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NOTE

Symbiont carbon and nitrogen assimilation in the *Cassiopea–Symbiodinium* mutualism

Christopher J. Freeman[1,*], Elizabeth W. Stoner[2], Cole G. Easson[3], Kenan O. Matterson[3], David M. Baker[4]

[1]Smithsonian Marine Station, 701 Seaway Drive, Fort Pierce, Florida 34949, USA
[2]Loxahatchee River District, 2500 Jupiter Park Drive, Jupiter, Florida 33458, USA
[3]Department of Biology, University of Alabama at Birmingham, 1300 University Blvd, Birmingham, Alabama 35294, USA
[4]The Swire Institute of Marine Science, School of Biological Sciences & Department of Earth Science, University of Hong Kong, Hong Kong, PR China

ABSTRACT: Symbiotic interactions in the marine environment have long been represented by mutualisms between photosymbionts and benthic marine invertebrates like corals and sponges. Although 'upside-down' epibenthic jellyfish in the genus *Cassiopea* also derive a substantial metabolic benefit from abundant communities of the dinoflagellate symbiont *Symbiodinium*, comparatively little is known about the efficiency of carbon (C) and nitrogen (N) assimilation within the *Cassiopea* holobiont. Using standardized 6 h incubations with ^{13}C- and ^{15}N- enriched compounds, we assessed symbiont C and N assimilation in both oral arm and bell tissue of *C. xamachana* under light and dark conditions. Carbon fixation was light dependent and highest in the photosymbiont-rich oral arm tissue. In contrast, $^{15}NO_3$ assimilation was light independent in both tissue types and was highest in bell tissue that was sparsely colonized by photosymbionts. This, coupled with higher bell tissue ^{15}N enrichment under dark conditions, implicates non-photosynthetic microbes in *Cassiopea* N metabolism. This zonation of microbial activity may allow *C. xamachana* to simultaneously fix C and assimilate ambient or porewater N released during *Cassiopea* pumping activity. Although *C. xamachana* may utilize symbiont-derived N, lower ^{15}N enrichment relative to C fixation suggests that *Cassiopea* may also rely on exogenous sources of N for growth. This study provides initial evidence that the efficiency of symbiont metabolism within *Cassiopea* jellyfish is comparable to, or exceeds, that of other common benthic marine invertebrates, supporting the contention that *Cassiopea* have an important role in the productivity and nutrient dynamics within their local environment.

KEY WORDS: Jellyfish · Symbiosis · Photosymbionts · Nutrient dynamics · Stable isotopes · Carbon fixation · ^{13}C and ^{15}N tracers

INTRODUCTION

By hosting abundant symbiont communities, many eukaryotes supplement heterotrophic feeding with diverse microbial metabolic pathways, allowing for survival and growth in nutrient-limited ecosystems (Moran 2007). These symbioses have long been represented by the mutualism between dinoflagellate symbionts within the genus *Symbiodinium* and diverse host species (Baker 2003) on shallow tropical coral reefs. In these systems, photosymbiont carbon (C) fixation often exceeds the energy requirements of the host, fueling the growth and proliferation of reef-building corals, octocorals, and sponges (Muscatine

*Corresponding author: freemanc@si.edu

& Porter 1977, Weisz et al. 2010, Freeman et al. 2013, Baker et al. 2015).

In addition to these common members of reef communities, *Symbiodinium* plays an important role in the ecology and evolution of other marine invertebrates (Baker 2003). For instance, *Cassiopea* spp. jellyfish host an abundant *Symbiodinium* community within their oral appendages (Verde & McCloskey 1998). These 'upside-down' jellyfish orient their oral arms towards the surface to expose their photosymbionts to downwelling sunlight, while their aboral (bell) surface pulses against the benthos to generate water flow and draw out sediment-locked nutrients (Welsh et al. 2009, Jantzen et al. 2010). In exchange for a safe, nutrient-rich habitat, these photosymbionts may satisfy ~160% of the host's metabolic C demand (Verde & McCloskey 1998) and support a growth rate of about 3% d^{-1} (Welsh et al. 2009).

