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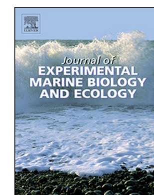

Juveniles of the Atlantic coral, *Favia fragum* (Esper, 1797) do not invest energy to maintain calcification under ocean acidification

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ABSTRACT

Ocean acidification (OA) threatens coral reef ecosystems by slowing calcification and enhancing dissolution of calcifying organisms and sediments. Nevertheless, multiple factors have been shown to modulate OA's impact on calcification, including the nutritional status of the coral host. In three separate experiments, we exposed juveniles of the Atlantic golf ball coral, *Favia fragum*, to elevated CO_2 and varied nutritional (light or feeding) conditions. Juveniles reared from planulae larvae were significantly larger and produced more $CaCO_3$ when fed, regardless of CO_2 level. However, corals subjected to elevated CO_2 produced less $CaCO_3$ per mm^2 regardless of feeding condition. Additionally, unfed corals reared under elevated light levels exhibited lower chlorophyll *a* and higher total lipid content, but light had no significant effect on coral calcification. Conversely, elevated CO_2 had a significant, negative affect on calcification, regardless of light condition but no detectable effect on physiological tissue parameters. Our results indicate that the sensitivity of juvenile *F. fragum* calcification to OA was neither modulated by light nor by feeding, despite physiological indications of enhanced nutritional status. This suggests that corals do not necessarily divert energy to maintain calcification under high CO_2, even when they have the energetic resources to do so.

1. Introduction

Ocean acidification (OA) is the decline in seawater pH caused by ocean absorption of increasing concentrations of atmospheric CO_2. This process shifts the balance of dissolved inorganic carbon (DIC) species in seawater, resulting in a reduction in the concentration of carbonate ions. Scleractinian corals utilize carbonate to produce $CaCO_3$ skeletons that serve as both structural reef scaffolding and $CaCO_3$ supply for reef building. The thermodynamic tendency for aragonite (the form of $CaCO_3$ produced by corals) to precipitate or dissolve is described by the saturation state (Ωar, $[Ca^{2+}][CO_3^{2-}]/Ksp(ar)$), with $CaCO_3$ dissolution favored when seawater is undersaturated (i.e., $\Omega < 1$). OA lowers Ωar, thus reducing the tendency for inorganic $CaCO_3$ precipitation and evoking concerns regarding the implications for coral reef ecosystems (Kleypas et al., 1999).

However, laboratory experiments and field studies of naturally low pH reefs suggest considerable variability in the sensitivity of coral calcification to OA (e.g., Gattuso et al., 1998; reviewed in Langdon et al., 2000; Hoegh-Guldberg et al., 2007; Fabry et al., 2008; Doney et al., 2009; Pandolfi et al., 2011). Coral nutrition and energetic status have been identified as factors that may contribute to this variability (e.g., Cohen and Holcomb, 2009; Ries et al., 2010; Holcomb et al., 2010; Edmunds, 2011; Rodolfo-Metalpa et al., 2011; Holcomb et al., 2012; Drenkard et al., 2013; Schoepf et al., 2013). Calcification is generally considered a metabolically costly process (e.g., active modulation of calcifying fluid pH, production of organic compounds that aid nucleation and crystal growth), which would become more energetically expensive under OA (Cohen and Holcomb, 2009). Corals obtain the nutrition they need to fuel calcification both through heterotrophic feeding and by consuming photosynthate obtained from their algal endosymbiosis (i.e., zooxanthellae). Numerous studies show that corals can produce more $CaCO_3$ when maintained under elevated nutritional conditions such as sufficient levels of photosynthetically active radiation (PAR) and heterotrophic feeding (e.g., reviewed in Gattuso et al., 1999; Ferrier-Pagès et al., 2011). Thus, it has been proposed that elevated nutritional or energetic status of the coral host may mitigate the negative impact of OA on calcification (e.g., Atkinson et al., 1995; Atkinson and Cuet, 2008; Cohen and Holcomb, 2009). Several studies have demonstrated this anticipated response with corals maintained under elevated CO_2 and elevated levels of inorganic nutrients (Langdon and Atkinson, 2005; Holcomb et al., 2010), PAR (Suggett et al., 2013; Dufault et al., 2013; Vogel et al., 2015) or

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heterotrophic feeding (e.g., Comeau et al., 2013; Towle et al., 2015) exhibiting reduced calcification sensitivity to CO_2. However, other studies show that, while corals that are fed (Edmunds, 2011; Drenkard et al., 2013) or maintained under elevated light conditions (Marubini et al., 2001; Comeau et al., 2014; Enochs et al., 2014) produce more $CaCO_3$, in total (relative to corals that are not fed or are maintained under lower light conditions), they still exhibit calcification sensitivity to CO_2. This variability in calcification responses under OA and nutritional enhancement suggests that energetic compensatory mechanisms such as increases in heterotrophic feeding rates (e.g., Towle et al., 2015), translocation of symbiont photosynthate to coral host (Tremblay et al., 2013), or biomass catabolism (Wall et al., 2017) may not be available to all species of scleractinian coral.

Here we present results from a series of experiments with juveniles of the Atlantic coral, *Favia fragum*, designed to further investigate the mechanistic role of heterotrophic and autotrophic nutrition in modulating coral calcification responses to OA. The first objective was to test the hypothesis that *F. fragum* will exhibit reduced calcification sensitivity to elevated CO_2 under heterotrophic feeding and/or elevated light (a stimulant of symbiont photosynthesis). The second objective was to ascertain whether this coral species exhibits a reduction in total tissue lipid content under elevated CO_2, which would be consistent with an investment of metabolic resources to maintain calcification under OA. In addition to prior knowledge of the timing of larval release in *F. fragum* (Szmant-Froelich et al., 1985; Goodbody-Gringley and de Putron, 2009; Goodbody-Gringley, 2010) and larval settlement behavior (Lewis, 1974; Goodbody-Gringley, 2010), we chose this test species because its brooded larvae are imbued with symbionts from their parent coral and, unlike scleractinian species that may exhibit more rapid juvenile polyp-budding (e.g., *Porites astreoides*, Lamarck, 1816; *personal obs.*), newly settled *F. fragum* persist as single polyps on the timescale of these experiments (i.e., 2–3 weeks). Together these factors eliminate potentially confounding effects due to differences in symbiont acquisition success, feeding effort capacity, and/or colonial resource sharing. Finally, since calcification begins after settlement, an additional advantage of using juvenile corals is that all skeletal accretion occurs under controlled experimental conditions. It is important to note that we did not follow a single, fully factorial experimental design that tested for the effects of CO_2, light and feeding because replicating eight experimental conditions was not feasible given our resources and experimental setup. Instead, experiments were fully factorial with respect to two variables (keeping the third constant). We compile those results here, rather than present each experiment in isolation, in order to facilitate a broader discussion of nutritional modulation of calcification response to OA.

2. Material and methods

Experiments were conducted at the Bermuda Institute of Ocean Sciences (BIOS) in St. George's, Bermuda during the summers of 2010, 2011 and 2012. Juvenile corals were reared from settlement (~48 h post larval release) over a range of CO_2 and light levels.

