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Reply

Reply to Kahng, S.E. Comment on "Lesser et al. Using Stable Isotope Analyses to Assess the Trophic Ecology of Scleractinian Corals. *Oceans* 2022, *3*, 527–546"

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Kahng [1] provided a comment on our recent paper [2] discussing the use of stable isotopic analyses (SIA) in the context of disentangling the roles of autotrophy versus heterotrophy in scleractinian corals from shallow to mesophotic depths. The comment provides no new insights, analyses, or understanding of the trophic ecology of corals over these depth gradients. Instead, Kahng [1] just restates many of the same objections, and some new ones, to our original work [3–5], and rejects the "multiple lines of evidence" approach proposed [2] to provide a "preponderance of available evidence" standard that would support, or refute, a transition to heterotrophy in scleractinian corals as depth increases into the mesophotic zone.

Accessibility to mesophotic coral reef ecosystems (MCEs, 30–150 m) is significantly more time consuming, costly, and technologically challenging compared to shallow coral reefs, limiting the available data for MCEs [6,7]. Given this limitation, the following important characteristics, and the premise for the original discussion on SIA [2], related to the trophic ecology of corals from shallow (<30 m) to mesophotic depths, is provided below. The attenuation of solar radiation, whether measured directly or modeled [7,8], with increasing depth results in a concomitant decrease in photosynthesis, the principal autotrophic input of carbon for scleractinian corals from shallow to mesophotic depths [9–11]. For many MCEs the decrease in irradiance between the 10% and 1% optical depths (i.e., midpoint to bottom of euphotic zone) coincides with a faunal break at ~60 m where a conspicuous transition from coral to suspension-feeder dominated communities occurs into the lower mesophotic (60–150 m) zone [12–14]. This transition in MCE communities is also accompanied by changes in trophic resources with depth. The availability of dissolved organic matter (DOM) generally declines with increasing depth, although it is still available in high concentrations (e.g., ~50 µmol C L^{-1} and ~3 µmol N L^{-1} at ~91 m [14]), while particulate organic matter (POM), primarily picoplankton, increases [14,15]. Zooplankton concentrations, however, vary widely with depth and are generally lower than on shallow coral reefs [16–18]. Corals can utilize DOM and live POM (i.e., picoplankton, nanoplankton, and meso-macroplankton) as heterotrophic carbon sources, in addition to photo-autotrophically derived carbon from their symbiotic algae (Symbiodiniacea) [19].

The acquisition of organic nitrogen, however, from these resources is more important stoichiometrically for their growth [19]. As previously described [2] the lower concentrations of zooplankton at mesophotic depths does not necessarily translate into lower feeding rates at mesophotic depths for corals. For example, mesophotic *Stylophora pistillata*, when experimentally provided the same amount of food as shallow-water conspecifics, feed at significantly higher rates [20]. At the community level, there may be more than enough heterotrophic sources of carbon and nitrogen overall to supply the requirements of

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the significantly lower population densities of mesophotic corals, e.g., [10]. Resolving the question of whether a coral is primarily autotrophic or heterotrophic has fallen largely on the interpretation of SIA data in the context of its environmental setting, and the physiology of the partners in the coral symbiosis. Below, we reply to a commentary by Kahng [1] on our recent contribution [2].

Examining SIA values in corals reveals a large degree of variability in the isotopic values of carbon and nitrogen in the hosts and symbionts of different species of corals from different locations under varying environmental conditions, with most corals being classified as mixotrophic [19,21,22]. Of the reasons stated by Kahng [1] as to why the interpretation of the SIA data in our previous work [2] is confounded, the work on host carbon retention by Tremblay et al. [23,24] is referenced. These studies represent isotopic, tracer-based studies on corals under different irradiances, feeding regimes, and bleaching conditions [23,24] under which the retention and internal carbon recycling between symbiotic partners is presented as an explanation for the increasingly negative $\delta^{13}C$ values of host tissues and symbionts of corals with increasing depth in *Stylophora pistillata* [25,26]. In practice, the experimental paper by Tremblay et al. [23] does not simulate the underwater light environment at ~30–40 m depth [10], nor at 60 m (~40–50 µmol quanta m^{-2} s^{-1}) or deeper depths [10]. Specifically, Tremblay et al. [24] is principally a study that examines the importance of heterotrophy during and after coral bleaching. Neither of these studies provides sufficient insights to offer a cogent counter argument to our interpretations of the SIA data on corals from shallow to mesophotic depths [2].

On mesophotic reefs, Alamaru et al. [25] observed that the changes in $\delta^{13}C$ of host tissues and symbionts of *S. pistillata* do suggest a close coupling and recycling of carbon between the two compartments. However, the values for both host and symbiont also decrease significantly with depth indicating that the original source of carbon, even when considering that carbon recycling is occurring, changes from an autotrophic to a heterotrophic source, and that reverse translocation from the host to symbiont may be occurring at mesophotic depths [25]. Additionally, the $\delta^{13}C$ of host and symbiont lipids both became significantly more negative with depth, with host tissues increasingly more negative than the symbionts [25] (Figure 3A), indicating a change to a heterotrophic source of carbon for lipid synthesis in the host. For the same species of coral, at the same location, Einbinder et al. [26] concluded that the change in $\delta^{13}C$ values for host and symbiont remain constant with depth and there is no indication of increasing heterotrophy with depth. Looking at the graph in Einbinder et al. [26] (Figure 2), however, reveals that both the host and symbiont $\delta^{13}C$ values decrease with depth, and this is confirmed with regression analyses of the data [2], an analysis that shows a significant depth effect on the $\delta^{13}C$ of host and symbiont compartments, with the rate of change in host tissues becoming increasingly more negative than symbionts beginning at 30 m. As the host uncouples its carbon requirements from the symbionts, the values become increasingly consistent with the $\delta^{13}C$ of zooplankton and POM. The interpretation of increasing heterotrophy with depth for *S. pistillata* is also supported with a SIBER (Stable Isotope Bayesian Ellipses in R) analysis (see below) of the isotope data, since the isotopic niche width overlap of host and symbionts decreased significantly with increasing depth [2].

