Coral Reefs (2013) 32:923–934
DOI 10.1007/s00338-013-1076-3

REPORT

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

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Received: 17 September 2012 / Accepted: 19 August 2013 / Published online: 30 August 2013

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* sp. 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, the polyunsaturated fatty acid docosahexaenoic acid (C22:6, n-3; DHA), and a variety of sterols. Prolonged exposure to high temperature causes the relative amount of unsaturated acids within the C18 fatty acids in *Symbiodinium* tissue to decrease. Thermal stress also causes a decrease in abundance of fatty acids relative to sterols, as well as the more specific ratio of DHA to an algal 4-methyl sterol. These shifts in fatty acid unsaturation and fatty acid-to-sterol ratios 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 *Symbiodinium* 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.

Keywords Lipid biomarkers · Thermal stress · *Symbiodinium* · Fatty acid unsaturation · Thermal sensitivity

Communicated by Biology Editor Dr. Anastazia Banaszak

Electronic supplementary material The online version of this article (doi:10.1007/s00338-013-1076-3) contains supplementary material, which is available to authorized users.

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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 are implicated in exacerbating coral diseases (Harvell et al. 1999; Cervino et al. 2004; Sutherland et al. 2004). Recent high-temperature bleaching episodes 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). Symptoms of bleaching include expulsion of *Symbiodinium* from host tissue and loss of photosynthetic pigment within 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 (Jones et al. 1998; Warner et al. 1999; Tchernov et al. 2011). The *Symbiodinium* heat-stress response 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 *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). Photosynthesis rates in cultured *Symbiodinium microadriaticum* increase at temperatures up to 30 °C but decrease drastically above 30 °C (Iglesias-Prieto et al. 1992). The mitotic index (percentage of cells dividing) in thermally stressed *Symbiodinium* cultures decreases within days of heat exposure (Bouchard and Yamasaki 2009). *Symbiodinium* growing within 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 1992). Intracellular nitric oxide production and caspase-like activity, implicated in programmed cell death, also increase after only 24 h of high-temperature exposure in cultured *S. microadriaticum* (Bouchard and Yamasaki 2009).

Changes in the composition of lipids have 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 1 or 2 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 4 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 15-ml glass test tube with 10 ml of f/2 growth medium. Cultures were maintained under

12/12-h light/dark cycles with ambient indoor fluorescent lighting. Light levels experienced by the cultures were not quantified, but this parameter was kept constant across experimental treatments. Cultures were nourished in f/2 growth medium at 26, 28, 30, 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 with 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's (1959) procedure. Frozen culture vials were allowed to just thaw, and the settled pellet of cells was pipetted into a precombusted glass centrifuge vial. If the pellet had not sufficiently settled, the entire culture was transferred to the centrifuge vial and samples were centrifuged at low speed for several minutes to concentrate the pellet. We then added to the cell pellet: 1 ml dichloromethane (DCM), 2 ml methanol, and 0.8 ml phosphate-buffered saline (PBS). The vials were sonicated for 5 min. Next, we added 1 ml DCM and 1 ml PBS. The vials were again sonicated 5 min. 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 precombusted sodium sulfate column to remove residual water. An aliquot of each extract was concentrated under gentle nitrogen and derivatized by adding 25 µl 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 chromatography–time-of-flight mass spectrometry (GC–TOF–MS) by comparison with 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., 10 µl DCM and 5 µl each of pyridine and BSTFA).

Saponification

Several extracts were saponified at 70 °C for 2 h in 0.5 N potassium hydroxide in methanol to liberate bound fatty acids. Basic and acidic fractions were extracted with hexane (3× each) and recombined prior to derivatization. Saponified fatty acids and sterols were analyzed by GC–FID as above.