2.1. Coral collection, spawning and larval settlement

Each year, mature colonies of the Atlantic brooding coral, *F. fragum* were collected at approximately 3–6 m depth from the inner lagoon Bailey's Bay patch reefs in Bermuda in early July, approximately one week prior to anticipated peak larval release date (Goodbody-Gringley and de Putron, 2009). Parent colonies were kept in outdoor flow-through seawater aquaria where they were exposed to ambient light and temperature conditions. Goodbody-Gringley (2010) reported average day-time summer light levels of approximately 240 µmol photons $m^{-2} s^{-1}$ in these outdoor aquaria which is less than half of the 516 µmol photons $m^{-2} s^{-1}$ PAR observed by de Putron et al. (2017) on one of the patch reefs (4–6 m depth) for the summer of 2009 (measurements made mid-day under clear skies). However, de Putron et al. (2017) point out that outdoor aquaria temperatures were, on average, only slightly higher than those observed on the patch reefs for a given summer (i.e., 2009, 2011, 2012).

Parent *F. fragum* colonies were isolated in glass jars at night in order to retain released larvae. All zooxanthellate larvae released per colony on each of the several peak release days were pooled together per day for settlement. For all experiments, we followed the settlement and sampling procedures described in Drenkard et al. (2013): collected larvae were settled on reef-conditioned terracotta tiles in recirculating seawater that was at pre-established CO_2 treatment levels. Following a 48-hour settlement period, tiles with metamorphosed larvae (referred to as “spat”) were allocated to experimental, recirculating tanks such that each aquarium contained approximately the same number of juvenile corals.

2.2. Experimental setup and conditions

Aquarium maintenance and the methods used to achieve desired CO_2 levels were identical each year and are reported in Drenkard et al. (2013). We regularly monitored the CO_2 concentration of the ambient and CO_2-enriched air that was bubbled into the tanks using a Qubit infrared CO_2 analyzer, and monitored the pH (NBS scale) of the tanks using an Orion electrode and pH meter. Tanks were maintained on a 12/12 h light-dark cycle using the same fluorescent aquarium lamps across experiments: low light (LL) conditions were achieved with 2-bulb, Perfecto Manufacturing aquarium lights while higher light (HL) conditions were attained with Sun Blaze T5 fixtures by Sunlight Supply, each containing four AquaSun T5 Very High Output tube lamps by UV Lighting; PAR for each experiment was measured with a LICOR probe/m and is reported below in irradiance units of µmol photons $m^{-2} s^{-1}$. To maintain consistent aquarium temperatures, all tanks were placed in water baths with 6 (4 in 2012) tanks per bath, which were thermally regulated by inline chiller/heater systems and individual aquarium temperatures were recorded at 15-minute intervals using HOBO temperature loggers (Onset Corp.); average treatment temperatures are reported in Table 1.

Prior to weekly water replacements (performed to prevent excessive accumulation of nitrogenous and other waste products), we collected discrete samples for salinity, alkalinity (Alk), dissolved inorganic carbon (DIC), and dissolved inorganic nitrogen and phosphorous from each aquarium. Salinity samples were analyzed at BIOS (Autosal salinometer) and Alk/DIC samples, poisoned with mercuric chloride during collection, were analyzed at the Woods Hole Oceanographic Institution (WHOI; Marianda VINDTA-3C system). These Alk and DIC values (not the Qubit pCO_2 readings or NBS pH data) were used to calculate the pH and Ωar for each treatment tank. The analytical methods used to determine the values for the variables reported in Table 1 and calculations for carbonate system parameters ($[HCO_3^-]$, $[CO_3^{2-}]$, Ωar) were the same each year and are described in Drenkard et al. (2013). The nutrient samples for all three experiments were analyzed at the WHOI Nutrient Analytical Facility and the results are reported in Table 2. The following subsections detail conditions that differed across experimental years.

2.2.1. Conditions specific to the 2010 experiment: 2 CO_2 levels, low light, fed and unfed

As described in Drenkard et al. (2013), the 3-week experiment in 2010 consisted of two CO_2 conditions (ambient and high) with partial pressure (mean ± SD) of 420 ± 20 ppmv, and 1670 ± 20 ppmv, respectively (Table 1), crossed against two feeding conditions: fed and unfed. Each CO_2-feeding treatment was conducted in triplicate (i.e., treatment average sample size = 3) for a total of 12, 5.5-gal aquaria (note: only unfed coral results from 2010 were analyzed further in this study). Light levels achieved an average PAR of 62 ± 8 µmol photons $m^{-2} s^{-1}$ (mean ± SD).

Table 1
Measured and calculated carbonate chemistry parameters. Average ± standard deviation among replicate tanks for given experimental treatment conditions. Average light levels, temperature and measured salinity, alkalinity, and DIC were used to calculate [HCO_3^-], [CO_3^{2-}], and aragonite saturation state (Ω_{ar}) for each aquarium using CO2SYS (Lewis and Wallace, 1998). We used Dickson and Millero's (1987) dissociation constants from the refit of Mehrbach et al. (1973) and the aragonite solubility of Mucci (1983). 2010 alkalinity, salinity and DIC data and calculated carbonate chemistry values are from Drenkard et al., 2013.

