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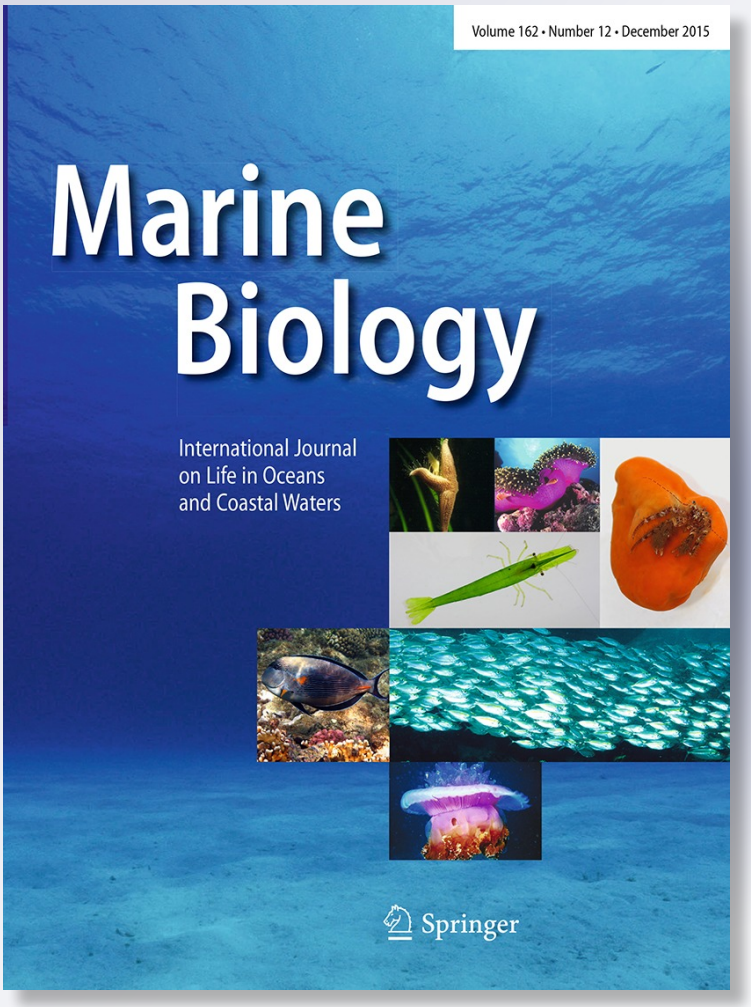

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FEATURE ARTICLE

Nitrogen transfer in a Caribbean mutualistic network

C. E. Cantrell[1] · R. P. Henry[1] · N. E. Chadwick[1]

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Abstract Coral reef symbioses are well-established models for studying multi-level networks of species interactions that provide nutritional benefits to partners. While the contributions of endosymbionts to cnidarian hosts have been extensively documented, relatively little is known about how exosymbionts contribute to nutrient cycling in coral reef cnidarians. We investigated exosymbiotic sources of ammonia and their contributions to physiological processes in Caribbean corkscrew sea anemones *Bartholomea annulata*. In laboratory experiments, anemones absorbed ammonia from seawater, which significantly enhanced the mitotic index of their endosymbiotic microalgae *Symbiodinium*, while anemone shrimp presence alone had no effect. Anemone shrimps excreted ammonia at much slower rates than anemones were able to absorb it, indicating that shrimp alone were not able to meet host nitrogen demand. Client fishes excreted ammonia ~10× more rapidly than did associated shrimps. On Caribbean coral reefs, anemone shrimps attract diverse client fishes through their parasite-cleaning behavior; these fishes excrete substantial ammonia near anemones while being cleaned. Exosymbiotic anemone shrimps thus may provide nutritional benefits to host anemones and microalgae indirectly, through their attraction of nitrogen-excreting fishes. This multi-level mutualistic network facilitates tight nutrient cycling among diverse species belonging to several phyla on coral reefs. While we assessed the rates and benefits of nutrient transfer under laboratory conditions, further research is needed to quantify the contributions of ammonia and other nutrients from client fishes through cleaner shrimps to host sea anemones in the field.

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Introduction

Coral reefs are highly productive ecosystems that contribute to local economies around the world and support levels of biodiversity comparable to those in tropical rainforests (Hubbell 1997). Questions remain about how they achieve the high primary productivity necessary for support of this biodiversity, especially in nutrient-poor environments with low levels of available nitrogen in the surrounding water column (<1 μM/L, Muscatine and Porter 1977). Examination of the tight nutrient cycling available within reefs through symbioses is one way of understanding how reef systems function as biodiversity hotspots even though they are surrounded by waters which contain almost no readily available nutrients (Linton and Warner 2003).

Symbiosis supports tight nutrient cycling due to intimate physical contact among partners. The major symbiosis on coral reefs that facilitates reef growth is the association between scleractinian corals and endosymbiotic microalgae *Symbiodinium* (zooxanthellae). Reef corals depend on the microalgae for most of their energy needs and for support of rapid skeletogenesis (Muscatine 1980); the nitrogen-limited microalgae in turn depend on their hosts for a constant supply of inorganic nutrients (Lee and Childress 1994). Nitrogen in the form of ammonia is readily taken up by endosymbiotic microalgae to synthesize glycerol, glucose, and amino acids among other compounds, which are translocated to host cnidarian storage pools for protein, lipid,

and carbohydrate synthesis (Lipshultz and Cook 2002). The highly competitive environment on coral reefs, where space and nutrients are limiting, causes many other types of benthic reef organisms to form symbioses (Trench 1979). Many soft-bodied cnidarians such as giant sea anemones host both microalgal endosymbionts and an array of obligate exosymbionts, primarily crustaceans and fishes, which provide an array of benefits to the host, including defense from predators (Godwin and Fautin 1992; Porat and Chadwick-Furman 2004; McCammon and Brooks 2014), ventilation services (Szczebak et al. 2013), and stimulation of tentacle expansion (Huebner et al. 2012). In particular, anemonefishes provide a constant stream of ammonia to host sea anemones (Roopin et al. 2008; Cleveland et al. 2011), which is utilized for host growth and reproduction (Holbrook and Schmitt 2005), as well as enhancement of symbiotic algal growth (Roopin and Chadwick 2009). The resulting increase in anemone body size creates habitat for occupation by more numerous and larger fish symbionts (Schmitt and Holbrook 2000; Hattori 2005; Ricciardi et al. 2010).

