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Lipid class composition of annually bleached Caribbean corals

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Abstract

Corals with high levels of total lipids are known to have increased resilience potential to bleaching, and lipid class management may shed further light on why some species are more resilient to, or are able to acclimatize to, annual bleaching stress. Here, we measured the lipid class composition of three species of Caribbean corals (*Porites astreoides*, *Porites divaricata*, and *Orbicella faveolata*) collected in July 2009 near Puerto Morelos, Mexico (20° 50′ N, 86° 52′ W) that were experimentally bleached 2 years in a row. Our results show that single bleaching can significantly alter lipid class composition in all species, while repeated bleaching can result in stable (i.e., acclimatized) or even more altered (i.e., not acclimatized) lipid class composition depending on the species. Specifically, *P. divaricata* and *O. faveolata* both had altered lipid class composition with losses in storage lipids following single bleaching, but maintained lipid class composition following repeated bleaching stress. However, both single and repeated bleaching altered the lipid class composition in *P. astreoides*, with changes persisting for the 6 weeks after repeated bleaching stress. This study provides evidence that lipid class management is part of the suite of variables associated with coral resilience, that *P. divaricata* and *O. faveolata* acclimatize their lipid class management in response to repeated bleaching stress, but that *P. astreoides* does not. Corals like *P. divaricata* and *O. faveolata* may, therefore, be more suitable for coral restoration efforts since they are more likely to persist under chronic repeat bleaching scenarios predicted for later this century.

Introduction

As seawater temperatures continue to rise, mass coral bleaching events are increasing in frequency and severity (Hughes et al. 2003; Hoegh-Guldberg et al. 2007). Tropical symbiotic Scleractinian corals have a mutualistic symbiotic relationship with photosynthesizing endosymbiotic algae (Symbiodiniaceae). Under a range of normal seawater conditions, the algal endosymbionts produce photosynthetic sugars that are transferred to the coral animal host. However, during periods of elevated seawater temperature, the symbiosis is disrupted and a large proportion of the algal endosymbionts are lost. When this happens, the corals have a white or bleached appearance, and photosynthesis and the corresponding photosynthetically fixed carbon production dramatically decline, putting the corals in a starved state (e.g., Glynn 1996; Brown 1997; Hoegh-Guldberg 1999; Grottoli et al. 2006). Prolonged bleaching stress often leads to declines in coral growth and reproduction, as well as increased incidence of disease and mortality (e.g., Hoegh-Guldberg 1999; Rosenberg and Ben-Haim 2002; Rodrigues and Grottoli 2007; Veron et al. 2009). Repeated bleaching

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can result in cumulative damage in some cases and induce acclimatization in others, turning some coral species "winners" into "losers" (Grottoli et al. 2014). Models had predicted that at the current rate of warming coral bleaching would occur annually in the Caribbean by 2025 and globally by midcentury (Donner et al. 2005; Donner 2009; van Hooidonk et al. 2013, 2014, 2015). However, back-to-back bleaching events have already been observed in Hawai'i, Florida, and the Great Barrier Reef. In 2016–2017, unprecedented repeated mass coral bleaching on the Great Barrier Reef resulted in 60% coral mortality (Hughes et al. 2019). Despite the devastating mortality and reef degradation from repeated bleaching events, some coral survive and recover from these events. Susceptibility to bleaching and the capacity to recover from bleaching varies among species and locations (e.g., Marshall and Baird 2000; Loya et al. 2001; Guest et al. 2012; Fine et al. 2013) and may be related to coral morphology (Wilkinson and Hodgson 1999; Loya et al. 2001), the species of Symbiodiniaceae hosted by the coral (e.g., Rowan et al. 1997; Warner et al. 1999; Berkelmans and van Oppen 2006; Grottoli et al. 2014; Howells et al. 2016), heterotrophic capacity or plasticity (Grottoli et al. 2006; Palardy et al. 2008; Levas et al. 2016), or energy reserves (e.g., Rodrigues and Grottoli 2007; Rodrigues et al. 2008; Anthony et al. 2009; Grottoli et al. 2014). Within the energy reserves, lipids appear to play a large role in resilience to, and survivorship from bleaching events (e.g., Grottoli et al. 2004; Tchernov et al. 2004; Rodrigues and Grottoli 2007; Anthony et al. 2009).

Most healthy corals can meet more than 100% of their metabolic demand with photosynthetically derived carbon (C) from their algal endosymbionts (e.g., Muscatine et al. 1984; Davies 1991; Grottoli et al. 2006, 2014). Excess fixed C can be assimilated in the form of lipids (Muscatine and Cernichiari 1969; Patton et al. 1977; Harland et al. 1993; Baumann et al. 2014). However, when corals are bleached they can no longer meet 100% of metabolic demand photosynthetically (Grottoli et al. 2006; Palardy et al. 2008; Grottoli et al. 2014) and must rely on other sources of fixed C to survive. Strategies for compensating for losses in photosynthetically derived C due to bleaching include: increased heterotrophy (i.e., feeding on zooplankton, particulate and dissolved organic C) (Grottoli et al. 2006; Palardy et al. 2008; Levas et al. 2016), decreased respiratory demand (Rodrigues and Grottoli 2007), decreased calcification (e.g., Jokiel and Coles 1977; Suzuki et al. 2003; Rodrigues and Grottoli 2006; Schoepf et al. 2015), catabolism of stored lipid reserves (e.g., Porter et al. 1989; Rodrigues and Grottoli 2007; Schoepf et al. 2015; Levas et al. 2018), or some combination of these.

Triacylglycerols and wax esters are considered as a significant source of stored C and energy, and can account for 46–73% of total coral lipids (Stimson 1987; Harland et al. 1993; Yamashiro et al. 1999; Oku et al. 2002; Rodrigues et al. 2008; Imbs and Yakovleva 2012). In addition, they are also the major lipid components of coral mucus (Benson and Muscatine 1974; Crossland et al. 1980; Crossland 1987) and eggs (Arai 1993; Padilla-Gamino and Gates 2012). When bleached, corals catabolize storage lipids (Yamashiro et al. 2005; Rodrigues et al. 2008; Imbs and Yakovleva 2012) and branching corals catabolize more storage lipids than mounding corals (Yamashiro et al. 2005). Structural lipids such as phospholipids and cholesterol are essential building blocks for cell membranes: their concentrations can fluctuate seasonally (Oku et al. 2003; Rodrigues et al. 2008) and as a result of short-term sedimentation stress (Niebuhr 1999). In bleached corals, phospholipids tend to decrease due to cell damage and cholesterol tend to increase in corals that increase their feeding on zooplankton, as it is one of the sources of cholesterol to the coral diet (Grottoli et al. 2004; Yamashiro et al. 2005; Rodrigues et al. 2008; Imbs and Yakovleva 2012). However, it is unknown how lipid classes vary in Caribbean corals in response to bleaching, or how lipid class management is affected by repeated bleaching in any coral species.

