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Photoautotrophic and heterotrophic carbon in bleached and non-bleached coral lipid acquisition and storage

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ABSTRACT

Lipids are key biomolecules within the coral holobiont, serving as energy reserves and promoting bleaching tolerance. To better understand the physiological implications of bleaching and recovery on lipid acquisition and storage, an isotope pulse-chase labeling experiment was conducted on treatment (bleached) and control (non-bleached) Hawaiian corals *Porites compressa* and *Montipora capitata* after 1 and 11 months on the reef. After 1 month on the reef, 75% of the carbon in newly acquired lipids in treatment corals of both species was heterotrophic in origin, which is two times higher than that in control corals. However, stored lipids in both treatment and controls were hugely dominated by heterotrophic C in *P. compressa* (i.e., 75–100%) and by photoautotrophic C in *M. capitata* (i.e., 90–100%). After 11 months on the reef, elevated levels of heterotrophic C input in lipid acquisition and storage were only seen in treatment *P. compressa*. Many previously measured physiological parameters are recovered in *P. compressa* after 8 months, suggesting that the need for increased heterotrophic C input for lipids appears to last beyond recovery of all other parameters, indicating that *P. compressa* is not yet fully recovered even after 11 months. In *M. capitata*, lipid acquisition and storage were 28–52% heterotrophic in origin and 48–72% photoautotrophic and, along with many previously measured physiological parameters, were fully recovered after 11 months on the reef. However, the evidence suggests that carbohydrate and possibly protein acquisition was not fully recovered even after 11 months. These species-specific differences in lipid acquisition and storage mechanisms, and response patterns to temperature stress indicate that corals may take more than 11 months to recover from bleaching and that heterotrophic carbon is critical to coral lipids, especially when bleached.

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1. Introduction

Coral reefs are declining globally due to a combination of direct and indirect human impacts, such as greenhouse gas emissions, agricultural runoff, overfishing, and habitat destruction (Frieler et al., 2013; Hoegh-Guldberg et al., 2007; Hughes et al., 2003; Veron et al., 2009). Mass coral bleaching, a phenomenon where whole communities of corals lose a significant proportion of their vital endosymbiotic dinoflagellates (commonly called zooxanthellae) and/or their algal photosynthetic pigments, is largely caused by elevated sea-surface temperatures (D'Croz et al., 2001; Glynn, 1996; Hoegh-Guldberg, 1999; Jokiel and Coles, 1990). Impacts of bleaching include: decreased growth in coral tissue and skeleton formation, reduction or cessation of gamete production and fertilization, and increased susceptibility to diseases (Fitt et al., 1993; Omori et al., 1999; Szmant and Gassman, 1990; Ward et al., 2000). Extended and/or extreme warming episodes can lead to mass coral mortality and ecosystem degradation (Stanley, 2003; Wilkinson, 2000). At the current rate of predicted global warming, mass bleaching events are expected to increase in frequency and intensity in all tropical oceans in the coming decades (Buddemeier et al., 2004; Hoegh-Guldberg, 1999; Wilkinson, 2000; Wooldridge et al., 2005) resulting in up to 60% coral mortality within the next few decades (Donner, 2009; Frieler et al., 2013).

Healthy corals acquire fixed organic carbon (C) by two means. First, the endosymbiotic algae photosynthetically fix C (photoautotrophy) in excess of their daily metabolic needs and translocate the majority of it to the coral host, thus supplying the host with up to 100% of its daily metabolic carbon requirements (Falkowski et al., 1993; Grottoli et al., 2006; Muscatine et al., 1981). Second, corals can acquire up to 60% of their fixed C by capturing zooplankton (including pico- and nanoplankton) heterotrophically (Goreau et al., 1971; Grottoli et al., 2006; Palardy et al., 2008; Rodrigues and Grottoli, 2006; Rodrigues et al., 2008b; Tremblay et al., 2012), via the uptake of dissolved organic carbon (DOC) (Levas, 2012; Levas et al., 2013), or particulate organic carbon (POC) (Anthony, 1999; Anthony and Fabricius, 2000).

During elevated temperature stress events, such as those that lead to coral bleaching, the symbiosis between the coral host and endosymbiont can break down. Corals lose significant numbers of their endosymbiotic

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algae and/or algal pigments causing decreases in photosynthesis and incorporation of photoautotrophically acquired C into coral tissues, as well as failure to meet metabolic demand through photosynthesis alone (Grottoli et al., 2006; Hughes et al., 2010; Lesser, 1997; Palardy et al., 2008; Rodrigues and Grottoli, 2007; Warner et al., 1996). To counteract decreases in photosynthetic C, corals can meet their daily metabolic energy requirements by doing one or more of the following: 1) catabolizing stored energy reserves (i.e.: lipids, proteins, carbohydrates) (Fitt et al., 1993; Grottoli and Rodrigues, 2011; Grottoli et al., 2006; Porter et al., 1989; Rodrigues and Grottoli, 2007), 2) increasing their feeding rates (i.e., heterotrophically acquired C) (Grottoli et al., 2006; Levas, 2012; Palardy et al., 2008; Tremblay et al., 2011), 3) decreasing metabolic rates (Levas, 2012; Rodrigues and Grottoli, 2007), and/or 4) decreasing calcification rates (Carricart-Ganivet et al., 2012; Leder et al., 1991; Levas et al., 2013; Rodrigues and Grottoli, 2006). In this study, we further explored how bleaching and recovery affect energy reserves, particularly lipid acquisition and storage.

Lipids are key biomolecules for growth and storage (Birsoy et al., 2013; Christie and Han, 2010; Patton et al., 1977; Rodrigues and Grottoli, 2007; Rodrigues et al., 2008b; Stimson, 1987), and play a significant role in the production of gametes and thus reproduction (Ward, 1995). Further, high lipid content promotes resilience to, and recovery from coral bleaching (Anthony et al., 2009). Therefore, physiological strategies that promote lipid acquisition and storage should promote coral resilience to bleaching. In principle, corals should be able to draw on fixed C (i.e., organic matter) for lipids from both photoautotrophically and heterotrophically acquired C. However, since bleaching significantly reduces photoautotrophically fixed C acquisition and allocation (Hughes et al., 2010), it is unclear how bleaching might affect lipid acquisition and storage, and how that might influence a coral's capacity to recover. If the majority of C allocated to lipid is photoautotrophic in origin, then recovery of lipid content and its associated physiological functions after coral bleaching should depend on recovery of the endosymbionts and their photosynthesis rates, which can take up to 4 months (Connolly et al., 2012; Levas et al., 2013; Rodrigues and Grottoli, 2007). However, if the majority of C allocated towards lipids is heterotrophic in origin, then recovery of lipid content in situ would depend on the heterotrophic plasticity of a given species when bleached (Grottoli et al., 2006; Hughes and Grottoli, 2013; Levas et al., 2013; Palardy et al., 2008). Coral species that are capable of increasing feeding rates when bleached could potentially recover their lipid levels very rapidly or even maintain lipid content (Grottoli et al., 2004; Hughes and Grottoli, 2013; Rodrigues and Grottoli, 2007). To date, no study has determined the proportionate contribution of both photoautotrophic and heterotrophic C to total holobiont lipids (henceforth referred to simply as lipids) in bleached and healthy corals after bleaching or during recovery. Here, we conducted a manipulative experiment in conjunction with carbon isotopic pulse-chase labeling to determine the contribution of photoautotrophically and heterotrophically acquired C to newly synthesized and stored lipids in corals. ^{13}C-isotope enrichment of the lipids in Hawaiian corals *Montipora capitata* and *Porites compressa* was measured after 1 and 11 months on the reef following bleaching to determine 1) the contribution of photoautotrophic vs. heterotrophic C to newly synthesized and stored lipids in bleached and healthy corals, and 2) the variability in the proportionate contribution of both sources of carbon to lipids over time and between species.

