Lipid biomarkers in *Symbiodinium* dinoflagellates: New indicators of thermal stress

J. Kneeland[1], K. Hughen[1,*], J. Cervino[2], B. Hauff[3], and T. Eglinton[4]

[1]*Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, MA, USA*

[2]*Guest Investigator, Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, MA, USA*

[3]*Michigan State University, Department of Zoology, East Lansing, MI, USA*

[4]*Geological Institute, Eidgenössische Technische Hochschule, Zürich, Switzerland*

**corresponding author*

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Abstract

Lipid content and fatty acid profiles of corals and their dinoflagellate endosymbionts are known to vary in response to high temperature stress. To better understand the heat stress response in these symbionts, we investigated cultures of *Symbiodinium goreauii* type C1 and *Symbiodinium* clade subtype 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, docosahexaenoic (C22:6, n-3) polyunsaturated fatty acid (PUFA), and a variety of sterols. The relative amount of unsaturated acids within the C18 fatty acids in *Symbiodinium* tissue decreases in response to thermal stress. Prolonged exposure to high temperature also causes a decrease in abundance of fatty acids relative to sterols. These shifts in fatty acids and sterols are common to both types C1 and D1, but the apparent thermal threshold of lipid changes is lower for type C1. This work indicates that ratios among free fatty acids and sterols in *Symbiodinium* can be used as sensitive indicators of thermal stress. If the *Symbdionium* lipid stress response is unchanged *in hospite*, the algal heat stress biomarkers we have identified could be measured to detect thermal stress within the coral holobiont.. These results provide new insights into the potential role of lipids in the overall *Symbiodinium* thermal stress response.

Introduction

Tropical reef-building corals around the world are threatened with bleaching and mortality due to high ocean temperatures (Wilkinson 2008), but a quantitative understanding of the thermal stress response remains incomplete. In recent years, anomalously high temperatures have been observed to correlate with coral bleaching (e.g., Goreau and Hayes 1994; Eakin et al. 2010) and may also be implicated in exacerbating coral diseases (Harvell et al. 1999; Cervino et al. 2004; Sutherland et al. 2004). High temperature bleaching episodes in recent years are among the most severe symptoms of a longer-term decline in reef health worldwide (Pandolfi et al. 2003).

High temperature negatively impacts corals and their dinoflagellate symbionts (*Symbiodinium* spp., sometimes referred to as zooxanthellae), and eventually leads to bleaching (Douglas 2003). The symptoms of bleaching include expulsion of the

Symbiodinium from host tissue and loss of photosynthetic pigment within the remaining *Symbiodinium* cells (Gates et al. 1992; Douglas 2003). Proposed mechanisms include damage to photosystem II proteins, disruption of carbon fixation pathways, or caspase-mediated apoptosis induced by reactive oxygen species (Warner et al. 1999; Jones et al. 1998; Tchernov et al. 2011). The heat stress response of the *Symbiodinium* appears to be more ecologically significant than the animal host response (Fitt et al. 2001), and thus is an important contributor to coral bleaching. Different clade subtypes of *Symbiodinium* display different ranges of thermal tolerance, which lends resilience to coral species with flexible symbioses (Baker 2003). The ecological pressure from thermal stress may favor more resilient taxa by "shuffling" within the symbiont population (Buddemeier and Fautin 1993). Repeated thermal stress may therefore result in a population with more heat-tolerant types of *Symbiodinium* (Jones et al. 2008).

Numerous biological symptoms of the *Symbiodinium* thermal stress response have been previously documented. Induction of heat shock proteins in thermally stressed *Symbiodinium* cells occurs in a matter of hours (Black et al. 1995). In less than one hour of high temperature exposure, photosynthesis rates in cultured *Symbiodinium microadriaticum* increased at temperatures up to 30°C and decreased drastically above 30°C (Iglesias-Prieto et al. 1992). The mitotic index (percent of cells dividing) in thermally stressed *Symbiodinium* cultures decreases within days of heat exposure (Bouchard and Yamasaki 2009). *Symbiodinium* growing within the tentacles and mesenterial filaments of the anemone *Anemonia viridis* initially grow faster when temperature is increased 10°C relative to controls. This high-temperature growth spurt occurs on a scale of days, and the faster-growing *Symbiodinium* may be preferentially expelled from the host (Suharsono and Brown 1992). Intracellular nitric oxide production and caspase-like activity, implicated in programmed cell death, also increase after only 24 hours of high-temperature exposure in cultured *Symbiodinium microadriaticum* (Bouchard and Yamasaki 2009).

Changes in the composition of lipids has been documented as part of the thermal stress response of *Symbiodinium* in both cultures and samples from the field (Tchernov et al.

2004; Bachok et al. 2006; Tolosa et al. 2011). The utility of fatty acid unsaturation as an indicator of taxonomic sensitivity to thermal stress was proposed by Zhukova and Titlyanov (2003) and explored in detail by Tchernov et al. (2004), who found that a ratio of specific fatty acids thought to reside in thylakoid membranes was diagnostic of thermally tolerant versus intolerant clades. Further work by Díaz-Almeyda et al. (2011) demonstrated increased melting temperature of thylakoid membranes after 1.5 weeks of thermal stress, associated with differences in membrane lipid composition. Indeed, the melting temperature of *Symbiodinium* membranes varies between different *Symbiodinium* clades and clade subtypes; more thermally susceptible varieties have thylakoid membranes with lower melting temperature (Díaz-Almeyda et al. 2011). Within a single *Symbiodinium* strain, thylakoid fatty acids in cells exposed to high temperature (31°C) for a week or more differ from cells grown at low temperature (24°C), though the compound-level changes remain ambiguous. In general, *Symbiodinium* heat-stress experiments typically terminate after one or two weeks of high-temperature exposure. On the reef scale, however, coral bleaching usually occurs after corals are exposed to unusually high temperature for periods of weeks or longer (Goreau and Hayes 1994), and the longer-term evolution of *Symbiodinium* biochemistry in heat-stressed cells remains unexplored.

Here we present a characterization of the effect of high temperature on *Symbiodinium* lipids over a period of four weeks. Consistent with the common use of ratios within the same compound class as proxies of environmental condition (e.g., Eglinton and Eglinton 2008), we identified ratios among the abundant *Symbiodinium* lipids that are sensitive to thermal stress. These lipid biomarker ratios are sensitive to heat stress over temperatures and timescales similar to bleaching episodes in the field. Analyses of free (unbound) lipid extracts were compared with saponified (total lipid) samples to show that the sense of change in the saponified lipid ratios agreed with the biomarkers characterized within the free lipids. These new thermal stress indicators could be used to diagnose symbiont stress within the coral holobiont, and contribute to understanding the thermal stress response in *Symbiodinium*.

Materials and Methods

Symbiodinium types and culture conditions

Clones of *Symbiodinium* spp. were obtained from the Bigelow Laboratory for Ocean Sciences in Boothbay, ME. Two strains with differing reported heat sensitivity were chosen for this experiment. Batch cultures of types C1 (*S. goreauii* strain CCMP 2466, originally isolated from the anemone *Discosoma sancti-thomae*; hereafter "type C1") and D1 (CCMP 2556, originally isolated from the coral *Montastraea faveolata*; hereafter "type D1") were employed. Type C1 is an ecologically and geographically abundant type (e.g., LaJeunesse et al. 2003) reported to be relatively sensitive to heat stress (e.g., LaJeunesse et al. 2010). *Symbiodinium* type D1 is reported to be thermally tolerant and is abundant in areas where temperatures are high or highly variable (e.g., Mostafavi et al. 2007).

Each *Symbiodinium* stock culture was initially maintained at 24°C. Each stock culture was homogenized and 500 µl was placed in each 15ml glass test tube with 10 ml of f/2 growth medium. Cultures were maintained under 12/12 hour light/dark cycles with ambient indoor fluorescent lighting. Light levels experienced by the cultures were not quantified, but this parameter was also kept constant among the experimental treatments. Cultures were nourished in f/2 growth medium at 26°C, 28°C, 30°C, and 32°C for a period of up to 4 weeks. Temperatures were regulated by thermal baths. The test tube cultures were supplemented with fresh f/2 medium twice weekly throughout the incubation period to maintain nutrient levels for growth and compensate for evaporation. Approximately 2 ml f/2 was added to the test tubes at each exchange; this amount was sufficient to maintain approximately equal volumes in all cultures. Samples of the stock culture (24°C) were frozen at the start of the experiment (t=0) as controls for comparison to the later time evolution of heat-treated samples. Within each *Symbiodinium* type, three individual test tubes (triplicate cultures) for each temperature treatment were collected for analysis each week. The test tubes were stored at -4°C prior to extraction for lipid analysis.

Lipid characterization

Lipids were extracted from the cultures with a modified Bligh and Dyer (1959) procedure. Frozen culture vials were allowed to just thaw, and the pellet of cells that settled to the bottom was carefully pipetted into a pre-combusted glass centrifuge vial. If the pellet had not sufficiently settled, the entire culture was transferred to the glass centrifuge vial and samples were centrifuged at low speed for several minutes to concentrate the cells in the bottom of the vial. We then added to the cell pellet: 1 ml dichloromethane (DCM), 2 ml methanol, and 0.8 ml phosphate-buffered saline (PBS). The centrifuge vials were sonicated for 5 minutes. Next we added 1 ml DCM and 1 ml PBS. The vials were again sonicated 5 minutes. The vials were finally centrifuged at low speed to aid in separating the polar (aqueous) and non-polar (organic) phases. The organic phase (total lipid extract or TLE) was carefully pipetted into a clean 4-ml glass vial. Extracts were concentrated by blowing down under a gentle nitrogen stream. Concentrated extracts were run through a pre-combusted sodium sulfate column to remove residual water. An aliquot of each extract was concentrated under gentle nitrogen and derivatized by adding 25µl each of DCM, pyridine and bis-(trimethylsilyl)trifluoroacetamide (BSTFA). Compound concentrations were quantified on an Agilent 6850 series gas chromatograph with flame ionization detection (GC-FID); compound identification was determined by gas chromatograph time-of-flight mass spectrometry (GC-TOF-MS) with comparison to spectra and retention times of authenticated standards. Where lipid concentrations were deemed inadequate for good quantitation, a fresh aliquot of the TLE was derivatized using a smaller total volume of solvent and derivatization agents (e.g., 10µl DCM and 5 µl each of pyridine and BSTFA).

Saponification

Several extracts were also 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. Saponified fatty acids and sterols were then analyzed by GC-FID as above.