Assimilation of dissolved inorganic nitrogen (DIN) by *Cassiopea* spp. and other symbiotic jellyfish also implies that these photosymbiont communities play a role in holobiont (unit including both host and symbiont cells; Zilber-Rosenberg & Rosenberg 2008) N metabolism and cycling (Welsh et al. 2009, Baker et al. 2013). Under saturating irradiance, DIN was removed from the surrounding seawater by *Cassiopea* spp. (Welsh et al. 2009) and assimilated and incorporated into the biomass of *Linuche unguiculata*, another symbiotic scyphozoan (Wilkerson & Kremer 1992). This latter study used ^{15}N-labeled DIN to show that NH_4^+ was taken up over 10× faster than NO_3 (Wilkerson & Kremer 1992). Uptake (Jantzen et al. 2010) and assimilation (Wilkerson & Kremer 1992) of NH_4^+ under dark conditions, however, also implicates non-photosynthetic microbes (Pitt et al. 2009) or host cells in NH_4^+ assimilation (Grover et al. 2002). This, coupled with a recent report ascribing NO_x release from *Cassiopea* individuals held in the dark to the presence of nitrifying bacteria (Welsh et al. 2009), suggests that holobiont N metabolism within symbiotic scyphozoans is complex.

Despite this previous research, little is known about the efficiency of symbiont C and N assimilation in the *Cassiopea* spp. holobiont (Pitt et al. 2009). To address this, we isolated the autotrophic pathway by incubating *C. xamachana* for 6 h in seawater spiked with $NaH^{13}CO_3$ and $Na^{15}NO_3$ following standardized methods previously applied to sponges (Freeman et al. 2013) and octocorals (Baker et al. 2015). Because *Symbiodinium* abundance is heterogeneous throughout *Cassiopea* spp. (Verde & McCloskey 1998, Estes et al. 2003), we assessed ^{13}C and ^{15}N assimilation in regions of *C. xamachana* where both dense and sparse photosymbiont communities were observed. In addition, because non-photosynthetic microbes may be present within the *Cassiopea* spp. holobiont, and may contribute to the overall holobiont C and N metabolism (Verde & McCloskey 1998), we investigated C and N assimilation under both light and dark conditions. Finally, we also followed the fate of assimilated C and N over time by measuring the ^{13}C- and ^{15}N- enrichment in *C. xamachana* maintained under natural conditions for 6 h and 18 h after exposure to enriched compounds. We hypothesized that ^{13}C and ^{15}N enrichment would be light dependent, highest in the oral arms where *Symbiodinium* abundance is greatest, and would be highest in both tissue types immediately after the initial 6 h exposure to enriched compounds.

MATERIALS AND METHODS

Collections and processing

Small (~8–11 cm bell width) *Cassiopea xamachana* were collected from a shallow (2–3 m) *Thalassia testudinum* bed (9° 18′ 19.96″ N, 82° 10′ 18.48″ W) between Solarte and Bastimentos islands in the Bocas del Toro archipelago of Panama. All individuals were placed into a 20 l bucket containing seawater for transit and then transferred to a flowing seawater tank at the Smithsonian Tropical Research Institute Station, where they were allowed to acclimate for 24 h prior to experiments. After 24 h, 5 individuals were sacrificed for measurements of the natural abundances of C and N isotopes ($\delta^{13}C$ and $\delta^{15}N$).

While *Symbiodinium* are found in both the oral arms and bell tissue of *Cassiopea* spp. hosts, these symbionts are generally in higher abundance in the oral arms compared to the bell (74.5% reside in the oral arms and 25.5% in the bell; Verde & McCloskey 1998). To investigate C and N assimilation in tissues containing both high and low abundances of photosymbionts, we sampled the ends of 4 to 6 oral arms and the center of the bell, respectively. These tissue samples were taken by first carefully severing the manubrium with a razor blade to separate the oral arms from the bell, and then snipping the ends of the oral arms with scissors and removing the center of the bell with a razor blade. Preliminary chlorophyll *a* (chl *a*) analyses (as in Freeman et al. 2014) on samples collected as part of this study verified that abundant photosymbiont communities were largely restricted to the oral arms (978 [± 81 SE] µg chl *a* g^{-1} dry mass in the oral arm vs. 78 [± 16] µg chl *a* g^{-1} dry mass in the bell tissue).

