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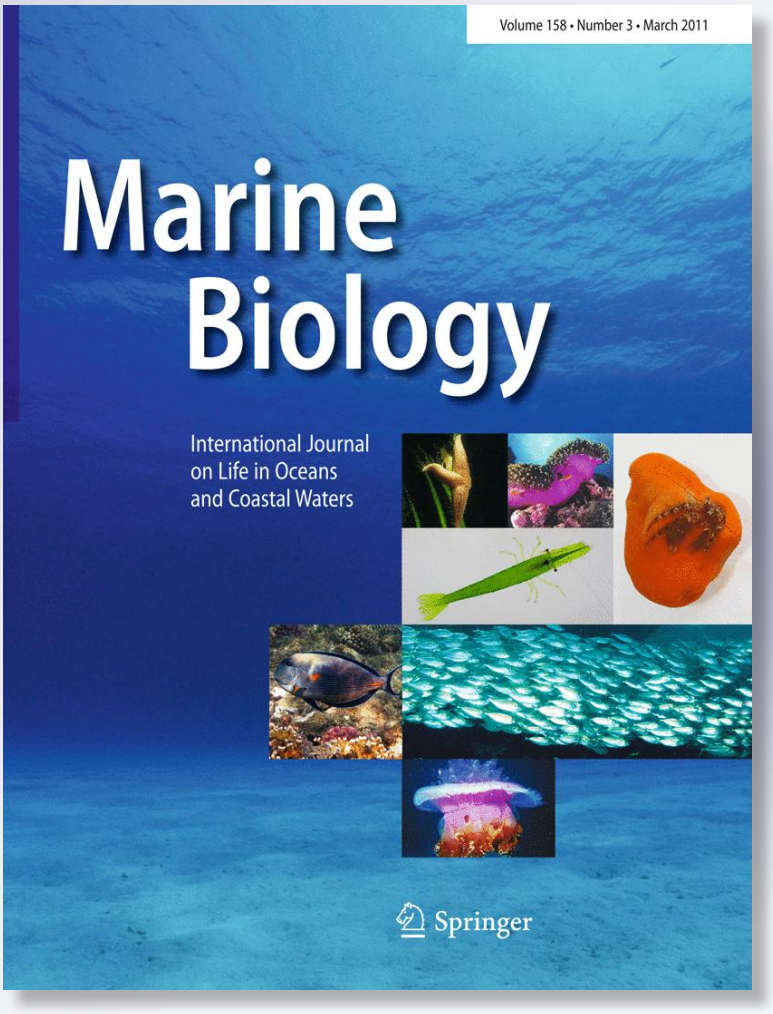


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ORIGINAL PAPER

# Nutritional exchange in a tropical tripartite symbiosis: direct evidence for the transfer of nutrients from anemonefish to host anemone and zooxanthellae

**Ann Cleveland · E. Alan Verde · Raymond W. Lee**

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**Abstract** The relationship between anemones and anemonefishes is an oft-cited and endearing example of a mutualistic symbiosis. Current research on mutualistic symbioses suggests these relationships are more commonplace and have greater importance at the ecosystem level on nutrient dynamics and evolutionary processes than previously thought. Using stable isotopes $^{15}N$ and $^{13}C$, both field and laboratory experiments were designed to investigate whether nutrient transfer from two species of resident anemonefishes (*Amphiprion perideraion* and *A. clarkii*) to host anemones (*Heteractis crispa*) occurs. Mass spectroscopy indicated that both $^{15}N$ and $^{13}C$ were significantly elevated in the tissues of anemonefishes and in both host anemone and zooxanthellae fractions. These experiments provide the first direct empirical evidence of nitrogen and carbon transfer from resident anemonefishes to host anemones and endosymbiotic zooxanthellae. Such transfer of elements within this intriguing tripartite association underscores the central role that nutrient dynamics contributes to the evolutionary processes of these marine symbioses.

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A. Cleveland (✉) · E. A. Verde
Corning School of Ocean Studies, Maine Maritime Academy, Castine, ME 04420, USA
e-mail: ann.cleveland@mma.edu

E. A. Verde
e-mail: alan.verde@mma.edu

R. W. Lee
School of Biological Sciences, Washington State University, Pullman, WA 99164, USA
e-mail: rlee@mail.wsu.edu

## Introduction

The symbiosis between cnidarians and endosymbiotic dinoflagellates ("zooxanthellae") has long been cited as the dominant reason for their combined success in tropical reef ecosystems that are notoriously nutrient poor (Davies 1992). The endosymbiotic dinoflagellates gain access to a protected space for photosynthesis and inorganic and organic molecules translocated from the host (Lipschultz and Cook 2002). The host cnidarians gain access to two metabolic pathways not available to animals—photosynthesis and the synthesis of essential amino acids (Wang and Douglas 1999). The algal/cnidarian symbiosis succeeds because the metabolic waste material from one partner is collectively utilized by the other partner via a tightly coupled recycling of both inorganic and organic carbon and nitrogen compounds (reviews by Yellowlees et al. 2008; Houlbrèque and Ferrier-Pagès 2009). Both carbon and nitrogen (Furla et al. 2005; Venn et al. 2008) are obtained by the zooxanthellae via metabolic byproducts from the host cnidarian and/or surrounding seawater. The cnidarian host, in turn, obtains both carbon (Muscatine 1990; Biel et al. 2007) and nitrogenous organic products (Wang and Douglas 1998; Wang and Douglas 1999) from its zooxanthellae and external seawater via DOM uptake and heterotrophy (Bachar et al. 2007; Houlbrèque and Ferrier-Pagès 2009). With regard to nitrogen, ammonia transfer between host cnidarian and endosymbiotic zooxanthellae has been demonstrated using $^{15}N$-labeled ammonia (Roberts et al. 1999; Tanaka et al. 2006).

The symbiotic relationship between cnidarians and zooxanthellae has thus been well characterized. One of the major metabolic pathways studied is the translocation of host nitrogenous waste to the endosymbiotic zooxanthellae (Muscatine and D'Elia 1978; Wilkerson and Trench 1986;

Roberts et al. 1999). The depletion-diffusion model of ammonium uptake suggests that zooxanthellae deplete ammonium from the host cytoplasm after inward diffusion of ammonium from the surrounding seawater (D'Elia et al. 1983). Once the zooxanthellae have gained access to ammonium, levels of photosynthesis, symbiont densities, and protein content of host and zooxanthellae all increase (Fitt and Cook 2001). The increase in photosynthesis and zooxanthella density then leads to increased growth rates of the cnidarian host (Meyer and Schulz 1985; Hoegh-Guldberg and Smith 1989; Fitt and Cook 2001). Any enhancement of ammonium availability to this dynamic system should therefore increase these physiological effects.

It has long been suspected that reef fishes and invertebrates facultatively or obligately associated with cnidarians may have a positive effect on their hosts (Pinnegar and Polunin 2006; Holbrook et al. 2008). Resident fishes have been shown to protect their hosts from predators (Fautin 1991, Fautin and Allen 1992; Godwin and Fautin 1992; Porat and Chadwick-Furman 2004), and fishes and invertebrates may mix stagnant water and remove sediments within the cnidarian's tentacles (Liberman et al. 1995; Goldshmid et al. 2004; Stewart et al. 2006), but recent studies have provided indirect evidence that fishes provide nitrogen to their hosts, and subsequently their endosymbiotic zooxanthellae as well. Arvedlund et al. (2006) suggested that juvenile cleaner wrasses *Thalassoma amblycephalum* may not only serve unusual clients, the anemones *Entacmaea quadricolor* and *Heteractis magnifica*, by removing mucus and necrotic tissue, but may also provide a possible ammonium benefit to the anemones. Meyer and Schultz (1985) found that coral heads associated with resting schools of grunts had higher growth rates and greater zooxanthella densities.

While the associations between grunts and coral heads, and cleaner wrasses and anemones are facultative, many pomacentrid fishes show obligate associations with cnidarians for at least some part of their life cycle. In these obligate associations between pomacentrids and cnidarians, the enhancement effects of associated fishes are even greater. Liberman et al. (1995) demonstrated that the coral *Stylophora pistillata* hosting the damselfish *Dascyllus marginatus* had increased polyp surface area and higher reproductive rates than corals without fish. The anemone *Entacmaea quadricolor* regenerated faster, had higher growth rates and survival, and greater zooxanthella densities, when associated with the obligate anemonefish *Amphiprion bicinctus* (Porat and Chadwick-Furman 2004, 2005). Holbrook and Schmitt (2005) examined the growth and survivorship of the sea anemone *Heteractis magnifica* associated with the orange-fin anemonefish *Amphiprion chrysopterus*; they found that growth rates, fission rates, and survivorship were greatest in anemones that had fish associated with them.