Statistical Analysis

For each *Symbiodinium* type, 3 culture vials (independent replicates) were collected and analyzed at each of 4 different times, under 4 different temperature regimes (16 experimental conditions). Some replicates were lost during processing, and the amount of lipid extracted from others was insufficient for calculation of the target lipid ratios, and thus the number of independent replicates was reduced. We analyzed multiple aliquots of some replicates to increase concentrations and to assess repeatability of the measurement. The total numbers of independent replicates (and aliquots within each independent replicate) for each experimental treatment are provided with the datasets in Supplemental Tables S1 and S2. For statistical analyses, data from all aliquots within each replicate culture were averaged together. To compare results between the 16 different experimental treatments, we performed a 4 x 4 factorial analysis of variance (4x4 ANOVA) using VassarStats. For each of the 3 lipid parameters calculated, the 4x4 ANOVA was used to assess significance of temperature, time, and temperature x time interaction effect. The significance of each experimental parameter is reported at the 95% ($p < 0.05$), 99% ($p < 0.01$), or 99.9% ($p < 0.001$) confidence level. Differences between temperatures, times and individual treatments were assessed for significance by comparing to Tukey's Honestly Significant Difference (HSD) at the 95% confidence level. Potential differences between the two *Symbiodinium* types were assessed conservatively by comparing to the larger of the two Tukey's HSD values for each parameter. Summary statistics (df, F ratio, p-value and Tukey HSD) for type C1 and type D1 lipid ratios are presented in Tables 1 and 2. For data plotting purposes, the mean and standard deviation were calculated for each of the 16 experimental treatments. Lipid ratios measured in the 24°C stock cultures are plotted at time 0; these data were not included in the statistical treatment.

Results

Lipid ratios

The most abundant lipids produced by *Symbiodinium* were saturated and unsaturated short-chain fatty acids, and a medium-chain PUFA, described below. The fatty acids included

in these lipid ratios represent approximately 50% of the total lipid extract in both *Symbiodinium* types. The sterols accounted for an average 39% of type C1 lipids and 32% of D1 lipids. The remaining 11% of C1 lipids and 18% of D1 lipids consisted of other sterols, fatty acids, and other lipid classes such as alkanes that were not further quantified due to low abundance. Lipid data for each compound as a percentage of lipid quantified in each aliquot is reported in Supplemental Table S1. The same data, averaged together for each experimental treatment, is reported in Supplemental Table S2. Further data analysis focused on lipid ratios calculated among the most abundant compounds, in order to identify biomarker proxies that are both easily measured, and sensitive to heat stress. Within the abundant fatty acids and sterols, several ratios stood out for their sensitive response to thermal stress.

Fatty acid unsaturation

The most abundant saturated fatty acids in *Symbiodinium* tissue were palmitic (C16:0) and stearic (C18:0) acids. Unsaturated fatty acids included palmitoleic (C16:1), oleic (C18:1), linolenic (C18:3) and stearidonic (C18:4) acids, as well as the PUFA docosahexaenoic acid (C22:6, n-3; DHA). Linoleic (C18:2) acid coeluted with oleic acid, and mass spectrometry showed it to be a minor contributor to the oleic acid peak in gas chromatograms. We focused further analyses on the most abundant fatty acids in the two cultured *Symbiodinium* strains.

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

In type C1, the temperature, time, and temperature x time interaction effects are all significant for C18 unsaturation ($p < 0.001$; Table 1). At low temperature conditions (26°C and 28°C), both *Symbiodinium* types showed high mean C18 fatty acid unsaturation ratios (88 ± 3% in type C1 [N=20] and 86 ± 2% in type D1 [N=23], 1 sigma), which did not change significantly over the 4-week experiment (Fig. 1). There were no significant differences between the two *Symbiodinium* types at 26°C and 28°C. However, at higher temperatures the C18 unsaturation ratio showed a strong decrease in response to thermal stress. In the more

thermally sensitive type C1, the C18 unsaturation began decreasing after 1 week of exposure to 32°C and continued declining to a low value around 30% unsaturated in weeks 3 and 4. At the more moderate thermal treatment of 30°C, the C18 unsaturation in type C1 first declined significantly below low temperature values after 2 weeks of high temperature exposure, and was statistically indistinguishable from the highest temperature values after 3 weeks of moderate thermal stress.

In the more thermally tolerant type D1, high temperature also caused a decline in C18 fatty acid unsaturation (Fig. 2). As with C1, the time, temperature, and interaction effects in type D1 were all significant ($p < 0.001$; Table 2) for C18 unsaturation. Exposure of D1 to 32°C caused a decline in C18 unsaturation after just one week of exposure, and values declined below 30% in weeks 3 and 4, as with type C1. However, at the intermediate 30°C treatment, type D1 showed no decrease in C18 unsaturation until week 3. After 4 weeks of exposure to 30°C, C18 unsaturation in type D1 dropped to a value around 30%, consistent with the highest temperature treatment. These data are consistent with a higher threshold for heat stress in type D1 compared to type C1. At the highest thermal stress conditions, however, C18 fatty acid unsaturation ratios in both types converged to the same low value.

Fatty acid to sterol ratios

We also observed a decrease in the concentration of fatty acids relative to sterols in heat-stressed cultures. This decrease was evident in several different ratios of fatty acids to sterols. Figure 3 shows the ratio of summed fatty acids (C16:0, C16:1, C18:0, C18:1, C18:3, C18:4, and C22:6) to summed sterols (cholesterol, dinosterol and the 4-methyl sterol, 4α,24-dimethyl-5α-cholestan-3β-ol). Fatty acid to sterol ratios were more variable within the low temperature cultures than the C18 unsaturation. Fatty acid to sterol ratios typically increased slightly in response to intermediate levels of heat stress (short time at the highest temperature, or moderate exposure time at moderately high temperature), but the fatty acid to sterol ratios rapidly decreased as the heat stress persisted.

Both *Symbiodinium* types showed slightly elevated fatty acid to sterol ratios at 28°C relative to 26°C (Figs. 3 and 4). However, there was no consistent temporal trend in fatty acid to sterol ratios at either 26°C or 28°C. At low temperatures, there was also no significant difference in fatty acid to sterol ratios between the two types.

Type C1 fatty acid to sterol ratios showed statistically significant temperature ($p < 0.01$), time ($p < 0.01$) and interaction ($p < 0.05$) effects (Table 1). In response to the highest temperature (32°C), fatty acid to sterol ratios decreased somewhat in the first week, and decreased significantly after 2 weeks of high temperature exposure. In the more moderate 30°C treatment, the fatty acid to sterol ratio initially increased, and then dropped after two weeks.

Type D1 fatty acid to sterol ratios also showed statistically significant temperature ($p < 0.001$), time ($p < 0.01$) and interaction ($p < 0.01$) effects (Table 2). In type D1, the fatty acid to sterol ratio increased slightly after 1 week at 32°C, and fell sharply in subsequent weeks. At 30°C, fatty acid to sterol ratios increased in the first two weeks and then decreased significantly after 4 weeks.

In the ratio of summed fatty acids to sterols, there again appeared to be a threshold type response. For the reportedly more sensitive type C1, the response occurred earlier and was more severe at intermediate thermal stress levels than for type D1. In contrast to C18 unsaturation, moderate thermal stress caused an initial elevation of fatty acid to sterol ratios.

The clear pattern of decrease in total fatty acid to sterol ratios at high heat stress was observed in a variety of different ratios of individual compounds as well. To develop a thermal stress indicator that could be used in whole coral (holobiont) samples, which may include confounding lipid signatures from coral animal host tissues, we investigated compounds, docosahexaenoic acid (DHA; C22:6(n-3) PUFA) and the 4-methyl sterol 4α,24-dimethyl-5α-cholestan-3β-ol, that are known to be concentrated in dinoflagellates but are not synthesized by coral (Volkman et al. 1998; Volkman 2003). The ratio of DHA to 4-methyl sterol displayed a thermal stress response similar to the bulk fatty acid to sterol ratio (Fig. 5).

This ratio of algal-specific compounds showed somewhat greater scatter at low temperatures. In fact, the time effect and temperature x time interaction effect were not statistically significant in type C1, though the temperature effect was significant ($p < 0.05$; Table 1). The DHA to 4-methyl sterol ratio nonetheless follows the same general pattern of initial increase at moderate temperatures and durations followed by a large decrease in response to greater heat stress. For type D1 (Fig. 6), the time, temperature, and interaction effects are all significant ($p < 0.01$; Table 2). The apparent thresholds for thermal stress determined by this individual compound ratio are comparable to those for the summed fatty acid to sterol ratio.

Other individual saturated and unsaturated fatty acids, when compared to cholesterol, 4α,24-dimethyl-5α-cholestan-3β-ol, or a sum of the most abundant sterols, show a similar pattern (data not shown). Exposure to moderately high temperature initially causes an increase in the fatty acid to sterol ratio, but after 1 to 3 weeks at high temperature the fatty acid to sterol ratios decrease dramatically. Some unsaturated fatty acids (e.g., C18:3) fall below detection limit at the highest temperature stress. 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 type C1 than for type D1.

Saponified (total) fatty acids

To test whether the heat stress indicators measured in free fatty acids represent the total lipid pool, we saponified a subset of extracts to release fatty acids bound in diglycerides, triglycerides, and wax esters. Since lipids from different portions of the cell would be included in this analysis, we did not expect absolute values of the lipid ratios to be comparable. Rather, we wanted to assess whether the magnitude and sense of change in reponse to thermal stress would be similar in this broader lipid pool. Saponified C18 fatty acids showed a decrease in unsaturation at high thermal stress (Fig. 7). The unstressed (26°C) C18 unsaturation value for the saponified fatty acids is slightly lower than in the free fatty acids (80-85% vs. 86-88%, respectively). Saponified C18 fatty acid unsaturation shows a decrease after 4 weeks of exposure at 32°C, although the magnitude of the decrease is not as

large as for free fatty acids. From this we conclude that bound fatty acid pools contain relatively more stearic acid (C18:0). Type C1 again shows a more severe response to high temperature stress than type D1 (Fig. 7).

Due to low sterol abundance in the saponified extracts, we report summed fatty acids relative to the most abundant sterol cholesterol only. The ratio of summed fatty acids to cholesterol in saponified samples declined at high temperature (Fig. 8). There was variability among the low temperature samples, but after 1 week at higher temperature (32°C) fatty acid to sterol ratios declined relative to low temperature (26°C) cultures. The total fatty acid to cholesterol ratio did not show clear differences between the two *Symbiodinium* types. Overall, the saponified samples indicated that the overall patterns of lipid stress signatures we observed are a robust feature of the cell's total lipid pool.

Discussion

Lipid profiles in cultured *Symbiodinium* showed that under heat stress sustained for a period of weeks, the relative amount of fatty acids decreased compared to sterols in a temperature, time, and type-dependent manner. Poly-unsaturated fatty acids (PUFAs) accounted for the majority of the C18 fatty acids, but their abundance relative to stearic acid (C18:0) also declined in heat-stressed cultures.

