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REPORT

Lipidome analysis of *Symbiodiniaceae* reveals possible mechanisms of heat stress tolerance in reef coral symbionts

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Abstract Climate change-induced global warming threatens the survival of key ecosystems including shallow water coral reefs. Elevated temperatures can disrupt the normal physiological functioning of photosynthetic organisms by altering the fluidity and permeability of chloroplast membranes that is defined and regulated by their lipid composition. Since the habitat-forming reef corals rely on the obligatory symbiosis with dinoflagellates of the family *Symbiodiniaceae*, their heat stress response can be expected to be strongly influenced by the symbiont's lipid metabolism. However, in contrast to the steady increase in the knowledge of the functioning of coral symbionts at the genomic and transcriptomic level, the understanding of their membrane lipid composition and regulation in response to temperature stress is lagging behind. We have utilised mass spectrometry-based lipidomic analyses to identify the key polar lipids that form the biological membranes of reef coral symbionts, comparing the thermotolerant species *Durusdinium trenchii* with the thermosensitive taxon *Cladocopium* C3, both hosted by *Acropora valida*. Our results indicate that the superior thermotolerance *D. trenchii* inside the host corals could be achieved through (1) the amount and saturation of sulfoquinovosyldiacylglycerols, in particular through putative photosystem II interactions, (2) the increased digalactosyldiacylglycerol to monogalactosyldiacylglycerol ratio with the potential to stabilise thylakoid membranes and integrated proteins, and (3) the chaperone-like function of lyso-lipids. Thereby, our study provides novel insights into the heat tolerance of coral symbionts, contributing to the understanding of the potential of coral reef ecosystems to respond and adjust to heat stress events that are becoming more frequent due to climate change. Finally, our identification of multiple mechanisms of heat tolerance in *Symbiodiniaceae* furthers the knowledge of the general stress physiology of photosynthetic organisms.

Keywords Heat tolerance · *Symbiodiniaceae* · Coral reefs · Global warming · Lipids · Mass spectrometry

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Introduction

Climate change is imposing significant stress on the physiological functioning of living organisms. In particular, episodes of elevated seawater temperatures exert a direct impact on the fluidity of biological membranes (Los and Murata 2004) and their melting point in poikilothermic organisms (Mansour et al. 2018). This disturbance can affect ion and solute transporters, induce changes in passive permeability and modify the function of membrane proteins via changes in their mobility and clustering (Hazel

1995; Welti et al. 2002). In photosynthetic cells, the effects of elevated temperature on the chloroplast membranes can cause inhibition of photosystem II (PSII) and result in a disruption of the electron transport chain and carbon fixation (Dörmann and Benning 2002).

Among the photosynthetic organisms that are severely endangered by temperature stress are dinoflagellates of the family *Symbiodiniaceae* (LaJeunesse et al. 2018). Members of different genera within this family form an obligatory symbiosis with reef corals responsible for building the calcareous framework of coral reef ecosystems thriving in tropical and subtropical regions around the globe.

Episodes of elevated seawater temperatures, exacerbated by light and unfavourable nutrient conditions, result often in the phenomenon of coral bleaching, the loss of the brownish-coloured symbionts that causes the white coral skeleton to shine through the transparent animal tissue (Brown 1997; Douglas 2003; Wiedenmann et al. 2013; Rosset et al. 2017). High-level mortality follows coral bleaching if the corals do not recover their symbionts within a relatively short period of time (Jokiel and Coles 1977; Glynn et al. 2001; Loya et al. 2001; Baird and Marshall 2002; Baker et al. 2008; Riegl et al. 2015; Hughes et al. 2018). Frequent mass bleaching events are occurring with increasing frequency as a consequence of global warming, threatening the survival of coral reefs (Donner et al. 2005; Baker et al. 2008; Hughes et al. 2018). Since $\sim$ 25% of all marine species are directly or indirectly dependent on coral reefs, their demise will have a substantial impact on marine biodiversity and result in a loss of ecosystem services for human societies (Hughes et al. 2003; Pandolfi et al. 2003).

Although the cellular mechanisms that underpin heat stress-mediated coral bleaching are not fully understood, it is clear that exposure to light and high temperature impairs the functioning of photosynthesis in the symbiotic algae (Lesser and Farrell 2004), associated with excess production of reactive oxygen species (Warner and Suggett 2016) and/or the loss of structural integrity of the thylakoid membranes (Warner et al. 1999; Tchernov et al. 2004; Díaz-Almeyda et al. 2011; Mansour et al. 2018). Therefore, the potential of corals to adjust to rising seawater temperatures will strongly depend on the capability of their algal symbionts to maintain their physiological function under changing conditions.

Recently, genetic analysis revealed a great taxonomic diversity among coral symbionts (Hume et al. 2016; LaJeunesse et al. 2018), but experiments with different *Symbiodiniaceae* species and genera in culture suggest that physiological traits cannot be directly explained by the taxonomic background in general (Goyen et al. 2017; Suggett et al. 2017; Mansour et al. 2018). However, ecological studies have identified the prevailing presence of specific symbiont species in heat tolerant corals. For example, in the hottest coral reef environment of the world, the Southern Persian/Arabian Gulf, *Symbiodinium thermophilum* is the prevalent symbiont of a range of host species (D'Angelo et al. 2015; Hume et al. 2016). The symbiont species *D. trenchii*, previously named *Symbiodinium trenchii* or clade D1a, is widely distributed in the world's oceans where its prevalence increased in the aftermath of heat stress anomalies, promoted by environmental degradation (LaJeunesse et al. 2014; Pettay et al. 2015). This species has the potential to increase the bleaching threshold of the host coral by $\sim$ 1–1.5 °C (Berkelmans and van Oppen 2006; Baker et al. 2008; LaJeunesse et al. 2014; Silverstein et al. 2017).

At present, the mechanisms underlying the increased thermal tolerance of *D. trenchii* are not fully understood. Earlier work that found a significantly higher saturation of 18-C fatty acids in thermally tolerant *Symbiodiniaceae* strains (Tchernov et al. 2004) led to the hypothesis that fatty acid saturation might be responsible for the increased thermal tolerance of *D. trenchii*. However, while a decrease in the proportion of unsaturated 18-C fatty acids was measured in cultured *Cladocopium* C1 (formerly clade C1) and *D. trenchii* after exposure to increased temperatures, there were no indications for a faster or stronger response in the latter (Kneeland et al. 2013). Equally, a comparative analysis of fatty acid content, their degree of saturation and associated thylakoid membrane integrity did not support a straightforward relationship between the saturation degree of fatty acids and increased thermal tolerance in cultured *Cladocopium* C1 and *Symbiodinium* A1 (Díaz-Almeyda et al. 2011).