Grottoli et al. (2014) showed that the Caribbean corals *Porites divaricata*, *Porites astreoides*, and *Orbicella faveolata*, exhibited contrasting physiological responses to experimentally induced single and repeated bleaching. *P. divaricata* catabolized its total lipids after both single and repeated bleaching, *O. faveolata* catabolized lipids only after repeated bleaching, while total lipids in *P. astreoides* were not catabolized in response to either single or repeated bleaching (Schoepf et al. 2015; Levas et al. 2018). Shifts in coral lipid class composition can reveal if corals catabolize their storage lipids (i.e., wax esters and triacylglycerols), incorporate more heterotrophic carbon into their tissues (i.e., increases in cholesterol), experience cellular damage (i.e., decreases in phospholipids), and if these response strategies vary with repeated bleaching or among species. In addition, changes in the proportionate contribution of each lipid class to coral lipid content can fluctuate, even when total lipids are unchanged. In effect, lipid class management can reveal underlying resilience strategies in corals. We know that the physiological responses to repeated stressors can differ vastly from those to single bleaching (Grottoli et al. 2014) and that lipids are key indicators of bleaching resistance capacity (e.g., Rodrigues and Grottoli 2007; Anthony et al. 2009; Schoepf et al. 2015; Levas et al. 2018). However, the lipid profiles of annually bleached corals have never been examined. Given the increasing frequency of bleaching events, understanding how lipid class composition varies in response to annual bleaching could be highly valuable to unlocking the underlying traits that impart resilience and susceptibility in corals. Here, we measured the lipid class composition (wax esters, triacylglycerols, phospholipids,

cholesterol, diacylglycerols, monoacylglycerols, and free fatty acids) in annually bleached Caribbean corals *P. divaricata*, *P. astreoides*, and *O. faveolata*.

Materials and methods

This study was conducted at Universidad Nacional Autonoma de Mexico's Instituto de Ciencias del Mar y Limnologia (UNAM-ICML, 20° 52′ N, 86° 52′ W). The maximum monthly mean (MMM) sea surface temperature on the reefs in the region occurs during August/September at 29 °C and the bleaching threshold sea surface temperature is 30.0 °C (i.e., MMM +1 °C) (NOAA Coral Reef Watch 2000). Satellite coral bleaching monitoring tools maintained by the U.S. National Oceanic and Atmospheric Administration (NOAA) have shown that 3–4 degree heating weeks (DHW = degrees celsius above MMM × number of weeks) are sufficient to induce bleaching in Caribbean corals (Liu et al. 2006; Eakin et al. 2010).

Experimental design

A detailed description of the experimental design is provided in Grottoli et al. (2014). Briefly, 10 coral ramets were collected from each of nine healthy colonies of *Porites divaricata*, *Porites astreoides*, and *Orbicella faveolata* (formerly *Montastraea faveolata*) in July 2009 from reefs near Puerto Morelos, Mexico (20° 50′ N, 86° 52′ W), totaling 90 fragments per species. One fragment from each colony was placed in one of 10 shaded outdoor flow-through seawater tanks. The corals were allowed to acclimate for 5 days. The temperature in five of the tanks was gradually elevated to 31.5 ± 0.20 °C over the course of 7 days, and subsequently maintained at that temperature (single bleaching treatment). The other five tanks received ambient reef water (30.6 ± 0.24 °C; controls). After 15 additional days (~ 3.2 DHW), one control and one treatment fragment from each parent colony were frozen at − 80 °C (0 weeks on the reef). The remaining fragments were transplanted back to the reef at 4.9-m depth (20° 52.815′ N, 86° 50.989′ W). After 6 weeks, one additional control and treatment fragment from each parent colony of each species were collected, frozen at − 80 °C, and the remaining fragments stayed on the reef for 1 full year.

The experiment was repeated the following summer by recollecting the remaining fragments from the reef. Coral fragments that were exposed to elevated temperatures the previous year were placed in tanks with elevated temperatures again (31.6 ± 0.24 °C) to simulate bleaching for a second year in a row (repeated bleaching). Coral fragments that were exposed to ambient temperatures the previous year were placed in tanks with ambient reef water again (30.4 ± 0.23 °C) as controls. After 17 days (~ 3.9 DHW), one treatment and one control fragment were collected and frozen at − 80 °C. The remaining fragments were transplanted back to the reef and then recollected after 6 weeks and frozen at − 80 °C.

Lipid class analyses

Total lipids were extracted with chloroform from a ground sub-sample of each coral fragment, as reported in Levas et al. (2018) and Schoepf et al. (2015). Archived total lipids were suspended in 5 mL of chloroform and stored at − 80 °C. Lipid class composition was measured on the archived total lipid samples using thin layer chromatography (TLC) according to the methods modified from Muñoz-Garcia and Williams (2005). Details pertaining to all chemicals and standards used are listed in Online Resource 1. In brief, prior to analyses, total lipid extracts were dried down under a stream of nitrogen gas over a water bath at 55 °C, and re-suspended in 80–500 µL of chloroform:methanol 2:1 (v/v) to which 50 mg L^{-1} of butylated hydroxytoluene was added to prevent oxidation of lipids. Silica gel plates were divided into thirteen 1-mm-wide lanes. Two sets of TLC plates were developed: one for non-polar lipids and another for the relatively polar lipids. To quantify each lipid class, two lipid standard solutions were prepared. For non-polar lipids, the standard solution contained wax esters, triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, and cholesterol. The standard solution of polar lipids contained phospholipids and cholesterol. Standard concentrations were prepared in chloroform:methanol 2:1 (v/v) with known concentrations of storage lipids ranging from 8.5 to 25 mg mL^{-1}, and structural lipids ranging from 5 to 8.5 mg mL^{-1}. The stock solution was serially diluted by a half three times to create four concentrations of the standards, which were loaded on every plate. Using a glass Hamilton gas tight syringe, 5–10 µL of each sample was loaded in duplicate in the pre-adsorbent layer of the plates. In addition, to validate our ability to quantify the lipids, we loaded a known concentration of cholesterol in chloroform:methanol 2:1 (v/v) in one lane on every plate. All plates were first washed using chloroform:methanol 2:1 (v/v) and activated for 30 min at 110 °C prior to sample loading.

To separate classes of non-polar lipids, a three-step development was used with 100% hexane, 100% toluene, and hexane:ethyl ether:acetic acid 70:30:1 (v/v/v), which were all run to the top of the plate. To separate classes of polar lipids, we used a two-step development in which solvent fronts of chloroform:methanol:water 40:10:1 (v/v/v) were allowed to travel to the top and to 10 cm of the plate, respectively. Between developments, plates were dried. The separated lipid bands were visualized by spraying the plates with a solution of 3% cupric acetate in 8% phosphoric acid

and then placing the TLC plates on hotplates or in an oven that were gradually heated to 220 °C over the course of 1 h.