Previous studies have suggested that a number of factors can influence lipid concentrations and distribution in corals and their symbionts, including availability of food in combination with both light intensity and short-term thermal stress (Treignier et al. 2008; Tolosa et al. 2011), severity of bleaching events (Bachok et al. 2006), and symbiont taxonomy (Zhukova and Titlyanov 2003; Tchernov et al. 2004). Treignier et al. (2008) found that high light intensity, and to a lesser extent high food availability, resulted in greater concentrations of lipids, including fatty acids and sterols. Similarly, Tolosa et al. (2011) showed that fed corals had increased concentrations of fatty acids and sterols and were much more resistant to thermal stress, with a smaller decrease in lipid content at high temperatures than starved corals.

Zhukova and Titlyanov (2003) investigated fatty acid distributions in polar lipids and triacylglycerols in *Symbiodinium* isolated from several coral species. They found distinct differences in fatty acid profiles of polar lipids between morphotypes of *Symbiodinium*, and suggested that fatty acids might be used to distinguish between symbiont taxa. Tchernov et al. (2004) measured fatty acids in *Symbiodinium* clones they categorized as either thermally sensitive or tolerant. They observed a higher ratio of C18:1 to C18:4 fatty acid in thermally tolerant types, i.e., the relative PUFA content was lower in sensitive types. Tchernov et al. (2004) interpreted the lower PUFA content in terms of a different composition in the thylakoid membranes of sensitive *Symbiodinium* strains, and they further noted that high PUFA content protects thylakoid membranes from oxidative damage by reactive oxygen species (ROS) during thermal stress. The lipids we measured generally corroborate the conclusion that PUFA content is high in unstressed cells. However, we observed that PUFA contents in unstressed cultures of type C1 and D1 show the same high value, and PUFA contents in highly stressed cultures of both types show the same low value. The observed similarity between the two types at low temperature corroborates the conclusion of Díaz-Almeyda et al. (2011) that lipid ratios in unstressed cultures are not sufficient to diagnose the thermal sensitivity of *Symbiodinium* varieties. PUFA content (and the C18:1/C18:4 ratio specifically, Supplemental Tables S1, S2) is thus not diagnostic of thermal sensitivity or tolerance in either unstressed or highly stressed *Symbiodinium*. Rather, these results indicate that differences in C18 unsaturation between types can instead be used to diagnose differences in the degree of thermal stress experienced by the symbionts.

Some of the changes we observed in fatty acid unsaturation may be attributable to varying contributions from membrane lipids *vs*. storage lipids. Unsaturated C18 fatty acids, and particularly C18:4 fatty acid, make up an unusually high percentage of the membrane lipids in cold-adapted dinoflagellates (LeBlond et al. 2006). On that basis, we may interpret the decreasing C18 fatty acid unsaturations either as a decrease in unsaturation within the membranes, or as a relative increase of more saturated energy storage lipids. It is understood that symbionts transfer their fatty acids to the host coral (Papina et al. 2003), and corals

harboring predominantly type C *Symbiodinium* contain a relatively greater amount of energy storage lipids compared to membrane lipids (Cooper et al. 2011). Both membrane and storage lipids contribute to the free fatty acids we measured. We cannot distinguish between a decrease in storage lipid, an increase in membrane lipid, or a change in membrane lipid unsaturation on the basis of these data. Further studies of intact polar lipids may help to resolve whether changes in unsaturation are driven primarily by membranes or storage lipids.

Fatty acid to sterol ratios consistently decreased in the high temperature cultures, and the relative magnitude of changes in individual fatty acids and sterols (Supplemental Tables S1, S2) indicates that we can rule out an increase in sterol content as the cause of these changes. We note that sterols are vital membrane components in all eukaryotic cells, including dinoflagellates (Volkman 2003). Sterols are associated with saturated fatty acids within lipid bilayer membranes (Pitman et al. 2004), and heterogeneous cholesterol content contributes to lipid raft formation (Simons and Ikonen 1997). Cholesterol in PUFA-rich membranes may also contribute to the disorder and fluidity of the membrane (Harroun et al. 2008). In starved corals (i.e. those relying primarily on symbiont photosynthate for nutrition), sterol content decreases, rather than increases, following temperature stress (Tolosa et al. 2011). Moreover, because the ratios of fatty acids to sterols are large in the low-temperature cultures, the magnitude of decrease in ratios for thermally stressed treatments would require a greater than 10-fold increase in sterol content, in contrast to only a 90% decrease in fatty acids. Given the lack of evidence for large changes in sterol concentrations, and the mobility of free fatty acids (discussed further below), we interpret the decline in fatty acid to sterol ratios as decreasing fatty acid concentrations.

Symbiodinium translocate fatty acids to the coral host (Papina et al. 2003), and thus coral lipid variations are likely explained by symbiont lipid changes. Studies that address lipid content in coral holobiont analyses often make no effort to separate symbiont tissue, and thus coral lipid content often includes a substantial contribution from *Symbiodinium* cells. In whole coral analyses, total lipid content generally decreases in response to thermal stress. Grottoli et al. (2004) observed a decrease in 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 content in bleached *Pavona frondifera* corals compared to healthy specimens. Taken together, these results suggest that the low relative abundance of fatty acids in the heat stressed *Symbiodinium* may be a result of decreased total production of lipid, particularly those rich in fatty acids.

The sharp decrease in unsaturated fatty acids is striking, and can also be considered in the context of coral field samples. Particularly, the *Symbiodinium* stress biomarkers we identified may help interpret otherwise conflicting reports of lipid changes in stressed zooxanthellate corals. Comparison between fatty acid profiles from coral host *Montipora digitata* and its symbionts showed that *Symbiodinium* produce more polyunsaturated fatty acids, particularly C18:3(n-3) (Papina et al. 2003). Both C18:3 and C18:4 PUFAs are abundant in the homogenized tissue of zooxanthellate octocorals, but not in an azooxanthellate species (Imbs et al. 2009). However, in another study, *Symbiodinium* isolated from 5 different coral species showed almost no contribution of C18:3, but rather much higher C18:4 (Zhukova and Titlyanov 2003). Papina et al. (2003) and Imbs et al. (2009) examined saponified (total) fatty acids, whereas Zhukova and Titlyanov (2003) analyzed free fatty acids; the present study confirms that different fatty acids are expected in free vs. saponified *Symbiodinium* extracts. These field studies found high abundance of PUFAs in coral symbionts, which is consistent with the high C18 unsaturation index observed in unstressed cultures. Extracts of whole-coral tissue in Japanese corals showed generally low C18 unsaturation, except in a few samples, notably *Porites lutea* (Yamashiro et al. 1999). If the dominance of unsaturated C18 acids observed in the *Symbiodinium* cultures is characteristic of all photosymbionts, this could indicate that the *P. lutea* coral specimen examined by Yamashiro et al. (1999) received a greater portion of its fatty acids from *Symbiodinium*, 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 *Symbiodinium* in those corals were experiencing thermal stress that was nonetheless below the threshold for visible bleaching.

High amounts of C18:3 may be indicative of rapid cell growth (Piorreck and Pohl 1984; Napolitano et al. 1997), since decreasing nutrient availability may cause a shift from predominantly polar lipids to neutral lipids (and thus a change in associated fatty acids). We regularly replenished f/2 growth medium throughout the culture period, and observed no consistent temporal change at low temperature. This suggests that we may rule out nutrient limitation in these cultures. In addition, the type-specific differences in thresholds for lipid response strongly support a thermal stress interpretation. Finally, the decrease in fatty acids relative to sterols is inconsistent with a build-up over time of neutral lipids, primarily triacylglycerols, which are rich in fatty acids. However, we cannot rule out the possibility that the addition of f/2 medium caused some physiological changes; differences in nutrient availability may have contributed to the scatter we observe between replicates. This study only addressed the potential affect of thermal stress on *Symbiodinium*. Other environmental stressors may also cause variability in the lipid ratios we observed. Light stress, in particular, is well known to impact corals (e.g., Brown et al. 1999; Fitt et al. 2001; Warner et al. 1999). Light stress may exacerbate the effects of thermal stress (e.g., Fitt et al. 2001), and thermal stress may slow the repair of cellular photosynthetic machinery (see discussion below). Ecological and environmental parameters such as the history of thermal exposure and *Symbiodinium*-coral association (Baker 2003; Cooper et al. 2011; Jones et al. 2008; LaJeunesse et al. 2010) may additionally impact the overall thermal stress response.

In order to detect *Symbiodinium* heat stress a variety of thermal stress indicators have been used, including photosynthetic efficiency (quantum yield of PS II ΔF/Fm; e.g., Brown et al. 1999; Fitt et al. 2001; Warner et al. 1999), heat shock proteins (e.g., Black et al. 1995), D1 protein within PSII (e.g., Warner et al. 1999), photosynthetic pigments (Hoegh-Guldberg and Smith 1989; Brown et al. 1999), and genetic markers (Vidal-Dupiol et al. 2009). Electron transport within the chloroplast and photochemical efficiency (Fv/Fm) both decrease in thermally stressed plants (Mishra and Singhal 1992). Quantum yield in clade C symbionts decreases during thermal stress and stays low during recovery, whereas stressed clade D *Symbiodinium* increases quantum yield (Rowan 2004). Rowan (2004) interpreted their results

as chronic photo-inhibition in the sensitive clade and photo-protection in the tolerant clade. The negative effect of thermal stress on PS II quantum yield in zooxanthellate corals may however be mitigated by adequate feeding (Borell and Bischof 2008; Tolosa et al. 2011). The timescales of change in photosynthetic efficiency experiments are generally shorter than this experiment and may not be applicable to longer-term bleaching events. Most importantly, measurements of photosynthetic efficiency require living cells; either a diving PAM fluorometer or immediate lab access is needed. D1 and heat shock proteins are also susceptible to degradation; in general the turnover time for proteins is faster than for lipids. Pigments are most amenable to later lab analysis, though normalization of pigment concentration within the holobiont is subject to uncertainty. All of these methods provide useful information, and lipids offer an additional, currently underutilized window on *Symbiodinium* and coral stress. Lipids degrade slowly and have long been used as environmental and ecological biomarkers (Eglinton and Eglinton 2008). In addition, the present results indicate that lipids in coral *Symbiodinium* respond to thermal stress over ecologically relevant timescales and thus may provide unique insights for understanding the cause and mechanism of coral bleaching.

Corals produce lipids, including saturated fatty acids and sterols (Oku et al. 2003; Papina et al. 2003), which could dilute or bias *Symbiodinium* stress indicators measured from lipids in the holobiont. To overcome this potential challenge to the usefulness of lipid-based stress indicators, we investigated compounds specific to dinoflagellates. In particular, the C22:6(n-3) PUFA DHA and 4-methyl sterol 4α,24-dimethyl-5α-cholesta-3β-ol are attributable to dinoflagellates (Volkman et al. 1998). The ratio of DHA to 4-methyl sterol makes a particularly good marker of *Symbiodinium* stress within the holobiont, because coral host lipids in the more complicated holobiont system would not impact the ratio. Measurable concentrations of 4α,24-dimethyl-5α-cholesta-3β-ol in the absence of DHA could indicate thermal stress in the *Symbiodinium in hospite.* This symbiont-specific heat stress marker may

also allow discrimination between *Symbiodinium* and coral host stress as the dominant factor in bleaching.

