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

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Abstract

Lipids are key biomolecules within the coral holobiont, serving as energy reserves and promoting bleaching resistance. To better understand the physiological implications of bleaching and recovery on lipid synthesis and storage, a 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. In both *P. compressa* and *M. capitata*, the role of heterotrophic carbon (C) in new lipid synthesis was higher in treatment compared to control corals after 1 month on the reef. However, after 11 months on the reef, increased heterotrophic C input was only seen in *P. compressa*. All previously measured physiological parameters are recovered in *P. compressa* after 8 months, suggesting that the need for increased heterotrophic C input for new lipid synthesis appears to last beyond recovery of all other parameters, indicating that *P. compressa* is not yet fully recovered even after 11 months. In contrast, all previously measured parameters and all the input of photoautotrophically acquired and heterotrophically acquired C are fully recovered in *M. capitata*. *P. compressa* relies heavily on heterotrophic C for stored lipids, while *M. capitata* relies mostly on photoautotrophic C for this purpose. These species-specific differences in lipid storage mechanisms, along with lack of recovery in *P. compressa* suggest that corals may take more than 11 months to recover from bleaching and that heterotrophic carbon is critical to coral lipid synthesis.
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 (Hughes et al. 2003; Hoegh-Guldberg et al. 2007; Veron et al. 2009; Frieler et al. 2013). 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, are largely caused by elevated sea-surface temperatures (Jokiel and Coles 1990; Glynn 1996; Hoegh-Guldberg 1999; D'Croz et al. 2001). Impacts of bleaching include: decreased growth in coral tissue and skeleton formation, reduction or cessation of gamete production and fertilization, and increased susceptibility to disease (Szmant and Gassman 1990; Fitt et al. 1993; Omori et al. 1999; Ward et al. 2000). Extended and/or extreme warming episodes can lead to mass coral mortality and ecosystem degradation (Wilkinson 2000; Stanley 2003). 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 (Hoegh-Guldberg 1999; Wilkinson 2000; Buddemeier et al. 2004; 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 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 (Muscatine et al. 1981; Falkowski et al.)
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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; Rodrigues and Grottoli 2006; Palardy et al. 2008; Rodrigues et al. 2008; 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, such as bleaching events, the symbiosis between the coral host and endosymbiont can break down. Corals lose significant numbers of their endosymbiotic algae causing decreases in photosynthesis and incorporation of photoautotrophically acquired C into coral tissues, as well as a failure to meet metabolic demand through photosynthesis alone (Warner et al. 1996; Lesser 1997; Grottoli et al. 2006; Rodrigues and Grottoli 2007; Palardy et al. 2008; Hughes et al. 2010). 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) (Porter et al. 1989; Fitt et al. 1993; Grottoli et al. 2006; Rodrigues and Grottoli 2007; Grottoli and Rodrigues 2011), 2) increasing their feeding rates (i.e., heterotrophically acquired C) (Grottoli et al. 2006; Palardy et al. 2008; Tremblay et al. 2011; Levas 2012), 3) decreasing metabolic rates (Rodrigues and Grottoli 2007; Levas 2012), and/or 4) decreasing calcification rates (Leder et al. 1991; Rodrigues and Grottoli 2006; Carricart-Ganivet et al. 2012; Levas et al. 2013). In this study, we further explored how bleaching and recovery affect energy
reserves, particularly lipid synthesis.

Lipids are key biomolecules for growth and storage (Patton et al. 1977; Stimson 1987; Rodrigues and Grottoli 2007; Rodrigues et al. 2008; Christie and Han 2010; Birsoy et al. 2013), 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 synthesis and storage should promote coral resilience to bleaching. In principle, corals should be able to draw on fixed C (i.e., organic matter) for lipid synthesis 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 synthesis and utilization, and how that might influence a coral’s capacity to recover. If the majority of C allocated to lipid synthesis 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 (Rodrigues and Grottoli 2007; Connolly et al. 2012; Levas et al. 2013).