Finally, a transcriptomic analysis of *D. trenchii* assigned a prominent role to a fatty acid desaturase in the evolutionary history of *Durusdinium* (clade D) (Ladner et al. 2012), which may indicate that desaturation of fatty acids might be involved in the response to thermal stress.

In summary, in spite of the accumulating evidence supporting a role of biological membranes in shaping the heat tolerance of coral symbionts (Tchernov et al. 2004; Díaz-Almeyda et al. 2011; Kneeland et al. 2013; Goyen et al. 2017; Mansour et al. 2018), there is no clear picture on how the lipid complement and fatty acid saturation might modulate the heat stress tolerance of *D. trenchii* and whether other lipid-mediated mechanisms might be utilised as well. An alternative response may involve the glycolipid sulfoquinovosyldiacylglycerol (SQDG), a key component of photosynthetic membranes, that has been previously assigned a critical role in modulating the heat and light stress tolerance of nutrient-deprived coral symbionts (Wiedenmann et al. 2013; D'Angelo and Wiedenmann 2014).

Therefore, in this study, we aimed to increase our understanding of the role of the lipid complement in the thermotolerance of coral symbionts. We applied high pressure liquid chromatography coupled with electrospray ionisation and tandem mass spectrometry (HPLC/ESI–MS/MS) analyses (Welti et al. 2002; Popendorf et al. 2013) to provide a comprehensive profile of the polar lipid compositions of two species of symbiotic cells selected by their differential thermotolerance. We used laboratory-cultured strains of the staghorn coral *Acropora valida* harbouring either *D. trenchii* or *Cladocopium* C3 (LaJeunesse et al. 2014, 2018) as dominant symbiont for experimentation to capture the in hospite functioning that is most representative of the natural environment. We compared the polar lipid profile of the two species with different thermal tolerance in the presence or absence of heat stress, to test our hypothesis that the constitution and remodelling of the membrane lipids are key processes associated with the heat stress response of the coral symbionts.

Materials and methods

Coral culture

Corals of Fijian origin, produced in local aquaculture, were obtained through Tropical Marine Centre London in 2009. The dominant symbionts harboured by different strains of *Acropora valida* were identified as *Symbiodiniaceae Cladocopium* C3 and *D. trenchii*, respectively, by analysis of the ITS2 rDNA sequences (Hume et al. 2013). Since then, these strains were asexually propagated within the experimental mesocosm at the University of Southampton (D'Angelo and Wiedenmann 2012). Repeated phylotyping confirmed that the corals did not change their dominant symbiont over time. Corals were cultured side-by-side at a constant temperature of 25 °C and light intensity of 150 µmol s^{-1} m^{-2} with a 12/12h light/dark cycle delivered by a metal halide lamp fitted with a 250 W Aqualine 10,000 burner (13,000 Kelvin) (Aqua Medic Bissendorf, Germany). Details of the setup are described in (D'Angelo and Wiedenmann 2012). Key water parameters during the relevant period were oxygen concentrations ~ 6.7 mg/l, pH ~ 8.2, salinity 33 and nitrate and phosphate were kept at replete levels (Wiedenmann et al. 2013; Rosset et al. 2017). Water current was generated by Turbelle Nanostream pumps (Tunze, Penzberg, Germany) with a flow rate 2400 l/h. The corals were placed in the main current of the pumps to ensure comparable exposure to water flow. Separate compartments of this experimental system with a comparable technical setup were used for the heat stress treatment. The experimental units were connected to the same recirculating water body, to ensure that major water parameters apart from temperature remained comparable.

Heat stress experiment

Replicate colonies containing either *D. trenchii* (control $n = 3$, heat stress $n = 4$) or *Cladocopium* C3 were used ($n = 3$ for each condition). Corals were kept in separate compartments of the experimental aquarium supplied with recirculated water from the same waterbody (~ 6000 l). Flow rates, current and light conditions were kept identical for the experimental tanks. In the treatment tanks, the temperature was gradually ramped up to 30 °C over a period of eight days. This elevated temperature was maintained for another eight days prior to sampling. A Diving Pulse Amplitude Modulated (PAM) Fluorometry (Waltz Diving PAM, Germany) was used to measure the maximum quantum yield (Fv/Fm) of the algal symbionts as an indicator of photodamage (Warner et al. 1999). After dark recovery for 11 h, corals were acclimated to low light (~ 5 µmol photons * m^{-2} s^{-1}) prior to taking the Fv/Fm measurements (Warner et al. 2010; Suggett et al. 2015). Great care was taken to keep the measuring probe at a comparable distance to the samples. By the end of the experiment, symbiont cell densities in the tissue of control and heat stress treated corals were determined as described below.

Isolation of symbiont cells

Symbiont cells were removed from defined areas of the coral colony by blasting the tissue using an airbrush. Algal cells were immediately separated from the host tissue homogenate by centrifugation at 2500×*g* (5 min, 4 °C). The pellets were then kept on ice. All samples were processed within a short timeframe (30 min), alternating the extraction of control and treatment samples. Cells were then re-suspended in ice-cold sterile seawater and an aliquot was removed for microscopic analysis. The remainder of the cells was again precipitated by centrifugation as described above. This wash step was repeated, and the cell pellets were immediately frozen at − 80 °C after removal of the supernatant. Cells in the separated aliquot were counted using a haemocytometer. To obtain cell density values for the corals, the determined cell numbers were normalised to the area of coral surface (cm^2) from which they were extracted.

Lipid extraction

Cell pellets were standardised to contain an equal cell number before extracting total lipids by a modified Bligh and Dyer protocol (Bligh and Dyer 1959). Cell pellets were

re-suspended in 800 µl Hank's balanced salt solution (HBSS) followed by the sequential addition of 1 ml dichloromethane, 2 ml methanol, 1 ml dichloromethane and 1 ml of demineralised water with vigorous mixing after the addition of each solvent. Organic and water phases were separated by centrifugation at 1000 g for 10 min. The lower organic phase was transferred into a glass vial, dried under a stream of nitrogen gas at 37 °C, and stored at − 20 °C.