Invertebrate exosymbionts such as barnacles and mussels also release inorganic waste products, mainly nitrogen and phosphorous, that are absorbed and utilized by the microalgae inside host coral tissues (Achituv and Mizrahi 1996; Mokady et al. 1998), likely leading to enhancement of host growth and provision of habitat space to the invertebrates. These mutually beneficial nutrient exchange relationships among diverse exosymbionts (fishes, crustaceans, molluscs), endosymbionts (microalgae) and host cnidarians (sea anemones, corals) may create positive feedback loops (diagrammed in Roopin et al. 2011; Huebner and Chadwick 2012a) that perpetuate multi-level symbiotic networks through evolutionary time. The contribution of exosymbionts to the cnidarian-zooxanthella symbiotic system has been understudied and is an important added dimension to the complexity of this mutualism which is central to coral reef formation and diversity. The assumption that close physical contact with exosymbionts results in guaranteed effective nutrient transfer to host cnidarians (and their algae) is based almost entirely on information collected on Indo-Pacific coral reefs. Alternate mechanisms and pathways may facilitate nutrient exchange on reefs in other geographic regions, but are not well understood.

In the Caribbean Sea, corkscrew sea anemones *Bartholomea annulata* are common reef inhabitants that host several types of obligate crustacean exosymbionts including spotted cleaner shrimp *Pedersoni yucatanicus* and Pederson's cleaner shrimp *Ancylomenes pedersoni* (Mahnken 1972; Briones-Fourzán et al. 2012). The latter functions as a key reef organism by removing parasites from a wide variety of Caribbean reef fishes (Bunkley-Williams and Williams 1998; Wicksten 1998; Sikkel et al. 2004; McCammon et al. 2010; Huebner and Chadwick 2012b). These shrimp are ammonotelic, and their constant excretion of ammonia (designated here as NH_3 but including both the gaseous and ionic form NH_4^+) potentially contributes to the nitrogen budget of host sea anemones. *P. yucatanicus* is commonly termed a cleaner shrimp; however, it does not appear to remove fish parasites (McCammon et al. 2010). The NH_3 excretion rate of *P. yucatanicus* has been determined (Spotte 1996), but neither the excretion rate of *A. pedersoni* nor the level of benefit from NH_3 excretion by either shrimp to the anemone host and microalgae is known. Individuals of *A. pedersoni* attract a steady stream of client fishes belonging to >16 families, which wait for cleaning services and then hover adjacent to host anemones (within a few cm) while the anemone shrimp remove their parasites (Huebner and Chadwick 2012a, b). The fishes attracted to these cleaning stations likely excrete large quantities of ammonia into the water column surrounding the anemones; however, the relative contribution of this additional nutrient source to the anemone–microalgal system also remains unknown.

Here, we quantify under laboratory conditions the benefits to host sea anemones *B. annulata* and their endosymbiotic microalgae *Symbiodinium* (clade C1, LaJeunesse 2002) from the nitrogen inputs provided both directly (through shrimp excretion) and indirectly (through the excretion of attracted client fishes) by the anemone shrimps *P. yucatanicus* and *A. pedersoni*. Objectives of the study were to: (1) determine rates of ammonia excretion by cleaner shrimps and selected client fishes and compare them with rates of ammonia uptake by sea anemones and (2) assess physiological benefits to the host anemones and their microalgae. We also discuss how the results reveal potential evolutionary drivers of this complex multi-level mutualistic network, and the mechanisms through which it functions ecologically.

Methods

Animal collection and maintenance

Corkscrew sea anemones *B. annulata*, spotted cleaner shrimp *Periclimenes yucatanicus*, and Pederson's cleaner shrimp *A. pedersoni* were collected by hand from nearshore habitats at 0–3 m depth along the upper and middle Florida Keys. They were placed in plastic bags of seawater, transferred to insulated plastic containers filled with aerated seawater changed approximately every 12 h, and transported to Auburn University within 1–3 days. Juvenile bicolor damselfish *Stegastes partitus*, yellowtail damselfish *Microspathodon chrysurus*, and longfin damselfish *Stegastes dienceaous* were ordered from a commercial

collector (Dynasty Marine, Marathon, FL) and shipped live to Auburn University. Damselfishes were used because they are common client fishes of Pederson's cleaner shrimp (Cheney and Coté 2001; Wicksten 1998; Huebner and Chadwick 2012b); juvenile fish were chosen as particularly amenable to laboratory studies because they had small body sizes (5–15 cm total length) and were omnivores, making them relatively easy to culture in closed-system laboratory aquaria, similar to other damselfishes previously cultured in our laboratory (Roopin and Chadwick 2009; Huebner et al. 2012).

For at least 1 month prior to laboratory experiments, all organisms were maintained in 80-L saltwater aquarium tanks connected to 80-L sumps. Lights were kept on 12:12 h timers, and salinity (34–36 ppt) and temperature (25–26° C) were monitored daily. Tanks were illuminated with 400 W Radium Metal Halide Lamps (Ocean Light 250, AquaMedic). Sea anemones were exposed to photosynthetically active radiation (PAR) at 66–73 μE m^{-2} s^{-1}, within the range of irradiance levels in their natural reef habitats (Nelsen 2008), as measured using a QSL-2001 Scalar PAR Sensor (Biospherical Instruments, San Diego, CA, USA; for details on culture regime, see Roopin and Chadwick 2009; Huebner et al. 2012). Nutrient levels in tank water were monitored regularly, and concentrations of NH_4^+, NO_2^-, and NO_3^{-2} were maintained at low levels (<0.5, 0.01, and 0.1 μmol L^{-1}, respectively) to mimic coral reef conditions (after Johannes et al. 1983). All shrimps and fishes were fed daily on a mixed diet of Formula One Marine Pellets (Ocean Nutrition San Diego, CA, USA) and a thawed slurry of frozen foods (Mysis Shrimp, Brine Shrimp, Emerald Entrée, and Marine Cuisine; San Francisco Bay Brand, Newark, CA, USA). Anemones were fed weekly with chopped raw cocktail shrimp (after Szczebak et al. 2013).

Due to the presence of their endosymbiotic microalgae, the anemones were able to withstand extended periods of starvation (Battey and Patton 1987). Thus, to ensure that they began experiments without large energy or nutrient reserves and were in a similar basal metabolic state so they potentially would respond to nutrient treatments, for an additional 1 month (following 1 month of initial regular laboratory culture) the anemones were starved and cultured separately from exosymbionts (after Godinot and Chadwick 2009; Roopin and Chadwick 2009). During regular laboratory culture (normal feeding regime, see above), all organisms were measured frequently; almost all (>95 %) appeared healthy and either maintained their original body sizes or grew.