2. Methods

2.1. Experimental design

The general experimental design and pulse-chase labeling methods for corals collected immediately following bleaching (0 months on reef) are outlined in detail in Hughes et al. (2010). In this study, we present findings from corals from the same study collected after 1 and 11 months on the reef. The same pulse-chase protocols were followed at 0, 1, and 11 months on the reef. Briefly, corals were collected from a fringing reef (2–4 meters depth) surrounding Moku O Lo'e Island at the Hawaii Institute of Marine Biology in Kaneohe Bay, Hawaii on 11 August, 2006. Five healthy colonies of the branching corals *M. capitata* and *P. compressa* were collected. Sixteen coral branch tip fragments (5 cm tall) were collected from each colony, attached to ceramic tiles, randomly assigned treatments, and placed into outdoor-flow-through seawater (filtered) tanks and allowed to acclimate for 7 days (Fig. 1). In half of the tanks the average daily temperature was 27.4 °C ± .08 (ambient controls). In the other half, temperature was slowly increased to 30.2 °C (±0.20) over the course of a week (approximately 0.5 °C per day) using aquarium heaters (bleaching treatment). These conditions were maintained for 3.5 weeks. Temperature was monitored every 15 min in each tank using Hobo UA-002-08 temperature loggers. As the tanks were outdoors, corals experienced exposure to natural sunlight, natural daily light–dark cycles, and natural weather conditions throughout the entire study. Midday light intensities in the tanks were reduced to that of collection depth (193 ± 35 μmol photons/m^2/s, as measured by Hobo UA-002-64 light loggers) by covering the tanks with 2 layers of neutral-density mesh. Throughout the tank experiment, corals were fed freshly caught zooplankton for 1 h at dusk every other night. Coral fragments were rotated within tanks daily, throughout the experiment, in order to avoid positional effects within a tank. In addition, all corals were rotated randomly among tanks of the same treatment every 4 days to prevent tank position effects. After 3.5 weeks the heaters were turned off (6 September, 2006), tank temperatures returned to ambient values (27.4 °C), and the bleached treatment and ambient control fragments were placed on the reef and allowed to recover for 1 month at 2–3 m depth (Fig. 1) prior to ^{13}C pulse-chase labeling. Fragments collected immediately following the 3.5 weeks in the tanks (i.e., 0 months on the reef) were not available for this study as they were destructively sampled for bulk tissue isotopic analyses (i.e., skeleton, host tissue, endosymbiont tissue) in Hughes et al. (2010).

After 1 and 11 months on the reef, two fragments of each colony and treatment were incubated in ^{13}C-labeled dissolved inorganic carbon (DI-^{13}C) in seawater for 8 h during the day in order to label the photoautotrophically acquired C. The fragments were then chased for one week in the outdoor tanks with unlabeled flow-through seawater to determine how much of the originally labeled photoautotrophic C was utilized for new lipid synthesis (i.e., detected in the first 24 h of the chase) and in stored lipids (i.e., detected after 168 h of the chase). Two additional fragments from each colony and treatment were incubated for 10 h during the night with ^{13}C-labeled rotifers in order to label the heterotrophically acquired C. The fragments were then chased for one week in the outdoor tanks with unlabeled flow-through seawater and without zooplankton or rotifers. The contribution of heterotrophic C utilized for new lipid acquisition (i.e., detected in the first 24 h of the chase) and in stored lipids (i.e., detected after 168 h of the chase) was determined.

2.2. DI-^{13}C: pulse-chase labeling of photoautotrophically acquired carbon

After 1 month on the reef, DI-^{13}C pulse incubations were conducted on 6 October for *P. compressa* and 9 October for *M. capitata*, respectively. Five 40 l glass aquaria were filled with 25 l of seawater at 07:30 h. The aquaria were placed in outdoor flow-through seawater (filtered) tanks with water circulating around them to maintain ambient temperature during the incubation. Treatment corals were placed in 2 of the 4 aquaria, ambient control corals were placed in 2 aquaria, with a 5th aquarium serving as a control (DI-^{13}C added, no coral, Fig. 1). At 08:00 h, 4.5 ml of 0.117 M of 98 at.% ^{13}C $NaHCO_3$ was added to the 4 coral-containing aquaria. Dissolved inorganic carbon (DIC) concentrations increased by 21 μmol kg^{-1} due to the $NaHCO_3$ addition — an increase of roughly 1%. The average initial incubation seawater $\delta^{13}C_{DIC}$ values were 911.90 ± 27.2‰ and 983.48 ± 8.9‰ for *M. capitata* and *P. compressa*, respectively. The incubations were performed for 8 h

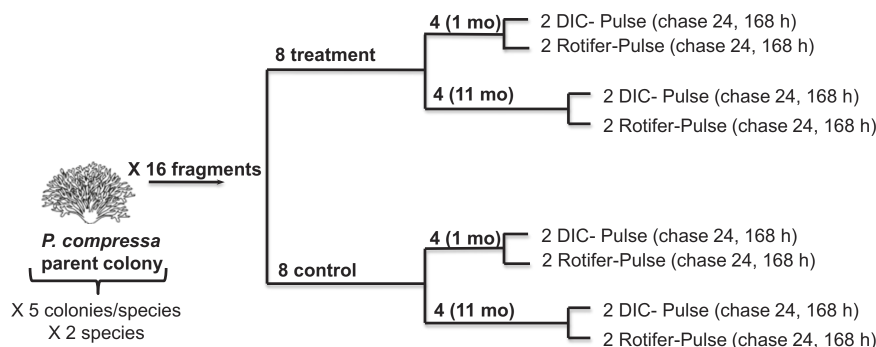

Fig. 1. Flow diagram of experimental design. This method was used for both *Porites compressa* and *Montipora capitata* corals. Mo = months on reef, DIC-pulse = ^{13}C-labeled dissolved inorganic carbon pulse-chase, Rot-pulse = ^{13}C-labeled rotifer pulse-chase, h = hours.
Figure is adapted from Hughes and Grottoli (2013).