Statistical analysis

For each *Symbiodinium* type, three culture vials (independent replicates) were collected and analyzed at each of four different times under four different temperature regimes (16 experimental conditions). Some replicates were lost during processing, and the amount of lipid extracted from others was insufficient for the 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 number of independent replicates for each experimental treatment is provided with the datasets in Electronic Supplemental Material, ESM Tables S1 and S2. For statistical analyses, data from all aliquots within each replicate culture were averaged. To compare results between the 16 different experimental treatments, we performed a 4 × 4 factorial analysis of variance (4 × 4 ANOVA) using VassarStats. For each of the three lipid parameters calculated, the 4 × 4 ANOVA was used to assess the significance of temperature, time, and temperature × 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's 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

Table 1 Statistical results of 4 × 4 factorial ANOVA for *Symbiodinium* type C1

4 × 4 factorial ANOVA: type C1				
	df	*F*	*p*	Tukey's HSD (0.05) level
C18 % unsaturation				
Temperature	3	67.46	<0.001	9.71
Time	3	14	<0.001	9.81
Temperature × 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 × 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 × time	9	1.53	NS	NS

Experimental treatments included four temperatures (26, 28, 30, and 32 °C) and four times (1, 2, 3, and 4 weeks from the start of temperature elevation). The 4 × 4 ANOVA determines the significance of temperature, time, and temperature × 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. A number of independent replicates for each of the 16 experimental treatments are given in Supplemental Table S1. *NS* not significant

Table 2 Statistical results of 4 × 4 factorial ANOVA for *Symbiodinium* type D1

4 × 4 factorial ANOVA: type D1				
	df	*F*	*p*	Tukey's HSD (0.05) level
C18 % unsaturation				
Temperature	3	48.52	<0.001	12.47
Time	3	8.78	<0.001	12.56
Temperature × 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 × 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 × time	9	4.17	<0.01	4.69

Experimental treatments included four temperatures (26, 28, 30, and 32 °C) and four times (1, 2, 3, and 4 weeks from the start of temperature elevation). The 4 × 4 ANOVA determines the significance of temperature, time, and temperature × 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. A number of independent replicates for each of the 16 experimental treatments are given in Supplemental Table S1. *NS* not significant

medium-chain PUFA, described below. The fatty acids included in these lipid ratios represent approximately 50 % of the TLE 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 lipids quantified in each aliquot are reported in ESM Table S1. The same data, averaged together for each experimental treatment, are reported in ESM Table S2. Further data analysis focused on lipid ratios calculated among the most abundant compounds, in order to identify biomarker indicators 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.

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 these acids plus the saturated C18:0 acid.

In type C1, the temperature, time, and temperature × time interaction effects are all significant for C18 unsaturation ($p < 0.001$; Table 1). At low-temperature conditions (26 and 28 °C), both *Symbiodinium* types showed high C18 fatty acid unsaturation ratios [88 ± 3 % in type C1 ($N = 20$) and 86 ± 2 % in type D1 ($N = 23$), 1σ], which did not change significantly over the 4-week experiment (Fig. 1). There were no significant differences between the two *Symbiodinium* types at 26 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 % in weeks 3 and 4. At the more moderate thermal treatment of 30 °C, the C18 unsaturation in type C1 first declined after 2 weeks of high-temperature exposure and was

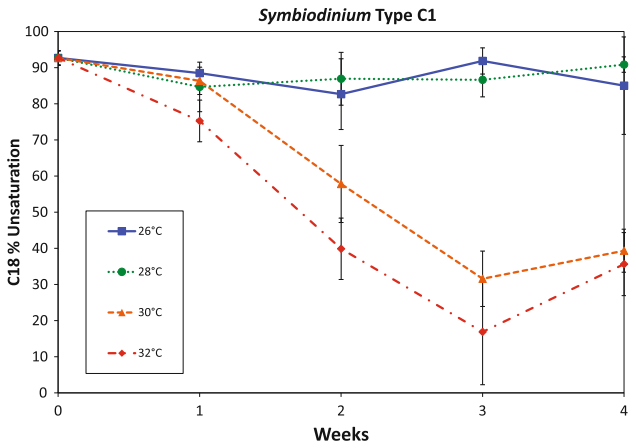

Fig. 1 Unsaturation ratio of C18 free fatty acids in *Symbiodinium* type C1 as a function of thermal stress. The C18 % unsaturation is defined as the ratio of the total unsaturated C18 fatty acids (C18:1, C18:2, C18:3, and C18:4) over the sum of all C18 fatty acids, including C18:0. Data are plotted 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 the number of replicates, see ESM Tables S1 and S2

