SYMBIOTIC BENEFITS TO SEA ANEMONES FROM THE METABOLIC

BYPRODUCTS OF ANEMONEFISH

Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

_____

Modi M. Roopin

Certificate of Approval:

_____	_____
Scott R. Santos Assistant Professor Biological Sciences	Nanette E. Chadwick, chair Associate Professor Biological Sciences
_____	_____
Raymond P. Henry Professor Biological Sciences	George T. Flowers Interim Dean Graduate School

SYMBIOTIC BENEFITS TO SEA ANEMONES FROM THE METABOLIC BYPRODUCTS OF ANEMONEFISH

Modi M. Roopin

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
December 17, 2007

SYMBIOTIC BENEFITS TO SEA ANEMONES FROM THE METABOLIC

BYPRODUCTS OF ANEMONEFISH

Modi M. Roopin

_____
Signature of Author

_____
Date of Graduation

THESIS ABSTRACT

SYMBIOTIC BENEFITS TO SEA ANEMONES FROM THE METABOLIC BYPRODUCTS OF ANEMONEFISH

Modi M. Roopin

Master of Science, December 17, 2007
(B.S., Biology, Tel-Aviv University, 2005)

156 Typed pages

Directed by Nanette E. Chadwick

Although anemonefishes and their giant sea anemone hosts became known to the western world in the late nineteenth century, and the first studies exploring these associations were published almost one hundred years ago, to this day the underlying benefits of this interaction to each partner are not fully understood. While benefits to the obligate anemonefishes are widely recognized, benefits to the anemone hosts have been quantified only recently. I describe here physiological benefits to the sea anemone host *Entacmaea quadricolor* from metabolic waste products excreted by the anemonefish *Amphiprion bicinctus*.

This project was conducted in three main stages: Initially, basal levels of excretion versus uptake of ammonia in laboratory-cultured sea anemones (*Entacmaea quadricolor*) and anemonefish (*Amphiprion bicinctus*) were quantified under varying levels of food and light. The nutritional balance sheet indicated that nitrogenous excretion by anemonefish potentially can supply >100% of the nitrogen requirements of sea anemone hosts. Secondly, a starvation experiment was conducted in laboratory aquaria to assess variation in the fitness traits of unfed anemones that were cultured either with 1-2 anemonefish, with artificial ammonia supplements, or with neither. The results indicated that ammonia excreted by resident anemonefish was the primary factor responsible for enhanced zooxanthellae density and reduced tissue loss of sea anemones that were cultured with fish. Thirdly, a field assessment of this symbiosis was conducted on coral reefs in the northern Red Sea. Analysis of water samples taken by scuba divers from among anemones tentacles versus from the water column a few meters away indicated that anemonefish alter the ammonia availability to their hosts by generating significant local enrichment around sea anemones. Examination of zooxanthella populations in field anemones showed that individuals of *E. quadricolor* exhibit a highly specific association with clade C *Symbiodinium* at all depths on the reef. In the Red Sea, sea anemones without anemonefish are extremely rare, and the density of their zooxanthellae is contingent primarily on variation in light availability among microhabitats.

In conclusion, resident anemonefish are important nutritional benefactors that provide ammonia, an essential limiting nutrient, to their benthic anemone hosts, and thus enhance their survival and fitness in nutrient-poor waters on coral reefs.

ACKNOWLEDGEMENTS

I would like to thank my advisor Nanette E. Chadwick, for her help with the development of this research, guidance and support. Nanette, your wise advice, and positive feedback gave me the motivation to see it through. I thank you for your open-minded approach and faith in my ability that inspired me to try new ideas. I also thank my other committee members, Raymond P. Henry and Scott R. Santos, for their helpful suggestions and comments that greatly improved this work. I have learned from all of you a lot of good science!

I would like to thank Daniel J. Thornhill and Mark R. Liles for their assistance with the DGGE analysis and Michael C. Wooten for statistical advice. Appreciation is also extended to the Israeli Nature and Parks Authority and to my friend and colleague Baraka Kuguru of the Interuniversity Institute for Marine Science, for his assistance with sampling during the field project.

Thanks also are extended to my valued laboratory assistants: Pamela Bellotti, Michael Greenmeyer, Sybil Glenos, Omar Mazher and Steven Scyphers. A special thanks to Omar Romagnoli; without your excellent ideas, experience and sense of humor, I could never have gotten our aquarium systems ready in time. Finally, I wish to thank my family and friends, especially my beautiful girlfriend Keren Ganon for her love and support and for putting up with my craziness for the last two years.

Style manual or journal used:
Auburn University Guide To Preparation And Submission Of Theses And Dissertations

Computer software used:
SPSS® 15.0 for Windows
Microsoft® Word
Microsoft® Excel
Microsoft® PowerPoint

TABLE OF CONTENTS

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER I. INTRODUCTION 1

CHAPTER II. ANEMONEFISH ARE A CONSTANTLY-REPLENISHED SOURCE OF INORGANIC NITROGEN TO THEIR SEA ANEMONE HOSTS 5

Introduction 6

Methods 10

Results 16

Discussion 19

CHAPTER III. NITROGEN EXCRETED BY ANEMONEFISH ENHANCES THE FITNESS OF SEA ANEMONE HOSTS AND THEIR ZOOXANTHELLAE UNDER LABORATORY CONDITIONS 35

Introduction 36

Methods 40

Results 49

Discussion 53

CHAPTER IV. ANEMONEFISH AFFECT THE AMMONIA AVAILABILITY TO GIANT SEA ANEMONES ON CORAL REEFS IN THE RED SEA 73

Introduction 74

Methods 77

Results 82

Discussion 83

CHAPTER V. PATTERNS OF ASSOCIATION BETWEEN THE BUBBLE TIP ANEMONE (*Entacmaea quadricolor*) AND ENDOSYMBIOTIC DINOFLAGELLATES (*Symbiodinium* spp.) ACROSS A DEPTH GRADIENT IN THE NORTHERN RED SEA 99

Introduction 100

Methods 103

Results 106

Discussion 107

CUMULATIVE BIBLIOGRAPHY 117

LIST OF TABLES

CHAPTER II.

Table 1. Variation in ammonia excretion rates among marine and brackish water fishes and invertebrates.....28

LIST OF FIGURES

CHAPTER II.

Figure 1. Variation in ammonia excretion rate with time after feeding in the anemonefish *Amphiprion bicinctus*.....31

Figure 2. Variation in ammonia uptake rate of the giant sea anemones *Entacmaea quadricolor* with external seawater ammonia concentration during the day.....32

Figure 3. Ammonia uptake in dark vs light conditions in giant sea anemones *Entacmaea quadricolor* 33

Figure 4A. Variation in ammonia excretion rates measured for anemonefish *Amphiprion bicinctus* alone versus with giant sea anemones *Entacmaea quadricolor* 34

Figure 4B. Ammonia buildup in experimental vessels with anemonefish *Amphiprion bicinctus* alone versus with giant sea anemones *Entacmaea quadricolor* 34

CHAPTER III.

Figure 1A. Effects of laboratory treatments on body size (tentacle crown diameter) variation in unfed giant sea anemones *Entacmaea quadricolor* 65

Figure 1B. Variation in final mean body sizes of unfed giant sea anemones *Entacmaea quadricolor* after 2 months of starvation (expressed as percentage of initial size) 65

Figure 2. Variation in abundances of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments: (A) control, (B) ammonia-enriched, (C) anemonefish. 66

Figure 3. Variation in the mean abundance of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments (changes are expressed as percentage of initial abundance) 68

Figure 4A. Patterns of zooxanthella cell division in giant sea anemones *Entacmaea quadricolor* 69

Figure 4B. Variation in the mitotic index of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* that were cultured in 3 treatments 69

Figure 5. Variation in chlorophyll *a* content per zooxanthella cell in unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments 70

Figure 6A. Variation in ammonia uptake rates by unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments..... 71

Figure 6B. Time course of ammonia uptake by unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments..... 71

Figure 7. Cladal identities of zooxanthellae (*Symbiodinium* spp.) in giant sea anemones *Entacmaea quadricolor* under laboratory conditions 72

CHAPTER IV.

Figure 1. Map of northern Gulf of Eilat (Aqaba) 92

Figure 2. Variation in host and zooxanthella parameters with depth among giant sea anemones *Entacmaea quadricolor*: (A) zooxanthellae abundance (B) zooxanthella mitotic index, (C) chlorophyll *a* level, (D) protein content.....93

Figure 3. Variation in number of resident anemonefish *Amphiprion bicinctus* per anemone among giant sea anemones *Entacmaea quadricolor* in the Red Sea 95

Figure 4. Variation in zooxanthella abundance within the tentacles of giant sea anemones *Entacmaea quadricolor* among microhabitats.....96

Figure 5. Variation in rates of ammonia excretion with time of day by adult anemonefish *Amphiprion bicinctus* in the field 97

Figure 6. Variation in dissolved ammonia concentration among anemone tentacles inhabited by adult anemonefish *Amphiprion bicinctus* versus that in the surrounding water column 98

CHAPTER V.

Figure 1. Map of northern Gulf of Eilat (Aqaba) 113

Figure 2. Variation in zooxanthella clades among host individuals of the giant sea anemone *Entacmaea quadricolor* at Eilat, northern Red Sea 114

Figure 3. Variation in RFLP patterns of zooxanthellae associated with the sea giant anemone *Entacmaea quadricolor* among depths at Eilat, northern Red Sea. 115

Figure 4. PCR-DGGE profiles of zooxanthellae from the giant sea anemone *Entacmaea quadricolor* 116

CHAPTER I

INTRODUCTION

The association between anemonefishes and sea anemones is a well-known example of symbiosis in the marine environment, and it is also a major component of coral reef ecosystems. Of nearly 1000 species of sea anemones, 10 serve as host to 28 species of damselfishes belonging to the genera *Amphiprion* and *Premnas*, and this relationship forms one of the most famous and recognized symbiotic associations in nature (Roughgarden 1975; Fautin 1991; Fautin and Allen 1997). Each anemone constitutes the territory of its fishes, which seldom venture far from it, retreating into its tentacles when threatened.

Experimental removal of either the anemonefish from the anemone or vice versa almost always results in the anemonefish becoming prey for larger fish (Mariscal 1970a,b; Allen 1972; Fautin 1991), indicating that the host is vital to the survival of these obligate partners, and as such enhances fish biodiversity on coral reefs. In addition to the obligate anemonefishes, sea anemones worldwide are known to form associations of varying types with over 70 other species of fishes and crustaceans (Randall and Fautin 2002; Patzner 2004; Chadwick and Arvedlund 2005). Some anemoneshrimps serve as cleaners of parasites for larger reef fish (Becker and Grutter 2004). Hence, sea anemones

can form centers for interactions among several trophic levels on the reef, and this symbiosis may have far-ranging effects on the coral reef ecosystem beyond direct effects on the species involved.

A high rate of endemism in anemonefishes has developed on some geographic regions, possibly due to the brief larval stage and limited ability of the adult fish to migrate long distances. Thus, isolated anemonefish populations have, over time, diverged into different species adapted to specific hosts and environmental conditions (Fautin and Allen 1997). For example, in the Red Sea, giant sea anemones are populated by an endemic anemonefish (*Amphiprion bicinctus*) that has developed a total dependence on the anemone host.

Many symbiotic associations are mutualistic, providing a variety of benefits to both partners. Protection mutualisms, in which one or both participants receive protection from natural enemies, are common in benthic marine systems (Gotto 1969). Anemonefish in the Red Sea take advantage of the toxic properties of the stinging cells ("nematocysts") of the sea anemone host, which deter potential fish predators and ensure an enemy-free living space. Conversely, adult anemonefish defend their host anemones from specialized cnidarians predators such as chaetodontid butterflyfish (Mariscal 1970 a,b; Fricke 1975; Fautin 1991; Godwin and Fautin 1992; Fautin and Allen 1997; Porat and Chadwick-Furman 2004). A second major type of mutualistic benefit involves the transfer of nutrients among symbiotic partners. All tropical anemones that host anemonefish also harbor microscopic, single-celled, dinoflagellates often referred to as "zooxanthellae" that belong to the genus *Symbiodinium* (Dunn1981; Fautin 1991). While some of the sugars from algal photosynthesis are used to fuel the algae's metabolism, most of them

"leak" to the anemone, and provide it with essential energy for growth and reproduction (D'Elia et al. 1983; Wilkerson and Muscatine 1984). Conversely, the metabolism of the symbiotic algae is believed to be limited by the low levels of carbon and nitrogen typically found in tropical waters. The ability of symbiotic algae to absorb and translocate inorganic nitrogen from seawater is consequently of great importance for host cnidarians that live in nutrient-poor marine waters (Muscatine and Porter 1977; Muscatine 1980; Shick and Dykens 1984; Cook et al. 1988; Achituv and Dubinsky 1990; Davies 1997; Whitehead and Douglas 2003). Thus, it has been proposed that elevated levels of ammonia (here and throughout this thesis defined as the combination of NH_3 gas and NH_4^+ ion) in the excretory waste products of fishes can augment the abundance of symbiotic zooxanthellae and therefore positively affect the growth rate and survival of cnidarian hosts (Meyer et al.1983; Meyer and Schultz 1985a,b; Porat and Chadwick-Furman 2005). The use of anemonefish excretory byproducts as a source of limiting inorganic nutrients may therefore be an additional benefit to host cnidarians, especially when capture of large quantities of zooplankton is not possible and nitrogen input from host feeding is poor.

The protective benefits to anemonefish from this symbiotic association are well-established, but those conferred on the anemone host by the fish do not occur in all species and are conferred mainly by adult fish (Fricke 1974, 1975; Fautin and Allen 1997; Porat and Chadwick-Furman 2004; Holbrook and Schmitt 2005). Thus, major benefits to the anemone host may instead come in form of nutrient transfer. Several models have been developed to explain how symbiotic associations form and to predict their properties based on the costs and benefits involved (Roughgarden 1975; Noe and Hammerstein 1995; Hoeksema and Schwartz 2001; Noe 2001). An intriguing approach is

the byproducts model, which states that mutualisms can evolve based on the use of byproducts from the physiological processes of each partner (Sachs et al. 2004). The toxic tentacles of the sea anemone which are equipped with thousands of small stinging cells and function primarily in prey capture and defense (Fautin and Allen 1997) are co-opted by the anemonefish to deter its own potential predators and to provide the fish with a predation-free microhabitat. In addition, waste products from the fish may be used as an important source of nutrients by the anemone host. As zooplanktivores (Fautin and Allen 1997), anemonefish import nutrients from the plankton to reef benthos. Thus, the ammonia excreted by these fish onto their sea anemone hosts also may serve as an important link through which nutrients in the plankton are transferred to the reef benthos. In this thesis, the scope and patterns of nutrient transfer from anemonefish to their anemone hosts are explored, especially in the form of excreted ammonia waste products.

CHAPTER II

ANEMONEFISH ARE A CONSTANTLY-REPLENISHED SOURCE OF INORGANIC NITROGEN TO THEIR SEA ANEMONE HOSTS

Abstract

The metabolic byproducts of symbiotic anemonefish potentially contribute to the nutritional budget of their host sea anemones. The anemonefish *Amphiprion bicinctus* is a closely-associated, obligate ectosymbiont of the giant sea anemone *Entacmaea quadricolor*. These fish excrete substantial amounts of ammonia during the day, thus potentially enriching the nutritional environment of the endosymbiotic zooxanthellae that dwell within host tissues. Additionally, anemonefish as zooplanktivores also may serve as an important link through which nutrients acquired from the plankton are transferred via excreted ammonia to benthic sea anemones. The symbiosis between giant sea anemones, zooxanthellae and anemonefish was examined under laboratory conditions to create a balance sheet for nitrogen production and transfer within this three-way symbiotic system. Sea anemones absorbed ammonia during the day at a rate of up to 4.15 μmole h^{-1} when in ammonia-enriched seawater (20μM), while at night their absorption rate declined to near zero, indicating that ammonia uptake was mainly a feature of

light-induced productivity by their zooxanthellae. Resident anemonefish excreted ammonia at a rate of 0.60 μmole g^{-1} h^{-1} after 24 hour of starvation, and 1.84 μmole g^{-1} h^{-1} at two hours following feeding. Since adult fish weighed an average of 11g under laboratory conditions, and in the field average approximately 20g, the ammonia produced by these anemonefish is more than enough to supply 100% of the nitrogen taken up by the anemone host and presumably used by the zooxanthellae. This ammonia supplement may substantially enhance the fitness of giant sea anemones, especially in the nutrient-poor waters on coral reefs.

Introduction

Costs and benefits of the association between anemonefishes and sea anemones were first explored almost one hundred years ago (reviewed in Fautin and Allen 1997), but to this day they remain poorly understood. Benefits of this association have been examined mostly for the anemonefish, and it is well recognized that these obligate guests benefit from sheltering among the anemone tentacles, which are packed with microscopic stinging cells ("nematocysts") that deter potential fish predators. In contrast, the only well-documented benefit of this association to the host anemone is protection from butterflyfish predators (Fricke 1974, 1979; Godwin and Fautin 1992; Porat and Chadwick-Furman 2004). Protection by resident anemonefish however, appears to be restricted to the adults of some fish species that exhibit high levels of territorial behavior and are large enough to aggressively drive off potential predators (Godwin and Fautin 1992; Fautin and Allen 1997; Randall and Fautin 2002). Cultured anemones that are not

exposed to predation stress also benefit from anemonefish presence (Porat and Chadwick-Furman 2005; Chapter 3), so the fish must confer benefits other than protection. One way that host anemones can profit from fish is hypothesized to involve generalized physiological benefits through the transfer of nutritive substances. This idea has been explored in preliminary studies, but has not been fully quantified (Cleveland et al. 2003, 2006; Porat and Chadwick-Furman 2005).

Coral reefs present a paradox of high diversity and productivity that persists in a highly oligotrophic marine desert (Sournia 1977; Entsch et al.1983; Crossland 1983). A key to the success of many reef cnidarians in this environment is their harboring of endosymbiotic algae, commonly referred to as zooxanthellae and belonging to the genus *Symbiodinium*, within their endodermal tissues. The two partners create an efficient nutrient cycling loop that reduces the overall loss of key nutrients from the system, and therefore increases the fitness of both host and symbiont (Muscatine and Porter 1977; Wilkerson and Trench 1986; Falkowski et al. 1993). All tropical anemones that host anemonefish possess endosymbiotic microalgae (Dunn 1981; Fautin 1991), which supply them with energy-rich photosynthate compounds for respiration, growth and reproduction (Shick and Dykens 1984; Steen 1988; Cook et al. 1988; Achituv and Dubinsky 1990; Davies 1997; Whitehead and Douglas 2003). Zooxanthellae are nitrogen-limited (Muscatine et al. 1989; Dubinsky et al. 1990; Davies 1997), and elevated levels of ammonia are known to stimulate their increased abundance in cnidarian tissues (Dubinsky et al. 1990; Falkowski et al. 1993) and to positively affect the growth rate and survival of the host (Meyer et al. 1983; Meyer and Schultz 1985b; Spotte 1996; Porat and Chadwick-Furman 2005). Inorganic nitrogen occurs at extremely low concentrations in

the waters surrounding coral reefs (e.g., typically below 0.5μM, Sournia 1977; Entsch et al. 1983; Crossland 1983), so any source of supplemental nitrogen could potentially enhances zooxanthella populations and consequently benefit the host cnidarian (Meyer and Schultz 1985a,b; Liberman et al. 1995; Spotte 1996; Mokady et al. 1998; Porat and Chadwick-Furman 2005).