Rates of ammonia excretion and uptake

To quantify rates of ammonia excretion by shrimps and fishes and of ammonia uptake by anemones, we used the phenol–hypochlorite method of Solorzano (1969) and protocols from Spotte (1996). Glass beakers (Fischer Scientific) were rinsed with 10 % HCl and then with microfiltered saltwater (MFSW, 0.45 μm) and placed in a polystyrene tub with heated water (25–26° C) and a water circulation pump. To measure shrimp excretion rates, each individual [*P. yucatanicus*: wet body mass = 0.27 ± 0.02 g (mean ± SE), range = 0.12–0.36 g, $N = 10$; *A. pedersoni:* 0.13 ± 0.02 g, range = 0.06–0.25 g, $N = 12$] was placed in a beaker with 250 mL of MFSW water within 2 h after feeding. All experiments included an empty beaker of MFSW in which the change in ammonia level was <1.0 % of maximum ammonia levels recorded in the beakers with shrimps, so adjustments were not made to compensate for atmospheric loss or bacteria utilization of ammonia. Water samples (5 mL) were taken from the beakers every 40 min for 2 h and immediately prepared for ammonia analysis (for methods, see Solorzano 1969; Roopin et al. 2008). To quantify rates of ammonia excretion by fishes, individuals of *S. partitus* (wet body mass = 5.6 ± 1.0 g, range = 1.9–9.6 g, $N = 8$), *M. chrysurus* (5.1 ± 0.4 g, range = 4.3–5.9 g, $N = 4$), and *S. diencaeus* (5.1 ± 0.5 g, range = 4.2–5.7 g, $N = 3$) each were incubated separately in 750-mL beakers of MFSW, with water samples taken every 20 min for 1 h. Sample intervals were more frequent than for shrimps because the fishes were larger and produced more ammonia per unit time. Excretion was measured for fishes both when starved (24 and 48 h without food) and fed (2 h after feeding, after Roopin et al. 2008). All excretion rates were measured during the daytime in aerated beakers (supplied with airstone bubbler) exposed to overhead ceiling lights in the laboratory.

Rates of ammonia uptake by sea anemones (wet body mass = 14.9 ± 3.0 g, range = 2.7–35.4 g, $N = 39$) also were measured during the daytime, because their microalgae photosynthesized and thus absorbed nitrogen during the day (Muscatine and D'Elia 1978; Wilkerson and Muscatine 1984; Lipschultz and Cook 2002). Light levels during ammonia uptake measurements for sea anemones were enhanced using halogen lamps suspended over the water bath, so that anemones were exposed to coral reef irradiance levels as described above. Each anemone used for the excretion effects experiment (see details below) was examined for ammonia uptake rate within 1 week after the experiment ended. Due to their sessile nature, sea anemones were placed individually in 1 L of MFSW in beakers and then allowed 45–60 min to attach their basal disks to the beaker wall and expand their tentacles. Water samples of 5 mL were taken from each beaker every 40 min for 2 h and immediately processed for quantification of total ammonia (Solorzano 1969). Anemone body size was determined a few days after the uptake measurements, by first measuring the length (L) and width (W) of

both the tentacle crown and oral disk with calipers, while the anemone was in its home tank, taking care to avoid contact and tentacle contraction. The tentacle crown surface area (TCSA) and oral disk surface area (ODSA) of each anemone were calculated using the equation for an oval (TCSA = 91.1 ± 17.7 cm^2, range = 25.5–496.7 cm^2; ODSA = 5.6 ± 1.8 cm^2, range = 1.7–10.9 cm^2, $N = 39$; after Hirose 1985; Hattori 2005; Mitchell and Dill 2005; Ricciardi et al. 2010; Huebner and Chadwick 2012a). Then, each anemone was manipulated gently by hand to induce contraction, removed from its home tank, blotted to remove excess water, and weighed on an electronic balance to obtain wet mass. Each anemone was exposed to air for <2 min; upon return to their home tanks, all anemones re-expanded and behaved normally within 4 h, indicating no long-term stress from the weighing procedure (after Roopin and Chadwick 2009). Anemone wet mass increased exponentially with both TCSA ($y = 01.84x^{0.47}$, where y = wet mass in g and x = TCSA in cm^2, $r^2 = 0.35$, $p < 0.05$) and ODSA ($y = 4.28x^{0.69}$, where y = wet mass in g and x = ODSA, $r^2 = 0.25$, $p < 0.05$; after Chadwick-Furman et al. 2000). Nitrogen excretion and uptake are reported as both mass-specific and whole-individual rates per min and per h, to facilitate among-species physiological comparisons (Spotte 1996; Porat and Chadwick-Furman 2004; Roopin et al. 2008), and discussion of ecological processes over varying timescales (Meyer and Schultz 1985; Huebner and Chadwick 2012b).

Effects of anemone shrimp excretion on sea anemones

To determine potential impacts of nitrogen excretion by shrimps on anemones, a laboratory experiment was conducted in which anemones were assigned randomly to each of nine culture tanks (tank details given above), and then each three tanks (4–5 anemones per tank) were assigned randomly to 1 of 3 treatments for 6 week ($N = 13$ anemones per treatment × 3 treatments = 39 anemones total): (1) shrimps: cultured with at least one individual of *P. yucatanicus* and 3 of *A. pedersoni* (based on social group sizes per anemone in natural populations, Mahnken 1972; Briones-Fourzán et al. 2012), (2) nutrients: cultured with supplements of NH_4Cl-spiked seawater (see details below), or (3) neither: control treatment, cultured with neither shrimps nor nutrient supplements (modified after Roopin and Chadwick 2009).

The NH_4^+ supplements (~30 µM) were administered 3× per week (about every other d) for 6 week, with each enrichment period lasting 1.5–2 h. This method differed somewhat from Roopin and Chadwick (2009), in which individuals of the sea anemone *Entacmea quadricolor* were placed in nutrient treatments (~10 µM) every day, because individuals of *B. annulata* are more delicate in body form, with more slender, longer columns, and elongated tentacles. Here, the nutrient treatments were reduced in frequency to every other day based on preliminary observations, to avoid damage to the delicate body structure of these anemones from frequent moving and disturbance. Ammonia supplement concentration was increased to compensate for the relative infrequency of nutrient baths. To control for effects of moving anemones to and from the nutrient enrichment containers, every other day the anemones in all treatments were removed from their tanks and each placed into a 1-L plastic container that floated inside the tank. Anemones in the shrimp and control treatments were exposed to MFSW with no added nutrients, while anemones in the nutrient treatment were placed in MFSW spiked to a concentration of 30 µM NH_4Cl.