While these studies provide good indirect evidence of the role fishes play in enhancing the growth and survivorship of their obligate cnidarian hosts, the mechanism is still not well-characterized. Roopin et al. (2008) demonstrated that under laboratory conditions, *A. bicinctus* excreted ammonium and the host anemone *E. quadricolor* absorbed ammonium. When members of these two species were incubated together, the buildup of ammonium in laboratory conditions was very low, strongly suggesting that the anemone, and potentially its endosymbiotic zooxanthellae, absorbed the excreted ammonium. Roopin and Chadwick (2009) further showed that ammonium supplements prevented long-term tissue loss in starved *E. quadricolor* to the same extent as did culturing the anemones with live anemonefish, indicating that excreted ammonium from fish symbionts augments anemone nutrition. Godinot and Chadwick (2009) additionally suggested that phosphate is readily excreted by anemonefish; however, laboratory studies suggest that excreted levels of phosphate are insufficient to supply the phosphate demand of anemones.

Muscatine and Kaplan (1994) and Roberts et al. (1999) have successfully demonstrated ammonium transfer between host cnidarians and endosymbiotic zooxanthellae using $^{15}$N-labeled ammonia. Natural levels of stable isotopes have also been used to elucidate trophic webs in marine ecosystems (Heikoop et al. 2000; Cocheret de la Morinière et al. 2003; Pinnegar and Polunin 2006; Ho et al. 2007). Thus, one promising method of demonstrating direct nitrogen transfer between resident anemonefishes, host anemones, and endosymbiotic zooxanthellae is the use of stable isotope $^{15}$N tracer studies.

In Negros Oriental, Philippines, six species of anemonefishes of the genus *Amphiprion* are found most commonly in association with three anemones (*Heteractis crispa, Entacmaea quadricolor, Stichodactyla* sp.). *Amphiprion clarkii* and *Amphiprion perideraion* are the dominant species of resident anemonefishes of *Heteractis crispa* at this site. Both species are planktivorous, actively feeding throughout the day in the water column, and return to their host anemones at night. The amount of time fish of each species spend in close contact with their host anemones (<25 cm from anemone) varies among species, with individuals of *A. perideraion* spending significantly more time ($\sim 4\times$) near their anemones than do those of *A. clarkii* (Cleveland et al. 2006). Consequently, there may be anemonefish-specific differences in the quantities of nutrients that are provided to the host anemones.

In the present study, we used stable isotopes $^{15}$N and $^{13}$C to provide the first direct evidence of nutrient transfer from resident anemonefishes to host anemones and subsequent transfer to endosymbiotic zooxanthellae. We hypothesized that these fishes provide a predictable and substantial source of nitrogen to their host anemones as well as the

symbiotic zooxanthellae that live within the anemones. We specifically asked whether (1) there is direct transfer of N- and C-containing products from the anemonefishes to the anemone hosts, and if so, (2) whether endosymbiotic zooxanthellae acquire these products as well. Both field and laboratory *A perideraion* and *A. clarkii* were fed a diet formulated with $^{15}N$ and $^{13}C$, and both fish and anemone tissues were analyzed for the presence of $^{15}N$ and $^{13}C$. The presence of any isotope in the anemone tissues was interpreted as direct transfer of C and/or N from resident fish to host anemone and endosymbiotic zooxanthellae.

## Methods

The experiments described elsewhere occurred May–August in multiple years (2004, 2007, and 2010). Laboratory feeding experiments were conducted at the Silliman University Marine Laboratory (SUML), Dumaguete, Negros Oriental, Philippines, and field feeding experiments were conducted in situ on patch reefs in front of the laboratory. Anemonefish sample sizes were unequal within treatments (Table 1) due to limited laboratory space for aquaria and limited anemonefish/anemone pairs that met field criteria (see below).

Anemone size can vary widely and is highly dependent on the water content of the coelenteron; we thus estimated the size of 108 haphazardly chosen anemones by measuring the tentacular crown diameter (surface area covered by the tentacles). The average (±SE) diameter of the tentacular crown (19.9 ± 0.6 cm) of *Heteractis crispa* was determined by measuring both the longest and shortest diameters perpendicular to each other and obtaining the mean value. The size distribution of anemones subsequently collected was assumed to reflect the distribution observed in the field.

### Collection and maintenance

Adult anemonefishes (*Amphiprion clarkii* and *A. perideraion*, Table 1) were collected from shallow patch reefs (<5 m) in front of SUML using SCUBA and hand nets. Fishes were collected from 1900 to 2200 by locating an anemone and gently probing until a fish was discovered. Fishes were immediately brought to a shaded (translucent roof) concrete table and placed in individual 38-L aquaria with running seawater (flushing rate $\sim$ 82 L $h^{-1}$). Prior to placement of fishes, each aquarium was provided with an artificial shelter (10 cm diameter × 20 cm length PVC tubing) and a single *H. crispa*. Fishes were maintained under ambient temperature conditions (range = 29 ± 1°C) and were fed twice daily on artificial diets (see below).

Anemones (*H. crispa*) were collected by hand using SCUBA. Where possible anemones were selected that were seen to host fishes, but behavioral studies of *A. perideraion* and *A. clarkii* suggest that mated pairs often inhabit more than one anemone, spending time in 2–5 different anemones over the course of the day (Cleveland et al. 2006). Individual *H. crispa* were haphazardly located by swimming over small patch reefs. The base of each anemone was buried in sand and shell gravel that was gently dug away until the diver could easily grasp the rock or coral rubble on which the anemone was attached. Both the rubble and the anemone were lifted from the sediment and placed in a submerged mesh bag and transported to the laboratory within 1 h of collection. The rock or coral stub to which the anemone attached was removed and anemones were allowed to attach to the glass bottom of the 38-L aquaria. One fish was added to each aquarium within 24 h of the anemone placement. We did not purposely keep the same anemonefish–anemone pairs from the field, although it is possible that some fish may have been reunited with their original anemone.

**Table 1** Biometric data (weight and standard length), sample sizes, and treatment conditions of the two anemonefish species used in this study

| Year | Treatment | Fish species | *N* | Weight (g) (Mean ± SE) | Standard length (mm) (Mean ± SE) |
|---|---|---|---|---|---|
| 2004 | Aquarium fed | *A. clarkii* | 9 | 24.7 ± 1.6 | 85.6 ± 1.8 |
| | | *A. perideraion* | 9 | 11.4 ± 0.9 | 68.0 ± 2.1 |
| 2004 | Field fed | *A. clarkii* | 15 | 20.8 ± 1.2 | 79.9 ± 1.4 |
| 2007 | Field fed | *A. clarkii* | 8 | 21.5 ± 3.2 | 76.6 ± 5.1 |
| | | *A. perideraion* | 13 | 9.4 ± 1.1 | 62.4 ± 2.8 |
| 2007 | Control | *A. clarkii* | 23 | 24.7 ± 1.6 | 85.3 ± 1.6 |
| | | *A. perideraion* | 22 | 11.4 ± 0.9 | 70.3 ± 1.8 |

Control anemonefishes include all fishes that were fed unlabeled food, in both laboratory aquaria and in situ, and wild-caught, unfed fish. As $\delta^{13}C$ and $^{15}N$ signatures were not statistically different (Tukey's HSD test, $p > 0.05$) between these control groups, the three classes of control fishes were combined

### Anemonefish diet formulation

We formulated an artificial diet that could be used for both control and isotope-labeled experiments. The control diet was made by grinding 30 g of commercial fish pellets to a fine powder and adding it to a mixture of 2 g agar and 100 ml boiling water (RO water). The resultant paste was spread out to a thickness of $\sim$1 mm and allowed to gel in a refrigerator. The food was refrigerated in a sealed container until use. Small pellets ($\sim 0.025 \pm 0.003$ g pellet$^{-1}$) were cut from the gelled food 5 min prior to each feeding. A fresh batch of food was made approximately every 5 days. The isotope-labeled diet was made as earlier, but 0.5 g of $^{15}$N- and $^{13}$C-labeled Isogro (Sigma–Aldrich) was added to the powdered food mixture prior to adding the boiling water.

### Laboratory feeding experiment

Laboratory fishes were acclimated to the aquaria and anemones for 24 h prior to the first day of feeding and then fed ad libitum on an artificial diet twice daily (0900 and 1500). Fishes were first trained to feed on an unlabeled diet for 10 days in the same aquaria as the anemones (however, feeding of fishes occurred at the opposite side of the aquarium from the anemones); all fishes accepted pellets by the end of the 10-day training period. Fishes were offered a single pellet at a time; if a fish refused a pellet, it was immediately removed from the aquarium by suction pipette. When a fish refused two pellets in sequence, feeding was stopped. After the training period, 11 *A. perideraion* and 9 *A. clarkii* were haphazardly selected and offered the $^{15}$N-$^{13}$C-labeled diet ad libitum twice daily for 5 days. The remaining 3 *A. perideraion* and 2 *A. clarkii* continued feeding on the unlabeled diet as control aquarium-fed fish. An additional 5 anemones were collected in July 2010 and were placed in individual 38-L aquaria as described above. Pellets were placed into each aquarium and allowed to sit for 5 min twice per day. This treatment served as an additional control to determine whether significant $^{15}$N and/or $^{13}$C transfer occurred directly from the pelleted food to the anemones.