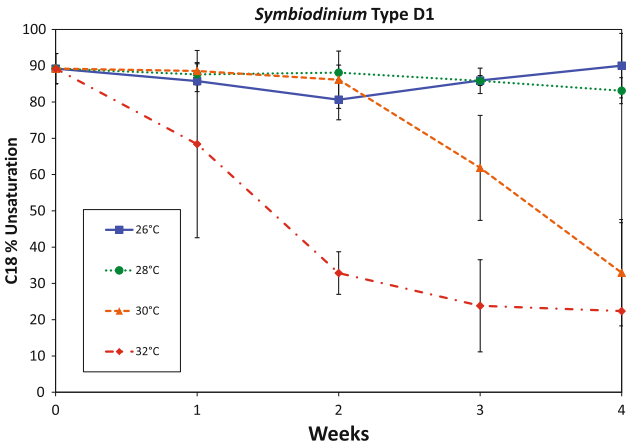

Fig. 2 Unsaturation ratio of C18 free fatty acids in *Symbiodinium* type D1 as a function of thermal stress. The C18 % unsaturation is defined as the ratio of the total unsaturated C18 fatty acids (C18:1, C18:2, C18:3, and C18:4) over the sum of all C18 fatty acids, including C18:0. Data are plotted 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 the number of replicates, see ESM Tables S1 and S2

statistically indistinguishable from the highest temperature values after 3 weeks.

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 1 week of exposure, and unsaturation 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, 4). However, there was no consistent temporal trend in fatty acid-to-sterol ratios at either 26 or 28 °C. At low

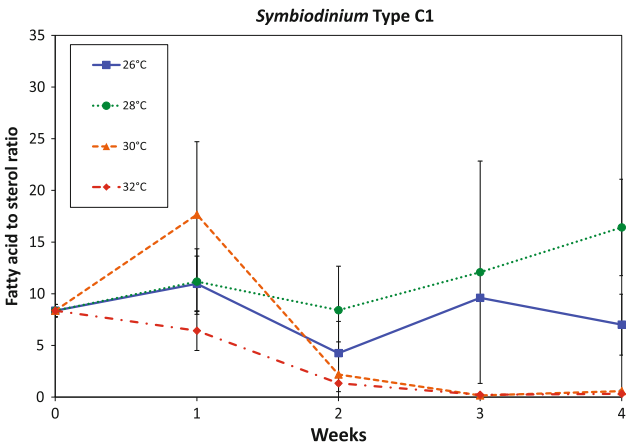

Fig. 3 Ratio of total fatty acids to sterols in *Symbiodinium* type C1 as a function of thermal stress. The fatty acid-to-sterol ratio is calculated as the sum of fatty acids [C16:0, C16:1, C18:0, C18:1, C18:3, C18:4, and C22:6(n-3)] to the sum of sterols (cholesterol, dinosterol, and 4α,24-dimethyl-5α-cholesta-3β-ol). 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 the number of replicates, see ESM Tables S1 and S2

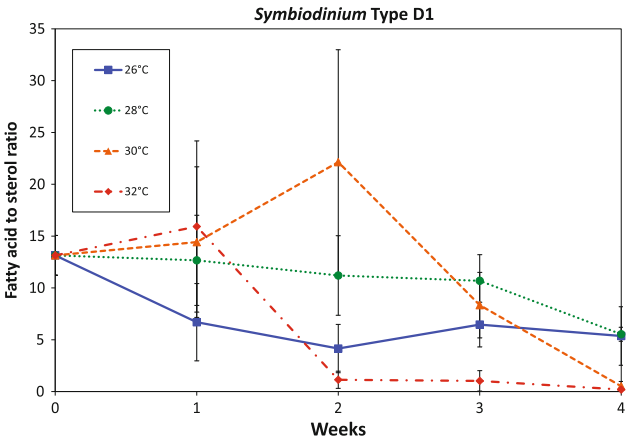

Fig. 4 Ratio of total fatty acids to sterols in *Symbiodinium* type D1 as a function of thermal stress. The fatty acid-to-sterol ratio is calculated as the sum of fatty acids [C16:0, C16:1, C18:0, C18:1, C18:3, C18:4, and C22:6(n-3)] to the sum of sterols (cholesterol, dinosterol, and 4α,24-dimethyl-5α-cholesta-3β-ol). 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 the number of replicates, see ESM Tables S1 and S2