Lipid analysis

Chromatography conditions

Dried lipid extracts were reconstituted in 100% methanol and separated using an Agilent 1100 LC system (Agilent Technologies) with a diol column (PrincetonSPHERE 100 DIOL 100 Å 5 µ, 150 × 2.1 mm), maintained at 22 °C. Mobile phases consisted of A) *n*- hexane/isopropanol/formic acid: 25% aqueous ammonium hydroxide (800:200:0.9:0.36 v/v); and B) isopropanol/water/formic acid: 25% aqueous ammonium hydroxide (900:100:0.9:0.36 v/v), as previously described (Popendorf et al. 2013). Chromatographic separation was achieved using a gradient of 100% A to 48% A over 20 min, then kept isocratic at 48% A for another 10 min. Solvent flow was set to 0.4 ml min^{-1}.

Mass spectrometry conditions and lipid analysis

Analysis of the lipids was performed using a triple quadrupole tandem mass spectrometer with electrospray ionisation interface (ESI-MS/MS) (Xevo, Waters, UK). Instrument parameters were set as follows: the source temperature was 150 °C, desolvation gas temperature was set at 200 °C, 3.22 kV to electrospray capillary, cone energy 50 V, argon was used as collision gas. The lipid classes monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), diacylglyceryl-carboxyhydroxymethylcholine (DGCC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) were identified by the use of precursor or neutral loss scans specific to diagnostic head group fragments (Table S1) (Popendorf et al. 2013).

All chromatograms and mass spectra were processed using MassLynx software version 4.0 (Waters, UK). For each chromatogram and mass spectra, background noise was subtracted and the data was smoothed prior to analysis. Each scan of a particular lipid class produced a total ion current chromatogram representative of all lipid species within the given class. The total abundance of each given lipid class was measured by integration of the chromatogram peak areas. The composition of each lipid class was determined by analysis of the mass spectra. The relative abundance of individual lipid species within each class was calculated by integration of the spectral peak areas and determination of the percent contribution of each peak area to the total. A threshold of 2% was set, with peak areas above this threshold considered to represent significant components of a respective lipid class.

The unsaturation index (UI) for each lipid class was calculated using the following formula:

$$\mathrm{UI}_x = \left[\sum y(\%\,\mathrm{lipid}_y \times \text{total number of double bonds lipid}_y)\right]/100$$

where y is every molecular lipid species belonging to the lipid class x.

MGDG and DGDG were quantified using lipid standards (Matreya LLC, PA, USA) dissolved in dichloromethane/methanol: 50 mM sodium acetate aqueous (300:665:35) using the precursor scan 243 ES+ (Welti et al. 2003). This was conducted by direct infusion with 5 µl/min flow rate and 58 V collision energy, using galactolipid standards as internal standards, allowing for direct comparison within the obtained mass spectra.

We compared the host lipid profiles with those of the symbionts using the same instrument settings and did not find indications of contaminations of the symbiont fraction above the detection threshold (data not shown).

Statistical analysis of data

The data is expressed as mean ± standard deviation (n = 3). Significant differences in lipid class abundance as well as in lipid class composition between the three samples were determined by two-way analysis of variance (ANOVA) followed by Tukey's test for pairwise comparison (Sigmaplot). Differences were considered to be significant with $P < 0.05$.

Principal component analysis

Principle component analysis (PCA) was performed using PAST software. For each lipid class, only lipid species that contributed more or equal to four per cent of the total composition of the given lipid class were included in the analysis. The values of signal intensity for each lipid species were used for this analysis rather than the percentage data to represent both differences in the composition of a lipid class as well as in the total abundance of a given lipid class among the experimental treatments. Three PCA runs were performed: the first including all analysed lipid classes, the second including only glycolipids, and the third including only betaine and phospholipids. Lipid species with loading values of ≥ 0.2 or ≤ − 0.2 were considered

to contribute prominently towards the principal components.

Results

In hospite response of symbiont species to heat stress

Replicate coral colonies of *A. valida* harbouring either *D. trenchii* or *Cladocopium* C3 as dominant symbionts were acclimated to the experimental aquarium conditions at 25 °C for < 6 months. The photosynthetic efficiency values (Fv/Fm) of ~ 0.65 characteristic for *D. trenchii* in hospite were higher compared to those of *Cladocopium* C3 (~ 0.55, Fig. S1a) at ambient temperature. In the high temperature treatment, only *Cladocopium* C3 symbionts showed a statistically significant reduction of Fv/Fm values compared to the control samples once 30 °C were reached after the 8 days of temperature ramping. Importantly, in temperature-treated *Cladocopium* C3 the lowered Fv/Fm values fell below 0.5 (Fig. S1a). In contrast, Fv/Fm values of *D. trenchii* never went below this value. The density of *Cladocopium* C3 zooxanthellae had dropped by ~ 50% at the end of the temperature treatment, whereas *D. trenchii* numbers remained essentially unaltered (Fig. S1b).

Differences in major lipid classes between the symbiont species

HPLC MS/MS analysis revealed the presence of lipid species belonging to the major polar lipid groups: glycolipids (MGDG, DGDG and SQDG), betaine lipids (DGCC), and phospholipids (PC, PG and PE). The minor class of phosphoserins (PS) and the betaine lipids diacylglyceryltrimethylhomoserine (DGTS) and diacylglyceryl-hydroxymethyl-N,N,N-trimethyl-beta-alanine (DGTA) were not detected using standard protocols (Popendorf et al. 2013; Cañavate et al. 2016). The comparative analysis between the two symbiont species indicated a different lipid composition of the membranes both under control conditions and after exposure to high temperature (Fig. 1a). Heat stress resulted in a change of total lipid composition only in *Cladocopium* C3 cells. These patterns were reproduced when only the glycolipids are included in the analysis (Fig. 1b). In contrast, no changes were obvious when the betaine lipids and phospholipids were analysed (Fig. 1c).

Differential accumulation of glycolipids in hospite

We compared the abundance of the chloroplast-located MGDG and DGDG lipids in the symbiont species under study. *Cladocopium* C3 symbionts accumulated slightly higher amounts of MGDG than *D. trenchii* both in the ambient controls and the temperature-treated samples (Fig. 2a). In contrast, the mean content of DGDG detected in *Cladocopium* C3 was lower compared to *D. trenchii* in both temperature conditions. This difference became statistically significant after exposure to high temperature (Fig. 2b). These patterns resulted in a significantly increased DGDG/MGDG ratio in *D. trenchii* as compared to *Cladocopium* C3 symbionts, both under control conditions as well as after the heat stress treatment (Fig. 2c).