Lipid classes were quantified from the absorbance of bands as a proxy for concentration. Following heating, TLC plates were scanned using an HP Scanjet 5590 and the absorbance of each band was quantified by photo densitometry using the image software Image Measurement and Analysis Lab (IMAL). Then, a standard curve for every lipid class was generated. To validate this method, we used the standard equations of each plate to calculate the actual concentration of cholesterol that was loaded in each plate. The average error, calculated as [(observed − actual)/actual], was of 3.36% ($n = 53$). Whenever error exceeded 10%, the samples were rerun. Lipid class values were reported as a proportion of the total lipid amount in Schoepf et al. (2015) and Levas et al. (2018).

Statistics

Non-metric multidimensional scaling (NMDS) plots were generated to graphically represent relationships between the overall lipid class compositions of each coral sample in the entire data set and to determine if there were any differences among species. Since 31 of the 189 samples had one or more lipid class values missing, they were excluded from the multi-variate analyses. A Bray–Curtis distance-based resemblance matrix was constructed using a square root transformation of the proportionate lipid classes from each sample. Vectors were added to NMDS plots (Pearson correlations > 0.1) to show the direction and magnitude of the influence of each lipid class to the distribution of the underlying data. To determine if species differed from each other in their overall lipid class composition, a one-way analysis of similarities (ANOSIM) was conducted. The ANOSIM pairwise test statistic R ranges from 0 (no difference and complete overlap between groups) to 1 (maximum difference and no overlap between groups) and is a strong indicator of separation among groups (Clarke and Gorley 2006). Differences were determined to be statistically significant at a $p < 0.05$. In addition, similarity percentages (SIMPER) analysis was conducted to determine the proportionate contribution of each lipid class to the dissimilarity among coral species. These analyses were then repeated on each species individually to determine if lipid class composition changed over time in bleached and control corals. Multivariate analyses were conducted using the software package Primer V6.

To further investigate how individual lipid classes (particularly those identified in the SIMPER analyses as most responsible for differences due to treatment) differed among treatments within each species, a three-way analysis of variance (ANOVA) was conducted on each lipid class for each species. Since ANOVA is robust to missing values, none of the data was removed from these analyses and all available data for all 189 samples was included. We considered treatment (controls, bleached corals) and time (0 and 6 weeks on the reef in 2009, 0 and 6 weeks on the reef in 2010) as fixed effects and fully crossed. Genotype was a random effect (nine genotypes) and was included in the ANOVA model to determine if any single genotype was systematically different from all others for a given lipid class. Because genotype was not a significant factor in any case, we removed it from the analyses. When a model was significant, post hoc slice tests were used to determine significant differences between average lipid class proportions between treatment and control corals within each time point. Normality was determined using a Shapiro–Wilk's test and homogeneity of variance by comparing plots of calculated expected vs. residual values prior to each ANOVA. If the distributions were not normal, a log or square root transformation of the data was used to achieve normality prior to ANOVA analyses. If the average of a given lipid class proportion in the control corals were significantly different from treatment corals immediately following bleaching (i.e. 0 weeks on the reef) and then no longer significantly differed after 6 weeks at ambient temperatures on the reef, that lipid class was considered to be recovered from bleaching. Since fragment pairs of each colony were included in all treatments and controls, differences between treatment and control for any variable were due to treatment alone, independent of genotype and seasonality. These univariate parametric analyses were conducted using SAS 9.4 and differences were considered as statistically significant at $p < 0.05$.

Results

A detailed record of average daily seawater temperatures on the reef and in experimental tanks as well as the visual appearance of the coral fragments throughout the study are described in Grottoli et al. (2014). In summary, following the first bleaching in 2009 (single bleaching), treatment fragments from all three species were visibly paler compared to control fragments with *O. faveolata* being the palest. Although the seawater temperatures in control tanks reached the bleaching threshold SST, the control fragments did not visibly bleach while in the tanks in either year. After 6 weeks on the reef, treatment fragments were not visibly different from their controls. However, a mild natural bleaching event occurred on the reef during late summer of 2009, which caused some paling in control fragments of *O. faveolata*, but did not visibly affect the control fragments of *P. astreoides* or *P. divaricata*. Following repeated bleaching in 2010, treatment *P. divaricata* fragments did not visibly differ from their controls, treatment *P. astreoides* were much paler than controls, and *O. faveolata* were slightly paler than controls. After 6 weeks of recovery on the reef, treatment *P.*

divaricata and *O. faveolata* did not visibly differ from controls, but *P. astreoides* were dramatically paler than controls. Summertime temperatures on the reef in 2010 were typical for the region and did not induce bleaching in control corals.

These discrepancies in bleaching susceptibility among species and between years were reflected in lipid class composition of all three species. Overall, lipid class composition of *P. astreoides* significantly differed from that of *P. divaricata* and *O. faveolata*, though with considerable overlap among all three species (Fig. 1, Table 1). Within the NMDS, the storage lipids of wax esters and triacylglycerols had correlation coefficients of at least 0.87 on the first axis while the structural lipids of phospholipids and cholesterol had correlation coefficients of at least 0.69 on the second axis (Fig. 1). This was consistent with SIMPER analysis which revealed that over 52% of the dissimilarity between *P. astreoides* and the other two species was due to the storage lipids wax esters and triacylglycerols (Table 2).

Within each species, wax ester and triacylglycerol storage lipids were the primary drivers of the distribution of the underlying data in NMDS space, and were orthogonal to the next most influential lipid classes—the structural lipids of phospholipids and cholesterol (Fig. 2). When non-bleached, wax esters and triacylglycerols accounted for 51%, 19%, and 50% of the total lipids in *P. divaricata*, *P. astreoides*, and *O. faveolata*, respectively.

Table 1 One-way analyses of similarities (ANOSIM) of percent lipid class composition with pairwise tests of differences between all three species

Species	*R* statistic	*p* value
P. astreoides vs. *P. divaricata*	**0.108**	**0.003**
P. astreoides vs. *O. faveolata*	**0.151**	**0.001**
P. divaricata vs. *O. faveolata*	0.029	0.127

The global model R was 0.103 with a *p* value of 0.001. The possible number of permutations was a large number and the actual number of permutations was 999 for all three comparisons. Significant *p* values for each pairwise comparison are bolded

Porites divaricata

Though the global ANOSIM test was not significant ($p = 0.064$), the overall lipid class composition significantly differed between treatment and control fragments after 6 weeks on the reef following single bleaching ($p = 0.017$, Table 3). SIMPER analysis revealed that wax esters and triacylglycerols accounted for over 58% of the dissimilarity and