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; Palardy et al. 2008; Hughes and Grottoli 2013; Levas et al. 2013). 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; Rodrigues and Grottoli 2007; Hughes and Grottoli 2013).
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 to determine the allocation (i.e., new lipid synthesis) and utilization of both photoautotrophically and heterotrophically acquired C to lipids in corals. $^{13}$C-isotope enrichment of the lipids in Hawaiian corals *Montipora capitata* and *Porites compressa* were measured after 1 and 11 months on the reef following bleaching to determine 1) the contribution of photoautotrophic vs. heterotrophic C to lipid synthesis 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 *Montipora capitata* and *Porites 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 ± 0.08 (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 minutes in each tank using Hobo UA-002-08 temperature loggers. Light intensities in the tanks were reduced to that of collection depth (midday: 235 ± 25.641 μmol photons/ m²/ sec, 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 hour 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 d 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 13C 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 13C-labelled dissolved inorganic carbon (DI-13C) in seawater, in order
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to label the photoautotrophically acquired C incorporated into lipids. The fragments were then chased for one week (168 hours) to track how the photoautotrophic C that was incorporated into the lipids during the incubation was utilized over the course of a week. Two additional fragments from each colony and treatment were incubated overnight with $^{13}$C-labeled rotifers after both 1 and 11 months on the reef in order to label the heterotrophic C incorporated into the lipids. They were also chased for one week to track how the heterotrophic C that was incorporated into the lipids during the incubation was utilized over the course of a week.

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

After 1 month on the reef, DI-$^{13}$C pulse incubations were conducted on 6 October for *Porites compressa* and 9 October for *Montipora 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 98 atom % $^{13}$C NaHCO$_3$ was added to the 4 coral-containing aquaria. Dissolved inorganic carbon (DIC) concentrations increased by 21µmol kg$^{-1}$ due to the NaHCO$_3$ addition – an increase of roughly 1%. 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 hours during the day to allow for uptake of DIC during maximum
photosynthesis. The incubation was 8 hours 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 and another at 168 hours 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 hours 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 hours. 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). Average initial incubation seawater δ\(^{13}\)C\(_{\text{DIC}}\) 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 heterotrophically acquired carbon

The incubations for *Porites compressa* were conducted on 6 October, and the incubations for *Montipora 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 hours prior to the incubations. Rotifer \(\delta^{13}\)C values for *M. capitata* and *P. compressa* were 3027.01±119.71‰ and 10051.03±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 hours 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 incubation was necessary to allow for assimilation and allocation of heterotrophic C to the lipids 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 hours and another after 168 hours 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 \(\delta^{13}\)C values during incubations for *M. capitata* and *P. compressa* following 11 months on the reef were 40601.71±4450.4‰ and 17316.67±2217.66‰, respectively.

### 2.4 Sample Analysis

Subsamples from branch tips were removed from whole coral samples of *M.*
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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. Tissue biomass was calculated as the sum of the sample ash free dry
weight and the dry lipid weight.

In preparation for isotopic analysis, subsamples of total lipid extracts were dried
down in tin capsules at 50°C under high-purity N₂ gas to a constant weight and analyzed
for δ¹³C as described in Grottoli and Rodrigues (2011). Each sample was combusted in a
Costech elemental analyzer (EA) and the resulting CO₂ gas was automatically analyzed
for δ¹³C with a Finnigan Delta IV stable isotope ratio mass spectrometer via a Finnigan
ConFlow III open-split interface. Lipid δ¹³C values (δ¹³C_L) values were reported relative
to Vienna Peedee Belemnite Limestone Standard (V-PDB) (δ¹³C = per mil deviation of
the ratio of stable C isotopes ¹³C:¹²C 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 repeated
measurements of coral lipid samples (n=12) had average differences of 1.86‰ with a
standard deviation of ±1.99‰.
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. Average baseline lipid δ\(^{13}\)C values for parent colonies of both *P. compressa* and *M. capitata* were -15.5 ± 0.07‰. The effects of treatment (bleached vs. non-bleached control), chase interval (first 24 hours vs. 168 hours), and genotype on the lipid δ\(^{13}\)C-enrichment of each species, label-type, and recovery interval were determined using a three-way ANOVA. Treatment and chase interval were fixed effects while genotype was a random effect. The purpose of including genotype in the ANOVA model was to determine if any single genotype was systematically different from all others for a given variable. A posteriori slice tests were used to determine if treatment and control averages significantly differed within a chase interval for each species, label-type, and recovery. Bonferroni corrections were not used due to increased likelihood of false negatives (Quinn and Keough 2002; Moran 2003). 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 heterotrophic rotifer-derived C to newly-synthesized lipids 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 lipids for each species and status after 1 and 11 months on the reef:

(1)

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

Where APE= atom % excess

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

(2)
The sum of equation 1 plus equation 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 equations 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.