### Field feeding experiment

Ten *A. perideraion* and 15 *A. clarkii* were chosen in 2004, and 13 *A. perideraion* and 8 *A. clarkii* were chosen in 2007, for in situ feeding experiments in the field at water depths of 3–5 m. To select the fishes, we observed the adult individuals of visually estimated average standard length (65–75 mm for *A. perideraion*, 75–85 mm for *A. clarkii*) for $\sim$10 min to determine the anemone each individual spent the most time in. Anemones were selected so that >5 m separated anemones of fishes to be fed. Individual fishes and their host anemones were identified by placing numbered flagging tape adjacent to the anemones. Fishes were trained to feed from a SCUBA diver twice daily (1000 and 1500); the diver would approach no closer than 2 m to the anemone and release artificial food pellets (see above) into the water column from a 50-ml capped vial. The training period was 5 days in 2004; *A. clarkii* quickly learned to accept food from divers during the training period such that they actively swam to the diver to receive the food. We were unsuccessful in feeding *A. perideraion* in 2004. In 2007, we switched from SCUBA to snorkel and extended the training period to 24 days and were then successful in feeding both *A. perideraion* and *A. clarkii* in the field. Once fishes were trained to feed from divers, $^{15}$N-$^{13}$C-labeled food was fed to the fishes for 7–8 days. Care was taken to feed 2–3 m from the host anemone so that no labeled food was consumed directly by the anemone. Uneaten food was quickly consumed by several wrasse species resident on the reef; indeed, the wrasses competed greatly with the anemonefishes for the food so we were only able to release 3–4 pellets at a time. Control fishes were fed unlabeled diet during this second feeding stage: 3 field *A. clarkii* in 2004 and 3 field *A. perideraion* and 4 field *A. clarkii* in 2007. Control fishes were located on the periphery of the patch reef $\sim$10 m away from where the feeding experiments occurred; this was to further minimize any possibility of the isotope-labeled pellets being eaten by control fishes. Additional unfed controls (14 *A. clarkii* and 16 *A. perideraion*) were collected from the reef $\sim$500 m away from the in situ experiment.

### Fish tissue processing

At the end of the $^{15}$N-$^{13}$C feeding trials, fishes were killed and liver, gill, and muscle tissue were removed (gonads were also removed when visible). Tissues were initially dried to constant weight at 63°C for 24–48 h and stored in airtight centrifuge tubes (SUML) and then further dried (90°C) and powdered using an aluminum mortar and motorized pestle at Maine Maritime Academy (MMA). The stable isotope analyses were performed at Washington State University (WSU) as described later.

### Anemone tissue processing

Zooxanthellae are known to concentrate in the tentacles and oral disc of host anemones (Verde unpubl data), so this section of the anemone was removed and processed. The tentacles and oral disc were homogenized in $\sim$100 ml of 200 mM $NaHPO_4$ (pH = 8) extraction buffer and filtered through a 100-μm mesh strainer to remove mesoglea. The filtered homogenate was brought to 250 ml with additional

extraction buffer and thoroughly mixed. Approximately 50 ml of this intact anemone homogenate was immediately removed to a plastic weigh boat and dried to constant weight at 63°C.

Two 50-ml aliquots of the remaining homogenate were centrifuged at ~7,000 rpm for 5 min; this separated each aliquot into a zooxanthellate algal pellet and an animal supernatant. The animal supernatant from each tube was combined into a single weigh boat and dried at 63°C. To obtain a clean algal fraction, the small amount of animal debris above each algal pellet was removed; the two algal pellets were then resuspended in 50 ml of extraction buffer and centrifuged a further 5 min at ~7,000 rpm. The supernatant and the animal tissue layer immediately above the algal pellet were discarded; the two resultant pellets were combined and resuspended a final time in 50 ml of extraction buffer, thoroughly mixed, and dried to constant weight at 63°C (SUML). The intact, animal, and zooxanthellate fractions of the sample were re-dried (90°C) and ground to a fine powder using an aluminum mortar and motorized pestle (MMA) and analyzed for $^{15}N$ and $^{13}C$ (WSU) as described later.

### Stable isotope analysis

Dried fish tissue or anemone fraction samples were added to tin capsules and combusted in a Costech (Valencia, USA) elemental analyzer in the laboratory of RWL. The resulting $N_2$ and $CO_2$ gases were separated by gas chromatography and admitted into the inlet of a GV Instruments (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for the determination of $^{15}N{\cdot}^{14}N^{-1}$ and $^{13}C{\cdot}^{12}C^{-1}$ ratios. Typical precision of analyses was ±0.5‰ for $\delta^{15}N$ and ±0.2‰ for $\delta^{13}C$ where $\delta(‰) = [(R_{sample} \times R_{standard}{}^{-1}) - 1] \times 1,000$ with $R = {}^{15}N{\cdot}^{14}N^{-1}$ or $R = {}^{13}C{\cdot}^{12}C^{-1}$. The standard for $\delta^{15}N$ was atmospheric nitrogen, and for $\delta^{13}C$, the standard was Peedee belemnite (PDB). Delta ($\delta$) values correlate with $^{15}N$ and $^{13}C$ content of samples with higher $\delta$ values corresponding to higher $^{15}N$ or $^{13}C$ content. Egg albumin was used as a daily internal reference material.

### Statistical analyses

Since all data sets met the assumptions of normality and homogeneity of variances (Barlett's test), parametric statistical analysis were conducted (Zar 1999). Student's *t* test, analysis of variance (one-, two-, and three-way ANOVA) and post hoc Tukey (HSD) tests were performed using the statistical package JMP 7.0 (SAS Institute). From year to year (between 2004 and 2007), $\delta^{15}N$ or $\delta^{13}C$ data sets for either control or treatment categories (for either fish or anemone tissues) that were not significantly different ($p > 0.05$) were subsequently pooled. The ensuing data were visualized using the SlideWrite Plus (Advanced Graphics Software) graphics package. Data are reported as means ± standard error (SE).

## Results

### Uptake of $^{15}N$ and $^{13}C$ labels

The isotopic labels $^{15}N$ and $^{13}C$ were taken up successfully in all treatments; this was true for both laboratory and field experiments. In all cases, $\delta^{15}N$ and $\delta^{13}C$ levels of experimental fishes, anemones, and zooxanthellae were significantly higher than those of control fishes, control anemones incubated with fishes, control anemones incubated without fishes, and control zooxanthellae ($p < 0.05$ for all comparisons, Tables 2 and 3 for fish tissues, Table 4 for anemone and zooxanthellae fractions). This provided the necessary evidence that the labeling technique was successful and also demonstrated that there was no significant difference in isotopic labels $^{15}N$ and $^{13}C$ between control anemones incubated with control fishes and control anemones without fishes. Because the isotopic levels were so low in control tissues relative to experimental tissues, control data do not appear in subsequent figures.

### Aquarium-fed fish

All fish tissues of *Amphiprion clarkii* and *A. perideraion* were significantly enriched with $^{15}N$ at the end of the aquarium feeding trials (Fig. 1a). $\delta^{15}N$ was highest in the liver for both species (two-way ANOVA, $F_{4,59\ \text{tissue}} = 67.3$, $p_{\text{tissue}} < 0.0001$); the isotopic signals were 6–7 times higher in the liver than those found in muscle tissue. $\delta^{15}N$ in gill tissues was also significantly higher than in muscle tissue for both species. The $\delta^{15}N$ of gonad tissue in fishes with developed gonads was intermediate between gill and muscle tissue. Both *A. clarkii* and *A. perideraion* showed similar patterns of $\delta^{15}N$, and there was no significant species effect (two-way ANOVA, $F_{1,59\ \text{species}} = 0.02$, $p_{\text{species}} = 0.89$, Fig. 1a) in $^{15}N$ accumulation in fish tissues.