Experimental setup

Before the start of the incubation, 1 l of filtered (Whatman GF/F) seawater amended with $NaH^{13}CO_3$ (98 atom percent [AP]^{13}C) and $Na^{15}NO_3$ (98 AP^{15}N) (as in Freeman et al. 2013, Baker et al. 2015) was added to each of 18 plastic containers (1.5 l capacity each), and these containers were placed into 2 seawater tanks (acting as constant temperature baths). To assess the role of photosymbionts in C and N assimilation within *C. xamachana,* 1 tank was exposed to ambient light ('light treatment'), while the other tank was covered by thick plywood, reducing light to <1% of ambient ('dark treatment'), as monitored by HOBO (Onset) data loggers deployed within both tanks over the course of the experiment. To begin the light/dark incubation, a single *C. xamachana* was added to each of the 9 containers in the dark treatment and 3 *C. xamachana* were added to each of the 9 containers in the light treatment (see below). After 6 h, a single *C. xamachana* was removed from each of the 9 containers under both light and dark treatments and processed as above to assess the ^{13}C and ^{15}N enrichment in each tissue type. After this initial 6 h incubation, plastic tubing was used to supply a constant flow of seawater to each of the 9 plastic containers (now each holding 2 *C. xamachana* individuals) within the light treatment, effectively flushing out the enriched incubation water. To follow the fate of the assimilated ^{13}C- and ^{15}N-labeled compounds over time, a single individual was removed from each of these containers after 6 h and 18 h and processed as above.

Stable isotope analysis

Tissue samples were prepared for isotope analyses, and stable isotope compositions were measured at the Stable Isotope Ratio Mass Spectrometry laboratory at the University of Hong Kong, as in Freeman et al. (2014). Natural abundance isotope values in tissues from wild *C. xamachana* and enriched experimental samples are expressed as atom percent (AP^{13}C and AP^{15}N) using equations outlined in Fry (2006) and Freeman et al. (2015). Isotope values are also expressed in delta (δ) notation in units per mil (‰) to compare isotopic composition between the current study and Freeman et al. (2013) and Baker et al. (2015). Precision of δ^{13}C and δ^{15}N was 0.02‰ and 0.6‰, respectively, as determined by repeated analysis of the internal acetanilide standard ('acet 6'; 70% C).

Data analysis

To test for differences in AP^{13}C and AP^{15}N values in each tissue type across treatments and between treatment and natural abundance samples, we used a general linear model (GLM). Pairwise comparisons of treatment effects were conducted using the Fisher's least significant difference (LSD) post hoc test. To test for differences in AP^{13}C and AP^{15}N values between bell and oral arm tissue of *C. xamachana* within each treatment, we used paired *t*-tests. Prior to analyses, residuals were tested for normality and homogeneity of variance. All data analyses were carried out using Systat (v.11).

RESULTS

We found substantial variation in ^{13}C and ^{15}N enrichment of the bell (general linear model [GLM]: $F = 28.8$, $p < 0.05$ and $F = 177.9$, $p < 0.05$ for ^{13}C and ^{15}N, respectively) and oral arm (GLM: $F = 113.0$, $p < 0.05$ and $F = 58.8$, $p < 0.05$ for ^{13}C and ^{15}N, respectively) tissues across treatments (Fig. 1 and the Appendix).