Year & light condition	Feeding treatment	CO_2 level	CO_2	pH	Temperature	Salinity	Alkalinity	DIC	HCO_3^-	CO_3^{2-}	Ω_{ar}
(μmol photon $m^{-2}s^{-1}$ ± sd)		(# tanks)	(ppmv CO_2 ± sd)	(NBS ± sd)	(°C ± sd)	(psu ± sd)	(μeq/kg ± sd)	(μmol/kg ± sd)	(μmol/kg ± sd)	(μmol/kg ± sd)	(± sd)
2010 Low light 62 ± 8	Unfed	Ambient (3)	420 ± 20	8.20 ± 0.02	27.7 ± 0.1	37.4 ± 0.3	2325 ± 20	1984 ± 16	1735 ± 13	239 ± 3	3.77 ± 0.03
		High (3)	1670 ± 20	7.72 ± 0.04	27.6 ± 0.1	37.0 ± 0.2	2326 ± 23	2207 ± 21	2069 ± 20	105 ± 5	1.66 ± 0.08
2011 High light 215 ± 21	Fed	Ambient (3)	420 ± 10	8.06 ± 0.12	27.4 ± 0.6	36.5 ± 0.9	2154 ± 110	1853 ± 70	1635 ± 68	207 ± 46	3.28 ± 0.71
		Intermediate (3)	1060 ± 10	7.78 ± 0.09	27.3 ± 0.5	36.6 ± 1.4	2200 ± 82	2054 ± 42	1907 ± 44	121 ± 33	1.92 ± 0.51
		High (3)	1720 ± 90	7.55 ± 0.12	27.6 ± 0.4	36.1 ± 0.6	2251 ± 67	2148 ± 72	2016 ± 74	95 ± 20	1.52 ± 0.32
		Very High (3)	2660 ± 30	7.37 ± 0.10	27.2 ± 0.4	36.3 ± 0.9	2406 ± 45	2350 ± 53	2220 ± 52	76 ± 12	1.21 ± 0.19
	Unfed	Ambient (3)	420 ± 10	8.06 ± 0.12	27.2 ± 0.4	36.2 ± 0.9	2160 ± 109	1852 ± 83	1629 ± 74	212 ± 31	3.38 ± 0.48
		Intermediate (3)	1060 ± 10	7.78 ± 0.09	27.5 ± 0.5	36.3 ± 1.0	2162 ± 86	2003 ± 64	1857 ± 53	123 ± 20	1.95 ± 0.31
		High (3)	1720 ± 90	7.56 ± 0.09	27.3 ± 0.6	36.8 ± 1.4	2315 ± 123	2222 ± 72	2084 ± 62	94 ± 31	1.48 ± 0.47
		Very High (3)	2660 ± 30	7.36 ± 0.10	27.5 ± 0.5	36.4 ± 1.0	2370 ± 69	2314 ± 69	2186 ± 66	75 ± 13	1.20 ± 0.21
2012 Low light 38 ± 5	Unfed	Ambient (4)	430 ± 20	8.16 ± 0.05	27.6 ± 0.3	36.8 ± 0.5	2352 ± 33	2018 ± 28	1772 ± 40	236 ± 11	3.72 ± 0.19
		High (4)	1920 ± 20	7.65 ± 0.05	27.7 ± 0.3	36.8 ± 0.5	2392 ± 69	2281 ± 63	2141 ± 58	103 ± 11	1.62 ± 0.17
2012 High light 227 ± 19	Unfed	Ambient (4)	430 ± 20	8.18 ± 0.03	27.6 ± 0.4	36.6 ± 0.2	2335 ± 27	2003 ± 17	1757 ± 11	234 ± 8	3.72 ± 0.12
		High (4)	1920 ± 20	7.67 ± 0.03	27.7 ± 0.4	36.7 ± 0.4	2357 ± 24	2254 ± 24	2119 ± 23	97 ± 8	1.54 ± 0.12

Table 2
Nutrient measurements. Average (± SD) seawater chemistry of replicate aquaria for a given experimental conditions (treatment). Tanks sampled after one week. These values are somewhat approximate; where values were indicated as "lower than a detection limit" the value was replaced with the next 0.005 μM lower for calculation purposes (e.g. < 0.05 μM became 0.045 μM).

Year	CO_2 treatment	Ammonium (μM NH_4^+ ± SD)	Phosphate (μM PO_4 ± SD)	Nitrite and nitrate (μM $NO_2 + NO_3$ ± SD)
2010	Fill Water	0.94 ± 0.53	0.11 ± 0.04	0.46 ± 0.23
Low Light	Ambient CO_2	6.31 ± 1.46	0.15 ± 0.10	0.46 ± 0.40
	High CO_2	3.26 ± 2.34	0.10 ± 0.03	0.29 ± 0.28
2011	Fill Water	0.56 ± 0.35	0.09 ± 0.05	0.85 ± 0.20
High Light	Ambient CO_2	0.44 ± 0.13	0.09 ± 0.02	0.08 ± 0.03
	High CO_2	0.33 ± 0.18	0.07 ± 0.01	0.06 ± 0.01
2012	Fill Water	< 0.05	< 0.05	0.71 ± 0.33
Low Light	Ambient CO_2	4.14 ± 0.38	< 0.05	0.45 ± 0.16
	High CO_2	4.81 ± 0.32	< 0.05	0.44 ± 0.15
High Light	Ambient CO_2	0.42 ± 0.54	< 0.05	< 0.05
	High CO_2	0.09 ± 0.09	< 0.05	< 0.05

2.2.2. Conditions specific to 2011 experiment: 4 CO_2 levels, higher light, fed and unfed

For the 3-week experiment in 2011, the partial pressures (mean ± SD) of the four CO_2 levels (ambient, intermediate, high, and very high) bubbled into recirculating experimental tanks were 420 ± 10 ppmv, 1060 ± 10 ppmv, 1720 ± 90 ppmv, and 2660 ± 30 ppmv, respectively (Table 1). Again, there were two feeding conditions (fed and unfed) following a crepuscular feeding schedule outlined in Drenkard et al. (2013): Corals were isolated on alternating nights for 3 h in 12.5 cm × 12.5 cm × 3 cm plastic containers filled with seawater from their respective treatment tanks; fed corals were provided with several milliliters of concentrated, seawater-suspended day-old *Artemia* (i.e., brine shrimp) nauplii, although ingestion rates were not measured. Each CO_2-feeding treatment was conducted in triplicate, for a total of 24, 5.5-gal aquaria. Average PAR was 215 ± 21 μmol photons $m^{-2}s^{-1}$ (mean ± SD). Skeletal data is presented for both fed and unfed corals but only unfed coral data is used for comparing CO_2-effect on tissue metrics.

2.2.3. Conditions specific to 2012 experiment: 2 CO_2 levels, higher and low light, unfed

The 2-week experiment in 2012 was designed to match the Ω_{ar} values achieved in 2010, with ambient and high CO_2 conditions maintained at 430 ± 20 ppmv and 1920 ± 20 ppmv respectively (Table 1). Note that although the Qubit measurements differed between experiments, these pCO_2 values were used only to monitor system stability, and not for carbonate chemistry calculations. The saturation state levels for high and ambient CO_2 tanks were generally comparable across experimental years (Table 1). We chose elevated CO_2 levels (for this and previous experiments) that were likely to affect *F. fragum* calcification (de Putron et al., 2011; Cohen et al., 2009). The corresponding Ω_{ar} values (~1.6 on average) are the low extreme predicted by global-scale (i.e., coarse) ocean-climate models for end-of-century, tropical reef conditions under high CO_2 emissions scenarios (e.g., Ricke et al., 2013). However, a number of present-day, low Ω_{ar} reef systems (e.g., Shamberger et al., 2014) may experience such conditions prior to 2100. Average PAR for low was 38 ± 5 (LL) and 227 ± 19 (HL) μmol photons $m^{-2}s^{-1}$ (mean ± SD). There were four replicates (i.e., treatment-average sample size = 4) for each CO_2-light treatment for a total of 16, 10-gal aquaria. No corals in this experiment were fed heterotrophically.

We chose sub-saturating and near-saturating light conditions, and as a result the light levels used in our experiments are lower than those observed on Bermuda patch reef collection sites (de Putron et al., 2017). While light saturation for *F. fragum* calcification is unknown,

studies using other coral species have demonstrated saturation occurring at levels comparable to our HL conditions. For example, Cohen et al. (2016) observed saturation for *Porites lutea* and *Acropora variabilis* calcification occurring at light (control and different spectral wavelength) intensities between 110 and 200 μmol photons $m^{-2} s^{-1}$), while Suggett et al. (2013) report saturating light intensities of 274 and 232 μmol photons $m^{-2} s^{-1}$ for *A. horrida* and *Porites cylindrica* calcification, respectively. Our two light levels fall within the exponential portion on most of the light saturation curves computed for several different coral species (Chalker (1981), where differences in light would be expected to have the greatest physiological impact. We also endeavored to avoid light-induced stress responses and maintain sufficient sample size of coral recruits: Levy et al. (2016) observed photo-oxidative stress in *Stylophora pistillata* at 500 μmol photons $m^{-2} s^{-1}$, Hoogenboom et al. (2012) hypothesize activation of photo-protective mechanisms in *Stylophora pistillata* and in *Turbinaria reniformis* at light intensities above 200 μmol photons $m^{-2} s^{-1}$ and Dufault et al. (2013) reported greatest survivorship of *Pocillopora damicornis* recruits at 122 μmol photons $m^{-2} s^{-1}$. Additionally, *F. fragum* larvae are known to seek out low-light reef crevices for settlement (Lewis, 1974) thus light levels that are lower than ambient reef conditions are relevant for early life stages of this particular coral species.