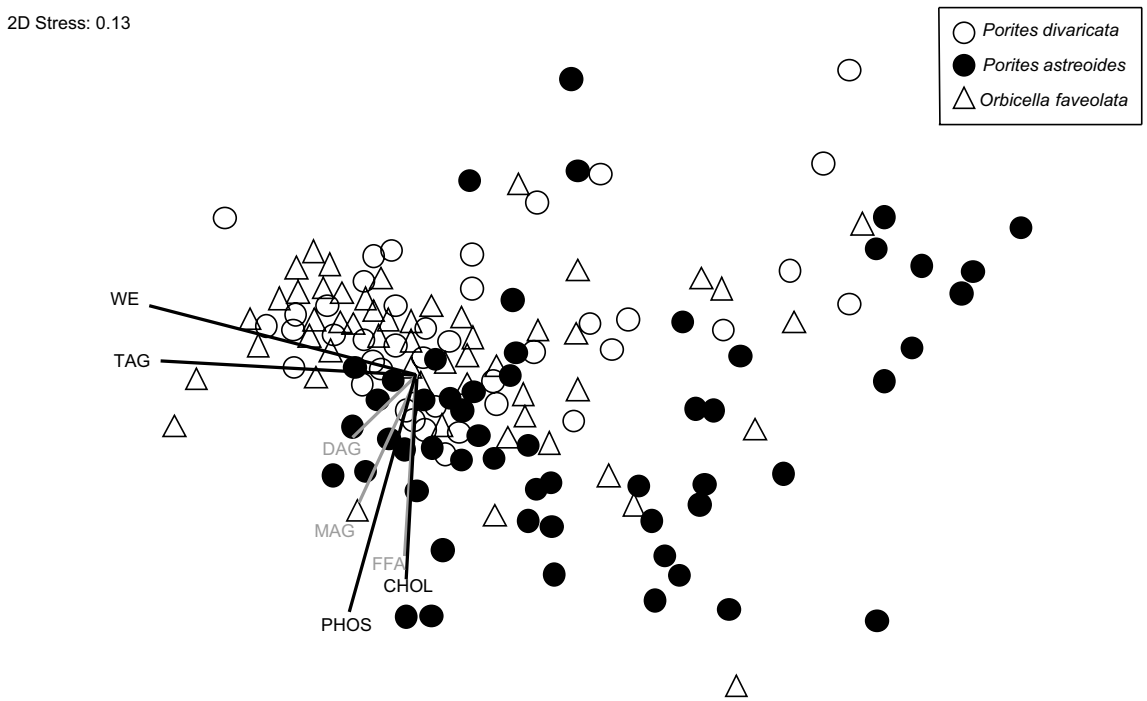

Fig. 1 Non-metric multidimensional scaling (NMDS) using lipid class composition values from all coral fragments. Each data point represents one coral fragment and its seven lipid class measurements. Vector overlay shows the relative influence of each lipid class to the distribution of the underlying data. Wax esters (WE), triacylglycerols (TAG), phospholipids (PHOS), and cholesterol (CHOL) vectors were the longest and shown in black. Remaining vectors are in gray. *DAG* diacylglycerols, *MAG* monoacylglycerols, *FFA* free fatty acids. Corresponding ANOSIMs and SIMPER analyses are in Tables 1 and 2. Unfilled circles = *P. divaricata* ($n = 41$), filled circles = *P. astreoides* ($n = 59$), triangles = *O. faveolata* ($n = 57$)

Table 2 Similarity percentages (SIMPER) analysis output for all three species

Species	Average dissimilarity	Lipid class	% Contribution
P. astreoides vs. *P. divaricata*	29.95	Wax esters	33.44
		Triacylglycerols	19.46
		Phospholipids	17.78
		Diacylglycerols	10.14
		Free fatty acids	8.11
		Cholesterol	5.90
P. astreoides vs. *O. faveolata*	29.11	Wax esters	37.09
		Triacylglycerols	20.32
		Phospholipids	15.23
		Diacylglycerols	9.28
		Free fatty acids	7.58
		Cholesterol	5.71
P. divaricata vs. *O. faveolata*	23.58	Wax esters	36.50
		Triacylglycerols	19.80
		Phospholipids	14.73
		Diacylglycerols	9.02
		Free fatty acids	8.22
		Cholesterol	6.67

phospholipids for another 20% (Fig. 2a; Table 3). Closer inspection of each individual lipid class revealed that wax esters, diacylglycerols and monoacylglycerols significantly declined in treatment compared to control corals immediately after single bleaching, but that after 6 weeks on the reef, phospholipids, cholesterol, wax esters, and monoacylglycerols were all significantly higher in treatment than in control *P. divaricata* (Fig. 3a–c, f; Online Resource 2). No significant differences in overall lipid class composition (Table 3) or individual lipid classes between treatment and controls (Fig. 3a–g; Online Resource 2) were detected following repeated bleaching. Seasonal differences were detected only in diacylglycerols (Fig. 3e).

Porites astreoides

Within each time point, the overall lipid class composition differed between treatment and control fragments after 6 weeks on the reef following single bleaching, with wax esters and triacylglycerols accounting for 40% of the dissimilarity and phospholipids accounting for 27% (Fig. 2b, Table 4). Closer inspection of each individual lipid class revealed that wax esters and monoacylglycerols had both declined at this time (Fig. 3j,m; Online Resource 3). After repeated bleaching, treatment and control fragment lipid class composition significantly differed from each other at both 0 and 6 weeks, with a higher degree of separation ($R = 0.787$ and 0.402, respectively) (Fig. 2b, Table 4). Wax esters and triacylglycerols accounted for 62% and 34% of the dissimilarity after 0 and 6 weeks on the reef, respectively, with phospholipids accounting for at least 14% of the dissimilarity at both time points (Table 4). Interestingly, storage lipid concentrations were higher in treatment than in control corals immediately following repeated bleaching (Fig. 3j–n; Online Resource 3). After 6 weeks on the reef, all of the storage lipids, except wax esters, and both structural lipid classes were higher in the treatment corals compared to the controls (Fig. 3h–n; Online Resource 3). Seasonal effects were observed in phospholipids, cholesterol, and free fatty acids (Fig. 3h, i, n) (Online Resource 3).

Orbicella faveolata

Overall lipid class composition differed between treatment and control *O. faveolata* fragments only after 6 weeks on the reef following single bleaching (Fig. 2c, Table 5). Wax esters and triacylglycerols accounted for 50% of the dissimilarity between treatment and control fragments and phospholipids and cholesterol accounted for another 26% at this time point (Table 5). Closer inspection revealed that both storage (wax esters, triacylglycerols, and monoacylglycerols) and structural lipids (phospholipids and cholesterol) declined in treatment corals compared to controls after 6 weeks following single bleaching (Fig. 3o–r, t; Online Resource 4).

The overall lipid class composition of repetitively bleached corals did not differ between treatment and controls (Table 5), though a decrease in wax esters was observed in treatment corals after 6 weeks on the reef (Fig. 3q, Online Resource 4). Seasonal variation of wax ester concentrations was also observed (Fig. 3q).