Compared to control conditions, C3 exposed to high temperature contained significantly lower amounts of SQDG. The level of these lipids in *Cladocopium* C3 increased in response to heat stress but reached only levels as low as in *D. trenchii* cells isolated from the control corals kept at 25 °C. In contrast, the accumulation of SQDG in *D. trenchii* was not affected by the 30 °C treatment (Fig. 2d).

Changes in betaine lipids and phospholipids

DGCC was the only betaine lipid detected in the *A. valida* symbionts. *D. trenchii* cells contained a significantly lower amount of this lipid as compared to *Cladocopium* C3, both under control conditions and after heat stress (Fig. 3a).

Among the identified phospholipids in this study, *Cladocopium* cells accumulated higher levels of PC (Fig. 3b) and PG (Fig. 3c) than *D. trenchii*, whereas both species had comparable content of PE (Fig. 3d). The amount of detected phospholipids did not vary upon exposure to elevated temperature.

Higher content of lyso-lipids in *S. trenchii* symbionts

Lyso-forms of DGCC and PC were observed in both taxa in hospite. *D. trenchii* cells had a higher content of lyso-DGCC compared to *Cladocopium* C3 symbionts when corals where maintained under control conditions (Fig. 4a). Levels in *D. trenchii* dropped upon exposure to high temperature but remained significantly higher than in *Cladocopium* C3. Lyso-PC contents followed a comparable pattern, although differences were not statistically significant (Fig. 4b).

Varying unsaturation indices within lipid classes

MGDGs

The analysis of lipid species composition revealed significant differences in the main constituents of the MGDGs between the *Symbiodinianaceae* taxa (Fig. S2). Whereas ~ 50% of the MGDG species in *Cladocopium* contained 38C fatty acids both under control conditions and after temperature treatment, this value reached ~ 71% in

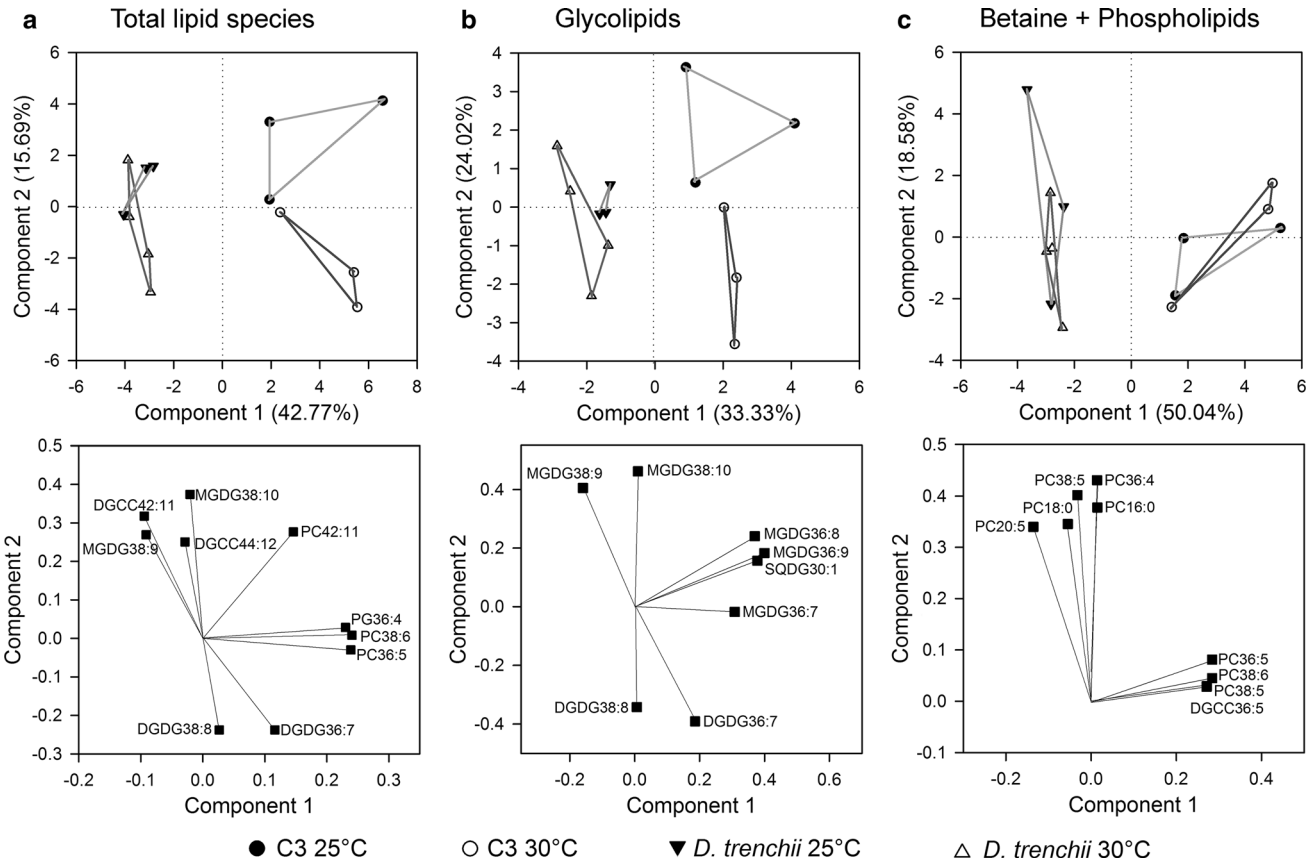

Fig. 1 Principal component analyses (PCA) of lipids identified in freshly isolated *D. trenchii* and *Cladocopium* C3 kept at 25 °C and after 30 °C treatment. **a** PCA of all lipid species identified, **b** PCA of glycolypids only, **c** PCA of betaine and phospholipids. The bottom graph of each panel show the major lipid species that contributed to the observed groupings in each particular analysis