### 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 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, lipid $\delta^{13}C$ enrichment in *P. compressa* was significantly lower in treatment than in control corals, and declined significantly over the 168 hour chase in control corals (Figure 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 hours 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 hours of the chase period, and no longer differed from controls after 168 hrs (Figure 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 newly synthesized coral lipids

Two general patterns were observed in the proportionate contribution of photoautotrophically and heterotrophically derived C to newly synthesized coral lipids (Fig 5) after 1 month on the reef. In the first 24, 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 new lipid synthesis was dominated by week old heterotrophically acquired carbon (Fig. 5A). Whereas, in *M. capitata* 70-100% of new lipid synthesis was dominated by week old photosynthetically acquired carbon (Fig. 5B). Finally, for *P. compressa* and for the first 24 hours 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 week old heterotrophically acquired C was detected in *M. capitata* lipids.

Three general patterns were observed after 11 months on the reef. In the first 24, controls 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 hours. In controls of both species, 39-40% of new lipid synthesis was dominated by week old heterotrophically acquired C. For comparison, in treatment corals of both species, 52-62% of new lipid synthesis was dominated by week old 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 synthesis, storage, and metabolism. Here, lipid C isotope enrichment in the first 24 hours sheds light on which sources of C (i.e., photoautotrophic vs. heterotrophic) are used for lipid synthesis and how that differs with temperature stress and with time on the reef. How lipids are stored over the short term is explored by examining the isotopic enrichment of lipids 168 hours after the pulse. Finally, overall lipid metabolism (lipid synthesis, maintenance, and/or catabolism) is assessed by examining all of the data as a whole.

4.1 Lipid Synthesis in Porites compressa

In healthy (i.e., non-bleached control) *P. compressa*, lipids were synthesized predominantly with photoautotrophic C (60-62%) (Fig 5 A, C) though heterotrophic C played a significant role (38-40%) (Fig 5 A, C). This pattern was consistent after 1 and 11 months on the reef, indicating that the proportionate contribution of photoautotrophic and heterotrophic C was consistent over time in healthy *P. compressa*. Previous findings have
shown that photoautotrophic and heterotrophic C are used for lipid synthesis in corals and anemones (Battey and Patton 1984; Bachar et al. 2007; Alamaru et al. 2009). However, this is the first study to show the importance of heterotrophic C in coral lipid synthesis over the course of bleaching and recovery. Heterotrophic C plays a vital role in coral biology and physiology (Anthony 1999; Grottoli and Wellington 1999; Anthony and Fabricius 2000; Grottoli et al. 2006; Palardy et al. 2006; Borell et al. 2008; Palardy et al. 2008; Rodrigues et al. 2008; Anthony et al. 2009; Houbréque and Ferrier-Pages 2009; Hughes et al. 2010; Connolly et al. 2012; Hughes and Grottoli 2013; Levas et al. 2013).

More specifically, heterotrophic C is used for tissue building and energy storage in general (Hughes et al. 2010) of which lipids are the largest component of energy reserves.

Even though heterotrophy only accounts for 30% of metabolic demand in this species (Palardy et al. 2008), our findings show that it allocates a disproportionately large share of that C to lipid synthesis.

In treatment corals, lipids were synthesized predominantly with heterotrophic C (75%) compared to 25% for photoautotrophic C (Fig. 5 A, C). This pattern was consistent after 1 and 11 months on the reef, indicating that the proportionate contribution of photoautotrophic and heterotrophic C persists for at least 11 months after the bleaching stress is removed. Thus, heterotrophic C is critical for lipid synthesis in bleached corals. However, the underlying mechanisms of lipid synthesis differ at 1 and 11 months. In recently bleached corals (i.e., after 1 month on the reef) heterotrophic C is the dominant source of C for lipid synthesis because photosynthesis and C acquired photoautotrophically are compromised (Fig 3A) (Rodrigues & Grottoli 2007; Hughes et
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al. 2010), not because heterotrophic feeding rates or allocation of heterotrophic C to lipid synthesis have increased (Fig 3A) (Grottoli et al. 2006; Palardy et al. 2008). However, after 11 months on the reef, the allocation of heterotrophically acquired C to corals (Hughes et al. 2013) and to lipids (Fig 4B) were dramatically higher than in controls even though by many other metrics such as photosynthesis rates, biomass, endosymbiont density, Chl a, energy reserves, and the allocation of photoautotrophic C to lipids, these corals had long since recovered (Fig 3B) (Rodrigues & Grottoli 2007). Hughes & Grottoli (2013) hypothesized that this heterotrophic compensation indicated that corals were still recovering after 11 months. The findings in this study indicate that lipids drive the bulk tissue enrichment observed in Hughes & Grottoli (2013) and support the hypothesis that bleached *P. compressa* corals are not fully recovered even after 11 months on the reef. Thus, *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).