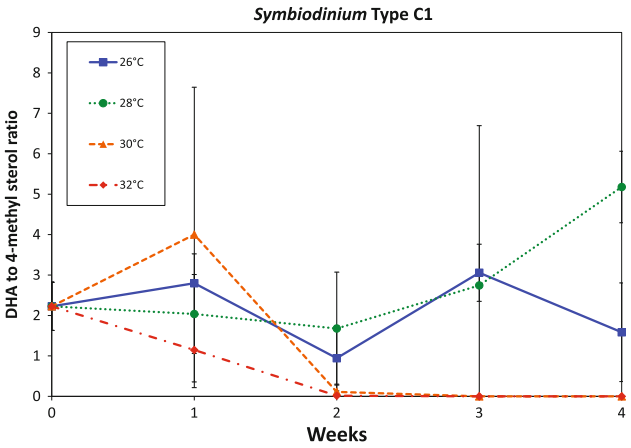

Fig. 5 Ratio of algal-specific fatty acid [docosahexaenoic acid C22:6(n-3)] to 4-methyl sterol (4α,24-dimethyl-5α-cholesta-3β-ol) in *Symbiodinium* type C1 as a function of thermal stress. Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ± SD),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

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 2 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 2 weeks and then decreased significantly after 4 weeks.

In the ratio of summed fatty acids to sterols, we again observed 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 × 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 1–3 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.

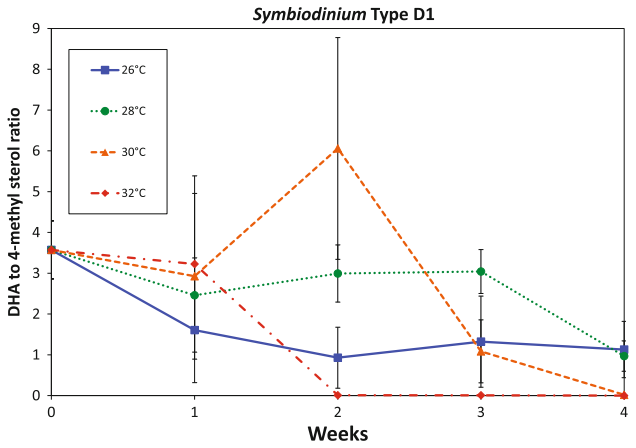

Fig. 6 Ratio of algal-specific fatty acid [docosahexaenoic acid C22:6(n-3)] to 4-methyl sterol (4α,24-dimethyl-5α-cholesta-3β-ol) in *Symbiodinium* type D1 as a function of thermal stress. Data are plotted as the average of all independent replicate cultures for each experimental treatment (mean ± SD), and data at time 0 are from 24 °C cultures. For the number of replicates, see ESM Tables S1 and S2

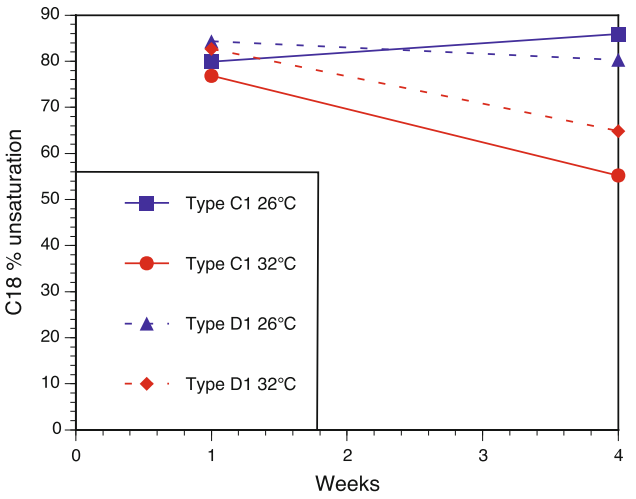

Fig. 7 C18 % unsaturation in saponified (total) fatty acids from *Symbiodinium* types C1 and D1 as a function of thermal stress. Saponifying lipid extracts releases fatty acids previously bound in diglycerides, triglycerides, and wax esters. Similar to the results for free fatty acids, the total C18 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