The excretions of fish are known to serve as an important source of limiting nutrients on coral reefs. For example, large schools of resident grunts (*Haemulon* spp.) that feed during the night and rest over colonies of the stony coral *Porites furcata* during the day have been shown to enhance the growth rates of the coral (Meyer and Schultz 1985a,b). Small groups of the damselfish *Dascyllus marginatus* that occupy the pocilloporid coral *Stylophora pistillata* also are known to enhance the growth rate of their coral host (Liberman et al. 1995). The importance of fish excretions has been demonstrated in freshwater ecosystems as well, in which they affect the flux of nutrients among benthic and pelagic habitats (Durbin et al. 1979; Vanni 2002; Glaholt and Vanni 2005). While large migratory fish schools can potentially alter nutrient flux between aquatic habitats, fish and other guests that form intimate ectosymbiotic associations with benthic organisms may affect the nutrient balance of their specific host. For example, ammonia excretion by the anemoneshrimp *Periclimenes yucatanicus* provides an important source of nitrogen for the sea anemone *Condylactis gigantea* on coral reefs in the Caribbean Sea (Spotte 1996), as do symbiotic barnacles (*Savignium milleporum*) living on the surfaces of fire corals (*Millepora dichotoma*) in the Red Sea (Achituv and Mizrahi 1996). Thus, the excretory products of obligate ectosymbiotic partners may provide an important nutritional benefit to host cnidarians.

The main nitrogenous excretion of marine fish is ammonia (75% - 85%), which rapidly binds to hydrogen ions to form ammonium ions in aqueous solution (Jobling 1981; Durbin and Durbin 1981; Tatrai 1981; Whitledge 1982; Meyer and Schultz 1985a). This form of nitrogen is readily assimilated by corals and other cnidarians (Muscatine and D'Elia 1978). Anemonefish are zooplanktivores (Fautin and Allen 1997) and the ammonia they excrete is thus imported to the reef from the plankton. These fish may serve as an important link through which nutrients are transferred to the benthos via excreted metabolic waste products that can be assimilated by giant sea anemones. Recent studies have demonstrated that anemonefish enhance the growth and survival of their sea anemone hosts (Porat and Chadwick-Furman 2004, 2005; Holbrook and Schmidt 2005), but the physiological mechanisms underlying these benefits are not fully understood.

The giant sea anemone *Entacmaea quadricolor* is the center of a 3-way symbiosis with endosymbiotic algae (*Symbiodinium* spp.) and the ectosymbiotic anemonefish *Amphiprion bicinctus*. Anemonefish may represent a reliable source of essential inorganic nutrients to their sea anemone hosts. Here I focus on this symbiosis as a model system to quantify the extent of anemonefish contribution to the nitrogen balance of anemone hosts.

Methods

Study organisms and maintenance

Giant sea anemones (*Entacmaea quadricolor*) were transported to Auburn University in 2006 from the Waikiki Aquarium (Hawaii, USA) where they had been cultured and propagated via clonal replication from individuals collected in Palau in 1985. Anemonefish (*Amphiprion bicinctus*) were acquired from Oceans Reefs and Aquariums (ORA), an aquaculture facility at Harbor Branch Oceanographic Institution at Fort Pierce, Florida, USA, in 2006. The acquired fish and anemones were distributed haphazardly among the 12 identical closed-system aquariums and were maintained under the conditions described below for at least 2 months prior to experiments.

Each aquarium system circulated ~160 liters of artificial sea water (Reef Crystals, Aquarium Systems, Inc., Ohio, USA) between an upper aquarium (71cm x 33cm x 35cm) and a lower sump (71cm x 33cm x 35cm). Water flowed into the upper aquarium from the sump alternately through two pipe outlets using a SCWD–wave maker (3iQ Ventures LLC, Manhattan Beach, CA, USA). Each system contained a protein skimmer, live rock and macroalgal cultures in the sump as mechanical and biological filters, and a layer of fine sand in both sump and aquarium. All systems were maintained at 34-35 ppt with a temperature of 26°C on a 12h light:12h dark photoperiod. Over each aquarium was suspended a six-bulb TEK-LIGHT™T5 high output fluorescent light, with a combination of three 39W T5 Midday 6000K, and three 39W T5 Pure actinic Giesemann PowerChrome fluorescent bulbs, which provided a constant irradiance of about 200 μE

m^{-2} s^{-1} at the bottom of the aquarium to 800 μE m^{-2} s^{-1} at the water surface as measured with a QSL-2101 Scalar PAR Sensor (Biospherical Instruments, San Diego, CA, USA). This level of irradiance was equivalent to that at 7-20m depth on coral reefs in the Red Sea (Stambler and Dubinsky 2004), which is the depth range of highest density of these organisms (Chadwick and Arvedlund 2005).

All the sea anemones that were used in the experiment were sized from photographs using the program Image Tool for Windows version 3.00 (UTHSCSA), and their average tentacle crown diameter (the area covered by the tentacles of each anemone) was 82 cm^2. Prior to the experiment anemones were fed weekly to satiation with small pieces of fish or shrimp, and anemonefish were fed daily (at about 10:00 h) to satiation with a combination of dry pellets (Formula one, AquaPet Americas, Utah, USA) and frozen (copepods, brine shrimp, mysids) foods. Feeding behavior was monitored carefully to ensure that all experimental fish ingested all proffered foods. See Godinot and Chadwick (2007) for additional details on culture conditions.

Experimental protocols

To establish a nutritional balance sheet, the resting ammonia (defined as the combination of NH_3 gas and NH_4^+ ion) flux of anemones and anemonefish was determined in both light and dark under controlled laboratory conditions. Rates of ammonia excretion/uptake were measured by placing each organism in a separate 1-2 L experimental vessel (glass beaker, Fisher Scientific) filled with 500-1000mL of seawater depending on animal size. Experimental vessels and all other glassware were thoroughly rinsed with 10% HCL and

double-distilled water and then rinsed again with filtered seawater prior to each experiment. Experiments were conducted in a water bath at a constant temperature of 26ºC under 400W Radium Metal Halide Lamps (Ocean Light 250, Aqua Medic), which provided irradiance equivalent to that in the holding aquaria (see above). The experimental vessels were aerated continuously using an airstone attached to a pump set to a low level that kept the water aerated and stirred, but produced as few bubbles as possible. All experimental trials also contained a control vessel with no animals, and either just seawater or ammonia-spiked water, depending on the experiment. Total incubation time of animals for each experiment was 100 min, and 5mL water samples were withdrawn for ammonia analysis every 20 min (Rainin pipette). Ammonia concentration in the experimental vessels was determined using the indophenol blue method (Solarzano 1969), scaled down 10-fold due to the small incubation volumes, on a GENESYS 5 Spectrophotometer (Thermo Electron, MA, USA). The modified procedure was tested repeatedly against a standard curve of 0-25 μM NH_4Cl and the results produced with the two procedures were not significantly different (Student's t-test, p = 0.802). Average ammonia flux (excretion or uptake) in the laboratory incubations was computed as the difference between the final and initial amounts of ammonia in the total volume of each experimental vessel. The amount of ammonia generated per hour per gram fish mass was calculated from the ammonia concentration, water volume in the experimental vessel and the wet mass of each fish (μmole g^{-1} h^{-1}). Changes in the water volume due to sampling, and the displacement of water due to the volumes of fish or anemone were incorporated into all calculations.

Fish excretion rates

Ammonia excretion rate was quantified for nine fish, each examined during four time periods (2, 6, 24 and 36 hours post-feeding). In order to avoid stressing the fish, only one excretion trial was performed per day on each fish; within 3 weeks, the ammonia assessments of all fish were completed. The above time periods were selected because *A. bicinctus* anemonefish are diurnal zooplanktivores (Fautin and Allen 1997), so in the field, the shortest time periods (2 and 6 hours post-feeding) measure excretion rates during the same day. In contrast, about ~8-20 hours post-feeding would occur at night, when the sea anemone hosts decrease their uptake rates significantly (see Results), so this time period was not examined. The time periods of 24 and 36 hours post-feeding revealed basal excretion rates when the fish were starved. Each experiment included a control vessel containing only seawater. Anemonefish were placed in experimental vessels and allowed to acclimate for 20 minutes prior to performing two complete changes of water to ensure that initial ammonia levels were close to zero when measurements began. Water samples (5mL) were taken every 20 min for 100 min (adapted from Wilkerson and Muscatine 1984; Spotte 1996) and at the end of each experimental trial, fish were removed from the experimental vessels, gently blotted and weighed in air to obtain their wet mass before being returned to their original aquaria. The fish appeared calm and swam normally during the time period in which excretion rate was monitored, and they did not appear to be stressed or breathing rapidly (according to opercular opening rates).

Changes in the concentration of ammonia were also measured in experimental vessels that each contained an anemonefish and a sea anemone (N = 9 pairs total), and these

were compared with changes in ammonia levels produced by the same anemonefish in the absence of an anemone. It was predicted that ammonia levels would be lower in the tests containing both fish and sea anemone than in those with fish alone since the anemones are predicted to absorb the excess ammonia. Food was withheld from fish 24 hours prior to this experiment to bring the fish as close to their basal excretion rate as possible. In each trial, an anemonefish was placed with its host anemone in a 2 L experimental vessel containing 1 L of seawater, and the ammonia level was quantified following the procedures described above.

Sea anemone uptake rates

Rates of ammonia uptake were quantified for 10 anemones, each incubated in seawater spiked with one of three concentrations of NH_4Cl: 0, 10 and 20 μM. Between tests at each concentration, the anemones were maintained under their normal feeding regime (see above) in their original tanks for at least three weeks to ensure no effects of the previous testing concentration. Uptake was measured as the reduction in ammonia concentration in the water in the experimental vessels. In each trial, a control was included, consisting of an experimental vessel with no animals but with seawater spiked to the same level as in the vessels containing the animals. At the end of each experimental run, the anemones were removed from the experimental vessels and returned to their original aquaria. This procedure did not appear to greatly affect the anemones: some contracted their tissues when transferred to the experimental vessels, but all re-expanded and attached their basal disks to the glass within the first 5-10 min of the experiment. Day and

night uptake experiments on anemones (N = 10) were also conducted at 10 μM NH_4Cl-spiked seawater to examine the effect of light on ammonia flux. Since these anemones were maintained under constant irradiance throughout the light period (12h) each day, it was assumed that any "time of day" effect would be minor, and thus all day experiments were conducted during mid-day. Night uptake rates were tested during the first hour after dark and then four hours after dark to determine whether ammonia absorption rate decreased during the night.

Ammonia in the seawater in experimental vessels can be eliminated by air bubbles or bacterial activity; this loss can cause an underestimate of the final dissolved ammonia concentration in the measured samples. Therefore, in all experiments described here, a continuously-aerated control vessel that contained only seawater (spiked or ambient depending on the experiment) was tested in addition to those with fish or anemones.

Statistical analyses

All statistical analyses were conducted using SPSS 15.0 for Windows. Normality and homogeneity of variances were tested using the Shapiro–Wilk statistical test for $n < 50$. Variation in fish ammonia excretion rate versus time-after-feeding was examined using one-way repeated-measures analyses of variance (ANOVA) with time as repeated variable, followed by Post-hoc pairwise comparisons between groups. Differences were considered significant at $P < 0.05$. Variation in sea anemone uptake rates with level of external ammonia concentration, and in levels of ammonia build-up in vessels with

anemonefish in the presence versus absence of anemones were tested using student's t-tests. Since the data consisted of one group of anemones that was repeatedly tested in two external ammonia treatments (10 μM and 20 μM), and a second group of anemones that was tested in zero ammonia (negative control) in an earlier experiment, a paired samples t-test was used to compare ammonia uptake in spiked water, and then t-tests for independent-samples were used to compare the negative control to uptake rates in each external concentration. The Bonferroni correction was applied to the significance level when more than one student's t-test was used. All results are presented as means ± one standard deviation unless otherwise indicated.

Results

In all control vessels, loss of ammonia represented <0.5% of maximum NH_4^+ concentration, and that pattern did not change significantly throughout the experiments (t-test, $t_{(5)} = 0.37$, $p = 0.71$). Thus, ammonia loss due to uptake by microbes or other causes was concluded to be negligible, and adjustments were not made to the data. Similar results were obtained by Haines and Wheeler (1978) and Bray et al. (1986).

Fish excretion rates

The rate of ammonia production by anemonefish was much higher for large than for small fish. The negative correlation between body size and ammonia excretion rate per gram was not strong (r^2=0.3, N=9), possibly due to the relatively small number of fish

examined compared to field studies that reported this trend (Jobting 1981; Meyer and Schultz 1985a; Schaus et al. 1997). Fish excretion rate decreased significantly vs. time after feeding (repeated measures ANOVA, $F_{(3,24)} = 66.9$, $p < 0.001$, $Eta^2 = 0.893$, Fig. 2.1). The Eta^2 value indicates that 89.3% of the variability in excretion rates was accounted for by the "time since feeding" effect. At approximately 24 hours after feeding, excretion appeared to reach a basal rate of 27.6 % of the maximal rate. The excretion rate decreased significantly from 1.84 ± 0.31 μmole g^{-1} h^{-1} at 2 hours after feeding to 1.07 ± 0.24 μmole g^{-1} h^{-1} at 6 hours after feeding (multiple comparisons test, $p < 0.001$), and significantly decreased further at 24 hours post-feeding to 0.60 ± 0.23 μmole g^{-1} h^{-1} ($p < 0.03$). After 36 hours, excretion rate decreased again to 0.42 ± 0.13 μmole g^{-1} h^{-1}, but this was not significantly different from excretion rate after 24 hour starvation ($p = 0.31$). An exponential fit of the data yielded a release rate coefficient (λ) of 0.0399 h^{-1} ($r^2 = 0.94$). Thus, excretion rate declined exponentially $Y = 1.6505e^{(-0.0399X)}$ throughout the first 24 hours after feeding, after which it stabilized to a constant basal rate (Fig. 2.1). Mean excretion at 24 h and 36 h after feeding was calculated as the basal excretion rate (0.50 μmole g^{-1} h^{-1}), and accounted for 27.6 % of the maximum excretion rate measured. The mass-specific ammonia excretion rate measured here was within the range of values known for a variety of marine organisms (Table 1.1).

Sea anemone ammonia uptake rates

Daytime rates of ammonia uptake by the sea anemone *Entacmaea quadricolor* varied with external ammonia concentration. The rate for sea anemones in seawater spiked with

20μM ammonia (4.15 ± 0.92 μmole h^{-1}) was significantly higher (paired t-test, $t_{(9)} = -9.72$, $p < 0.001$) than that in 10μM ammonia spiked SW (1.78 ± 0.50 μmole h^{-1}, Fig. 2.2). In an earlier experiment, the resting ammonia flux of sea anemones was evaluated during the day in non-spiked seawater (negative control) using a different group of individuals: ammonia levels in the experimental vessels stayed close to zero (uptake rate, -0.13 ± 0.13 μmole h^{-1}), which suggested that ammonia uptake by the endosymbiotic zooxanthellae may masked ammonia excretion by the host anemone. As expected, ammonia uptake in this negative control was significantly slower than uptake in 10 μM (t-test, $t_{(18)} = -11.53$, $p < 0.001$) and 20 μM (t-test, $t_{(18)} = -14.53$, $p < 0.001$, Fig. 2.2).

The uptake rate by sea anemones in 10μM ammonia was significantly lower during darkness (nighttime conditions) than when illuminated (daytime conditions, t-test, $t_{(18)} = -5.29$, $p < 0.001$). During the night, uptake rates stayed consistently low and did not differ significantly between 1 and 4 hours after the onset of dark conditions (0.55 ± 0.94 μmole h^{-1} versus 0.31 ± 0.84 μmole h^{-1} respectively, t-test, $t_{(9)} = 0.693$, $p = 0.506$, Fig. 2.3).

Anemonefish ammonia excretion rates in the presence of anemones

When tested in the presence of sea anemone hosts, the rate of increase in ammonia in test vessels (= fish excretion – anemone uptake) was significantly lower (0.32 ± 0.13 μmole g^{-1} h^{-1}) than ammonia increase in vessels with fish alone (0.64 ± 0.27 μmole g^{-1} h^{-1}, paired t-test, $t_{(8)} = -3.08$, $p < 0.015$, Fig. 2.4A). The average net increase in ammonia

concentration in the experimental vessel containing both a fish and anemone (8.05μM) was only 19.7% of that generated by a fish alone (40.86μM, Fig 2.4B), indicating that >80% of the ammonia released by the fish was absorbed by the sea anemone at an average rate of about 5.3 μmole h^{-1}.

Discussion

The data presented here show that during the day, adult anemonefish generate substantial amounts of ammonia that can be absorbed and presumably utilized by the sea anemone host and/or its endosymbionts, and that host sea anemones rapidly take up ammonia from the surrounding seawater. Thus, an anemonefish can potentially supply >100% of the nitrogenous nutritional requirements of host anemones. In their natural habitat, adult *Amphiprion bicinctus* reach ~14cm in length (Allen 1991) and average approximately 20g in mass, with some individuals exceeding 50g (Roopin unpublished data). These fish are diurnal zooplanktivores (Fautin 1985; Fautin and Allen 1997), so their ammonia contribution via gill excretion is far greater during the day (when they are constantly feeding) than at night when they rest. In the laboratory setting, fed adult anemonefish excreted ammonia at a rate of 1.84 μmole g^{-1} h^{-1}, so in the wild an average 20g adult anemonefish can potentially generate an ammonia load of about 883.2 μmole day^{-1}. This ammonia contribution greatly enriches the host anemone's immediate environment above the low background ammonia levels in the Red Sea of 0.05 – 0.5μM (The Israel National Monitoring Program (NMP), May 2006, available in: http://www.iui-eilat.ac.il/NMP /indexE.htm), and < 0.1 from my field monitoring (see Chapter 4). In addition, sea

anemones in the wild usually are occupied by a pair of adult anemonefish plus 2-4 smaller individuals (Fautin and Allen 1992, 1997). This group occupancy of hosts further increases the ammonia available to the host anemone. Porat and Chadwick-Furman (2005) reported a mean excretion rate of 0.61 μmole g^{-1} h^{-1} when they examined *A. bicinctus* anemonefish in the field, similar to the excretion rate produced by a 24-hour starved fish in the above laboratory experiment. The amount of food consumed prior to testing by fish under field conditions varies with parameters such as site, depth and season, which likely contributed to differences between laboratory and field excretion rates. Time-scaled, controlled laboratory quantifications such as those generated by the present study, provide an important point of reference for comparison of excretion rates measured in the field. This type of laboratory study also allows assessment of fish nutritional status, maximal ammonia contribution, and inference of the potential magnitude of fish contribution to the well-being of the host and to the stability of the symbiotic interaction in general.