2.3. Sample collection of skeletons, total tissue lipids, symbiont counts, and pigments

At the experiments' conclusions, corals were individually removed from the tiles using a razor blade, for soft tissue analyses. Due to the juveniles' small size (i.e., single polyp), several analyses required pooling multiple individuals (combined in a micro-centrifuge tube) from a given tank to ensure sufficient material for detection: total lipid content (from both coral and symbiont; 10 spat per sample), symbiont counts (5 spat per sample in 2010, 1 spat per sample in 2011 and 2012), and pigment (5 spat per sample). We then standardized each result by the number of pooled spat to yield value-per-coral; the number of samples per tank average is reported in Table S1. Samples collected for total lipid and symbiont counts were frozen at −80 °C and − 20 °C respectively. The samples used for pigment (chlorophyll) analysis were originally intended for genetic studies and thus frozen at −20 °C in RNAlater (QIAGEN). This is not the preferred method for storing pigment samples, but samples from all years were subjected to the same storage conditions, so while the exact values for pigment densities may be underestimates, the comparisons among experiments and treatments are informative. Tiles with corals remaining were placed in 10% bleach/ seawater solution to remove the tissue, thus exposing the coral skeleton.

2.4. Quantification and analysis of coral calcification and physiology

Skeletal (corallite) parameters were measured following the methods in Drenkard et al. (2013): Bleached corallites were digitally photographed, removed from tiles, and weighed on a Metro-Toledo micro-balance (Cohen et al., 2009; de Putron et al., 2011). We used Spot Imaging software to analyze skeletal size, which we defined as the average primary septa (i.e., radially extending $CaCO_3$ plate) length, and skeletal weight being the total corallite mass (assumed entirely composed of $CaCO_3$). The strong correlation between corallite size and weight ($R = 0.94$; Fig.1) suggests that these two parameters are not independent. Therefore, to better isolate the influence of CO_2 and nutrition on the calcification rate, we area-normalized skeletal weight by dividing individual corallite mass by its area (as defined by average septal diameter).

We used symbiont and chlorophyll *a*real-density to estimate the coral host's potential to utilize light as a form of nutrition, and area-normalized, total tissue lipid as an indicator of stored energetic reserves available to the organism to better withstand environmental stressors (i.e., OA). These analyses all commenced with homogenizing a given number (Section 2.3) of whole coral polyps (i.e., tissue and skeleton), which was necessary due to the limited amount of sample material available in newly settled spat. As in Drenkard et al. (2013), we followed the solvent extraction methods outlined by Folch et al. (1957) and Cantin et al. (2007) for gravimetric quantification of total lipid content. For symbiont analyses, the ground polyp slurry was centrifuged and the resultant pellet was re-suspended in 250 μL (2010; samples composed of 5 spat) or 50 μL (2011 and 2012; samples composed of a single spat) filtered seawater. Symbionts from multiple (6–9 in 2010; 3–4 in 2011 and 2012) aliquot subsamples were counted (typically 4 replicates per aliquot) on a known-volume hemocytometer grid. Following homogenization, chlorophyll samples were sonicated in 100% methanol to release pigments, and spiked with a known amount of canthaxanthin, which served as an internal standard before analysis using HPLC techniques outlined in Wright et al. (1991). We assumed that chlorophyll *a* degradation products (on average, ~28% of the chlorophyll *a* signal when including pheophytin) were the result of the preservation process because the chlorophyll *a* signal from a set of 2010 coral samples that were flash frozen was comprised of, on average, < 5% degradation products with no detectable amounts of pheophytin (data not shown). Therefore, we corrected for and included these degradation product concentrations in the total reported amount of chlorophyll *a*. Data for tissue lipid content, symbiont counts, and chlorophyll mass were normalized by circular area, which is defined by the average primary septa diameter from a sample's respective tank (tissue sampling procedures prevented obtaining exact skeletal measurements from the individual corallites used in these analyses).

All statistical analyses were performed on tank-average values (sample sizes for average calculations are reported in Table S1; statistical sample sizes are the number of tanks in a given treatment) using MYSTAT/SYSTAT (Systat Software, Inc. Chicago, IL, USA). Together, the following portions of our 2010 and 2011 experiments approximately replicate our 2012 experiment of 2 CO_2 × 2 light conditions: 2010 (unfed, both CO_2 treatments, LL) and 2011 (unfed, ambient and 1720 ppmv CO_2 treatments, HL). Statistical analyses were only conducted on coral samples from the same year because inter-annual experimental differences (e.g., CO_2 levels, light conditions, parental effects) prevent meaningful, across-year comparison. However, we can speak to the reproducibility of CO_2 effect significance on unfed coral physiological parameters under LL or HL conditions. This required different statistical tests for each year due to the number of treatment conditions analyzed (i.e., subset vs. entire experiment). The following sections detail the specific tests. Some data were transformed prior to analysis in order to homogenize variance; the specific transformations are reported parenthetically following mention of the respective dependent variable.

2.4.1. Statistical tests specific to 2010 experiment

In order to understand *F. fragum* calcification and physiological responses to OA under LL conditions, we conducted four individual *t*-tests for CO_2 effect on area-normalized skeletal weight, symbiont counts, and chlorophyll *a*nd lipid content of 2010 unfed corals. These results (Table S2) are discussed in the context of the CO_2 effects determined for the same parameters in 2012 unfed corals.