**4.2 Lipid Synthesis in Montipora capitata**

In healthy (i.e., control) *M. capitata*, lipids were synthesized predominately with photoautotrophic C (72-83%), while heterotrophic C played a relatively small (17-28%), but potentially vital role (Fig 5B, D). This pattern was consistent after 1 and 11 months and is similar to the patterns observed in healthy *P. compressa*. Thus when healthy, both species of corals build most of their lipids with photoautotrophically acquired C, with
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heterotrophically acquired C playing a measurable and non-trivial role (17-40%). The relatively low contribution of heterotrophically acquired C in newly synthesized lipids in M. capitata is consistent with 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 treatment corals, lipids were synthesized primarily with heterotrophic C (71%) after 1 month on the reef (Fig 5B) and dropped to 43% after 11 months on the reef (Fig 5D). Differences between patterns seen at 1 and 11 months on the reef are driven by a variety of factors. In recently bleached corals (1 month on the reef) heterotrophic C was the dominant C source for lipid synthesis because photosynthesis and photoautotrophically acquired C in lipids were compromised (Fig 3C) (Rodrigues & Grottoli 2007; Grottoli et al. 2006; Hughes et al. 2010) and because feeding rates and CHAR dramatically increase (Grottoli et al. 2006; Palardy et al. 2008). Since there is no evidence that this increase in heterotrophy leads to increases in heterotrophic C to bulk tissue (Hughes and Grottoli 2013) or lipids (Fig. 4C), it stands to reason that the excess heterotrophic C is catabolized to meet metabolic demand. After 11 months on the reef, heterotrophic C was no longer the dominant source of C for lipid synthesis because the coral had largely, but not fully, recovered from bleaching the previous summer. While Chl a and total energy reserves recover within 8 months (Rodrigues and Grottoli 2007) and the proportionate contribution of heterotrophic C to lipid synthesis is similar between treatment and control corals after 11 months on the reef (Fig 5D), treatment host and endosymbiont tissue are still dramatically enriched in heterotrophic C compared to
control corals of this species after 11 months on the reef (Hughes & Grottoli 2013). Thus, heterotrophic C is utilized in protein and/or carbohydrate synthesis. Hughes and Grottoli (2013) hypothesized that increased lipid synthesis from heterotrophically acquired C in P. compressa after 11 months on the reef indicated that lipid synthesis had not yet fully recovered (despite recovery of total lipid values (Rodrigues and Grottoli 2007). As this increase is not seen in M. captiata, it is likely that new lipid synthesis was fully recovered after 11 months in this species, but protein and/or carbohydrate synthesis was not.

4.3 Lipid Storage and Metabolism in Porites compressa

Stored lipids were dominated by heterotrophic C in both control (77%) and treatment (100%) corals (Fig 5A) after 1 month on the reef. Thus, lipids synthesized from heterotrophic C are preferentially stored relative to lipids synthesized from photoautotrophic C in both control and treatment corals. These findings are consistent with Fig 3A and Fig 4A, in which photoautotrophic C in lipids decreased over the course of the week long chase and heterotrophic C remained constant in treatment corals. Lipids synthesized from heterotrophic C may be preferentially utilized for structural lipid synthesis (i.e., phospholipids and cholesterol) making them less likely to be catabolized. The loss of lipids synthesized with photoautotrophic C over the course of a week may have been due to one or more of the following mechanisms: 1), preferential catabolism of those lipids, 2) preferential loss of those lipids through mucus secretions (Brown and Bythell 2005), and/or 3) dilution of those lipid δ\(^{13}\)C with newly synthesized non-labelled lipids. First, lipid classes are not equally catabolized in P. compressa corals (Rodrigues et
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al 2008; Grottoli & Rodrigues 2011) 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 need in the cnidarian Aiptasia sp. (Bachar et al. 2007) and in P. compressa (Hughes et al. 2010).