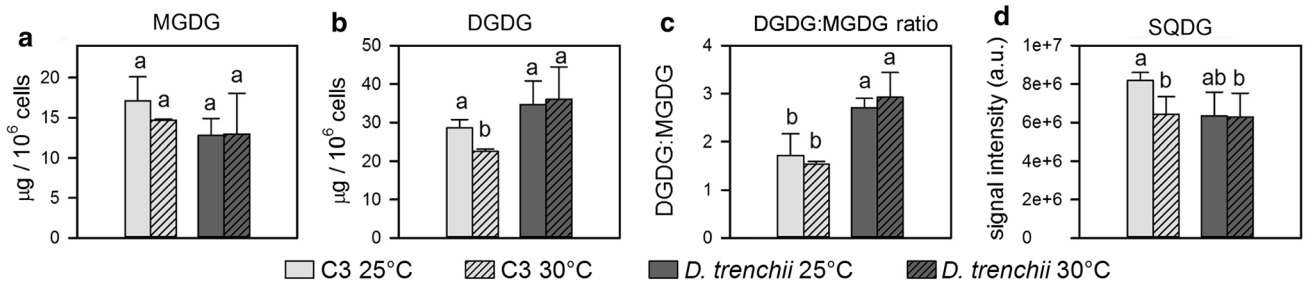

Fig. 2 Glycolipids in *D. trenchii* and *Cladocopium* C3 from *A. valida* corals kept at 25 °C and after 30 °C treatment. Content of MGDG (**a**) and DGDG (**b**) were calculated per million cells, quantification was performed using commercially available standards. **c** Ratio of DGDG to MGDG. **d** Amount of SQDG in symbiont cells expressed as the integrated HPLC chromatogram peak values. Bars display average values and error bars represent standard deviation. Lowercase letters indicate significant (*a/b*, $P < 0.05$) or nonsignificant (*a/a*, *b/b*) differences; *P* values from statistical analyses are given in Table S2

D. trenchii. In both taxa, the fatty acids in this lipid class exhibited a high degree of unsaturation, characterised by 8, 9, or 10 double bonds per corresponding MGDG molecule. Three different MGDG species with 36-C fatty acids were characterised by a total of 7, 8, or 9 double bonds per molecule. They represented ~ 37% of the total MGDG content in *Cladocopium* C3 cells. In contrast, these values were significantly lower in *D. trenchii* (control: 22.0 ± 1.2, treatment: 18.3 ± 1.8 (mean ± SD)) (Fig. S2, Table S2). The different composition of MGDGs between the taxa resulted in a significantly higher unsaturation index (UI) in *D. trenchii* as compared to *Cladocopium* C3 (Fig. 5, Tables S2 and S3).

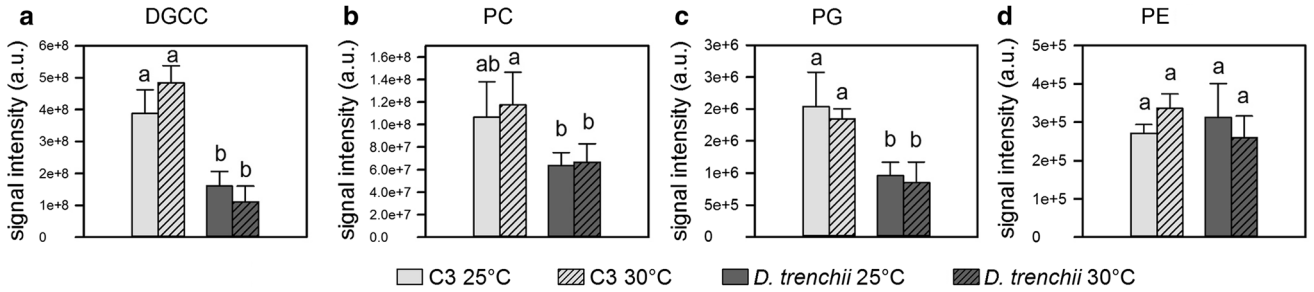

Fig. 3 Betaine lipids and phospholipids in *D. trenchii* and *Cladocopium* C3 from *A. valida* corals after kept at 25 °C or after incubation at 30 °C. Amounts of DGCC (**a**), PC (**b**), PG (**c**) and PE (**d**) measured in symbiont cells are expressed as the integrated peak values of HPLC chromatograms. Bars display average values and error bars represent standard deviation. Lowercase letters indicate significant (*a/b*, $P < 0.05$) or nonsignificant (*a/a*, *b/b*) differences; *P* values from statistical analyses are given Table S2

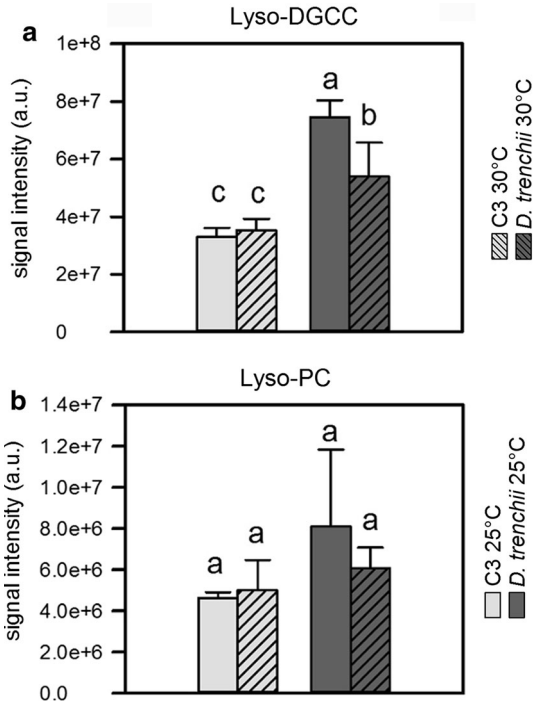

Fig. 4 Lyso-lipids in *D. trenchii* and *Cladocopium* C3 from *A. valida* corals kept at 25 °C or after incubation at 30 °C. Amounts of lyso-DGCC (**a**), lyso-PC (**b**) in symbiont cells are expressed as the integrated peak values of HPLC chromatogram. Bars display average values and error bars represent standard deviation. Lowercase letters indicate significant (*a/b*, $P < 0.05$) or nonsignificant (*a/a*, *b/b*) differences; *P* values from statistical analyses are given Table S2

DGDGs

The composition of DGDGs was similar in *Cladocopium* C3 and *D. trenchii* cells under control conditions. Out of all the DGDG molecules identified, ~ 20% contained 36-C and ~ 60% 38-C in their fatty acids in both species (Fig. S2). These major components did not vary after exposure to high temperature. The UI in *Cladocopium* cells kept at 25 °C was comparable to D. *trenchii* kept at 25 °C or after exposure to high temperature. Changes in the DGDG species composition of *Cladocopium* C3 upon heat stress exposure resulted in a significant decrease in the UI (Fig. 5, Table S2 and S4).