Measurement of physiological parameters in sea anemones

The following parameters were measured at the start and end of the 6-week experiment: (1) the body size (TCSA and ODSA, as described above) of each whole anemone, (2) animal protein content within the anemone tentacles, and (3) the abundance, chlorophyll a content, and mitotic index of *Symbiodinium* microalgae in the tentacles (after Wilkerson et al. 1983; Kuguru et al. 2007; Roopin and Chadwick 2009). To determine physiological parameters in the anemone tentacles, tissue samples were obtained by removing five tentacle tips from each anemone (each tip 1–2 cm length and 0.012–0.025 g wet mass). The samples were obtained from haphazardly selected long tentacles attached to the inner oral disk, avoiding the smaller outer tentacles, with no single tentacle sampled twice, to enhance ease of collection and minimize damage to the anemones. The tentacle tips from each anemone were homogenized in 1 mL of MFSW and centrifuged at 5 g for 5 min at ~24° C, following a standard protocol for cnidarian tissue and algal separation (see below). Supernatant containing the homogenized animal tissue was saved for immediate protein analysis. The remaining pellet of microalgal cells was vortexed thoroughly before being diluted for cell counts, mitotic index, and chlorophyll analysis (after Porat and Chadwick-Furman 2005; Roopin et al. 2011). Analyses of protein content in the animal fraction of the tentacle samples were performed using the Bradford method (BioRAD Quick start, after Roopin et al. 2011). Serial dilutions were made for the standard curve using instructions provided with the Bio-RAD kit. Protein samples were analyzed immediately after tentacle homogenization (5000 rpm for 5 min) at 590 nm using a Genesys 5 spectrophotometer.

To assess microalgal cell abundances in the tentacles, five subsamples of each diluted algal slurry were taken, and *Symbiodinium* cells were counted using a Hausser

Scientific hemacytometer under 400× magnification using a phase contrast microscope. Preliminary 24-h studies of *B. annulata* revealed no clear diel pattern of algal cell division, similar to many Caribbean corals (Wilkerson et al. 1983), but unlike *E. quadricolor* and some other sea anemones (Wilkerson et al. 1988; Roopin et al. 2011). Therefore, tentacle samples were taken haphazardly between the hours of 10:00 and 16:00. Mitotic index was calculated as a percentage of doubling cells per 1000 cells. Doubling times and growth rates were calculated using equations in Wilkerson et al. (1983).

Chlorophyll analysis also was performed on the resuspended algal pellet; chlorophyll a was extracted using 90 % acetone solution chilled to 4 °C for 24 h. The resulting slurry was later centrifuged at 5 g for a minimum of 5 min or until all debris was pelleted out. The supernatant was extracted and analyzed using a Genesys 5 spectrophotometer at 630, 664, 690, and 750 nm (after Roopin and Chadwick 2009). An acetone blank was used to zero the spectrophotometer before each measurement was taken. Chlorophyll a content was quantified using equations from Jeffery and Humphries (1975).

Statistical analyses

All statistical analyses were conducted using R 3.0.2. Variation in excretion rates between the two shrimp species was analyzed with Mann–Whitney–Wilcoxon tests, while fish excretion rates were analyzed using repeated measures ANOVA, with time as a repeated variable, because they were assessed at three times since feeding. Data from the laboratory experiment were analyzed using repeated measures ANOVA, with time as a repeated variable for all treatments. Data were examined for homogeneity of variance prior to application of parametric tests. Tukey's post hoc tests were utilized to identify which groups differed, and differences between groups were considered significant at $p < 0.05$ for all statistical tests. All results are presented as means ± 1 standard error unless otherwise noted.

Results

Ammonia excretion by shrimps and fishes

Fed individuals of the spotted cleaner shrimp *P. yucatanicus* ($N = 10$) excreted ammonia at a rate of 3.44 ± 0.61 μM g^{-1} h^{-1} (range = 1.03–5.96), about 3× as rapidly on a mass-specific basis as did fed individuals of Pederson's cleaner shrimp *A. pedersoni* ($N = 12$), which excreted at a rate of 1.16 ± 0.12 μM g^{-1} h^{-1} (range = 0.74–1.72, Mann–Whitney *U* test, $U = 8.81$, $p < 0.01$, Fig. 1). Individuals of *P. yucatanicus* also were about twice as large as those of *A. pedersoni* (mean wet body mass = 0.27 vs. 0.13 g, see Methods), so on a whole-individual basis they excreted approximately 6× the total amount of ammonia per unit time as did *A. pedersoni* (0.93 μM h^{-1} or 0.015 μM min^{-1} vs. 0.15 μM h^{-1} or 0.003 μM min^{-1}, respectively).

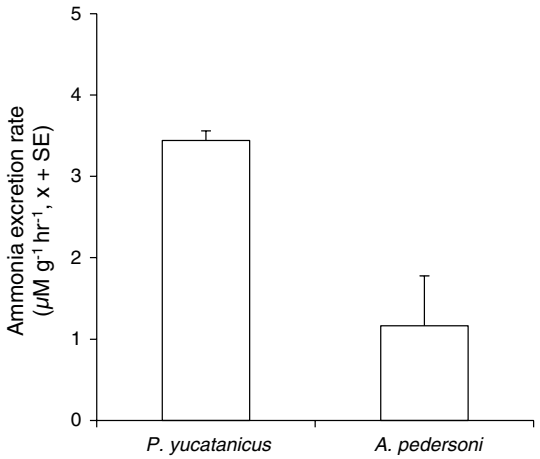

Fig. 1 Variation in rates of ammonia excretion between two species of obligate anemone shrimps: spotted cleaner shrimp *P. yucatanicus* ($N = 10$) and Pederson cleaner shrimp *A. pedersoni* ($N = 12$)

Rates of ammonia excretion by bicolor damselfish *Stegastes partitus* ($N = 8$) at 2 h after feeding were about the same as those for *P. yucatanicus* on a per-mass basis, at 3.75 ± 0.17 μM g^{-1} h^{-1}. Excretion rate did not vary significantly with time since feeding, remaining at 2.27 ± 0.20 μM g^{-1} h^{-1} at 24 h after feeding, and 3.66 ± 0.31 μM g^{-1} h^{-1} at 48 h after feeding (repeated measures ANOVA, $F_{(1,10)} = 0.012$, $p = 0.92$, Fig. 2a). However, due to their much larger body sizes than the shrimps (mean body mass = 5.6 g, see "Methods" section), the average whole-individual excretion rates of ammonia by these fish were an order of magnitude greater than shrimp excretion rates (see above), at approximately 0.21 μM min^{-1} for individuals <5 g wet body mass ($N = 4$) and 0.41 μM min^{-1} for individuals >5 g ($N = 4$, using basal excretion rate after 48 h starvation).