Therefore, it is likely that both control and treatment P. compressa catabolize newly synthesized lipids assimilated from photoautotrophic C to meet daily metabolic demand after 1 month on the reef. Second, since existing C stores (as opposed to newly fixed C) are the source of dissolved organic C (DOC) secretions in corals (Tanaka et al. 2008) and lipids are a source of the C for DOC and mucous secretions (Brown and Bythell 2005), it is unlikely that the rapid decrease in enrichment is primarily due to the loss of C through mucous secretions. Lastly, any dilution of the lipid $\delta^{13}$C with newly synthesized unlabeled lipids would further enhance the decrease observed over the 168hrs. Thus, lipids derived from photoautotrophic C are catabolized, while lipids built with heterotrophic C are stored in treatment P. compressa after 1 month on the reef.

After 11 months on the reef, photoautotrophic C dominated (60%) in stored lipids and heterotrophic C accounted for a significant portion (40%) in control corals. This mimics the patterns seen in lipid synthesis (62% photoautotrophic C, 38% heterotrophic C) (Fig 5C). 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 (Fig 5A, C). This difference is most likely due to seasonal variation related to spawning. While spawning does not have an effect on lipid concentrations in P. compressa (Stimson 1987), it appears to reduce the
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proportionate contribution of heterotrophic C to stored lipids. Thus we hypothesize that heterotrophic C is disproportionately allocated to the lipids in released spawn.

In treatment corals, heterotrophically acquired C no longer accounted for 100% of the C in stored lipids after 11 months on the reef, accounting instead for 62% (Fig. 5A, C). Some of this decrease is most likely due to seasonal variability associated with 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. However, the trend of higher heterotrophic input in stored lipids compared to that of controls (Fig 4C) and enhanced heterotrophic C in bulk host and endosymbiont tissue (Hughes and Grottoli 2013), suggests that the synthesis of storage lipids had not fully recovered, despite the full recovery of total lipid reserves, tissue biomass, and a suite of other physiological parameters (Grottoli et al. 2004; Grottoli et al. 2006; Rodrigues and Grottoli 2007; Rodrigues et al. 2008). This is consistent with evidence from Caribbean corals indicating that full recovery from bleaching can take longer than one year (Fitt et al. 1993). In this case, it appears that lipids synthesized from photoautotrophic C are responsible for recovery of the bulk lipid concentration, but that corals are still synthesizing lipids with heterotrophic C, possibly a specific class (or several classes) of lipids, long after the coral appears to be otherwise recovered. This indicates that recovery is perhaps more complicated than previously thought and that annual bleaching (as discussed in Grottoli et al. (2014)) may prove extremely troubling for reefs, as large scale recovery has been measured to take between 2 and 40 years (Baker et al. 2008).
4.4 Lipid Storage and Metabolism in Montipora capitata

Photoautotrophically acquired C accounted for most (92%) of the C in stored lipids after 1 month on the reef, in control corals (Fig 5B). This indicates that lipids built with photoautotrophically acquired C are 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 Dl^{13}C (Fig 3B) and the depletion of lipid enrichment values when pulse-labeled with Dl^{13}C-Rotifers (Fig 4B). A small amount lipids derived from heterotrophically acquired C (8%) were stored as well, highlighting that heterotrophically acquired C is utilized in lipids of healthy corals. However, in control M. capitata, most lipids built with heterotrophically acquired C were catabolized.

In treatment corals, the contribution of photoautotrophic C to lipid storage increased to 100% after 1 month on the reef (Fig 5C). Interestingly, M. capitata increases its feeding rate dramatically after bleaching (Grottoli et al. 2006; Palardy et al. 2008), yet heterotrophically acquired C was not enriched in the bulk tissue of the host or symbiont immediately after bleaching (Hughes et al. 2010), or in the lipids after 1 month on the reef (Fig 4C, 5C). In fact, it appears that lipids built with heterotrophic C make up only a small portion of total lipids (very low enrichment value in Fig 4C), and that regardless of bleaching status, these lipids are almost entirely catabolized. It appears that only a small portion of the heterotrophic C taken in during feeding is allocated to energy reserves, with the large majority of it being used immediately to meet metabolic demand and that
this preference for storing photoautotrophically derived lipids is independent of feeding rate. Taking all of the evidence together, heterotrophic carbon is critical for lipid synthesis after 1 month on the reef (first 24 hours, Fig 5B), and that the vast majority of it is rapidly catabolized to meet metabolic demand while lipids built with photoautotrophic C are stored in *M. capitata* (168 hours, Fig 5B). This is in contrast to *P. compressa* where heterotrophic C is critical for both lipid synthesis and for lipid storage (Fig 5A). Thus, species-specific differences in lipid storage mechanisms and C utilization may determine which corals remain prevalent or dominant on reefs as the climate continues to change.