Rates of ammonia excretion by *Amphiprion bicinctus* here are within the range of rates for other types of fishes and invertebrates (Table 1.1), and are very similar to those of reef fish that have been shown to contribute nutrients to the growth and survival of other benthic organisms (Meyer and Schultz 1985a; Bray et al. 1986). The temporal pattern of ammonia excretion exhibited here by *A. bicinctus* is characteristic of diurnal fishes (Brett and Zala 1975; Jobling, 1981; Meyer and Schultz, 1985a; Bray et al. 1986). Hence, temporal variation in excretion rate consists of two phases: the first begins when the fish gut is full, and declines exponentially as digestion proceeds and the gut empties. The second phase occurs when the fish gut is completely empty; this represents the basal

excretion rate. The decline in ammonia excretion rates throughout the night is typical for diurnal fish that do not feed during this interval (Brett and Zala 1975; Jobling 1981), and has been quantified for Atlantic menhaden *Brevoortia ryrannus* (Durbin and Durbin 1981) and juvenile grunts *Haemullon* spp.(Meyer and Shultz 1985a). Therefore, in the wild, ammonia excretion by diurnal zooplanktivores such as *A. bicinctus* is highest during morning and midday hours, after the fish have begun feeding, and declines throughout the night (Meyer and Schultz 1985a).

Large anemonefish are of much greater value to anemone hosts than are small ones. In all measurements reported here, large fish always excreted much greater total amounts of ammonia then did small fish. Thus, even if a large fish excretes ammonia at a lower rate per gram of fish mass than does a small fish, the overall ammonia contribution to the symbiosis of a large fish is always greater. Furthermore, in the wild, the rigid hierarchical social structure of anemonefish ensures that larger fish consume more food than do smaller ones and occupy space closer to the center of the anemone (Fautin and Allen 1997), further increasing the importance of ammonia contribution by large adult anemonefish compared to small ones that occupy the same anemone (Holbrock and Schmitt 2002, 2004).

The contribution of anemonefish to the ammonia budget of host anemones may be of greater fitness value than those of many other organisms that associate with cnidarians. For example, although the excretion rates of the anemonefish *A. bicinctus* fall within the range reported by Meyer and Schultz (1985a,b) for schools of resting grunts (*Haemulon* spp.), their actual contribution may be different because anemonefish and grunts differ in their behavior. Anemonefish are obligate, close associates with their

hosts, unlike juvenile grunts, which migrate around the reef. Anemonefish directly contact the tissues of host anemones for many hours during the day and all hours at night, while grunts rest near coral colonies only during the day. Also, in contrast to the corals that grunt schools rest over, some host sea anemones (in this case, *Entacmaea quadricolor)* occur in deep crevices or holes on the reef (Fautin and Allen 1992, 1997; M. Roopin pers. obser.) that potentially reduce the dilution rate of ammonia supplements excreted by the anemonefish (see Bray 1986) and consequently making them more readily available for the anemone. Finally, ammonia excretion rates for grunts were calculated on a dry weight basis, while in the present study *A.bicinctus* ammonia excretion rates were calculated using wet weight, essentially underestimating the actual excretion rate of anemonefish compared to grunts. Invertebrate associates such as the spotted anemone shrimp (*Periclimenes yucatanicus*), which is a frequent ectosymbiont of giant sea anemones in the Caribbean Sea, also can supply their host with supplemental nitrogen through their ammonia excretions (Spotte 1996). Anemonefish excrete ammonia at a lower rate per gram body mass (1.84 μmole g^{-1} h^{-1}) than do spotted anemone shrimps (2.36 μmole g^{-1} h^{-1}); nevertheless, the ammonia contribution of anemonefish is of much greater magnitude, because anemonefish average greater than 100 times the mass of an anemoneshrimp (0.14 ± 0.02 g wet mass, Spotte 1996). Anemoneshrimps remain close to the anemone at all times, and may consume anemone tentacles as food (Fautin et al. 1995). In contrast, anemonefish forage for zooplankton during the day, and range at times several meters from their host (Fricke 1974, 1979; Meroz and Fishelson 1997; Porat and Chadwick-Furman 2004). Thus, in contrast to invertebrate associates, anemonefish also serve as a link between the

planktonic and benthic ecosystems by consuming nutrients from open ocean plankton and releasing these nutrients near their benthic hosts, resulting in uptake and assimilation in the reef benthos. This cross-ecosystem nutrient transfer extends the ecological importance of anemonefish from benefactors in a specific symbiotic interaction, to organisms that affect nutrient cycling on a higher level.

The absorption of dissolved inorganic nutrients from the surrounding seawater by the sea anemones that was observed here is of similar pattern to uptake that was observed for other cnidarian that host symbiotic zooxanthellae (Kawaguti 1953; D'Elia and Webb 1977; Muscatine and D'Elia 1978; Muscatine and Marian 1982; Burris 1983; Wilkerson and Trench 1986; D'Elia and Cook 1988; Roberts et al. 2001; Grover et al. 2002). However, while many past studies have demonstrated uptake and retention of inorganic nutrients by host cnidarians, ony a few have quantified nutrient flux in the symbiosis. All cnidarians take up ammonia, especially under similar environmental conditions (ie: light), but individual uptake rates vary widely across species (Muscatine and D'Elia 1978; Wilkerson and Muscatine 1984; Rahav et al. 1989; Hoegh-Guldberg and Williamson 1999; Grover et al. 2002). Due to this variation, the uptake rates of each type of cnidarian host must be quantified to determine the potential importance of supplemental ammonia contributions by ectosymbionts. Quantification of ammonia flux is highly important since it is the primary limiting inorganic nutrient cycled in cnidarian symbioses, and the major form of nitrogen in fish excretory products, which are available for uptake by cnidarian hosts (Muscatine and D'Elia 1978).

The capacity of the sea anemones examined here to absorb ammonia was directly related to exposure to light, and most probably is driven by the symbiotic algal partner,

similar to the pattern observed in investigations of other cnidarians (Muscatine and D'Elia 1978; Wilkerson and Muscatine 1984; Domotor and D'Elia 1984; Cook and D'Elia 1988; Gunnersen et al. 1988; reviewed by Miller and Yellowlees 1989). Uptake rate by *E. quadricolor* as a function of external ammonia concentration tended to behave linearly, but in these experiments did not exhibit an exact saturation kinetic pattern (ie: ceasing of uptake) that is typical for freshly isolated zooxanthellae (D'Elia et al. 1983). Diversion of cnidarians from standard Michaelis-Menten kinetics for ammonia uptake is common (Muscatine and D'Elia 1978; Muscatine and Marian 1982; Burris 1983; Wilkerson and Muscatine 1984), and has been proposed to result from a diffusive component of uptake through the animal tissue (D'Elia et al. 1983). In addition, since ammonia uptake rate is a function of the algal partner, zooxanthella abundance within the host tissues potentially can affect ammonia uptake dynamics. Spotte (1996) speculated that a higher ammonia uptake rate exhibited by anemones cultured with shrimps than without was due to relatively higher zooxanthella densities in these anemones tissues. My anemones were maintained with ectosymbiotic anemonefish, which in other studies have been shown to induce higher levels of zooxanthellae in host anemones tissues (Porat and Chadwick 2005; see Chapter 3.). Thus, it is hypothesize that 20μM is an insufficient external ammonia concentration for these anemones to demonstrate saturation, partly due to the relatively high densities of zooxanthella symbionts in these anemones (Roopin, unpublished data). This hypothesis should be tested in future studies by quantifying the uptake rates of anemones with different zooxanthella abundances in various external ammonia concentrations. The results here also indicate that supply of inorganic nutrients by anemonefish may be sufficient to create nutritional conditions under which sea

anemones can increase and sustain high zooxanthella densities. Thus, in this way, anemonefish may indirectly affect the ammonia uptake dynamics of their hosts, resulting in more energy translocated to the host from the zooxanthellae and increased growth rate of the host tissues. The connection between algal densities in host tissues, ammonia uptake dynamics, and retention capabilities have not been examined in detail, and may be a potentially important factor in elucidating cnidarian-algal-ectosymbiont symbiotic interactions.

The bulk of the incorporation of supplemental nutrients by anemones from the surrounding seawater took place in the light, coinciding temporally with maximal ammonia excretion levels by the anemonefish partner. Dark inhibition of uptake rates observed here also has been reported for other species, but in contrast to these findings, an extended period of dark incubation was required to hinder ammonia uptake (Muscatine and D'Elia 1978; Wilkerson and Muscatine 1984; Rahav et al. 1989). In free-living algae, a high rate of nutrient uptake in the dark is an indicator of nutrient deficiency (Syrett 1981). Thus, the immediate decrease of ammonia uptake in response to dark by the anemones examined here may indicate a sufficient nutrient supply in anemones that are kept with resident fish (see Chapter 3). During the night, anemonefish continue to supply a considerable amount of ammonia that is more available to the host than during the day, since the fish sleep among the host tentacles. So, although in my study anemones absorbed ammonia at a very slow rate in the dark, ammonia-starved anemones likely increase their dark ammonia uptake, and can absorb fish-excreted ammonia throughout the night.

Anemones took up ammonia at a rapid rate (5.3 μmole h^{-1}) when tested in the presence of anemonefish. This finding appears to be due to the constant gradual addition of ammonia by anemonefish that maintained an overall higher concentration in the vessel (concentrations increased to an averaged maximum of ~ 41μM at 100 min, see Fig. 2.4A) compared to uptake experiments with the anemone alone, in which the initial ammonia spike (20 μM, max) declined rapidly during anemone uptake. Constant mixing of the water around the anemone from anemonefish swimming (Dennison and Barnes 1988; Fautin 1991; Porat and Chadwick-Furman 2004) may also have contributed to higher uptake rate by reducing the boundary layer and increasing CO_2 availability for zooxanthellae photosynthesis. Alternately, the anemones may exhibit a physiological or behavioral reaction to fish presence that allows them to more efficiently absorb the ammonia then when they are alone. For example, anemones are known to expand more fully in the presence, rather than in the absence, of anemonefish (Porat and Chadwick-Furman 2004).

Although the direct translocation of fish waste into host anemones was not traced, it is demonstrated here that anemonefish can rapidly enrich an area the size of a typical anemone hole with greater than 10 times the ambient ammonia concentration in the Red Sea. In response, the sea anemones absorbed 80% of this enrichment at a rate of 5.3 μmole h^{-1}. These results join a growing body of evidence that fish and invertebrates living in symbiotic association with zooxanthellae cnidarians provide substantial nutritional benefits to the host holobiont. I conclude that supplements to the nutritional budget of the host are ecologically important as a corridor connecting trophic levels within the reef, and the reef with the surrounding oceanic ecosystem. To verify these laboratory results in the field, labeled nitrogen studies are needed to trace the path of

ammonia from anemonefish to host anemones and zooxanthellae (Cleveland et al. 2003). Future studies are also expected to quantify the amounts of fish-contributed ammonia that are actually assimilated by anemones under various environmental and nutritional conditions in the field.

Acknowledgements

We thank the students in the Chadwick laboratory group at Auburn University, USA, for assistance in aquarium tank setup and animals care. Funding was provided by start-up funds to N.E. Chadwick from Auburn University and by NSF IBN 02-30005 to RPH.

Table 1.1. Variation in ammonia excretion rates among marine and brackish water fishes and invertebrates after feeding. Studies with unfed fish were excluded. Excretion rates are shown per wet mass except those indicated by an asterisk, which are per dry mass.

Species	System	Method	Ammonium excretion rate (μmole g^{-1} h^{-1})	Source
Two band anemonefish (*Amphiprion Bicinctus*)	Marine	Laboratory	1.84	Present study
French grunt (*Haemulon flavolineatum*)	Marine	Field*	1.7*	Meyer and Schultz 1985a
White grunt (*Haemulon plumieri)*	Marine	Field*	2.5*	Meyer and Schultz 1985a
Various marine fishes	Marine		0.17 – 12.5*	Meyer & Schultz 1985a (table 3)
Blacksmith (*Chromis puncctipinnis*)	Marine	Field*	2.13 - 3.26	Bray et al. 1986
Variety of Pacific fish	Marine	Field	0.39*	Wood 1958
Various brackish water fishes	Brackish		1.29 - 3.49	Haertel-Borer et al. 2004
Five demersal fish	Marine	Field	2.1 – 10.64*	Whitledge 1982
Blue sharks (*Prionace glauca*)	Marine	Field	1.02*	Whitledge 1982
Mytilid bivalve (*Lithophaga simplex*)	Marine	Laboratory	0.36-0.81	Mokady et al. 1998
Common octopus *Octopus vulgaris*	Marine	Field	1.8*	Whitledge 1982
Spotted anemone shrimp (*Periclimenes yucatanicus*)	Marine	Field	2.36	Spotte 1996

Figure Legends

Fig 2.1. Ammonia excretion vs time after feeding in anemonefish *Amphiprion bicinctus*. (N=10), T = 26°C. Times with different letter values denote significance at the 0.05 level (ANOVA followed by post-hoc pairwise comparisons of marginal means).

Fig 2.2. Ammonia uptake vs external seawater ammonia concentration by giant sea anemones *Entacmaea quadricolor* during the day. Mean ± SD (N=10). Anemones examined under laboratory conditions of irradiance (~800 μE m^{-2} s^{-1}) similar to levels on coral reef. Letter values denote significance at the 0.05 level.

Fig 2.3. Ammonia uptake vs light conditions in giant sea anemones *Entacmaea quadricolor* in 10μM NH_4Cl-spiked seawater. Mean ± SD (N=10). T = 26°C.

Fig. 2.4A. Variation in ammonia excretion rates measured for anemonefish *Amphiprion bicinctus* alone versus with giant sea anemones *Entacmaea quadricolor*. The same fish were used with versus without the anemone treatment (= paired replicates). N=9 replicates examined per treatment. Mean ± SD (N=9). T = 26°C. Letter values denote significance at the 0.05 level.

Fig. 2.4B. Ammonia build-up in experimental vessels with anemonefish *Amphiprion bicinctus* alone versus with giant sea anemones *Entacmaea quadricolor*. The same fish

were used with versus without the anemone treatment (= paired replicates). Mean ± SD (N=9). T = 26°C.

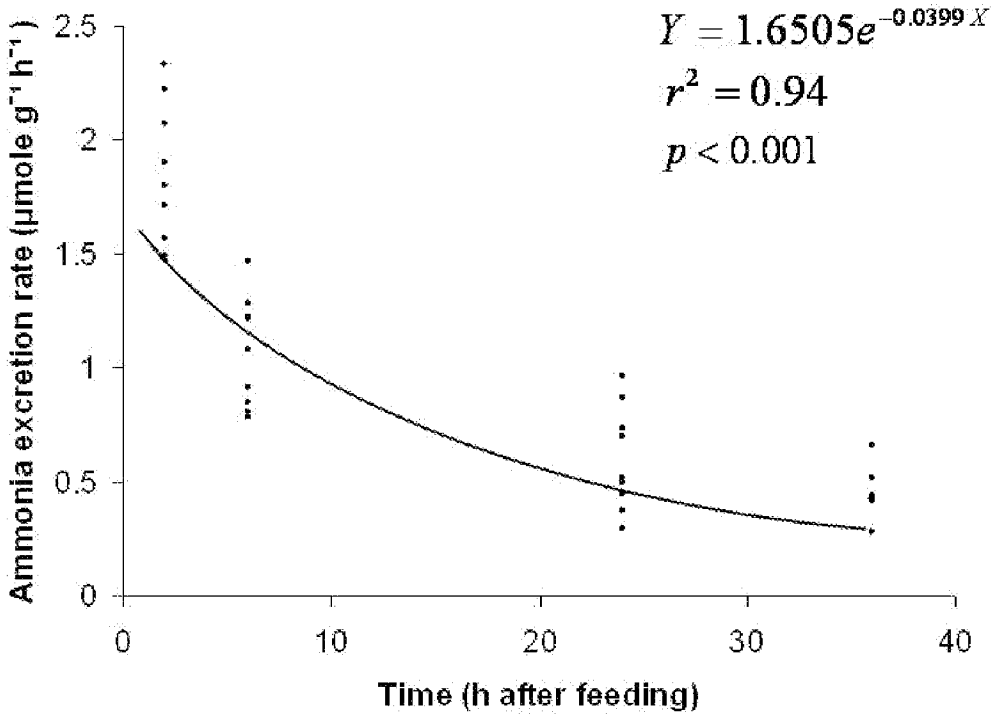
$Y = 1.6505e^{-0.0399X}$
$r^2 = 0.94$
$p < 0.001$
Ammonia excretion rate (µmole g⁻¹ h⁻¹)
2.5
2
1.5
1
0.5
0
0
10
20
30
40
Time (h after feeding)

Fig 2.1.

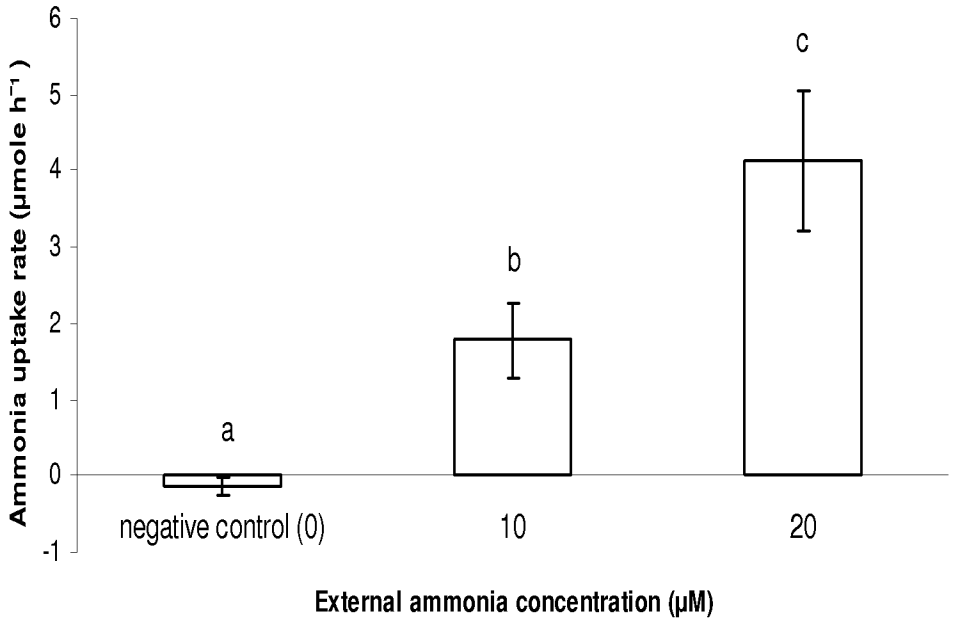
Ammonia uptake rate (µmole h⁻¹)
6
5
4
3
2
1
0
-1
a
b
c
negative control (0)
10
20
External ammonia concentration (µM)

Fig 2.2.