The pattern for $\delta\ ^{13}C$ in the fish tissues of *A. clarkii* and *A. perideraion* was similar to that of $\delta\ ^{15}N$ (two-way ANOVA, $F_{4,59\ \text{tissue}} = 43.8$, $p_{\text{tissue}} < 0.0001$, $F_{1,59\ \text{species}} = 0.4$, $p_{\text{species}} = 0.51$, Fig. 1b). Liver $\delta^{13}C$ was highest for both fish species, followed by gill $\delta^{13}C$ and muscle $\delta^{13}C$. $\delta^{13}C$ of gonadal tissue was again intermediate between gill and muscle tissue. There was no significant difference in

**Table 2** $\delta^{15}$N levels in five fish tissue types (liver, gill, muscle, eggs, and testes)

| Fish species | Tissue | Control $\delta^{15}$N (Mean ± SE) | *N* | Aquarium Fed $\delta^{15}$N (Mean ± SE) | *N* | Field Fed$_{2004}$ $\delta^{15}$N (Mean ± SE) | *N* | Field Fed$_{2007}$ $\delta^{15}$N (Mean ± SE) | *N* |
|---|---|---|---|---|---|---|---|---|---|
| *A. clarkii* | Liver | 8.3 ± 0.2 | 23 | 929.2 ± 40.0 | 9 | 305.3 ± 54.8 | 15 | 208.2 ± 48.2 | 8 |
| | Gill | 9.1 ± 0.2 | 23 | 414.7 ± 33.4 | 9 | 184.2 ± 31.5 | 15 | 105.1 ± 25.3 | 8 |
| | Muscle | 10.1 ± 0.2 | 23 | 144.4 ± 9.9 | 9 | 74.6 ± 13.9 | 15 | 31.4 ± 6.9 | 8 |
| | Eggs | 8.6 ± 0.2 | 8 | 339.7 ± 48.0 | 4 | 316.4 ± 126.4 | 5 | 301.4 ± 101.7 | 3 |
| | Testes | 11.4 ± 3.4 | 9 | 369.5 ± 47.5 | 4 | 177.0 ± 66.5 | 5 | 130.7 ± 89.1 | 3 |
| *A. perideraion* | Liver | 7.6 ± 0.5 | 22 | 866.0 ± 80.8 | 8 | | | 378.5 ± 93.6 | 13 |
| | Gill | 7.6 ± 0.3 | 22 | 466.4 ± 69.2 | 9 | | | 152.1 ± 36.3 | 13 |
| | Muscle | 8.2 ± 0.3 | 22 | 172.7 ± 14.5 | 8 | | | 52.8 ± 11.9 | 13 |
| | Eggs | 6.3 ± 0.5 | 17 | 419.5 ± 71.2 | 3 | | | 655.6 ± 188.8 | 7 |
| | Testes | 6.9 ± 0.4 | 3 | 245.0 ± 20.1 | 2 | | | 161.2 ± 78.9 | 3 |

In all cases, tissues from experimental treatments had significantly higher $\delta^{15}$N (Tukey's HSD, $p < 0.05$, factors were treatment versus control, species, tissue) than control tissues. Data for control fish from 2004 and 2007 were combined as they did not differ significantly (Student's *t* test, $p > 0.05$) between 2004 and 2007. *A. perideraion* failed to feed in the field in 2004 so there are no data for that interval

**Table 3** $\delta^{13}$C levels in five fish tissue types (liver, gill, muscle, eggs, and testes)

| Fish species | Tissue | Control $\delta^{13}$C (Mean ± SE) | *N* | Aquarium Fed $\delta^{13}$C (Mean ± SE) | *N* | Field Fed$_{2004}$ $\delta^{13}$C (Mean ± SE) | *N* | Field Fed$_{2007}$ $\delta^{13}$C (Mean ± SE) | *N* |
|---|---|---|---|---|---|---|---|---|---|
| *A. clarkii* | Liver | −16.9 ± 0.4 | 23 | 205.8 ± 11.0 | 9 | 63.7 ± 16.2 | 15 | 23.5 ± 7.6 | 8 |
| | Gill | −15.4 ± 0.3 | 23 | 88.2 ± 10.0 | 9 | 24.2 ± 7.3 | 15 | 8.9 ± 8.2 | 8 |
| | Muscle | −15.4 ± 0.2 | 23 | 29.2 ± 3.2 | 9 | 4.7 ± 4.3 | 15 | −7.8 ± 2.3 | 8 |
| | Eggs | −16.0 ± 0.5 | 8 | 70.9 ± 12.8 | 4 | 35.3 ± 27.1 | 5 | 48.7 ± 26.9 | 3 |
| | Testes | −14.2 ± 0.7 | 9 | 84.3 ± 9.4 | 4 | 28.9 ± 16.6 | 5 | 13.4 ± 27.2 | 3 |
| *A. perideraion* | Liver | −15.7 ± 0.6 | 22 | 188.4 ± 24.1 | 8 | | | 37.5 ± 15.5 | 13 |
| | Gill | −13.6 ± 0.2 | 22 | 91.8 ± 17.1 | 9 | | | 9.0 ± 6.3 | 13 |
| | Muscle | −14.2 ± 0.1 | 22 | 38.2 ± 4.6 | 8 | | | −2.5 ± 3.2 | 13 |
| | Eggs | −14.6 ± 0.3 | 17 | 65.0 ± 23.6 | 3 | | | 101.9 ± 32.3 | 7 |
| | Testes | −14.0 ± 0.2 | 3 | 59.1 ± 3.9 | 2 | | | 20.1 ± 17.5 | 3 |

In all cases, tissues from experimental treatments had significantly higher $\delta^{13}$C (Tukey's HSD test, $p < 0.05$, factors were treatment versus control, species, tissue) than control tissues. Data for control fish from 2004 and 2007 were also combined as they did not differ significantly (Student's *t* test, $p > 0.05$) between 2004 and 2007. *A. perideraion* failed to feed in the field in 2004 so there are no data for that interval

label uptake between the two species; this repeated the pattern seen with $\delta^{15}$N.

## Field-fed fish

Only *A. clarkii* was successfully trained to feed on labeled food in 2004. $\delta^{15}$N was again significantly higher in liver tissue (one-way ANOVA, $F_{4,51} = 4.62$, $p = 0.003$, Fig. 2a) and also in egg tissue than it was in muscle tissue. Although $\delta^{15}$N was tended to be higher in liver and egg tissue than in gill tissue and testes, this difference was not significant. This pattern was essentially repeated for $\delta^{13}$C, with the highest level of isotope in the liver (one-way ANOVA, $F_{4,51} = 3.8$, $p = 0.009$, Fig. 2b) and the lowest level in muscle tissue. $\delta^{13}$C in gill, eggs, and testes were intermediate between liver and muscle and did not differ significantly from either tissue.

Both *A. clarkii* and *A. perideraion* were fed successfully on labeled diet in 2007, and there was a significant difference in $\delta^{15}$N between species (two-way ANOVA, $F_{1,73\ \text{species}} = 4.5$, $p_{\text{species}} = 0.04$, Fig. 3a) with *A. perideraion* having higher $\delta^{15}$N levels. There was considerably more variation in tissue $\delta^{15}$N in field-fed fishes than in aquarium-fed fishes, most likely due to variation in the amount of labeled pellets that individual fish consumed as it was not possible to feed fishes to satiation in situ. As a result of this variability, patterns in the tissue accumulation of $^{15}$N were harder to discern but still showed significant variation (two-way ANOVA, $F_{4,53\ \text{tissue}} = 7.2$, $p_{\text{tissue}} < 0.0001$, Fig. 3a). In contrast with what we saw with the aquarium-fed fishes, the $\delta^{15}$N signal was not highest in the liver but rather in the egg tissue. For *A. perideraion*, $\delta^{15}$N in egg tissue was significantly greater than in gill or muscle tissue but did not differ significantly from liver tissue or testes.

