PHOSPHATE EXCRETION BY ANEMONEFISH AND UPTAKE BY GIANT SEA ANEMONES: DEMAND OUTSTRIPS SUPPLY

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ABSTRACT

High biodiversity on coral reefs results in part from tight nutrient cycling among symbiotic organisms, such as within the obligate associations among some damselfishes, cnidarians, and zooxanthellae. Some anemonefish excrete ammonia 20–50× faster than host anemones can absorb this nutrient, leading to significant growth of the anemones and their zooxanthellae. In contrast, little is known about phosphate transfer in this major coral reef mutualism. We determined rates of phosphate excretion by anemonefish and uptake by giant sea anemones under laboratory conditions, and compared them with known rates of ammonia transfer in this symbiosis. Immediately after feeding with a phosphate-rich diet, anemonefish excreted phosphate at slow rates of 0.07 ± 0.01 μmol P g^{-1} d^{-1}, which did not vary significantly with body size. Starved anemones that had been cultured with phosphate supplements absorbed phosphate at a significantly slower rate (0.18 ± 0.03 μmol P g^{-1} d^{-1}) than did those cultured with either no supplements or with anemonefish, which absorbed phosphate at similarly rapid rates (0.54 ± 0.01 and 0.51 ± 0.14 μmol P g^{-1} d^{-1} respectively). We conclude that under laboratory conditions, anemones absorb phosphate up to 6.6× faster than the rate at which it is excreted by their anemonefish, and thus fish do not appear to provide sufficient phosphate to their hosts through this pathway. Anemones may get most of their phosphorus via ingestion of fish feces and/or mucus, or via the ingestion of prey.

Coral reefs support the highest biodiversity of all marine ecosystems, despite extremely low concentrations of dissolved nutrients in the oligotrophic waters surrounding tropical reefs (Crossland, 1983; Chadwick-Furman, 1996; Davies, 1997). This paradox may be explained in part by the tight recycling of essential nutrients among organisms that form complex, multi-level symbioses on coral reefs (reviewed in Roopin et al., 2008). Many species of damselfishes form obligate shelter symbioses with coral reef cnidarians, especially among the branches of stony corals (Holbrook et al., 2008) and the tentacles of giant sea anemones (Fautin and Allen, 1997; Randall and Fautin, 2002). These associations are ecologically important, in that the damselfish consume zooplankton and thus import nutrients from the open sea to the reef benthos, and also serve as major prey for piscivorous reef fishes (Holbrook and Schmitt, 2002).

Fishes release essential nutrients such as phosphorus and nitrogen via excretion and defecation. Due in part to the central ecological roles played by fishes that associate symbiotically with cnidarians on coral reefs, nutrient dynamics in these symbioses are being investigated. Here we explore rates of phosphorus excretion by anemonefish and of uptake by sea anemones. Past studies have shown that excretion of nutrients by damselfish possibly enhances the growth rate, reproductive output, and survival of corals and sea anemones (Liberman et al., 1995; Holbrook and Schmitt, 2005; Holbrook et al., 2008). Haemulid fish augment the growth of corals over which they rest diurnally between migrations, probably via released nutrients (Meyer and Schultz, 1985). Some anemonefish also excrete substantial amounts of ammonia (defined here as the combination of NH_3 gas and NH_4^+ ion) that contrib-

ute to sea anemone growth and survival (Porat and Chadwick-Furman, 2004, 2005a; Roopin and Chadwick, 2009).