Rates of ammonia excretion by the yellowtail damselfish *M. chrysurus* ($N = 4$) were slightly more elevated at 2 h after feeding (4.85 ± 0.42 μM g^{-1} h^{-1}). However, they decreased significantly with time since feeding, to 2.35 ± 0.16 μM g^{-1} h^{-1} at 24 h and 1.65 ± 0.1 μM g^{-1} h^{-1} at 48 h (repeated measures ANOVA, $F_{(1,10)} = 43.68$, $p < 0.001$, Fig. 2b). All pair-wise comparisons differed significantly (Tukey's post hoc tests, $p < 0.05$) except for those between 24 and 48 h ($p = 0.20$). Whole-animal excretion rates ranged from about 0.14 μM min^{-1} for individuals <5 g body mass ($N = 2$) to 0.44 μM min^{-1} for individuals >5 g ($N = 2$).

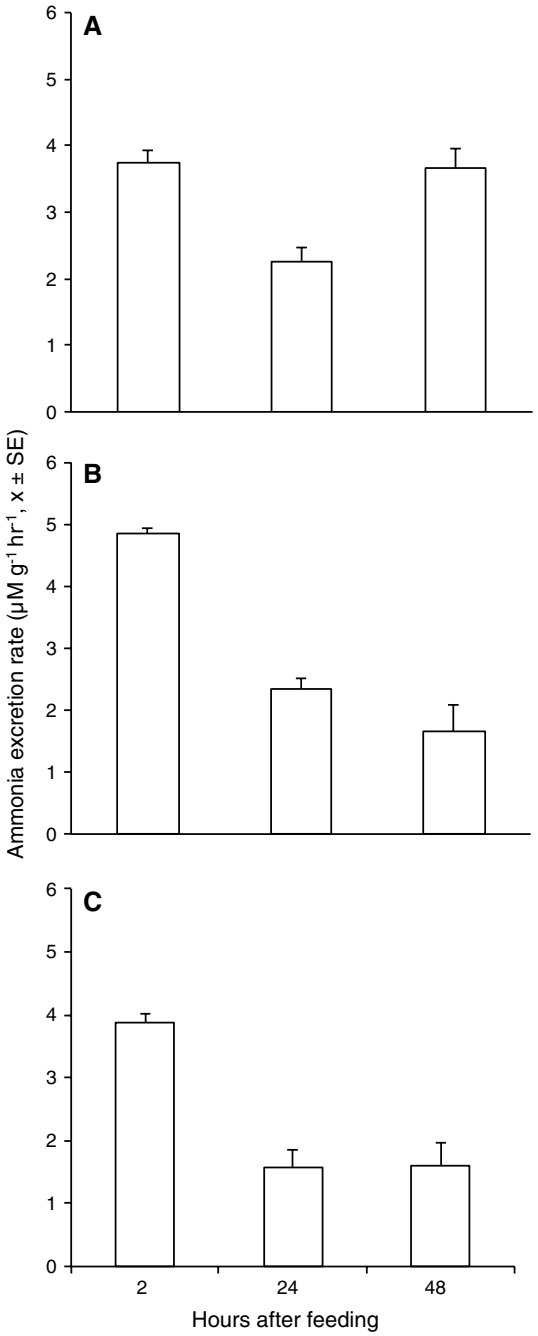

Fig. 2 Variation in rates of ammonia excretion with time since feeding, among three common coral reef damselfishes that visit cleaner shrimps on Caribbean sea anemones (Huebner and Chadwick 2012a): **a** bicolor damselfish *Stegastes partitus* ($N = 8$), **b** yellowtail damselfish *M. chrysurus* ($N = 4$), and **c** longfin damselfish *Stegastes diencaeus* ($N = 3$)

Individuals of the longfin damselfish *Stegastes diencaeus* ($N = 3$) excreted ammonia at similar rates of 3.89 ± 0.13 µM g^{-1} h^{-1} at 2 h after feeding, decreasing to 1.58 ± 0.26 µM g^{-1} h^{-1} at 24 h and 1.60 ± 0.36 µM g^{-1} h^{-1} at 48 h. The excretion rates for this species varied significantly with time since feeding (repeated measures ANOVA, $F_{(1,10)} = 12.62$, $p < 0.05$, Fig. 2c), except for between the two starved states (Tukey's post hoc test, $p = 0.99$, Fig. 2c). Whole-animal excretion rates were similar to those of the other two damselfish species, at about 0.13 µM min^{-1} for individuals <5 g wet body mass ($N = 2$) to 0.33 µM min^{-1} for the one individual that was >5 g.

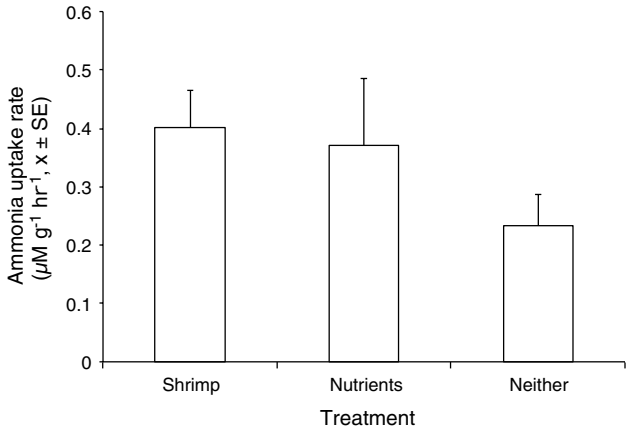

Fig. 3 Variation in the daytime ammonia uptake rates of corkscrew sea anemones *B. annulata*, among individuals after exposure to 6 week of three laboratory treatments ($N = 13$ anemones per treatment), in which they were cultured with: (1) shrimps, (2) nutrients, or (3) neither (control group)