during the day to allow for uptake of DIC during maximum photosynthesis. The incubation was 8 h long in order to take advantage of peak photosynthesizing daylight hours and allowing sufficient time for C assimilation and allocation. Coral fragments were removed from the glass incubation aquaria after 8 h, and returned to unlabeled, natural flow-through seawater. One fragment from each colony and treatment was removed during the first 24 h and another at 168 h and immediately frozen at −50 °C (Fig. 1). Previous work on these corals shows that the δ^{13}C enrichment values in the bulk host tissue, endosymbiont tissue, and skeleton do not differ significantly over the first 24 h following DI-^{13}C incubations (Hughes et al., 2010), so it was assumed that δ^{13}C lipid enrichment values also do not differ over the course of the first 24 h. The same procedure was repeated for both species after 11 months on the reef (16 August, 2007 for *M. capitata* and 18 August, 2007 for *P. compressa*, Fig. 1). The average initial incubation seawater $\delta^{13}C_{DIC}$ values were 848.11 ± 8.1‰ and 860.23 ± 10.6‰ for *M. capitata* and *P. compressa*, respectively.

2.3. ^{13}C-rotifer: pulse-chase labeling of heterotrophically acquired carbon

The incubations for *P. compressa* were conducted on 6 October, and the incubations for *M. capitata* were conducted on 9 October, 2006. As with the DI-^{13}C labeling, 40 l glass aquaria were filled with 16 l of filtered seawater and placed in outdoor flow-through seawater tanks. Treatment corals were placed into 2 tanks, ambient corals were placed into 2 tanks, 1 tank served as a ^{13}C rotifer control (^{13}C-rotifer added, no coral, Fig. 1), and 2 additional tanks served as seawater controls (no ^{13}C-rotifer, no coral). Labeled rotifers were added to the coral-containing aquaria when it was dark (20:00 h), at a density of 10–15 rotifers ml^{-1} of seawater. The rotifers were ^{13}C-labeled by feeding them ^{13}C-labeled *Nanocropsis* paste for 96 h prior to the incubations. Rotifer δ^{13}C values for *M. capitata* and *P. compressa* were 3027.01 ± 119.71‰ and 10,051.03 ± 115.27‰ respectively. Rotifer enrichment values varied between incubations because they were prepared on different days with different batches of rotifers. This did not affect our ability to statistically evaluate isotope enrichment patterns within each species. In addition, it did not affect our ability to calculate the mass balances (see details below) as the different rotifer enrichment values were taken into account.

Corals were incubated with ^{13}C-labeled rotifers for 10 h during the night as corals naturally feed on zooplankton at night, and then placed back into unlabeled, flow-through seawater prior to sunrise. A 10-hour night incubation was necessary to allow for assimilation of heterotrophic C during peak feeding times while not exposing the corals to sunlight, which would have confounded the results because photosynthetic uptake of DIC occurs when the corals are exposed to sunlight. One fragment from each colony and treatment was removed within the first 24 h and another after 168 h and immediately frozen at −50 °C. The same procedure was followed for both species after 11 months on the reef (August 16, 2007 for *M. capitata* and August 18, 2007 for *P. compressa*, Fig. 1). Rotifer δ^{13}C values during incubations for *M. capitata* and *P. compressa* following 11 months on the reef were 40,601.71 ± 4450.4‰ and 17,316.67 ± 2217.66‰, respectively.

2.4. Sample analysis

Subsamples from branch tips were removed from whole coral samples of *M. capitata* and *P. compressa*. Approximate surface areas were calculated based on caliper measurements and attributing the most appropriate geometric shape to the tip (i.e., cone, cylinder, or prism). The subsamples were then ground whole (skeleton + animal tissue + endosymbiont) with a mortar and pestle. Total lipids were extracted from ground samples with 2:1 chloroform:methanol, washed in 0.88% KCl, followed by 100% chloroform solution, and another KCl wash (Grottoli et al., 2004). Total lipid extracts were dried to a constant weight, resuspended in 100% chloroform, and stored in 2:1 chloroform: methanol at −80 °C.

In preparation for isotopic analysis, subsamples of total lipid extracts were dried down in tin capsules at 50 °C under high-purity N_2 gas to a constant weight and analyzed for δ^{13}C as described in Grottoli and Rodrigues (2011). Each sample was combusted in a Costech elemental analyzer (EA) and the resulting CO_2 gas was automatically analyzed for δ^{13}C with a Finnigan Delta IV stable isotope ratio mass spectrometer via a Finnigan ConFlow III open-split interface. Lipid δ^{13}C ($\delta^{13}C_L$) values were reported relative to the Vienna Peedee Belemnite Limestone Standard (V-PDB) (δ^{13}C = per mil deviation of the ratio of stable C isotopes ^{13}C:^{12}C relative to V-PDB). Repeated measurements of the USGS-40 standard (n = 28) had a standard deviation of ±0.03‰, repeated measurements of the USGS-41 standard (n = 26) had a standard deviation of ± 0.06‰, and duplicate analyses of coral lipids (n = 12) differed by 1.86 ± 1.99‰ (standard deviation).

2.5. Statistical analysis

Average baseline lipid δ^{13}C values were measured from the whole lipid extracts of the parent colonies collected from the reef at the beginning of the experiment. All lipid δ^{13}C values were reported as enrichment values relative to baseline values. The average baseline lipid δ^{13}C values for parent colonies of both *P. compressa* and *M. capitata* were −15.5‰ ± 0.07. Three-way analysis of variance (ANOVA) was used to test for significant status (bleached vs. non-bleached control), chase (first 24 h vs. 168 h), and genotype effects of the lipid δ^{13}C-enrichment for each species and label-type after 1 and 11 months on the reef. Status and chase (two levels: first 24 h, 168 h) were fixed effects and fully crossed, while genotype was a random effect (5 levels: one for each genotype). In cases where significant genotype effects were

detected, Tukey's tests showed that the distribution of genotype average values overlapped such that no one genotype was completely different from all of the others. As a result, we concluded that the selected colonies represented the natural variation of the population, as no single or group of genotypes was consistently different from all of the others. This is reassuring, as genotypes were not replicated within cells, so full exploration of genotype interaction terms was not possible. Thus, interaction terms involving genotype were not included in the ANOVAs. A posteriori slice tests (e.g., tests of simple effects (Winer, 1971)) were used to determine if treatment and control averages significantly differed within a chase interval for each species, label-type, and recovery and allowed for more detailed exploration of the data. Bonferroni corrections were not used due to increased likelihood of false negatives (Moran, 2003; Quinn and Keough, 2002). We realized that multiple ANOVAs without Bonferroni corrections have inherent limitations, but in this case, they are more informative and have fewer weaknesses than using a Bonferroni correction (Moran, 2003) or using multivariate approaches with this dataset. Prior to ANOVA analysis, all data were tested for homogeneity of variance using Shapiro–Wilk's test. Any data set not meeting this assumption was log transformed with the addition of a constant prior to transformation when necessary. Statistical analyses were generated using SAS software, Version 9.03 of the SAS System for Windows. Values of $p < 0.05$ were considered significant. Due to logistical limitations, it was not possible to pulse-chase both species simultaneously, so pulse-chase labeling experiments were conducted on each species on sequential days. As a result, species could not be compared using statistical approaches, but qualitative comparisons between the trends seen in each species were still possible.