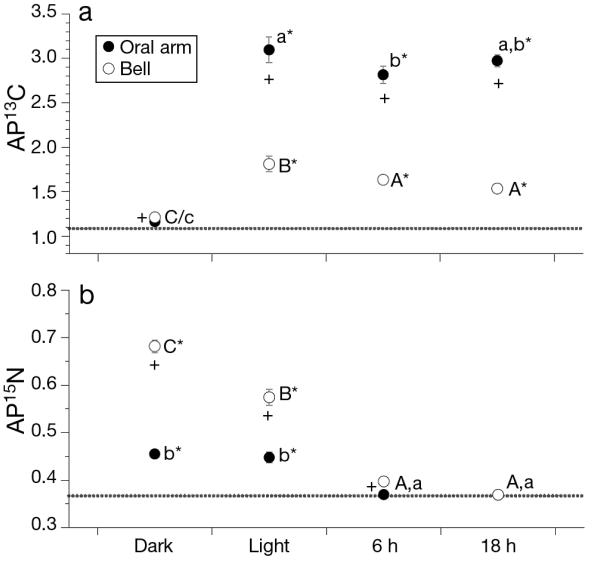

Fig. 1. Mean (±SE) (a) atom percent (AP)^{13}C and (b) AP^{15}N values in bell (open circles) and oral arm (filled circles) tissue from *Cassiopea xamachana* individuals incubated for 6 h under light and dark conditions, and individuals maintained under natural conditions for 6 and 18 h after exposure to enriched compounds. The dotted line indicates the AP^{13}C or AP^{15}N value in natural abundance samples. Letters indicate significant differences across treatments in bell (upper case letters) or oral arm (lower case letters) tissue; *indicates significant enrichment relative to natural abundance tissue samples; + indicates a significant difference between bell and oral arm tissues within a single treatment. N = 9 for all experimental samples, and N = 5 for natural abundance samples

Although there was some assimilation of ^{13}C label in the dark (Appendix), $AP^{13}C$ values of both tissue types from the dark treatment were similar to natural abundance values (GLM with LSD multiple comparisons test: $p > 0.05$; Fig. 1a), and were significantly lower than the $AP^{13}C$ values under all 3 light treatments (GLM with LSD multiple comparisons test: $p < 0.05$; Fig. 1a). $AP^{13}C$ values were significantly higher in oral arm tissue than bell tissue across all light treatments (paired *t*-test: $p < 0.05$), but were higher in the bell under dark conditions (paired *t*-test: $p < 0.05$). Although ^{13}C enrichment was generally highest in individuals sampled right after the initial 6 h exposure, both fractions remained enriched relative to natural abundance samples, even after 18 h (Fig. 1a).

In contrast, $AP^{15}N$ values of both tissue types from the dark treatment were significantly higher than initial, natural abundance values, and $AP^{15}N$ values of the bell tissue were highest in the dark treatment (GLM with LSD multiple comparisons test: $p < 0.05$; Fig. 1b). $AP^{15}N$ values were elevated in the bell tissue relative to the oral arms in the dark treatment, following the initial 6 h exposure period, and after 6 h (paired *t*-test $p < 0.05$) in flowing seawater. Unlike $AP^{13}C$, the $AP^{15}N$ values from both tissue types were similar to natural abundance values just 6 h post-enrichment (Fig. 1b).

DISCUSSION

We provide evidence supporting the contention that photosymbionts (i.e. *Symbiodinium*; LaJeunesse 2002, Coffroth & Santos 2005, Thornhill et al. 2006) within the oral arms and bell tissue of *Cassiopea xamachana* are capable of efficient C fixation. Enrichment of oral arm tissue was striking (mean ± SE $\delta^{13}C$ values of ~1850 ± 137‰; Appendix) and was over 6× greater than the average photosymbiont-hosting Caribbean sponge (range of ~100–800‰; average of ~300‰; Freeman et al. 2013) and octocoral (range of <100–500‰, average of ~220‰; Baker et al. 2015). Even bell tissue was enriched ($\delta^{13}C$ values of ~650 ± 82‰) relative to most members of these other functional groups, suggesting that *C. xamachana* holobionts from our study are functioning as autotrophs under ambient light conditions and are fixing more C than is required for respiration alone (Verde & McCloskey 1998). Indeed, with a daily gross photosynthesis to respiration ratio of 2.04 (Welsh et al. 2009), photosymbiont productivity in *Cassiopea* is well above the compensation point of 1.0 and higher than that of Caribbean sponges and octocorals (Freeman et al. 2013, Baker et al. 2015).