**Table 4** $\delta^{15}$N and $\delta^{13}$C levels in three anemone fractions (intact anemone, animal fraction, zooxanthellae fraction)

| Fish species | Tissue | Control $\delta^{15}$N (Mean ± SE) | *N* | Aquarium Fed $\delta^{15}$N (Mean ± SE) | *N* | Field Fed$_{2004}$ $\delta^{15}$N (Mean ± SE) | *N* | Field Fed$_{2007}$ $\delta^{15}$N (Mean ± SE) | *N* |
|---|---|---|---|---|---|---|---|---|---|
| *A. clarkii* | Intact | 5.0 ± 0.6 | 6 | 80.3 ± 9.6 | 9 | 15.3 ± 2.6 | 10 | 17.5 ± na | 1 |
| | Animal | 5.2 ± 0.6 | 6 | 60.0 ± 7.1 | 9 | 14.5 ± 2.5 | 10 | 12.5 ± 1.7 | 2 |
| | Zooxanthellae | −3.8 ± 0.6 | 6 | 171.7 ± 23.0 | 9 | 25.3 ± 5.0 | 10 | 31.8 ± 15.4 | 2 |
| *A. perideraion* | Intact | 7.9 ± 2.4 | 8 | 58.0 ± 7.2 | 11 | | | 26.1 ± 6.2 | 7 |
| | Animal | 8.5 ± 3.0 | 8 | 39.1 ± 3.3 | 11 | | | 23.6 ± 7.1 | 6 |
| | Zooxanthellae | 0.3 ± 1.3 | 7 | 104.9 ± 12.1 | 11 | | | 39.2 ± 9.8 | 7 |
| Fish species | Tissue | Control $\delta^{13}$C (Mean ± SE) | *N* | Aquarium Fed $\delta^{13}$C (Mean ± SE) | *N* | Field Fed$_{2004}$ $\delta^{13}$C (Mean ± SE) | *N* | Field Fed$_{2007}$ $\delta^{13}$C (Mean ± SE) | *N* |
| *A. clarkii* | Intact | −12.7 ± 0.5 | 6 | −7.5 ± 1.0 | 9 | −12.1 ± 0.2 | 10 | −12.9 ± na | 1 |
| | Animal | −12.7 ± 0.3 | 6 | −7.7 ± 1.0 | 9 | −12.1 ± 0.2 | 10 | −12.1 ± 0.7 | 2 |
| | Zooxanthellae | −16.3 ± 0.7 | 6 | −8.2 ± 1.0 | 9 | −12.4 ± 0.3 | 10 | −13.0 ± 2.0 | 2 |
| *A. perideraion* | Intact | −12.5 ± 0.5 | 8 | −10.5 ± 0.3 | 11 | | | −10.1 ± 0.8 | 7 |
| | Animal | −12.2 ± 0.7 | 8 | −10.1 ± 0.4 | 11 | | | −9.9 ± 1.1 | 6 |
| | Zooxanthellae | −15.8 ± 0.5 | 7 | −11.7 ± 0.5 | 11 | | | −12.8 ± 0.3 | 7 |

In all cases, tissues from experimental treatments had significantly higher $\delta^{15}$N (Tukey's HSD test, $p < 0.05$, factors were treatment versus control, species, tissue) than control tissues. *A. perideraion* failed to feed in the field in 2004 so there are no data for that interval. For control anemones, a total of 6 anemones that hosted *A. clarkii* and 8 anemones that hosted *A. perideraion* were sampled during 2004 and 2007, but as no significant difference was detected (Student's *t* test, $p > 0.05$) between the 2 years, the data were combined

$\delta^{15}$N of *A. clarkii* tissues did not differ statistically from each other, although the pattern of their signals mimicked those of *A. perideraion*. This contrasted sharply with the 2004 results for *A. clarkii* where we were able to discern significant differences in label concentrations between liver, eggs and muscle.

In contrast to the significant species effect on $^{15}$N signals in field-fed fishes, a species effect was not significant in $\delta^{13}$C accumulation in tissues (two-way ANOVA, $F_{1,73\ \text{species}} = 2.3$, $p_{\text{species}} = 0.13$, Fig. 3b). There was a significant tissue effect (two-way ANOVA, $F_{4,73\ \text{tissue}} = 6.9$, $p_{\text{tissue}} < 0.0001$, Fig. 3b) with $\delta^{13}$C levels being highest in the egg tissue of *A. perideraion*, followed by egg tissue of *A. clarkii* (although egg $\delta^{13}$C in *A. clarkii* was not significantly different from $\delta^{13}$C in the other tissues). While $\delta^{13}$C levels in liver, gill and muscle tissues were not significantly different from each other, they followed the pattern of accumulation seen thus far in that liver signals tended to be highest and muscle signals tended to be lowest, with gill tissue signals intermediate.

## Anemone hosts of aquarium-fed fish

All three anemone fractions (intact anemone, animal, zooxanthellae) were significantly enriched in $^{15}$N and $^{13}$C relative to controls. $\delta^{15}$N in these fractions was at least an order of magnitude greater than control $\delta^{15}$N (Table 4) with the highest concentration evident in zooxanthellae (two-way ANOVA, $F_{2,56\ \text{tissue}} = 32.7$, $p_{\text{tissue}} < 0.0001$, Fig. 4a). $\delta^{15}$N in zooxanthellae was 2–3 times greater than $\delta^{15}$N in either intact or animal fractions in the aquarium experiment, and there was a significant species effect (two-way ANOVA, $F_{1,56\ \text{species}} = 15.2$, $p_{\text{species}} = 0.003$, Fig. 4a). Anemones with resident *A. clarkii* had significantly higher levels of $\delta^{15}$N in zooxanthellae than did anemones with resident *A. perideraion.*

There was no significant difference (two-way ANOVA, $F_{2,56\ \text{tissue}} = 1.26$, $p_{\text{tissue}} = 0.29$, Fig. 4b) in $\delta^{13}$C among intact anemone, animal, and zooxanthellae fractions in anemones of aquarium-fed fishes, but the levels were significantly elevated relative to control anemones ($\delta^{13}$C was lower than that of the PDB standard for both experimental and control anemones so values are negative). There was a significant species effect (two-way ANOVA, $F_{1,56\ \text{species}} = 24.6$, $p_{\text{species}} < 0.0001$, Fig. 4b) with $\delta^{13}$C tending to be higher in anemones with resident *A. clarkii*.

## Anemone hosts of field-fed fishes

As seen in the anemone hosts of the aquarium-fed fishes, $\delta^{15}$N and $\delta^{13}$C of intact anemone, animal, and zooxanthellate fractions were significantly higher than controls for host anemones of field-fed fishes. However, the magnitude of the increase was much less than that seen for aquarium-fed fishes. In 2004, only *A. clarkii* ate the labeled pellets, but in 2007 both fish species fed successfully. While the results of the aquarium feeding experiments demonstrated significant differences in $\delta^{15}$N between the different

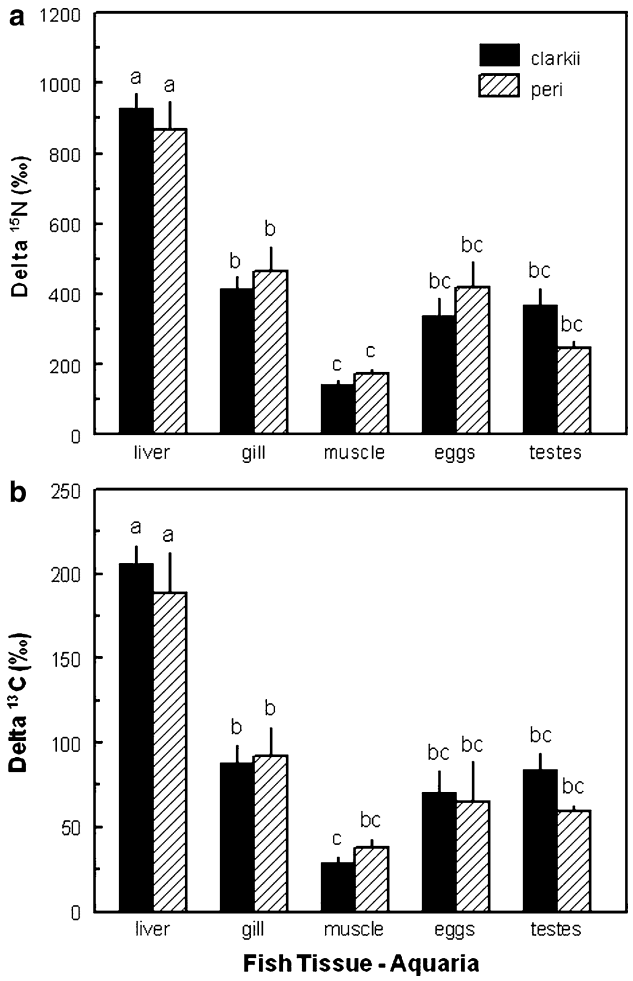


**Fig. 1** $\delta^{15}$N (**a**) and $\delta^{13}$C (**b**) in selected fish tissues of *A. clarkii* and *A. perideraion* after 5 days of ad libitum feeding on a $^{15}$N-$^{13}$C-labeled diet. Fish were maintained in 38-L aquaria with running seawater. *Histogram bars* that share letters are not significantly different (Tukey's HSD test, $p > 0.05$). Data are mean ± SE. For fish sample sizes (*N*), refer to appropriate column in Tables 2 and 3