2.6. Percent contribution of photoautotrophic and heterotrophic carbon to lipids

In order to further understand the physiological implications of bleaching on lipids, the percent contribution of photosynthetically derived and heterotrophically rotifer-derived C to newly-synthesized lipids (i.e., lipid isotope enrichment in the first 24 h) and stored lipids (i.e., lipid isotope enrichment after 168 h) of each species and status (treatment or control) at each chase and recovery interval was calculated. The following formula was used to calculate the percent contribution of photosynthetically derived C to newly synthesized and stored lipids for each species and status after 1 and 11 months on the reef:

$$\frac{\left[\frac{\text{Coral APE}_{DIC}}{\text{Seawater APE}}\right]}{\left[\frac{\text{Coral APE}_{DIC}}{\text{Seawater APE}} + \frac{\text{Coral APE}_{Rot}}{\text{Rotifer APE}}\right]} \qquad (1)$$

where APE = atom % excess.

To calculate the percent contribution of heterotrophically derived C to the newly synthesized and stored lipids, the following formula was used:

$$\frac{\left[\frac{\text{Coral APE}_{Rot}}{\text{Rotifer APE}}\right]}{\left[\frac{\text{Coral APE}_{DIC}}{\text{Seawater APE}} + \frac{\text{Coral APE}_{Rot}}{\text{Rotifer APE}}\right]}. \qquad (2)$$

The sum of Eq. (1) plus Eq. (2) equals 100% for each species, status, and chase. This calculation assumes a linear relationship between the enrichment of the coral and the labeled concentration of DIC or rotifers. Detailed descriptions of terms in Eqs. (1) and (2) can be seen in Table 1. Error associated with the average coral atom % values was propagated through the equation. Assessment of these results was qualitative only as statistical approaches were not possible. As a result, only large scale trends can be interpreted.

Table 1
Explanation of terms used in formulas (1) & (2).

Term	Definition
R_{std}	The published standard R value for isotope standards (V-PDB) = 0.0112240 (deGroot, 2004)
$\delta^{13}C_{sample}$	$\delta^{13}C$ value of each sample
R_{sample}	The R value calculated for each sample. $R_{sample} = \left[\left(\frac{\delta^{13}C_{sample}}{1000}\right) + 1\right] \times R_{std}$
Atom $\%_{Postdose}$	Atom % values of source materials (seawater DIC and rotifers) after isotope labeling dosage (pulse). $\text{Atom }\%_{Postdose} = \left[1 + \left[\frac{1}{\left(\frac{\delta^{13}C_{sample}}{1000}\right)+1}\right] \times R_{std}\right]^{-1}$
Atom $\%_{Baseline}$	Atom % values of source materials (seawater DIC and rotifers) at natural abundance. $\text{Atom }\%_{Baseline} = \left[1 + \left[\frac{1}{\left(\frac{\delta^{13}C_{sample}}{1000}\right)+1}\right] \times R_{std}\right]^{-1}$
APE	Atom % excess = Atom $\%_{Postdose}$ − Atom $\%_{Baseline}$
Coral APE_{DIC}	Average APE value for corals incubated in labeled DIC (photoautotrophic label).
Coral APE_{Rot}	Average APE value for corals incubated with labeled rotifer (heterotrophic label).
Seawater APE	Average APE value for the labeled seawater DIC.
Rotifer APE	Average APE value of the labeled rotifers.
% contribution of photosynthetically acquired C to new lipids	$\frac{\left[\frac{\text{Coral APE}_{DIC}}{\text{Seawater APE}}\right]}{\left[\frac{\text{Coral APE}_{DIC}}{\text{Seawater APE}} + \frac{\text{Coral APE}_{Rot}}{\text{Rotifer APE}}\right]}$
% contribution of heterotrophically acquired C to new lipids	$\frac{\left[\frac{\text{Coral APE}_{Rot}}{\text{Rotifer APE}}\right]}{\left[\frac{\text{Coral APE}_{DIC}}{\text{Seawater APE}} + \frac{\text{Coral APE}_{Rot}}{\text{Rotifer APE}}\right]}$

3. Results

The average water temperatures in the treatment and control tanks were 30.2 °C (±0.20 SE) and 27.4 °C (±0.08 SE), respectively (Hughes and Grottoli, 2013). Immediately following 3.5 weeks in the tanks (i.e., 0 months on reef) treatment fragments of both species were visibly white and controls remained brown. After 1 month on the reef, the corals from the treatment tanks of both species were still visibly bleached (Fig. 2) and average chlorophyll a (Chl *a*) values of the treatment corals were significantly lower than those of the control corals (Hughes and Grottoli, 2013). After 11 months on the reef, treatment and control fragments appeared to have similar brown coloration (Fig. 2) and had the same Chl *a* concentrations in both species (Hughes and Grottoli, 2013).

3.1. Photoautotrophically acquired carbon in lipids

After 1 month on the reef, post hoc analyses revealed that lipid $\delta^{13}C$ enrichment in *P. compressa* was significantly lower in treatment than in control corals after both 24 and 168 h of chase, and declined significantly over the 168 hour chase in control corals (Fig. 3A, Table 2). However after 11 months on the reef, treatment and control lipid $\delta^{13}C$ enrichment values did not differ, nor did they change over the week-long chase period (Fig. 3B, Table 2). In comparison, after 1 month on the reef, *M. capitata* lipid $\delta^{13}C$ enrichment values were significantly lower in treatment than in control corals in the first 24 h of the chase period and then no longer differed from controls after that (Fig. 3C, Table 2). After 11 months on the reef lipid $\delta^{13}C$ enrichment values did not differ between treatment and control corals and decreased significantly over the course of the chase in the control corals (Fig. 3D, Table 2).

3.2. Heterotrophically acquired carbon in lipids

After 1 month on the reef, lipid $\delta^{13}C$ enrichment did not differ significantly between treatment and control *P. compressa* and *M. capitata*

corals, nor did they change over the course of the 168 hour chase period in *P. compressa* (Fig. 4A, C Table 3). Lipid $\delta^{13}C$ enrichment values slightly (but significantly) decreased over the course of the chase in *M. capitata*. However, after 11 months on the reef, lipid $\delta^{13}C$ enrichment values were higher in treatment *P. compressa* corals relative to control corals in the first 24 h of the chase period, and no longer differed from controls after 168 h (Fig. 4B, Table 2). No significant treatment or chase effects were detected in *M. capitata* after 11 months on the reef (Fig. 4D, Table 3).

