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KINETIC AND METABOLIC ISOTOPE EFFECTS IN CORAL SKELETAL CARBON ISOTOPES: A RE-EVALUATION USING EXPERIMENTAL CORAL BLEACHING AS A CASE STUDY

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ABSTRACT

Coral skeletal $\delta^{13}C$ can be a paleo-climate proxy for light levels (i.e., cloud cover and seasonality) and for photosynthesis to respiration (P/R) ratios. The usefulness of coral $\delta^{13}C$ as a proxy depends on metabolic isotope effects (related to changes in photosynthesis) being the dominant influence on skeletal $\delta^{13}C$. However, it is also influenced by kinetic isotope effects (related to calcification rate) which can overpower metabolic isotope effects and thus compromise the use of coral skeletal $\delta^{13}C$ as a proxy. Heikoop et al. (2000) proposed a simple data correction to remove kinetic isotope effects from coral skeletal $\delta^{13}C$, as well as an equation to calculate P/R ratios from coral isotopes. However, despite having been used by other researchers, the data correction has never been directly tested, and isotope-based P/R ratios have never been compared to P/R ratios measured using respirometry. Experimental coral bleaching represents a unique environmental scenario to test this because bleaching produces large physiological responses that influence both metabolic and kinetic isotope effects in corals. Here, we tested the $\delta^{13}C$ correction and the P/R calculation using three Pacific and three Caribbean coral species from controlled temperature-induced bleaching experiments where both the stable isotopes and the physiological variables that cause isotopic fractionation (i.e., photosynthesis, respiration, and calcification) were simultaneously measured. We show for the first time that the data correction proposed by Heikoop et al. (2000) does not effectively remove kinetic effects in the coral species studied here, and did not improve the metabolic signal of bleached and non-bleached corals. In addition, isotope-based P/R ratios were in poor agreement with measured P/R ratios, even when the data correction was applied. This suggests that additional factors influence $\delta^{13}C$ and $\delta^{18}O$, which are not accounted for by the data correction. We therefore recommend that the data correction not be routinely applied for paleo-climate reconstruction, and that P/R ratios should only be obtained by direct measurement by respirometry.

1. INTRODUCTION

The analysis of stable carbon and oxygen isotopes of coral skeletal carbonate are powerful tools that have significantly advanced our understanding of past climate. Coral skeletal $\delta^{13}C$ ($\delta^{13}C_s$) has been established as a proxy for light levels or cloud cover, seasonality, and nutrient/zooplankton levels (e.g. Fairbanks and Dodge, 1979; Gagan et al., 1994; Grottoli, 1999, 2002; Grottoli and Wellington, 1999; Swart et al., 1996b), whereas coral skeletal $\delta^{18}O$ ($\delta^{18}O_s$) records sea surface temperature (SST) and salinity (SSS) (e.g. Druffel, 1997; Fairbanks and Dodge, 1979; Gagan et al., 1994; Grottoli and Eakin, 2007; Swart et al., 1996a). However, coral aragonite does not precipitate in equilibrium with the isotopic composition of seawater, and is typically depleted in both $\delta^{13}C_s$ and $\delta^{18}O_s$ relative to seawater (McConnaughey, 1989a; Swart, 1983) (Fig. 1). Two patterns of isotopic disequilibrium are common in biological carbonates: (1) metabolic isotope effects related to photosynthesis and respiration, which modulate the isotopic composition of the internal dissolved inorganic carbon (DIC) pool from which carbonate is precipitated (McConnaughey, 1989a; McConnaughey et al., 1997; Swart, 1983), and (2) kinetic isotope effects related to CO_2 hydration and hydroxylation during calcification (McConnaughey, 1989a, 1989b).

In tropical scleractinian corals, metabolic isotope effects are thought to dominate coral $\delta^{13}C_s$, which allows for their use as proxies for light levels or cloud cover, seasonality, and nutrient/zooplankton levels (e.g. Fairbanks and Dodge, 1979; Gagan et al., 1994; Grottoli, 1999, 2002; Grottoli and Wellington, 1999; Swart et al., 1996b). However, given the common presence of symbiotic dinoflagellates (*Symbiodinium* spp.) in tropical corals, rapid calcification resulting from this symbiosis (Gattuso et al., 1999; Goreau and Goreau, 1959) also favors strong kinetic effects that may mask underlying metabolic effects (Allison et al., 1996; Cohen and Hart, 1997; Heikoop et al., 2000; McConnaughey, 1989a, 1989b). Thus, coral $\delta^{13}C_s$ can be challenging to use as climate proxy.

While metabolic isotope effects influence only carbon isotopic composition, kinetic isotope effects influence both carbon and oxygen isotopic composition (McConnaughey, 1989a). Generally, kinetic isotope effects result in the simultaneous depletion of $\delta^{18}O_s$ and $\delta^{13}C_s$ in an

approximate ratio of 1:3 (McConnaughey, 1989a). As a consequence, a strong correlation between $\delta^{18}O_s$ and $\delta^{13}C_s$ (due to their simultaneous depletion) can be used as a diagnostic tool to detect the presence of strong kinetic effects (McConnaughey, 1989a). In contrast, the presence of metabolic isotope effects is indicated by the lack of a strong correlation between $\delta^{18}O_s$ and $\delta^{13}C_s$ (McConnaughey, 1989a). These characteristic relationships between $\delta^{18}O_s$ and $\delta^{13}C_s$ have been used to propose a simple data correction that removes kinetic isotope effects and reveals potentially hidden metabolic effects in the $\delta^{13}C_s$ signature (Heikoop et al., 2000). Assuming that all corals in a study are grown under the same environmental conditions, variability in $\delta^{18}O_s$ should only be caused by kinetic isotope effects and can be used to remove variability in $\delta^{13}C_s$ that is due to kinetic isotope effects (see section 2.4.3 for mathematical details). Any remaining variability in $\delta^{13}C_s$ would therefore be the result of metabolic isotope effects alone, separating the isotope effect of interest from the "unwanted" kinetic effects.

Kinetic and metabolic isotope effects are best assessed when visualized in $\delta^{18}O_s$ vs. $\delta^{13}C_s$ space (Fig. 1). In the absence of kinetic and metabolic fractionation effects, carbonates should precipitate in isotopic equilibrium with seawater (Fig. 1). Both metabolic isotope effects and kinetic isotope effects (KIE hereafter) cause significant offsets from isotopic equilibrium. Given the depletion of $\delta^{18}O_s$ and $\delta^{13}C_s$ in an approximate ratio of 1:3 (McConnaughey, 1989a), corals typically plot along or parallel to the KIE line when kinetic isotope effects dominate (Fig. 1). Faster growing corals are expected to plot further away from isotopic equilibrium composition than slower growing corals due to more pronounced KIE effects (Fig. 1) (Allison et al., 1996; McConnaughey, 1989a). In contrast, metabolic isotope effects cause offsets from the KIE line towards both more enriched and more depleted $\delta^{13}C_s$ (Fig. 1). This is because photosynthesis enriches the internal DIC pool from which the skeleton is precipitated as photosynthesis preferentially removes ^{12}C, whereas respiration leads to the incorporation of isotopically depleted metabolic C. Generally, photosynthesis affects $\delta^{13}C_s$ more strongly (up to 11‰) than respiration (about 1.5‰) because symbiotic corals calcify mainly during the day when photosynthetic CO_2 uptake is several times faster than respiratory CO_2 release (McConnaughey, 1989a; McConnaughey et al., 1997). Since high photosynthesis rates are generally related to high