By hosting symbionts, *C. xamachana* holobionts are able to assimilate $^{15}NO_3$ (Wilkerson & Kremer 1992, Pitt et al. 2009, Niggl et al. 2010). In fact, after 6 h, *C. xamachana* were as enriched as, or more enriched than, some photosymbiont-hosting sponge species (*C. xamachana* $\delta^{15}N$ values of ~200–800‰ [Appendix] vs. sponge range of ~60–2300‰; average of ~600‰; Freeman et al. 2013). Interestingly, although the assimilation of NH_4^+ and NO_x by symbiotic scyphozoans is commonly ascribed to photosymbiont metabolism (Wilkerson & Kremer 1992, Jantzen et al. 2010, Niggl et al. 2010), light-independent ^{15}N enrichment in both the bell and oral arm tissue in our study also implicates non-photosynthetic microbial taxa in *C. xamachana* N metabolism (Welsh et al. 2009, Jantzen et al. 2010). While dark assimilation by photosymbionts may account for some of this enrichment, efficient dark $^{15}NO_3$ assimilation in the bell tissue, where photosymbiont abundance is lowest (Verde & McCloskey 1998), further supports the role of non-photosynthetic microbes in *C. xamachana* N metabolism.

Our enrichment data suggest a zonation of microbial activity within the *C. xamachana* holobiont, pairing abundant and productive photosymbiont communities (Verde & McCloskey 1998, Estes et al. 2003) in the oral arms with microbes capable of efficient N metabolism in the bell tissue. With photosymbiont-derived C potentially fueling the assimilation of ambient or porewater N released during *Cassiopea* pumping activity (Verde & McCloskey 1998, Welsh et al. 2009, Jantzen et al. 2010), this zonation may optimize nutrient acquisition in oligotrophic waters. Although nitrifying bacteria within *Cassiopea* have been implicated in the release of NO_x in the dark (Welsh et al. 2009), investigations into the *Cassiopea* microbiome and the distribution of microbial groups within and on the surface of a *Cassiopea* individual are certainly warranted to better elucidate the metabolic pathways available to the host (Pitt et al. 2009) and to better understand the potential coupling between photosymbiont-derived C and transformations of N in the bell and animal–sediment interface.

With low levels of $^{15}NO_3$ assimilation relative to ^{13}C fixation in the current study and a low total N (NH_4^+ and NO_x) uptake rate relative to photosynthetic rate (Welsh et al. 2009), *Cassiopea* might rely on exogenous sources of N such as zooplankton or dissolved organic N via predation for growth (Wilkerson & Kremer 1992, Pitt et al. 2009, Welsh et al. 2009). In addition, the loss of ^{15}N signal only 6 h after the initial in-

cubation implies that microbially derived ^{15}N atoms are rapidly processed within the *C. xamachana* holobiont. Although the loss of DIN from *Cassiopea* is negligible (Pitt et al. 2009, E. Stoner unpubl. data), the ^{15}N signal may have been diluted by heterotrophic feeding on natural sources of N in the incubation containers once flowing seawater was added, the recycling of host-derived N in the *Cassiopea–Symbiodinium* symbiosis (Pitt et al. 2009), or the loss of ^{15}N-labeled organic matter in mucus (Niggl et al. 2010). In contrast, the strong ^{13}C signal in both bell and oral arm tissue remained or was only slightly diminished, even after 18 h in flowing seawater. While this certainly supports the contention that photosymbiont-derived C is integrated into *C. xamachana* holobiont biomass and is a dominant source of C to these jellyfish, additional research is needed that follows the integration and loss of ^{13}C and ^{15}N over shorter (1–5 h) and longer (days) time periods to better understand C and N processing within *C. xamachana*.