Ammonia uptake by sea anemones

The sea anemones ($N = 39$) absorbed ammonia at a mass-specific rate of 0.34 ± 0.06 µM g^{-1} h^{-1} that did not vary significantly among individuals in the 3 treatment groups [cultured with either shrimps (0.40 ± 0.07 µM g^{-1} h^{-1}), nutrients (0.37 ± 0.12 µM g^{-1} h^{-1}), or neither (0.23 ± 0.05 µM g^{-1} h^{-1}), $N = 13$ anemones per group, see "Methods" section; ANOVA, $F_{(2,27)} = 0.098$, $p = 0.91$, Fig. 3]. The rate of whole-individual uptake of ammonia was approximately 5.07 µM h^{-1} or 0.08 µM min^{-1}, based on a mean wet body mass of 14.9 g (see "Methods" section). Thus, on a whole-individual basis, host sea anemones absorbed ammonia approximately 5–25× more rapidly than anemone shrimps were able to excrete it, while large juvenile client fishes supplied ammonia about 2.5–5× faster than the sea anemones could absorb it.

Effects of anemone shrimp excretion on sea anemones

Most of the examined measures of sea anemone physiology did not vary significantly among the experimental treatments. Anemone body size (TCSA) varied widely among individuals within treatment but not significantly among

Table 1 Variation in physiological parameters of corkscrew sea anemones *B. annulata*, before and after 6 week of laboratory experimental treatments ($N = 13$ anemones per treatment): (1) shrimps (cultured with symbiotic anemone shrimps), (2) nutrients (cultured with ammonia supplements), or (3) neither (control group, cultured with neither)

Physiological parameter	Treatment and period						Results of repeated measures ANOVA	
	Shrimps		Nutrients		Neither			
	Before	After	Before	After	Before	After	*F* value	*p* value
Anemone tentacle crown surface area (TCSA, cm^2)	100.30 (27.26)	96.84 (24.15)	97.06 (14.82)	68.90 (9.08)	103.17 (14.62)	61.02 (8.85)	1.14	0.33
Anemone oral disk surface area (ODSA, cm^2)	6.04 (1.23)	7.67 (1.48)	7.08 (1.80)	4.70 (0.61)	7.00 (1.30)	5.34 (1.18)	0.73	0.49
Anemone tissue protein concentration (mg g^{-1})	36.45 (2.30)	36.51 (3.80)	37.20 (1.74)	35.60 (3.10)	36.80 (1.78)	36.67 (2.92)	0.08	0.93
Microalgal cell abundance (# cells $g^{-1} \times 10^8$)	1.14 (0.15)	2.00 (0.24)	1.60 (0.16)	1.87 (0.16)	1.28 (0.14)	2.00 (0.23)	0.20	0.82
Microalgal chlorophyll a concentration (pg $cell^{-1}$)	1.01 (0.22)	0.22 (0.11)	0.71 (0.16)	0.20 (0.07)	0.78 (0.17)	0.31 (0.14)	1.54	0.22
Microalgal mitotic index (MI, % cells dividing)	5.97 (0.56)	7.90 (1.38)	6.14 (0.86)	11.8 (0.77)	5.16 (0.64)	5.80 (1.00)	7.47	<0.001
Microalgal cell division rate (μ, day^{-1})	0.13 (0.01)	0.16 (0.03)	0.13 (0.02)	0.24 (0.02)	0.11 (0.01)	0.12 (0.02)	7.40	<0.001

Values are presented as means ± 1 standard error (in parentheses). See text for details

treatments, either before or after the experiment (Table 1). During the 6-week starvation experiment, anemone TCSA declined in all three treatment groups: on average by 4 % in anemones cultured with shrimps, 29 % in anemones cultured with nutrient supplements, and 41 % in those cultured with neither. However, due to large variation in shrinkage rates among individuals within each treatment, they did not differ significantly among the three treatments (Table 1). Similar to TCSA, sea anemone oral disk surface area (ODSA) did not vary significantly with treatment either before or after the experiment. On average, the sea anemones cultured with shrimps grew in ODSA (by ~27 %), while those cultured with nutrient supplements or with neither, shrank (by about 34 and 24 %, respectively). As such, in terms of both types of measures, the presence of shrimps appeared to preserve mean anemone body size, but the trends were not significant. Mass-specific protein content of the anemones varied much less among individuals than did body size, and remained more stable throughout the experiment than did any other physiological variable measured, with no significant variation observed among treatments (Table 1).

The abundance of *Symbiodinium* cells in the anemone tentacles did not vary significantly with treatment; on average, it increased slightly in all treatments. However, chlorophyll a levels per microalgal cell declined precipitously in all three treatments, more so than for any other parameter measured, and paralleled the observed declines in host body size. On average, per-cell chlorophyll content declined by ~78 % in anemones cultured with shrimps, 72 % in those with nutrient supplements, and 60 % in those cultured with neither (no significant difference between groups, Table 1).

Conversely, the percent of *Symbiodinium* cells observed dividing (mitotic index, MI) increased significantly in the anemones exposed to nutrient supplements; it did not change significantly in either the shrimp or control treatments (Table 1, latter two groups not significantly different from each other, Tukey's post hoc tests, $p = 0.40$). The estimated time required for the microalgae to double in abundance (doubling time, T) varied significantly with treatment, but only between the nutrient and control groups ($p < 0.05$). The division rate (μ, day^{-1}) of *Symbiodinium* in the anemones exposed to anemone shrimp or to neither treatment likewise rose slightly, while microalgae in the nutrient treatment almost doubled their division rate on average (significant variation among groups, with differences only between the nutrient and control groups, $p < 0.05$).

Discussion

Our previous studies demonstrated that clownfishes in obligate symbioses with sea anemones are able to supply 100 % of the host anemone's nitrogen uptake through excretion of ammonia waste (Roopin and Chadwick 2009; Roopin et al. 2011). This ability does not appear to hold for the shrimp–anemone symbiotic system examined here. Individual shrimps are small

(average of 0.13–0.27 g wet mass, depending on species) and therefore contain much less metabolically active tissue than do most fishes. Average-sized individuals of the two anemone shrimp species examined here produced only 0.003–0.015 μM NH_3 min^{-1}, roughly 4–18 % of the ammonia that an average-sized anemone absorbed from ambient seawater over this time period. The observed patterns of microalgal division rates and doubling times within the sea anemones also revealed that ammonia supplements were beneficial to these symbiotic partners, but that shrimp presence was not; hence, shrimp alone do not appear to provide enough nutrients to significantly impact the algae within their hosts. Because ammonia is soluble and diffuses rapidly in water, the anemones likely receive at least some of the ammonia excreted by shrimps, as evidenced by the slight upticks in mitotic index and density of symbiotic zooxanthellae in anemones cultured with shrimps (Table 1).