Kahng [1] also references two studies to support the view that the dissolved inorganic carbon (DIC) pool of seawater, both pCO_2 and HCO_3, can affect $\delta^{13}C$ values, but no explanation as to how that might occur is provided in the text. The readers must presume that this is a result of the depletion of metabolic CO_2 at high rates of photosynthesis (i.e., high irradiances), where the symbionts must then draw on CO_2 from the internal HCO_3^- pool, and when that is depleted, HCO_3^- from the ambient seawater must be drawn upon as described by Muscatine et al. [27], for which the delivery is then facilitated by a CO_2 concentrating mechanism made possible by the activities of carbonic anhydrase [28]. Carbon isotopic fractionation is then reduced as photosynthetic rate increases with changes in the ^{13}C values of the internal DIC pool affecting the ^{13}C values of both the host tissues and skeleton, where $\delta^{13}C$ values would be enriched (i.e., less negative) [29]. The other

referenced study [30] is on sea anemones which do not calcify, depend to a greater degree on heterotrophy than corals, and show more negative ^{13}C values of the host and symbiont as pCO_2 increases [30]. How the isotopic signature of the DIC pool might be relevant to mesophotic corals specifically is addressed below.

Kahng [1] also re-states that nutrient concentrations can confound the interpretation of $\delta^{13}C$ values without responding to our previous, and detailed, discussion of this possibility. Because experiments that reportedly demonstrated confounding effects of nutrient concentrations on $\delta^{13}C$ values [31] were conducted at a constant irradiance of ~200 µmol quanta m^{-2} s^{-1} and incorporated unbalanced treatments with N:P ratios of 101 and 63 for two species of coral, we rejected these data as providing any indication of nutrient effects on the $\delta^{13}C$ values of corals at mesophotic depths without additional evidence [2]. We even provided nutrient data [2] (Figure 1) from the site where our studies on *Montastraea cavernosa* were conducted [10] which showed that the confounding effects of nutrients were not possible. No counter argument to these data were presented in Kahng [1]. Kahng [1] also argues that other confounding factors affect the interpretation of $\delta^{13}C$ data; below, we address these arguments in the order presented.

Light dependent fractionation rates

Kahng [1] suggests that interpreting declining $\delta^{13}C$ coral host values in the context of increasing heterotrophy is confounded by the co-occurring fractionation of $\delta^{13}C$ by light. To support this, experimental data from the tests of symbiotic foraminifera are referenced [32], which precipitate calcite, not aragonite, and do show a "vital effect" on both carbon and oxygen isotopes as irradiance changes. In contrast, work by Spero and Deniro [33] on another symbiotic foraminiferan showed that irradiance has no effect on $\delta^{18}O$ values, but did cause changes in $\delta^{13}C$ that were related to changes in photosynthesis. However, the original data by Spero and Deniro [33] were not evaluated using a statistical analysis, and no new analyses of the data are presented by Kahng [1]. For corals that precipitate aragonite, the work of Nahon et al. [34] is also referenced but that study does not include any irradiance data. Instead, their $\delta^{13}C$ values of host minus symbiont ($\delta^{13}C_{h\text{-}s}$) do indicate a high reliance on autotrophy, consistent with their location in a shallow lagoon and where even turbid environments might allow sufficient light levels to saturate photosynthesis. These data suggest that high rates of net productivity by the symbionts and translocation of photosynthate to the host are occurring. It is not surprising, therefore, that there was little evidence for an increase in heterotrophy for most corals in these shallow lagoon environments. Swart [35], in work based on corals precipitating argonite, specifically states that at the time there was no evidence to suggest light-dependent fractionation of $\delta^{13}C$, and Swart [36], using a single coral species, quantified changes in the isotopic composition of respired CO_2, demonstrating a seasonal cycle based on changing irradiances as evidence for light-dependent fractionation for corals in shallow water (i.e., 8 m). These data were used to calculate a carbon isotopic fractionation value assuming that respiration in the day was the same as in the night, which is known to be incorrect [37]. Also, assuming a value of one for the photosynthetic (PQ) and respiratory (RQ) quotients of corals exposed to seasonally varying irradiances is questionable for RQ and incorrect for PQ [38,39].

Despite these concerns, Swart et al. [36] clearly state, in a discussion of autotrophy versus heterotrophy for their experimental corals, that "In this regard, a further explanation for the changes in the $\delta^{13}C$ of the respiratory CO_2 and the organic tissues may be that during periods of more negative $\delta^{13}C$, the corals derive more of their food from heterotrophy." As previously reported, at least for the data on *Montastraea cavernosa*, there is no significant relationship between the $\delta^{13}C$ and $\delta^{18}O$ values of the coral skeleton [2], and therefore no simultaneous depletions of $\delta^{13}C$ and $\delta^{18}O$, or evidence of strong kinetic effects [40]. This indicates that metabolic effects on isotopic fractionation dominate as depth increases in *M. cavernosa*.