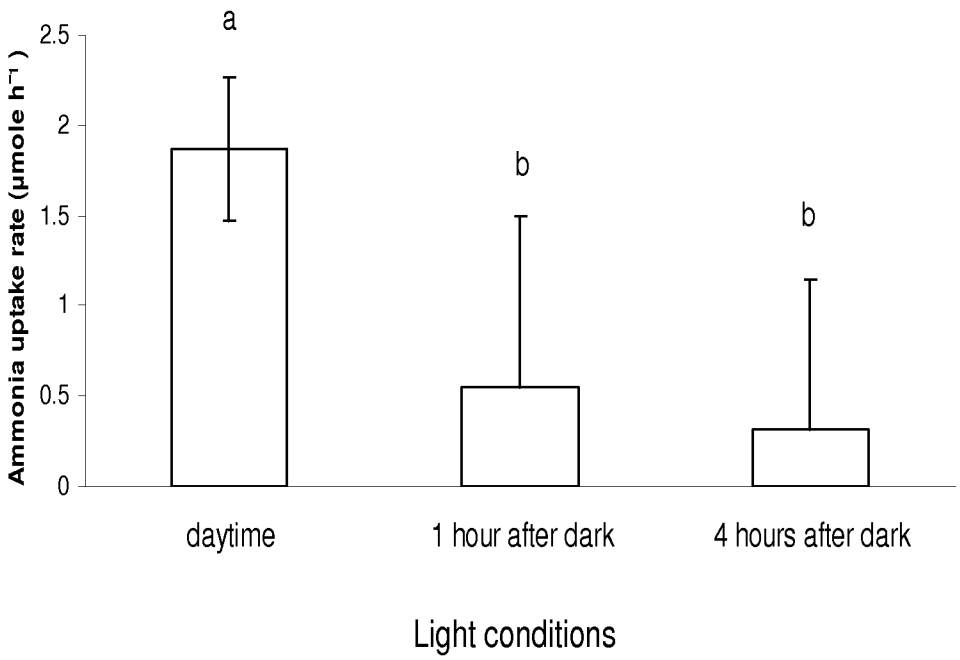
a
b
b
2.5
2
1.5
1
0.5
0
Ammonia uptake rate (µmole h⁻¹)
daytime
1 hour after dark
4 hours after dark
Light conditions

Fig 2.3.

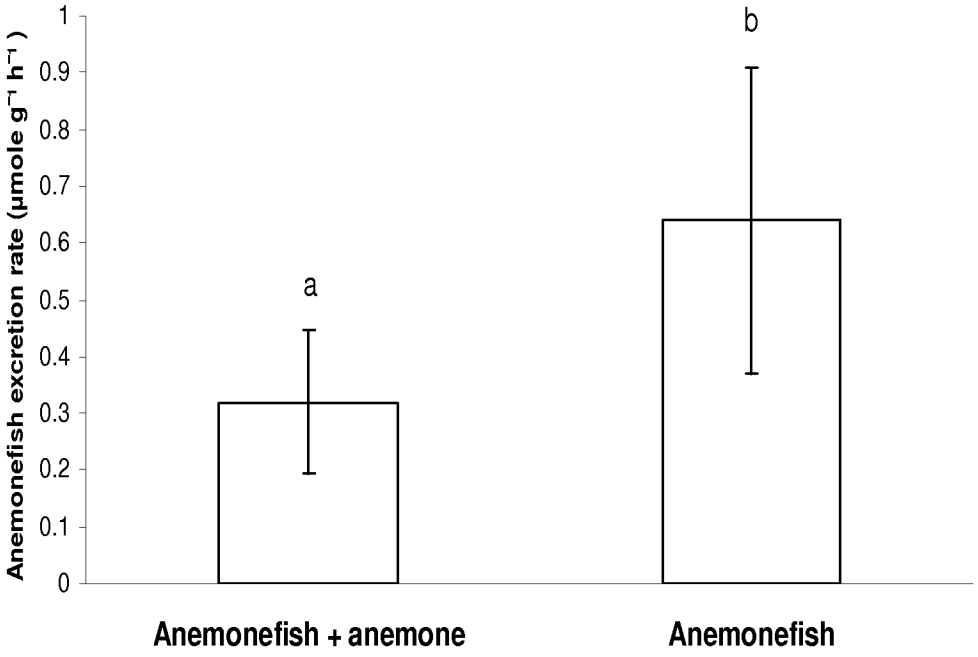

Fig. 2.4A

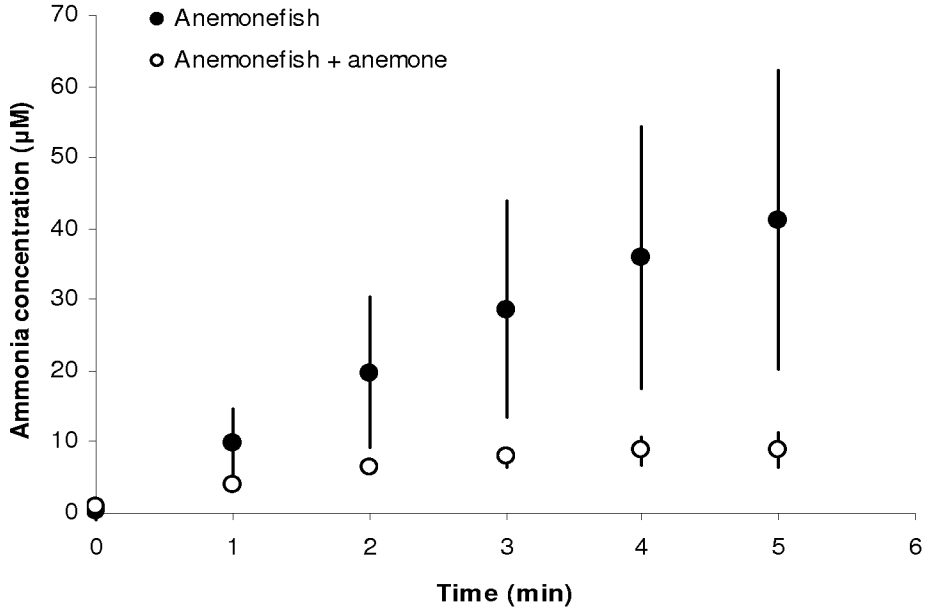

Fig. 2.4B

CHAPTER III

NITROGEN EXCRETED BY ANEMONEFISH ENHANCES THE FITNESS OF SEA ANEMONE HOSTS AND THEIR ZOOXANTHELLAE UNDER LABORATORY CONDITIONS

Abstract

The costs and benefits of symbiotic associations between benthic invertebrate hosts and ectosymbiotic guests are not well understood, especially with respect to the possible effects of local nutrient enrichment by the guests on the host organisms. In the present study, I estimated the fitness parameters of sea anemone hosts (*Entacmaea quadricolor*) as a function of the presence of adult anemonefish guests (*Amphiprion bicinctus*) under laboratory conditions. Starved sea anemones were maintained with either anemonefish, ammonia (defined as the combination of NH_3 gas and NH_4^+ ion) enrichment, or neither for >2 months. All sea anemones responded to starvation by significantly altering the abundance of their endosymbiotic microalgae (zooxanthellae), but although anemones in all treatments exhibited a similar pattern of initial reduction followed by a gradual increase in zooxanthellae densities, the level of response and overall changes in

zooxanthellae abundance varied significantly among treatments. An external supply of inorganic ammonia (both from the anemonefish and artificial supplementation) allowed anemones to maintain higher abundances of zooxanthellae in their tentacles, thus alleviating the effects of starvation. Anemones cultured with both ammonia supplements and resident anemonefish exhibited extremely low ammonia uptake rates compared to control anemones, indicating that their zooxanthellae contained sufficient reserves of nitrogen. All anemones decreased in size due to starvation, but tissue loss was much less severe for anemones with ammonia or anemonefish supplements. This pattern indicates that their abundant zooxanthellae supplied them with extra energy, essentially reducing depletion of host reserves. I conclude that anemonefish supply a continuous ammonia supplement in the form of metabolic waste products to their host anemones. This indirect supplementation of the anemone's energy budget reduces the dependence of their zooxanthellae on anemone host feeding, and therefore mediates the adverse impacts of variation in food availability on the stability of this symbiosis.

Introduction

Symbiotic interactions are ubiquitous in nature, but many of the mechanisms underlying these interactions remain poorly understood, including the potential benefits to each symbiotic partner, which often are not well quantified (Bronstein 1994; Bruno et al 2003). In the association between anemonefishes (28 species of damselfishes in the genera *Amphiprion* and *Premnas*) and host sea anemones (10 species in the Order Actiniaria; Fautin and Allen 1997), the obligate fishes cannot survive in nature without

the safe haven they find among the anemone's tentacles. The sea anemone hosts, on the other hand, are not obligate-partners and appear able to survive without anemonefish under both field (Moyer 1980; Godwin and Fautin 1992) and laboratory (Fautine and Allen 1997; M. Roopin pers. obser) conditions. Although anemones have enhanced survival and higher growth rates when they harbor anemonefish (Porat and Chadwick-Furman 2004; Holbrook and Schmitt 2005), the various mechanisms of benefit to the anemone hosts are not well understood, and only the mechanism of protection from specialized cnidarian predators is well documented (Mariscal 1970 a,b; Fricke 1975; Fautin 1991; Godwin and Fautin 1992; Fautin and Allen 1997; Porat and Chadwick-Furman 2004; Hattori 2006). Anemonefish protection of their hosts from predation has been observed only for the adult fish of some species (Mariscal 1970a,b; Fricke 1974, 1975; Moyer 1980; Fautin 1991; Godwin and Fautin 1992; Fautin and Allen 1997). Thus, hypotheses regarding more generalized physiological benefits to host anemones through the utilization of resident anemonefish waste products have been proposed (Fautin 1991; Cleveland et al. 2003; Porat and Chadwick-Furman 2005; holbrook and Scmitt 2005). To date, the magnitude of the potential nitrogen contribution by anemonefishes to this association has not been well quantified, and the effects of such contributions on the symbiosis have not yet been rigorously examined.

Coral reefs represent a paradox of highly diverse and productive communities that persist in a highly oligotrophic marine desert (Sournia 1977; Entsch et al.1983; Crossland 1983). Algal-cnidarian symbioses thrive in this environment through efficient metabolic exchange between the endosymbiotic algae ("zooxanthellae") that are located in the host cnidarian tissues. The algae provide the host with photosynthetic energy, and the

cnidarian host provides a source of nitrogen for the algae. Efficient recycling within this symbiosis reduces the overall loss of key nutrients, and therefore increases the fitness of both the host and symbiont (Muscatine and Porter 1977; Wilkerson and Trench 1986; Falkowski et al. 1993). All tropical sea anemones that host anemonefish also harbor zooxanthellae (Dunn 1981; Fautin 1991), which supply them with energy-rich photosynthate compounds for respiration, growth and reproduction (Shick and Dykens 1984; Steen 1988; Cook et al. 1988; Achituv and Dubinsky 1990; Davies 1997; Whitehead and Douglas 2003). Zooxanthellae predominately belong to the genus *Symbiodinium* (Freudenthal 1962; Taylor 1974) and recent studies have demonstrated that the genus is comprised of eight distinct strains (= clades A through H; reviewed in Pochon et al. 2006) that exhibit a range of physiological responses (reviewed in Coffroth and Santos 2005). The specific clade(s) that a host species harbors may affect its distribution, reaction to extreme environmental conditions, and ability to survive and recover after a disturbance (reviewed in Baker 2003).

Fish constantly excrete nitrogenous waste through their gills with most of it (75% - 85%) being in the form of ammonia (Jobling 1981; Durbin and Durbin 1981; Tatrai 1981; Whitledge 1982; Meyer and Schultz 1985a), a form of nitrogen that is readily available for cnidarians uptake (Muscatine and D'Elia 1978). Numerous studies in aquatic ecosystems have shown that fish affect nutrient cycles through the transport and recycling of materials such as ammonia. On coral reefs, large schools of resident grunts (*Haemulon spp.*) that feed during the night and rest over colonies of the stony coral *Porites furcata* during the day have been shown to excrete large quantities of ammonia which appear to enhance coral growth rates (Meyer and Schultz 1985a,b). The important

role of fish excretions has been demonstrated in freshwater ecosystems as well, in which fish can affect the flux of nutrients between benthic and open water habitats (Durbin et al. 1979; Vanni 2002; Glaholt and Vanni 2005). While large migratory fish schools can potentially alter nutrient flux among habitats, symbiotic fish and other marine guests that associate with benthic host organisms may affect the nutrient balance of their hosts. For example, the anemoneshrimp *Periclimenes yucatanicus* is an important source of nitrogen for the sea anemone *Condylactis gigantea* (Spotte 1996). The excretory byproducts of obligate ectosymbiotic partners likely provide an important nutritional benefit to many types of host cnidarians, especially large corals and sea anemones that host abundant fish and crustacean guests.

In algal-cnidarian symbioses, zooxanthellae obtain inorganic nutrients from various sources: host catabolism (Szamant-Froelich and Pilson 1984), host holozoic feeding (Steen 1986), and the surrounding sea water (Muscatine 1980; Wilkerson and Trench 1986). The net excretory ammonia of host cnidarians often is insufficient to solely sustain zooxanthellae (Szmant-Froelich and Pilson 1984; Wilkerson and Muscatine 1984; Cook et al. 1988; McAuley and Cook 1994). For example, in the Red Sea coral *Stylophora pistillata*, excretory ammonia can potentially support only about one-third of the maximum specific growth rate of its zooxanthellae at steady state (Rahav et al. 1989). Cook and D'Elia (1987) first proposed that zooxanthellae may be nutrient limited *in hospite.* Several lines of evidence support this nitrogen-limitation theory, including enhancement of dark carbon fixation by hosts with high symbiont densities and no supplemental ammonia (Cook et al. 1994), an increase in cnidarian zooxanthella densities with the addition of inorganic nitrogen (Hoegh-Guldberg and Smith 1989b; Muscatine et

al. 1989; Stambler et al. 1991; Stimson and Kinzie 1991; Hoegh-Guldberg 1994; Muller-Parker et al. 1994b), and the inability of unfed anemones (*Aiptasia pallida*) to support their zooxanthella populations without ammonia supplements (Cook et al. 1988). Thus, the contribution of ammonia waste products by anemonefishes (see Chapter 2.) can augment the capacity of sea anemone hosts to support and potentially increase zooxanthella density, which in term will provide more photosynthetic energy to the host, particularly when heterotrophic food sources are limited (Meyer and Schultz 1985a,b; Spotte 1996; Mokady et al. 1998; Roberts et al 1999a,b; Porat and Chadwick-Furman 2005).

A direct path of nutrient transfer from the anemonefish to the sea anemone host tissues has been shown by Cleveland et al. (2003, 2006) using stable isotope markers. However, the physiological mechanisms of this potential benefit and quantification of the extent to which it contributes to the host remain unknown. In the present study, I demonstrate that the ammonia contributions of anemonefish have a substantial effect on the body condition, growth and zooxanthella population dynamics of starved hosts, and may enhance the tolerance of this symbiosis to starvation stress.

Methods

Study organisms and maintenance

Giant sea anemones *Entacmaea quadricolor* were transported to Auburn University in 2006 from Waikiki Aquarium, Hawaii, USA, where they had been propagated via clonal

replication from individuals collected in Palau in 1985. Anemonefish *Amphiprion bicinctus* were transported to Auburn in 2006 from Oceans Reefs and Aquariums (ORA), an aquaculture facility of Harbor Branch Oceanographic Institution at Fort Pierce, Florida, USA. One anemone was placed on each side of a rigid plastic screen (2cm mesh) in each of 12 closed-system aquaria (N = 24 anemones total), together with 1-2 anemonefish per anemone (N = 30 anemonefish total). All fish and anemones were acclimated for at least four months prior to experiments. Each closed-system aquarium circulated 160 liters of artificial seawater (Reef Crystals, Aquarium System, Inc., Ohio, USA) between an upper tank (77cm x 32cm x 33cm) containing the animals, and a lower sump (77cm x 32cm x 33cm) with filters. Water flowed into the upper tank from the sump alternately through two pipe outlets using a SCWD–wave maker (3iQ Ventures LLC, Manhattan Beach, CA, USA). Each sump contained a protein skimmer, live rock, and macroalgal cultures as filters, and both upper tanks and sumps contained a layer of fine sand. Anemones were allowed to attach to pieces of flat rock (old coral skeletons) that were placed in each upper tank. All systems were maintained at 34-35 ppt salinity, temperature of 26°C, and a 12h light:12h dark photoperiod. Over each aquarium was suspended a six bulb TEK-LIGHT™T5 high output fluorescent light, with a combination of three 39W T5 Midday 6000K, and three 39W T5 Pure actinic Giesemann PowerChrome fluorescent bulbs, which provided irradiance of about 200 $\mu E\ m^{-2}\ s^{-1}$ at the bottom of the aquarium to 800 $\mu E\ m^{-2}\ s^{-1}$ at the water surface (QSL-2101 Scalar PAR Sensor, Biospherical Instruments, San Diego, CA, USA), which is equivalent to irradiance at 7-20m depth on coral reefs in the Red Sea where this symbiosis occurs (Stambler and Dubinsky 2005).

Experimental design

Under culture conditions, all anemones were fed weekly to satiation with small pieces of fish or shrimp. Anemonefish were fed each morning to satiation with a combination of dry pellets (Formula one, AquaPet Americans, Utah, USA) and frozen foods (copepods, brine shrimps, mysids).

To assess the effects of ammonia enrichment on the fitness of starved sea anemones, each individual was assigned haphazardly to one of three treatments for a period of two months: (1) without anemonefish in nutrient-poor seawater, referred to as the "control treatment" (N = 8 anemones), (2) with 1-2 anemonefish in nutrient-poor seawater, referred to as the "anemonefish treatment" (N = 8 anemones and N = 13 anemonefish), and (3) without anemonefish in ammonia-enriched seawater (daily ammonia treatment of ~10μM ammonia for 1-1.5 hours), referred to as the "ammonia treatment" (N = 8 anemones). Identical treatments were assigned to both sides of each aquarium system (see above) to avoid effects from one side of the mesh barrier to the other. Each aquarium system was assigned randomly to a treatment using a random number generator. During the experiment, food was withheld from all anemones, but the anemonefish were fed as usual (see above). The feeding behavior of the fishes was monitored carefully to ensure that they ingested all proffered foods, and uningested food was removed from the tanks. After two months, treatments were reversed in terms of nutrient supply as follows: (1) anemonefishes were removed from the "anemonefish treatment", (2) daily ammonia supplements were stopped for the "ammonia treatment" group, and (3) 1-2 anemonefish per anemone were added to the "control group"

anemones. These reversed treatments were maintained for an additional three weeks before all animals were returned to their original culture conditions.

Unfed anemones sizes

To assess patterns of tissue loss during starvation, the body size of each sea anemone was determined from photographs at the start of the experiment and after two months of treatment (prior to treatment reversal). Sea anemones that appeared contracted were rephotographed up to three times to obtain expanded sizes. Three individuals, one in each treatment, were excluded from the size analysis since they consistently remained contracted or hidden. Photographs were scanned into a computer, and the software program Image Tool (Ver.3.00, UTHSCSA) was used to determine the long and short axial lengths of the sea anemones (tentacle tip to tentacle tip, an approximation of tentacle-crown diameter). The area covered by the tentacles of each anemone was regarded as an oval and was estimated as (long axial length) X (short axial length) X $\pi/4$ (after Hirose 1985). Sea anemones tissue loss was estimated by calculating final body size as a percentage of initial size.