These lipid measurements demonstrate that thermal stress causes significant declines in PUFAs, both relative to saturated fatty acids and relative to sterols. One explanation for a decreased PUFA inventory is an increase in PUFA degradation. ROS produced in thermally stressed cells may degrade PUFAs in the thylakoid membrane (Tchernov et al. 2004). PUFAs may be further degraded to poly-unsaturated aldehydes (PUAs) (Catalá 2009). In particular, ROS, known to be abundant in thermally stressed cells (Lesser 2006), could catalyze the PUFA to PUA reaction through a lipid peroxidation intermediate (Catalá 2009). PUAs from algal cells have been implicated in apoptosis induction (Andrianasolo et al. 2008) and could be responsible for ultimate *Symbiodinium* mortality following thermal stress. Peroxidation of thylakoid PUFAs has been documented in response to light and heat stress in plants (Mishra and Singhal 1992). PUFAs may also be important for the cell's ability to regulate membrane fluidity and tolerate thermal stress (Stubbs and Smith 1984). 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; the difference was attributed to PSII repair efficiency (Gombos et al. 1994). The capacity to efficiently repair thermally damaged PSII structures may also determine thermal stress tolerance in *Symbiodinium* (Takahashi et al. 2009). Recent experiments with *Arabidopsis thaliana* mutants demonstrated that tri-unsaturated fatty acids are abundant in wild-type thylakoid membranes and are required for thermal tolerance (Routaboul et al. 2012). The faster decline in fatty acid unsaturation observed in the type C1 cultures compared to type D1 may likewise account for increased heat sensitivity in type C1. We find that lipids extracted from cultured *Symbiodinium* types C1 and D1 show systematic and significant changes in response to thermal stress. With prolonged heat exposure, the relative amounts of unsaturated C18 fatty acids decrease and overall fatty acid to sterol ratios decrease. The dramatic declines in PUFA concentration relative to saturated fatty acids and sterols in heat-stressed samples are consistent with a *Symbiodinium* molecular cascade mechanism ultimately leading to PUA production and an

apoptosis-like response. The lipid stress response occurs in both types C1 and D1, but type C1 responds earlier and at lower temperature, in agreement with the higher thermal tolerance reported for type D1 (Baker 2003). We have identified lipid ratios for unstressed and highly stressed *Symbiodinium* cultures that do not differ between the two *Symbiodinium* types with very different reported thermal tolerance. However, the difference in stress response between types is clear in the offset timing of lipid changes at intermediate levels of stress. The temporal evolution of this thermal stress response corresponds to timescales expected to cause thermal bleaching in the coral reef environment, although the full development of this heat stress response would not be evident in experiments of shorter duration. This study is the first to demonstrate this longer-term lipid response in two ecologically important *Symbiodinium* types of widely different thermal tolerance.

From this work we anticipate that these *Symbiodinium* stress signals should be measurable in corals. Careful culturing of zooxanthellate corals, addressing the potential effects of other stressors such as light and feeding status, will help test these biomarker indicators for eventual field use. Finally, investigation of intact polar lipids may help to identify the cellular source of these stress indicators within specific lipid classes (e.g., phospholipids and glycolipids). In-depth analysis of lipids from separated coral animal tissue and *Symbiodinium* growing *in hospite* could determine whether lipid profiles from the holobiont system are useful as markers of *Symbiodinium* heat stress.

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Figure legends

Figure 1. Unsaturation ratio of C18 free fatty acids in *Symbiodinium* type C1 as a function of thermal stress. The C18 % unsaturation is defined as the ratio of the total unsaturated C18 fatty acids (C18:1, C18:2, C18:3, and C18:4) over the sum of all C18 fatty acids, including C18:0. Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ±SD), and data at time 0 are from 24°C cultures. For number of replicates, see Supplemental Tables S1 and S2.

Figure 2. Unsaturation ratio of C18 free fatty acids in *Symbiodinium* type D1 as a function of thermal stress. The C18 % unsaturation is defined as the ratio of the total unsaturated C18 fatty acids (C18:1, C18:2, C18:3, and C18:4) over the sum of all C18 fatty acids, including C18:0. Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ±SD), and data at time 0 are from 24°C cultures. For number of replicates, see Supplemental Tables S1 and S2.

Figure 3. Ratio of total fatty acids to sterols in *Symbiodinium* type C1 as a function of thermal stress. 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 C22:6(n-3)) to the sum of sterols (cholesterol, dinosterol and 4α,24-dimethyl-5α-cholesta-3β-ol). Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ±SD), and data at time 0 are from 24°C cultures. For number of replicates, see Supplemental Tables S1 and S2.

Figure 4. Ratio of total fatty acids to sterols in *Symbiodinium* type D1 as a function of thermal stress. 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 C22:6(n-3)) to the sum of sterols (cholesterol, dinosterol and 4α,24-dimethyl-5α-cholesta-3β-ol). Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ±SD), and data at time 0 are from 24°C cultures. For number of replicates, see Supplemental Tables S1 and S2.

Figure 5. Ratio of algal-specific fatty acid (docohexaenoic acid C22:6(n-3)) to 4-methyl sterol (4α,24-dimethyl-5α-cholesta-3β-ol) in *Symbiodinium* type C1 as a function of thermal stress. Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ±SD), and data at time 0 are from 24°C cultures. For number of replicates, see Supplemental Tables S1 and S2.

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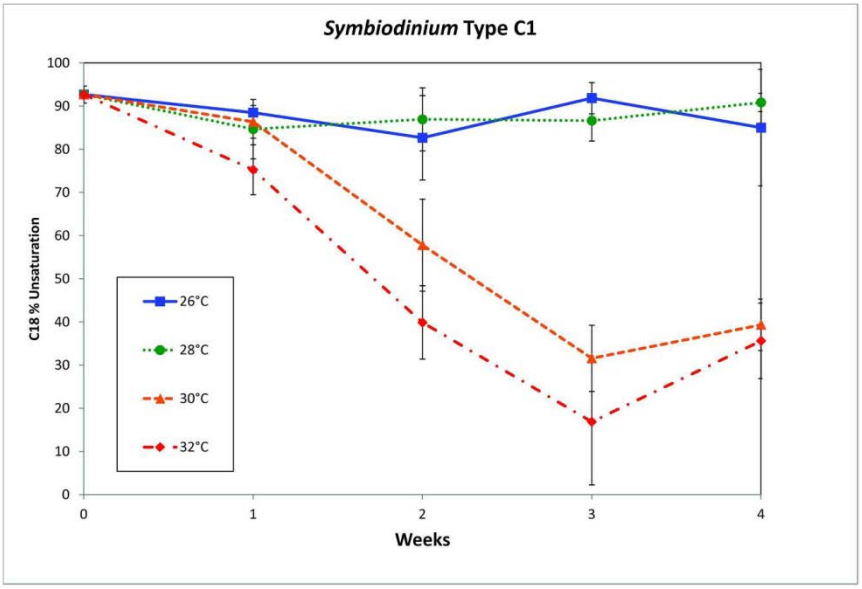
Figure 7. C18 % unsaturation in saponified (total) fatty acids from *Symbiodinium* types C1 and D1 as a function of thermal stress. Saponifying lipid extracts releases fatty acids previously bound in diglycerides, triglycerides, and wax esters. Similar to results for free fatty acids, the total C18 fatty acid pool shows a decrease in unsaturation due to high thermal stress, here 32°C for 4 weeks. A single extract was saponified and measured for each treatment.

Figure 8. Total fatty acid to sterol ratios for saponified lipid extracts from *Symbiodinium* types C1 and D1 as a function of thermal stress. Saponifying lipid extracts releases fatty acids previously bound in diglycerides, triglycerides, and wax esters. The ratio of summed fatty acids to cholesterol in saponified samples shows a decline in fatty acids at high temperature for both clades C1 and D1, similar to results for free lipids. A single extract was saponified and measured for each treatment.



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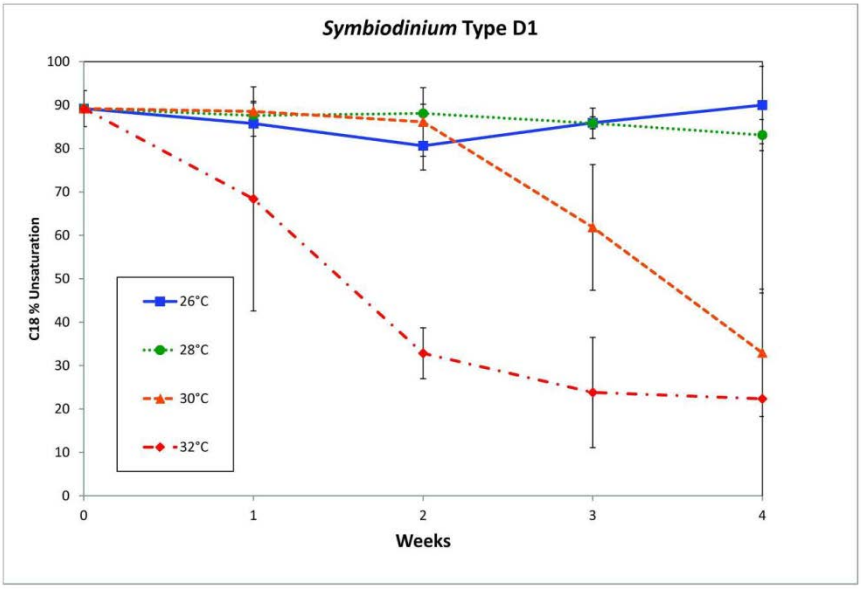

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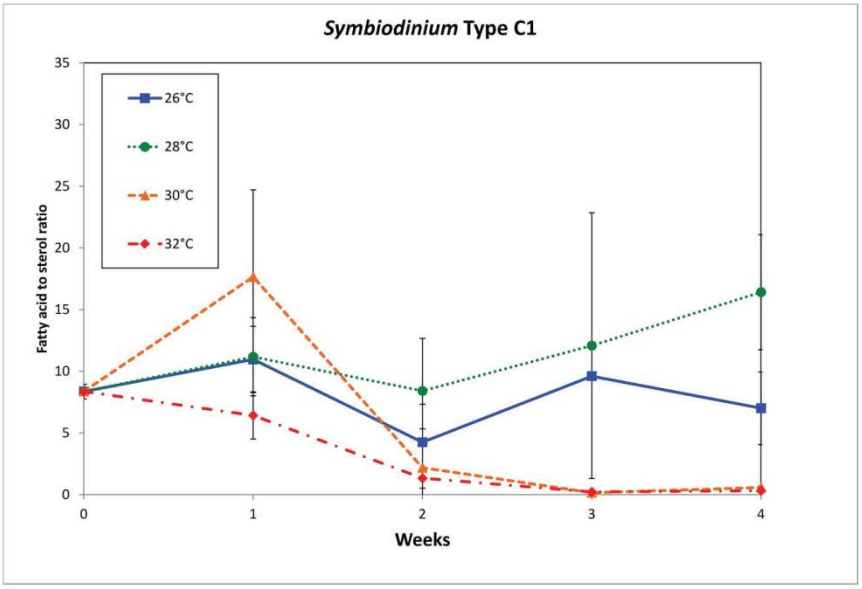