Supporting this interpretation, a study by Omata et al. [41] examined the patterns of carbon isotopic fractionation based on irradiance and, in the absence of access to heterotrophic resources, showed that the metabolic isotopic fractionation of carbon resembles

a photosynthesis–irradiance curve producing more positive $\delta^{13}C_s$ values as irradiance increases, while $\delta^{13}C$ fractionation through kinetic effects causes more negative values as growth rates increase [40,41]. Given that rates of calcification and growth in most corals decline with decreasing irradiance [9,42,43], the $\delta^{13}C$ values of host tissues, symbionts, and skeleton in mesophotic corals would appear to be primarily affected by metabolic effects, including increasing dependence on heterotrophy for carbon with increasing depth. We recommend, again, that skeletal $\delta^{13}C$ and $\delta^{18}O$ values are essential, in combination with host and symbiont isotopic data, to unravel the question of transitions from autotrophy to heterotrophy in corals [2]. For *Montastraea cavernosa*, additional support includes a clear decrease in the contribution of autotrophic carbon from the symbionts with depth, as indicated by the crossover depth between the host and symbiont $\delta^{13}C$ regression lines and the increasing differences between $\delta^{13}C$ values of the host and symbionts (i.e., $\delta^{13}C_{h\text{-}s}$) with depth [2] (Figure 2B,D).

Kahng [1] presents the isotopic data from Crandall et al. [44] as additional evidence against the transition to heterotrophy in corals with increasing depth. As previously described [2], the $\delta^{13}C$ values for sterols followed the exact same trend with depth as the bulk $\delta^{13}C$ values for coral tissues (i.e., increasingly negative with depth) and indicated that both *M. cavernosa* and *Agaricia* spp. are mixotrophic [44]. The $\delta^{13}C$ values for cholesterol, a biomarker of heterotrophy, showed more negative values in both *M. cavernosa* and *Agaricia* spp. from mesophotic depths compared to shallow depths [44] (Figure 2). This indicates that, integrated over time, these corals acquired more carbon from heterotrophic sources at mesophotic depths than their shallow-water conspecifics as was also indicated by the changes in the bulk tissue $\delta^{13}C$ values. The actual differences in the mass of cholesterol, or its statistical analysis, were not reported between depths with only an $n = 2$ for shallow and $n = 3$ for mesophotic depths, and there was no statistical analysis reported, either in the original study or in Kahng [1] for the $\delta^{13}C$ values of cholesterol for either coral species as a function of depth. Not having samples of *M. cavernosa* from depths deeper than 60 m (i.e., the lower mesophotic zone; [14]) also limited the ability to detect a transition from autotrophy to heterotrophy relative to the lower mesophotic zone [44], demonstrating that sampling throughout the range of occurrence for any coral is essential to fully understand changes in their trophic status, if any, with depth.

$\delta^{13}C_{h\text{-}s}$ As a metric for autotrophy and heterotrophy

This commonly used metric provides one indication for an increase in heterotrophic contributions to corals as indicated by a decrease in the $\delta^{13}C_{h\text{-}s}$ ratio with depth [2]. Its general utility for corals under a range of environmental conditions has recently been supported [45,46], including changes in irradiance with increasing depth, e.g., [2] (Figure 2B,D). Despite support for the metric, Kahng [1] suggests that the variability in the metric is too high to unravel autotrophic versus heterotrophic contributions to corals generally, and, one assumes, for corals along the shallow-to-mesophotic depth gradient. Kahng [1] also suggests several factors that may contribute to this variability, and references Alamaru et al. [25] where SIA data for *Stylophora pistillata* are presented as evidence for the lack of any transition to heterotrophy with increasing depth. Both host and symbiont $\delta^{13}C$ values in that study [25] become more negative with depth, and the $\delta^{13}C_{h\text{-}s}$ value is also significantly affected by depth, but the uncoupling between host and symbiont observed in other studies [2] is weak, suggesting autotrophy is important at least down to the maximum depth of the study (i.e., 60 m). However, the differences in the $\delta^{13}C$ of host tissues and algal lipids increased significantly with depth indicating a change to a heterotrophic source of carbon for lipid synthesis [25]. Also, as discussed above, Crandall et al. [44] only measured $\delta^{13}C$ in the bulk tissues so it was not possible to calculate $\delta^{13}C_{h\text{-}s}$. In addition, the corals from Nahon et al. [34] are in different lagoons, presumably under different irradiances, but no irradiance data are reported, and the corals might still have been saturated relative to photosynthesis, with the contribution of autotrophy being more important in these habitats as indicated by their $\delta^{13}C_{h\text{-}s}$ values. Hoogenboom et al. [47] was a laboratory study examining the role of feeding on energy allocation in several species of coral, where $\delta^{13}C_{h\text{-}s}$

values were compared to a metric examining the contribution of heterotrophic feeding to animal respiration (CHAR) on corals exposed to a fixed irradiance. Feeding was found to increase the density of symbionts in experimental corals with significant differences between host and symbiont found in only one of five species for $\delta^{15}N$, and for fed versus unfed in one of five, and host versus symbiont in three of five coral species for $\delta^{13}C$. These results have little relevance for understanding the application of stable isotopes on corals along a shallow-to-mesophotic depth gradient and for understanding how $\delta^{13}C_{h\text{-}s}$, as it is related changes in irradiance, can be used to better understand the trophic biology of these corals.

Lastly, symbiont genotypes as a function of depth are invoked as a confounding factor for utilizing $\delta^{13}C_{h\text{-}s}$ values on corals. Wall et al. [48], a study undertaken in a tropical estuary at a depth of 6 m, is referenced for support. This study compares Symbiodiniaceae from the genera *Cladocopium* spp. and *Durusdinium glynnii*, known to be generalist symbionts and a stress-tolerant symbiont, respectively, in a system that has experienced several coral bleaching events. The study of the physiological differences between these symbionts, given the dynamic environmental conditions they experience with their host, *Montipora capitata*, is inherently interesting and important. Understanding the changes in the contribution of autotrophy and heterotrophy to carbon, nitrogen, and energy budgets of these corals as it relates to the symbiont genotype is also important. But, the results of this study, and others like it [49], cannot be extrapolated to corals in the mesophotic zone. There are just too many habitat-related differences between MCEs and tropical estuaries. Nonetheless, the utility of $\delta^{13}C_{h\text{-}s}$ values on corals within each habitat is completely valid [45,46]. While Price et al. [46] reported divergent results between techniques, in particular between $\delta^{13}C_{h\text{-}s}$ and $\delta^{15}N_{h\text{-}s}$, results that may have been influenced by health status, locality, and species differences were not significantly different in their estimates of the contribution of autotrophy versus heterotrophy. Given previous discussions on the problem of interpreting $\delta^{15}N$ in corals because of internal nitrogen cycling [2,50], this is not surprising. The take home message here is that we have never conducted among-species comparisons, and do not advocate doing so because of the variability in multiple phenotypic traits between coral species [51]. The variability in baseline values in $\delta^{13}C$ and $\delta^{15}N$ for corals under similar nutrient, light, and trophic resources clearly illustrate this point [21].