After 11 months on the reef, photoautotrophic C still accounted for the majority (61%) of C in stored lipids in control corals, but heterotrophic input was increased (39%) relative to corals sampled after 1 month on the reef (8%) (Fig 5B, D). This increase mimics the trend seen in lipid synthesis in after 11 months and is likely related to seasonal variation due to spawning (as in *P. compressa*). While spawning does not affect overall lipid concentration in *M. capitata* (Padilla-Gamino and Gates 2012), it does appear to affect the percent contribution of photoautotrophic and heterotrophic C to lipids.

In treatment corals, heterotrophically acquired C and photoautotrophically acquired C each account for about 50% of C in stored lipids (52% and 48% respectively). Again, input of heterotrophically acquired C in stored lipids was increased (52%) after 11 months, compared to treatment corals after 1 month on the reef (0%). Since *M. capitata* spawns normally the year following a bleaching event (Cox 2007), it is likely that
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spawning occurred in August 2007. As a result, it is likely that this increase is due to seasonal variation related to spawning. Due to the similarities seen in percent contribution of photoautotrophic and heterotrophic C between treatment and control corals after 11 months on the reef (Fig 5D), it is likely that lipid synthesis and storage were fully recovered in treatment *M. capitata* corals after 11 months on the reef. Taken together with previous evidence that total energy reserves, biomass, Chl a, and endosymbiont density are recovered by 8 months after bleaching at the latest (Rodrigues and Grottoli 2007), it appears that *M. capitata* is fully recovered from bleaching after 11 months on the reef. Due to this recovery, it is unlikely that the relative increase in heterotrophic input to stored lipids after 11 months was related to increased feeding rates, again supporting the fact that this increase was related to spawning.

4.5 Summary

Overall, it is clear that heterotrophic C plays a role in both new lipid synthesis and lipid storage in both healthy and bleached corals. In both *P. compressa* and *M. capitata*, the role of heterotrophic C in new lipid synthesis was higher in treatment compared to control corals after 1 month on the reef. In *P. compressa*, this increase is seen again after 11 months on the reef, whereas in *M. capitata*, input of heterotrophic C input was similar in treatment compared to control corals after 11 month on the reef. As all other physiological parameters are recovered in *P. compressa* after 8 months, it stands that the need for heterotrophic C for new lipid synthesis appears to last past the recovery of all other parameters, indicating that this
species may not be fully recovered even 11 months after bleaching. Alternatively, new lipid synthesis, lipid storage, photoautotrophic, and heterotrophic input to lipids do not vary much between control and treatment *M. capitata* corals after 11 months on the reef. Added together with all previously measured physiological parameters, which all recover to normal levels by 8 months, corals of this species appear to be fully recovered after 11 months on the reef. Spawning appears to play a significant role in percent contribution of photoautotrophic and heterotrophic C to lipid storage; leading to decreased heterotrophic C input in *P. compressa* and increased heterotrophic C input in *M. capitata*. In addition, *P. compressa* relies heavily on heterotrophic C to build lipids that are stored, while *M. capitata* depends mostly on photoautotrophic C. Both spawning effects and lipid storage mechanisms are different in each species, suggesting that there are species-specific differences in C allocation and utilization. As a result, *P. compressa* and other corals that require elevated input of heterotrophic C to lipids long after a bleaching event, and rely heavily on heterotrophic C for lipid storage, are less resilient than *M. capitata* and corals from other species that do not have this requirement. Thus, *M. capitata* is likely more resilient to bleaching than *P. compressa* and, as such, may fare better on future reefs as the oceans continue to warm.
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605 School of Earth Sciences Friends of Orton Hall fellowship.
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### Table 1: Explanation of terms used in formulas 1-5.