Zooxanthella abundance and mitotic index

To assess the physiological performance of anemones in each treatment during starvation, I determined the protein content of host tissues, zooxanthellae abundance, chlorophyll *a* content and zooxanthellae cell division rate (mitotic index) every 2-3 weeks during the

study. During each sampling period, tissue from several tentacle tips (2-3 cm) on each anemone were removed and immediately analyzed. Sampling did not appear to adversely affect the anemones as they each possess dozens of tentacles and are able to rapidly regenerate lost tips (Porat and Chadwick-Furman 2005). Each tentacle tip was blotted dry and weighed to obtain its wet mass, homogenized in 2mL of seawater with a 5mL Wheaton tissue grinder, and the homogenate was centrifuged at high speed (~5000rpm) on a 5415D eppendorf-centrifuge for 5 minutes to pellet the algal cells. The supernatant was removed by Pasteur pipet to a separate vial, and the algal pellet in the original vial was re-suspended in 2mL of seawater. This procedure was repeated at least three times to produce a pellet consisting of mostly zooxanthellae and almost no animal tissue (after Cook et al. 1988). The combined supernatants from the samples were used to determine total protein content in the host tissue, and 1mL sub-sample of the stock cell suspension was transferred to a separate vial for chlorophyll *a* analysis (see below). The remaining cell suspensions were diluted with seawater to produce densities between 3×10^5 and 7.4×10^6 cells mL^{-1} and aliquots were removed for cell counts. Zooxanthella numbers were determined from 3 replicate hemacytometer counts using a Hausser Scientific hemacytometer (after Stambler and Dubinsky 1987; D'Elia and Cook 1988; Spotte 1996).

The proportion of cells undergoing division in each sample was recorded and used to calculate a zooxanthella mitotic index (McDuff and Chisholm 1982). Algae were counted under phase contrast (400×), and cells were be scored as dividing if they were doublets up to the stage of separation of daughter cells (after Wilkerson et al.1983; Cook et al. 1988). In a preliminary experiment, the mitotic activity of zooxanthellae in fed *Entacmaea quadricolor* anemones was repeatedly (4-hr intervals) measured during a

24-hour period, revealing that the zooxanthellae peaked in division rate at about 7:30 in the morning. Therefore, tentacle sampling was routinely conducted during the experiment during 7:30-8:00 in the morning.

Host protein content and zooxanthella chlorophyll a levels

The total protein content of the animal tissue homogenates was determined using the Bradford technique: the combined supernatant from a sample (animal protein) was re-homogenized and the Bio-Rad procedure (Bio-Rad, Richmond, California; Bradford 1976) was applied with bovine serum albumine (BSA) as a standard. All samples were prepared with filtered sea water (FSA) and yielded absorbance in the range of 0.2 to 1.2 at 595 nm. Standard curves were produced using the seven pre-diluted standards (at concentrations 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 mg/mL) that were supplied with the Bio-Rad Quick start kit (Bio-Rad, Richmond, California).

Chlorophyll *a* levels were assessed by extracting a 1mL aliquot of cell suspension (described above) with 90% acetone overnight at 4°C, centrifuging the acetone extract, and reading absorbance at 750, 664 and 630 nm with a GENESYS 5 Spectrophotometer (Thermo Electron Spectronic, MA, USA). The equations of Jeffrey and Humphrey (1975) were used to calculate chlorophyll *a* from the absorbance scores.

Sea anemone ammonia uptake rates

To determine effects of the above treatments on ammonia uptake rates by the anemones, measurements were made after 2 months of starvation, prior to treatment reversals. Ammonia flux in 4 haphazardly-selected sea anemones from each of the above 3 treatment groups was determined in the light under laboratory conditions. Each organism was placed into a separate 1 L experimental vessel (glass beaker, Fisher Scientific) and filled with 500mL of NH_4Cl-spiked seawater (~10μM, Fisher Chemical A649-500). Prior to each experiment, all glassware was rinsed thoroughly with 10% HCL and double-distilled water, and then rinsed again with filtered seawater. The experimental vessels were placed in a water bath at 26°C, under 400W Radium Metal Halide Lamps (Ocean Light 250, Aqua Medic), which provided irradiance equivalent to that in the holding aquaria (see above). The experimental vessels were aerated continuously using an airstone attached to a pump set to a low level that kept the water aerated and stirred, but produced as few bubbles as possible. All runs also contained a spiked seawater control vessel with no animals. Total incubation time for each run was 100min, and 5mL water samples were withdrawn for ammonia analysis every 20 min. Ammonia concentration in the experimental vessels was determined using the indophenol blue method (Solarzano 1969), scaled down 10-fold due to the small incubation volumes, on a GENESYS 5 Spectrophotometer (Thermo Electron Spectronic). The modified procedure was tested repeatedly against a standard curve of 0-25 μM NH_4Cl: results produced with the two procedures were not significantly different (t-test, $p = 0.802$).

Average ammonia flux was computed as the difference between the final and initial amounts of ammonia in the total volume of each experimental vessel. Changes in water volume due to sampling and the displacement of water due to the volumes of anemones were incorporated into all calculations. At the end of each experimental run, anemones were removed from the experimental vessels and returned to their original aquaria. This procedure did not appear to greatly affect the anemones: some contracted their tissues when transferred to the experimental vessels, but all re-expanded and attached their basal disks to the glass within the first 5-10 min of the experiment. Since the anemones were maintained under constant irradiance throughout the light period (12h) each day, it was assumed that any "time of day" effect would be minor, and thus all experiments were conducted during mid-day.

Zooxanthella cladal analysis

The cladal identity of the zooxanthella populations in all anemones was determined at both the beginning and end of the starvation experiment. Total nucleic acids were extracted from anemone tissue samples according to Coffroth et al. (1992), then *Symbiodinium* 18S-rDNA was amplified by PCR using the primer set ss5 (5' GGTT GATCCTGCCAGTAGTCATATGCTTG-3') and ss3z (5'-AGCACTGCGTCAGTC CGAATAATTCACCGG-3'), as described in Rowan and Powers (1991b). *Symbiodinium* 18S-rDNA PCR products were digested with *TaqI* restriction enzyme (Rowan and Powers 1991b) that generates distinctive restriction fragment length polymorphism (RFLP) patterns for several large *Symbiodinium* clades [i.e., *Symbiodinium* A, B, C

(Rowan and Powers 1991a, b), D (Carlos et al. 1999), and E (*Symbiodinium californium*; LaJeunesse and Trench 2000; LaJeunesse 2001)]. Digested products were separated on 2% sodium-borate (SB) agarose gels and visualized with ethidium bromide under UV light. The cladal identity of each sample was determined comparing the RFLP patterns to those of *Symbiodinium* cloned (i.e., known) standards or to the literature.

Statistical analyses

All statistical analyses were conducted using SPSS 15.0. Normality and homogeneity of variance were tested using the Shapiro–Wilk statistical test for $n < 50$. Differences in ammonia uptake rates among the treatment groups were tested using one-way analysis of variance (ANOVA), followed by post-hoc pairwise comparisons (LCD and Sidak). Differences in zooxanthellae abundance, chlorophyll levels, and cell division rates among treatment groups were tested using repeated-measures ANOVA with time as the repeated measure, followed by post-hoc pairwise comparisons between groups. The Bonferroni correction was applied to significance levels. All results were considered significant at $P < 0.05$. All results are presented as means ± one standard deviation unless otherwise indicated.

Results

Unfed anemones sizes

All of the unfed anemones shrank significantly during the two month starvation period (Repeated measures ANOVA, $F_{(1,18)} = 21.72$, $p < 0.005$). In addition, the final sizes of these anemones expressed as a percentage of their original sizes (Fig. 3.1B) varied among the three treatments (ANOVA, $F_{(2,18)} = 7.094$, $p < 0.005$). Post hoc analyses indicated that anemone size reduction in the "control treatment" (64.75 ± 13.2 % of original size) was significantly larger than in the other two treatments (anemonefish, 31.4 ± 17.6 % of original size, $p < 0.004$ and ammonia, 30.45 ± 25.4 % of original size, $p < 0.005$, Fig. 3.1A).

Zooxanthella abundance and mitotic index

Zooxanthella abundance in starved anemones varied significantly among the three treatments at the end of the eight week starvation period ($F_{(3,66)} = 21.45$, $p < 0.0005$, partial $Eta^2 = 0.494$). In addition, the rate of change in zooxanthella abundance throughout the experiment (the time effect) significantly differed among the treatments ($F_{(1,22)} = 1136.48$, $p < .0005$). There was a significant interaction between the effects of time starved and treatment group ($F_{(6,66)} = 6.654$, $p < 0.005$, partial $Eta^2 = 0.377$). The Eta^2 values indicate that 87.1% of the variability in zooxanthellae abundance was accounted for by the combination of the time and treatment effects. Although food

depravation significantly affected zooxanthella abundances in all treatments ("control treatment", $F_{(3,20)} = 6.362$, $p < 0.003$, "ammonia treatment" $F_{(3,20)} = 16.431$, $p < 0.0005$ and "anemonefish treatment" $F_{(3,20)} = 7.972$, $p < 0.001$), the dynamics of zooxanthella abundances over time significantly differed among treatments (Post hoc analyses LCD, $p < 0.005$ and Sidak, $p < 0.005$) (Fig. 3.2). Zooxanthella abundance in the "control treatment" significantly decreased from original levels ($18.3\pm3.7\times10^7$ to $12\pm4.0\times10^7$) during the first three week period ($F_{(3,20)} = 6.362$, $p < 0.003$); this initial decrease was evident in the increasingly pale color of these anemones. In contrast, zooxanthella abundance in the "ammonia treatment" did not vary significantly during the first three weeks of starvation, and even increased from $13.2\pm2.8\times10^7$ at week 3 to $22.3\pm4.4\times10^7$ at week 6 of starvation ($F_{(3,20)} = 16.431$, $p < 0.0005$). Anemones in the "anemonefish treatment" did not exhibit a significant change in zooxanthellae abundances during the first three weeks of starvation and their zooxanthella abundance significantly increased from $25.3\pm6.0\times10^7$ at week 6 to $30.9\pm5.0\times10^7$ at week 8 ($F_{(3,20)} = 7.972$, $p < 0.001$). Following treatment reversal (Fig. 3.3), all groups exhibited significant changes in zooxanthellae abundances with an opposite pattern to that prior to reversal (Pairwise comparisons of the estimated marginal means, Bonferroni adjusted, $p < 0.005$). Addition of anemonefish yielded a significant increase in zooxanthellae abundance in the "control treatment" anemones, and both ceasing the ammonia treatment and removing anemonefish caused significant decreases in zooxanthellae abundance (Pairwise comparisons of the estimated marginal means, Bonferroni adjusted, $p < 0.005$).

The diel pattern of cell division by zooxanthellae in fed anemones (prior to the above starvation experiment) followed a strong cycle, with a division peak approximately

one hour after first light, in agreement with patterns described for other sea anemones under field (Cook et al. 1988) and laboratory (Cook et al.1988; Smith and Muscatine 1999; Fitt and Cook 2001) conditions. Zooxanthellae mitotic activity begun to increase approximately one hour before first light and declined to minimum levels around 10AM (Fig. 3.4A). Average mitotic index (MI) ranged from 1.5 to 4.9% and specific growth rate (μ) was 0.0478 d^{-1} based on the maximum mitotic index value using the equations of Wilkerson et al. (1983). All anemones exhibited a clear peak in zooxanthella cell division around 7:30AM, and although no samples were observed with zero dividing cells during the off-peak hours, the zooxanthellae were interpreted as displaying phased division.

During starvation, while mitotic index of the "control treatment" anemones remained constant throughout the experiment, treatments with a supply of inorganic nitrogen (artificial or through fish excretion) exhibited a temporary, but nonsignificant, increase in mitotic index after three weeks (Fig. 3.4B). By week 6, the mitotic indices of all 3 groups declined, and by week 8, the "anemonefish" and "ammonia" treatments were below original levels. A repeated measures analysis of variance (ANOVA) indicated that time during starvation significantly affected the mitotic indices ($F_{(3,66)} = 3.59$, $p < 0.018$) while differences among treatment groups after eight weeks were not significant ($F_{(2,22)} = 26.25$, $p = 0.07$).

Host protein content and zooxanthella chlorophyll a levels

Except for a slight and nonsignificant increase in the "control" and "anemonefish" treatments during the first few days, protein levels in all treatment groups remained

unchanged throughout the experiment (repeated measures ANOVA, $F_{(3,66)} = 1.60$, $p = 0.197$). Protein levels in the "control" and "anemonefish" treatments quickly returned to original levels and remained stable until the end of the experiment (ranged from 33.2±2.4 to 40.7±2.4 μg/gr tissue, 33.7±2.5 to 35.6±5.4 μg/gr tissue, and 29.9±2.3 to 36.5±3.9 μg/gr tissue in the "control", "ammonia" and "anemonefish" treatments respectively).

Values of chlorophyll *a* per zooxanthella cell in the "control" and "ammonia" treatments were slightly but not significantly lower during the first three weeks of the experiment (from 2.86±0.60 to 2.31±0.99 pg chl *a* cell^{-1} and 2.79±0.91 to 2.18±1.14 pg chl *a* cell^{-1} respectively), while chlorophyll *a* levels in the anemonefish treatment were slightly higher (2.82±0.84 to 2.98±1.06 pg chl *a* cell^{-1}, Fig. 3.5). By week 6, chlorophyll levels in the "control" and "ammonia" treatments gradually increased and remained near their original levels throughout the experiment. In contrast, values of chlorophyll *a* per zooxanthella cell in the anemones kept with resident anemonefish were consistently but not significantly higher than original values throughout the experiment (from 2.82±0.83 to 3.26±1.30 pg chl *a* cell^{-1}, Fig. 3.5). Differences among the three treatments over time were not significant (repeated measures ANOVA, $F_{(3,66)} = 1.302$, $p = 0.28$).

Sea anemone ammonia uptake rates

In control vessels without animals, the loss of ammonia represented < 0.5% of the maximum NH_4^+concentration, which did not change significantly throughout the experiments (t-test, $t_{(5)} = 0.37$, $p = 0.71$). Thus, ammonia loss due to uptake by microbes

or other reasons was negligible and adjustments were not made to the data. Similar conclusions were obtained by Haines and Wheeler (1978) and Bray et al. (1986).

Daytime uptake rates of sea anemones in ammonia-enriched seawater (10μM) varied significantly with treatment group (ANOVA, $F_{(2,9)} = 7.63$, $p < 0.0012$). Post hoc analyses indicated that the ammonia uptake rate of "control treatment" anemones (mean = 2.17 ± 0.37 μmole h^{-1}) was significantly higher than of anemones that were kept with anemonefish (0.9 ± 0.41 μmole h^{-1}, $p < 0.01$, Fig. 3.6).

Zooxanthella cladal analysis

All sea anemones examined in this study (N = 24 total) harbored *Symbiodinium* belonging to clade C (Fig. 3.7). None of the anemones altered their zooxanthella type in response to the two months of starvation under varying nutrient availability.

Discussion

It is demonstrated here that anemonefish contribute substantial amounts of limiting nutrients in the form of ammonia to their sea anemone hosts, which may increase the range of environmental disturbances that host anemones can withstand and still maintain a stable metabolism. In contrast to the reduced numbers of zooxanthellae supported by control anemones, those with resident anemonefish were able to increase and support higher densities of zooxanthellae that are thought to partly compensated for the lack of heterotrophic input to the hosts.

Responses of algal symbiont densities to host cnidarian feeding regimes can vary among associations, and may result in: increases in symbiont density (Pool 1976; Douglas and Smith 1984; Muller-Parker 1985; Hoegh-Guldberg and Smith 1989b; Muller-Parker et al. 1994b), no change (Kevin and Hudson 1979; Fitt et al. 1982), or decreases in the abundance of zooxanthellae in host tissues (Taylor 1969; Szmant-Froelich and Pilson 1980; Clayton and Lasker 1984; Cook et al. 1988; Titlyanov et al. 2000). Here it was observed that in the initial response to starvation, only anemones without ammonia supplements reduced their zooxanthella abundance, and also that long-term changes depended on the treatment (Fig. 3.3). When host feeding ceased, zooxanthellae were initially lost faster than they were replaced, leading to a reduction in abundance. Although this reduction occurred in all treatment groups, it was significant only in the control anemones. As the experiment progressed, a gradual increase in the zooxanthellae populations was evident, implying that higher zooxanthellae abundance is a key feature in the host's adaptation to its nutritional status. Following 2 months of starvation, only those anemones with anemonefish or ammonia treatments exhibited a significant net zooxanthellae increase, of 140% and 180% respectively. In contrast, control treatment anemones were able to recover only 80% of their original zooxanthellae populations. Similar patterns of changes in algal abundances were observed for the anemonefish and ammonia treatments, indicating that ammonia (or lack of ammonia) was the primary factor limiting zooxanthellae abundance in starved sea anemones. Thus, anemonefish benefit their host anemone (at least in part) through ammonia contributions.

During the reversal period, addition of anemonefish to the control anemones resulted in a rapid recovery of their zooxanthella populations. In contrast, the opposite

pattern occurred in the other reversal treatments, again implicating ammonia availability as a factor contributing to the significant variation in anemone performance among treatments (Fig. 3.3). When food is scarce, anemone host feeding and catabolism may not be sufficient to supply all of the nitrogen requirements of growing zooxanthellae (Rahav et al. 1989). As such, external nutrient sources can contribute substantially to the stability of this association. Since host cnidarians depend on zooxanthella photosynthesis to meet their energy requirements (Porter 1980; Muscatine 1980), the external ammonia contributions of resident anemonefish may "relax" the dependence of zooxanthellae on host feeding, thereby allowing starved anemone hosts to maintain higher zooxanthellae densities to supplement their energy input (Summons et al.1986; Hoegh-Guldberg and Smith 1989b; Marubini and Davies 1996). Hoegh-Guldberg and Smith (1989b) demonstrated that the stony corals *Seriatopora hystrix* and *Stylophora pistillata* were nitrogen-limited at their highest zooxanthellae populations, but with supplemental external ammonia, these corals are able to maintain these high zooxanthellae densities, that provided a maximum rate of net photosynthetate delivery to the host. Thus, anemonefish that are obligate residents of giant sea anemone represent a constantly-replenished source of inorganic nutrients that indirectly increase the net energy available to a starved host through enhancement of its zooxanthellae populations.

The temporary stimulation of zooxanthellae division rates that was observed here in some of the treatments also has been reported for other cnidarians exposed to external ammonia supplements (Cook et al. 1988; Muscatine et al. 1989; Fitt et al. 1993a; Hoegh-Guldberg 1994; Smith and Muscatine 1999; Fitt and Cook 2001). Since the division rates of the zooxanthellae remained low during the remainder of the experimental

period in all treatments, it appears that both external ammonia and the nutritional status of the host have little effect on algal division *in situ*. The lack of a significant MI increase may have resulted from the long time intervals among sampling periods (2-3 weeks), which could have missed an early peak of high zooxanthella division rate. Also, the values of MI in *Entacmaea quadricolor* were low compared with those reported by Cook et al. (1988) for *Aiptasia pallida.* Thus, the small changes in MI observed here may have been sufficient to cause the larger changes in zooxanthella population size, although they remained undetected due to the low signal/noise ratio in my measurements.