While these shrimps may not directly supply much of the nitrogen needs of algae within the anemone hosts, they may do so indirectly by attracting client fishes that provide adequate nitrogen. Cleaner shrimps attract the members of >16 families of client fishes to host anemones (Wicksten 1995, 1998; Huebner and Chadwick 2012b); the anemones thus serve as cleaning stations at which the shrimp clean the clients of parasites from their gills, around and in clients' mouths, fins and bodies (Wicksten 1998; Grutter 2003). Reef fishes accumulate significant parasite loads overnight; then, beginning in the early morning they visit cleaning stations throughout the daytime (Johnson and Ruben 1988; Bunkley-Williams and Williams 1998; Sikkel et al. 2004). They are attracted by the visual cue of the sea anemone and approach the anemone host and then pose for cleaning, possibly in response to the behavioral display of the cleaner shrimp; they then remain stationary for 10–20 s on average, while the shrimp consume their exoparasites, with reported cleaning durations reaching upward of 120 s in some cases (Huebner and Chadwick 2012b). Client fishes position themselves so that their head and gill areas are in close proximity to the anemone and its shrimp, and during this cleaning pose, they excrete ammonia across their gills continuously. The rates and absolute amounts of ammonia excreted by client fishes appear to be large enough to supply >100 % of the ammonia uptake by a typical anemone host. For example, damselfish excrete ammonia at 0.21–0.41 μM min^{-1} for fish below and above 5 g, respectively. That translates to an absolute amount of 0.42 and 0.82 μM NH_3 supplied per fish, assuming that they each spend 2 min at the anemone being cleaned of parasites, more than twice the ammonia (~0.16 μM) that a typically sized anemone (14.9 g) absorbs from ambient seawater over this period. Adult damselfishes are frequent visitors to anemone shrimp cleaning stations on Caribbean reefs (Cheney and Coté 2001; Sikkel et al. 2000, 2004, 2005). Even larger fishes such as groupers, which can weigh up to 2 kg, excrete up to 70 μM NH_3 min^{-1}, which means that large individuals could potentially supply well over 100 % of the nitrogen taken up by an anemone in a very short time (reviewed in Table 1 of Roopin et al. 2008). Large piscivorous fishes that visit cleaning stations, such as those in the family Serranidae (groupers, Sluka et al. 1997), could especially benefit microalgal populations in host sea anemones through delivering higher *P*/*N* ratios and thus more phosphorous load than do herbivores (Allgiers et al. 2014). Microalgal *Symbiodinium* populations require both *N* and *P* and perform optimally when well supplied with both of these essential nutrients (Muller-Parker et al. 1994; Godinot and Chadwick 2009; Cleveland et al. 2011). Large piscivores also provide the cleaner shrimp with more food in the form of gill and skin parasites than do smaller-bodied herbivorous fishes (Poulin and Grutter 1996), leading to a potentially positive feedback cycle of longer cleaning interactions per host (Silvano et al. 2012), enhanced shrimp growth and abundance, more fish excretion in the vicinity of host, and an increase in host anemone body size. Large client fish body size and feeding guilds of piscivorous fishes therefore appear to be important to this symbiotic system on coral reefs.

As populations of large-bodied fishes decline (Hughes 1994; Holmlund and Hammer 1999), their loss could have negative effects on the abundance of anemone-based fish cleaning stations. Shrimps spend less time cleaning smaller-bodied versus larger-bodied fish clients (Silvano et al. 2012), potentially leading to a negative feedback loop of fewer fish-excreted nutrients for host anemones, smaller anemones with fewer shrimp (Huebner and Chadwick 2012a), and eventually more fish parasites (Bshary 2003; Grutter 2003). This decline is worrisome because fish biomass contributes significantly to the *N*/*P* ratios that sustain coral reefs and their adjacent environments (Allgiers et al. 2014).

In this mutualistic network, the shrimp's primary contribution to the nitrogen balance of the host anemone may not be to provide ammonia nitrogen directly from its own metabolism and excretion, but rather to attract client fishes to the anemone. These fishes then excrete ammonia while being cleaned, at rates in excess of what is taken up by the anemone. This type of mutualistic network occurs among diverse plant and animal taxa (Fig. 4) in both the Caribbean (Wicksten 1995, 1998) and Indo-Pacific (Grutter 2003; Chadwick et al. 2008). Client fishes may feed in nearby seagrass beds or on zooplankton (reviewed in Huebner et al. 2012) and then import nutrients in the form of excreted

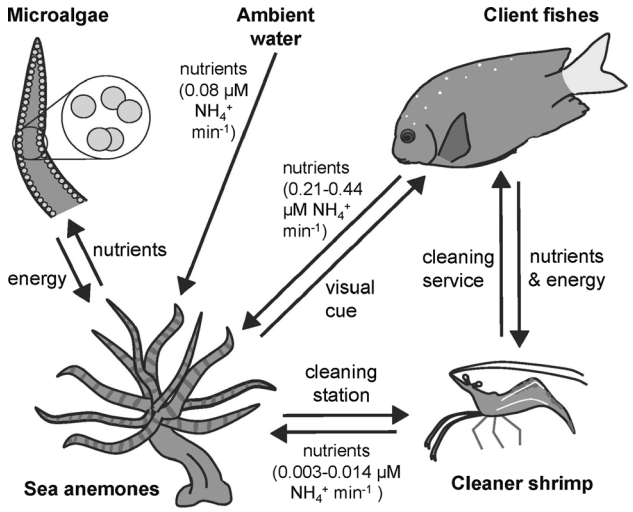

Fig. 4 Nutrient and energy transfer in Caribbean anemone shrimp cleaning symbioses. Client fishes use sea anemones as visual cues to locate cleaning stations of anemone shrimp (Huebner and Chadwick 2012a). Cleaner shrimp receive nutrients and energy by consuming exoparasites from client fishes (Cheney and Coté 2001; Sikkel et al. 2004). Cleaner shrimp excrete nutrients too slowly to impact anemones, but client fishes excrete nutrients at much more rapid rates while at the station than sea anemones can absorb them from ambient sea water (present study). Sea anemones provide nutrients to endosymbiotic microalgae, which in return release energy-rich compounds that support host anemone maintenance of body size (Stambler and Dubinsky 1987; Roopin and Chadwick 2009). Because larger anemones attract more client fishes than do smaller anemones (Huebner and Chadwick 2012a), nutrients supplied by client fishes to sea anemones may create a positive feedback loop in which anemone growth is enhanced, further attracting more client fishes. Nutrient exchange rates are presented as whole-body excretion or uptake per minute, because each cleaning interaction occurs over a timescale of seconds to minutes (Huebner and Chadwick 2012b). Figure design: Lindsay Huebner