3.3. Proportionate contribution of photoautotrophically and heterotrophically derived C to coral lipids

Two general patterns were observed in the proportionate contribution of photoautotrophically and heterotrophically derived C to coral lipids (Fig. 5) after 1 month on the reef. In the first 24 h, control corals of both species synthesized 16–40% of lipids with heterotrophically acquired C, but treatment corals synthesized 70–75% of their lipids with heterotrophically acquired C. A week later, species-specific trends were observed in both treatment and control corals. In *P. compressa*, 77–100% of stored lipids were dominated by week old heterotrophically acquired carbon (Fig. 5A). Whereas in *M. capitata*, 70–100% of stored lipids were dominated by photosynthetically acquired carbon (Fig. 5B). Finally, for *P. compressa* and for the first 24 h of *M. capitata*, incorporation of heterotrophically acquired C into lipids was greater in treatment corals compared to their paired control (Fig. 5A, B). Of note, after 1 week no heterotrophically acquired C was detected in *M. capitata* stored lipids.

Three general patterns were observed after 11 months on the reef. In the first 24 h, control corals of both species synthesized 28–38% of lipids with heterotrophically acquired C, but treatment corals synthesized 43–75% of lipids with heterotrophically acquired C (Fig. 5C, D). A week later, patterns in both species were similar to those after 24 h. In controls of both species, 39–40% of stored lipids were dominated by heterotrophically acquired C. For comparison, in treatment corals of both species, 52–62% of stored lipids were dominated by heterotrophically acquired C (Fig. 5C, D). Finally, incorporation of heterotrophically acquired C into lipids was always greater in treatment corals compared to their paired control.

4. Discussion

Understanding the effects of temperature stress on lipids requires careful examination of lipid acquisition, storage, and metabolism. Here, lipid C isotope enrichment in the first 24 h sheds light on which sources of C (i.e., photoautotrophic vs. heterotrophic) are allocated to lipids and how that differs with temperature stress and with time on the reef (i.e., 1 vs. 11 months). How lipids derived from each C source are then stored over the short term is explored by examining the isotopic enrichment of lipids 168 h after the pulse. Finally, overall lipid metabolism (lipid acquisition, maintenance, and/or catabolism) is assessed by examining all of the data as a whole. These findings are particularly informative, as this study was conducted using natural seawater and zooplankton under natural, outdoor sunlight conditions complete with a day/night cycle.

4.1. Newly acquired lipids in P. compressa

The proportionate contribution of photoautotrophic and heterotrophic C was consistent over time in healthy (i.e., non-bleached control) *P. compressa*, as lipids were built predominately with photoautotrophic C (i.e., 60–62%) after 1 and 11 months on the reef (Fig. 5A, C). Previous studies have shown that lipids are derived from photoautotrophic and heterotrophic C in anemones (Bachar et al., 2007). In corals, photoautotrophic C has been shown to be used for lipids (Battey and Patton, 1984), and natural abundance isotopic data suggests that heterotrophic C is incorporated into coral lipids (Alamaru et al., 2009). This paper builds on previous work and is the first to *directly* show the importance of heterotrophic C in coral lipid acquisition. Heterotrophic C plays a vital role in coral biology and physiology (i.e., Anthony et al., 2009; Grottoli

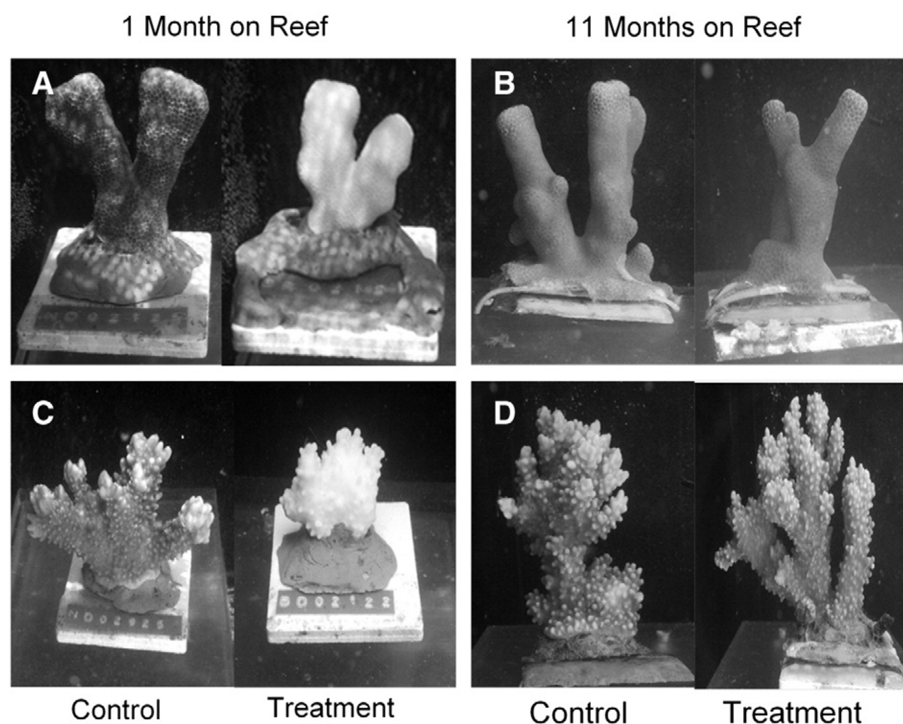

Fig. 2. Photographs of representative coral fragments of control and treatment *Porites compressa* after A) 1 month and B) 11 months on the reef and of *Montipora capitata* following C) 1 month and D) 11 months on the reef.
Photographs by A.G. Grottoli and A. Hughes.