The mechanisms of maintaining symbiont densities in cnidarian hosts are poorly understood, and have been proposed to involve regulation by the host so that neither of the symbiotic partners outgrows each other (Drew 1972; Cook 1985; Smith and Muscatine 1999). Muscatine and Pool (1979) have proposed three pathways through which this regulation may occur: (1) symbiont expulsion (2) digestion of excess zooxanthellae, and/or (3) pre-mitotic inhibition of zooxanthella growth via restricted access to essential nutrients, or via the production of growth-inhibiting factors (Muscatine 1967). Since division rates of zooxanthellae in the experimental anemones were consistently low, I conclude that the gradual increases observed in zooxanthellae abundance per gram host tissue resulted from decreases in either host biomass, or expulsion rates of zooxanthellae, or both (Stimson 1988; Stimson and Kinzie 1991, Muller-Parker and Davy 2001). The intertidal sea anemone *Anthopleura elegantissima* has been suggested to control its symbiont population via expulsion (McCloskey et al. 1996), but in most associations this path does not appear to be important in zooxanthella regulation (Fitt and Trench 1983, Hoegh-Guldberg et al. 1987, Hoegh- Guldberg and Smith 1989b; Muscatine et al. 1989).

As such, a hypothetical reduction along with a low algal expulsion rate (Hoegh-Guldberg and Smith 1989b; Muscatine et al. 1989) is not likely to facilitate any substantial increase in net zooxanthellae numbers. The possibility remains that some cnidarians may partly regulate symbiont density by digesting their algae (Titlyanove et al. 1996, 2000), but there is currently no strong evidence to suggest that this mechanism is a dominant regulatory mechanism in giant sea anemones. Thus, I conclude that during early starvation, temporary ammonia-stimulated algal growth occurred in the anemonefish and ammonia treatments, which delayed any decrease in algal numbers due lack of host food. Later, when algal growth rates returned to normal, the observed increases in zooxanthella density likely were due to a combination of the loss of anemone tissue (ie: decrease in algal habitat size) during shrinkage and to the supplemental nutrients that allowed the maintenance of high zooxanthella densities. To date no evidence has unequivocally demonstrated active host control of zooxanthella numbers. Therefore, these data are in agreement with the conclusions of Smith and Muscatine (1999) that no single mechanism is likely to regulate the standing stock of zooxanthellae. Rather, a combination of processes interact to regulate zooxanthella biomass accumulation and cell division, maintaining levels compatible with the metabolic state of the host and nutrient availability in the external environment.

The magnitude of responses by giant sea anemone hosts to anemonefish nutrient supplementation likely varies among habitats and species combinations. It may also be influenced by variation in physiological performance among *Symbiodinium* clades, involved in the symbiosis (Chang et al. 1983; Hoegh-Guldberg and Smith 1989b; Muller-Parker et al. 1994b; Goulet et al. 2005). The sea anemone *Entacmaea quadricolor*

harbored *Symbiodinium* clade C and starvation stress or supplemental inorganic nutrients did not change this association. Thus, nutrient cycling patterns in these experiments were not influenced by changes in the genetic identity of the symbiont population.

The lack of variation in host protein levels throughout the experiment is similar to the patterns observed in other studies (Fitt and Pardy 1981; Muller-Parker 1985; Muscatine et al. 1989; Hoegh-Guldberg and Smith 1989). This indicates that the cnidarian host conserves nitrogen through the catabolism of translocated symbiont carbon (Rees and Ellard 1989; Szmant et al. 1990; Wang and Douglas 1998). In this way, the host benefits from high zooxanthella densities due to an increased supply of photosynthetic carbon that supplements its energy budget (Dubinsky et al. 1984; Porter et al. 1984; Muscatine et al. 1984) and reduces the depletion of its own reserves (Fitt and Pardy 1981; Tytler and Davies 1986; Zamer and Shick 1989; Cikala et al. 1999).

The slower loss of animal biomass in those treatments with supplemental nutrients compared to the starved controls with no external ammonia source indicate that the latter rapidly depleted nutrient reserves and therefore experience a more severe energetic loss. Similar conclusions have been drawn for the sea anemone *Anemonia viridis* (Beaver 1996; Roberts et al. 1999a), starved individuals that were kept with a constant nutrient supplementation (20μM ammonia) exhibited a positive daily growth opposed to negative growth for anemones with no nutrient supplementation. Different concentration and ammonia availability, as well as inherited differences among species, may account for the anemone growth reported by Roberts et al. (1999a). Nevertheless, although in my experiments all anemones showed negative growth, the trend in which nutrient supplementation slowed host tissue loss is similar for both studies. Ammonia

supplied by anemonefish excretion substantially delayed the effects of starvation under laboratory conditions, and it likely also enhances the growth rates of unstarved individuals under natural conditions (Meyers and Schultz 1985b; Holbrock and Schmitt 2005; Porat and Chadwick Furman 2004, 2005).

Zooxanthellae in unfed cnidarian hosts are known to absorb and retain external inorganic nutrients at a significantly faster rate than those in fed hosts (Szamant-Froelich and Pilson 1984; D'Elia and Cook 1988; Muller-Parker et al. 1990). Thus, the observed higher uptake rate for ammonia (2.17 μmole h^{-1}) in the control anemones, compared to that of fed individuals (1.77 μmole h^{-1}, see Chapter 2.), indicate a nitrogen deficit in these anemones. Sea anemones that received supplemental ammonia possessed a much higher zooxanthella standing stock, but absorbed ammonia at a significantly lower rate (1.58 μmole h^{-1}). Interestingly, anemonefish appeared to contribute even more to host nutrition than did the artificially added ammonia, as indicated by the exceptionally low ammonia uptake rate of anemones cultured with fish (0.9 μmole h^{-1}, less than 50% of control anemones). Thus, the presence of anemonefish appears to contribute a substantial elevation in inorganic nutrients availability that leads to the accumulation of nutrient stores by host zooxanthellae, in addition to the production of new algal cells (Muscatine et al. 1989; Muller-Parker et al. 1994a). The more-or-less constant supply of anemonefish-released nutrients appears to represent an easily-utilized nitrogen source than a once-daily ammonia spike, and likely contributes to the nutritional stability of this association. Furthermore, since dark carbon fixation is enhanced in starved hosts in the presence of elevated ammonia concentrations (Cook et al. 1992), nutritional benefits from anemonefish may extend to starved hosts throughout the night (see Chapter 2.).

Anemones in the anemonefish treatment also remained in their holes and rarely changed location; in contrast, to behaviors such as climbing on the tank walls, retraction of tentacles, and frequent changes of location within the tank, which were exhibited mostly by the control anemones (and near the end of the starvation experiment, also by the ammonia-treated anemones; M. Roopin pers. obs.). These differences suggest that anemones cultured with fish behave differently from those lacking fish, and are less likely to exhibit tentacle contraction and other behaviors associated with a need to conserve energy (Sebens and DeRiemer 1977).

In conclusion, anemonefish represent a renewable source of limiting inorganic nutrients to sea anemone hosts. Since variation in environmental conditions and in the host nutritional state can reduce zooxanthella population densities and destabilize the symbiosis (Smith and Muscatine 1999), the slow but constant fertilization provided by resident anemonefish may therefore buffer environmental impacts that reduce rates of growth and reproduction in giant sea anemones. Although ammonia supply appears to be a major physiological benefit of hosting anemonefish, the host anemone may also acquire substantial benefits from other activities of guest fish, such as their swimming activities and rubbing against the host tentacles, which may mix the usually stagnant water among the tentacles (Dennison and Barnes 1988; Fautin 1991; Porat and Chadwick-Furman 2004), and thus, may augment the effects of added nutrients by reducing the boundary layer for mass transport (Summons et al. 1986; Snidvongs and Kinzie 1994).

Acknowledgements

I thank the students in the Chadwick laboratory group at Auburn University, USA, for assistance in tanks and animals care. I also thank Raymond Henry for his help with nitrogen measurements. Funding was provided by start-up funds from Auburn University to N. E. C.

Figure Legends

Fig. 3.1A. Effects of laboratory treatments on body size (tentacle crown diameter) variation in unfed giant sea anemones *Entacmaea quadricolor*. White bars (X ± SD) represent the average initial size. Black bars X ± SD represent final size after 2 months. N=7 anemones per treatment. Initially, 8 individuals were in each treatment, but one in each treatment was excluded from size analyses due to constant contraction and inability to accurately measure body size.

Fig. 3.1B. Variation in the final mean body sizes (black bars, tentacle crown diameter) of giant sea anemones *Entacmaea quadricolor* after 2 months of starvation among 3 laboratory treatments, expressed as a percentage of their initial mean sizes (white bars). N=7 anemones per treatment.

Fig. 3.2. Variation in abundances of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments: (A) control (N = 8), (B) ammonium-enriched (N = 8), (C) anemonefish (N = 8). Vertical bars = X ± SD

Fig. 3.3. Variation in the mean abundance of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* cultured in 3 treatments. Changes are expressed as percentage of initial abundance. Open symbols are during treatments (weeks 0-8) and closed symbols are after the treatments were reversed (8-11 weeks). N=8 anemones per treatment,

Fig. 3.4A. Patterns of zooxanthella cell division in giant sea anemones *Entacmaea quadricolor* that were fed weekly and subjected to a 12 h light: 12 h dark cycle, in which light began at 06:00 hours and ceased each day at 18:00 hours. N=3 anemones examined during each time interval. Data are presented as X ± SD.

Fig. 3.4B. Variation in the mitotic index of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* that were cultured in 3 treatments. Mitotic indices (MI) are expressed as a percentage of initial MI prior to starvation. N=8 anemones examined per treatment.

Fig. 3.5. Variation in chlorophyll *a* content per zooxanthella cell in unfed giant sea anemones *Entacmaea quadricolor*. N=8 anemones tested per treatment. Data are presented as X ± SD.

Fig. 3.6A. Variation in ammonia uptake rates by unfed giant sea anemones *Entacmaea quadricolor* cultured for 2 months in 3 treatments. N=4 anemones tested for ammonia uptake from each treatment. Vertical bars = X ± SD.

Fig. 3.6B. Time course of ammonia uptake by unfed giant sea anemones (*Entacmaea quadricolor).* Lines were fitted by regression analysis. (○) individuals that received a daily ammonium treatment ($y = -0.0574x + 8.5193$, $r^2 = 0.99$), (□) individuals kept with resident anemonefish ($y = -0.0304x + 8.5323$, $r^2 = 0.91$), (Δ) control anemones that

received no supplements and no fish ($y = -0.0694x + 7.7011$, $r^2 = 0.8594$). N=4 anemones tested for ammonia uptake from each treatment.

Fig. 3.7. Cladal identities of zooxanthellae (*Symbiodinium* spp.) in giant sea anemones *Entacmaea quadricolor* under laboratory conditions. L = DNA 100bp size ladder, A - C = *Symbiodinium* clade standards. Zooxanthella cladal identities were identical both before and after 2 months of anemone starvation in the following experimental treatments: (a) control (31,41), (b) ammonium supplement (34,50), and (c) anemonefish (33,40,48,49,51). See text for treatment details.

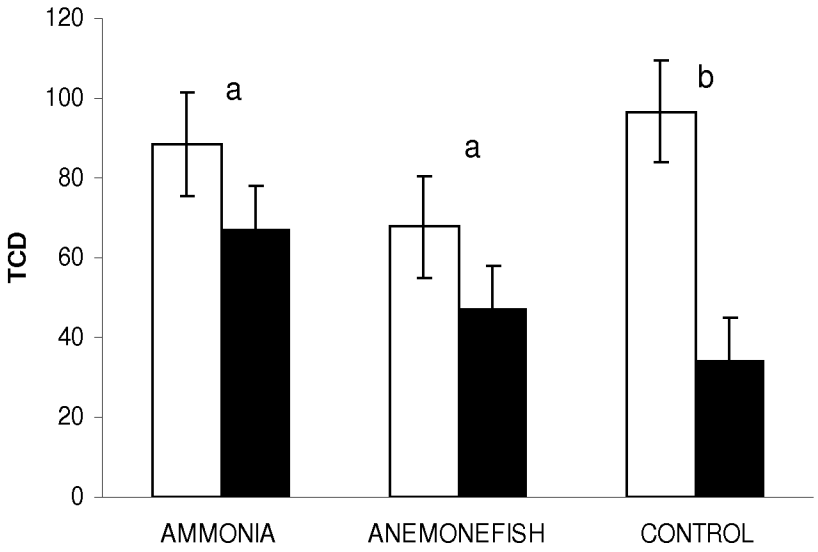

Fig. 3.1A

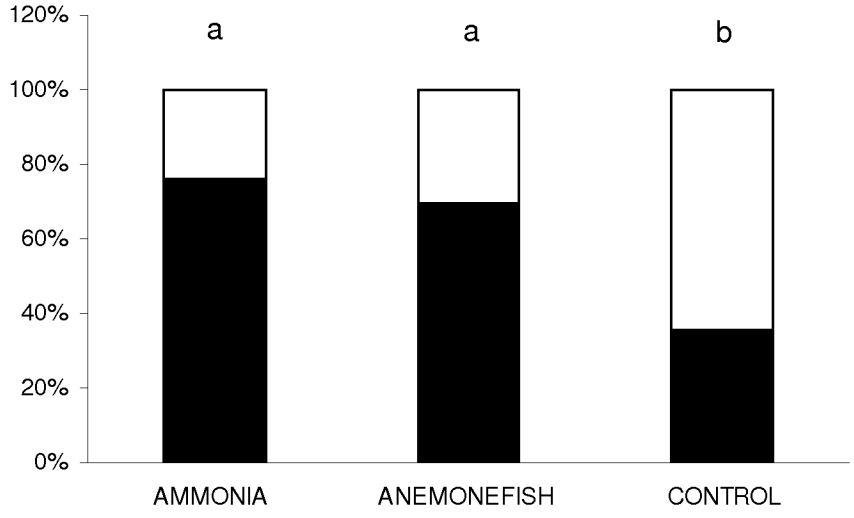

Fig. 3.1B

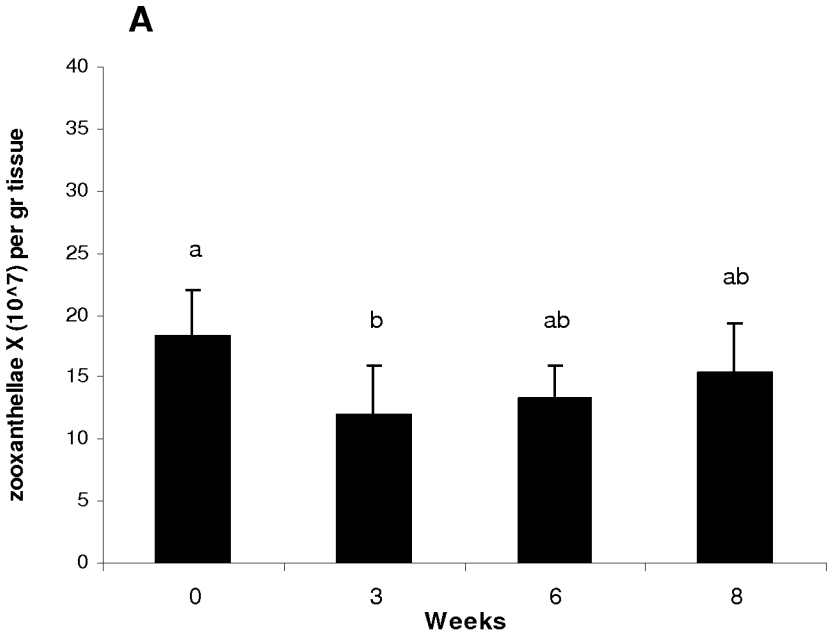

Fig. 3.2A

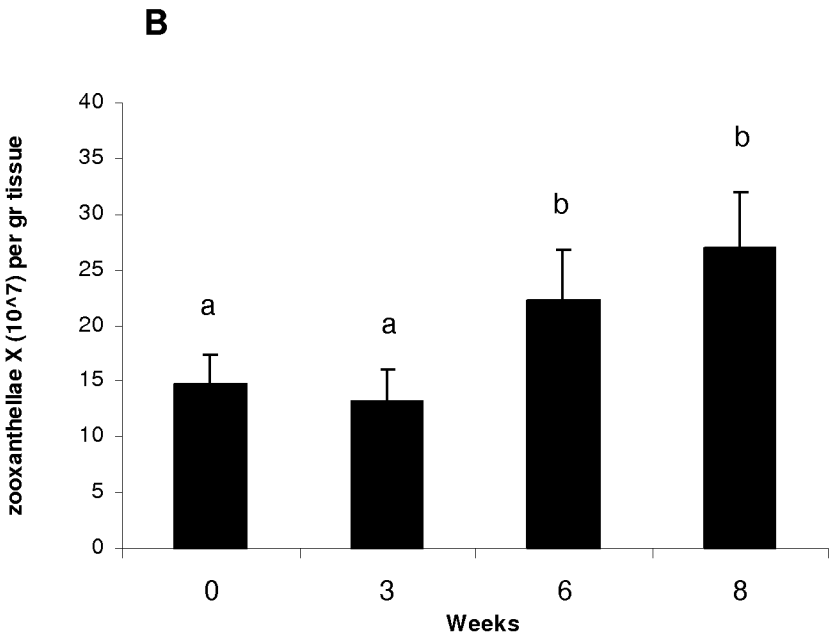

Fig. 3.2B

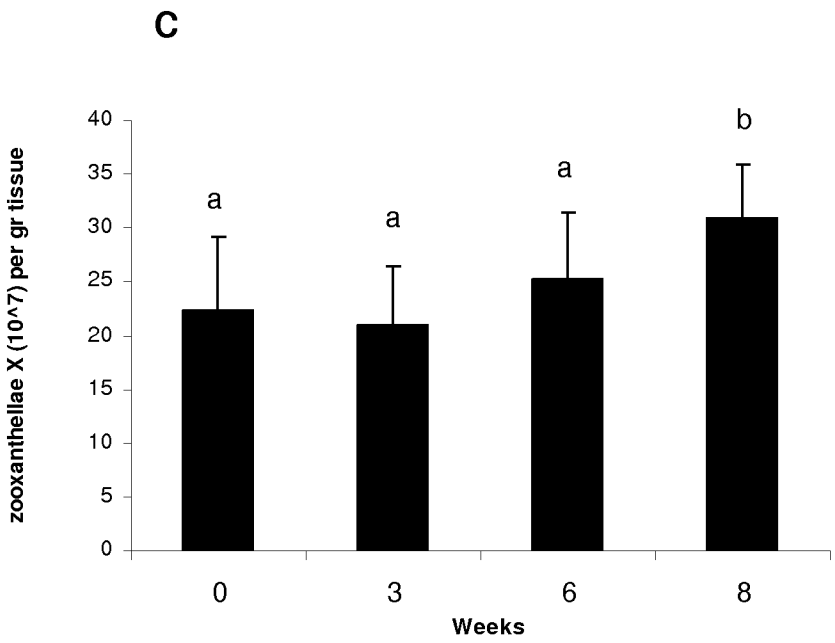
C
zooxanthellae X (10^7) per gr tissue
40
35
30
25
20
15
10
5
0
a
a
a
b
0
3
6
8
Weeks