112 calcification rates, fast growing healthy corals are expected to plot towards more enriched $\delta^{13}C_s$
113 and more depleted $\delta^{18}O_s$ values, respectively (Fig. 1).
114 The data correction proposed by Heikoop et al. (2000) has been used to improve correlation
115 of $\delta^{13}C_s$ with environmental variables (Heikoop et al., 2000) and as a correction for kinetic
116 isotope effects in core records (Ourbak et al., 2008). Beyond its implications for paleo-climate
117 reconstruction, the concept of metabolic and kinetic isotope effects is also a valuable tool to
118 detect changes in coral metabolism, the degree of auto- vs. heterotrophy, and changes in
119 calcification rates. They can thus be used to infer ecological and physiological plasticity in corals
120 (Maier et al., 2003), and to trace physiological changes during a variety of environmental
121 scenarios, including coral bleaching (Suzuki et al., 2003), haze events (Risk et al., 2003), and
122 different water flow conditions (Suzuki et al., 2008). Further, $\delta^{13}C_s$ has been used to estimate the
123 ratio of photosynthesis to respiration (P/R ratio) based on where corals plot in the space of $\delta^{18}O_s$
124 vs. $\delta^{13}C_s$ (Maier et al., 2003) or based on calculations using coral skeletal and tissue isotopes
125 (Heikoop et al., 2000; Kaandorp et al., 2005; Lesser et al., 2010; Maier, 2004). However,
126 isotope-based P/R ratios have never been compared to ratios measured directly by respirometry.
127 Therefore, it is unknown if they are reliable proxies for P/R ratios in corals.
128 Although some have challenged McConnaughey's model of metabolic and kinetic
129 isotope effects (e.g. Adkins et al., 2003), it is generally widely accepted and has been supported
130 and applied by many studies (Allison et al., 1996; Cohen and Hart, 1997; Heikoop et al., 2000;
131 e.g. McConnaughey, 1989b; Omata et al., 2005; Omata et al., 2008; Suzuki et al., 2003; Suzuki
132 et al., 2005). However, the data correction proposed by Heikoop et al. (2000) has never been
133 tested using controlled culturing experiments where the extent of both isotope effects is
134 quantified by simultaneous measurement of the physiological variables that cause fractionation
135 (i.e., photosynthesis, respiration, and calcification) and the paired skeletal stable isotopes values.
136 More specifically, such a comparison is needed to determine if (1) the data correction proposed
137 by Heikoop et al. (2000) effectively removes kinetic isotope effects, (2) the data correction can
138 be applied to a wide range of coral species under a range of environmental scenarios that
139 influence both metabolic and kinetic isotope effects, and (3) isotope-based P/R ratios are reliable
140 proxies for P/R ratios measured by respirometry.

One such environmental scenario that affects both metabolic and kinetic isotope effects is coral bleaching, which is largely caused by periods of elevated seawater temperature (Baker et al., 2008; Brown, 1997; e.g. Glynn, 1996; Hoegh-Guldberg, 1999). During bleaching, corals lose significant amounts of their algal endosymbionts and/or photosynthetic pigments (e.g. Fitt et al., 2001; Hoegh-Guldberg and Smith, 1989; Jokiel and Coles, 1990), which renders them pale or "bleached" in appearance. This can result in dramatic declines in photosynthesis (e.g. Grottoli et al., 2006; Levas, 2012; Porter et al., 1989; Rodrigues and Grottoli, 2007), thus causing changes in metabolic isotope effects. Further, calcification rates of bleached corals are often reduced (Allison et al., 1996; Levas et al., 2013; e.g. Porter et al., 1989; Rodrigues and Grottoli, 2006; Schoepf, 2013), thus affecting kinetic isotope effects. In $\delta^{18}O_s$ vs. $\delta^{13}C_s$ space, bleached corals are expected to plot closer to the KIE line than healthy corals due to reduced photosynthesis, and also closer to equilibrium as calcification rates are often compromised (Fig. 1). However, bleached corals do not always show this expected trend (i.e., a decrease in $\delta^{13}C_s$) (Hartmann et al., 2010; Leder et al., 1991; Levas, 2012; Rodrigues and Grottoli, 2006), which is potentially due to strong kinetic effects masking changes in metabolism. The application of a data correction to remove kinetic isotope effects (Heikoop et al., 2000) might therefore reveal the masked metabolic isotope effects, and thus improve accuracy and interpretation of skeletal isotopes in bleached corals.

Here, we present physiological and isotopic data from a controlled bleaching experiment using three Caribbean coral species. In addition, we reanalyzed previously published data from the bleaching experiments of Rodrigues and Grottoli (2006) and Levas et al. (2013) to include three Pacific coral species in the dataset. In these bleaching experiments, corals were either bleached by exposure to elevated seawater temperature for 2.5 – 4 weeks or were kept at ambient temperatures as controls. Pacific corals were exposed to elevated temperature once, whereas Caribbean corals were repeat bleached by exposing them to elevated temperature in two consecutive summers. This was done to assess any potential effects of frequent thermal stress on coral isotopes, which could have implications for the reconstruction of past bleaching events from coral skeletons. Short and long term recovery was assessed over 8-11 months, and P/R ratios were measured in addition to their tissue (animal host and algal endosymbiont) and

skeletal isotopes at relevant time points. A suite of other physiological measurements were performed on these corals (Aschaffenburg, 2012; Levas et al., 2013; McGinley, 2012; Rodrigues and Grottoli, 2006, 2007; Rodrigues et al., 2008; Schoepf, 2013), providing a rich background of physiological information within which to interpret our findings. Although the original P and R rates and isotopic data of Rodrigues and Grottoli (2006) and Levas et al. (2013) are published, they have never been transformed using the data correction proposed by Heikoop et al. (2000), and isotope-based P/R ratios were never calculated based on tissue and skeletal isotopes in these studies. Therefore, the re-analyzed data presented here will provide novel insight into the nature of metabolic and kinetic isotope effects in bleached and non-bleached Pacific coral species. Further, the incorporation of three Pacific coral species into our dataset allowed for a rigorous testing of the proposed hypotheses across six coral species originating from two ocean basins.

We hypothesize that (1) the correlation between $\delta^{13}C_s$ and animal host tissue $\delta^{13}C$ ($\delta^{13}C_h$) improves when $\delta^{13}C_s$ values are corrected ($\delta^{13}C_{scorr}$) according to Heikoop et al. (2000), and (2) as photosynthesis and calcification decline with bleaching, $\delta^{13}C_{scorr}$ values move towards the upper left quadrant in $\delta^{18}O_s$ vs. $\delta^{13}C_s$ space. Further, we hypothesize that isotope-based calculated P/R ratios (Heikoop et al., 2000; Kaandorp et al., 2005) are significantly correlated with P/R ratios measured by respirometry. If these hypotheses are supported, the correction proposed by Heikoop et al. (2000) could be routinely applied to paleo-climate reconstruction and improve the accuracy of coral proxy records. With improved metabolic signals in bleached corals, the reconstruction of past bleaching events from coral skeletons may be more effective. Finally, isotope-based P/R ratios could be used to infer coral metabolism in mesophotic environments, where respirometry cannot be easily performed. Overall, this approach of combined physiological and isotopic analyses should significantly promote the understanding of the functional processes underlying isotopic proxy signals in coral skeletons.

2. MATERIAL AND METHODS

2.1 Hawaii Bleaching Experiments

A detailed description of the first bleaching experiment in Hawaii can be found in Rodrigues and Grottoli (2006). Briefly, coral fragments from branching *Porites compressa* and branching *Montipora capitata* were collected from Point Reef, Kaneohe Bay, Hawaii (21°26.18'N; 157°47.56'W) in late August 2003 from 2 m depth. After allowing them to acclimate for two weeks, half of all fragments were placed in shaded outdoor tanks with ambient seawater (26.8°C ± 0.04 SE) (non-bleached controls), while the other half were placed in tanks with elevated temperature seawater (30.1°C ± 0.05 SE) (bleached corals) (Fig. 2A). Temperature was gradually elevated over the course of three days. Corals were not fed during the experiment, and inflow pipes were fitted with a 50 μm-filter. To minimize positional effects, corals were rotated within and among tanks of the same treatment daily. After one month, 25% of all treatment and control fragments were collected and frozen for isotopic analyses (= 0 month recovery), whereas the remaining corals were placed back on the reef to recover *in situ* (Fig. 2A). To assess short and long term recovery, a third of all remaining treatment and control corals were collected after 1.5, 4, and 8 months, respectively (Fig. 2A). Photosynthesis and respiration rates were measured at each recovery interval before corals were frozen for isotopic analyses. Coral fragments were stained with alizarin at each recovery interval.