Proximate biomass composition

Kahng [1] references a study by Wall et al. [52] that studied the changes in the $\delta^{13}C$ values of bulk tissue, symbiont, and host compartments as affected by co-occurring changes in proximate biochemical composition (PBC: carbohydrate, protein, and lipid) pre and post coral bleaching on shallow (<3 m) corals. Changes in PBC, despite no change in the mass of individual compounds between bleached and non-bleached colonies, affected the $\delta^{13}C$ values of corals during recovery from bleaching in largely unknown ways. Kahng [1] offers the readers no mechanism(s) by which we should apply the results on bleached and recovering corals to understand the transitions from autotrophy to heterotrophy for corals on MCEs.

Stable Isotope Bayesian Ellipses in R (SIBER)—Isotopic niche width evidence

While Kahng [1] is correct in stating that the isotopic niche and trophic niche are not the same, the isotopic niche width provides important ecological information about the individuals, populations, and communities they represent [53]. For multicompartmental systems like corals, Conti-Jerpe et al. [22] used SIBER, and the overlap between host and symbiont standard ellipse area corrected for sample size (SEA_c), as a proxy for trophic niche. This overlap, or the lack of overlap, indicates the degree of linkage between the carbon or nitrogen content of the consumed organic material utilized by these compartments, with the $\delta^{15}N$ values of host and symbionts providing the most discriminatory power [22]. Where there is no overlap, this is interpreted as an uncoupling between host and symbiont compartments, with host heterotrophy driving those differences [22]. If the SIA data for corals contain errors from confounding factors, then one would expect those errors to

propagate into other analyses such as SIBER. Kahng [1] suggest this is the case for the data from *Montasraea cavernosa*. However, we have already provided evidence that metabolic effects predominate over kinetic effects for *M. cavernosa*, that nutrient concentrations could not have affected the SIA values, and that multiple analyses of the SIA data support a transition to heterotrophy with increasing depth [2]. Additionally, despite the lack of significance in the $\delta^{15}N_{h\text{-}s}$ values with depth for *M. cavernosa* [2], the values increased from 3 m ($\delta^{15}N_{h\text{-}s}$ 0.65 ‰ ± 0.57 [SE]) to 61 m ($\delta^{15}N_{h\text{-}s}$ 1.49 ‰ ± 0.21 [SE]). And even with the absence of $\delta^{15}N$ data for 76 and 91 m [10], SIBER modeling provided another supportive piece of data on the interpretation of the patterns in the $\delta^{15}N$ SIA data. With regard to the $\delta^{13}C$ values, expected trophic enrichment is ~1‰ between trophic levels, which is entirely consistent with the results of the SIBER analysis where it is clear that increasingly negative $\delta^{13}C$ values of the host occur from shallow (3, 10, 15, and 23 m) depths ($\delta^{13}C$ −14.16‰ ± 1.64 [SE]), to upper (30 and 46 m) mesophotic ($\delta^{13}C$ −15.03‰ ± 0.84 [SE]) depths, and to lower (61 and 91 m) mesophotic ($\delta^{13}C$ −17.76‰ ± 1.74 [SE]) depths [2] (Figure 3) as photo-autotrophic carbon inputs significantly decrease [2,10], and corals consume more organic carbon from heterotrophic sources. So, here, changes in both $\delta^{13}C$ and $\delta^{15}N$ values with depth contribute to the SIBER modeling outputs [2]. For *Stylophora pistillata*, the SIBER analysis indicates that there is no overlap in the isotopic niche width between host and symbionts for either shallow or mesophotic populations. The SIBER analysis for *S. pistillata* combines the SIA data from 5, 10, 15, and 20 m for the shallow category, while 30, 50, and 65 m depths are combined for the mesophotic category. To better appreciate the SIBER analysis, one should simultaneously examine the regressions for depth-dependent changes in the $\delta^{13}C$ values of the host and symbionts, as well as the $\delta^{13}C_{h\text{-}s}$ values, where the uncoupling between host and symbiont $\delta^{13}C$ values occurs at ~15 m, indicating a significant decrease in carbon translocation between symbiont and host at this unexpectedly shallow depth [2]. From that point, both host and symbiont $\delta^{13}C$ values continue to diverge as the host consumes more organic material from heterotrophic sources. Again, the multiple layers of data, including the SIBER analysis, are complimentary and demonstrate transitions from autotrophy to heterotrophy with increasing depth [2].