2.4.2. Statistical tests specific to 2011 experiment

We first investigated the effects of OA and heterotrophic nutrition on *F. fragum* calcification under HL conditions using a multivariate analysis of variance (MANOVA; Tables S3 & S4) to test for the effects of CO_2 and feeding on both corallite size (log transformed) and weight (negative inverse transformed). We then tested the effects of OA and autotrophic nutrition on size-adjusted calcification using a two-way ANOVA (Table S5) of area-normalized skeletal weight (log transformed). Ambient and 1720 ppmv CO_2 conditions yielded saturation states most similar to those achieved in 2010 and 2012. Therefore, we

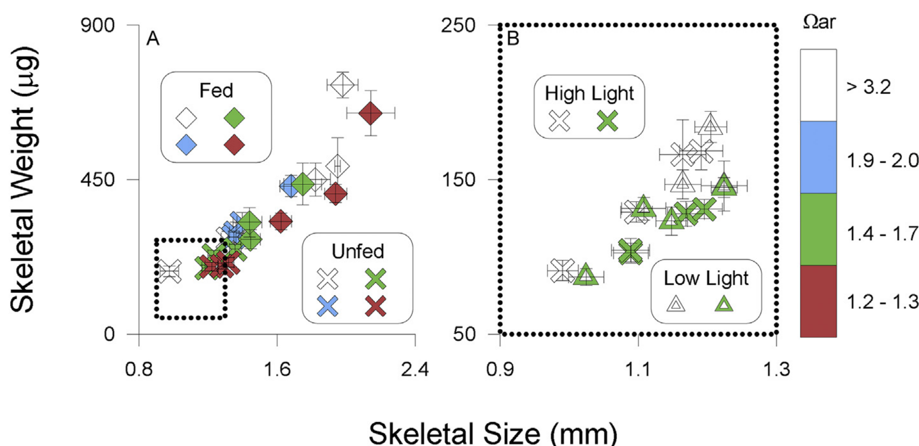

Fig. 1. Juvenile coral skeletal weight vs. diameter (i.e., size) from a) 2011 and b) 2012. The linear regression (Weight = 470.25* Diameter – 385.81) and correlation (R = 0.94) between skeletal size and weight were computed using data from both experiments but are separated by year to improve data readability; the dash-lined area in panel a. indicates the size-by-weight region that would be occupied by the 2012 data (panel b). Each symbol represents the average weight and size for a given treatment tank. Diamonds, x's and hollow triangles indicate fed-HL (2011), unfed-HL (2011 & 2012) and unfed-LL (2012) experimental conditions, respectively; symbol coloration represents the saturation state range. Error bars indicate ± 1 standard error where n = 3 (4) for 2011 (2012).

included an a priori test in the aforementioned ANOVA to specifically test for the effect due to CO_2 under HL on area-normalized skeletal weight of unfed coral exposed to only these CO_2 levels.

In order to also understand *F. fragum* physiological responses to OA under HL conditions for comparison with 2012 findings, we conducted three individual t-tests (Table S2) for CO_2 effect on the following area-normalized dependent variables of unfed corals reared under ambient and 1720 ppmv CO_2: symbiont counts, and chlorophyll *a*nd lipid content. These results, and the result of the a priori test for CO_2 effect on area-normalized skeletal weight, are discussed in the context of the CO_2 effects determined for the same parameters in 2012 unfed corals.

2.4.3. Statistical tests specific to 2012 experiment

We first investigated the effects of OA and autotrophic nutrition on *F. fragum* calcification using a MANOVA (Tables S6 & S7) to test for the effects of CO_2 and light on both corallite size and weight (log transformed). We then tested the effects of OA and autotrophic nutrition on size-adjusted calcification and physiology by conducting four individual, two-way ANOVAs (Tables S8 & S9) of the following area-normalized dependent variables: skeletal weight (negative inverse transformed), zooxanthellae counts, chlorophyll *a* and tissue lipid content (log-transformed).

3. Results

3.1. Skeletal size and weight

Fed corals in the 2011 experiments were significantly larger and produced more $CaCO_3$ than their unfed counterparts (Fig. 1a); there was no detectable effect due to CO_2 or the interaction between CO_2 and feeding on skeletal size or weight. Conversely, area-normalized skeletal weight was significantly lower under elevated CO_2 conditions, but there was no significant effect due to feeding or due to the interaction between CO_2 and feeding (Fig. 2a). Similarly, in 2010 (LL) and 2011 (HL), corals reared under ambient CO_2 produced more $CaCO_3$ per mm^2 than corals reared under high CO_2 (Ω_{ar} = ~1.5–1.7) conditions (Fig. 3a & b).

In the 2012 experiment, corals reared under ambient CO_2 conditions generally produced more $CaCO_3$ than those subjected to elevated CO_2 conditions, but there was no significant effect due to light treatment or the interaction between light and CO_2 (Fig. 1b). When corallite diameter and weight were analyzed individually, the effects of CO_2 and light treatments were not significant. However, area-normalized weight was significantly lower in the high CO_2 level (Fig. 2b), but there was no significant effect on area-normalized weight due to light, and no significant interaction between CO_2 and light (Fig. 3c & d).

3.2. Symbiont, pigment and lipid density

For both 2010 (LL) and 2011 (HL) experiments, area-normalized symbiont density (Fig. 4a & b), chlorophyll *a* content (Fig. 5a & b) and lipid content (Fig. 6a & b) were not significantly different between unfed corals reared under ambient vs. elevated CO_2 conditions.

Among 2012 corals, those reared under HL conditions exhibited significantly lower area-normalized chlorophyll *a* content (Fig. 5c vs. d) and significantly greater area-normalized total tissue lipid content (Fig. 6c vs. d) than those reared under LL conditions, regardless of CO_2 level. We did not detect a significant effect due to light on area-normalized symbiont densities (Fig. 4c & d), nor were there significant effects due to either CO_2 or the interaction between light and CO_2 on area-normalized symbiont counts, pigment content, and lipid content.

4. Discussion

We assessed calcification, the capacity to acquire nutrition via symbiont photosynthesis, and the energetic reserves (approximated by total lipid content) of recently settled *F. fragum* corals subjected to two levels of CO_2 and nutrition via heterotrophic feeding or light. These results help characterize the extent to which nutrition may impact the calcification response of *F. fragum* to OA: Heterotrophic feeding appears to be the dominant driver of corallite size in corals from the 2011 experiment, with no significant effect due to CO_2 in either the 2011 or the 2012 experiment (Fig. 1; Tables S4 & S7). This is consistent with the septa diameter results of Drenkard et al. (2013). We surmise that OA has minimal impact on coral tissue extent, which we assume drives lateral corallite size (i.e., the larger the area covered by calcifying tissue, the larger the diameter of the accreted corallite; Davies, 1984). However, unfed HL corals in the 2012 experiment were not significantly larger than unfed LL corals, suggesting that a coral's physiological (and possibly metabolic) response differs depending on the type of nutrition available (i.e., feeding vs. light). This is consistent both with Comeau et al. (2013)'s finding that light intensity does not significantly affect biomass and Davies' (1984) hypothesis that, although symbiont photosynthesis may provide the coral with considerable metabolic fuel, this carbon-rich "junk food" (Falkowski et al., 1984) does not provide the nitrogen-rich compounds required to increase coral biomass.