A second, similar bleaching experiment was performed in summer 2006 to assess bleaching impacts on mounding *Porites lobata* (Fig. 2B). A detailed description of the experimental design can be found in Levas et al. (2013). Briefly, coral fragments were collected from Sanpan Channel, Kaneohe Bay, Hawaii (21°26.18'N; 157°47.56'W) in August 2006 from 10-12 m depth. After allowing them to acclimate for two weeks, half of all fragments were placed in shaded outdoor tanks with ambient seawater (27.5°C ± 0.08 SE) (non-bleached controls), while the other half were placed in tanks with elevated temperature seawater (30.2°C ± 0.20 SE) (bleached corals) (Fig. 2B). Temperature was gradually elevated over the course of seven days. Corals were fed freshly caught zooplankton for 1 h at dusk every other night. To minimize positional effects, corals were rotated within and among tanks of the same treatment daily. After 23 days, 25% of all treatment and control fragments were collected and frozen for isotopic analyses (= 0 month recovery), whereas the remaining corals were placed back on the reef to recover *in situ* (Fig. 2B). To assess short and long term recovery, a third of all remaining

treatment and control corals were collected after 1, 5, and 11 months, respectively (Fig. 2B). Photosynthesis and respiration rates were measured at each recovery interval before corals were frozen for isotopic analyses.

2.2 Mexico Bleaching Experiment

Coral fragments of mounding *Orbicella faveolata* (formerly *Montastraea faveolata* (Budd et al., 2012)), encrusting to mounding *Porites astreoides*, and branching *Porites divaricata* were collected in July 2009 from shallow reefs (3-8 m) near Puerto Morelos Reefs National Park, Mexico (20°50'N, 86°52'W). After allowing them to acclimate for 5 days, half of the coral fragments were placed in tanks with ambient seawater temperature (30.66 ± 0.24°C) (non-bleached controls), while the other half were placed in tanks with elevated temperature seawater (31.48 ± 0.20°C) (bleached corals) (Fig. 2C). Seawater temperature in the treatment tanks was gradually elevated over the course of seven days. Corals were not fed, but had access to unfiltered seawater. Fragments were rotated daily within and among tanks of the same treatment to minimize any positional effects. After a total of 15 days, temperature in all tanks was returned to ambient levels, and all coral fragments were placed on the back reef to recover *in situ* for one full year (Fig. 2C).

In July 2010, the bleaching treatment was repeated using the same experimental protocol. All coral fragments that had recovered on the back reef for 1 year were recollected and thoroughly cleaned. All corals that had served as ambient control fragments the previous summer were placed in tanks with ambient seawater (30.40 ± 0.23°C) (non-bleached corals), while all corals that had been used as treatment fragments were maintained in tanks with elevated temperature seawater (31.60 ± 0.24°C) (repeat bleached corals) (Fig. 2C). After 17 days, all tanks were returned to ambient temperature levels. During the last days of the repeat bleaching treatment, photosynthesis and respiration rates were measured on one ambient control and one treatment coral fragment of each colony and species (i.e., n=9 fragments per species and treatment) (6-7 August 2010), and were then frozen for additional physiological and isotopic analyses (= 0 month of recovery) (Fig. 2C). All remaining fragments were placed on the back

reef to recover *in situ*. To assess short- and long-term recovery from repeat bleaching, one fragment from each colony and treatment was recollected from the reef after 1.5 and 11 months of recovery (Fig. 2C), and then frozen for isotopic analyses. Photosynthesis and respiration rates were not measured at these recovery intervals.

2.3 Photosynthesis to Respiration Ratios

Net photosynthesis (P) and day respiration (R) rates were measured by quantifying changes in dissolved oxygen by incubating non-bleached and bleached corals in UV-transparent acrylic chambers under light and dark conditions (Coles and Jokiel 1977). Oxygen production or consumption was measured over 10 – 40 min intervals during the day. Net P and day R rates were calculated as μmol O_2 min^{-1} and then standardized to coral ash-free dry tissue biomass (for *O. faveolata, P. astreoides, P. divaricata, P. compressa, M. capitata*) or surface area (for *P. lobata*). P/R ratios were then calculated as follows:

$$P/R = (\text{net P} + \text{day R}) / (\text{day R}) = (\text{gross P}) / (\text{day R}) \quad (1)$$

A detailed description of the P and R measurement methods for the Pacific corals are given in Rodrigues and Grottoli (2007) and Levas et al. (2013).

P/R ratios derived from measured P and R values were then compared to P/R ratios calculated from coral skeletal and tissue isotopes (see methods below). Although P/R ratios calculated from isotopes reflect a longer time period (i.e., the most recent growth period) than P/R ratios measured via respirometry, the values are nevertheless comparable because the respirometry measurements capture the cumulative effect of treatment conditions experienced by the coral up to this point. While it would be ideal to perform P/R measurements repeatedly over the growth period represented by isotopes, this was logistically not possible due to the high degree of replication in these studies (i.e., up to three species per experiment and up to nine fragments per treatment).

2.4 Isotopic Analyses

2.4.1 Seawater Dissolved Inorganic Carbon (DIC) Isotopes

A total of nine filtered seawater samples from Kaneohe Bay, Hawaii, were collected throughout 2006/07 for $\delta^{13}C_{DIC}$ analyses. They were preserved with anhydrous HgCl. In the laboratory, each sample was acidified on a vacuum extraction line under high-purity helium flow, with the resulting CO_2 gas cryogenically isolated under vacuum, and the DIC concentration was determined (McNichol et al. 1994). The CO_2 from each DIC sample was sealed in Pyrex ampoules and introduced into a Finnigan Delta IV Stable Isotope Ratio Mass Spectrometer (SIRMS) via an automated 10-port inlet. All $\delta^{13}C$ values were reported as per mil values relative to Vienna-Pee Dee Belemnite limestone standard (v-PDB). $\delta^{13}C_{DIC}$ analyses were not performed for seawater from Puerto Morelos, Mexico. The standard deviation of repeated measurements of an internal standard was ± 0.03‰ (n = 37).

2.4.2 Tissue and Skeletal Isotopes

A detailed description of the isotopic analyses for the Pacific corals can be found in Rodrigues and Grottoli (2006) and Levas et al. (2013), and for the Caribbean corals in Schoepf (2013). Briefly, coral tissue was removed from the skeleton using an airbrush, homogenized, and separated into animal host and algal endosymbiont fractions by centrifugation. The two fractions were then individually transferred onto pre-baked (450°C) GF/F filters and combusted in an Elemental Analyzer coupled to a Finnigan Delta IV SIRMS. For skeletal isotopes, only the uppermost layer of the dried skeleton were gently shaved with a diamond-tipped Dremel tool and ground to fine powder using established methods (Grottoli et al. 2004, Rodrigues and Grottoli 2006). For branching corals, only branch tips were shaved. About 80-100 µg of the untreated (Grottoli et al., 2005) skeletal powder were analyzed for $\delta^{13}C$ and $\delta^{18}O$ using an automated Kiel Carbonate Device coupled to a Finnigan Delta IV SIRMS. Samples were acidified under vacuum with 100% ortho-phosphoric acid. The carbon isotopic composition of the animal host ($\delta^{13}C_h$), algal endosymbiont ($\delta^{13}C_e$), and skeleton ($\delta^{13}C_{sorig}$) were reported as the per mil deviation of the stable isotopes ^{13}C:^{12}C relative to v-PDB. Skeletal oxygen isotopes ($\delta^{18}O_{sorig}$) were reported as

the per mil deviation of the ratio of stable isotopes ^{18}O:^{16}O relative to v-PDB. For both organic and skeletal isotopes, approximately 10% of all samples were run in duplicate. The standard deviation of repeated measurements of internal standards for each dataset can be found in Table S1.

2.4.3 Data Correction

Coral skeletal carbon isotopes ($\delta^{13}C_{sorig}$) were corrected ($\delta^{13}C_{scorr}$) using skeletal oxygen isotopes ($\delta^{18}O_s$) to remove kinetic effects according to the equation developed by Heikoop et al. (2000):

$$\delta^{13}C_{scorr} = \delta^{13}C_{sorig} - (3*(\delta^{18}O_s - \delta^{18}O_{s\ average})) \quad (2)$$

Here, $\delta^{18}O_{s\ average}$ was calculated individually for each treatment and recovery interval for each species.