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<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{std}}$</td>
<td>The published standard R value for isotope standards (V-PDB) = 0.0112240 (deGroot 2004)</td>
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<tr>
<td>$\delta^{13}C_{\text{sample}}$</td>
<td>$\delta^{13}$C value of each sample.</td>
</tr>
<tr>
<td>$R_{\text{sample}}$</td>
<td>The R value calculated for each sample. $R_{\text{sample}} = \left(\frac{\delta^{13}C_{\text{sample}}}{1000} + 1\right) \times R_{\text{std}}$</td>
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<td>Atom %$_{\text{Postdose}}$</td>
<td>Atom % values of source materials (seawater DIC and rotifers) after isotope labelling dosage (pulse). $\text{Atom %}<em>{\text{Postdose}} = \left[1 + \left(\frac{1}{\left(\frac{\delta^{13}C</em>{\text{sample}}}{1000} + 1\right)}\right) \times R_{\text{std}}\right]^{-1}$</td>
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<td>Atom %$_{\text{Baseline}}$</td>
<td>Atom % values of source materials (seawater DIC and rotifers) at natural abundance. $\text{Atom %}<em>{\text{Baseline}} = \left[1 + \left(\frac{1}{\left(\frac{\delta^{13}C</em>{\text{sample}}}{1000} + 1\right)}\right) \times R_{\text{std}}\right]^{-1}$</td>
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<td>APE</td>
<td>Atom % Excess = $\text{Atom %}<em>{\text{Postdose}} - \text{Atom %}</em>{\text{Baseline}}$</td>
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<td>Coral APE$_{\text{DIC}}$</td>
<td>Average APE value for corals incubated in labeled DIC (photoautotrophic label).</td>
</tr>
<tr>
<td>Coral APE$_{\text{Rot}}$</td>
<td>Average APE value for corals incubated with labelled rotifer (heterotrophic label).</td>
</tr>
<tr>
<td>Seawater APE</td>
<td>Average APE value for the labelled seawater DIC.</td>
</tr>
<tr>
<td>Rotifer APE</td>
<td>Average APE value of the labelled rotifers.</td>
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</table>

% contribution of photosynthetically acquired C to new lipids

$$\left[\frac{\text{Coral APE}_{\text{DIC}}}{\text{Seawater APE}}\right] \times \frac{\text{Coral APE}_{\text{DIC}} + \text{Coral APE}_{\text{Rot}}}{\text{Seawater APE} + \text{Rotifer APE}}$$

% contribution of heterotrophically acquired C to new lipids

$$\left[\frac{\text{Coral APE}_{\text{Rot}}}{\text{Rotifer APE}}\right] \times \frac{\text{Coral APE}_{\text{DIC}} + \text{Coral APE}_{\text{Rot}}}{\text{Seawater APE} + \text{Rotifer APE}}$$
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 hrs vs. 168 hrs), and coral genotype on \(\delta^{13}\)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).

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<td>p</td>
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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 hours vs. 168 hours), and coral genotype on $\delta^{13}$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).

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Figure 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, hrs = hours. Figure adapted from Hughes and Grottoli (2013).
Figure 2: Photographs of representative coral fragments of treatment and control Porites compressa and Montipora capitata following 1 and 11 months on the reef. Photographs by A.G. Grottoli and A. Hughes.
Figure 3: Photoautotrophic C in *Porites compressa* and *Montipora capitata* lipids. Average (± 1 S.E.) lipid $\delta^{13}C$ enrichment of newly synthesized holobiont lipids 24 and 168 hours after pulse-labeling with $^{13}C$-labeled dissolved inorganic carbon in seawater. *Porites compressa* after A) 1 month and B) 11 months on the reef and *Montipora 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. * indicate significant differences between control and treatment within a chase interval. † indicate significant differences between 24 and 168 hour chase intervals within a status (treatment or control).
Figure 4: Heterotrophic C in *Porites compressa* and *Montipora capitata* lipids. Average (± 1 S.E.) lipid $\delta^{13}C$ enrichment of newly synthesized holobiont lipids 24 and 168 hours after pulse-labeling with $^{13}C$-labeled rotifers. *Porites compressa* after A) 1 month and B) 11 months on the reef and *Montipora 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. * indicate significant differences between control and treatment within a chase interval. † indicate significant differences between 24 and 168 hour chase intervals within a status (treatment or control).
Figure 5: Average (± 1 S.E.) proportionate contribution of photoautotrophically (DIC pulse-chase labeling) and heterotrophically (rotifer pulse-chase labeling) acquired carbon to holobiont total lipids 24 and 168 hours after pulse-labeling 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.