Drenkard et al. (2013) proposed that fed corals produce more $CaCO_3$ in total because of their larger polyp size (i.e., area) over which $CaCO_3$ is accreted. In other words, given two corals of different sizes, calcifying at equal rates (mass $CaCO_3$ area^{-1} time^{-1}) for a set amount of time, the larger coral would produce more $CaCO_3$ because its calcification is integrated over a larger area. This is consistent with the observed correlation between corallite size and weight (R = 0.94; Fig. 1) and our 2011 findings, which show that when skeletal weight

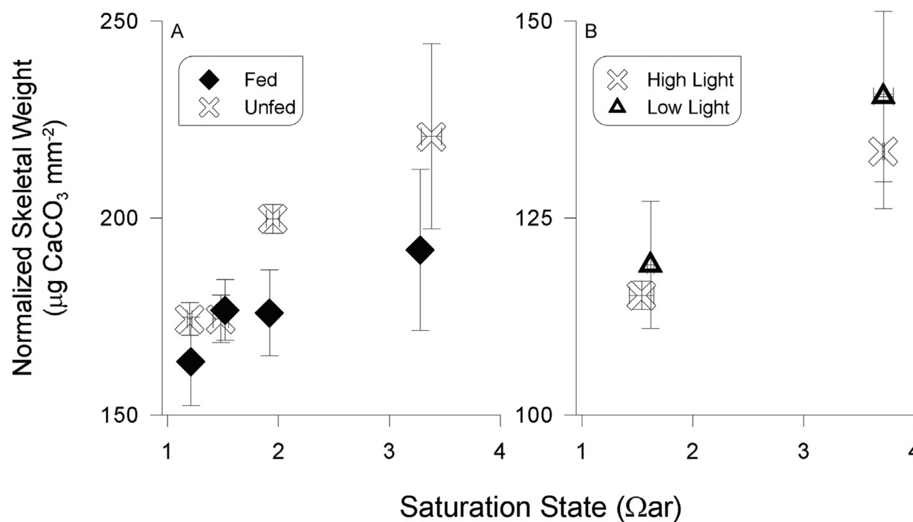

Fig. 2. Area-normalized skeletal weight from a) 2011 and b) 2012 experiments plotted against the average aragonite saturation state of their respective treatment conditions. Error bars indicate ± 1 standard error where n = 3 (4) for 2011(2012).

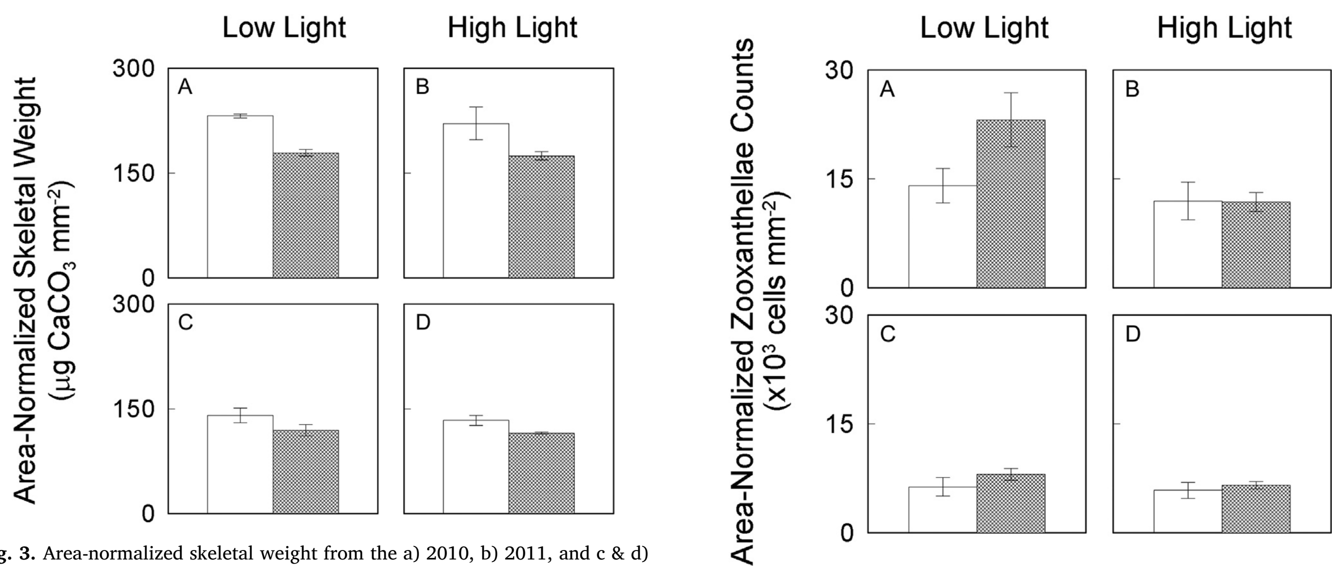

Fig. 3. Area-normalized skeletal weight from the a) 2010, b) 2011, and c & d) 2012 experiments. Average light levels (± SD) were: 62 ± 8 (LL2010), 215 ± 21 (HL2011), 38 ± 5 (LL2012), and 227 ± 19 (HL2012) μmol photons $m^{-2}s^{-1}$. White bars represent ambient (Ωar > 3.2), while shade bars represent elevated (1.4 < Ωar < 1.7) CO_2 conditions; Error bars indicate ± 1 standard error where n = 3 for 2010, 2011 and 2012 LL, ambient CO_2 conditions; *n* = 4 for other 2012 experimental conditions.

Fig. 4. Area-normalized symbiont densities from the a) 2010, b) 2011, and c& d) 2012 experiments. Average light levels (± SD) were: 62 ± 8 (LL 2010), 215 ± 21 (HL 2011), 38 ± 5 (LL 2012), and 227 ± 19 (HL 2012) μmol photons $m^{-2}s^{-1}$. White bars represent ambient (Ωar > 3.2), while shaded bars represent elevated (1.4 < Ωar < 1.7) CO_2 conditions; Error bars indicate ± 1 standard error where n = 3 for 2010, 2011 and 2012 LL, ambient CO_2 conditions; n = 4 for other 2012 experimental conditions.

was normalized to polyp size (i.e., basal area), there was no longer a significant effect due to feeding (Fig. 2a; Table S5). That is, nutrition via heterotrophic feeding did not significantly alter the rate of coral calcification, but instead increased the area over which calcification occurred, thus resulting in more $CaCO_3$ production overall. While this contrasts with studies that demonstrate elevated calcification rates among fed vs. unfed corals (e.g., Edmunds, 2011), it is consistent with the species-specific (reviewed in Ferrier-Pagès et al., 2011), and sometimes conditional nature (e.g., Towle et al., 2015) of scleractinian calcification response to heterotrophic feeding.

Similar to feeding, elevated light levels did not significantly impact area-normalized skeletal weight, which was unexpected given historical reports of light-enhanced calcification (e.g., Barnes, 1982; Gattuso et al., 1999; Marubini et al., 2001). This may be due to our use of total skeletal weight to investigate OA-impacts on skeletogenesis: in doing so, we integrate both light- and dark-calcification, which Chan and Connolly (2012) suggest may obscure subtler light-intensity responses of specific calcification phases. Alternatively, this response could be a reflection of coral life stage: recently settled spat may not yet be replete with algal symbionts, which would affect any zooxanthellae-dependent mechanisms (reviewed in Gattuso et al., 1999) of light enhanced calcification.

Coral calcification in both 2011 and 2012 experiments exhibited a significant effect due to CO_2 but no significant interaction with nutritional condition, which suggests that neither form of nutrition reduced calcification sensitivity to OA. This result differs from the findings of previous OA/light studies: Comeau et al. (2013) observe no significant impact due to CO_2 on *Porites rus* calcification, regardless of nutrition provided via light or feeding. Conversely, calcification rates in two coral species (*Acropora horrida* and *Porites cylindrica*) are elevated under saturating light conditions and OA-sensitivity is reduced relative to conspecifics maintained under LL conditions (Suggett et al., 2013).