2.4.4 Carbon Isotopic Equilibrium

Carbon isotopic equilibrium ($\delta^{13}C_{eq}$) for aragonite was calculated following the precedence of McConnaughey et al. (1997) and Heikoop et al. (2000) using the equation of Romanek et al. (1992):

$$\delta^{13}C_{eq} = \delta^{13}C_{DIC} + 2.7 \quad (3)$$

For Pacific corals, an average $\delta^{13}C_{DIC}$ of +0.12 ‰ ± 0.44 SD (n=9) was measured in Kaneohe Bay in 2006 and 2007 (Table S2), and $\delta^{13}C_{eq}$ was therefore estimated to be +2.82‰. For Caribbean corals, $\delta^{13}C_{DIC}$ is unknown for Puerto Morelos and was therefore estimated to be +1.15‰ based on literature values from other locations in the Caribbean (Grossman and Ku, 1986; Maier, 2004; Maier et al., 2010; Moyer and Grottoli, 2011; Swart et al., 1996b; Watanabe et al., 2002). Thus, an average $\delta^{13}C_{eq}$ of +3.85‰ was calculated for Puerto Morelos.

2.4.5 Oxygen Isotopic Equilibrium

Two different methods, Grossman and Ku (1986) and Maier (2004), exist in the literature to calculate oxygen isotopic equilibrium in carbonates ($\delta^{18}O_{eq}$), where only Grossman and Ku (1986) incorporate temperature-dependent fractionation. To facilitate comparisons across studies, both methods were used in this study. First, $\delta^{18}O_{eq}$ was calculated using the equation by Grossman and Ku (1986):

$$(\delta^{18}O_{eq} - \delta^{18}O_{seawater}) = 4.75 - 0.23*T\ (°C) \qquad (4)$$

where $\delta^{18}O_{eq}$ and $\delta^{18}O_{seawater}$ are expressed on the same isotopic scale.

For Pacific corals, $\delta^{18}O_{seawater}$ of Kaneohe Bay is unknown and was estimated to be +0.4‰ (SMOW) or +0.13‰ (v-PDB) based on values from the global ^{18}O database (Epstein and Mayeda, 1953; Ostlund et al., 1987; Schmidt et al., 1999). Seawater temperature ranged from 23.0 – 28.0°C in Kaneohe Bay (Levas, 2012; Rodrigues and Grottoli, 2006), which resulted in an average $\delta^{18}O_{eq}$ of -1.24‰ when the temperature of the bleaching treatments were included (i.e., 30.1°C for *P. compressa* and *M. capitata*, and 30.2°C for *P. lobata*). This average was used to calculate the end point of the KIE line in all figures to represent the range of $\delta^{18}O_{eq}$ across treatments and seasons. However, for all correlation analyses, $\delta^{13}C_{scorr}$ was calculated using $\delta^{18}O_{eq}$ values that were computed individually for each treatment and recovery interval, thus taking into account temperature differences due to treatment or season.

For Caribbean corals, $\delta^{18}O_{seawater}$ is unknown for Puerto Morelos and was therefore estimated to be +0.85‰ (SMOW) based on literature values from the Caribbean (Leder et al., 1996; Maier, 2004; Watanabe et al., 2002). This value was then converted to v-PDB scale by subtracting 0.27‰ (Schmidt, 1999), resulting in an average $\delta^{18}O_{seawater}$ of +0.58‰ (v-PDB). Seawater temperature was monitored using HOBO temperature loggers throughout the study, with an annual range of 25.5 – 30.4°C, resulting in an average $\delta^{18}O_{eq}$ of -1.24‰ when the temperature of the bleaching treatment (31.6°C) was included. As for Pacific corals, this average was used to calculate the end point of the KIE line in all figures to represent the range of $\delta^{18}O_{eq}$ across treatments and seasons. However, for all correlation analyses, $\delta^{13}C_{scorr}$ was calculated

using $\delta^{18}O_{eq}$ values that were computed individually for each treatment and recovery interval, thus taking into account temperature differences due to treatment or season.

Second, $\delta^{18}O_{eq}$ was calculated according to Maier (2004) where $\delta^{18}O_{eq}$ equals $\delta^{18}O_{seawater}$ after conversion to v-PDB isotopic scale:

$$\delta^{18}O_{eq}\,(\text{v-PDB}) = \delta^{18}O_{seawater}\,(\text{SMOW}) - 0.27 \qquad (5)$$

This equilibrium value is independent of seawater temperature. For Pacific and Caribbean corals, $\delta^{18}O_{eq}$ was therefore +0.13‰ and +0.58‰ (v-PDB), respectively.

2.4.6 Isotope-based P/R Ratios

P/R ratios were calculated from skeletal and tissue isotopes according to the following equations (Kaandorp et al., 2005; Maier, 2004):

$$M_{offset} = (\delta^{13}C_{sorig} - \alpha\,(\delta^{18}O_s - \delta^{18}O_{eq}) - \delta^{13}C_{eq}) \qquad (6)$$

$$P/R = ((M_{offset} - \rho)/\rho) \,/\, (\delta^{13}C_e / \delta^{13}C_h) \qquad (7)$$

where M_{offset} is the metabolic offset from the kinetic isotope effect (KIE) line, α is the slope of the relationship between $\delta^{18}O_s$ to $\delta^{13}C_{sorig}$ which is estimated to be 0.33 based on the simultaneous depletion of $\delta^{18}O_s$ and $\delta^{13}C_{sorig}$ in an approximate ratio of 1:3 due to KIE effects (Heikoop et al., 2000; McConnaughey, 1989a), ρ is the offset of $\delta^{13}C_{sorig}$ from the KIE line due to respiration which is estimated to be -1.5‰ (Heikoop et al., 2000; McConnaughey et al., 1997), $\delta^{13}C_e$ is the carbon isotopic composition of the algal endosymbiont, and $\delta^{13}C_h$ is the carbon isotopic composition of the animal host. Absolute values of ρ were used for calculations. Isotope-based P/R ratios were calculated two ways: once using $\delta^{18}O_{eq}$ calculated after Grossman and Ku (1986) and once after Maier (2004). When $\delta^{18}O_{eq}$ was calculated after Grossman and Ku (1986), it was calculated individually for each treatment and recovery interval, thus taking into account temperature differences due to treatment or season.

2.5 Statistical Analyses

To determine the presence of kinetic isotope effects, correlations between uncorrected original $\delta^{13}C_s$ (i.e., $\delta^{13}C_{sorig}$) and $\delta^{18}O_s$ were calculated using Spearman's correlation coefficient (*r*). Following Heikoop et al. (2000), $\delta^{13}C_{sorig}$ was considered to be dominated by kinetic isotope effects when the correlation was statistically significant (*p*-values≤0.05).

To test the effectiveness of the data transformation in removing kinetic isotope effects, two methods were used. First, using a quantitative approach following (Heikoop et al., 2000), correlations were computed for $\delta^{13}C_{sorig}$ vs. $\delta^{13}C_{h,}$ and for corrected $\delta^{13}C_s$ (i.e., $\delta^{13}C_{scorr}$) vs. $\delta^{13}C_h$. Since coral tissue isotopes are not affected by kinetic isotope effects associated with calcification, they can be used to assess if the data correction was effective. Thus, a significant correlation between $\delta^{13}C_{scorr}$ and $\delta^{13}C_h$ would indicate that metabolic isotope effects dominate the corrected skeletal isotope signal, and that kinetic isotope effects were successfully removed. While Heikoop et al. (2000) used whole tissue (i.e., animal host + algal endosymbiont) isotopes for this comparison, we chose to use animal host isotopes as the coral tissue is made up of much more animal host cells compared to algal cells. Nevertheless, both $\delta^{13}C_h$ and $\delta^{13}C_e$ versus $\delta^{13}C_{sorig}$ and $\delta^{13}C_{scorr}$ were evaluated (see Table S3 for correlations with $\delta^{13}C_e$). However, correlations with $\delta^{13}C_e$ were generally weaker than those with $\delta^{13}C_h$ and so were not further evaluated. Correlation analyses were computed (1) for each geographical location pooled across all treatments and recovery intervals, (2) for each species and treatment pooled across all recovery intervals, and (3) individually for each species, treatment, and recovery interval as long as sample size was at least four. The data correction was considered to be effective in removing kinetic isotope effects when (1) the correlation was statistically significant and (2) Spearman's *r* was higher for $\delta^{13}C_{scorr}$ vs. $\delta^{13}C_h$ compared to $\delta^{13}C_{sorig}$ vs. $\delta^{13}C_h$ (Heikoop et al., 2000).