SQDGs

Three different species of SQDGs ([30:1], [30:0] and [32:0]) were identified in the *Symbiodianaceae* taxa in this study (Fig. S2). Out of the SQDG molecules identified in symbionts from corals kept under controlled conditions, 30.3 ± 7.4% (*Cladocopium* C3) and 25.7 ± 0.5% (*D. trenchii*) contained [30:1] fatty acids substituents (Table S5). Those values were significantly lower (21.0 ± 2.2% and 4.8 ± 2.0, respectively) when the corals were exposed to high temperature. A reversed pattern was observed for [32:0] molecules that were less abundant in symbionts from corals kept at 25 °C (Fig. S2, Table S5). The differences in the SQDG composition resulted in a significantly lower UI in *D. trenchii* cells as compared to *Cladocopium* C3 cells in both temperature conditions (Fig. 5, Table S2).

DGCCs

In both symbiont taxa, the pool of DGCCs was characterised by their content of long-chain fatty acids with a high degree of unsaturation. However, the lipid species composition was markedly different (Fig. S2, Table S6). In *D. trenchii*, the three main representatives of the DGCC class were molecules with [38:6] (43.3 ± 9.6%), [44:12] (29.2 ± 6.5%), and [42:11] (11.5 ± 3.0%). In contrast, the three most abundant lipid species of this class in *Cladocopium* cells were [38:6] (52.3 ± 2.5%), [36:5] (23.0 ± 2.4%), and [44:12] (11.09 ± 3.9%). These differences result in a UI of DGCC that is significantly higher in *D. trenchii* cells, both under control conditions and after heat stress (Fig. 5, Table S2).

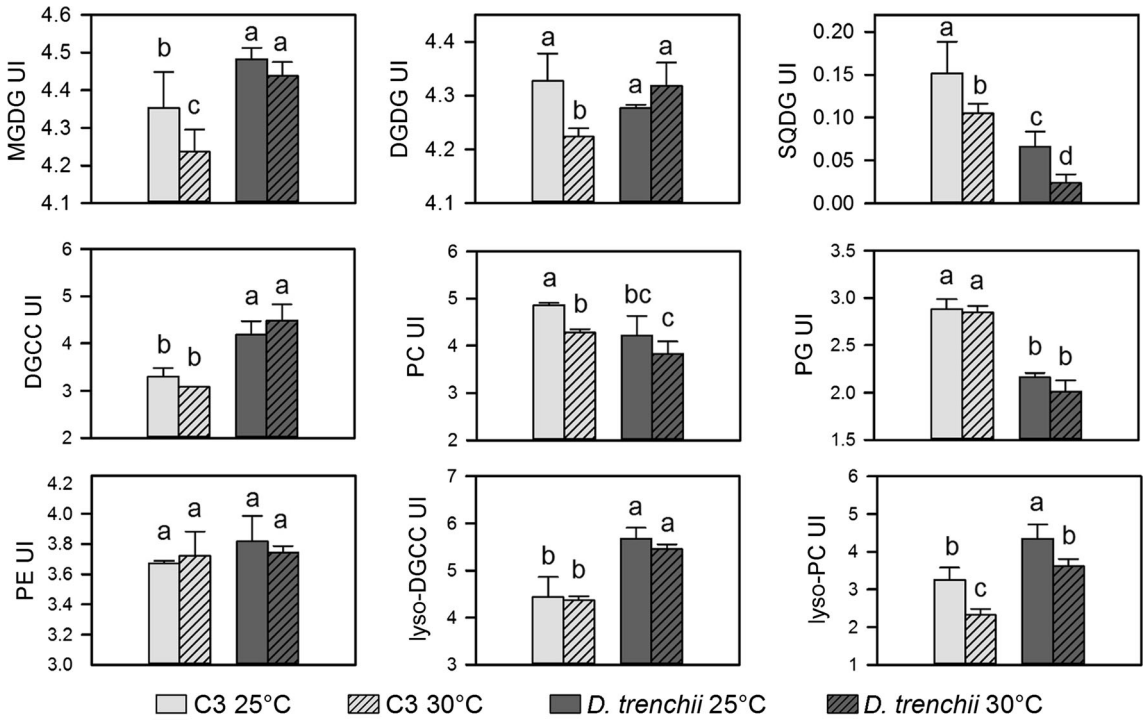

Fig. 5 Unsaturation index (UI) from lipids identified in *D. trenchii* and *Cladocopium* C3 from *A. valida* corals kept under 25 °C or after incubation at 30 °C. Bars display average values and error bars are standard deviations. Lowercase letters indicate significant (*a/b/c*, $P < 0.05$) or nonsignificant (*a/a*, *b/b*, *c/c*) differences; *P* values from statistical analyses are given Table S2

PCs, PGs and PEs

Two of the main species identified within DGCCs, namely [44:12] or [38:6], were also detected in PCs in both taxa (Fig. S2). Respectively, they contributed 59.1 ± 1.8% and 14.7 ± 1.7% to the total PC species in *Cladocopium* C3 and 49.6 ± 9.5% and 9.0 ± 1.8% in *D. trenchii* (Table S7). While the composition of PC species remained unaltered in *D. trenchii* in response to heat stress, in *Cladocopium* C3 the representation of individual PC molecules changed and resulted in a decrease of the UI to a value comparable to that of *D. trenchii* under control conditions (Fig. 5, Table S2).

Both under control and temperature stress conditions, a lower UI was calculated for PGs present in *D. trenchii* cells as compared to *Cladocopium* C3 (Fig. 5, Fig. S2, Tables S2 and S8). In contrast, PE was characterised by the same UI values in both taxa (Fig. 4g, Fig. S2, Tables S2 and S9). Among these lipids classes, only the PCs in *Cladocopium* C3 showed a significant reduction of the UI after exposure of the corals to high temperature.