Phosphorus is a limiting nutrient for the growth of coral reef sea anemones, and controls in part the abundance of their endosymbiotic microalgae (zooxanthellae; Muller-Parker et al., 1990). Autotrophs, and especially zooxanthellae, can mainly use "biologically available phosphorus" (BAP), which consists of dissolved phosphate and a small biolabile fraction of dissolved organic phosphorus (Karl and Björkman, 2002). Concentrations of dissolved phosphate available for uptake by anemones on coral reefs are extremely low (0.15 μM–0.6 μM; Pilson et al., 1972). Due to the ability of fish to excrete phosphate as a waste product of metabolism (Meyer and Schultz, 1985; Schaus et al., 1997), and the rapid absorption of dissolved phosphate by sea anemones and their zooxanthellae (Muller-Parker et al., 1990), anemonefish potentially serve as important sources of phosphate for sea anemones. Previous research concluded that fish release most of their waste nitrogen via excretion, but most phosphorus (in the form of molybdate reactive phosphorus) through leaching of their feces (Meyer and Schultz, 1985). However, because fish also release some phosphorus (in the form of dissolved phosphate) by excretion through their gills (Meyer and Schultz, 1985; Schaus et al., 1997), and the importance of excreted nitrogen in the anemonefish symbiosis recently has been quantified (Roopin and Chadwick, 2009), we determine the relative contribution of excreted phosphorus as a pathway for nutrient transfer in this association.

We focus on the dynamics of dissolved inorganic phosphorus (phosphate), as this phosphorus form is the one chiefly used by photoautotrophs, and thus is the main form of interest for rapid uptake of nutrients within the symbiotic association between cnidarians and their zooxanthellae (Muller-Parker et al., 1990). We describe rates of phosphate excretion by anemonefish and of phosphate uptake by sea anemones under laboratory conditions, and discuss this process in relation to nitrogen transfer in this major coral reef mutualism.

Methods

Aquacultured individuals of two-band anemonefish *Amphiprion bicinctus* Rüppel, 1828 and bulbtip anemones *Entacmaea quadricolor* Rüppel and Leuckart, 1828 were maintained in closed-system aquaria at Auburn University, using methods described in Roopin and Chadwick (2009). Immediately prior to examination of phosphate dynamics, the body sizes of anemonefish and anemones were determined using methods described in Roopin et al. (2008). In addition, the relationship between wet and dry fish mass was assessed by thawing 12 frozen fish, blotting them to remove excess water, recording wet mass to the nearest 0.01 g on an electronic balance, oven-drying at 95 °C for 24 hrs, and then recording dry mass. Fish were frozen individually in vacuum-sealed plastic bags at –25 °C for about 6 mo prior to these measurements. The relationship between tentacle crown diameter (TCD, measured as in Roopin et al., 2008) and wet mass of the anemones also was determined by removing each anemone from its aquarium, gently handling it to cause the anemone to contract its tentacles and expel excess water, blotting it with an absorbent cloth, and then weighing each anemone on an electronic balance to the nearest 0.01 g. Each anemone was out of water for < 1 min and expanded normally after return to its aquarium.

To assess effects of nutritional state on the rate of phosphate uptake by anemones, we performed a five-week laboratory experiment (modified after Roopin and Chadwick, 2009). Three anemones (1 small 6.4 ± 0.2 cm, 1 medium 12.1 ± 0.1, and 1 large individual 16.0 ± 0.3 cm TCD, mean ± SE) were assigned to each of three treatments: (1) with three anemonefish,

(2) without anemonefish, and (3) with added phosphate. Anemones in all treatments were unfed for the duration of the experiment, to detect effects of phosphate addition when starved (after Muller-Parker et al., 1990). Each of the nine experimental anemones was housed in a separate closed-system 80 L aquarium equipped with a water pump, protein skimmer, and sump filter system (described in Roopin et al., 2008). Three anemonefish [1 small (mean fork length, FL ± SE) 5.1 ± 0.1 cm, 1 medium 6.8 ± 0.1 cm, and 1 large individual 7.9 ± 0.1 cm] were maintained with each of the three anemones (1 small 6.0 cm, 1 medium 12.0 cm, and 1 large individual 16.5 cm TCD) in treatment (1). Anemones and fish were not matched for size: each anemone received a small, a medium, and a large fish. The fish were fed daily with 0.01 g of Formula One Marine Pellets per aquarium (Aqua Pets Americas, Salt Lake City), which were ascertained during preliminary observations to support rapid fish growth. These pellets consisted of mostly shrimp and fish meal, and thus we inferred that their N:P ratio was about 16:1, similar to the phosphorus content of zooplankton (Anderson and Hessen, 1991; Downing, 1997) upon which these anemonefish feed in the wild (Fautin and Allen, 1997). Care was taken to monitor the fate of all proffered pellets, and any pellets not ingested by the fish within 3 min after feeding were removed from the aquaria. In treatment (3), the concentration of dissolved phosphate was raised each day as a short-term pulse of 2.0 μM phosphate, by placing each anemone in a separate plastic bucket containing 2.5 L of aerated phosphate-enriched seawater from its aquarium for 30 min (similar to treatments used for ammonia by Roopin and Chadwick, 2009).