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 response 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 was slightly lower than in the free fatty acids (80–85 vs. 86–88 %, respectively). Saponified C18 fatty acid unsaturation decreased after 4 weeks of exposure at 32 °C, although the magnitude of the decrease was 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 showed 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 patterns of lipid stress signatures we observed are a robust feature of the cell's total lipid pool.

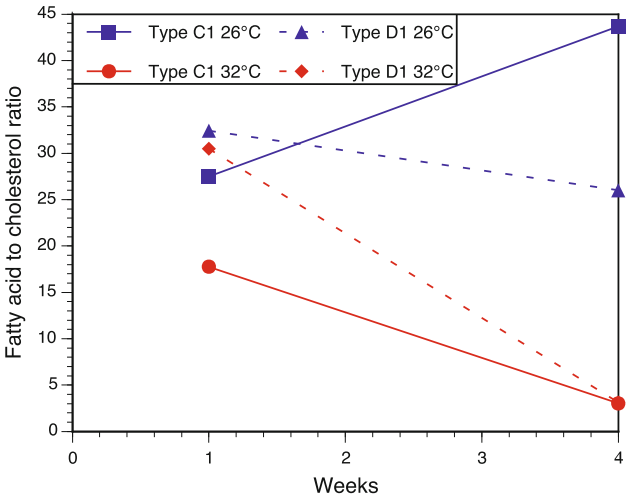

Fig. 8 Total-fatty acid-to-sterol ratios for saponified lipid extracts from *Symbiodinium* types C1 and D1 as a function of thermal stress. Saponifying lipid extracts releases fatty acids previously bound in diglycerides, triglycerides, and wax esters. The ratio of summed fatty acids to cholesterol in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

Discussion

Lipid profiles in cultured *Symbiodinium* showed that under heat stress sustained for a period of weeks, fatty acid unsaturation and the relative amount of fatty acids compared to sterols decreased in a temperature-, time-, and type-

dependent manner. Polyunsaturated fatty acids (PUFAs) accounted for the majority of the C18 fatty acids, and their abundance relative to stearic acid (C18:0) declined in heat-stressed cultures. In addition, concentrations of all fatty acids, including saturated acids and PUFAs, decreased relative to sterols following thermal stress.

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, ESM 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 instead indicate differences in the degree of thermal stress experienced by *Symbiodinium*.

Some of the changes we observed in fatty acid unsaturation may be attributable to varying contributions from membrane lipids versus storage lipids. Unsaturated C18 fatty acids, and particularly C18:4 fatty acid, make up a high percentage of the membrane lipids in cold-adapted dinoflagellates (LeBlond et al. 2006). On this basis, we may interpret the decreasing C18 fatty acid unsaturations either as a decrease in unsaturation within the membranes, or as a relative increase in more saturated energy storage lipids. Symbionts transfer fatty acids to the host coral (Papina et al. 2003), and corals harboring predominantly type C *Symbiodinium* contain relatively more 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 decreased storage lipid, increased membrane lipid, and a change in membrane lipid unsaturation based on these data. Further studies on 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 (ESM 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). 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 following temperature stress (Tolosa et al. 2011). Moreover, because the ratios of fatty acids to sterols are large in low-temperature cultures, the magnitude of decrease in ratios for thermally stressed treatments would require a >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 declining 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. Coral lipid content generally decreases in response to thermal stress. Grottoli et al. (2004) observed a lipid decrease 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 heat-stressed *Symbiodinium* may be a result of decreased total production of lipids, 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 confusing coral lipid data. Comparison between fatty acid profiles from coral host *Montipora digitata* and its symbionts showed that *Symbiodinium* produce more PUFAs, particularly C18:3(n-3) (Papina et al. 2003). Both C18:3 and C18:4 PUFAs are abundant in zooxanthellate octocorals, but not in an azooxanthellate species (Imbs et al. 2009). However, in another study, *Symbiodinium* from five coral species showed almost no 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 versus saponified *Symbiodinium* extracts. These field studies found high abundance of PUFAs in coral symbionts, which is consistent with the high C18 unsaturation observed in unstressed cultures. Differences in C18 unsaturation between different species of Japanese corals (Yamashiro et al. 1999) might be explained by intermediate levels of thermal stress experienced by the symbionts.