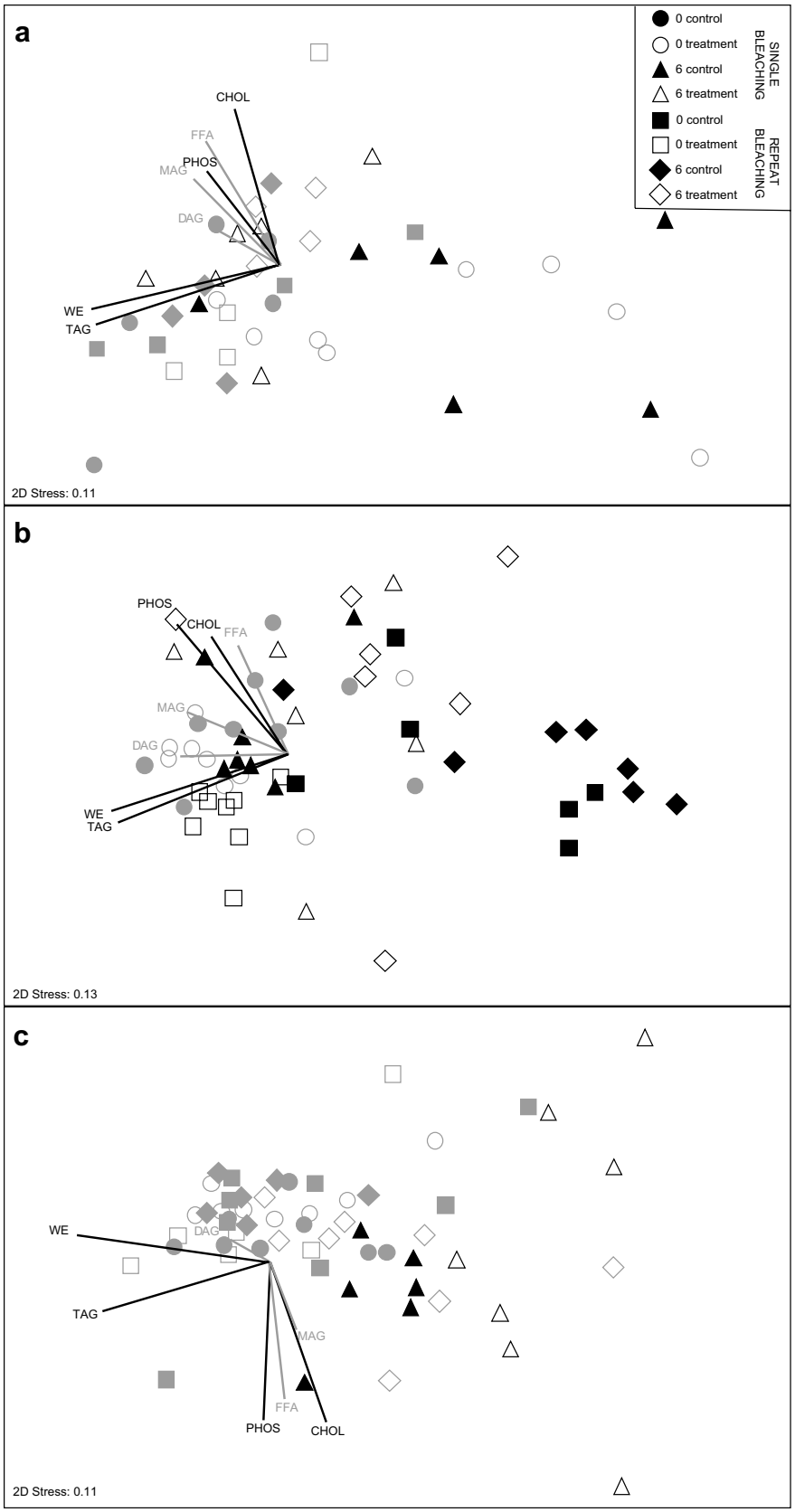

Fig. 2 Non-metric multidimensional scaling (NMDS) plot of **a** *Porites divaricata* ($n=41$), **b** *Porites astreoides* ($n=59$), and **c** *Orbicella faveolata* ($n=57$). Control (filled shapes) and treatment (unfilled shapes) corals after 0 and 6 weeks following single bleaching (2009) (circles and triangles) and repeat bleaching (2010) (squares and diamonds). Black shapes correspond to time points where control and treatment corals differed significantly from each other based on ANOSIMs analyses. If control and treatment corals did not differ within a time point, the symbols are gray. Vector overlay shows the relative influence of each lipid class to the distribution of the underlying data. Wax esters (WE), triacylglycerols (TAG), phospholipids (PHOS), and cholesterol (CHOL) vectors were typically the longest and shown in black. Remaining vectors are in gray. *DAG* diacylglycerols, *MAG* monoacylglycerols, *FFA* free fatty acids. Corresponding ANOSIM and SIMPER analyses are in Tables 3, 4 and 5

Table 3 One-way analyses of similarities (ANOSIM) (global $R = 0.094$, $p = 0.064$) and similarity percentages (SIMPER) analyses of the lipid composition of *Porites divaricata*

ANOSIM						SIMPER		
Bleaching	Weeks on reef	*R* statistic	*p* value	Possible # of permutations	Actual # of permutations	Average dissimilarity	Lipid class	% Contribution
Single	0	0.163	0.105	1287	999			
	6	**0.298**	**0.017**	462	462	27.86	Wax esters	40.12
							Phospholipids	20.66
							Triacylglycerols	18.41
							Cholesterol	4.49
							Diacylglycerols	5.46
Repeat	0	-0.042	0.657	35	35			
	6	0.219	0.171	35	35			

When pairwise tests of differences between treatment and control at each time point (0 and 6 weeks on the reef following single bleaching in 2009 and repeat bleaching in 2010) were significant, the percent contribution of each lipid class to the dissimilarity between treatment and control corals was determined using SIMPER analysis. Significant *p* values are bolded

Discussion

This study is the first to examine lipid class composition of bleached Caribbean corals, and the first to examine the lipid class composition of annually bleached corals of any species. Given the importance of total lipids to coral resilience to bleaching (e.g., Rodrigues and Grottoli 2007; Anthony et al. 2009; Schoepf et al. 2015; Levas et al. 2018) and the growing frequency and intensity of bleaching events (e.g., Spalding and Brown 2015; Heron et al. 2016; Hughes et al. 2017), understanding lipid class management in annually bleached corals should offer some clues as to how corals might acclimatize (or not) to future ocean conditions. Overall, our results do show that annual bleaching does significantly alter lipid class management strategies in a way that differs from single bleaching, and is species specific.