Second, using a qualitative approach, $\delta^{13}C_{scorr}$ was plotted for each species, treatment, and recovery interval in $\delta^{18}O_s$ vs. $\delta^{13}C_s$ space. It was then visually assessed to determine if bleached corals plotted more towards lower photosynthesis and lower calcification rates (i.e., towards the upper left in $\delta^{18}O_s$ vs. $\delta^{13}C_s$ space – see Fig. 1) compared to non-bleached corals, as would be

expected based on measured changes in physiology and calcification (Aschaffenburg, 2012; Levas et al., 2013; Rodrigues and Grottoli, 2006, 2007; Schoepf, 2013). This qualitative approach also allows for visualization of how the data correction transforms the original $\delta^{13}C_s$ data.

Lastly, to compare measured and isotope-based P/R ratios, correlations were calculated for each species for the following comparisons: (1) measured P/R vs. isotope based P/R using $\delta^{13}C_{sorig}$ and $\delta^{18}O_{eq}$ after Grossman and Ku (1986), (2) measured P/R vs. isotope based P/R using $\delta^{13}C_{scorr}$ and $\delta^{18}O_{eq}$ after Grossman and Ku (1986), (3) measured P/R vs. isotope based P/R using $\delta^{13}C_{sorig}$ and $\delta^{18}O_{eq}$ after Maier (2004), and (4) measured P/R vs. isotope based P/R using $\delta^{13}C_{scorr}$ and $\delta^{18}O_{eq}$ after Maier (2004). In addition, paired t-tests were computed for the same four comparisons to test if the averages of the measured and isotope-based P/R ratios differed significantly from one another. Treatments and recovery intervals were pooled for each species. For t-test analyses, data were first tested for normality using residual plots and Shapiro-Wilk's test and when necessary, data were transformed to meet the assumption of normality. Bonferroni corrections were not applied because they increase the risk of false negatives (Moran, 2003; Quinn and Keough, 2002).

The bleached coral fragments were determined to be fully recovered for a given variable once the average bleached isotopic value no longer significantly differed from the non-bleached control. Since all fragments were exposed to identical conditions, except temperature, during the tank portion of the experiment, any differences in the measured variables between bleached and non-bleached control fragments were due to the experimental temperature effects alone, independent of natural seasonal variation. A total of three outliers (two from *P. compressa*, one from *M. capitata*) were excluded from all statistical analyses but are clearly indicated in the figures. Statistical analyses were performed using SAS software, Version 9.2 and 9.3 of the SAS System for Windows.

3. RESULTS

3.1 Isotope Correlations

3.1.1 All Coral Data Combined

When all of the data were combined, the skeletal carbon and oxygen isotopes ($\delta^{13}C_{sorig}$ and $\delta^{18}O_s$, respectively) of all Pacific and Caribbean coral species plotted parallel to the KIE line, but were slightly offset towards more depleted $\delta^{18}O_s$ values or more enriched $\delta^{13}C_{sorig}$ values (Fig. 3). They were much closer to the KIE line that used oxygen isotopic equilibrium ($\delta^{18}O_{eq}$) calculated using Grossman and Ku (1986) compared to that using $\delta^{18}O_{eq}$ calculated using Maier (2004).

3.1.2 Pacific Corals

Quantitative Assessment. $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ were highly correlated in all Pacific corals, even when the data set was analyzed by species, and by treatment within species (Table S4A). The only exceptions were *P. compressa* (all corals) and non-bleached *M. capitata* (Table S4A). $\delta^{13}C_{sorig}$ and $\delta^{13}C_h$ were significantly correlated in five cases, but none of them showed improved correlations when computed with $\delta^{13}C_{scorr}$ (Table S4A).

When correlations were calculated by treatment within species at each recovery interval, significant correlations between $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ were only observed in four of 22 cases (Table S4B). Furthermore, only three of the 16 cases showed significant correlations between $\delta^{13}C_{sorig}$ and $\delta^{13}C_h$ (Table S4B). Of these, none of the correlation coefficients improved with $\delta^{13}C_{scorr}$. However in one case, the correlation with $\delta^{13}C_{scorr}$ was significant even though it was not significant with $\delta^{13}C_{sorig}$ (Table S4B).

Qualitative Assessment. Figure 4 shows how the relationship of $\delta^{13}C_{sorig}$ versus $\delta^{18}O_s$ (Fig. 4A-F) is modified by the data correction to $\delta^{13}C_{scorr}$ (Fig. 4G-L) in the Pacific corals. In $\delta^{13}C_{sorig}$ vs. $\delta^{18}O_s$ space, all of the values plotted below both KIE Lines (Fig. 4A-F). When plotted with $\delta^{13}C_{scorr}$, all of the values plotted below the KIE line using $\delta^{18}O_{eq}$ calculations of Maier (2004) (Fig. 4G-L). However, several values plotted above or on the KIE line produced using the $\delta^{18}O_{eq}$ calculations of Grossman and Ku (1986) (Fig. 4G-H). In addition, bleached $\delta^{13}C_{scorr}$ values at 0 and 1.5 months of recovery were depleted by similar amounts as non-

488 bleached corals (Fig. 4G-L). Further, bleached $\delta^{13}C_{scorr}$ values showed no clear trend associated
489 with longer recovery times, although at 4 and 8 months of recovery they appear somewhat more
490 enriched than values at 0 and 1.5 months of recovery in both *P. compressa* and *M. capitata* (Fig.
491 4H-L). Similarly, bleached $\delta^{13}C_{scorr}$ values did not show any clear trend in their offset from the
492 KIE lines with longer recovery times (Fig. 4H-L).
493
494 ***3.1.3 Caribbean Corals***
495 **Quantitative Assessment.** All Caribbean corals showed significant correlations between
496 $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$, even when the data set was analyzed individually by species and treatment
497 (Table S5A). The only exception was non-bleached *P. divaricata* corals (Table S5A). In
498 addition, $\delta^{13}C_{sorig}$ and $\delta^{13}C_h$ were significantly correlated in 8 of 10 cases, two of which had
499 stronger correlations with $\delta^{13}C_{scorr}$ than $\delta^{13}C_{sorig}$ (Table S5A).
500 When correlations were calculated by treatment within species at each recovery interval,
501 $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ were highly correlated in 8 of 17 cases (Table S5B). Furthermore, only three
502 of the 17 cases showed significant correlations between $\delta^{13}C_{sorig}$ and $\delta^{13}C_h$ of which only one had
503 a stronger correlation when $\delta^{13}C_{scorr}$ was used (Table S5B).
504 **Qualitative Assessment.** Figure 5 shows how the relationship of $\delta^{13}C_{sorig}$ versus $\delta^{18}O_s$
505 (Fig. 5A-F) is modified by the data correction to $\delta^{13}C_{scorr}$ (Fig. 5G-L) in the Caribbean corals. In
506 $\delta^{13}C_{sorig}$ vs. $\delta^{18}O_s$ space, all of the values plotted below both KIE lines (Fig. 5A-F). When plotted
507 with $\delta^{13}C_{scorr}$, all of the values plotted below the KIE line using $\delta^{18}O_{eq}$ calculations of Maier
508 (2004) (Fig. 5G-L). However, several of the *O. faveolata* values plotted above the KIE line
509 produced using the $\delta^{18}O_{eq}$ calculations of Grossman and Ku (1986) (Fig. 5G-H). In addition,
510 $\delta^{13}C_{scorr}$ values of repeat bleached *O. faveolata* at 1.5 months of recovery were more depleted
511 than $\delta^{13}C_{scorr}$ at 0 and 11 months of recovery (Fig. 5H). They were also more depleted than
512 $\delta^{13}C_{scorr}$ values of non-bleached corals at 1.5 months of recovery (Fig. 5G). Further, $\delta^{13}C_{scorr}$
513 values of repeat bleached *O. faveolata* at 0 months of recovery appeared to be less offset from
514 the KIE lines than $\delta^{13}C_{scorr}$ values of non-bleached corals at that recovery interval (Fig. 5G, H).
515 This was, however, not observed at 1.5 months of recovery (Fig. 5G, H).