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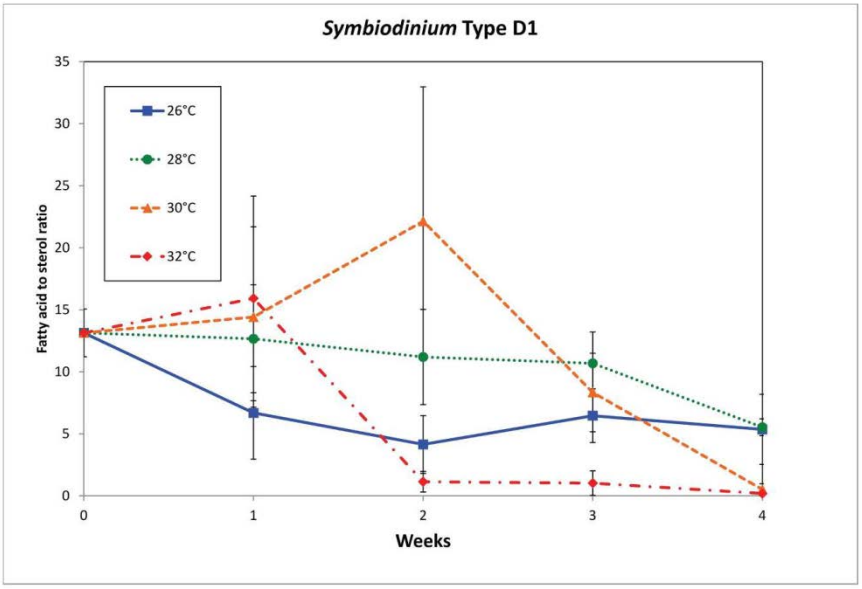

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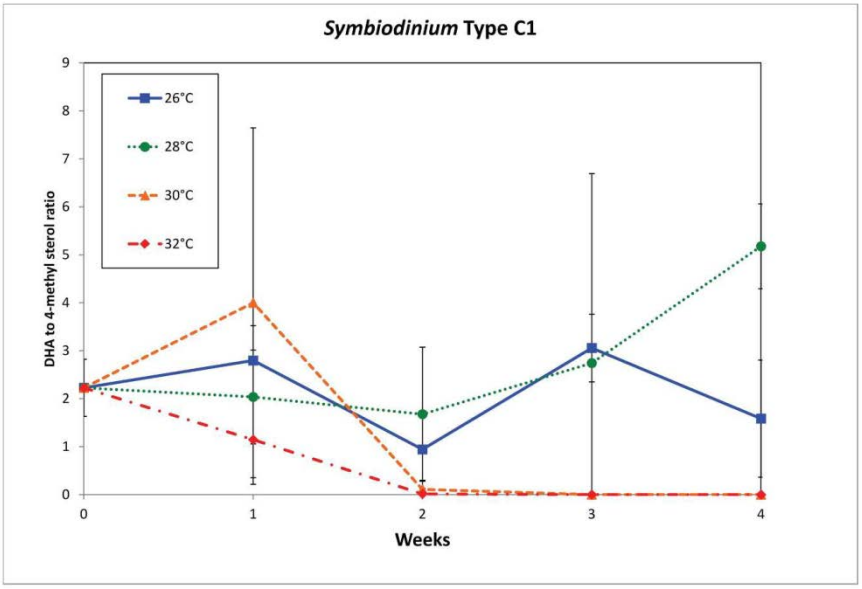

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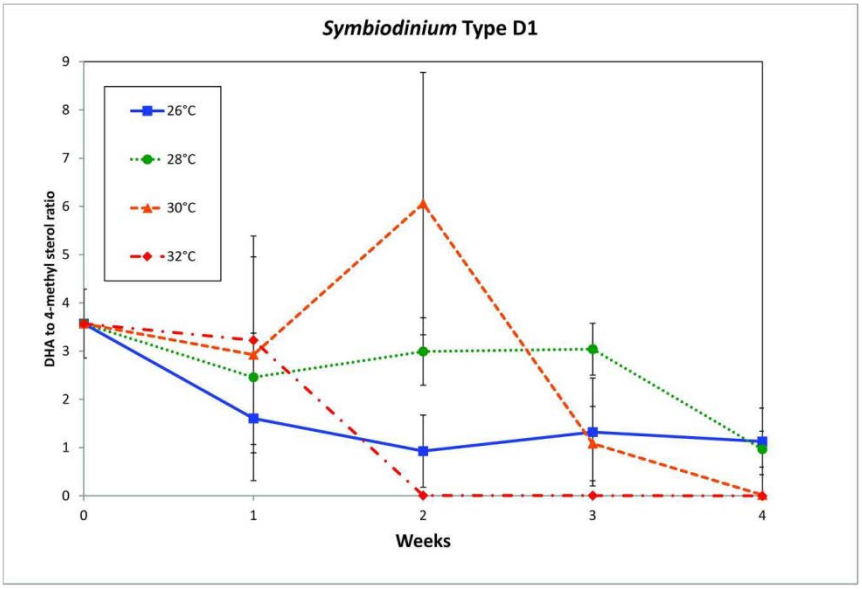

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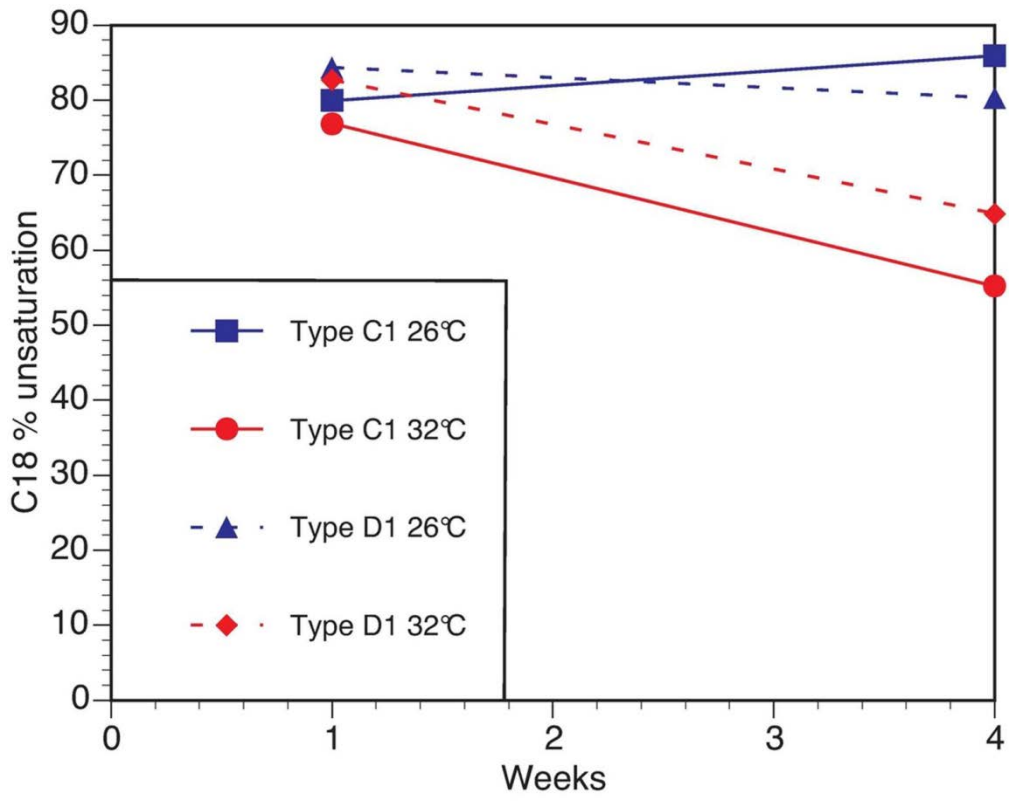

Figure 7. C18 % unsaturation in saponified (total) fatty acids from Symbiodinium types C1 and D1 as a function of thermal stress. Saponifying lipid extracts releases fatty acids previously bound in diglycerides, triglycerides, and wax esters. Similar to results for free fatty acids, the total C18 fatty acid pool shows a decrease in unsaturation due to high thermal stress, here 32°C for 4 weeks. A single extract was saponified and measured for each treatment.
98x78mm (300 x 300 DPI)

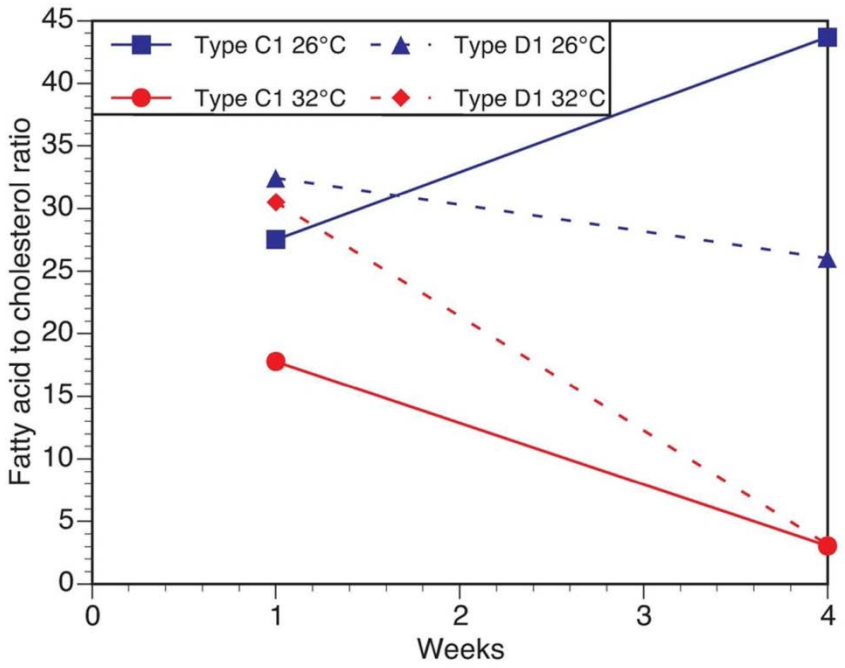

Figure 8. Total fatty acid to sterol ratios for saponified lipid extracts from Symbiodinium types C1 and D1 as a function of thermal stress. Saponifying lipid extracts releases fatty acids previously bound in diglycerides, triglycerides, and wax esters. The ratio of summed fatty acids to cholesterol in saponified samples shows a decline in fatty acids at high temperature for both clades C1 and D1, similar to results for free lipids. A single extract was saponified and measured for each treatment.
98x65mm (300 x 300 DPI)

Tables for Kneeland et al. revision to Coral Reefs

4x4 Factorial ANOVA: Type C1

C18 % unsaturation	df	F	P	Tukey HSD [0.05] level
Temperature	3	67.46	<0.001***	9.71
Time	3	14	<0.001***	9.81
Temperature x time	9	10.59	<0.001***	28
Fatty acid/sterol				
Temperature	3	7.5	<0.01**	5.47
Time	3	5.78	<0.01**	5.53
Temperature x time	9	2.55	<0.05*	15.77
DHA/4-methyl sterol				
Temperature	3	3.6	<0.05*	2.12
Time	3	2.11	NS	NS
Temperature x time	9	1.53	NS	NS

Table 1. Statistical results of 4 x 4 factorial ANOVA for Symbiodinium type C1. Experimental treatments included 4 temperatures (26°C, 28°C, 30°C and 32°C) and 4 times (1, 2, 3, and 4 weeks from start of temperature elevation). The 4x4 ANOVA determines the significance of temperature, time, and temperature x time interaction effect separately. Tukey's HSD value represents the minimum difference of each parameter between temperatures, times, or individual treatments that is statistically significant ($p < 0.05$); Tukey's HSD is a conservative post-hoc test. Numbers of independent replicates for each of the 16 experimental treatments are given in Supplemental Table S1. NS = not significant.