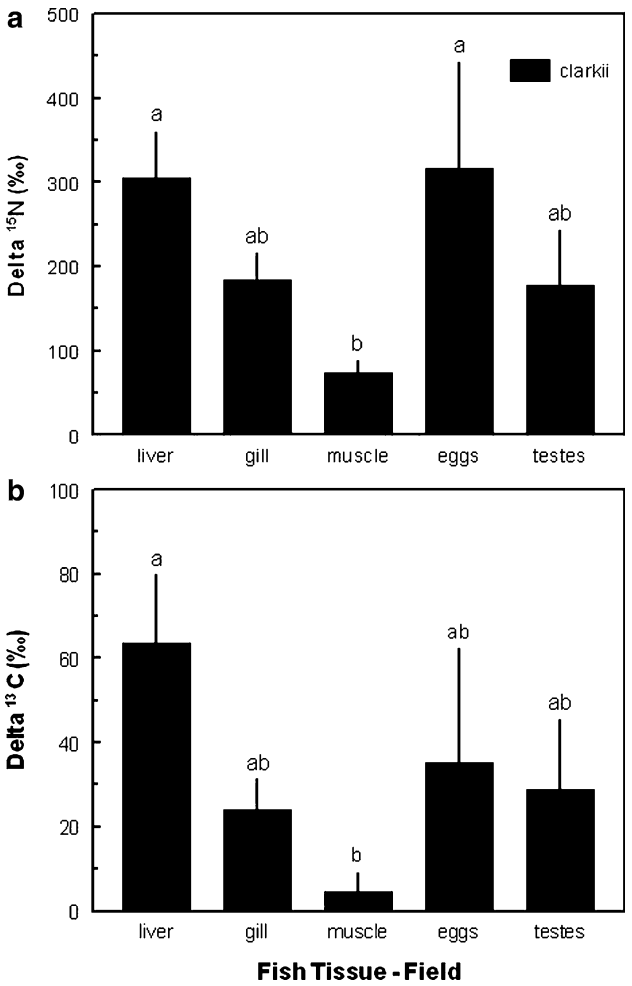


**Fig. 2** $\delta^{15}$N (**a**) and $\delta^{13}$C (**b**) in selected fish tissues of *A. clarkii* trained to feed on a $^{15}$N-$^{13}$C-labeled diet in the field in 2004. Fish were fed labeled diet for 7–8 consecutive days prior to collection. *Histogram bars* that share letters are not significantly different (Tukey's HSD test, $p > 0.05$). Data are mean ± SE. For fish sample sizes (*N*), refer to appropriate column in Tables 2 and 3

anemone fractions, we did not see significant differences among fractions for the field experiments. However, the trend toward increased $\delta^{15}$N in zooxanthellae fractions was still apparent. In 2004, $\delta^{15}$N in zooxanthellae was almost twice that of $\delta^{15}$N in intact anemone and animal fractions (one-way ANOVA, $F_{2,28} = 0.8$, $p = 0.07$, Fig. 5a) for host anemones of *A. clarkii*. In 2007, again $\delta^{15}$N in zooxanthellae tended toward a value twice that of the other fractions (two-way ANOVA, $F_{2,21\ \text{tissue}} = 1.2$, $p_{\text{tissue}} = 0.31$, $F_{1,21\ \text{species}} = 0.7$, $p_{\text{species}} = 0.39$, Fig. 5b). The variation in the amount of food ingested by field-fed fishes could have led to the high variation seen in $\delta^{15}$N in anemone fractions and thus potentially masks significant fractionation among tissue types.

While $\delta^{13}$C was significantly higher in all anemone tissue fractions of experimental anemones relative to controls, no pattern was revealed between the different fractions. There was no significant difference in $\delta^{13}$C among intact, anemone, or zooxanthellate fractions in 2004 (one-way ANOVA, $F_{2,28} = 0.79$, $p = 0.47$, Fig. 6a) when only *A. clarkii* were successfully fed, nor in 2007 when both fish species fed successfully (two-way ANOVA, $F_{2,21\ \text{tissue}} = 1.4$, $p_{\text{tissue}} = 0.28$, $F_{1,21\ \text{species}} = 2.6$, $p_{\text{species}} = 0.12$, Fig. 6b). These results replicated those of the aquarium experiments where significant label uptake occured but no anemone fraction differences were seen.

## Discussion

All three anemone fractions (intact anemone, animal, zooxanthellae) were significantly enriched in $^{15}$N and $^{13}$C relative to controls in both the laboratory and in situ; these data provide the first direct evidence of $^{15}$N and $^{13}$C

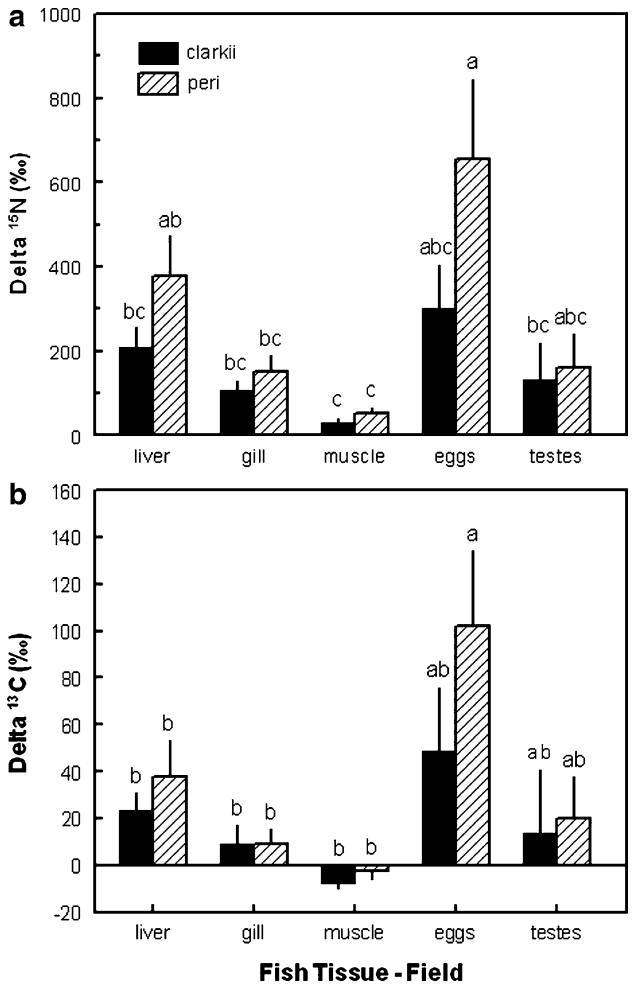


**Fig. 3** $\delta^{15}$N (**a**) and $\delta^{13}$C (**b**) in selected fish tissues of *A. clarkii* and *A. perideraion* trained to feed on a $^{15}$N-$^{13}$C-labeled diet in the field in 2007. Fish were fed labeled diet for 7–8 consecutive days prior to collection. *Histogram bars* that share letters are not significantly different (Tukey's HSD test, $p > 0.05$). Data are mean ± SE. For fish sample sizes (*N*), refer to appropriate column in Tables 2 and 3

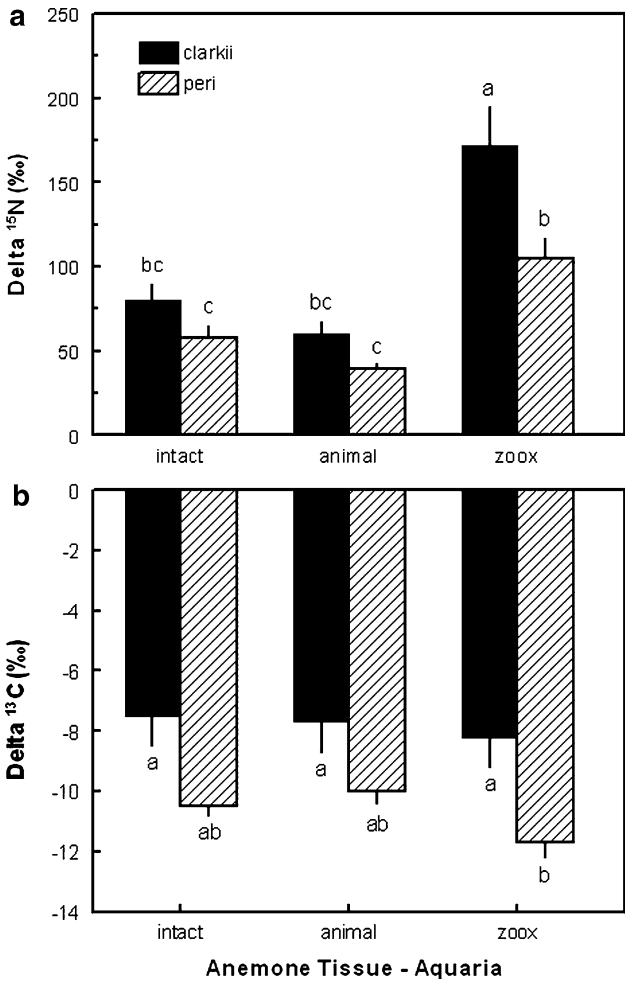


**Fig. 4** $\delta^{15}$N (**a**) and $\delta^{13}$C (**b**) in intact anemone (intact), animal, and zooxanthellae (zoox) fractions of anemones incubated for 5 days with *A. clarkii* and *A. perideraion* fed ad libitum with a $^{15}$N-$^{13}$C-labeled diet; fishes and anemones were housed in 38-L aquaria with running seawater. *Histogram bars* that share letters are not significantly different (Tukey's HSD test, $p > 0.05$). Data are mean ± SE. For anemone sample sizes (*N*), refer to appropriate column in Table 4