Fig. 3.2C

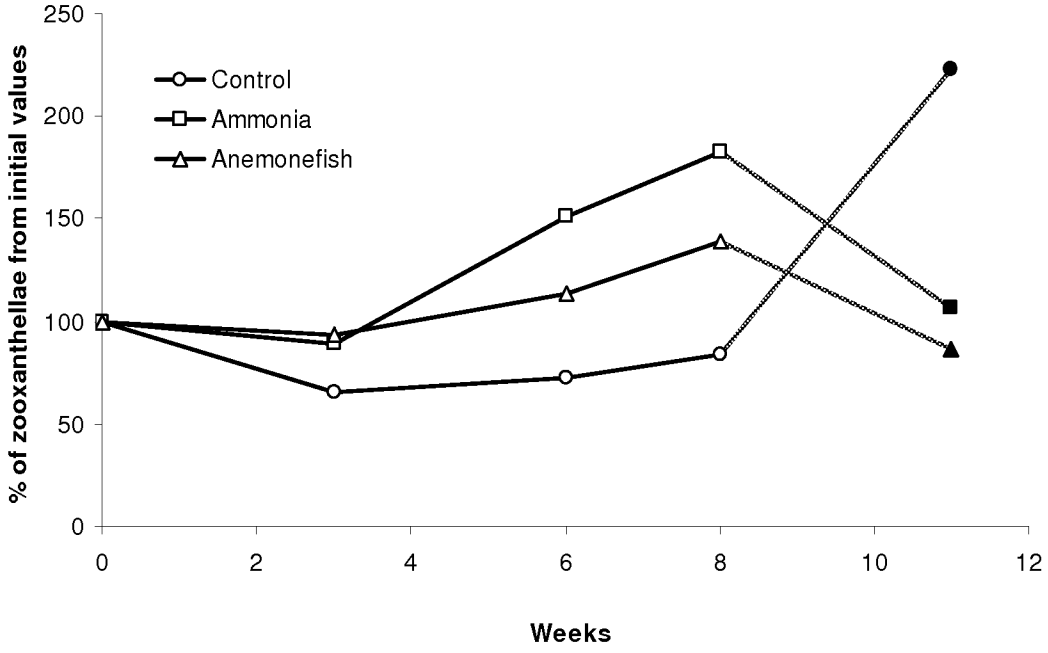
250
200
150
100
50
0
% of zooxanthellae from initial values
Control
Ammonia
Anemonefish
0
2
4
6
8
10
12
Weeks

Fig. 3.3

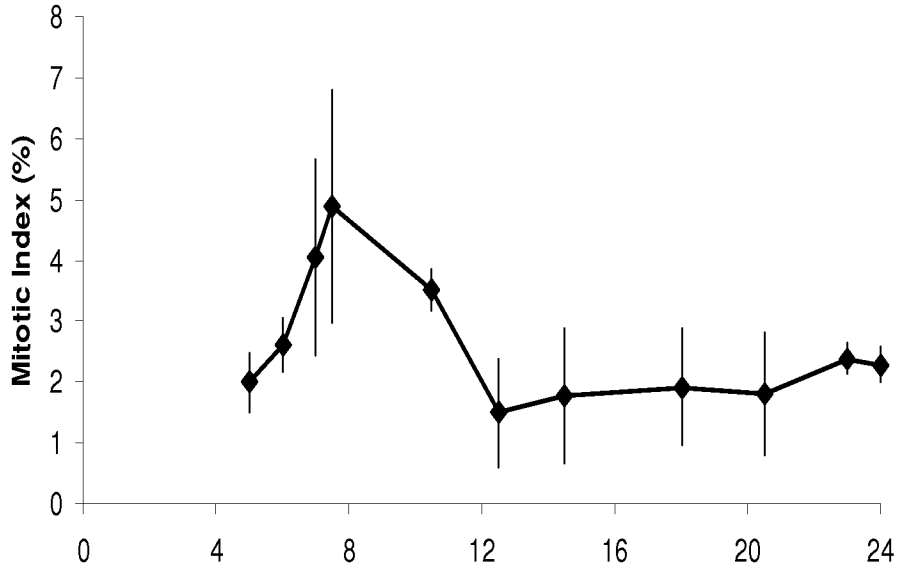

Fig. 3.4A

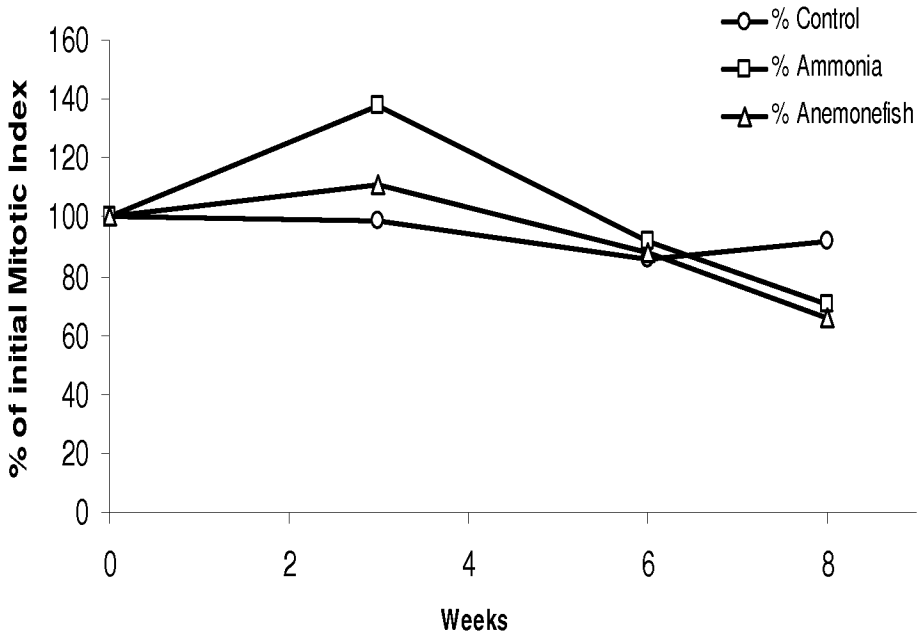

Fig. 3.4B

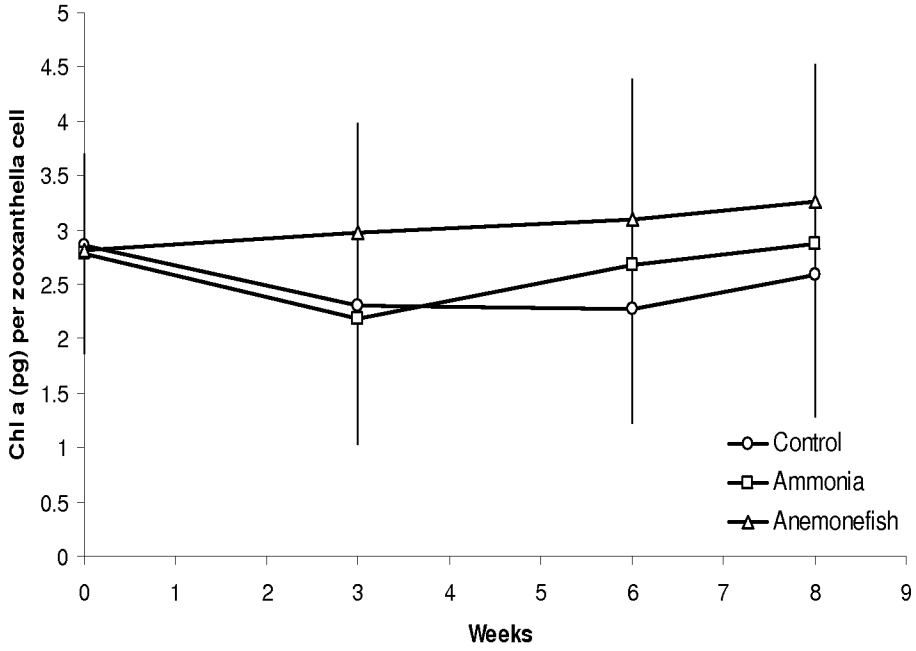
Chl a (pg) per zooxanthella cell
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
8
9
Weeks
Control
Ammonia
Anemonefish

Fig. 3.5.

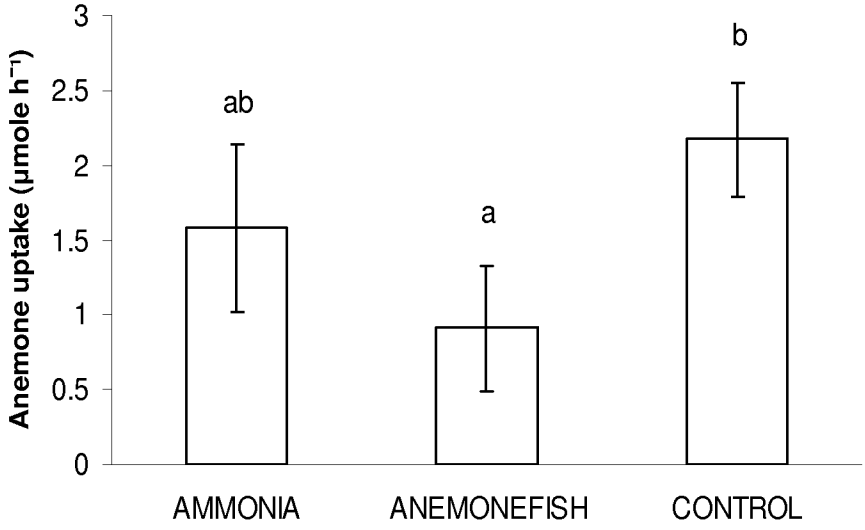

Fig. 3.6A

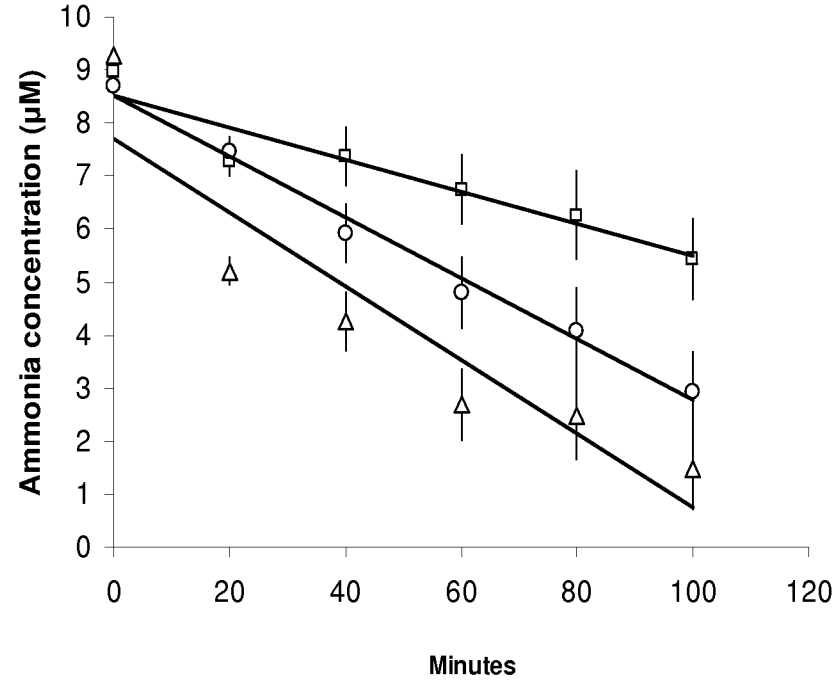

Fig. 3.6B

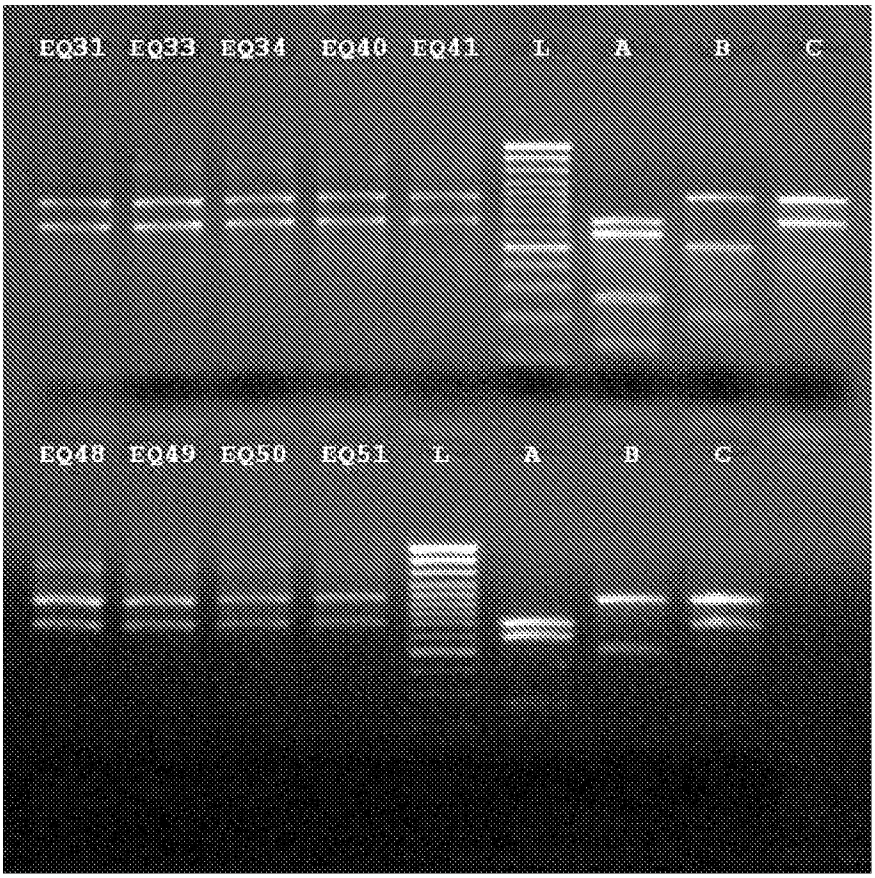

Fig. 3.7.

CHAPTER IV

ANEMONEFISH AFFECT THE AMMONIA AVAILABILITY TO GIANT SEA ANEMONES ON CORAL REEFS IN THE RED SEA

Abstract

Anemonefish may provide nutrient enrichment to giant sea anemone hosts, but their rates of nitrogenous waste excretion under field conditions are not known. Field measurements in the Red Sea indicated that anemonefish excreted ammonia (defined as the combination of NH_3 gas and NH_4^+ ion) at a rate of 0.63 μmole $g^{-1}h^{-1}$ on average during the daytime, and generated a significant enrichment of 0.34 μM above background reef ammonia concentrations in the water among host anemone tentacles. Artificial enhancement of nutrient availability to cultured sea anemones has been shown to stimulate an increase in their zooxanthellae standing stock, especially when starved. However, evaluation of the physiological properties of host anemones in the Red Sea with respect to depth, irradiance, and the number of resident anemonefish indicated that local irradiance conditions was the primary factor causing variation in zooxanthella abundance among hosts. Thus, effects of local ammonia enrichment by anemonefish likely were masked by the photoadaptation of host anemones. Current conditions indicate sufficient nutrition to

host sea anemones at Eilat. However, should food conditions change, the number of resident anemonefish harbored by the host may become a dominant factor in host nutritional physiology. Habitat choice within the structurally complex coral reef may serve as a behavioral mechanism allowing sea anemones to control levels of irradiance available for their algal symbionts, and also may mask the effects of fish local nutrient enrichment on host zooxanthella density. I conclude that in the Red Sea, anemonefish substantially augment dissolved inorganic nutrients available to host anemones, and that host physiological parameters are influenced by a complex array of environmental factors, primarily levels of irradiance.

Introduction

The association between anemonefishes and giant sea anemones is a well-known marine mutualism and an important component of coral reefs throughout the Indo-Pacific region. Anemonefish shelter among the sea anemone's lethal tentacles (Fautin and Allen 1997), and in turn provide the host with nutritional benefits (Cleveland et al. 2003, 2006; Holbrook and Schmitt 2005; Porat and Chadwick 2005) and protection against some specialized anemone predators (Mariscal 1970 a,b; Fricke 1975; Fautin 1991; Godwin and Fautin 1992; Fautin and Allen 1997; Porat and Chadwick-Furman 2004; Hattori 2006). Sea anemones can be abundant macro-invertebrates on some Indo-Pacific and Caribbean coral reefs, and in the process substantially contribute to the structural complexity and biodiversity of reefs. Like most reef cnidarians, all tropical sea anemones that harbor symbiotic anemonefish also associate with algal endosymbionts

("zooxanthellae" in the genus *Symbiodinium*) in a relationship that conveys nutritional advantages to both partners. The host receives from the algae photosynthetically-derived energy products (Muscatine and Porter 1977; Falkowski et al. 1984; Muscatine et al. 1984; Steen 1988; Rahav et al. 1989; Achituv and Dubinsky 1990; Davies 1997; Whitehead and Douglas 2003) and serves as a source of limiting inorganic nutrients (Szamant-Froelich and Pilson 1984) that are essential for the growth of the algae and that are typically scarce in the oligotrophic waters of coral reefs (Sournia 1977; Entsch et al.1983; Crossland 1983). In a related study (see Chapter 2) I have quantified the magnitude of nutrient waste production by anemonefish, and demonstrated that under laboratory conditions anemonefish added large amounts of ammonia to the water, a form of nitrogen readily available for uptake by cnidarians hosts (Muscatine and D'Elia 1978). However, under field conditions, it is not known whether ammonia addition by anemonefish is sufficient to generate ammonia-enriched microenvironments around their anemone hosts.

Almost all studies of ammonia excretion by fishes have been conducted in the laboratory (Forster and Goldstein 1969; McCarthy and Whitledge 1972; Brett and Zala 1975; Durbin and Durbin 1981; Jobling 1981; Tatrai 1981) and little is known about ammonia release rates by reef fishes in the field. Webb et al. (1975) speculated that increased ammonia concentrations around isolated coral pinnacles were due in part to resident damselfishes (*Dascyllus* sp.). More recently, Meyer and Schultz (1985a,b) demonstrated that during the day, schools of juvenile grunts (*Haemulon* spp.) release nitrogen and phosphorus that may be utilized by corals. If ectosymbiotic fish release dissolved and particulate wastes near their cnidarian hosts, nutrient amounts available to

the host would depend in part on water movement, fish diets, and other environmental factors. In addition, the typical microhabitat parameters and behavioral patterns of anemone hosts may affect the magnitude of nutritional benefits they derive from fish wastes. The reef crevices and holes in which these anemones often occur (Fautin and Allen 1997) possess structural properties that may delay the natural dilution of fish-added nutrients. This habitat type could allow accumulation of external ammonia concentrations that can stimulate an increase in zooxanthella densities and enhanced uptake and retention of nutrients by the symbiosis (Muscatine and D'Elia 1978; Muscatine and Marian 1982; Wilkerson and Trench 1986; see Chapter 3.). In turn, this process can lead to an increase in the overall supply of photosynthetically-derived energy compounds to the host (Dubinsky et al. 1984; Porter et al. 1984; Muscatine et al. 1984).