In repeat bleached *P. astreoides*, $\delta^{13}C_{scorr}$ values at 1.5 months of recovery were more enriched than values of non-bleached corals at this time interval (Fig. 5I, J). In *P. divaricata*, repeat bleached $\delta^{13}C_{scorr}$ values at 1.5 months of recovery were generally more depleted than at 11 months of recovery (Fig. 5L), but no different from non-bleached corals at the same time interval (Fig. 5K). In both species, no clear trend was observed regarding the offset of repeat bleached $\delta^{13}C_{scorr}$ values from the KIE lines at any recovery interval or compared to non-bleached corals (Fig. 5I, K, J, L).

3.2 Measured and Isotope-Based P/R Ratios

3.2.1 Pacific Corals

In 8 of 12 cases, measured P/R ratios of Pacific corals were not correlated with any isotope-based P/R ratios (Table S6, Fig. 6). However in *P. compressa*, measured P/R ratios were negatively correlated with isotope-based P/R ratios using $\delta^{18}O_{eq}$ calculated after Maier (2004), independent of whether original or corrected $\delta^{13}C_s$ was used (Table S6, Fig. 6A, D). In *P. lobata*, measured P/R ratios were positively correlated with isotope-based P/R ratios using $\delta^{18}O_{eq}$ calculated after Grossman and Ku (1986), independent of whether original or corrected $\delta^{13}C_s$ was used (Table S6, Fig. 6C, F).

Paired t-tests indicated that isotope-based P/R ratios were significantly different from measured P/R ratios in Pacific corals (Table S7). Despite the significant correlations of measured and isotope-based P/R ratios using $\delta^{18}O_{eq}$ calculated after Maier (2004) in *P. compressa*, t-tests indicated that they were significantly different (Table S7). In contrast, t-tests confirmed that isotope-based P/R ratios using $\delta^{18}O_{eq}$ calculated after Grossman and Ku (1986) in *P. lobata* were not significantly different from measured P/R ratios (Table S7). Further, isotope-based P/R ratios using corrected $\delta^{13}C_s$ and $\delta^{18}O_{eq}$ calculated after Grossman and Ku (1986) in *M. capitata* were not significantly different from measured P/R ratios (Table S7), even though they were not significantly correlated (Table S6).

3.2.2 Caribbean Corals

Measured and isotope-based P/R ratios were not significantly correlated in any Caribbean coral species, independent of the type of $\delta^{18}O_{eq}$ and whether original or corrected $\delta^{13}C_s$ was used (Fig. 7, Table S8). Similarly, paired t-tests showed that isotope-based P/R ratios differed significantly from measured P/R ratios in the majority of cases (Table S9). However, in both *O. faveolata* and *P. astreoides*, isotope-based P/R ratios using $\delta^{18}O_{eq}$ calculated after Grossman and Ku (1986) did not significantly differ from measured P/R ratios (Table S9). This was independent of whether $\delta^{13}C_{sorig}$ or $\delta^{13}C_{scorr}$ was used. Generally, all isotope-based P/R ratios using $\delta^{18}O_{eq}$ calculated after Grossman and Ku (1986) resulted in negative P/R ratios in these species (Table S9).

3.2.3 Influence of $\delta^{18}O$ Equilibrium On Isotope-Based P/R Ratios

Generally, isotope-based P/R ratios calculated using $\delta^{18}O_{eq}$ after Grossman and Ku (1986) tended to underestimate P/R ratios in both Pacific and Caribbean coral species, sometimes resulting in negative values, whereas isotope-based P/R ratios calculated using $\delta^{18}O_{eq}$ after Maier (2004) were typically higher than measured P/R ratios (Figs. 6, 7, Tables S7, S9). Further, isotope-based P/R ratios calculated using $\delta^{18}O_{eq}$ of Grossman and Ku (1986) sometimes resulted in negative P/R ratios, but measured P/R ratios were always greater than zero (Figs. 6, 7).

4. DISCUSSION

In the present study, we re-evaluated metabolic and kinetic isotope effects in coral skeletal $\delta^{13}C_s$ of non-bleached and bleached Pacific and Caribbean corals that exhibit different growth morphologies. We show for the first time that although all coral species showed significant kinetic isotope effects, the data correction proposed by Heikoop et al. (2000) did not improve the metabolic signal from these corals. Further, independent of whether the data correction was used or not, isotope-based P/R ratios differed significantly from P/R ratios measured by respirometry.

4.1 Presence of Kinetic Isotope Effects

Both Pacific and Caribbean coral species showed highly significant correlations between $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ (Fig. 3, Tables S4, S5). While this was independent of morphology, correlation coefficients were generally higher in Caribbean compared to Pacific corals. Significant correlations between $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ is consistent with other studies for a wide range of both Pacific and Caribbean corals (Heikoop et al., 2000; Maier et al., 2003; McConnaughey, 1989a; McConnaughey et al., 1997; Omata et al., 2005; Risk et al., 2003; Suzuki et al., 2003; Suzuki et al., 2008). Overall, this indicates that the isotopic signal of the corals studied here showed strong kinetic isotope effects (Heikoop et al., 2000; McConnaughey, 1989a; McConnaughey et al., 1997).

Theoretically, the observed variability in $\delta^{18}O_s$ - which was used to remove kinetic isotope effects from $\delta^{13}C_{sorig}$ (Heikoop et al., 2000) - could have been caused by factors other than kinetic isotope effects, such as variations in seawater temperature, salinity, and seawater $\delta^{18}O$. However, salinity and seawater $\delta^{18}O$ were identical for both bleached and non-bleached controls throughout all studies, and temperature only differed between bleached and non-bleached controls during the 2.5-4 weeks of the bleaching treatment. Thus, temperature could have influenced $\delta^{18}O_s$ at the 0 month recovery interval. However, no environmental effects can account for $\delta^{18}O_s$ kinetic isotopic fractionation during any other recovery interval.

4.2 Evaluation of the $\delta^{13}C_s$ Data Correction

Two methods were used to evaluate the effectiveness of the $\delta^{13}C_s$ correction. First, $\delta^{13}C_{sorig}$ versus $\delta^{13}C_h$ correlations were compared to $\delta^{13}C_{scorr}$ versus $\delta^{13}C_h$ correlations. Second, $\delta^{18}O_s$ versus $\delta^{13}C_{scorr}$ plots of non-bleached and bleached corals were visually compared for each species to determine if measured changes in photosynthesis, respiration, and calcification due to bleaching were reflected in the theoretically expected changes in the isotopic composition of the skeleton.

Using the first method, we were able to show that the data correction proposed by Heikoop et al. (2000) to remove kinetic isotope effects was generally not effective in any of the six coral species studied here (Tables S4, S5). Heikoop et al. (2000) provided evidence for the efficacy of the data correction by correlating both original and corrected $\delta^{13}C_s$ with whole tissue carbon isotopes as they are not affected by kinetic isotope effects related to calcification. They showed that $\delta^{13}C_{scorr}$ versus $\delta^{13}C_{tissue}$ correlations were stronger in both Pacific and Caribbean corals collected over depth and light gradients compared to the same correlations using original $\delta^{13}C_s$. However, our data overwhelmingly showed that for 49 of 53 cases evaluated, $\delta^{13}C_{scorr}$ resulted in no change or weaker correlations with $\delta^{13}C_h$ than with $\delta^{13}C_{orig}$ (Tables S4, S5).

These results were confirmed using a second, qualitative method of assessment, where plots of $\delta^{18}O_s$ vs. $\delta^{13}C_{scorr}$ did not produce results consistent with the measured changes in photosynthesis, respiration, chlorophyll *a* concentrations, and calcification in either Pacific or Caribbean corals (Figs. 4G-L, 5G-L). During the first two months of recovery, bleached corals were expected to have more depleted $\delta^{13}C_s$ and more enriched $\delta^{18}O_s$ values (i.e., to plot in or near the upper left quadrant) compared to non-bleached corals as photosynthesis, chlorophyll *a* concentrations, and calcification rates were significantly lower in bleached corals in most coral species during this time (Levas et al., 2013; Rodrigues and Grottoli, 2006, 2007; Schoepf, 2013). However, this was not reflected in the $\delta^{13}C_{scorr}$ values as expected. For example in Pacific *M. capitata*, chlorophyll *a* concentrations and calcification rates were significantly lower in bleached corals than in non-bleached corals for at least 4 months after the bleaching treatment (Rodrigues and Grottoli, 2006, 2007), yet their $\delta^{13}C_{scorr}$ values were not more depleted and did not show a greater offset from the KIE line than in non-bleached corals (Fig. 4I, J). Similarly, in Caribbean *P. astreoides*, chlorophyll *a* concentrations and calcification rates of bleached corals were significantly lower than in non-bleached corals after 1.5 months of recovery (Schoepf, 2013), but their $\delta^{13}C_{scorr}$ values were more enriched than the non-bleached corals at this time point (Fig. 5I, J) – this is the opposite of what is expected from the model proposed by Heikoop et al. (2000).