First, we found that shifts in lipid class composition and storage lipid content parallel shifts in coral bleaching susceptibility. This pattern may reveal a previously unknown physiological mechanism of how repeated bleaching can turn "winners" into "losers" (Grottoli et al. 2014), and vice versa. Namely, lipid class compositions of both *Porites divaricata* and *Orbicella faveolata* shifted following single, but not repeated bleaching (Fig. 2a, c; Table 6), which is consistent with previously reported bleaching susceptibility being more intense following single bleaching than after repeated bleaching in both species (Grottoli et al. 2014; Schoepf et al. 2015; Levas et al. 2018). However, lipid class composition shifted following both single and repeated bleaching in *Porites astreoides* (Fig. 2b; Table 6), and bleaching severity increased after repeated bleaching in this species (Grottoli et al. 2014). Thus, the two coral species that acclimatized to repeated bleaching, *P. divaricata* and *O. faveolata*, maintained a more stable lipid class composition following repeated bleaching stress and had higher proportions of storage lipids (wax esters + triacylglycerols, 51% and 50%, respectively); whereas, the more susceptible species *P. astreoides* did not and had lower proportions of storage lipids (19%).

Second, we found that lipid class management, specifically with respect to storage lipids, differs between Caribbean and Indo-Pacific corals when bleached. Lipid class composition shifts in all three Caribbean coral species were mainly driven by the most abundant lipid classes: wax esters, triacylglycerols, and phospholipids (Tables 3, 4, 5, 6). Interestingly, wax esters were consistently catabolized in all three species of corals at some point during the first 6 weeks following single bleaching, but triacylglycerols typically were not (Fig. 3c, d, j, k, q, r). This differs from singly bleached Hawaiian, Japanese, and other Indo-Pacific corals where declines in triacylglycerols are always observed (Grottoli et al. 2004; Yamashiro et al. 2005; Rodrigues et al. 2008; Imbs and Yakovleva 2012). Here, only *O. faveolata* showed significant decreases in triacylglycerols 6 weeks following single bleaching (Fig. 3r). This suggests that the mechanism for storage lipid class catabolism in Caribbean corals differs from that of Pacific corals. Additional patterns in lipid class composition of each Caribbean species are further explored below.

Porites divaricata

Immediately after single bleaching, wax esters declined by 51% (Fig. 3c) along with total lipids and calcification (Levas et al. 2018). At the same time, this species increased its heterotrophic uptake of dissolved organic carbon (Levas et al. 2016). This could potentially account for the increase in cholesterol after 6 weeks on the reef, assuming that DOC is rich in cholesterol like other heterotrophic sources of carbon (i.e., zooplankton) (Fig. 3b). In fact, the overall lipid

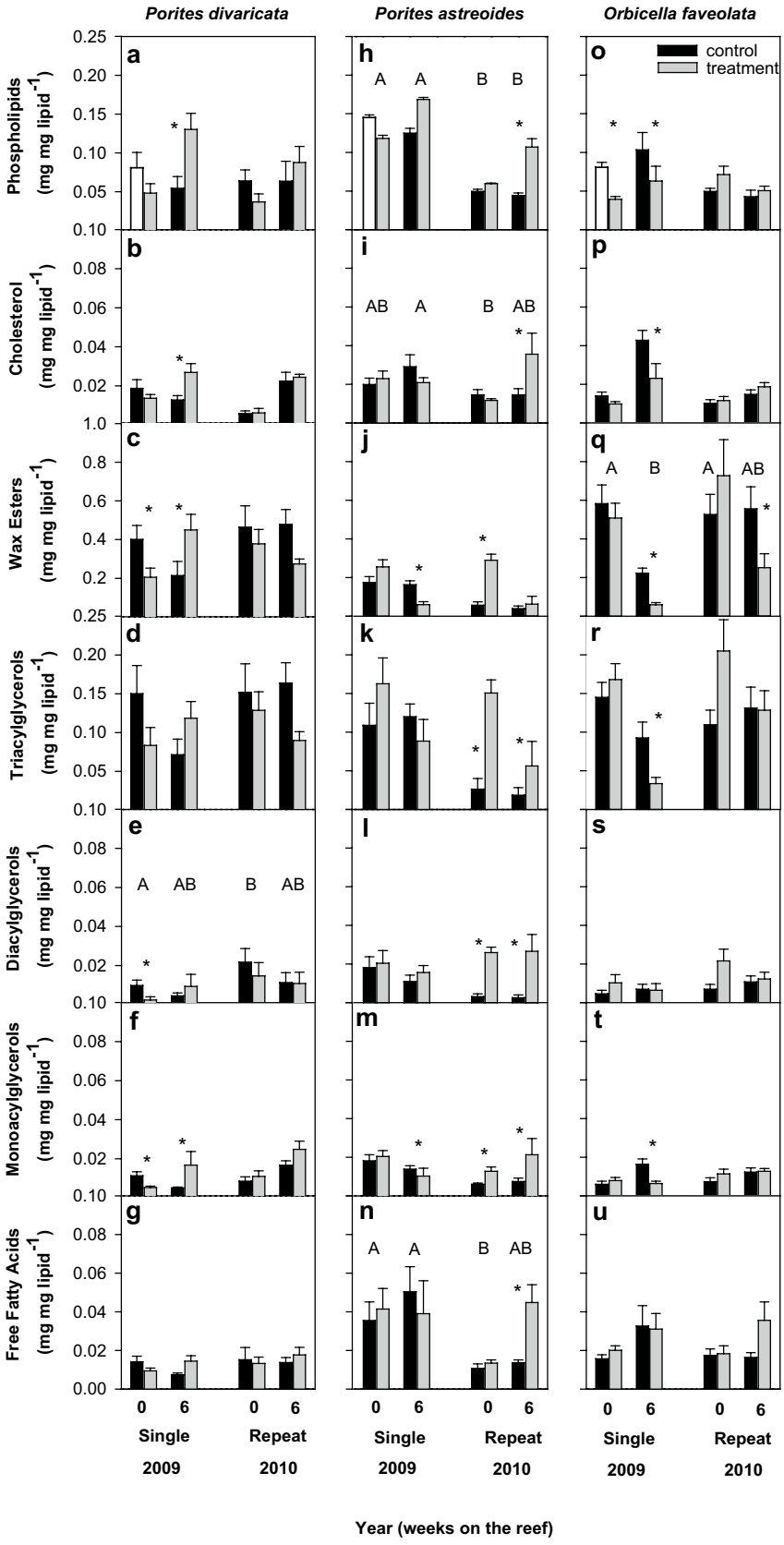

Fig. 3 Coral lipid class composition. Average proportion of lipid classes relative to total lipids (±1 standard error) of control (black bars) and treatment (gray bars) *Porites divaricata* (**a**–**g**), *Porites astreoides* (**h**–**n**), and *Orbicella faveolata* (**o**–**u**) corals after 0 and 6 weeks on the reef in 2009 (single bleaching) and 2010 (repeat bleaching). *Significant difference between treatment and control averages within a given time point. Time points with the same letter are not significantly different. Corresponding analyses of variance (ANOVA) results are presented in Online Resources 2–4. Samples sizes for each average are shown in Online Resource 5