δ18Oskel-Corrected δ13Cskel as a proxy for photosynthesis:respiration (P:R) ratios

Kahng [1] suggests that alternate models have "replaced" Heikoop et al. [29] that better explain offsets from isotopic equilibrium for skeletal isotopic values (e.g., pH of extracellular calcifying fluid), but those remain largely untested for corals under a variety of environmental conditions [49]. Additionally, those studies were for paleoclimate reconstructions and either use azooxanthellate coral, or use a single coral sample from ~2.5 m that shows relatively constant $\delta^{13}C_{skel}$ values across the sampled skeleton under environmental conditions that are not described. Neither study addresses the use of $\delta^{13}C_{skel}$ to calculate P:R ratios. Kahng [1] also lists a series of conditions that are required for an isotopic correction technique to be used on the skeletons of corals for the purposes of estimating P:R ratios. The isotopic correction of $\delta^{13}C_{skel}$ for kinetic effects was developed to differentiate metabolic from kinetic effects present in the $\delta^{13}C_{skel}$ [29]. Coral samples from Jamaica were used for the development of the correction and collected from 1, 5, 12, 22, and 30 m, a reasonable range to capture significant differences in irradiance with depth that would vary daily, seasonally, and annually at each depth [6–8] to see the effects on $\delta^{13}C_{skel}$ in the absence of kinetic effects. The $\delta^{18}O_{skel}$ data are used to calculate transformed $\delta^{13}C_{skel}$ values because it is assumed that only kinetic effects (i.e., calcification) influence $\delta^{18}O_{skel}$. The transformed $\delta^{13}C_{skel}$ values are not on the PDB isotopic scale and are therefore specific for each study [29]. Heikoop et al. [29], using the transformed $\delta^{13}C_{skel}$ values, were able to improve their regression analyses between $\delta^{13}C_{skel}$ values and irradiance producing more robust patterns consistent with changes in irradiance and models of metabolic carbon fractionation in corals. The reported values for transformed $\delta^{13}C_{skel}$ were not due to changes in seawater temperature as the water column was well mixed and isothermal in the depth range of coral collection, as it is for *Montastraea cavernosa* [10] (see Figure 1B and the absence of a pycnocline from surface waters to 95 m depth). The lack

of any pycnocline, and therefore any barriers to a well-mixed water column, would also eliminate significant variability in $\delta^{13}C$ values of DIC or $\delta^{18}O$ for the ambient seawater, at least for this reef at the time of sampling. As a result, the conditions outlined in Kahng [1] have been met for this dataset [10]. For all the species examined in the development of the isotopic correction, a strong correlation between $\delta^{18}O_{skel}$ and $\delta^{13}C_{skel}$ was observed indicating the presence of strong kinetic effects [29]. No such relationship was detected for *M. cavernosa* from shallow to mesophotic depths [2,10], but this does not mean that kinetic effects were not occurring, only that metabolic effects predominated over kinetic effects in *M. cavernosa*. Using the calcification-related kinetic effects in the $\delta^{18}O_{skel}$ data to transform the skeletal $\delta^{13}C_{skel}$ of *M. cavernosa* reveals the metabolic effects, and can also provide estimates of P:R along the depth gradient [2,10]. It has also been reported that rates of growth, or skeletal extension, also strongly influence kinetic effects such that faster growing corals should exhibit lower, more negative $\delta^{18}O_{skel}$ values [40,54], but that is not evident for *M. cavernosa* [2], where slower growing colonies at mesophotic depths would have minimal kinetic effects on $\delta^{18}O_{skel}$ values [2]. Regarding the effects that symbiont type might have on $\delta^{18}O_{skel}$ values; neither of the references provided by Kahng [1] have any information on symbiont genotypes, or their possible effects on skeletal isotopes. It is not unreasonable, however, to hypothesize that the novel symbiont genotypes identified in several mesophotic corals [10,55], in addition to population level genetic differences and cryptic speciation in the hosts [56,57], might all affect the patterns of kinetic and metabolic fractionation in the host, symbiont, and skeleton in currently unknown ways.

The reported inverse relationship between $\delta^{18}O_{skel}$ values and P:R ratios for *Montastraea annularis*, now *Orbicella annularis*, [58] is based on corals (n = 12) maintained at 5.5 m under seasonal changes in irradiance that do not reflect the steep changes in irradiance with depth that occur along the shallow to mesophotic depth gradient [6,7]. Corals for isotopic analysis and physiological measurements (n = 4) were collected at different timeframes during the experiment. Daily P:R ratios were calculated based on short-term (~3–4 h) incubations of photosynthesis and respiration, with its inherent problems [39], and were not only averaged across the diel cycle, but respiration was assumed to be the same in the light as in the dark. The $\delta^{13}C_{skel}$ values were also interpolated to monthly values, for comparison with P:R ratios which introduces additional error. The mechanism that causes the inverse relationship between $\delta^{18}O_{skel}$ values and P:R ratios cannot be determined definitively [58], but possibilities include the seasonal changes in the $\delta^{13}C$ of DIC, changes in the partitioning of carbon between host and symbiont, kinetic fractionation due to changes in rates of growth, and changes in the dependence between autotrophy and heterotrophy [58]. The data for Swart et al. [36] are equally problematic as discussed above, in addition to also being conducted in shallow water (i.e., 8 m), on 18 ramets from an unreported number of genets, where only an n = 2 corals were processed for SIA of the skeleton. The statement that there is no relationship between P:R ratios and $\delta^{13}C_{skel}$ is not justified without any statistical analysis. P:R ratios change directly with changes in irradiance [36] (Figure 3) despite the reported lag period. Changes in $\delta^{13}C_{skel}$ also respond directly to changes in irradiance and P:R ratios from April to November and again from December to March [36] (Figure 8). The main problem is that there is a sampling mismatch between irradiance and $\delta^{13}C_{skel}$, with irradiance being significantly under sampled (i.e., monthly) to make any quantitative statements. The data would be amenable to a segmented regression approach, especially if more irradiance data are added. More importantly, these data do not provide any insight into the physiology and isotopic biogeochemistry of mesophotic corals. At best, they may lay the framework for asking important ecological and physiological questions with well-replicated, quantitative measurements, within an ecological framework, related to mesophotic corals. We did not "dismiss" results as suggested by Kahng [1]; instead, we critically evaluated the data as presented, and in the context of their utility, to understand the trophic biology of corals from shallow to mesophotic depths.