transfer from resident anemonefishes to host anemones and their intracellular zooxanthellae. Zooxanthellate fractions in our study had the highest $\delta^{15}$N; this result concurs with that of Roberts et al. (1999) who found that zooxanthellae became enriched with $^{15}$N at up to 17 times the rate of the host *Anemonia viridis*. Many studies have indirectly demonstrated the role resident fishes may play in the nitrogen budget of their host corals (Liberman et al. 1995; Meyer and Schultz 1985) or anemones (Porat and Chadwick-Furman 2004, 2005; Holbrook and Schmitt 2005; Roopin et al. 2008; Roopin and Chadwick 2009). Heikoop et al. (2000) examined the $^{15}$N signals in corals subject to sewage disposal and found the corals consumed wastewater nutrients. Their study successfully used stable isotopes in wastewater to demonstrate that cnidarians are capable of uptake of nitrogenous waste and the evidence that fishes could thus directly provide nitrogenous waste was tantalizingly close. Fish metabolic waste would be expected to be taken up by cnidarians, as our study suggests.

$\delta^{15}$N and $\delta^{13}$C were highest in the liver tissues of *Amphiprion clarkii* and *A. perideraion*, as expected. The role of the liver in digestion and processing of proteins, carbohydrates, and lipids (Gerking 1994) would make it a likely organ for $^{15}$N and $^{13}$C to be found in high concentration. Similarly, the significant isotope signal level at the gills was expected as the gills are the primary site of nitrogen excretion, generally in the form of ammonium, and carbon excretion in the form of bicarbonate ion (Marshal and Grosell 2006). The $\delta^{15}$N and $\delta^{13}$C in muscle tissue was often lower than those in liver and gill tissue for two reasons: the duration of the feeding experiment was relatively short to trigger increased muscle growth in the

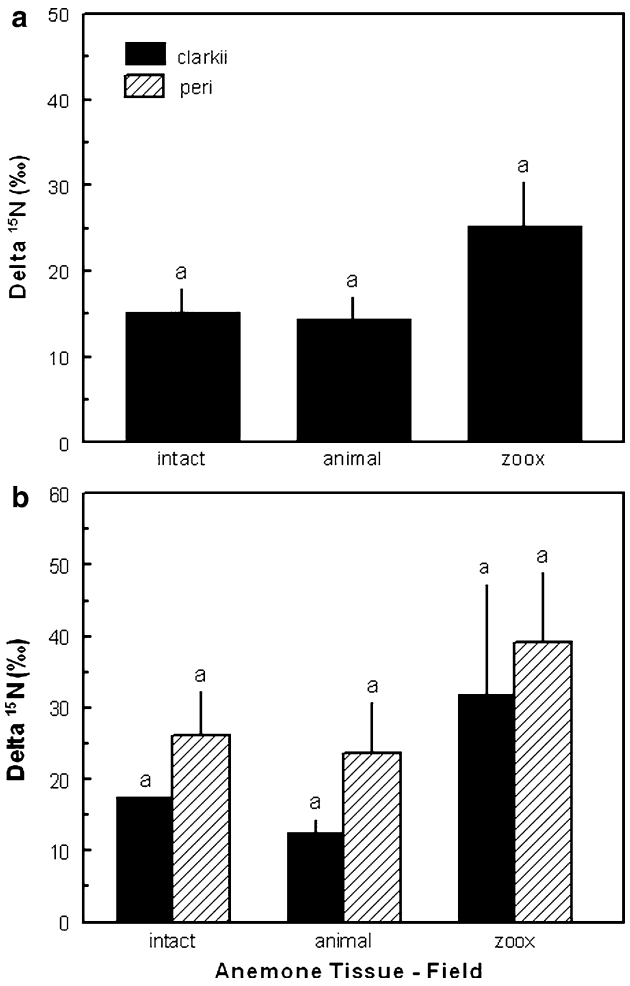


**Fig. 5** $\delta^{15}$N in intact anemone (intact), animal, and zooxanthellae (zoox) fractions of host anemones of **a** field-fed *A. clarkii* fed 7–8 days on a $^{15}$N-$^{13}$C-labeled diet in 2004 and **b** field-fed *A. clarkii* and *A. perideraion* fed 7–8 days on a $^{15}$N-$^{13}$C-labeled diet in 2007. *Histogram bars* that share letters are not significantly different (Tukey's HSD test, $p > 0.05$). Data are mean ± SE. For anemone sample sizes (*N*), refer to appropriate column in Table 4

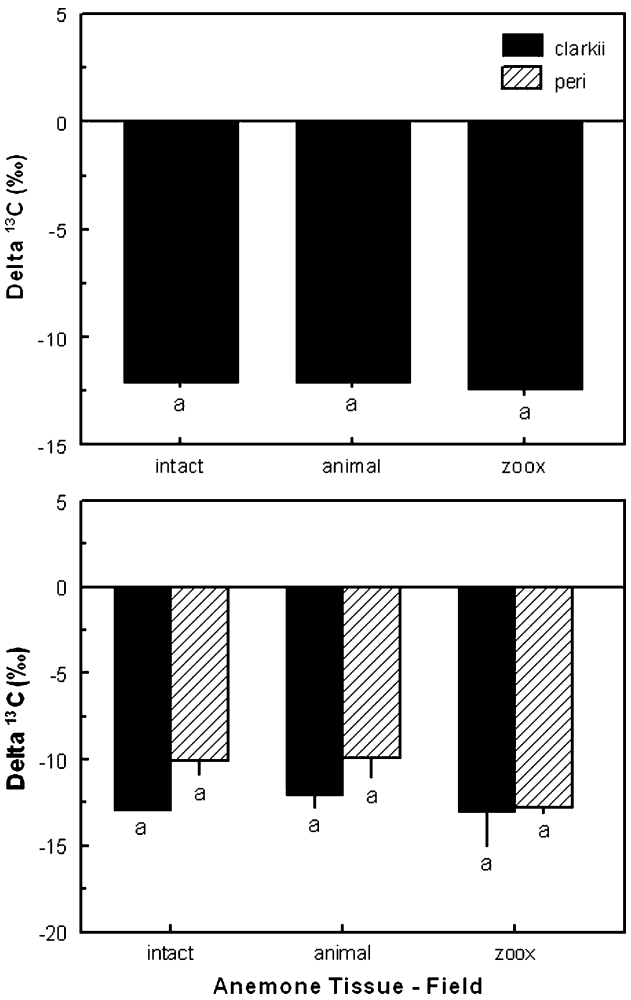


**Fig. 6** $\delta^{13}$C in intact anemone (intact), animal, and zooxanthellae (zoox) fractions of host anemones of **a** field-fed *A. clarkii* fed 7–8 days on a $^{15}$N-$^{13}$C-labeled diet in 2004 and **b** field-fed *A. clarkii* and *A. perideraion* fed 7–8 days on a $^{15}$N-$^{13}$C-labeled diet in 2007. *Histogram bars* that share letters are not significantly different (Tukey's HSD test, $p > 0.05$). Data are mean ± SE. For anemone sample sizes (*N*), refer to appropriate column in Table 4

adult fishes and growth rates of anemonefishes are highly socially controlled (Buston 2003). Because of the strict social control over growth rate, it is more likely that excess food energy is directed toward gonadal development and may explain the elevated isotopic signals of both eggs and testes. Anemonefishes are capable of breeding year round (Fautin and Allen 1992; Richardson et al. 1997), and the number of breeding cycles per year may be correlated with energy intake in pomacentrids (Tyler and Stanton 1995).