Table 4 One-way analyses of similarities (ANOSIM) (global $R=0.399$, $p=0.001$) and similarity percentages (SIMPER) analyses of the lipid composition of *Porites astreoides*

ANOSIM						SIMPER		
Bleaching	Weeks on reef	*R* statistic	*p* value	Possible # of permutations	Actual # of permutations	Average dissimilarity	Lipid class	% Contribution
Single	0	0.005	0.353	24,310	999			
	6	**0.287**	**0.011**	1716	999	36.53	Phospholipids	27.85
							Wax esters	22.69
							Triacylglycerols	17.97
							Diacylglycerols	11.24
							Free fatty acids	9.09
							Cholesterol	6.28
Repeat	0	**0.787**	**0.001**	3003	999	36.24	Wax esters	33.89
							Triacylglycerols	28.16
							Phospholipids	13.81
							Diacylglycerols	13.8
							Monoacylglycerols	3.89
	6	**0.402**	**0.005**	1716	999	36.53	Phospholipids	19.72
							Triacylglycerols	19.08
							Diacylglycerols	15.92
							Wax esters	14.53
							Free fatty acids	13.68
							Cholesterol	9.66

When pairwise tests of differences between treatment and control at each time point (0 and 6 weeks on the reef following single bleaching in 2009 and repeat bleaching in 2010) were significant, the percent contribution of each lipid class to the dissimilarity between treatment and control corals was determined using SIMPER analysis. Significant *p* values are bolded

Table 5 One-way analyses of similarities (ANOSIM) (global $R=0.245$, $p=0.001$) and similarity percentages (SIMPER) analyses of the lipid composition of *Orbicella faveolata*

ANOSIM						SIMPER		
Bleaching	Weeks on reef	*R* statistic	*p* value	Possible # of permutations	Actual # of permutations	Average dissimilarity	Lipid class	% Contribution
Single	0	0	0.076	0.16	6435			
	6	6	**0.396**	**0.003**	1716	28.93	Wax esters	31.57
							Triacylglycerols	19.07
							Phospholipids	16.5
							Cholesterol	9.8
							Free fatty acids	9.43
							Diacylglycerols	7.76
Repeat	0	−0.008	0.406	3003	999			
	6	0.167	0.055	3003	999			

When pairwise tests of differences between treatment and control at each time point (0 and 6 weeks on the reef following single bleaching in 2009 and repeat bleaching in 2010) were significant, the percent contribution of each lipid class to the dissimilarity between treatment and control corals was determined using SIMPER analysis. Significant *p* values are bolded

class profile of bleached *P. divaricata* significantly differed from that of controls only after 6 weeks on the reef (Fig. 2a, Table 6) due to increases in phospholipids, cholesterol, wax esters, and monoacylglycerols (Fig. 3a–c, f). The elevated phospholipids could be indicative of cellular repair, cell growth, and reacquisition of endosymbionts because endosymbionts typically have higher levels of phospholipids than the coral host (Patton et al. 1977; Imbs et al. 2010) and algal cell density had fully recovered by this time (Levas et al. 2018). Dramatic increases in phospholipids have also been

Table 6 Summary of the results of analyses of similarities (ANOSIM) and analyses of variance (ANOVA) tests

Species	Time		Overall (ANOSIM)	Storage (wax esters and/or triacylglycerols	Structural (phospholipids and/or cholesterol)
Porites divaricata	Single bleaching	0	–	**Decrease**	–
		6	**Different**	**Increase**	**Increase**
	Repeat	0	–	**–**	–
		6	–	**–**	–
Porites astreoides	Single	0	–	–	–
		6	**Different**	**Decrease**	–
	Repeat bleaching	0	**Different**	**Increase**	–
		6	**Different**	**Increase**	**Increase**
Orbicella faveolata	Single bleaching	0	–	–	**Decrease**
		6	**Different**	**Decrease**	**Decrease**
	Repeat bleaching	0	–	–	–
		6	–	**Decrease**	–

Overall significant differences in lipid class composition between treatment and control corals of each species within a time point based on ANOSIMs (Tables 3, 4, 5) are indicated as "different". Significant increases or decreases in storage and structural lipids of treatment corals compared to controls based on ANOVA analyses (Online Resources 2–4) are also bolded. Time points are 0 and 6 weeks following single bleaching (2009) and repeat bleaching (2010). "–" no significant differences

observed during recovery from bleaching in the coral *M. capitata* in Hawai'i (Grottoli et al. 2004; Rodrigues et al. 2008). The combination of elevated wax esters and cholesterol after 6 weeks of recovery may indicate that these corals allocate resources to rebuilding energy reserves and cells as an acclimatization response to thermal stress and that the C allocated to lipids could be at least in part heterotrophically derived. This is consistent with work by Baumann et al. (2014) showing that heterotrophic C is the primary source of fixed C for lipid synthesis during recovery from bleaching in the morphologically similar Hawaiian coral *P. compressa.* However, *P. divaricata* and *P. compressa* differ in their lipid class management in two key ways. Firstly, *P. divaricata* has detectable levels of di- and monoacylglycerol while *P. compressa* does not (Fig. 3e, f) (Grottoli et al. 2004; Rodrigues et al. 2008), suggesting that *P. divaricata* may be more conservative in its storage lipid catabolism, while *P. compressa* immediately catabolizes triacylglycerols to free fatty acids and glycerols. Secondly, while harboring similar proportions of triacylglycerols, *P. divaricata* stores proportionately more wax esters than *P. compressa* (Grottoli et al. 2004; Rodrigues et al. 2008), which could underlie the fast recovery of *P. divaricata*.

Although total lipids decreased following repeated bleaching (Schoepf et al. 2015), the proportions of the underlying lipid classes did not change significantly (Figs. 2a, 3a–g, Table 6). This is consistent with isotopic evidence indicating that the balance between photoautotrophic and heterotrophic sources of C was similar between repetitively bleached and control corals (Schoepf et al. 2015). It also suggests that the shift in the dominant endosymbiont type from the more thermally sensitive *Cladocopium* C47 at the beginning of the study to the more thermally tolerant *Symbiodinium* A4 following repeated bleaching (Grottoli et al. 2014) may have influenced not just thermal tolerance, but also lipid class composition stability. Additional study is needed to confirm this finding but if true would mean that endosymbiont-type shuffling influences host lipid physiology. Overall, *P. divaricata* appears to stabilize its lipid class profile following repeated bleaching, providing additional evidence of acclimatization to annual bleaching.

Porites astreoides

Porites astreoides has been increasing in abundance in the Caribbean over the past few decades (Green et al. 2008). However, previous work has shown that while this species is resistant to single bleaching (Warner et al. 2006; Grottoli et al. 2014), it is very sensitive to repeated bleaching and therefore less likely to persist in the future compared to the other two species (Grottoli et al. 2014; Schoepf et al. 2015; Levas et al. 2018). Total lipid concentrations of *P. astreoides* did not differ between treatment and controls at any time point following single or repeated bleaching (Schoepf et al. 2015; Levas et al. 2018), but lipid class composition did dramatically change within 6 weeks after both bleaching stresses (Figs. 2b, 3h–n, Table 6).