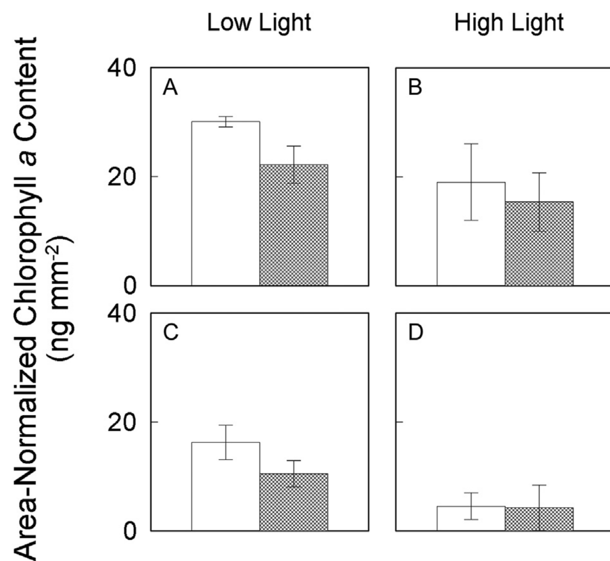

Fig. 5. Area-normalized chlorophyll *a* content from the a) 2010, b) 2011, and c & d) 2012 experiments. Average light levels (± SD) were: 62 ± 8 (LL 2010), 215 ± 21 (HL 2011), 38 ± 5 (LL 2012), and 227 ± 19 (HL 2012) μmol photons $m^{-2}s^{-1}$. White bars represent ambient (Ωar > 3.2), while shaded bars represent elevated (1.4 < Ωar < 1.7) CO_2 conditions; Error bars indicate ± 1 standard error where n = 3 for 2010, 2011 and 2012 LL, ambient CO_2 conditions; n = 4 for other 2012 experimental conditions.

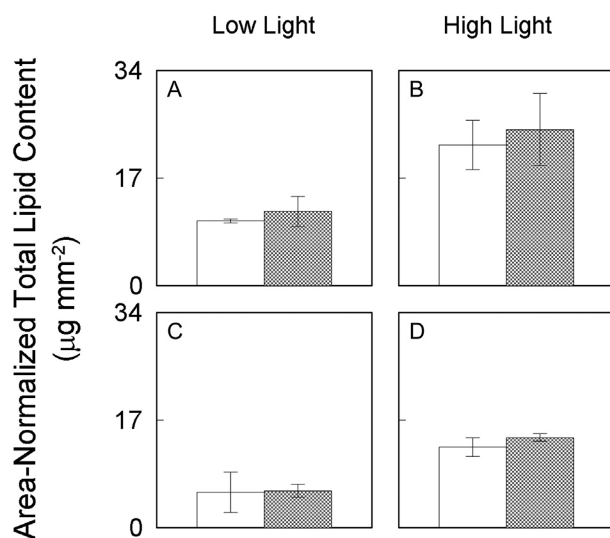

Fig. 6. Area-normalized total lipid content from the a) 2010, b) 2011, c & d) 2012 experiments. Average light levels (± SD) were: 62 ± 8 (LL 2010), 215 ± 21 (HL 2011), 38 ± 5 (LL 2012), and 227 ± 19 (HL 2012) μmol photons $m^{-2}s^{-1}$. White bars represent ambient (Ωar > 3.2), while shaded bars represent elevated (1.4 < Ωar < 1.7) CO_2 conditions; Error bars indicate ± 1 standard error where n = 3 for 2010, 2011 and 2012 LL, ambient CO_2 conditions; n = 4 for other 2012 experimental conditions.

Similarly, *Acropora millepora* exhibited greater CO_2-sensitivity under low vs. high light conditions (Vogel et al., 2015).

Differences in experimental light conditions may contribute to the discrepancies among these studies. The experimental light levels (~50 and 220 μmol photons $m^{-2}s^{-1}$) did not represent the upper range of light intensity that adult *F. fragum* might experience on the reef, although they may be relevant to *F. fragum* spat, as larvae seek out low-light niches for settlement (Lewis, 1974). Comeau et al. (2013) hypothesize that *P. rus* do not exhibit a significant light-effect because calcification is not light-limited at either ~215 or 1000 μmol photon $m^{-2}s^{-1}$. In order to elicit a measurable physiological light-response (successfully achieved, as evidenced by the significant light-effect on photosynthetic and metabolic metrics discussed below), we chose two light conditions that were estimated to be sub-saturating and near-saturating. Though we do not know the exact saturating light level for our particular coral species, Suggett et al. (2013) report saturating light intensities of 274 and 232 μmol photons $m^{-2}s^{-1}$ for *A. horrida* and *P. cylindrica* calcification, respectively, and our two light levels fell within the exponential portion on most of the light saturation curves for corals computed by Chalker (1981). Similar to this study, Suggett et al. (2013) and Vogel et al. (2015) observe different physiological responses under LL (sub-saturating) vs. HL (saturating/near-saturating) conditions, specifically 100 vs. 400 μmol photon $m^{-2}s^{-1}$, and 35 vs. 150 μmol photon $m^{-2}s^{-1}$, respectively. However, unlike those two studies, we did not observe light-induced reductions in calcification sensitivity to CO_2.

Our results also differ from those of Holcomb et al. (2010) and Langdon and Atkinson (2005) wherein nutritional enhancement via inorganic nutrients reduce differences in calcification rate between corals under ambient and high CO_2 conditions. Notably, the result for DIN-enriched coral from Holcomb et al. (2010) is attributable to elevated calcification rates under high pCO_2 as well as lower calcification under ambient pCO_2 conditions, which may be explained by the ability of light and nutrients to stimulate symbiont photosynthesis, causing carbon limitation under ambient CO_2 levels and providing more photosynthate under high CO_2 levels (Marubini and Davies, 1996; Langdon and Atkinson, 2005; Holcomb et al., 2010; Suggett et al., 2013). It is important to note that each of the aforementioned studies use different coral test species, which may vary in nutritional requirements and survival strategies. Suggett et al. (2013) suggest that species effects may explain dissimilar degrees of light-mitigation observed in *A. horrida* vs. *P. cylindrica* calcification, and it is possible that responses in this study are particular to *F. fragum*. Additionally, unlike the above-cited studies, we used newly settled coral recruits: a life history stage that might have exhibited different physiological responses to OA from those observed in mature adult corals. With regard to age, it is worth noting that corals from the 2012 experiment appeared to exhibit lower values across responses metrics (i.e., area-normalized weight, symbiont count, and chlorophyll *a*nd lipid content) than corals from the 2010 and 2011 experiments (Figs. 3–6). We assume this can be explained by the fact that 2012 spat were 2, not 3 (2010 and 2011) weeks of age at sampling. Younger corals would, for example, have had skeletal weight than older corals (assuming similar size and calcification rate) because the measurement represents $CaCO_3$ accretion integrated over a shorter amount of time. However, it is also possible that other factors such as discrepancies in tank conditions or parental effects (e.g., variable allocation of larval energetic reserves) could have driven inter-annual experimental differences. For this reason we did not formally compare results between years with inter-annual statistical analyses. Rather, we emphasize that, for a given response metric under similar light conditions, the same within-year CO_2 response significance manifested across years despite potentially differentiating, inter-annual factors.