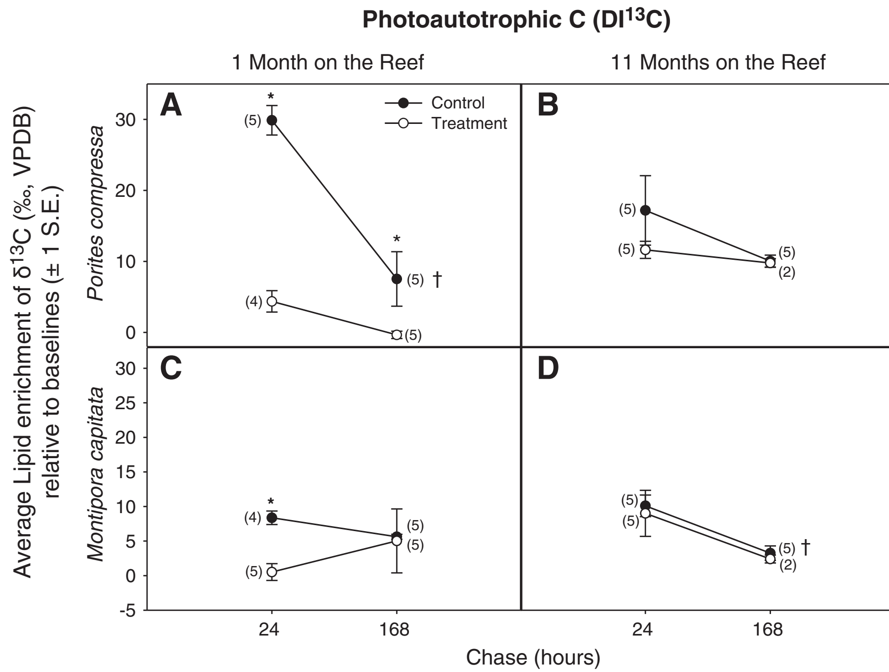

Fig. 3. Photoautotrophic C in *Porites compressa* and *Montipora capitata* lipids. Average (± 1 S.E.) δ^{13}C enrichment of holobiont total lipids 24 and 168 h after pulse-labeling with ^{13}C-labeled dissolved inorganic carbon in seawater. *P. compressa* after A) 1 month and B) 11 months on the reef and *M. capitata* after C) 1 month and D) 11 months on the reef. Treatment = open circles, control = filled circles, VPDB = Vienna Peedee Belemnite Limestone Standard. Numbers in parenthesis are sample size (n) for each average. * indicates significant differences between control and treatment within a chase interval. † indicates significant differences between 24 and 168 hour chase intervals within status (treatment or control).

et al., 2006; Houlbreque and Ferrier-Pages, 2009; Levas et al., 2013). More specifically, heterotrophic C is heavily incorporated into coral tissues (Hughes et al., 2010) and can play a critical role in coral carbon budgets for up to 11 months following bleaching (Hughes and Grottoli, 2013). Even though heterotrophy only accounts for 30% of metabolic demand in this species (Palardy et al., 2008), our findings show that it allocates a disproportionally large share of that C to lipids.

In treatment corals, lipids were derived predominantly from heterotrophic C (75%) (Fig. 5A, C) throughout the study, which indicates the long-term importance of this C source. In contrast, lipids were derived predominately from photoautotrophic C in control corals, indicating that treatment corals had not fully recovered after 11 months on the reef. The high contribution of heterotrophic C to lipids of treatment corals appears to be driven by decreases in photosynthesis (Rodrigues and Grottoli, 2007) and the acquisition of photoautotrophic C (Fig. 3A) (Hughes et al., 2010), and not a result of increases in feeding rates (Grottoli et al., 2006; Palardy et al., 2008), or heterotrophic input to lipids (Fig. 3A). After 11 months on the reef, heterotrophic input still dominated the lipids (Fig. 4B) and coral tissues in general (Hughes and Grottoli 2013) in spite of the fact that all other measured physiological parameters had fully recovered (Rodrigues and Grottoli, 2007; Rodrigues and Grottoli, 2008a,b). Thus, *P. compressa* is still recovering and heterotrophic C is vital to that recovery even 11 months following a bleaching event. Consequently, *P. compressa* and other coral species with similar physiology would be compromised if bleaching events were to occur annually, as has been predicted to occur in the second half of this century (Donner et al., 2007; Frieler et al., 2013; van Hooidonk et al., 2013), as they would potentially have insufficient time to fully recover between bleaching events.

4.2. Newly acquired lipids in *M. capitata*

In healthy (i.e., control) *M. capitata*, lipids were derived predominately from photoautotrophic C (72–83%) while heterotrophic C played

Table 2
Montipora capitata and *Porites compressa* DI^{13}C enrichment. Three-way analysis of variance (ANOVA) of the effect of status (treatment vs. control), chase (first 24 h vs. 168 h), and coral genotype on δ^{13}C enrichment above baseline in DI^{13}C pulse-labeled *P. compressa* after 1 (n = 19) and 11 (n = 17) months on the reef and *M. capitata* after 1 (n = 19) and 11 (n = 17) months on the reef. Significant effects are in bold (p < 0.05).

Time on reef source	1 month				11 months			
	SS	DF	F	p	SS	DF	F	p
P. compressa								
Model	2743.17	7	13.05	**0.0002**	419.77	7	2.06	0.1544
Status	1228.35	1	40.89	**0.0001**	54.43	1	1.87	0.2046
Chase	893.51	1	29.74	**0.0002**	105.53	1	3.63	0.0893
Geno	83.16	4	0.69	0.6127	261.31	4	2.25	0.1442
Status ∗ chase	328.32	1	10.93	**0.0070**	7.66	1	0.26	0.6202
M. capitata								
Model	2.04	7	3.37	**0.0355**	251.68	7	2.16	0.1400
Status	0.82	1	9.53	**0.0103**	0.14	1	0.01	0.9286
Chase	0.03	1	0.32	0.5829	86.67	1	5.20	**0.0485**
Geno	0.92	4	2.66	0.0893	98.81	4	1.48	0.2857
Status ∗ chase	0.05	1	0.58	0.4612	4.82	1	0.29	0.6037

Table 3
Montipora capitata and *Porites compressa* ^{13}C-rotifer enrichment. Three-way analysis of variance (ANOVA) of the effect of status (treatment vs. control), chase (first 24 h vs. 168 h), and coral genotype on δ^{13}C enrichment above baseline in ^{13}C-rotifer labeled *P. compressa* after 1 (n = 17) and 11 (n = 17) months on the reef, and *M. capitata* after 1 (n = 16) and 11 (n = 15) months on the reef. Significant effects are in bold (p < 0.05).

Time on reef source	1 month				11 months			
	SS	DF	F	p	SS	DF	F	p
P. compressa								
Model	4978.76	7	0.87	0.5612	42,983.52	7	1.92	0.1776
Status	1429.77	1	1.75	0.2179	15,946.94	1	5.00	0.0522
Chase	426.24	1	0.52	0.4879	8844.40	1	2.77	0.1303
Geno	2060.85	4	0.63	0.6520	7767.09	4	0.61	0.6668
Status ∗ chase	378.63	1	0.46	0.5126	4000.00	1	1.25	0.2919
M. capitata								
Model	38.78	7	5.59	**0.0136**	2660.68	7	1.47	0.3131
Status	2.91	1	2.94	0.1249	386.86	1	1.49	0.2414
Chase	16.99	1	17.1	**0.0033**	486.23	1	1.88	0.2312
Geno	15.17	4	3.82	0.0504	1341.98	4	1.29	0.3584
Status ∗ chase	0.00	1	0.00	0.9604	252.34	1	.097	0.3567

a relatively small, but potentially vital role after both 1 and 11 months on the reef (Fig. 5B, D). This pattern is similar to the pattern observed in healthy *P. compressa*. Thus, in healthy corals of both species, lipids are derived predominately from photoautotrophically acquired C, with heterotrophically acquired C playing a much smaller role. The relatively low contribution of heterotrophically acquired C in newly derived lipids in healthy *M. capitata* is consistent with the low percent contribution of heterotrophically acquired C to daily animal respiration (CHAR) values and low feeding rates previously seen in healthy corals of this species (Grottoli et al., 2006; Palardy et al., 2008).