4x4 Factorial ANOVA: Type D1

C18 % unsaturation	df	F	P	Tukey HSD [0.05] level
Temperature	3	48.52	<0.001***	12.47
Time	3	8.78	<0.001***	12.56
Temperature x time	9	5.5	<0.001***	34.65
Fatty acid/sterol				
Temperature	3	6.51	<0.001***	5.44
Time	3	7.44	<0.01**	5.48
Temperature x time	9	3.53	<0.01**	15.11
DHA/4-methyl sterol				
Temperature	3	6.05	<0.01**	1.79
Time	3	6.03	<0.01**	1.8
Temperature x time	9	4.17	<0.01**	4.69

Table 2. Statistical results of 4 x 4 factorial ANOVA for Symbiodinium type C1. Experimental treatments included 4 temperatures (26°C, 28°C, 30°C and 32°C) and 4 times (1, 2, 3, and 4 weeks from start of temperature elevation). The 4x4 ANOVA determines the significance of temperature, time, and temperature x time interaction effect separately. Tukey's HSD value represents the minimum difference of each parameter between temperatures, times, or individual treatments that is statistically significant ($p < 0.05$); Tukey's HSD is a conservative post-hoc test.

Numbers of independent replicates for each of the 16 experimental treatments are given in Supplemental Table S1. NS = not significant.

Electronic Supplementary Material

Lipid biomarkers in *Symbiodinium* dinoflagellates: New indicators of thermal stress

Kneeland J, Hughen K, Cervino J, Hauff B, Eglinton T

Table S1. Fatty acid and sterol content as a percent of analyzed lipid for each measured aliquot of *Symbiodinium* cultures. Aliquots include both analyses of replicate cultures from the same experimental treatment (temperature, time), as well as replicate analyses (injections onto the GC) of the same culture sample.

Table S2. Fatty acid and sterol content as a percent of analyzed lipid, averaged for each experimental treatment. Where multiple aliquots of a single culture replicate were analyzed, those measurements were averaged separately first, before averaging across replicate culture samples for each treatment. Standard deviations (STD) were calculated based on the number of replicate cultures for each experimental treatment.

Type	Temp. (°C)	Week	Replicate	C16:1 fatty acid area (%)	C16:0 fatty acid area (%)	C18:4 fatty acid area (%)	C18:3 fatty acid area (%)	C18:1 fatty acid area (%)	C18:0 fatty acid area (%)	C22:6 fatty acid area (%)	Cholesterol area (%)	C29 Δ22---4---methyl--stanol area (%)	4α,24---dimethyl---5α cholesta--3β--ol area (%)	Dinosterol area (%)
C1	24	0	a	11.6	24.9	6.7	22.2	4.9	2.1	13.3	3.2	3.4	5.0	2.6
C1	24	0	b	9.5	32.6	4.3	21.9	5.3	3.0	10.7	3.0	2.7	5.9	1.0
C1	26	1	a	8.3	33.5	4.8	19.9	5.5	4.0	11.8	3.6	2.5	5.2	0.9
C1	26	1	b	9.3	33.7	5.8	21.1	5.0	4.1	11.9	2.4	2.1	3.6	1.0
C1	26	2	a	6.2	20.9	0.0	20.1	3.6	3.2	8.2	9.9	8.7	16.1	3.1
C1	26	2	b	7.7	28.0	8.4	22.5	3.3	4.4	11.4	3.3	3.3	6.1	1.6
C1	26	2	c	11.7	32.2	3.5	5.6	4.7	5.5	5.3	7.6	7.5	11.9	4.5
C1	26	3	a	8.5	17.8	8.8	30.1	4.3	2.6	15.6	3.4	3.0	4.5	1.5
C1	26	3	a	8.5	17.8	8.9	30.0	4.3	2.6	15.6	3.4	3.0	4.3	1.5
C1	26	3	a	8.5	17.8	8.9	30.0	4.3	2.6	15.7	3.4	3.0	4.4	1.4
C1	26	3	b	6.2	25.8	9.7	26.5	2.4	4.6	12.7	2.9	3.2	4.9	1.1
C1	26	4	a	5.1	22.1	11.1	31.8	2.1	4.1	11.8	3.0	3.4	4.4	1.2
C1	26	4	a	5.2	22.1	11.1	31.8	2.1	4.1	11.8	2.9	3.4	4.3	1.2
C1	26	4	a	5.1	22.1	11.1	31.8	2.1	4.1	11.8	3.0	3.4	4.3	1.2
C1	26	4	b	5.1	42.0	3.3	11.7	4.7	8.6	4.8	6.3	3.3	8.3	1.8
C1	26	4	b	5.4	44.5	1.9	14.0	4.9	9.1	0.1	6.2	4.2	8.1	1.7
C1	26	4	c	14.0	31.0	7.9	9.2	1.7	1.2	15.3	2.1	5.6	8.8	3.2
C1	28	1	a	9.6	36.1	5.0	14.3	5.4	9.2	7.5	3.0	2.1	4.5	3.3
C1	28	1	a	10.3	44.9	3.6	15.6	5.3	3.7	4.6	3.4	3.1	4.4	1.1
C1	28	1	b	12.1	37.6	5.1	17.0	6.2	3.3	9.6	2.3	2.2	3.5	0.9
C1	28	2	a	7.8	25.7	7.1	21.1	3.8	2.9	12.3	5.3	4.2	7.6	2.2
C1	28	2	a	7.5	25.9	6.8	20.5	3.9	2.9	11.2	5.4	4.7	8.2	3.0
C1	28	2	a	7.8	25.9	6.9	20.8	3.9	2.9	12.0	5.3	4.4	7.6	2.5
C1	28	2	b	10.7	33.2	6.0	21.0	5.4	3.3	11.3	2.3	2.3	3.6	1.0
C1	28	2	c	14.3	43.2	3.3	8.4	7.2	4.6	4.3	4.0	3.3	5.8	1.6
C1	28	2	c	15.2	53.8	1.5	3.8	5.5	3.3	0.0	4.3	4.0	6.8	1.9
C1	28	3	a	5.0	11.4	3.3	13.4	7.1	3.1	0.8	12.9	10.0	16.1	17.1
C1	28	3	b	11.0	32.5	6.2	21.8	4.8	3.6	14.2	1.5	1.6	1.9	0.7
C1	28	3	c	16.8	42.4	6.8	9.2	7.4	5.4	2.9	2.6	2.1	3.2	1.2
C1	28	4	a	10.3	22.2	8.6	27.5	4.9	3.4	13.9	2.7	2.4	3.0	1.1
C1	28	4	a	10.3	22.4	8.6	27.8	4.1	3.4	14.1	2.7	2.4	3.1	1.1
C1	28	4	a	10.2	22.2	8.5	27.6	4.8	3.4	13.9	2.7	2.4	3.1	1.1
C1	28	4	b	9.9	32.1	7.0	23.1	4.9	4.2	12.1	1.9	1.9	2.1	0.8
C1	30	1	a	10.7	21.6	0.0	22.6	7.5	3.9	22.6	3.2	2.2	4.6	1.2