Similarly, the work of Shoepf et al. [49] should be evaluated in its own right and whether it is applicable to mesophotic corals. Given our concern that coral bleaching is

an extreme physiological insult [59], also recognized by the authors [49], these results do not provide additional insights into the trophic ecology of corals at mesophotic depths. Kahng [1] states that the validity of the Schoepf et al. [49] study, for understanding how these results apply to mesophotic corals, lies in the results from the control corals. First, the experiments were conducted under an irradiance regime simulating 2 m depth, or ~175–223 µmol quanta m^{-2} s^{-1} [60], in what is a tropical estuary for these Pacific corals and where these irradiances would be equivalent to ~30 m in the Bahamas [10]. In the same study [49], experiments on corals from the Caribbean are conducted on collections from 3 to 8 m and do not report any irradiance data. In other experiments for this system, midday irradiances at 4–5 m were reported to be ~800–1000 µmol quanta m^{-2} s^{-1} [61] in the Bahamas [10]. These data represent shallow water conditions and are not informative regarding mesophotic corals which often see irradiances between 10% and 1% of surface irradiances. The calculation of P:R ratios are also based on oxygen flux measurements of <1 h with its potential problems as previously discussed [39]. The results of the study showed very little agreement between directly measured and isotope-based P:R ratios notwithstanding the experimental issues discussed above, but of note is the complete absence of any analysis on the correlation between directly measured and isotope-based P:R ratios that separated treatment effects (i.e., bleached versus non-bleached corals), because treatments and/or recovery intervals for bleached and non-bleached colonies were pooled for each species comparison [49] (Tables S6–S9). Any analysis suggesting that the "data for corals are self-evident" as advocated by Kahng [1] is non-existent. What is clear from this study is that $\delta^{18}O_{skel}$-corrected $\delta^{13}C_{skel}$, as a proxy for P:R ratios, is apparently not applicable for corals in bleaching studies. Given these points, even Schoepf et al. [49] state that; "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". In fact, Lesser et al. [10] discuss that the transition to increased dependence on heterotrophy may have occurred as shallow as 23 m and likely by 45 m.

Stylophora pistillata

Muscatine et al. [27] recognized that corals can shift their trophic reliance from being primarily autotrophic to a greater dependence on heterotrophy for their carbon requirements, as light decreases with increasing depth, by exploiting a mixotrophic strategy to suit their species-dependent phenotypes. This paper showed that the increasingly depleted $\delta^{13}C$ signature of the host tissue of corals, and its divergence from the $\delta^{13}C$ of their symbionts, was evidence of increasing heterotrophy in the presence of decreasing translocation of photosynthate. These heterotrophic sources included zooplankton ($\delta^{13}C$ values of −18.0 to −19.8‰; [62]) and POM ($\delta^{13}C$ values of −17.8 to −27.7‰; [63]). The seminal works on *Stylophora pistillata* referenced by Kahng [1] are important papers. The assertion by Kahng [1] that we "misinterpreted" or "contradicted" the conclusions of these papers is incorrect. We provided both a re-analysis and different interpretations of the data consistent with our increased understanding of the trophic ecology and isotopic geochemistry of corals and our responsibility for the application of the scientific method as an iterative process. Kahng [1] states that the $\delta^{13}C_h$ values for *S. pistillata* in Einbinder et al. [26] reaches −22.7‰ at mesophotic depths, which exceeds the value of zooplankton and POM at 65 m referenced as –19.6 to 20.9‰ from their plankton collections. But Kahng [1] fails to recognize, as did the authors [25], that collecting "plankton" with a 100 µm net eliminates

many categories of heterotrophic food resources, including a broad range of zooplankton and POM (i.e., picoplankton, nanoplankton, and meso-macroplankton), that mixotrophic corals can consume [19]. These sources have $\delta^{13}C$ values [63] well within the range of $\delta^{13}C_h$ values observed for *S. pistillata* at mesophotic depths [26]. As an additional point, Alamaru et al. [25] used a POM value of −21.0‰, and no $\delta^{13}C_h$ value exceeds this cutoff for mesophotic *S. pistillata* from the same location [25] (Figure 2A).

Regarding the compound-specific isotopic analysis of amino acids (CSIA-AA), Kahng [1] makes several assertions based on the original design and analysis of the Martinez et al. [64] study. Lesser et al. [2] recognized the shortcomings of the experimental design, a reciprocal transplant that lost a treatment group and was thus compromised by its non-orthogonal, unbalanced nature. As a result, only the $\delta^{13}C_h$ data of the five essential amino acids (i.e., valine, leucine, isoleucine, methionine, and phenylalanine) for corals from 5 and 60 m, and not the remaining transplant treatment, were re-analyzed. The $\delta^{13}C$ of essential versus non-essential amino acids can be used effectively to unravel trophic sources and sinks without the confounding influence of trophic fractionation. The isotopic values for different amino acid carbon source end members in CSIA-AA are therefore faithfully maintained and provide a source "fingerprint". The CSIA-AA studies on *S. pistillata* [64,65] analyze the $\delta^{13}C$ of essential amino acids, with one study creating three treatment groups, autotrophy, mixotrophy, and heterotrophy [65]. This study shows that the CSIA-AA of these essential amino acids revealed no significant differences between the $\delta^{13}C_h$ and $\delta^{13}C_s$ for any amino acid, while significant treatment effects were reported for methionine and isoleucine that were significantly more negative and interpreted as indicating an increase in heterotrophy [65] (Table 2). Additionally, a PERMANOVA and an nMDS plot of all essential amino acids from the autotrophy and heterotrophy treatment groups showed a significant difference between these treatment groups [65] (Figure 5), and for the analysis associated with that figure, the authors state that "It showed a clear separation between the two treatments (PERMANOVA $p = 0.002$) but not between compartments (PERMANOVA $p = 0.85$) pointing to different carbon sources between treatments with similar carbon sources within the compartments." This shows the utility of the $\delta^{13}C$ of essential amino acids to identify autotrophic versus heterotrophic conditions under this experimental design. Given these results, the re-analyzed data from Martinez et al. [64] shows that, of the essential amino acids studied, mesophotic corals had significantly lower $\delta^{13}C$ values for valine and isoleucine compared to shallow corals, while phenylalanine did not show a significant effect of depth. These results also indicate an increasing dependence on heterotrophic resources with increasing depth [2].