We measured the phosphate uptake rates of the sea anemones after 5 wks, as well as the excretion rates of the anemonefish. We determined the excretion rates of eight large anemonefish (mean ± SE = 7.24 ± 0.72 g wet mass, range = 4.39–11.57 g wet mass, 6.29–7.53 cm FL) within 30–60 min after feeding, since phosphate excretion in fishes is known to be highest then (Schaus et al., 1997). Each animal was placed in a separate beaker containing aerated artificial seawater (250 ml for fish, and 200–350 ml for anemones, depending on their size), inside a water bath maintained at 26–27 °C (after Roopin et al., 2008). 400W Radium Metal Halide lamps provided 200–400 μmol photons m^{-2} s^{-1} of irradiance, equivalent to that at 9–15 m depth on the coral reef (Stambler and Dubinsky, 2005). To measure maximal uptake rates by the anemones, the water in each beaker was aerated with an air stone, and, after an acclimation period of 45 min, the phosphate level was raised by 9 μM. After animals were allowed to acclimate (45 min for anemones, 20 min for fish, based on preliminary observations), a water sample of 15 ml was taken from each beaker every hour for 4 hrs (after Muller-Parker et al., 1990; Spotte, 1996; Porat and Chadwick-Furman, 2005b; Roopin et al., 2008). The seawater removed during sampling was not replaced. A beaker with no animal served as a control for changes in phosphate concentration due to microbial activity or adsorption onto the surface of the beakers. An additional control for the effect of feeding regime on phosphate uptake by the anemones consisted of a beaker containing an anemone that had been fed weekly in the normal culture routine (weekly feeding with pieces of shrimp; see Roopin et al., 2008 for details on culture conditions). Animals did not appear to be stressed during excretion and uptake measurements (e.g., fish exhibited regular opercular ventilation and slow swimming behavior, and anemones expanded their tentacles fully). Levels of phosphate were determined using the MAGIC method (Karl and Tien, 1992), which produced a precipitate that was analyzed using the ascorbic acid technique (Murphy and Riley, 1962) under the conditions described by Greenberg et al. (1992). Preliminary testing showed that this method produced linear results for phosphate concentrations of 0.5–500 μM. We corrected for the small amount of phosphate removed during water sampling, then determined the amount of phosphate absorbed by each anemone or excreted by each fish during the 4 hr sampling period, multiplied by 24 hrs d^{-1}, and normalized to the wet mass of each organism, to obtain rates of phosphate uptake or excretion in μmol g^{-1} wet mass d^{-1}. All data are reported as means ± 1 SE, unless otherwise indicated.

Results

All individuals grew for about a year under the culture conditions, prior to the phosphate measurements described here (anemonefish increased by 40%–250% and sea anemones by 10%–270% in wet mass yr^{-1}, depending on initial size). Anemonefish wet mass increased exponentially with FL (wet mass in g = 0.02 FL in $cm^{3.15}$, N = 9 fish, $r^2 = 0.99$, Spearman correlation test: $P < 0.01$), and linearly with dry mass (wet mass in g = 3.04 dry mass in g, N = 12 fish, $r^2 = 0.99$, $P < 0.01$). In addition, the wet mass of the sea anemones increased exponentially with TCD (wet mass in g = 0.20 TCD in $cm^{2.10}$, N = 9 anemones, $r^2 = 0.87$, $P < 0.05$).