Initially, lipid classes did not change after single bleaching (Table 6). This may be due to the increase in heterotrophic feeding on zooplankton and DOC that supplemented fixed carbon when singly bleached, thus minimizing the need to immediately catabolize any storage lipids (Grottoli

et al. 2014; Levas et al. 2016). Only after 6 weeks on the reef did lipid classes significantly change with a 50% decline in wax esters (Fig. 3j, Table 6). This is consistent with the catabolism of wax esters in bleached Hawaiian *P. compressa* (Grottoli et al. 2004; Rodrigues et al. 2008). Despite increased heterotrophy following single bleaching (Grottoli et al. 2014; Levas et al. 2016), increased cholesterol levels were not observed in *P. astreoides* (Fig. 3i) as it was in *P. divaricata* (Fig. 3b) and in the Hawaiian coral *M. capitata* (Rodrigues et al. 2008). This suggests that heterotrophically acquired carbon was not used for lipid synthesis as has been previously observed in Hawaiian corals (Baumann et al. 2014), but was perhaps catabolized to meet metabolic demand after single bleaching (Hughes et al. 2010).

When repetitively bleached, *P. astreoides* conserved its structural lipids and over-assimilated all of its storage lipids (Fig. 3h–n), despite the detrimental effects that repeated bleaching had on this species overall health (Grottoli et al. 2014; Schoepf et al. 2015) including a 68% loss in its endosymbiotic algae (Grottoli et al. 2014). In fact, *P. astreoides* continued to bleach once the repeated thermal stress was removed and had not fully recovered after 11 months (Schoepf et al. 2015). The fragility of this species to repeated bleaching stress is in stark contrast to its robustness to single isolated experimental bleaching (Grottoli et al. 2014; Schoepf et al. 2015; Levas et al. 2018) and its current increase in abundance on Caribbean reefs (Green et al. 2008). Two factors related to lipids could be contributing to the cumulative damage effect repeated bleaching has on *P. astreoides*. First, this species has lower total lipid concentrations (Schoepf et al. 2015; Levas et al. 2018) and lower proportion of storage lipids (wax esters + triacylglycerols = 19%) than either *P. divaricata* or *O. faveolata* (51% and 50%, respectively) suggesting that lipids do not constitute a large source of energy reserve for *P. astreoides*. Models shows that survival following bleaching is a function of high initial energy reserves and heterotrophic feeding (Anthony et al. 2009). With low levels of total and storage lipids, *P. astreoides* is at an energetic disadvantage when exposed to annual bleaching stress. Second, the sharp increases in storage lipids following repeated bleaching, namely wax esters and triacylglycerols, could be an effort to produce eggs in one last attempt at reproduction in the event of impending mortality. This species' long reproductive season, spanning from January to September (Szmant 1986), results in the chronic loss of lipids through gamete release and could account for the low total and storage lipid reserves. This potential life history tradeoff strategy of increasing reproductive effort in the face of life-threatening stress (Zera and Harshman 2001) has been observed in other invertebrates under extreme environmental conditions (Spicer and Gaston 2009) and may apply to *P. astreoides* as well. The over-assimilation of storage lipids following repeated bleaching stress also coincided with dramatic declines in overall physiology and a lack of any shuffling in the species of Symbiodiniaceae (Grottoli et al. 2014; Schoepf et al. 2015). Our findings add to a growing body of evidence that *P. astreoides* does not acclimatize to repeated bleaching stress and is likely to decrease in abundance in the Caribbean once annual bleaching events become the norm later this century.

Orbicella faveolata

Total lipid concentrations of *O. faveolata* did not differ between treatment and controls following single bleaching (Levas et al. 2018); however, lipid class composition did (Fig. 2c, Table 6). After 6 weeks on the reef following single bleaching, treatment corals had 41% less structural lipids (cholesterol and phospholipids) than non-bleached controls (Fig. 3o, p). This coincided with a 70% loss of endosymbionts (Levas et al. 2018), which would have contributed to cell damage and loss of structural lipids. In addition, the dramatic 71% loss of the main storage lipids, wax esters and triacylglycerols (Fig. 3q, r) is consistent with a coral that was failing to meet metabolic demand despite an increase in heterotrophically acquired DOC (Grottoli et al. 2014; Levas et al. 2018). Despite some paling of control corals after 6 weeks on the reef in 2009, structural and storage lipids of treatment corals were significantly lower than in controls after 6 weeks on the reef (Fig. 3o–r, t). Therefore, the effect of bleaching on the lipid classes in *O. faveolata* is a conservative estimate and had the controls not paled, the difference between the treatment and control lipid classes would most likely have been greater.

Following repeated bleaching, lipid class composition did not differ between treatment and control corals (Fig. 2c, Table 6) in spite of an initial decline in total lipids and endosymbiont density (Schoepf et al. 2015; Grottoli et al. 2014). Within 6 weeks, total lipids and endosymbiont density had recovered to control levels (Schoepf et al. 2015; Grottoli et al. 2014) though the inability to meet metabolic demand (Grottoli et al. 2014) appears to have been supported by catabolism of wax esters (Fig. 3q). The switch of the dominant endosymbionts from the thermally sensitive *Cladecopium* C7 and *Breviolum* B17 at the beginning of the study to the thermally tolerant *Durusdinium trenchii* D1a and *Symbiodinium* A3 by second bleaching (Grottoli et al. 2014) may have contributed to stabilizing lipid class composition after repeated bleaching stress. Despite its listing as endangered by the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species, this study

supports the growing body of evidence that this species may be able to acclimatize to future ocean conditions.

Implications

We show that corals that acclimatize to repeated bleaching have higher storage lipid content (i.e., wax esters and triacylglycerols) and more stable lipid class composition (*P. divaricata and O. faveolata*) than corals that do not acclimatize (*P. astreoides*). In areas in which annual bleaching occurs, *P. divaricata* and *O. faveolata* could be prioritized for propagation in coral nurseries and for restoration efforts, as they may be more likely to persist in the future. However, this study examined only the effects of elevated seawater temperatures on lipid class composition and does not account for the potential interactive effects of temperature and ocean acidification, overfishing, pollution, etc. on acclimatization potential. Ultimately, the capacity of these three Caribbean species to acclimatize to future ocean conditions will not depend on one genetic or phenotypic parameter, but a suite of strategies including effective lipid class management.

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Data availability Data deposited at http://www.bco-dmo.org/project/516103.

Compliance with ethical standards

Conflict of interest No competing interests declared.

Ethical approval All collections and experiments were conducted following the rules and regulations of Mexico and imported to the USA under CITES permits held by UNAM-ICML and the Ohio State University.

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