Trends in ^{13}C and ^{15}N enrichment between the bell and oral arm tissue may reflect the rapid translocation of biomolecules from symbionts concentrated in the oral arms to host cells in the bell tissue (Verde & McCloskey 1998). However, because this translocation would also be reflected in the continued transfer of ^{13}C- and ^{15}N-labeled compounds after the initial incubation, leading to diminished enrichment in the oral arm tissue and higher enrichment in the bell tissue, we suggest that this is unlikely. Additional studies investigating the transfer of ^{13}C- and/or ^{15}N-labeled compounds from symbiont to host are certainly warranted. Unfortunately, although previous studies have successfully isolated *Symbiodinium* (Verde & McCloskey 1998, Stat et al. 2008) or *Symbiodinium* and animal cells (Wilkerson & Kremer 1992) from symbiotic scyphozoans via homogenization followed by centrifugation, the organic matter content of these organisms is low (< ~3%; Pitt et al. 2009), and previous research using these methods found no indication of the translocation of ^{15}N-labeled compounds, even after 33 h (Wilkerson & Kremer 1992). Future studies coupling ^{13}C- and ^{15}N-enriched compounds with higher-resolution technology like NanoSIMS may better elucidate this transfer (Kopp et al. 2013).

This study provides initial evidence that symbiont metabolism within *C. xamachana* is comparable to other common benthic marine invertebrates, supporting the contention that *Cassiopea* holobionts have an important role in the productivity and nutrient dynamics of their local environment. As a common member of shallow, benthic communities in tropical and subtropical areas worldwide, with locally high densities in some areas (Jantzen et al. 2010, Niggl et al. 2010, Stoner et al. 2011), *Cassiopea* may drive benthic photosynthetic oxygen production and act as a sink for local inorganic nutrients (Welsh et al. 2009). While *Cassiopea* consume organic sources of N, members of their symbiont community also allow for DIN assimilation, potentially contributing to higher growth rates in response to elevated nutrients (E. Stoner unpubl. data) and their proliferation and larger size in areas in close proximity to human populations and anthropogenic inputs (Stoner et al. 2011). Finally, through the release of copious amounts of mucus, symbiont-derived nutrients may ultimately act as a trophic link between benthic and pelagic food webs in these systems (Verde & McCloskey 1998, Niggl et al. 2010).

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Appendix

Mean (± SE) isotope values in delta notation ($\delta^{13}C$ and $\delta^{15}N$) and atom percent ($AP^{13}C$ and $AP^{15}N$) from *Cassiopea xamachana* oral arm and bell tissue from initial (natural abundance) and experimental conditions (individuals incubated for 6 h under light or dark conditions and individuals maintained under natural conditions for 6 and 18 h after exposure to enriched tracers)

	$\delta^{13}C$		$\delta^{15}N$		$AP^{13}C$		$AP^{15}N$	
	Oral arm	Bell	Oral arm	Bell	Oral arm	Bell	Oral arm	Bell
Initial	−18.2 (0.37)	−16.8 (0.39)	0.14 (0.22)	2.48 (0.18)	1.09 (<0.01)	1.09 (<0.01)	0.37 (<0.01)	0.37 (<0.01)
Dark	53.1 (3.27)	96.9 (11.9)	243.8 (13.5)	867.9 (36.5)	1.16 (<0.01)	1.21 (0.01)	0.46 (<0.01)	0.68 (0.01)
Light	1859.7 (136.8)	650.0 (81.9)	223.6 (30.5)	571.9 (45.6)	3.1 (0.14)	1.81 (0.09)	0.45 (0.01)	0.57 (0.02)
6 h	1593.0 (90.4)	486.0 (33.6)	9.3 (0.43)	84.8 (14.7)	2.82 (0.10)	1.63 (0.04)	0.37 (<0.01)	0.40 (<0.01)
18 h	1742.0 (63.9)	394.9 (43.6)	7.28 (0.39)	8.37 (0.87)	2.97 (0.07)	1.54 (0.05)	0.37 (<0.01)	0.37 (<0.01)

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