While both *A. clarkii* and *A. perideraion* showed significant incorporation of $^{15}$N and $^{13}$C, not all of ingested nutrients can be assimilated by the fishes. Studies of other pomacentrid fish species indicate a range of assimilation efficiency for ingested protein [two Gulf of California damselfishes *Microspathodon dorsalis* and *Stegastes rectifraenum* 57–67% (Montgomery and Gerking 1980), jewel damselfish *Plectroglyphidodon lacrymatus* 83% (Polunin 1988), *S. apicalis* > 50% (Klumpp and Polunin 1989), threespot damselfish *S. planifrons* and dusky damselfish *S. dorsopunicans* > 95% (Cleveland and Montgomery 2003)] and carbohydrate [*Microspathodon dorsalis* and *S. rectifraenum* 37–44% (Montgomery and Gerking 1980), jewel damselfish *Plectroglyphidodon lacrymatus* 56% (Polunin 1988), threespot damselfish *S. planifrons* and dusky damselfish *S. dorsopunicans* > 90% (Cleveland and Montgomery 2003)]. Ingested nutrients that are not assimilated are excreted as fecal material and calcium carbonate/magnesium carbonate precipitates (Walsh et al. 1991; Wilson et al. 2009) and ammonium and bicarbonate ions at the gills (Marshal and Grosell 2006); it is these excreted nutrients that are hypothesized to facilitate growth

and reproduction in cnidarians and zooxanthellae. Meyer et al. (1983) indicated that 80–99% of nitrogen excreted by French and white grunts, *Haemulon flavolineatum* and *H. plumier*, is in the form of ammonium; this is the form of nitrogen most readily taken up by cnidarians (Muscatine and Porter 1977; D'Elia and Webb 1977; Muscatine and D'Elia 1978). Roopin et al. (2008) estimated the mean ammonium excretion rate for a pair of anemonefish *A. bicinctus* at 10–74 μmol $h^{-1}$ under laboratory conditions. Lower excretion rates (12.6 μmol $h^{-1}$ $fish^{-1}$) are estimated in the field yet still represent an enrichment over background ammonia levels (Roopin et al. 2008).

There was a significant species effect in $\delta^{15}N$ seen in the anemone fractions in the aquarium experiment with host anemones of *A. clarkii* having significantly higher concentrations of nitrogen. A trend to the opposite was seen in the field experiment where $\delta^{15}N$ tended to be higher in anemone fractions with resident *A. perideraion* although this difference was not significant due to limited sample size (several *A. clarkii* samples were lost in transit). These results lead to an intriguing question of whether one anemonefish species may be a better symbiont to its host anemone. Roopin et al. (2008) suggested that the time an anemonefish spends away from its host anemone can reduce the potential nutrient contribution of ammonium and fecal material. In our aquarium studies, both *A. clarkii* and *A. perideraion* were confined to the same size aquaria. As *A. clarkii* is the larger species on average, (*A. clarkii* mean weight = 23.7 ± 6.6 g, *A. perideraion* mean weight = 10.9 ± 3.6 g, for all fishes used in our study) and ingested more of the labeled pellets, it is likely that the species effect we saw in the aquarium experiments was due to the higher feeding and subsequent excretion rates of *A. clarkii*. However, the behavior of *A. clarkii* and *A. perideraion* in the field is significantly different from the forced behavior of the aquarium environment. Under natural field conditions, female *A. clarkii* spend significantly more time away from their host anemone, and are associated with significantly more anemones, than are female *A. perideraion*. Cleveland et al. (2006) found that female *A. clarkii* spend on average about 10% of their time within 25 cm of a host anemone, and each female associates with 4-5 anemones within her home range, although one anemone is usually the preferred host. In contrast, female *A. perideraion* spend about 50% of their time within 25 cm of their host anemone and generally are only associated with one anemone (although some females associate with more). Male *A. clarkii* and *A. perideraion* show similar behaviors (Cleveland unpubl data). This ranging pattern was also documented by Hattori (2002); *A. clarkii* had a larger home range, sometimes including two or more hosts, whereas *A. perideraion*'s home range was not much larger than the extent of the host anemone. These behavioral differences between species suggest that *A. perideraion*, while still the smaller fish species, may provide more nutrient benefit to the host anemone due to the higher amount of time spent in close proximity to their host anemones. Excretion of ammonium from the gills is likely not voluntarily controlled by the fish and thus is likely to occur whether the fish is within its host anemone or away. This interpretation suggests that *A. perideraion* could provide more nitrogen to the anemone via ammonium excretion. Some fishes are seen to control where they defecate, and both *A. clarkii* and *A. perideraion* may provide direct fecal input to their host anemone when spending time within their tentacles. Indeed, only rarely were *A. clarkii* observed defecating in the field, and *A. perideraion* never were, which suggests they may defecate directly within the tentacular crowns of their hosts.

The significant elevation of $\delta^{15}N$, but not $\delta^{13}C$, in the zooxanthellae suggests that nitrogen is a limiting nutrient that must be conserved, while carbon at least in the form of $^{13}CO_2$ is not. Inorganic carbon, in the form of bicarbonate, is in abundance in seawater and presumably does not limit photosynthesis. In contrast, the organic $^{13}C$-containing compounds associated with fecal material, and with fish mucus, may play a more significant role in zooxanthellae nutrition. Fish mucus, which is rich in glycoproteins (Nakagawa et al. 1988), is a significant source of nutrition in many reef symbioses (e.g. Arnal et al. 2001). Rinkevich et al. (1991) demonstrated that photosynthetic products from zooxanthellae in the branching coral *Stylophora pistillata* were incorporated into coral mucus that was then grazed by the symbiotic xanthid crab *Trapezia cymodoce*. Arnal et al. (2001) suggested that the protein content of fish mucus is not negligible (39–78% dry weight) and is a reliable source of energy for cleaner wrasses and cleaner shrimp. Lastly, Brooks and Mariscal (1984) hypothesized that anemonefishes secrete protective mucus to avoid being stung by their host anemones. It is therefore likely that the mucus coating of the fishes in our experiment incorporated $^{13}C$ and $^{15}N$, which was then transferred to the anemone by either direct ingestion or transmembrane absorption by ectoderm and/or endoderm layers (Ferguson 1982; Gomme 1982; DeFreese and Clark 1991); subsequent transfer to zooxanthellae would then be possible.

The ecological significance of symbioses in the coral reef ecosystem cannot be overstated, nor must we risk oversimplification by focusing solely on one exchange at a time. Significant work has been done on algal–sponge (Davy et al. 2002), algal–cnidarian (see Table 2 of Venn et al. 2008), algal–mollusk (Belda et al. 1993), and shrimp–cnidarian (Fautin et al. 1995) symbioses, as well as fish–cnidarian interactions (Porat and Chadwick-Furman 2004, 2005; Holbrook and Schmitt 2005; Holbrook et al. 2008; Roopin et al. 2008; Roopin and Chadwick 2009) but the

links are much more complex. Davies (1992) suggested that the ecological success of coral reefs in an otherwise nutrient-poor tropical ocean is largely due to the multitude of symbiotic relationships among coral reef species. Tropical fishes of the family Pomacentridae have been found to have the highest rate of body mass growth per week of reef fish species examined (Depczynski et al. 2007), reflecting their importance for energy transfer among trophic levels. Anemonefishes, as planktivores, are net importers of nitrogen from the water column to the benthic environment (Fricke 1979; Fautin and Allen 1992), and their high feeding rates and biomass production confer benefits not only to their host anemone and endosymbiotic zooxanthellae but potentially serve as mediators that affect nutrient cycling at a higher level (Roopin et al. 2008).

Consider this scenario. The growth, fission rate, and reproductive capacity of anemones are tied to their endosymbiotic zooxanthellae (Holbrook and Schmitt 2005). Increased nutrient levels, particularly nitrogen (Roopin et al. 2008; Roopin and Chadwick 2009), facilitate increased photosynthesis and mitotic division in zooxanthellae (Fitt and Cook 2001). As zooxanthellae densities increase, substantially more essential amino acids and other organic compounds are translocated to the host anemone, which facilitates the anemone's growth (as shown for corals by Meyer and Shultz 1985; Hoegh-Guldberg and Smith 1989; Fitt and Cook 2001). Larger anemones can shelter more anemonefish, as well as increase the number of *Dascyllus* permitted as cohabitants (Schmitt and Holbrook 2003). As the number of resident anemonefishes and *Dascyllus* increase, the positive feedback of ammonium excretion and fecal input to anemone growth will facilitate more anemone reproduction and/or fission, which in turn can provide additional habitat for other anemonefishes, as well as the myriad other organisms that such a community supports.

While the links in this system have long been suspected, yet only recently investigated (Porat and Chadwick-Furman 2005; Roopin et al. 2008; Roopin and Chadwick 2009), our study provides the first definitive $^{15}N$ and $^{13}C$ link from resident anemonefish to host anemone and to endosymbiotic zooxanthellae. The complex interactions of the coral reef environment, of which this tripartite symbiosis plays a part, are under increasing threat from overharvesting, pollution, bleaching, and climate change (Roberts et al. 2002). Shuman et al. (2005) found that close to 60% of the total aquarium trade catch in the Philippines consisted of anemones and anemonefishes. Our data suggest that anemonefishes play a significant role in host cnidarian nutrition and may protect against or mitigate bleaching events. The implications of this study are relevant to the basic understanding of nutrient cycling in coral reef ecosystems and underscore the complex interactions in a marine ecosystem that contains more than 25% of global marine biodiversity.

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