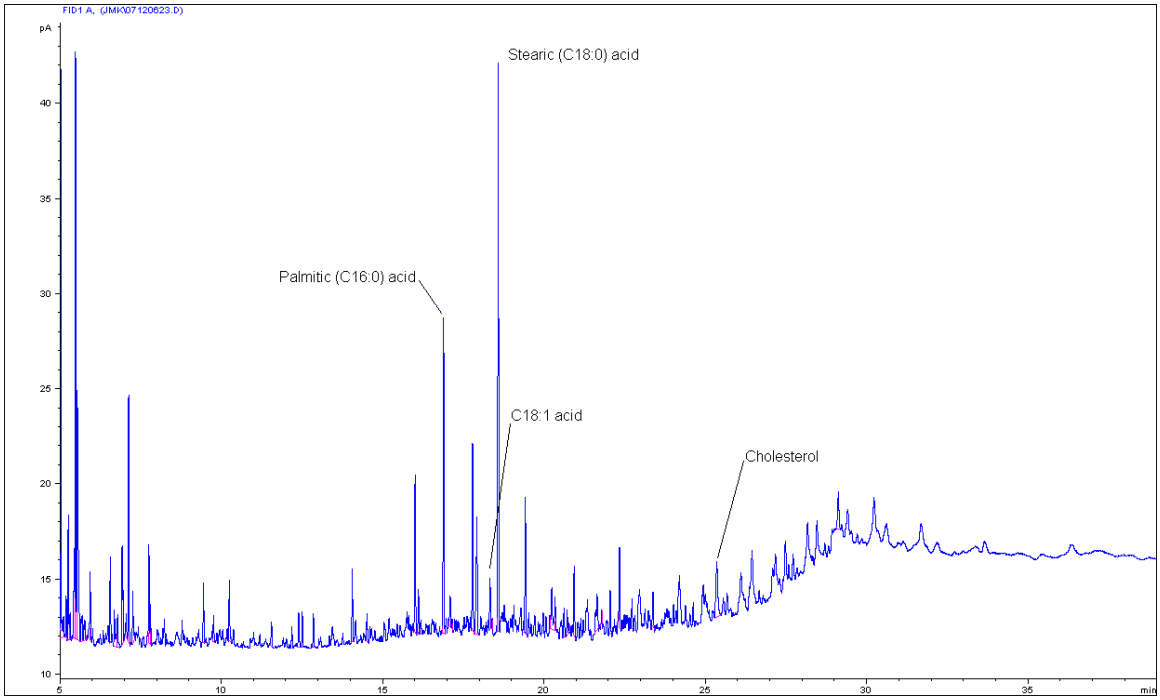

Figure A.2. GC-FID chromatogram of total lipid extract from *Montastraea faveolata* coral skeleton collected at Sapodilla Caye, Belize. In contrast to the cultured *Symbiodinium* lipids, stearic acid (C18:0) is the most abundant lipid. Palmitic acid is also present, but unsaturated acids are much less abundant in coral aragonite than in cultured *Symbiodinium*. Sterols are measurable, but are generally different compounds than those in *Symbiodinium*, with the exception of cholesterol.