Phosphate levels of 0.08 ± 0.01 µM were measured in the artificial seawater used in uptake and release experiments (control in Fig. 1A). There was no significant difference in phosphate concentration among sample times in the control beakers during the experiments (Kruskal-Wallis tests: P = 0.41 for control in Fig. 1A, and P = 0.56 for control in Fig. 2).

The anemonefish excreted phosphate slowly but steadily over 4 hrs (Fig. 1A), at a rate of 0.075 ± 0.006 µmol P g^{-1} wet mass d^{-1} (0.025 ± 0.002 µmol P g^{-1} dry mass d^{-1}). This slow rate of phosphate excretion did not vary significantly with fish size (Spearman correlation test: P = 0.96, Fig. 1B).

After 5 wks under experimental conditions, the rate of phosphate uptake by anemones varied significantly among the treatments (Kruskal-Wallis test: H = 5.60, $P < 0.05$). Anemones that had been cultured without anemonefish absorbed phosphate at a slightly but not significantly greater rate (0.54 ± 0.01 µmol P g^{-1} wet mass d^{-1}, i.e., 9.77 ± 0.18 µmol P $anemone^{-1}$ d^{-1}) than did those that had been cultured with anemonefish (0.51 ± 0.14 µmol P g^{-1} wet mass d^{-1}, i.e., 11.73 ± 3.21 µmol P $anemone^{-1}$ d^{-1}, Bonferroni multiple comparison test: P = 0.91, Fig. 2). In contrast, anemones that had been cultured with phosphate supplements absorbed significantly less phosphate (0.18 ± 0.03 µmol P g^{-1} wet mass d^{-1}, i.e., 4.46 ± 0.74 µmol P $anemone^{-1}$ d^{-1}) than did those in the other two treatments (Bonferroni multiple comparison test: $P < 0.05$). A control sea anemone that had been fed throughout the 5 wk also absorbed very little phosphate (0.14 µmol P g^{-1} wet mass d^{-1}, i.e., 3.14 µmol P $anemone^{-1}$ d^{-1}).

Thus, based on the presence of three fish per anemone, each anemone received up to 1.77 µmol P d^{-1} from the excretion products of their fish, while they each absorbed between 3.14 and 11.73 µmol P d^{-1}. The anemones in treatment (2) therefore absorbed phosphate up to 6.6× faster than the rate at which it was excreted by their anemonefish.

Discussion

We demonstrated that starved sea anemones absorb phosphate up to 6.6× faster than the rate at which it is excreted by symbiotic anemonefish. Prior exposure to phosphate enrichment strongly influences the rate of phosphate uptake by anemones, while fish presence does not. Thus, anemonefish do not appear to provide enough excreted phosphate to meet the nutritional requirements of anemones and their zooxanthellae. The opposite pattern occurs in terms of the transfer of excreted nitrogen in this symbiosis: under the same experimental conditions, anemonefish excrete nitrogen 20–50× faster than anemones are able to absorb it (Porat and Chadwick-Furman, 2005a; Roopin et al., 2008).