In contrast, treatment coral lipids were derived primarily from heterotrophic C (71%) after 1 month on the reef (Fig. 5B) due to the compromised nature of photosynthesis (Grottoli et al., 2006; Rodrigues and Grottoli, 2007) and photoautotrophically acquired C to coral tissues (Hughes et al., 2010) and lipids (Fig. 3C), and not because the heterotrophically acquired C in lipids (Fig. 4C) or bulk tissue (Hughes and Grottoli, 2013) enrichment increased. However, feeding rates and CHAR do dramatically increase in *M. capitata* when bleached (Grottoli et al., 2006; Palardy et al., 2008), which indicates that the majority of heterotrophic C is catabolized to meet daily metabolic demand. After 11 months on the reef, the contribution of heterotrophic C to lipids dropped (43%) and no longer differed from controls (Fig. 5B) while at the same time Chl *a* and energy reserves had fully recovered (Rodrigues and Grottoli, 2007). However, treatment host and endosymbiont tissue were still dramatically enriched in heterotrophic C compared to control corals of this species after 11 months on the reef (Hughes and Grottoli, 2013). Together, these findings indicate that heterotrophic C must be utilized in carbohydrate and possibly even some protein building and that *M. capitata* is not fully recovered after 11 months on the reef.

4.3. Lipid storage and metabolism in P. compressa

Stored lipids were dominated by heterotrophic C (77–100%) after 1 month on the reef in treatment and control *P. compressa* (Fig. 5A). Thus, lipids derived from heterotrophic C were preferentially stored relative to those derived from photoautotrophic C in this species. These findings are consistent with the observed decrease in photoautotrophic C in lipids in control corals and the trend of declining photoautotrophic C in lipids of bleached corals (Fig. 3A, Table 2) while maintaining heterotrophic C in lipids (Fig. 4A, Table 3) over the week-long chase.

The preferential loss of lipids derived from photoautotrophic C over the course of a week (Fig. 5A) may have been due to one or more of the following mechanisms: 1) preferential loss of those lipids through mucus secretions (Brown and Bythell, 2005), 2) preferential catabolism of those lipids, and/or 3) dilution of those lipids with newly synthesized non-labeled lipids. First, because existing C stores (as opposed to newly fixed ones) are the source of C to mucous secretions (Brown and Bythell, 2005), the rapid decrease in enrichment is not likely due to mucous secretions. Second, lipid classes are not equally catabolized in *P. compressa* corals (Grottoli and Rodrigues, 2011; Rodrigues et al, 2008b) which could account for the preferential catabolism of lipids synthesized from photoautotrophic C. In addition, photoautotrophically acquired C has been shown to be preferentially used for short-term respiratory needs in the cnidarian *Aiptasia* sp. (Bachar et al., 2007) and in *P. compressa* (Hughes et al., 2010). Therefore, it is likely that *P. compressa* preferentially catabolized newly synthesized lipids assimilated from photoautotrophic C to meet daily metabolic demand while lipids built with heterotrophic C were preferentially stored. Lastly, any dilution of the lipid $\delta^{13}C$ with newly acquired unlabeled lipids would further enhance the decrease observed over the 168 h and could account for the observations in Fig. 5A.

After 11 months on the reef, photoautotrophic C dominated (60%) stored lipids in control corals while heterotrophic C dominated (62%) stored lipids in the treatment corals (Fig. 5C). This mimics the pattern seen for lipid synthesis in the first 24 h and reveals that both photoautotrophically and heterotrophically derived lipids are catabolized to meet metabolic demand, and the proportionate contribution of both sources of C to storage lipids in treatment corals had not recovered to control levels after 11 months on the reef. These findings are consistent with Hughes and Grottoli (2013) as well as Figs. 3 and 4, where

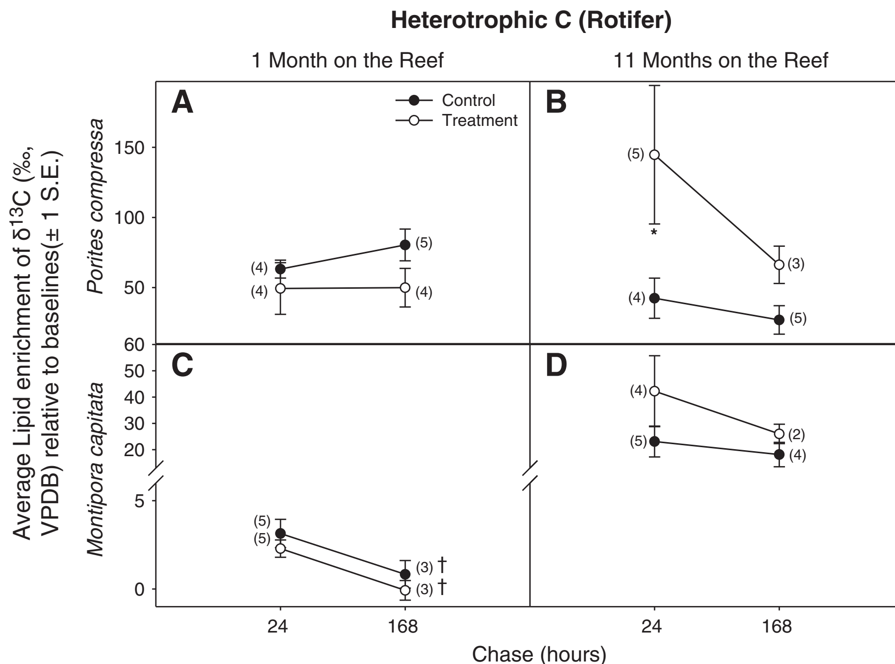

Fig. 4. Heterotrophic C in *Porites compressa* and *Montipora capitata* lipids. Average (±1 S.E.) $\delta^{13}C$ enrichment of holobiont total lipids 24 and 168 h after pulse-labeling with ^{13}C-labeled rotifers. *P. compressa* after A) 1 month and B) 11 months on the reef and *M. capitata* after C) 1 month and D) 11 months on the reef. Treatment = open circles, control = filled circles, VPDB = Vienna Peedee Belemnite Limestone Standard. Numbers in parenthesis are sample size (n) for each average. * indicates significant differences between control and treatment within a chase interval. † indicates significant differences between 24 and 168 hour chase intervals within a status (treatment or control).