C1	30	1	b	13.2	39.4	0.0	17.2	7.8	3.2	13.4	1.4	1.7	1.9	0.7
C1	30	1	c	18.1	50.4	2.1	8.2	10.4	4.5	0.0	1.9	1.5	2.1	0.8
C1	30	2	a	0.9	12.4	0.0	1.8	1.1	3.5	0.0	25.1	17.5	31.1	6.6
C1	30	2	b	5.9	55.3	2.8	0.9	7.7	7.5	2.2	5.9	3.5	6.7	1.6
C1	30	2	c	2.1	11.3	0.0	5.3	2.4	3.8	0.0	23.6	16.2	29.3	6.0
C1	30	3	a	0.3	6.3	0.0	0.7	0.5	2.0	0.0	26.4	19.1	38.0	6.7
C1	30	3	b	0.0	7.5	0.0	0.0	1.4	3.8	0.0	21.6	22.0	36.5	7.2
C1	30	4	a	0.8	17.0	0.0	1.1	2.9	5.3	0.0	19.3	14.4	32.6	6.6
C1	30	4	a	0.0	19.9	0.0	0.0	3.3	6.8	0.0	18.8	13.9	31.7	5.5
C1	30	4	a	0.0	19.7	0.0	0.0	3.2	6.7	0.0	18.7	13.8	31.9	5.9
C1	30	4	a	0.0	19.5	0.0	0.0	3.2	6.6	0.0	18.7	14.0	32.1	5.9
C1	30	4	b	1.4	17.8	2.4	0.0	4.3	8.7	0.0	13.2	12.6	22.6	16.9
C1	32	1	a	6.4	29.3	7.1	17.0	4.1	6.2	12.8	3.5	4.3	7.7	1.4
C1	32	1	b	6.1	44.2	3.5	10.0	5.1	7.4	9.8	3.7	3.5	5.7	1.0
C1	32	1	c	9.5	41.1	5.9	8.2	4.5	7.1	0.7	5.5	5.6	9.8	1.9
C1	32	2	a	2.4	27.1	0.0	0.0	2.8	6.4	0.8	16.4	14.1	24.5	5.6
C1	32	2	a	2.3	26.7	0.0	0.0	2.8	6.5	0.8	16.2	13.6	25.6	5.6
C1	32	2	a	2.2	26.7	0.0	0.0	2.8	6.5	0.8	16.5	14.4	24.7	5.3
C1	32	2	b	0.0	9.9	2.3	0.0	1.5	4.6	0.0	12.3	32.1	31.1	6.2
C1	32	2	b	2.5	35.3	2.9	0.0	3.3	7.5	0.7	13.1	11.5	19.7	3.5
C1	32	2	c	3.0	39.0	0.0	5.5	3.8	11.5	0.0	10.1	9.4	15.3	2.3
C1	32	3	a	0.0	10.3	0.0	0.0	1.5	4.5	0.0	24.9	14.3	37.7	6.7
C1	32	3	a	0.0	10.2	0.0	0.0	1.5	4.4	0.0	25.1	14.4	38.0	6.5
C1	32	3	a	0.0	10.3	0.0	0.0	0.0	4.4	0.0	25.5	14.6	38.7	6.4
C1	32	4	a	0.0	11.1	0.0	0.0	3.2	4.5	0.0	22.1	13.9	38.7	6.5
C1	32	4	a	0.0	10.8	0.0	0.0	3.0	4.5	0.0	22.2	13.9	39.0	6.6
C1	32	4	a	0.0	11.1	0.0	0.0	2.3	4.3	0.0	22.4	14.1	39.5	6.3
C1	32	4	b	0.0	11.7	0.0	0.0	2.0	5.7	0.0	19.2	14.8	39.2	7.4
C1	32	4	c	1.0	13.1	0.0	2.1	2.0	5.5	0.0	17.4	17.4	36.8	4.6
D1	24	0	a	12.0	30.2	5.5	23.2	6.0	2.7	11.4	2.7	2.0	3.7	0.5
D1	24	0	a	14.7	25.1	5.8	24.6	6.5	2.2	12.1	2.5	2.2	3.7	0.7
D1	24	0	b	14.9	37.4	3.2	12.3	7.4	2.9	13.8	2.4	2.0	3.1	0.5
D1	24	0	c	13.5	37.6	2.3	13.3	6.7	3.0	15.4	2.4	2.3	3.1	0.3
D1	24	0	c	9.4	41.9	3.4	13.8	5.1	4.2	12.0	3.4	1.5	4.6	0.8
D1	24	0	c	9.4	41.9	3.4	13.6	5.1	4.2	12.0	3.4	1.5	4.6	0.9
D1	24	0	c	9.2	40.8	3.3	13.3	6.3	4.1	11.7	3.3	1.5	4.8	1.8
D1	26	1	a	11.3	25.3	6.8	10.7	3.7	4.5	2.9	7.9	8.7	14.4	3.7
D1	26	1	b	8.7	32.5	9.1	19.0	2.7	4.3	9.4	2.6	3.3	5.0	3.3
D1	26	1	c	8.2	34.9	5.8	17.4	4.7	4.1	13.0	3.5	2.7	4.8	0.9
D1	26	1	c	8.3	35.0	5.8	17.4	4.7	4.1	12.9	3.5	2.8	4.8	0.8

D1	26	1	c	8.2	35.0	5.8	17.4	4.7	4.1	13.0	3.5	2.7	4.7	0.9
D1	26	2	a	5.3	33.0	5.5	10.6	0.0	5.3	3.8	9.2	8.5	15.7	3.1
D1	26	2	b	7.9	32.2	6.4	19.3	3.3	4.6	10.3	3.6	3.6	6.0	2.9
D1	26	2	c	6.0	30.7	4.7	11.8	2.1	4.5	9.9	5.6	7.9	13.1	3.7
D1	26	2	c	6.2	31.8	4.9	12.2	2.2	4.6	10.2	5.7	8.3	11.6	2.3
D1	26	2	c	6.1	31.6	4.9	12.0	2.1	4.6	10.0	5.7	8.2	12.0	2.7
D1	26	3	a	11.4	37.0	7.8	9.2	3.0	2.9	3.0	6.4	7.0	9.5	2.7
D1	26	3	b	7.8	30.0	7.7	18.2	3.3	5.2	8.7	2.5	9.0	7.7	0.0
D1	26	3	c	6.5	29.2	6.9	20.0	2.7	5.1	15.0	3.9	3.5	5.9	1.5
D1	26	3	c	6.5	29.1	6.9	20.0	2.6	5.1	15.0	3.9	3.5	5.9	1.4
D1	26	3	c	6.4	29.1	7.0	20.0	2.6	5.1	15.0	4.0	3.5	6.0	1.3
D1	26	4	a	6.0	27.5	7.6	20.3	3.6	6.5	7.2	4.9	6.0	8.6	1.8
D1	26	4	b	5.8	27.8	9.0	24.7	3.0	5.4	10.1	4.0	3.8	5.2	1.2
D1	26	4	c	9.8	44.8	0.0	4.1	0.0	0.0	8.8	8.2	6.9	14.0	3.2
D1	28	1	a	9.7	39.7	5.3	15.7	6.5	5.1	7.8	2.6	2.7	4.2	0.6
D1	28	1	b	10.6	37.2	7.5	19.2	4.8	3.5	9.7	2.0	2.0	2.8	0.6
D1	28	1	c	9.6	37.1	5.9	16.5	4.7	3.5	9.9	3.3	3.3	4.7	1.5
D1	28	1	c	9.6	37.2	5.9	16.4	4.7	3.5	10.0	3.3	3.2	4.9	1.3
D1	28	1	c	9.4	36.2	5.8	15.9	4.6	3.4	9.8	3.2	3.8	5.0	2.9
D1	28	2	a	9.8	35.0	4.9	16.0	5.9	3.6	12.8	2.8	2.9	4.8	1.4
D1	28	2	a	9.9	35.1	4.9	16.0	5.9	3.6	12.9	2.8	2.8	4.7	1.3
D1	28	2	a	9.8	35.0	4.9	15.9	5.9	3.6	12.9	2.8	2.8	4.7	1.8
D1	28	2	b	11.5	35.7	6.2	17.4	6.2	3.3	11.6	1.9	2.2	3.1	0.9
D1	28	2	c	9.2	37.2	4.3	14.0	5.4	3.7	12.9	3.6	3.1	5.2	1.5
D1	28	2	c	9.2	37.5	4.3	13.9	4.8	3.9	12.9	3.5	3.1	5.3	1.6
D1	28	2	c	9.3	37.5	4.3	13.9	4.7	3.8	12.9	3.5	3.0	5.2	1.8
D1	28	3	a	11.4	31.3	5.3	17.1	6.0	4.1	12.4	3.4	3.2	4.6	1.2
D1	28	3	a	11.1	31.3	5.3	17.3	6.0	4.1	12.7	3.4	3.2	4.5	1.0
D1	28	3	a	11.1	31.2	5.4	17.2	6.0	4.1	12.7	3.4	3.2	4.6	1.1
D1	28	3	b	9.4	34.3	6.9	20.1	4.3	4.2	11.6	2.3	2.6	3.2	1.2
D1	28	3	c	9.3	38.3	4.1	12.7	5.1	4.8	13.0	3.7	2.8	4.8	1.3
D1	28	3	c	9.0	38.3	4.1	12.8	5.2	5.0	13.1	3.7	2.8	4.8	1.3
D1	28	3	c	9.0	38.3	4.1	12.8	5.1	4.9	13.1	3.7	2.8	4.9	1.3
D1	28	4	a	7.1	32.3	4.0	16.2	4.5	6.0	9.9	5.8	4.1	8.1	1.9
D1	28	4	a	6.9	32.5	4.0	16.3	4.4	5.9	10.0	5.8	4.2	8.0	1.9
D1	28	4	a	6.9	32.3	4.1	16.2	4.5	6.0	9.9	5.8	4.1	8.1	2.0
D1	28	4	b	7.0	30.7	7.5	25.6	5.2	5.8	0.7	5.1	3.5	7.0	2.1
D1	28	4	b	6.8	30.2	5.9	19.9	4.2	5.6	9.0	4.6	5.3	6.8	1.7
D1	30	1	a	13.0	26.0	4.4	21.9	8.5	3.4	7.1	2.3	6.9	6.6	0.0
D1	30	1	b	11.1	43.1	0.0	16.5	7.5	3.7	11.3	1.4	2.6	2.2	0.5

D1	30	1	c	9.9	37.2	2.9	15.5	5.5	3.3	12.1	1.7	2.5	4.4	5.0
D1	30	1	c	10.1	37.7	3.1	16.0	5.6	3.3	12.4	2.0	4.6	5.2	0.0
D1	30	1	c	10.1	37.5	3.0	16.1	5.6	3.3	12.4	2.1	5.1	4.6	0.0
D1	30	2	a	12.0	33.9	4.5	19.9	6.8	2.9	13.8	1.4	2.0	2.2	0.6
D1	30	2	b	12.7	37.9	4.5	18.7	5.8	3.3	12.8	1.1	1.4	1.5	0.4
D1	30	2	c	9.5	44.8	0.0	11.2	6.4	4.7	12.9	2.5	3.1	3.6	1.3
D1	30	2	c	9.2	43.8	0.0	10.9	6.2	4.6	12.7	2.5	3.8	4.4	1.7
D1	30	2	c	9.7	46.1	0.0	5.8	7.7	4.8	13.4	2.6	4.6	4.3	1.2
D1	30	3	a	9.5	51.0	0.0	6.1	6.9	5.8	4.7	3.9	4.4	6.2	1.4
D1	30	3	b	8.9	48.8	0.0	10.5	6.2	6.8	7.8	2.8	3.5	4.0	0.8
D1	30	3	c	2.8	57.9	2.7	0.7	4.3	9.4	3.9	5.8	4.2	7.3	1.1
D1	30	3	c	2.8	58.1	2.7	0.7	4.3	9.4	3.9	5.8	4.1	7.2	0.9
D1	30	3	c	2.8	58.0	2.7	0.7	4.3	9.4	3.9	5.8	4.2	7.2	0.9
D1	30	4	a	0.0	6.9	0.0	0.0	0.0	0.0	0.0	18.4	15.7	54.1	5.0
D1	30	4	a	1.3	11.2	0.0	0.4	1.0	4.7	0.0	17.5	16.8	41.8	5.2
D1	30	4	b	0.0	20.8	1.7	0.0	5.9	10.0	1.1	16.2	13.8	27.7	2.9
D1	32	1	a	3.9	62.7	0.0	1.0	4.4	8.6	4.4	3.6	3.8	5.9	1.7
D1	32	1	b	10.3	43.6	5.4	14.5	6.0	4.1	10.2	1.3	2.0	2.2	0.5
D1	32	1	c	8.7	46.0	3.3	10.0	8.0	4.4	12.1	2.0	2.1	2.9	0.7
D1	32	1	c	9.5	50.0	3.5	2.1	8.7	4.7	13.2	2.1	2.2	3.1	0.8
D1	32	1	c	8.8	46.3	3.3	10.1	7.1	4.5	12.2	2.0	2.1	3.0	0.7
D1	32	2	a	1.3	14.8	0.0	1.5	2.3	5.4	0.0	20.3	14.3	34.2	5.8
D1	32	2	a	0.0	14.2	0.0	0.0	4.4	6.7	0.0	20.8	12.0	36.6	5.2
D1	32	2	a	0.0	14.7	0.0	0.0	2.7	7.0	0.0	21.2	12.1	37.0	5.4
D1	32	2	a	0.0	14.6	0.0	0.0	4.9	6.7	0.0	20.5	11.9	36.4	5.0
D1	32	2	b	0.0	14.6	0.0	0.0	4.9	6.7	0.0	20.5	11.9	36.4	5.0
D1	32	2	c	1.7	30.5	0.0	5.3	1.9	39.9	0.0	8.3	2.1	9.5	0.8
D1	32	2	c	2.4	33.4	4.2	0.0	0.0	8.5	0.9	12.7	11.4	23.5	3.0
D1	32	2	c	2.3	33.6	4.2	0.0	0.0	8.8	0.9	12.8	11.3	23.2	3.0
D1	32	2	c	1.7	32.4	4.1	0.0	0.0	12.5	0.8	12.5	10.7	22.6	2.6
D1	32	3	a	0.8	10.1	0.0	0.3	1.3	3.7	0.0	22.7	15.8	38.2	7.1
D1	32	3	b	2.5	15.2	0.0	5.1	4.7	16.2	1.3	13.7	11.1	25.7	4.5
D1	32	3	b	0.0	14.9	0.0	0.0	3.1	8.8	0.0	18.9	13.0	36.5	4.9
D1	32	3	c	0.0	37.4	0.0	10.4	2.8	34.6	0.0	4.1	4.1	6.7	0.0
D1	32	3	c	0.0	12.3	0.0	0.0	0.0	4.4	0.0	22.3	12.6	41.8	6.7
D1	32	3	c	0.0	12.2	0.0	0.0	0.0	4.7	0.0	23.4	13.4	41.1	5.2
D1	32	3	c	0.0	12.2	0.0	0.0	0.0	0.0	0.0	24.2	13.5	42.5	7.5
D1	32	4	a	0.0	8.1	0.0	0.0	0.0	5.3	0.0	20.8	10.8	48.4	6.7
D1	32	4	a	0.0	7.7	0.0	0.0	0.0	5.0	0.0	20.6	10.9	48.8	6.9
D1	32	4	a	0.0	7.5	0.0	0.0	0.0	5.1	0.0	20.9	11.1	48.7	6.7