Lyso-lipids

In *D. trenchii*, ~ 80% of the lyso-DGCCs contained [22:6] fatty acids as substituents (Fig. S2), whereas this species contributed only ~ 60% of lyso-DGCCs in *Cladocopium* C3 (Table S10). Thus, a higher UI characterised lyso-DGCCs in *D. trenchii* as compared to *Cladocopium* C3 cells (Table S2). No changes of this parameter were measured after the stress treatment (Fig. 5). In contrast, the UI of lyso-PC, also higher in *D. trenchii* than in *Cladocopium* C3 cells, decreased after exposure to elevated temperature in both symbiont taxa (Fig. 5). The most abundant lyso-PC species in both taxa contained the [22:6] fatty acid (Fig. S2, Table S11).

Discussion

Despite ample evidence demonstrating the higher thermal tolerance of corals when they are associated with *D. trenchii* (Berkelmans and van Oppen 2006; Baker et al. 2008; Pettay et al. 2015; Silverstein et al. 2017), the cellular mechanisms involved in the superior performance of this coral symbiont species under heat stress are not well understood. In this work, we used HPLC ESI–MS/MS to examine the polar lipid composition in symbiotic dinoflagellates comparing different species in hospite. Specifically, we used different strains of *A. valida* containing either thermotolerant *D. trenchii* or *Cladocopium* C3 which is representative of a thermosensitive taxon

(LaJeunesse et al. 2003, 2014, 2018) for qualitative and quantitative analyses of the polar lipids of the dinoflagellate cells. The symbionts were extracted from host *A. valida* kept under control conditions and after exposure to heat stress. PCA of the key lipid classes reveal fundamental differences in the lipidomes of the two symbiont species already at ambient temperatures. Heat stress resulted in a change of total lipid composition only in *Cladocopium* C3 cells. The same patterns could be observed when the glycolipids were analysed in isolation but not when the betaine lipids and phospholipids were analysed by themselves. These data indicates that the observed changes in the total lipid complement are mostly driven by changes in glycolipids.

Here, we argue that the increased performance of the *D. trenchii* under study at elevated temperatures could result from several distinct strategies to modulate their membrane composition, and we propose lipid-mediated mechanisms of thermotolerance that have not yet been discussed for coral symbionts.

Heat stress response of corals harbouring *D. trenchii* and *Cladocopium* C3

The efficiency of PSII after dark recovery measured as Fv/Fm in vivo was used as an indicator for the degree of photodamage of the symbionts (Warner et al. 1999; Gorbunov et al. 2001; Smith et al. 2013). In the absence of heat stress, Fv/Fm values were notably higher for *D. trenchii* ($\sim$ 0.65) than for *Cladocopium* ($\sim$ 0.55), a difference that is expected between different species of *Symbiodianacea* (Suggett et al. 2015). However, while in heat-stressed *Cladocopium* C3 the Fv/Fm after dark recovery consistently dropped, reflecting a typical response of a stress sensitive species, the Fv/Fm of *D. trenchii* did not show a statistically significant change in response to the temperature treatment (Warner et al. 1999, 2010). At the same time, symbiont densities reduced by 50% in heat-stressed *Cladocopium* C3-containing corals, whereas no loss of symbionts was detected in animals harbouring *D. trenchii.* Our data suggests that the photosynthetic reaction centre pools of *D. trenchii* in hospite is less compromised under heat stress (Goyen et al. 2017) compared to those of *Cladocopium* C3. This may indicate that that *D. trenchii* is less affected by the temperature treatment and help to explain why symbiont numbers remain high despite the exposure to elevated temperatures. These findings are in line with increased bleaching tolerance reported for corals hosting *D. trenchii* from natural reefs (Berkelmans and van Oppen 2006; Jones et al. 2008; Silverstein et al. 2017).

Saturation of fatty acids in response to high temperature

Our study shows that out of the nine lipid classes detected in *Symbiodiniaceae* cells in hospite, five (MGDG, DGDG, SQDG, PC, lyso-PC) have a higher saturation of their fatty acids in the heat sensitive *Cladocopium* C3 after exposure to elevated temperature. This can be considered as a classical response of the cells in photosynthetic organisms to prevent leakiness of the biological membranes occurring at high temperatures (Gombos et al. 1994). The absence of a similar response in *D. trenchii* could indicate that the temperature stress used in our experiment was not severe enough to trigger a comparable mechanism in the thermotolerant symbiont or that the thermal acclimation response of this species relies on alternative strategies.

In line with the latter, major lipid classes (MGDGs, DGCC, lyso-DGCC and lyso-PCs) in *D. trenchii* revealed a higher degree of unsaturation as compared to *Cladocopium* C3, independently from the experimental temperature. In our study, only PCs, PGs and SQDGs were significantly more saturated in *D. trenchii*, both at ambient temperature and under heat stress.

An increased fatty acid saturation in *D. trenchii* in response to elevated temperature was detected only among SQDGs and lyso-PCs. These results suggest that the higher thermotolerance of *D. trenchii* in hospite is not accomplished by a generalised increased saturation of lipids in their biological membranes. At the same time, our findings suggest a central role of the saturation of SQDGs in the response to temperature stress as their unsaturation index is already reduced in *D. trenchii* at ambient temperature and the difference to *Cladocopium* C3 is further increased under temperature stress.

A key role of SQDGs in the thermotolerance of coral symbiont species

Fatty acids of SQDGs in free-living dinoflagellates and symbiotic forms from non-coral hosts include saturated or monounsaturated forms (Leblond and Chapman 2000; Awai et al. 2012; Garrett et al. 2013). In the coral symbiont species examined in this study, we have identified the SQDG molecules containing fatty acids with combined [C-chain lengths: double bonds] of [30:0], [30:1] and [32:0]. The decreased UI calculated for the SQDG class of *D. trenchii* after exposure of corals to high temperature is due to a reduced content of one species, [30:1], and the concomitant increase in the percent of [32:0] molecules. This type of modification, in which the single double bond is replaced to produce a fully saturated molecule, has been

shown to have a disproportionately high effect on the environment of the molecule and on the lipid melting point, and thus the biophysical properties of the membrane (Quinn et al. 1989). Moreover, although they do not reach the values measured in *D. trenchii*, a reduced UI of SQDGs after the high temperature treatment was also detected in the heat sensitive *Cladocopium* C3 symbionts, pointing to a general response strategy in symbiotic dinoflagellates.