ammonia, enhancing connectivity between adjacent ecosystems (Verweij et al. 2006; Nagelkerken et al. 2008). Similar transfer is performed by migrating fish that rest over reefs and deliver nutrients from nearby seagrass beds (Meyer and Schultz 1985) and by fish activities in other aquatic communities, leading to enhanced system resilience (Holmlund and Hammer 1999; Allgiers et al. 2014).

The extra nitrogen provided by client fishes also could serve as a positive feedback mechanism to perpetuate this symbiosis through evolutionary time. In our experiment, the host anemones were not significantly impacted by shrimp presence alone, but the data show a pattern of increasing mean oral disk size in anemones cultured with shrimps, compared with a decrease in the anemones cultured both without shrimp or nutrients and those with nutrients only. This trend relates to previous work, showing that larger sea anemones may be more visible to client fishes (Huebner and Chadwick 2012a). Higher visibility to potential clients could enhance the number of fishes in general that a station receives, thus increasing the ammonia available in the immediate surroundings of the anemone, leading to a positive feedback loop of fitness benefits (Fig. 4).

Most of the physiological parameters recorded here were within the ranges of values known for other species of sea anemones (Table 2), indicating that many characteristics of *B. annulata* and of its endosymbiotic microalgae are representative of coral reef sea anemones in general. While excess nutrients did not have a significant effect on sea anemone body size over the timescale examined here, they impacted microalgal populations, as known for other cnidarians subjected to nutrient supplements (Fitt and Cook 2001; Hoegh-Guldberg 2006; Roopin and Chadwick 2009). The most obvious effect of nitrogen supplements was increased microalgal mitotic index within the anemone tissues, indicating enhanced algal growth and replication. The lack of significant change in total algal abundance suggests a potentially high cell turnover rate of these microalgal populations, leading to regular expulsion of the excess algae (Hoegh-Guldberg et al. 1987; Fitt 2000).

Several changes could be made to this type of experiment to allow detection of further potential impacts on host anemones from anemone shrimp and/or client fish nutrient excretions. The experimental duration could be extended; 6 week may not be long enough for enhanced microalgal mitotic rates to translate into augmented anemone body size, especially as recorded in tentacle crown surface area for an anemone species with long, thin, undulating tentacles. Also due to high levels of variation among anemone individuals in growth rate and body size, larger sample sizes might reveal more consistent physiological effects of applied treatments. Client fishes could be added to the experimental tanks, or the entire experiment could be conducted in larger macrocosm tanks. To definitively demonstrate an effect under coral reef conditions, field experiments are needed to explore the indirect impacts of client fish visits on host sea anemone body size and fitness through cleaner shrimp services.

This study provides laboratory-based evidence that nutrient cycling in a coral reef symbiosis potentially facilitates the transfer of nitrogen from non-obligate associates to a cnidarian host. Unlike previous studies on anemonefish–anemone symbioses, in which the fish leaves the anemone, feeds in the water column, and returns, directly translocating nitrogen from the water column to the host (reviewed in Roopin et al. 2011), anemone shrimp remain with the host and attract client

Table 2 Variation in physiological characteristics among species of actiniarian and corallimorpharian sea anemones and their endosymbiotic microalgae

Sea anemone order and species	Anemone nitrogen uptake rate ($\mu M\ g^{-1}\ h^{-1}$)	Anemone tissue protein concentration ($mg\ g^{-1}$)	Microalgal cell abundance (# cells $g^{-1} \times 10^8$)	Microalgal chlorophyll a concentration ($pg\ cell^{-1}$)	Microalgal mitotic regularity	Microalgal mitotic index (MI)	Microalgal cell division rate (μ, day^{-1})	Microalgal cell doubling time (T, days)	Source
Actiniaria									
Aiptasia pallida	–	5.0	–	–	–	–	–	–	Muller-Parker (1987)
A. pulchella	–	4.3–14.9	0.02–0.03	1.7–3.0	Random	<1	0.02	42.0	Wilkerson et al. (1983); Muller-Parker (1987)
Anemonia sulcata	–	–	0.4–0.6	–	–	–	–	–	Stambler and Dubinsky (1987)
Anthopleura elegantissima	–	–	–	–	Random	3.0–5.0	0.06–0.10	6.9–11.2	Wilkerson et al. (1983)
Aulactinia stelloides	–	20.0	–	–	–	2.2–4.4	–	–	Smith (1986)
B. annulata	0.23–0.40	35.1–37.2	1.3–1.6	0.4–1.0	Random	5.0–6.0	0.11–0.24	6.0–7.7	Present study
Bunodeopsis globulifera	–	–	–	–	–	2.0–7.0	0.05	–	Day (1994)
Condylactis gigantea	0.03–0.04[a]	–	1.3–1.6	–	–	–	–	–	Spotte (1996)
Entacmaea quadricolor	0.12–0.60	22.2–27.4	0.2–3.0	0.2–3.0	Phased (peak at 08:00)	1.0–6.0	–	–	Porat and Chadwick-Furman (2005); Roopin and Chadwick (2009); Dixon et al. (2014)
Corallimorpharia									
Rhodactis rhodostoma	–	–	0.1–0.2	0.5	Phased	1.0–5.0	–	–	Kuguru et al. (2007)

Shown are ranges of reported values, for individuals under laboratory conditions or in shallow marine habitats (<10 m depth below sea surface; deepwater individuals were not included)

[a] Values reported in units of $\mu M/L\ min^{-1}$

fishes from the water column, thus indirectly influencing the possible transfer of ammonia nitrogen to the host.

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