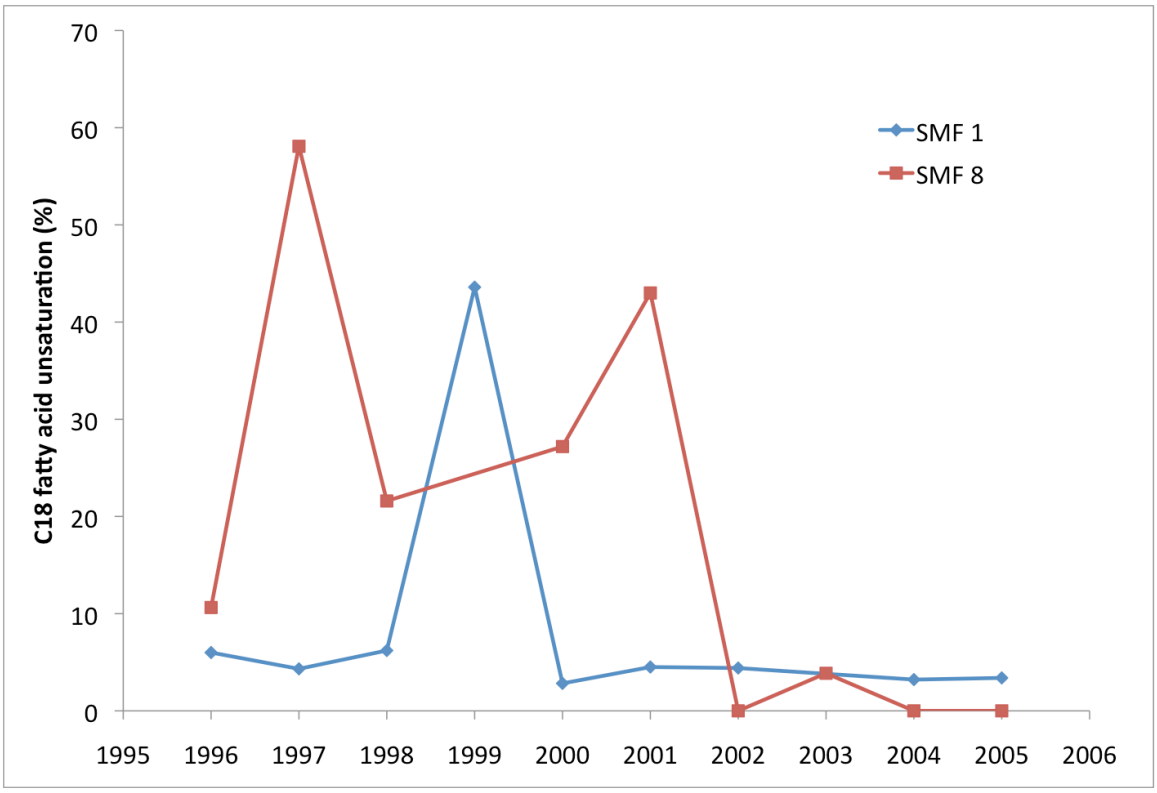

Figure A.3. C18 free fatty acid unsaturation ratio from intracrystalline lipids extracted from skeletal aragonite of two *Montastraea faveolata* colonies.

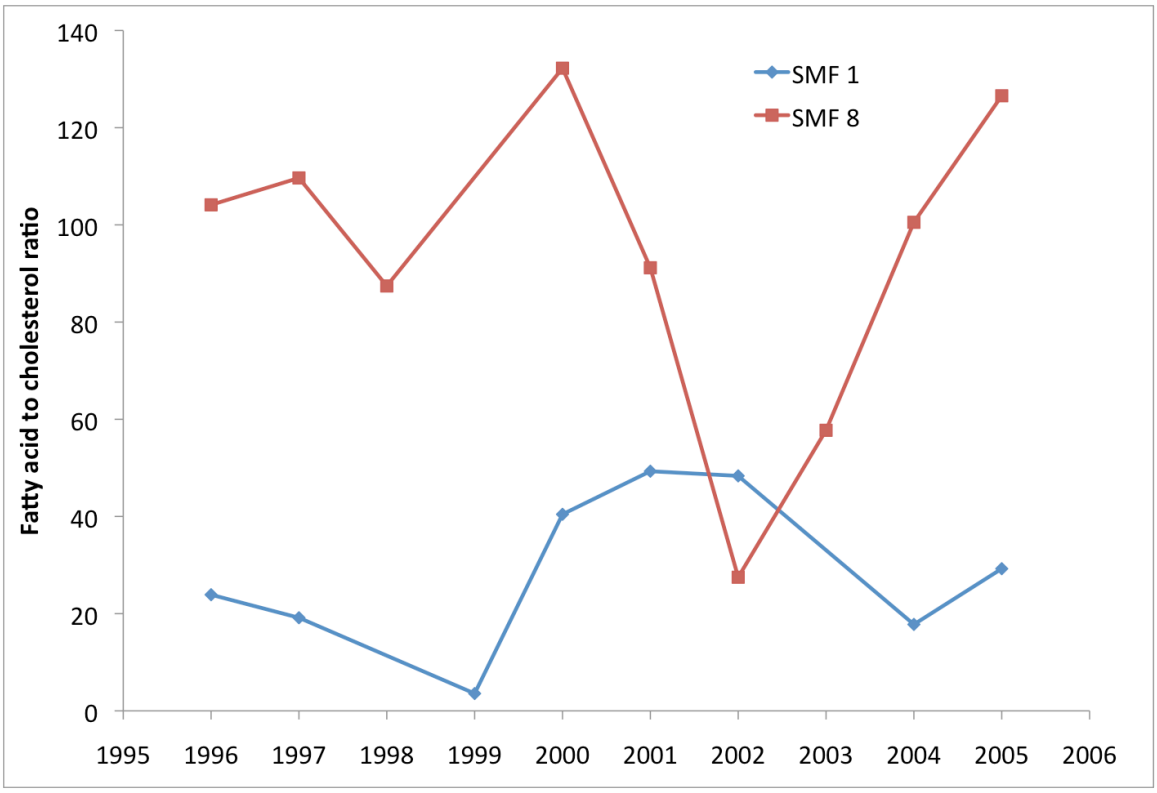

Figure A.4. Fatty acid to cholesterol ratio from intracrystalline lipids extracted from skeletal aragonite of two *Montastraea faveolata* colonies.

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