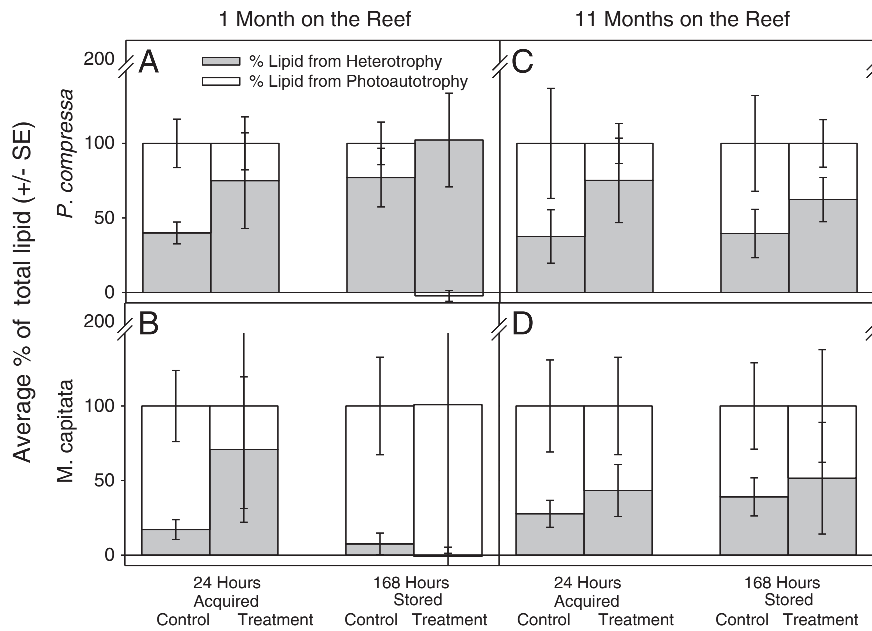

Fig. 5. Average (±1 S.E.) proportionate contribution of photoautotrophic (DIC pulse-chase labeling) and heterotrophic (rotifer pulse-chase labeling) carbon to newly acquired holobiont total lipids (i.e., first 24 h of chase) and stored holobiont total lipids (i.e., after 168-hour chase) in *Porites compressa* after A) 1 month and B) 11 months on the reef and in *Montipora capitata* after C) 1 month and D) 11 months on the reef.

heterotrophic C in bulk host and endosymbiont tissue, and lipids, respectively, remained enhanced after 11 months on the reef. Therefore, storage lipids had not fully recovered, despite the full recovery of total lipid reserves and a suite of other physiological parameters (Rodrigues and Grottoli, 2007). This is consistent with evidence from Caribbean corals indicating that full recovery from bleaching can take longer than one year (Fitt et al., 1993).

Interestingly, the heterotrophic C input in stored lipids after 11 months on the reef was about half of what it was after the first month in both control and treatment corals (Fig. 5A, C). This difference is most likely due to spawning. *P. compressa* has reduced gamete production, but not cessation, following bleaching (Sudek et al., 2012). So spawning should have occurred in August 2007, the same month that the 11 month samples were collected. While spawning does not have an effect on total lipid concentrations in *P. compressa* (Stimson, 1987), it appears to reduce the proportionate contribution of heterotrophic C to stored lipids. Thus we hypothesize that heterotrophic C is disproportionately allocated to the lipids in released larvae.

4.4. Lipid storage and metabolism in M. capitata

In control corals, photoautotrophically acquired C accounted for most (92%) of the C in stored lipids after 1 month on the reef (Fig. 5B), indicating that lipids derived from photoautotrophically acquired C were preferentially stored relative to those built with heterotrophically acquired C in this species after 1 month on the reef. This is consistent with the maintenance of lipid enrichment values when pulse-labeled with DI^{13}C (Fig. 3B) and the depletion of lipid enrichment values when pulse-labeled with DI^{13}C-Rotifers (Fig. 4B) and with the known low feeding rates and CHAR values in this species when healthy (Grottoli et al., 2006; Palardy et al., 2008).

In treatment corals, the contribution of photoautotrophic C to lipid storage increased to 100% after 1 month on the reef (Fig. 5B). In fact, it appears that lipids derived from heterotrophic C make up only a small portion of total lipids (Fig. 4C), and that regardless of bleaching status, these lipids are almost entirely catabolized. Taking all of the evidence together, heterotrophic carbon is critical for lipid acquisition after 1 month on the reef (first 24 h, Fig. 5B) and is rapidly catabolized to meet metabolic demand while lipids acquired with photoautotrophic C are stored in *M. capitata* (168 h, Fig. 5B). Conversely, heterotrophic C is critical for both lipid synthesis and lipid storage in *P. compressa* (Fig. 5A). Thus, species-specific differences in lipid storage mechanisms and C utilization may determine which corals remain prevalent or dominant on reefs in the future. After 11 months on the reef, photoautotrophic C still accounted for the majority (61%) of C in stored lipids in control corals, though a smaller majority than it did after 1 month on the reef (Fig. 5B, D), this difference is likely due to the effects of spawning.

In treatment corals, heterotrophically acquired C and photoautotrophically acquired C each accounted for about 50% of C in stored lipids after 11 months on the reef (Fig. 5D). This is in stark contrast to the lack of any heterotrophically derived C in storage lipids after 1 month on the reef (Fig. 5B), likely due to spawning. Since *M. capitata* spawns normally the year following a bleaching event (Cox, 2007), it is likely that spawning occurred in August 2007 as well. While lipid concentrations (Rodrigues and Grottoli, 2007) and lipid acquisition (Fig. 5D) were fully recovered in treatment *M. capitata* corals after 11 months on the reef, bulk tissue values were still dramatically enriched (Hughes and Grottoli, 2013). Thus, carbohydrate acquisition and possibly even protein acquisition had not fully recovered yet. As a result, *M. capitata* corals were not fully recovered, even 11 months after bleaching.

4.5. Conclusions

Overall, it is clear that heterotrophic C plays an important role in both new lipid acquisition and lipid storage in both healthy and bleached corals. In *P. compressa* the contribution of heterotrophic C in lipid acquisition and storage was higher in treatment compared to control corals throughout the study. Thus bleached corals appear to highly depend on heterotrophy for at least a year for their lipids. *M. capitata* was also dependent of heterotrophic C for lipid acquisition when bleached, but catabolized these lipids instead of storing them. Within a year, the acquisition and storage of lipids were recovered in bleached *M. capitata* corals, but evidence suggests that carbohydrates and possibly protein may not be recovered. Taken together, recovery of coral physiology (specifically, energy acquisition and usage) appears to continue long after visible recovery and the recovery of most

parameters. This indicates that more frequent bleaching events (i.e., annual or biannual) could prevent full recovery, potentially leading to mortality and loss of coral biodiversity.

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