After 8-11 months of recovery, photosynthesis, chlorophyll *a* concentrations, and calcification rates were fully recovered in most species (Levas et al., 2013; Rodrigues and

Grottoli, 2006, 2007; Schoepf, 2013). Therefore, bleached corals at this time point were expected to have more enriched $\delta^{13}C_{scorr}$ values and greater offsets from the KIE line (reflecting recovered photosynthesis and calcification) than bleached corals at 0-1.5 months of recovery. However, this was not always the case. For example, in Pacific *P. compressa*, $\delta^{13}C_{scorr}$ values of bleached corals at 8 months of recovery were similar to values at 1.5 and 4 months of recovery (Fig. 4G, H). This occurred despite fluctuations in chlorophyll *a* concentrations with significantly lower, the same, and higher chlorophyll *a* concentrations in bleached compared to non-bleached corals at 1.5 months, 4 months, and 8 months of recovery, respectively (Rodrigues and Grottoli, 2007). Similarly, in Caribbean *O. faveolata*, $\delta^{13}C_{scorr}$ values of bleached corals at 11 months of recovery plotted in the same space as their values at 0 month of recovery (Fig. 5G, H), even though chlorophyll *a* concentrations and calcification rates were significantly compromised at 0 month but not at 11 months of recovery (Schoepf, 2013). Thus, both methods used to test the effectiveness of the data correction proposed by Heikoop et al. (2000) demonstrated convincingly that (1) the data correction does not effectively remove kinetic isotope effects, and (2) it does not improve the metabolic signal in bleached corals. This was surprising given that two of the species (i.e., *Porites lobata* and *Porites astreoides*) were the same as those studied by Heikoop et al. (2000), and a third one was the same genus (i.e., *Orbicella* (formerly *Montastraea*)).

Several factors may have contributed to the observed differences between the Heikoop et al. (2000) and the present study. First, Heikoop et al. (2000) used whole tissue (animal host + algal endosymbiont) $\delta^{13}C$ for the correlations with $\delta^{13}C_{sorig}$ and $\delta^{13}C_{scorr}$, while $\delta^{13}C_h$ was used in the present study because whole tissue $\delta^{13}C$ was not available. Correlations were evaluated for both $\delta^{13}C_h$ and algal endosymbiont $\delta^{13}C$, but the latter correlations were generally even weaker than those with $\delta^{13}C_h$ for both $\delta^{13}C_{sorig}$ and $\delta^{13}C_{scorr}$ (Table S3). Second, the small sample sizes available to calculate correlations by treatment within species at each recovery interval may have biased the observed results. However, when $\delta^{13}C_{scorr}$ vs. $\delta^{13}C_h$ correlations were pooled by species and treatment, similar results were obtained (Tables S4A, S5A). Third, the type of samples selected could also have played a role. Importantly, corals in this study were exposed to elevated temperature resulting in coral bleaching, whereas Heikoop et al. (2000) collected corals

659 across natural light and depth gradients. Specifically, coral bleaching may produce stress-related
660 responses that affect coral isotopic fractionation in unknown ways.
661 While methodological differences between studies may account for some of the observed
662 inconsistencies, the small number of significant correlations between $\delta^{13}C_{sorig}$, $\delta^{13}C_{scorr}$, and
663 $\delta^{13}C_h$, and the poor agreement with physiological measurements strongly suggest that the
664 assumptions underlying the data correction are not as predictable as previously assumed. This
665 finding is strengthened by the fact that two of the species studied here (one Pacific and one
666 Caribbean) as well as one genus are the same as in Heikoop et al. (2000). The key assumption
667 used for the Heikoop et al. (2000) data correction is that variability in $\delta^{18}O_s$ is entirely due to
668 kinetic isotope effects (provided that corals were grown under similar environmental conditions).
669 However, if other factors influence $\delta^{18}O_s$ in addition to KIE, the data correction would not be as
670 effective. For example, it has been shown that linear extension rate can influence $\delta^{18}O_s$ (Felis et
671 al., 2003; Maier et al., 2004). Since it is uncertain whether the primary variable affecting kinetic
672 isotope effects is extension rate, calcification rate, or density (Heikoop et al., 2000), the data
673 correction may or may not account for this discrepancy among different qualitative
674 measurements for coral growth. Further, changes in endosymbiont type may influence $\delta^{18}O_s$
675 (Carilli et al., 2013). Coral bleaching can be accompanied by changes in endosymbiont type (e.g.
676 Jones et al., 2008), and this could have potentially played a role in our experiments. Significant
677 changes in endosymbiont type occurred in all Caribbean coral species (McGinley, 2012), in
678 particular for *O. faveolata* and *P. divaricata*, and may have influenced $\delta^{18}O_s$.
679 Other methods have estimated the relative intensities of metabolic and kinetic isotope
680 effects using a vector approach (Omata et al., 2005; Omata et al., 2008). Although they were able
681 to separate metabolic and kinetic isotope effects, this approach is also based on McConnaughey's
682 isotope fractionation model (McConnaughey, 1989a, 1989b) and is therefore similar to the
683 Heikoop et al. (2000) data correction approach and assumptions. As a consequence, the vector
684 approach does not account for any other potential factors that might influence $\delta^{13}C_s$ and $\delta^{18}O_s$.
685 Several authors have challenged McConnaughey's model of kinetic and metabolic
686 isotope effects and proposed that $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ offsets from isotopic equilibrium are caused
687 by a biologically-induced pH gradient in the extracellular calcifying fluid (ECF) rather than

kinetic isotope effects related to the rate of $CaCO_3$ production (Adkins et al., 2003; Rollion-Bard et al., 2003). Direct measurements of pH at the site of calcification have confirmed the presence of biologically-induced pH gradients in corals (e.g. Al-Horani et al., 2003; Venn et al., 2011), but the isotopic composition of the calcifying fluid is still a matter of debate (e.g. McConnaughey, 2003). If pH-gradients in the ECF indeed affect $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$, any data correction or vector approach trying to isolate metabolic isotope effects would also have to account for pH effects at the site of calcification. Further studies are required to identify the specific drivers of variability in coral skeletal $\delta^{13}C_{sorig}$ and $\delta^{18}O_s$ to more effectively isolate kinetic from metabolic isotope effects.

4.3 Comparison of Measured and Isotope-based P/R Ratios

Measured and isotope-based calculated P/R ratios were generally in poor agreement independent of how oxygen isotopic equilibrium was calculated or whether $\delta^{13}C_s$ was corrected or not. Further, isotope-based P/R ratios calculated using $\delta^{18}O_{eq}$ after Grossman and Ku (1986) tended to underestimate P/R ratios in all coral species, sometimes even resulting in negative values, whereas isotope-based P/R ratios calculated using $\delta^{18}O_{eq}$ after Maier (2004) were typically higher than measured P/R ratios (Figs. 6, 7). This was particularly evident in the Caribbean coral species (Fig. 7, Tables S8, S9), where isotope-based P/R ratios were not significantly correlated with measured P/R ratios in any of the three species.

Estimating carbon and/or oxygen isotopic equilibrium values based on literature values may have confounded the isotope-based P/R ratios. Direct measurements of seawater $\delta^{13}C_{DIC}$ and $\delta^{18}O$ from Puerto Morelos, Mexico during the study period were not performed, and equilibrium values were estimated based on literature values from other locations in the Caribbean. However, $\delta^{13}C_{DIC}$ was measured for Pacific corals in 2006/07, and isotope-based P/R ratios were nevertheless in poor agreement with measured P/R ratios. Further, it is possible that the skeletal material that was sampled for isotopic analysis may have reflected slightly different time periods in some of the coral species since alizarin staining was only used for *P. compressa* and *M. capitata*. However, agreement between isotope-based and measured P/R ratios was not better in

these coral species compared to those that had not been stained. Therefore, it is unlikely that these factors caused the significant differences observed in this study.