Kahng [1] suggests that the $\delta^{13}C$ values of the essential amino acids in corals are independent of their trophic ecology based on the experimental data from Ferrier-Pagès et al. [65]. Despite the experimental support demonstrating heterotrophy using CSIA-AA discussed above, the experimental design of Ferrier-Pagès et al. [65] is also compromised. First, it is pseudo-replicated by taking multiple ramets ($n = 18$) from fewer ($n = 6$) genets and not keeping track of ramets and genets as a treatment factor. Second, there are two main factors manipulated, light and food. The three treatments heterotrophy, autotrophy, and mixotrophy should have been replicated in both the dark and at 200 µmol quanta m^{-2} s^{-1}, the fixed irradiance of the experiment, resulting in a 2 × 3 matrix for six treatment groups. This would have created autotrophic and mixotrophic treatment groups that were the same except for the time frames for the heterotrophic (6 weeks) and the mixotrophic and autotrophic (12 weeks) treatments which introduces time as another factor and further confounds the experiment. And there was no consideration for interactive effects in most analyses (e.g., Table 2). In spite of these significant experimental design problems, the $\delta^{13}C$ value of phenylalanine is invariant in the laboratory study of Ferrier-Pagès et al. [65] as we observed in our re-analysis of the Martinez et al. [64] data. The original analysis from the unbalanced design in Martinez et al. [64], however, does show a significant effect of depth for phenylalanine, suggesting an increase in heterotrophy for mesophotic corals. These data do not provide compelling evidence for using phenylalanine alone as a marker of whether

corals are transitioning to heterotrophy, and clearly show the value of better experimental designs. The real value of phenylalanine as a source amino acid, however, is in its $\delta^{15}N$ values and the calculation of trophic position (TP). Both in Martinez et al. [64] and our reanalysis there is no significant difference between the TP of the shallow and mesophotic corals, the values for which fall between primary producers and consumers, while Ferrier-Pagès et al. [65] show a clearer transition from primary producer to consumer with a TP of 1.1 to 2.3 among their treatment groups. Taken together, for *S. pistillata*, the $\delta^{13}C$ and $\delta^{15}N$ of the host and symbiont, and other metrics calculated from that data, along with the CSIA-lipid and CSIA-AA data on samples from shallow and mesophotic depths, provide a more than reasonable interpretation of increased heterotrophy at mesophotic depths.

Energetic balance

The first paragraph in Kahng [1] on bioenergetics refers to the importance of considering both sides of the inputs and demands of a balanced energetic budget approach. We agree, and one of us has written about how important this is [39] and what approaches and considerations must be made in conducting such a study to acquire empirical data to incorporate into the calculations of Scope for Growth or that could be incorporated into energetic models (e.g., Dynamic Energy Budget) [39]. But we never argued the transition of corals from autotrophy to heterotrophy from an energetic perspective, but on carbon based trophic strategies [2]. Carbon budgets are not energetic budgets, especially not stable isotopes of carbon [39].

Subsequently, Kahng [1] discusses some of the fundamental physiological changes that occur with depth for corals in general energetic terms such as inputs and outputs (i.e., photosynthesis and respiration) that should lead to, but is not stated, not only changes in an energetic budget but changes in isotopic composition of the coral host, symbionts, and skeletons as well as the calculation of metrics such as P:R ratios. Central to this issue is the decrease in irradiance with depth [14] which can vary significantly with reef topography [8] and, in combination with coral morphology, can significantly affect photosynthesis [8,11]. Irradiance metrics associated with changes in physiology and community composition with depth include the calculation of optical depths which include estimates of the midpoint and bottom of the euphotic zone. These are used as reference points contextualizing site-specific community transitions and faunal breaks based on decreasing irradiances with depth, decreasing rates of primary productivity, and water column optics for photoautotrophic organisms [7,8,11]. Despite claims made by Kahng [1] that corals have superior light harvesting capabilities compared to terrestrial plants, the data show that both corals and terrestrial plants absorb the same fraction of incident light but that corals reach these high efficiencies using lower chlorophyll densities and the presence of a highly scattering skeleton. Even with increased light-harvesting capabilities, daily integrated gross productivity of mesophotic corals is linearly related to daily irradiance and decreases with depth, but the absolute rate of productivity is morphology dependent which is reflected in their minimum quantum requirements [11]. Nonetheless, compared to corals at shallow depths, corals at mesophotic depths show rates of integrated gross productivity that are over 20-times lower for *Montastraea cavernosa* with P:R ratios of one or less [10,11]. These physiological data, based on the integrated signal of stable isotopes from multiple compartments, is consistent with the calculation of the compensation point (~81 m) for these Bahamaian reefs using optical depths [7]. This, of course, will vary significantly among coral species.