High amounts of C18:3 may indicate rapid cell growth and nutrient effects (Piorreck and Pohl 1984; Napolitano et al. 1997). We regularly replenished f/2 growth medium and observed no effects at low temperature. This suggests that we may rule out nutrient limitation in these cultures. However, we cannot rule out the possibility that nutrient addition caused some physiological changes; differences in nutrient availability may have contributed to scatter between replicates. This study only addressed the potential effect of thermal stress on *Symbiodinium*. Other environmental stressors may cause variability in the lipid ratios we proposed. Light stress, in particular, is well known to impact corals (e.g., Brown et al. 1999; Warner et al. 1999; Fitt et al. 2001). 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. Ecological and environmental parameters such as thermal history and *Symbiodinium*–coral association (Baker 2003; Jones et al. 2008; LaJeunesse et al. 2010; Cooper et al. 2011) may additionally impact the thermal stress response.

A variety of thermal stress indicators have been used previously, including photosynthetic efficiency (quantum yield of PS II $\Delta F/Fm$; e.g., Brown et al. 1999; Warner et al. 1999; Fitt et al. 2001), 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 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). 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). The present results indicate that lipids in *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 good marker of *Symbiodinium* stress within the holobiont, because coral host lipids 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 *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 decreased PUFA inventory is increased PUFA degradation. ROS from stressed cells may degrade PUFAs in the thylakoid membrane (Tchernov et al. 2004). PUFAs may further degrade to polyunsaturated aldehydes (PUAs) (Catalá 2009). ROS, known to be abundant in thermally stressed cells (Lesser 2006), 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 influence the cell's ability to regulate membrane fluidity and tolerate thermal stress (Stubbs and Smith 1984). *Synechocystis* cells genetically unable to produce PUFAs were 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 damaged PSII structures may also determine thermal stress tolerance in *Symbiodinium* (Takahashi et al. 2009). Recent experiments with *Arabidopsis thaliana* mutants demonstrated that triunsaturated fatty acids, abundant in wild-type thylakoid membranes, 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. Lipids in cultured *Symbiodinium* types C1 and D1 show systematic and significant changes in response to thermal stress. The dramatic declines in PUFA concentration relative to saturated fatty acids and sterols in heat-stressed *Symbiodinium* are consistent with a 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). Lipid ratios for unstressed and highly stressed *Symbiodinium* cultures do not differ between the two *Symbiodinium* types. However, the difference in stress response between types is clear in the offset timing of lipid changes at intermediate stress. Timescales of lipid response are similar to timescales expected to cause thermal bleaching in the coral reef environment, although the full development of lipid changes would not be evident in shorter experiments. 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 indicators should be measurable in corals. Careful culturing of zooxanthellate corals, addressing potential effects of other stressors such as light and feeding status, will help test these indicators for eventual field use. Finally, investigation of intact polar lipids may help to identify the source of these stress indicators within specific lipid classes (e.g., phospholipids and glycolipids). In-depth analysis of lipids from separated coral tissue and *Symbiodinium* growing *in hospite* could determine whether lipid profiles from the holobiont are useful markers of *Symbiodinium* heat stress.

Acknowledgments Daniel Montluçon and Nick Drenzek assisted with lipid analyses. This research was supported by Award No. USA 00002 to K. Hughen made by King Abdullah University of Science and Technology (KAUST). This manuscript benefitted significantly from the helpful comments of two anonymous reviewers.

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