Notably, the present paper demonstrated marked differences between the two symbiont species not only regarding the degree of saturation, but also concerning the overall content of SQDG. We found that in the absence of heat stress *D. trenchii* contained lower amounts of these lipids as compared to *Cladocopium* C3. Upon exposure to elevated temperatures, *Cladocopium* C3 cells reduced SQDGs to levels comparable to *D. trenchii*. A previous study revealed a link between reduced thermal tolerance with a relative increase in the cellular SQDG content in coral symbionts in response to nutrient stress (Wiedenmann et al. 2013), suggesting the reduced SQDG content in *D. trenchii*, along with the higher saturation, might be a mediator of its thermal tolerance.

In plant cells, SQDGs are found exclusively in chloroplast membranes. They are bound to photosystem II-related complexes and contribute directly to the maintenance of structure and function of the photosynthetic apparatus (Sato et al. 2003). Structural disorder of the D1 protein within the PSII complex caused by the complete loss of SQDG has been suggested to interfere with the functioning of the oxygen-evolving complex and electron transfer (Frentzen 2004). Accordingly, the regular conformation of the PSII complex supported by the association with SQDG is considered to be indispensable for the heat and light stress tolerance of PSII in green algae (Minoda et al. 2002; Sato et al. 2003; Frentzen 2004). Importantly, PSII damage in *Symbiodiniaceae* and the resulting photoinhibition is a key determinant of coral bleaching (Warner et al. 1999; Warner and Suggett 2016). In this context, it has been proposed that among the various physiological impairment mechanisms operating at different extents across different taxa, heat stress may for instance aggravate photoinhibition by reducing the repair of PSII (Takahashi et al. 2004; Warner and Suggett 2016).

Alternative composition of thylakoid membranes as a strategy to cope with high temperatures

The galactolipids MGDG and DGDG are the main components of the thylakoid membranes of photosynthetic cells (Dörmann and Benning 2002; Harwood and Guschina 2009; Moellering and Benning 2011). Our results show that these lipids in *D. trenchii* and *Cladocopium* C3 in hospite are characterised mainly by their polyunsaturated fatty acids (PUFAs), in agreement with previous analyses of the lipid composition of dinoflagellates including *Symbiodiniaceae* (Leblond and Chapman 2000; Guella et al. 2003; Gray et al. 2009; Awai et al. 2012; Flaim et al. 2012; Leblond et al. 2015). Interestingly, we measured a significantly higher ratio of DGDG/MGDG in *D. trenchii* as compared to *Cladocopium* symbionts. This difference is remarkable due to the contrasting biophysical properties of these molecules, with DGDGs being bilayer-forming and MGDGs non-bilayer lipids (Quinn et al. 1989; Dörmann and Benning 2002). Consequently, the ratio of DGDG/MGDG in the membrane is crucial for proper physiological functioning and it is thus tightly regulated by photosynthetic cells (Bruce 1998). In *S. microadriaticum,* an increase in the DGDG/MGDG ratio was detected after exposure to elevated temperatures (Leblond et al. 2015). The critical effect of such a ratio change was demonstrated by the analysis of *Arabidopsis* mutants defective in the capacity to acquire thermotolerance (Chen et al. 2006). While wild-type plants survived heat stress exposure when they had previously been exposed to moderately high temperatures, the mutants died during comparable treatments. This lack of acquired thermotolerance was directly related to the incapability of the mutant to increase the DGDG/MGDG ratio in their chloroplasts in response to a pre-treatment with moderate heat stress (Chen et al. 2006).

The ratio of bilayer-forming to non-bilayer-forming lipids affects the insertion of proteins in the membrane and the intracellular protein trafficking (Dörmann and Benning 2002). Accumulating evidence indicates that this ratio might play a direct role in assisting the proper folding or renaturation of integral membrane proteins (Bogdanov and Dowhan 1999; Bogdanov et al. 2010). Therefore, our results suggest that the increased thermotolerance of *D. trenchii* in hospite could be directly related to the higher ratios of DGDG/MGDG even in the absence of heat stress. In contrast, the susceptibility of *Cladocopium* C3 could be related to the generally lower DGDG/MGDG ratios coupled to the incapability to remodel the galactolipid profiles of its chloroplasts in response to elevated temperatures. Future research should investigate to which extent the observed difference in the changes in the lipid profiles are an adaptive response to mitigate the impact of stress or an indicator of stress-induced damage to the organism.

The polar lipid profile of Symbiodiniaceae and the role of lyso-lipids

At present, there is no comprehensive characterisation of the polar lipid profile of coral-hosted *Symbiodiniaceae* available. Our study therefore fills an important knowledge gap and complements reports on some lipid molecules characteristic in other symbiotic and non-symbiotic dinoflagellates

(Gray et al. 2009; Awai et al. 2012; Flaim et al. 2012; Imbs et al. 2015; Leblond et al. 2015). With the exception of PEs, all lipids detected in our work exhibited pronounced differences in their cellular content or degree of saturation when comparing *D. trenchii* and *Cladocopium*. In contrast, the amount of these lipids remained essentially unaltered by the heat stress treatment in each species.

Interestingly, compared to *Cladocopium*, *D. trenchii* was characterised by higher levels of lyso-lipids, in particular by lyso-DGCC, showing also differences in their saturation degree. Lyso-lipids are upregulated in bacteria exposed to elevated temperature, where they facilitate the renaturation of proteins by a mechanism similar to that of molecular chaperones (Kern et al. 2001). The chaperon-like function of lyso-lipids could be confirmed by in vitro studies using synthetic model systems (Kern et al. 2001). Therefore, the increased accumulation of lyso-lipids may constitute a separate mechanism involved in the heat stress tolerance of *D. trenchii*.

While corals in nature can experience acute heat stress episodes comparable to our experimental treatment (Riegl et al. 2019), they are also frequently exposed to longer lasting chronic heat stress and therefore it would be important to extend these studies to evaluate the comparative responses of lipid remodelling between acute and chronic stress.

In summary, our work provides a comprehensive characterisation of the polar lipid content of dinoflagellate symbionts of reef-building corals in hospite, contributing knowledge urgently required to understand the physiological functioning of this association. We provide evidence that the lipid complement in hospite of the heat tolerant *D. trenchii* is profoundly different compared to the more susceptible *Cladocopium* C3.

The results of our study indicate that symbiotic algae can apply multiple strategies involving the lipid constitution of their membranes to cope with elevated temperature. These insights not only further our knowledge on the capacity of reef corals to adjust to the impact of climate change but also have a broad significance to understand heat stress responses in photosynthetic organisms.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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