Although skeletal $\delta^{13}C_{sorig}$ has often been viewed as an indicator of P/R ratios (e.g. Grottoli and Wellington, 1999; Swart et al., 1996b; Swart et al., 2005), this is the first time that the accuracy of calculated isotope-based P/R ratios was tested by comparing them to measured P/R ratios. While the findings of this study clearly demonstrate that isotope-based P/R ratios are not reliable proxies for measured P/R ratios and are significantly affected by the choice of $\delta^{18}O_{eq}$, they may nevertheless be useful to estimate *relative* changes in P/R over extreme environmental gradients. For example, Lesser et al. (2010) calculated isotope-based P/R ratios for *Montastraea cavernosa* ranging from 3 to 91 m depth. They found that P/R ratios significantly decreased with depth, and that P/R was greater than 1 up to a depth of 61 m. This relative decrease with depth as well as the transition towards heterotrophy below a specific depth (60 m) is certainly realistic. However, given the findings from this study, it is likely that their reported P/R ratios significantly overestimated P/R because they calculated $\delta^{18}O_{eq}$ after Maier (2004). As a consequence, the transition towards heterotrophy in their study likely occurred at a depth shallower than 60 m.

4.4 Implications for Paleo-Climate Reconstruction

Overall, the findings of this study demonstrate that the data correction proposed by Heikoop et al. (2000) did not effectively remove kinetic isotope effects in the Caribbean and Pacific coral species studied here, and that the metabolic effect of the bleaching signal did not improve with the data correction. It is therefore unlikely that the data correction can improve the accuracy of skeletal $\delta^{13}C_{sorig}$ as a paleo-climate proxy or the reconstruction of past bleaching events from coral skeletons, as was suggested by Heikoop et al. (2000). Since both *O. faveolata* and *P. lobata* are mounding coral species commonly used for paleo-climate reconstruction, this is disappointing news. While the data correction may nevertheless be useful in improving correlations of skeletal $\delta^{13}C_{sorig}$ with environmental variables in some species and/or locations (Heikoop et al., 2000), a routine application without evaluation of its effectiveness (Ourbak et

al., 2008) cannot be recommended. Further, isotope-based P/R ratios should be interpreted with great caution, especially when seawater $\delta^{13}C$ and $\delta^{18}O$ are unknown.

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5. FIGURE LEGENDS

Figure 1. Idealized scheme of kinetic and metabolic isotope effects in $\delta^{18}O_s$ vs. $\delta^{13}C_s$ space. KIE marks the line along which kinetic isotope effects occur. Isotopic equil. represents isotopic equilibrium with seawater. Resp. and Photosynthesis indicate the carbon isotopic offset from the KIE line due to respiration and photosynthesis, respectively. Slow Calc. and Fast Calc. refer to slow and fast calcification rates. NB indicates non-bleached, healthy corals, whereas BL indicates bleached corals. Arrows represent hypothetical trends only and do not indicate magnitude of effects or specific $\delta^{18}O_s$ or $\delta^{13}C_s$ values. See text for more detail.

Figure 2. Experimental design of the three bleaching experiments for the Pacific corals (A) *Porites compressa, Montipora capitata*, and (B) *Porites lobata*, and (C) for the Caribbean corals *Orbicella faveolata, Porites astreoides,* and *Porites divaricata*. White rectangles indicate time spent in tanks maintained at ambient or elevated temperatures and the average temperature of each, whereas grey rectangles indicate time spent on the reef at ambient temperatures. Duration of time spent in tanks was approximately 3-4 weeks for the Pacific corals and 2.5 weeks for the Caribbean corals. Months of recovery refer to time points when coral fragments were collected from the reef.

Figure 3. Plot of skeletal oxygen isotopes ($\delta^{18}O_s$) vs. original skeletal carbon isotopes ($\delta^{13}C_{sorig}$) for (A) non-bleached and singly bleached Pacific corals, and (B) non-bleached and repeat bleached Caribbean corals.
KIE (=kinetic isotope effects) marks the line along which kinetic isotope effects occur. Eq. represents isotopic equilibrium composition based on two different methods to calculate $\delta^{18}O_{eq}$ (Grossman and Ku 1986, Maier 2004). Wi and Su represent winter and summer isotopic equilibrium composition, respectively. Resp. and P indicate the carbon isotopic offset from the KIE line due to respiration and photosynthesis, respectively. Slow and Fast refer to calcification rates. NB = non-bleached control, BL = bleached, PC = *Porites compressa*, MC = *Montipora capitata*, PL = *Porites lobata* OF = *Orbicella faveolata*, PA = *Porites astreoides*, PD = *Porites divaricata*. *r* = Spearman's correlation coefficient, NS = not significant ($p>0.05$).

Figure 4. Plots of skeletal $\delta^{18}O$ ($\delta^{18}O_s$) vs. (A-F) original skeletal $\delta^{13}C$ ($\delta^{13}C_{sorig}$) and (G-L) corrected skeletal $\delta^{13}C$ ($\delta^{13}C_{scorr}$) for non-bleached and singly bleached Pacific coral species *Porites compressa, Montipora capitata,* and *Porites lobata* throughout 8-11 months of recovery.

● = 0 month of recovery, ● = 1 or 1.5 months of recovery, ● = 4 or 5 months of recovery, and ● = 8 or 11 months of recovery. NB = non-bleached control, BL = singly bleached. slow and fast refer to calcification rates. – = KIE line leading to $\delta^{18}O_{eq}$ after Grossman and Ku (1986), --- = KIE line leading to $\delta^{18}O_{eq}$ after Maier (2004). Δ = outliers excluded from statistical analyses.

Figure 5. Plots of skeletal $\delta^{18}O$ ($\delta^{18}O_s$) vs. (A-F) original skeletal $\delta^{13}C$ ($\delta^{13}C_{sorig}$) and (G-L) corrected skeletal $\delta^{13}C$ ($\delta^{13}C_{scorr}$) for non-bleached and repeat bleached Caribbean coral species *Orbicella faveolata, Porites astreoides,* and *Porites divaricata* throughout 11 months of recovery.

● = 0 month of recovery, ● = 1.5 months of recovery, and ● = 11 months of recovery. NB = non-bleached control, BL = repeat bleached. – = KIE line leading to $\delta^{18}O_{eq}$ after Grossman and Ku (1986), --- = KIE line leading to $\delta^{18}O_{eq}$ after Maier (2004). P = carbon isotopic offset from KIE line due to photosynthesis, slow and fast refer to calcification rates.

Figure 6. Correlations of measured and isotope-based P/R ratios for non-bleached and bleached Pacific coral species *Porites compressa, Montipora capitata,* and *Porites lobata*.

Isotope-based P/R ratios were computed with both (A-C) $\delta^{13}C_{sorig}$ (original P/R) and (D-F) $\delta^{13}C_{scorr}$ (corrected P/R). Further, they were computed using $\delta^{18}O_{eq}$ values either after Grossman and Ku (1986) (filled symbols) or after Maier (2004) (open symbols). Dotted line indicates 1:1 agreement of isotope-based and measured P/R ratios. Treatments and recovery intervals were pooled for each species. *r* = Spearman's correlation coefficient when correlation was statistically significant. + = outliers excluded from statistical analyses.

Figure 7. Correlations of measured and isotope-based P/R ratios for non-bleached and repeat bleached Caribbean coral species *Orbicella faveolata, Porites astreoides,* and *Porites divaricata* at 0 month of recovery. Isotope-based P/R ratios were computed with both (A-C) $\delta^{13}C_{sorig}$ (original P/R) and (D-F) $\delta^{13}C_{scorr}$ (corrected P/R). Further, they were computed using $\delta^{18}O_{eq}$ values either after Grossman and Ku (1986) (filled symbols) or after Maier (2004) (open symbols). Dotted line indicates 1:1 agreement of isotope-based and measured P/R ratios. Treatments were pooled for each species. None of the correlations were statistically significant.

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