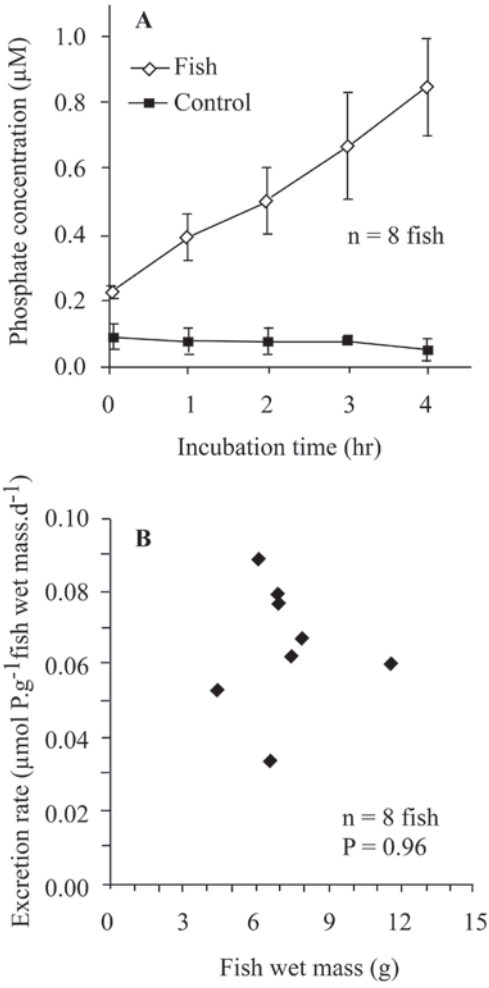

Figure 1. Phosphate excretion by anemonefish (*Amphiprion bicinctus*) after feeding under laboratory conditions. (A) Changes in phosphate concentration over 4 hrs. The upper line is for incubation beakers that each contained a fish. The lower line is for a control beaker of artificial seawater with no fish. The two types of beakers displayed different phosphate levels at time 0 due to a small amount of phosphate buildup during the 20 min acclimation period for fish that preceded time 0 (see text for details). Data are presented as means ± SE. (B) Excretion rates for 8 fish that varied in wet mass.

Due to the limited number of individuals measured in this laboratory study, we may not have estimated accurately rates of natural variation in the transfer of dissolved phosphate in this symbiosis. However, the amount of variation observed here in rates of phosphate uptake and release were similar to those observed in related studies on ammonia using larger sample sizes (Roopin and Chadwick, 2009; Roopin et al., 2008). Also, under field conditions, after consuming a natural diet of zooplankton on coral reefs, anemonefish excrete ammonia at similar rates to those measured in our laboratory studies (Roopin et al., 2009). Under field conditions, the rate of transfer of excreted nutrients from fish to hosts may be slower than we ob-

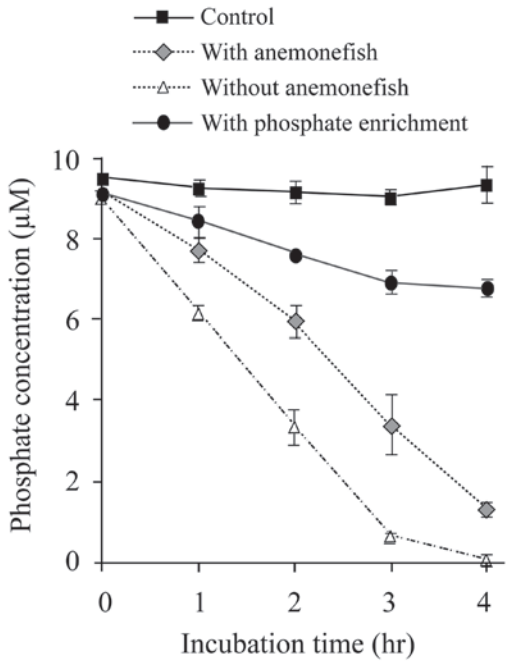

Figure 2. Comparison of phosphate uptake over 4 hrs by starved sea anemones (*Entacmaea quadricolor*) after 5 wks in laboratory treatments. The upper line (black squares) represents a phosphate-spiked control beaker with no anemones. Data are presented as means ± SE. N = 3 for each treatment. See text for treatment details.

served under laboratory conditions, because the fish range up to several meters from anemones during diurnal feeding on zooplankton (Fautin and Allen, 1997; Porat and Chadwick-Furman, 2004). In addition, water currents may carry off dissolved nutrients excreted by fish before they reach host tissues. However, field measurements indicate that anemonefish cause significant elevation of dissolved ammonia concentrations among anemone tentacles on Red Sea coral reefs (Roopin et al., 2009).