D1	32	4	a	0.3	7.6	0.0	0.0	1.1	4.1	0.0	18.9	18.6	41.7	7.7
D1	32	4	b	0.7	9.9	0.0	0.6	1.0	2.5	0.0	19.3	17.0	42.2	6.8

				C16:1 fatty acid		C16:0 fatty acid		C18:4 fatty acid		C18:3 fatty acid		C18:1 fatty acid		C18:0 fatty acid		C22:6 fatty acid		Cholesterol		C29 Δ22--4-methyl--stanol		dimethyl---5α---cholesta--3β---ol		Dinosterol	
Type	Temp. (°C)	Week	Replicates	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD
C1	24	0	2	10.5	1.5	28.7	5.4	5.5	1.7	22.0	0.2	5.1	0.3	2.6	0.6	12.0	1.8	3.1	0.1	3.1	0.5	5.5	0.7	1.8	1.1
C1	26	1	2	8.8	0.7	33.6	0.1	5.3	0.7	20.5	0.8	5.2	0.3	4.0	0.1	11.8	0.0	3.0	0.8	2.3	0.3	4.4	1.1	1.0	0.1
C1	26	2	3	8.5	2.9	27.0	5.7	4.0	4.2	16.0	9.2	3.9	0.7	4.4	1.1	8.3	3.1	7.0	3.3	6.5	2.8	11.3	5.0	3.1	1.4
C1	26	3	2	7.4	1.6	21.8	5.6	9.3	0.6	28.3	2.6	3.3	1.3	3.6	1.5	14.1	2.1	3.2	0.4	3.1	0.2	4.7	0.4	1.3	0.2
C1	26	4	3	8.1	5.1	32.1	10.6	7.2	4.3	17.9	12.1	2.9	1.7	4.7	3.9	9.9	6.7	3.8	2.2	4.3	1.2	7.1	2.4	2.0	1.0
C1	28	1	2	11.0	1.6	39.0	2.0	4.7	0.6	16.0	1.4	5.8	0.6	4.9	2.2	7.8	2.6	2.8	0.6	2.4	0.3	4.0	0.7	1.6	0.9
C1	28	2	3	11.1	3.6	35.8	11.6	5.1	2.4	15.9	8.5	5.2	1.3	3.4	0.5	8.4	5.4	3.9	1.6	3.5	1.1	5.9	2.1	1.8	0.8
C1	28	3	3	10.9	5.9	28.8	15.8	5.4	1.9	14.8	6.4	6.4	1.4	4.0	1.2	5.9	7.2	5.7	6.3	4.6	4.7	7.1	7.8	6.4	9.3
C1	28	4	2	10.1	0.3	27.2	7.0	7.7	1.1	25.4	3.2	4.7	0.3	3.8	0.6	13.0	1.3	2.3	0.6	2.2	0.3	2.6	0.7	0.9	0.2
C1	30	1	3	14.0	3.7	37.1	14.5	0.7	1.2	16.0	7.3	8.6	1.6	3.9	0.7	12.0	11.3	2.1	0.9	1.8	0.4	2.9	1.5	0.9	0.3
C1	30	2	3	3.0	2.6	26.3	25.1	0.9	1.6	2.7	2.3	3.7	3.5	4.9	2.3	0.7	1.3	18.2	10.7	12.4	7.7	22.4	13.6	4.7	2.8
C1	30	3	2	0.1	0.2	6.9	0.9	0.0	0.0	0.4	0.5	0.9	0.6	2.9	1.3	0.0	0.0	24.0	3.4	20.6	2.0	37.2	1.0	6.9	0.4
C1	30	4	2	0.8	0.9	18.4	0.9	1.2	1.7	0.1	0.2	3.7	0.8	7.5	1.7	0.0	0.0	16.0	4.1	13.3	1.0	27.4	6.7	11.5	7.8
C1	32	1	3	7.4	1.9	38.2	7.9	5.5	1.8	11.7	4.7	4.5	0.5	6.9	0.6	7.8	6.3	4.3	1.1	4.5	1.0	7.8	2.1	1.4	0.5
C1	32	2	3	2.2	0.9	29.5	8.5	0.9	1.5	1.8	3.2	3.0	0.7	8.0	3.0	0.4	0.4	13.0	3.2	15.1	6.2	21.9	5.7	4.2	1.7
C1	32	3	1	0.0		10.3		0.0		0.0		1.0		4.4		0.0		25.2		14.4		38.1		6.6	
C1	32	4	3	0.3	0.6	11.9	1.1	0.0	0.0	0.7	1.2	2.3	0.5	5.2	0.7	0.0	0.0	19.6	2.4	15.4	1.8	38.3	1.4	6.2	1.4
D1	24	0	3	12.9	2.3	35.2	6.7	4.0	1.4	16.6	6.4	6.5	0.8	3.1	0.7	12.8	1.0	2.7	0.4	1.9	0.2	3.7	0.6	0.7	0.2
D1	26	1	3	9.4	1.7	30.9	5.0	7.3	1.7	15.7	4.4	3.7	1.0	4.3	0.2	8.4	5.1	4.7	2.8	4.9	3.3	8.0	5.5	2.6	1.6
D1	26	2	3	6.4	1.4	32.2	0.8	5.6	0.8	14.0	4.7	1.8	1.7	4.8	0.4	8.1	3.7	6.1	2.8	6.7	2.7	11.3	4.9	3.0	0.1
D1	26	3	3	8.5	2.6	32.0	4.3	7.5	0.5	15.8	5.8	3.0	0.3	4.4	1.3	8.9	6.0	4.3	2.0	6.5	2.8	7.7	1.8	1.4	1.4
D1	26	4	3	7.2	2.3	33.4	9.9	5.5	4.8	16.4	10.9	2.2	1.9	4.0	3.5	8.7	1.4	5.7	2.2	5.6	1.6	9.3	4.4	2.0	1.0
D1	28	1	3	9.9	0.6	37.9	1.6	6.2	1.2	17.1	1.9	5.3	1.1	4.0	0.9	9.1	1.1	2.6	0.7	2.7	0.7	4.0	1.1	1.1	0.8
D1	28	2	3	10.2	1.2	36.0	1.2	5.2	1.0	15.8	1.7	5.7	0.6	3.5	0.3	12.5	0.7	2.8	0.8	2.7	0.5	4.3	1.1	1.4	0.4
D1	28	3	3	9.9	1.1	34.6	3.5	5.4	1.4	16.7	3.7	5.1	0.9	4.4	0.4	12.4	0.7	3.1	0.8	2.9	0.3	4.2	0.9	1.2	0.1
D1	28	4	2	6.9	0.0	31.4	1.4	5.4	1.9	19.5	4.6	4.6	0.2	5.8	0.2	7.4	3.6	5.3	0.7	4.3	0.2	7.5	0.8	1.9	0.0
D1	30	1	3	11.3	1.5	35.5	8.7	2.5	2.3	18.1	3.3	7.2	1.5	3.5	0.2	10.2	2.8	1.9	0.5	4.5	2.2	4.5	2.2	0.7	0.9
D1	30	2	3	7.1	3.7	52.6	4.8	0.9	1.5	5.8	4.9	5.8	1.3	7.3	1.9	5.4	2.1	4.2	1.5	4.0	0.5	5.8	1.7	1.0	0.3
D1	30	3	3	7.1	3.7	52.6	4.8	0.9	1.5	5.8	4.9	5.8	1.3	7.3	1.9	5.4	2.1	4.2	1.5	4.0	0.5	5.8	1.7	1.0	0.3
D1	30	4	2	0.3	0.5	14.9	8.3	0.9	1.2	0.1	0.1	3.2	3.8	6.2	5.4	0.5	0.8	17.1	1.2	15.0	1.7	37.8	14.3	4.0	1.6
D1	32	1	3	7.8	3.4	51.2	10.1	2.9	2.7	7.6	6.7	6.1	1.7	5.7	2.5	9.0	4.2	2.3	1.2	2.6	1.0	3.7	1.9	1.0	0.6
D1	32	2	3	0.8	1.1	20.5	10.3	1.0	1.8	0.6	0.7	3.0	2.3	10.2	6.3	0.2	0.4	17.6	5.2	11.1	1.9	30.7	9.5	4.3	1.7
D1	32	3	3	0.7	0.6	14.6	4.3	0.0	0.0	1.8	1.3	2.0	1.7	9.0	4.7	0.2	0.4	19.2	3.3	12.9	2.6	34.1	3.7	5.5	1.3
D1	32	4	2	0.4	0.4	8.8	1.5	0.0	0.0	0.3	0.4	0.6	0.5	3.7	1.7	0.0	0.0	19.8	0.7	14.9	3.0	44.6	3.3	6.9	0.1