The calculation of optical depths estimates the midpoint and bottom of the euphotic zone. As previously reported, and depending on the optical properties of the mesophotic habitat, the values for optical depths can vary; the 10% optical depth ranges between 29 and 51 m (mean $\pm$ SD: 42 $\pm$ 7 m), whereas the 1% optical depth ranges between 58 and 102 m (mean $\pm$ SD: 85 $\pm$ 15 m) [14]. It is an optical description of the changes in irradiances of the water column, which in turn effects the photo-physiology of multiple functional groups. Photoautotrophs, specifically corals and macroalgae, can be found below the compensation point for any specific system [66,67], and specific mechanisms of photo-acclimatization to extremely low light levels have been identified in corals [68],

coralline algae [69], and macroalgae [70]. The question remaining is whether these capabilities, which increase the efficiency of photon capture and utilization at low irradiances, can actually maintain net positive photosynthesis at lower mesophotic depths. While P:R ratios calculated for *Montastraea cavernosa*, and based on gross productivity, do not go below 1 until 91 m [10], other corals have been shown to exhibit depth-dependent decreases in respiration down to 60 m, in a system where the 1% optical depth is ~80 m, and can potentially offset decreases in photosynthesis with depth leading to increased P:R ratios at 60 m [71].

Whether the photo-acclimatization capabilities of corals are generally superior to macroalgae and coralline algae, or heterotrophy facilitates reverse translocation of organic carbon to their symbionts, or if most corals can maintain net positive photosynthesis and P:R ratios at depths >60 m is unknown. In one of the rare studies to measure oxygen fluxes on mesophotic corals in situ, Fricke et al. [72], measuring photosynthesis and respiration on *Leptoseris fragilis*, showed that net productivity at ~105 m was rarely positive throughout the day except at maximum midday surface irradiances during the summer [72] (Figure 6) and the break between positive and negative net production occurred between 54 m and 95 m [72] (Figure 5). Similarly, modeling of gross productivity in three scleractinian corals, based on empirical data down to 100 m, shows significantly lower rates of gross productivity at depths below 20 m throughout the day [11]. The ϕ_m data used in the calculation of GPP for *M. cavernosa* [10] are extrapolated from 50 m to deeper depths [2,11] because there are no other ϕ_m data available for most corals at lower mesophotic depths. In the absence of these data, and other important photophysiological data collected in situ, this is the best available means to make these calculations. Where presented, these data were used to demonstrate that the productivity of *M. cavernosa* declines with depth as a prelude to interpreting the SIA data and transitions to heterotrophy, not to build a balanced energetic budget on this coral, or any other coral from shallow to mesophotic depths, where we do not have the data required [2,39].

Another reason that Kahng [1] presents to support his statement regarding obligate autotrophy in mesophotic corals is that the host has lost critical amino acid (i.e., essential amino acids) biosynthetic pathways, and that heterotrophy cannot supply these amino acids. Therefore, they must be obtaining these amino acids from their symbionts. However, Fitzgerald and Szmant [73] does provide tracer-based data to show that scleractinian coral hosts can synthesize de novo eight of the essential amino acids considered to be critical for metazoans.

Facultative heterotrophy

We did not specifically advocate any of the three scenarios on facultative heterotrophy [2] presented by Kahng [1]. We simply took the approach that to answer important questions on various aspects of heterotrophy in corals, and because of the technical difficulties in assessing heterotrophy using SIA, a multi-layered approach was needed to understand that corals exhibit a range of distinct trophic strategies [74]. As it relates to corals having the ability to regulate heterotrophic feeding, we know they can. The studies of Grottoli et al. [75] demonstrate an increase in heterotrophic feeding after corals bleach and lose their photoautotrophic carbon source, to meet the costs of their metabolic energy requirements. And mesophotic *Stylophora pistillata* feed at significantly higher rates compared with shallow conspecifics when provided similarly sized rations [20]. Now, whether that same capability to increase feeding rates occurs as productivity declines along the shallow-to-mesophotic depth gradient is unknown. It should also be recognized that facultative heterotrophy would vary significantly among species and habitats [76]. One could test the hypothesis that certain coral species can replace their autotrophic losses by increasing their heterotrophic feeding and are therefore trophic generalists (i.e., mixotrophic) with a wide depth distribution into the lower mesophotic zone. Other species, however, may not be able to adjust their feeding rates in response to decreasing autotrophy and have a depth distribution limited to the upper mesophotic zone, while others maintain autotrophic capabilities into the lower mesophotic but maintain some heterotrophic capacity to obtain

essential nutrients such as nitrogen, for protein synthesis and growth, albeit at a slow rate. The point of Lesser et al. [2] was to present an approach to decipher which corals are doing what in the context of depth-related transitions in trophic strategies.

Concluding thoughts on SIA and trophic analysis

Mesophotic coral reef ecosystems, especially the lower mesophotic zone, are ecosystems with unique communities that have evolved to environmental conditions fundamentally different than their shallow-water counterparts [77]. In writing the original contribution [2], we sought out the literature and datasets to assess an approach for investigators to use SIA in their studies of multi-compartmental, symbiotic, scleractinian corals. We recognized in a series of publications [3–5] that specific statements, that we believed were incorrect, were repeatedly being used regarding the SIA analysis of mesophotic corals. We wrote a paper that addressed those statements and re-analyzed, and where appropriate re-interpreted, previously published data to provide new insights and recommend a multi-layered approach for using standard SIA analysis of corals from shallow to mesophotic depths to assess transitions from autotrophy to heterotrophy. The literature we chose was either the most comprehensive for our questions and/or incorporated the broadest shallow-to-mesophotic depth gradient. There are few such datasets available using standard SIA approaches, let alone more informative techniques such as CSIA of lipids or amino acids, for this type of review and analysis. Multiple lines of evidence using standard SIA techniques, and metrics calculated from those data, support the transition from autotrophy to heterotrophy in many, but not all, scleractinian corals from shallow to mesophotic depths which can be dependent on how well sampled corals are through the depth gradient. We do not need a "reality check" and feel that the underpinnings of Kahng's comments [1] to our original paper [2] are wanting and his conclusions not sufficiently supported in the context of the trophic strategies of corals from shallow to mesophotic depths.

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