We focused on the excretion of BAP (phosphate) through anemonefish gills, because this is the most rapidly-available form of phosphorus for zooxanthella cells (Muller-Parker et al., 1990; Karl and Björkman, 2002). Thus, we underestimate here the total amount of phosphorus potentially provided to sea anemones by anemonefish, which likely includes supply via fish feces and/or mucus secretions. Much of the phosphorus added to coral reefs by fishes is in particulate form in feces, and probably becomes available only slowly via leaching and bacterial activity (Meyer and Schultz, 1985). This process is not well understood, in part because the leaching of fish fecal material may occur mostly in the sediment surrounding coral reefs, rather than in the water near cnidarians (Meyer and Schultz, 1985). Due to their physically intimate association with anemonefish, giant sea anemones may obtain some phosphorus and other nutrients by ingesting the fecal pellets of the fish. However, hosting of anemonefish did not reduce the phosphate uptake rates of the anemones examined here, indicating a deficiency of phosphorus even though the hosts were exposed to fish feces. Thus, these anemones may rely more on ingestion of food items to obtain this nutrient, than on either the excretion or elimination products of fish. Ingestion of

particulate phosphorus in food items is known to contribute substantially more than does absorption of dissolved phosphate to the nutrition of tropical sea anemones (Muller-Parker et al., 1990).

Excretion rates of fish vary with temperature, diet, and body size, but in general, marine fishes excrete ammonia about 50× more rapidly than they do phosphate (Meyer and Schultz, 1985). Freshwater fishes are more variable, and excrete ammonia 3–100× more rapidly than phosphate (Schaus et al., 1997). Thus, according to known rates of ammonia excretion by these anemonefish of 43.8–134.2 mmol g^{-1} dry mass d^{-1} (= 0.60–1.84 μmol g^{-1} wet mass hr^{-1} × 3.04 g wet mass g^{-1} dry mass × 24 hr d^{-1}, Roopin et al., 2008), expected rates of phosphate excretion are about 1.4–3.5 mM g^{-1} dry mass d^{-1} (= 4.3–10.5 mM g^{-1} wet mass d^{-1}), whereas the observed rate was only 0.075 mM g^{-1} wet mass d^{-1}, about 55–140× lower than expected, but within the range of rates known for lake fishes (Schaus et al., 1997). Rates of phosphate excretion recorded here also did not vary significantly with fish mass within species, similar to the pattern reported by Meyer and Schultz (1985) for much larger reef fish. The observed rates of phosphate excretion for anemonefish were 190–590× lower than rates of ammonia excretion by these fish (Roopin et al., 2008). Thus anemonefish appear to have a much lower stoichiometry of N:P nutrient supply compared to other reef fishes.

The mean rate of phosphate uptake by all anemones in our study (8.48 mM d^{-1} per anemone (based on mean wet mass of 22.4 g) was 200× higher than that reported for the sea anemone *Aiptasia pallida* Verrill, 1864 after starvation for 4 wks (0.045–0.075 μM d^{-1}; Muller-Parker et al., 1990). This difference likely is due to the longer starvation time in our experiment (5 wks), the much larger body size of our anemones compared to that of *A. pallida*, and the higher concentration of phosphate provided for uptake (9 μM vs 0.66–1.26 μM). Phosphate fluxes in cnidarians are known to depend in part on ambient phosphate concentrations (D'Elia, 1977; Muller-Parker et al., 1990).

The slow rates of phosphate uptake by both the fed control anemone and the phosphate-enriched anemones indicate that these conditions provided enough phosphate to meet their phosphorus demands. In contrast, the relatively rapid rate of phosphate uptake by the starved anemones that were cultured both with and without anemonefish reveals that they became depleted in phosphorus during the 5-wk experiment. We conclude that under laboratory conditions, anemonefish do not appear to be an important source of phosphate to sea anemones and their zooxanthellae. This study indicates that the stoichiometry of N:P nutrient supply by anemonefish may not match that of N:P demand by anemones and their zooxanthellae, and that phosphorus may be obtained mainly from other sources, such as through direct consumption of zooplankton prey by sea anemones.

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