We used measurements of symbiont areal-densities, and chlorophyll and tissue lipid content to determine whether our two light conditions elicited different photosynthetic responses because we were not equipped to directly measure the photosynthetic rate of individual juvenile corals. Symbiont areal-densities under different light conditions did not differ significantly in the 2012 light experiment, but chlorophyll *a* concentrations under LL were considerably higher than under HL conditions. This is consistent with studies that show higher chlorophyll concentrations (i.e., photoadaptation) among corals living at depth and other light-limited environments (Falkowski and Dubinsky, 1981; Porter et al., 1984). We cannot say whether our results reflect a light-compensation response or DIN availability. In both HL experiments (2011 and 2012), there was an apparent drawdown of DIN: nitrate and nitrite concentrations were reduced by an order of magnitude relative

to the original fill water conditions and the ammonium buildup in LL tanks did not occur in the HL tanks (Table 2). This was likely due to algal growth in HL tanks, whereas nutritional competition and ammonium recycling by external algal species was probably lower under LL conditions and therefore these nutrients were more available to the coral symbiont to supplement their photosynthetic machinery. However, the fact that symbiont densities were not significantly higher is an argument for photoadaptation over DIN enrichment: Smith and Muscatine (1986) and Muscatine et al. (1989) demonstrate that ammonium additions significantly increase symbiont densities in *Stylophora pistillata* both in situ and in experimental settings, with an increase in the fixed nitrogen content of symbiont tissue.

Despite lower photosynthetic capacity (i.e., chlorophyll *a* densities), HL corals still exhibited significantly greater total tissue lipid content (Fig. 6). Falkowski et al. (1993) propose that the addition of inorganic nitrogen disrupts the nutrient limitation imposed by the coral host on its algal symbionts, effectively allowing the zooxanthellae to retain their photosynthate for growth and division and reducing the amount transferred to the host coral, which the host could then metabolize to maintain proton pumping (Muscatine et al., 1989). Conversely, elevating light levels does not increase the availability of substrate for photosynthesis (i.e., CO_2) or symbiont structural materials (i.e., DIN), but, until saturating light levels are reached, it does provide additional energy to drive the photosynthetic process. Under continued host-imposed nutrient limitation, symbionts would be unable to utilize and retain this excess, carbon-rich photosynthate for growth and division, thus increasing the transfer to the coral host. Our tissue lipid results (Fig. 6) were consistent with this hypothesis, suggesting that HL corals are receiving additional carbohydrate resources. However, the fact that these corals were not significantly larger than corals reared under LL conditions indicates that this lipid material was not contributing to expanding tissue extent, but rather was being stored by the coral as a metabolic fuel reserve. This is consistent with several previous studies that found elevated concentration of storage lipid content in corals maintained under elevated light conditions (e.g., Stimson, 1987; Oku et al., 2003). Additional analysis of the lipid composition, specifically structural vs. storage lipid content, would help test this hypothesis.

Critically, total tissue lipid was not reduced under elevated CO_2 conditions (Fig. 6), which might be expected if the corals were actively utilizing metabolic resources to offset OA-driven impacts on calcification (e.g., Ries, 2011; McCulloch et al., 2012). Unfed, LL (effectively starved) corals may have had insufficient energetic reserves to invest in OA-compensation responses such as proton pumping or other active calcification accelerants. However, HL corals clearly had more metabolic resources (i.e., significantly higher total lipid content) than LL corals, yet there was no apparent, relative consumption of lipids under elevated CO_2 condition (Fig. 6). The importance of coral energetic reserves has been emphasized in the literature: corals with higher lipid content and those that are metabolically flexible generally have lower mortality risk and are better able to survive stress events such as bleaching (e.g., Rodrigues and Grottoli, 2007; Anthony et al., 2009). Wall et al. (2017) show reduced tissue lipid and energy content in *Pocillopora acuta* under elevated CO_2 but no significant reduction in calcification, suggesting a metabolic compensation mechanism for this particular coral species to maintain calcification under elevated CO_2 conditions. However, studies showing that heterotrophically fed corals do not appear to catabolize total lipid content to offset the impacts of CO_2 on calcification (Schoepf et al., 2013), despite maintaining significantly higher lateral growth than unfed corals (here; Drenkard et al., 2013), suggests that maintaining calcification rates may not be 1) a metabolic priority or even 2) an available mechanism for all scleractinian species (e.g., McCulloch et al., 2012). With regard to the first point, other calcifying organisms prioritize growth (i.e., increasing size) over $CaCO_3$ production (e.g., Krumhardt et al., 2017). Also, corals at early life history stages (this study) may have had less flexibility in allocating energetic resources to offsetting the impact of OA on calcification. Indeed, Spalding et al. (2017) hypothesize that larval and juvenile calcifiers may be most vulnerable to the increased energetic demands imposed by OA, which has crucial implications for future reef recruitment success. To the second point, if the specific process by which OA impairs *F. fragum* calcification is not mitigable by a mechanism entailing coral investiture of additional energy (e.g., Edmunds et al., 2016), we would not necessarily have expected to observe an effect on energetic reserves (such as total lipid content) and resultant alleviation of OA-impact on calcification under fed or HL conditions.

It is possible that our nutritional conditions were not high enough to elicit an energetically driven, CO_2-compensation response in coral calcification. We do not know, for instance, how the brine shrimp in our "fed coral" condition compared to plankton concentration and composition on the patch reefs, thus we may not have fully saturated *F. fragum* heterotrophic response. Furthermore, our "unfed" condition is probably ecologically unrealistic as it omitted naturally occurring, baseline zooplanktivory. Similarly, our HL conditions were approximately half the level of PAR reported for ambient patch reef conditions (de Putron et al., 2017). If our HL levels were much below saturating, we would have expected higher experimental light levels to elicit a stronger physiological response (e.g., greater total lipid content) than observed, which in turn may have provided sufficient autotrophically-derived energy to modulate calcification response to OA. Additional experiments, analyzing a broader range of realistically available coral nutrition for *F. fragum* would address these uncertainties. Studies investigating these response variables in other species of newly settled scleractinians would also clarify whether the implications derived in this study are applicable to other reef-building recruits, which has important implications for drivers of future reef species composition under OA.

5. Conclusions

Both heterotrophic feeding and elevated light levels elicited physiological responses among juvenile *F. fragum* in the form of significantly increased corallite size (feeding), and reduced chlorophyll *a* and increased tissue lipid content (light). However, under both forms of nutrition, area-normalized calcification still exhibited OA-sensitivity, and there was no indication that corals were actively investing energetic reserves (i.e., consuming total lipid content) to offset CO_2-induced reductions in $CaCO_3$ production. This suggests that maintaining calcification under OA may be a lower metabolic priority or an unavailable mechanism to this particular coral species and/or life stage and thus elevated nutritional status may not mitigate OA stress.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2018.07.007.

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