To investigate the extent of ammonia contribution by anemonefish to their host anemones under field conditions, excretion rates of fish were quantified in the field and the localized nutrient levels around hosts assessed. This symbiotic association was also examined over a wide depth range (1-22m) to determine whether the physiological parameters of host anemones vary with light availability, number of resident fish, and habitat structure.

Methods

Variation in sea anemone and zooxanthella parameters

Giant sea anemones (*Entacmaea quadricolor*) and their resident anemonefish (*Amphiprion bicinctus*) were examined during May to July 2006 on coral reefs adjacent to the Interuniversity Institute for Marine Science (IUI) near Eilat, Israel (Fig. 4.1) at the northern tip of the Gulf of Aqaba, Red Sea (29°30N, 34°55E). A preliminary survey of the 3km of coastline near the IUI revealed that all host anemones contained resident anemonefish. Thus, 35 anemones that varied in the number of guest fish (1 - 3 per host) were selected haphazardly over a depth range of 1-22m and tagged by scuba divers. Small, unequal sample sizes of anemones were sampled at each depth, since the Israeli coastline in the Gulf of Aqaba is a protected nature reserve, and the collecting permit limited the number of anemones to be sampled.

Notes were taken on the degree of shading within each individual anemone microhabitat (deep in crevice, semi-shaded by rocks, or fully exposed to light), which allowed the ranking of anemones into three discrete irradiance categories (low, medium, high, corresponding to the above three habitat categories).

To assess the physiological properties of these sea anemones, tissue samples from several tentacle tips (2-3 cm) on each tagged individual were removed for determination of the protein content of host tissue, and abundance, division rate, and chlorophyll *a* content of their zooxanthellae. Tissue samples were analyzed within 2 hour after collection. Each tentacle tip was blotted dry and weighed to obtain wet mass and then

homogenized in 2mL of seawater with a 5mL Wheaton tissue grinder. The homogenate was centrifuged at high speed (~5000rpm) on a 5415D eppendorf-centrifuge for 5 minutes to pellet the zooxanthellae. Supernatant was removed by Pasteur pipet to a separate vial, and the algal pellet in the original vial re-suspended in 2 mL of seawater. This procedure was repeated at least three times to produce a pellet consisting of zooxanthellae with almost no animal tissue (after Cook et al. 1988). The final zooxanthella pellet was suspended in 2mL of seawater. The combined supernatants were used to determine total protein content in the host anemone tissue. A 1mL sub-sample of the zooxanthella cell suspension was transferred to a separate vial for chlorophyll *a* analysis (described below). The remaining zooxanthella cell suspensions were diluted with seawater to produce densities between 3×10^5 and 7.4×10^6 cells mL^{-1} and aliquots were removed for cell counts. The numbers of zooxanthellae were determined from three replicate haemacytometer counts per tentacle tip, obtained using a Hausser Scientific haemacytometer (after Stambler and Dubinsky 1987; D'Elia and Cook 1988; Spotte 1996). The proportion of cells undergoing division in each sample was recorded and used to calculate the zooxanthella mitotic index (MI, McDuff and Chisholm 1982). Algae were counted under phase contrast (400×) and cells were scored as dividing if they were doublets up to the stage of separation of daughter cells (after Wilkerson et al. 1983; Cook et al. 1988). Preliminary observations on the mitotic activity of zooxanthellae in fed cultured *Entacmaea quadricolor* anemones repeated at 4hr intervals over a 24-hour period (see Chapter 3.) indicate that zooxanthellae peak in division rate around 7:30 AM. Thus, in the present study, tentacles were routinely sampled between 7:30 and 8:30AM.

Chlorophyll *a* levels were assessed by extracting 1mL aliquot of zooxanthella cell suspension with 90% acetone overnight at 4°C, centrifuging the acetone extract, and reading absorbance at 750, 664 and 630 nm. The equations of Jeffrey and Humphrey (1975) were used to calculate chlorophyll *a* from the absorbance scores. Total protein content of the animal tissue homogenate was determined using the Bradford technique: the combined supernatant from a sample (animal protein) was re-homogenized and the Bio-Rad procedure (Bio-Rad, Richmond, California; Bradford 1976) was applied with bovine serum albumine (BSA) as a standard. All samples were prepared with filtered sea water (FSA) to yield absorbance in the range of 0.2-1.2 at 595 nm. Standard curves were produced using seven pre-diluted standards at concentrations that were supplied with the Bio-Rad Quick Start Kit (Bio-Rad, Richmond, California).

Nutrient levels and fish excretion rates in the field

Anemonefish (N = 5) were removed from non-tagged host anemones in the field at dusk, and their ammonia excretion rates quantified 1) immediately after capture; and, 2) the following morning after spending the night in an aquarium. Ammonia excretion rate was measured by placing each fish in a separate 2 L experimental vessel (glass aquarium) filled with 1 L of seawater. Prior to each experiment, experimental vessels and all other glassware were rinsed thoroughly in 10% HCL and double-distilled water, and then rinsed again with filtered seawater. During ammonia monitoring, experimental vessels were placed in a running flow-through seawater bath to maintain temperature at 26ºC, under fluorescent lights that provided low irradiance levels similar to those on the reef

during dusk and sunrise when measurements were taken. Experimental vessels were aerated continuously using an airstone attached to a pump set to a low level that kept the water aerated and stirred, but produced as few bubbles as possible. All runs contained a control vessel with seawater and no animals. Anemonefish were placed in the experimental vessel and allowed to acclimate for 20 minutes prior to performing two complete changes of water using inflow and outflow tubing, to ensure that ammonia levels were close to zero when measurement began. The total incubation time of each animal was 100min, and 5mL water samples were withdrawn for ammonia analysis every 20 min (adapted from Wilkerson and Muscatine 1984; Spotte 1996). Ammonia concentration in the experimental vessels was determined using the indophenol blue method (Solarzano 1969), scaled down 10-fold due to the small incubation volumes involved. The modified procedure was tested repeatedly against a standard curve of 0-25μM NH_4Cl and results from the two procedures did not differ significantly (Student's t-test, $p = 0.802$). Average ammonia flux was computed as the difference between the final and initial amounts of ammonia in the total volume of each experimental vessel. The amount of ammonia generated per hour per gram fish mass was calculated from the ammonia concentration, the water volume in the experimental vessel, and the wet mass of each fish (μmole g^{-1} h^{-1}). Changes in the water volume due to sampling and water displacement by fish volume were incorporated into all calculations. At the end of each experimental run, fish were removed from the experimental vessels, gently blotted and weighed in air to obtain their wet mass. Fish were maintained overnight in a flow-through seawater system, and after measurement of their excretion rates in the morning, they were released back into their original anemone hosts on the reef. The fish appeared calm and

swam normally during excretion measurements, and did not appear to be stressed or breathing rapidly as indicated by opercular (gill cover) opening rates.

Levels of dissolved ammonia near sea anemones in the field were determined from water samples collected by scuba divers near 13 haphazardly-selected tagged individuals. Each anemone was sampled on two occasions during evening and morning slack tides. On each occasion, one sample was taken from among the anemone tentacles, and one from the nearby water column (5-7m away). A 20mL Luer Lock polypropylene syringe with a 0.22μm Millipore syringe filter (Millex GP) was used to collect 10-15mL of seawater that was analyzed within the hour. Following collection, samples were chilled in an icebox to prevent ammonia loss. The ammonia concentration in each sample was determined using the indophenol blue method (Solarzano 1969) as described above.

Statistical analyses

Statistical analyses of the data were conducted using SPSS 15.0. Normality and homogeneity of variance were tested using the Shapiro–Wilk statistical test for $n < 50$. Variation in sea anemone and zooxanthella parameters across depths were examined using one-way analysis of variance (ANOVA), followed by post-hoc pairwise comparisons with the LCD and Sidak post hoc criterion for significance. Differences in ammonia concentration among anemone tentacles versus the nearby water column were examined using a paired student's t-test. The significance level for all tests was set at $p < 0.05$. Results are presented as means ± one standard deviation unless otherwise indicated.

Results

Variation in sea anemone and zooxanthella parameters

Zooxanthella abundance varied widely among anemones within each depth range, and although individuals in deeper sites exhibited higher average zooxanthellae density this trend was not significant (ANOVA, $F_{(2,32)} = 0.798$, $p = 0.46$, Fig. 4.2A). Other physiological parameters such as host protein levels, zooxanthella mitotic index, and chlorophyll *a* level per zooxanthella cell also were highly variable among anemones, and did not vary significantly with depth (ANOVAs, $F_{(2,32)} = 0.094$, $p = 0.91$, and $F_{(2,32)} = 0.126$, $p = 0.88$, and $F_{(2,32)} = 0.686$, $p = 0.51$, respectively, Fig. 4.2B,C,D). In addition, none of the above four parameters, varied significantly with the number of resident fish per anemone (ANOVAs, $F_{(2,32)} = 0.242$, $p = 0.786$, $F_{(2,32)} = 1.705$, $p = 0.201$, $F_{(2,32)} = 2.967$, $p = 0.066$, and $F_{(2,32)} = 1.596$, $p = 0.218$, respectively). However, this relationship was difficult to determine under field conditions since most anemones contain 1-2 fish which vary in size, and potentially can generate similar level of enrichments (Fig. 4.3).

Zooxanthella abundance decreased significantly with the level of irradiance in the anemone microhabitat (one-way ANOVA, $F_{(2,31)} = 15.76$, $p < 0.005$). Multiple comparison analyses using the LCD ($p < 0.01$) and Sidak ($p < 0.03$) post hoc tests indicated that zooxanthella densities differed significantly among the three irradiance levels (Fig. 4.4).

Nutrient levels and fish excretion rates in the field

The rate of ammonia production by anemonefish was significantly higher (paired student's t-test, $t_{(4)} = 5.06$, $p < 0.008$, Fig. 4.5) at dusk (0.63±0.10 μmole g^{-1} h^{-1}) than at dawn (0.35±0.13 μmole g^{-1} h^{-1}). While ammonia concentration among anemones tentacles with resident anemonefish ranged from 0.93-1.06 μmole L^{-1} during the morning and evening slack tides, respectively (Fig. 4.6). These levels were significantly higher than the ambient ammonia concentrations 5-7m away in the surrounding seawater (0.76 μmole L^{-1}) during both morning (student's t-test, $t_{(12)} = 2.5$, $p < 0.028$) and evening periods (student's t-test, $t_{(12)} = 3.05$, $p < 0.01$). The highest concentration of ammonia recorded among an anemone's tentacles was 1.97 μmole L^{-1} during an evening measurement. Although local enrichment of ammonia among the anemone tentacles was highest at dusk, it was not significantly higher than the lower enrichment levels measured in the morning (student's t-test, $t_{(11)} = -1.78$, $p = 0.103$). Thus, high ammonia levels were maintained around the anemones during day, and appeared to decline only slightly at night when the resident anemonefish did not feed.

Discussion

Here it is shown that, on coral reefs in the Red Sea, anemonefish excrete sufficient nitrogenous waste to increase the ammonia concentrations around their host anemones significantly above ambient reef levels. Thus, previously documented enhancement of host growth, survival and zooxanthella abundance when individuals harbor anemonefish

(Spotte 1996; Porat and Chadwick-Furman 2005; Holbrock and Schmitt 2005; see Chapter 3.), appears to be due in part to the local enrichment of their microenvironment by fish waste products.

Due to the high recruitment rate of the anemonefish *A. bicinctus* in the Red Sea, individuals of the main host anemone *E. quadricolor* without resident fish are very rare (Fautin 1986, 1991; Meroz and Fishelson 1997; Chadwick and Arvedlund 2005). In numerous survey dives along the cost during this study, no uninhabited sea anemones of this species have been observed. In contrast, uninhabited individuals of a less preferred giant sea anemone (*Heteractis crispa*), which is an alternative host for *A. bicinctus*, were ubiquitous. Thus, it appears that association with *E. quadricolor* hosts may present greater advantages to *A. bicinctus* than association with *H. crispa* (Chadwick and Averdlund 2005). Because no anemones (*E. quadricolor*) were uninhabited in the field, it was not possible to detect significant physiological variation among anemones with the number of adult symbiotic fish.

The light environment can have a major effect on the productivity, physiology and ecology of zooxanthellate cnidarians (Dustan 1982; Dubinsky et al. 1984; Porter et al. 1984; Falkowski et al. 1990; Muller-Parker and Davy 2001). Since light decreases exponentially with depth, roughly following the Beer–Lambert law, effects of light availability often correlate with depth. However, the 3-dimensional complexity of the reef can produce some shaded habitats at all depths (Sheppard 1981; Chang et al. 1983), and these effects can offset the general correlation between depth and light level in coral reef microenvironments. Many cnidarians may acclimate to attenuated irradiance by maintaining higher zooxanthella densities and/or the algae may increase pigment

concentration per cell (Falkowski and Dubinsky 1981; Titlyanov et al. 2000; Kuguru et al. 2007). At Eilat, most *Entacmaea quadricolor* giant sea anemones resided in shaded or partly shaded microhabitats, in which regardless of depth, light availability was the dominant environmental factor affecting zooxanthella abundance in the anemone tissues. This is supported by the observation that at shallow sites where irradiance was high, individuals exposed to direct sunlight exhibited significantly lower zooxanthella densities than shaded individuals at the same depth.

It is well established that many cnidarians depend on zooxanthella photosynthesis to meet their energy requirements (Porter 1980; Muscatine 1980; Gatusso and Jaubert 1990). Thus, light is an essential resource to these symbioses, but extremely high irradiance may also damage zooxanthellae and many cnidarians are harmed by high light levels (Warner et al. 1999; Fitt et al. 2001; Loya et al. 2001; Bhagooli and Hidaka 2003; Rowan 2004) through inhibition of photosynthesis (Winters et al. 2003). In the northern Red Sea, host sea anemones are exposed to some of the highest irradiances levels on earth (e.g., http://ww w.eosweb.larc.nasa.gov /sse/), and may need to (1) actively shield their zooxanthellae from photoinhibition at high irradiance, and (2) otherwise regulate light levels available to their zooxanthellae in order to adjust algal productivity to levels compatible with host resources. Coral reef anemones are capable of habitat shifts and movement among micro-habitats (Muller-Parker and Davy 2001; Hattori 2006), and thus can regulate light levels arriving to their zooxanthellae simply by moving away from or toward light (Sebens 1976; Warner et al. 1999). This type of locomotory control on light exposure potentially allows sea anemones to maximize the nutritional benefits they derive from their endosymbiotic algae within a range of environmental conditions. When

anemones retreat into shaded habitats on the reef, their zooxanthellae invest energy (at the host's expense) in the production of new algal cells and the synthesis of additional chlorophyll as an effort to capture more light energy and maintain a similar rate of photosynthesis. To successfully photoadapt to shade, the nitrogen-limited zooxanthellae (Cook and D'Elia 1987; Muscatine et al. 1989; Cook et al. 1994; Hoegh-Guldberg 1994; Muller-Parker et al. 1994b) require a greater supply of inorganic nutrients, normally available to them through host feeding and catabolism (Clayton and Lasker 1984; Steen 1986; Rahav et al. 1989). For example, the absence of certain photoadaptive responses in some shade-dwelling colonies of reef-building corals (Redjale 1976; Falkowski and Dubinsky 1981) and symbiotic sea anemones (Svoboda and Porrmann 1980; Muller-Parker 1985) may be due to a deficit of nutrients and/or zooplanktonic food (reviewed in Titlianov et al. 2000). Thus, in the symbiosis between *E. quadricolor* and *A. bicinctus*, local nutrient enrichment through anemonefish waste products may relax the dependence of zooxanthellae on host feeding. Therefore, enhancing the ability of sea anemones to photoadapt to shade, compared to other cnidarian hosts that do not associate with anemonefish.

The present study is the first demonstration in the field that resident anemonefish measurably increase local ammonia levels available to their hosts. In the field, anemonefish were observed to generate a constant ammonia gradient of 0.34 μM above background concentrations. The highest ammonia gradient measured around a sea anemone was 1.33 μM above background concentration, which is a much higher ammonia level than the 0.05 – 0.5μM measured by The Israel National Monitoring Program (NMP) during May 2006 on reefs at Eilat (available in: http://www.iui-

eilat.ac.il/NMP /indexE.htm). This indicates that local enrichment around an anemone is highly variable and probably contingent on the number and size of fish present, their feeding history, the current velocity, and the shape and size of crevices in which the anemones reside (see Chapter 2.). The average ammonia excretion rates measured for fish in the field were similar to those determined by Porat and Chadwick-Furman (2005) at the same field site. However, as shown here, anemonefish release ammonia at significantly lower rates in the morning after fasting all night than during the evening after feeding all day. In any case, this lower ammonia release rate appears sufficient to maintain a significant nutrient gradient around the host during the early morning hours.

Laboratory and field experiments have demonstrated that sea anemones lacking resident anemonefish grow more slowly and are more susceptible to nutritional deficits than those with fish (Porat and Chadwick-Furman 2005; Holbrock and Schmitt 2005; Chapter 3.). Here, no significant differences in host physiology were detected between sea anemones with one versus >1 anemonefish, implying that the nutritional contribution of even one anemonefish is sufficient to support host nitrogen requirements under field conditions at Eilat. This surplus of ammonia release by each fish is similar to results from laboratory measurements, in which an anemonefish releases ammonia at a much faster rate than its anemone host can absorb (see Chapter 2.). Environmental stress to zooxanthellate cnidarians in terms of nutrition or irradiance usually is evident in zooxanthella abundance: therefore, the similar average zooxanthella densities of field sea anemones (~17×10^7) and laboratory-cultured individuals that were maintained for several months with 1-2 anemonefish under optimal conditions (~18×10^7, see Chapter 3.)

indicates that the anemones at Eilat are in "good" nutritional condition and are not currently experiencing environmental stress.

This work establishes that in their natural habitat, resident anemonefish (*A. bicinctus*) positively alter ammonia availability to their preferred host anemone (*E. quadricolor*). I have demonstrated elsewhere here (Chapter 3.) that anemonefish benefit their hosts via nutrient excretion. Therefore, I conclude that anemonefish-induced nutrient enrichment appears advantageous to the host under natural conditions at Eilat. However, the direct physiological benefits from enhanced ammonia availability appear to be masked by effects of irradiance level. While symbiont photoadaptation limits the niche diversity of many cnidarians (Iglesias-Prieto and Trench 1994), behavioral mechanisms exhibited by sea anemones (i.e., mobility and niche selection) to avoid extremely high light may ease environmental constraints on this specific association. Anemonefish-induced local ammonia enrichment also may foster the persistence of sea anemones in shaded habitats that require higher zooxanthella levels that these large anemones could not otherwise support. In order to better understand the nutrient dynamics of reef communities, additional research is needed on patterns of release of ammonia and other nutrients by reef fish and invertebrates that are obligate or facultative associates of reef cnidarians.

Acknowledgements

I thank Baraka Kuguru of the Interuniversity Institute for Marine Science for assistance with anemone tagging in this project. This work was supported by start-up funds to NEC

and a graduate research award to MR, both from Auburn University. This research is submitted in partial fulfillment of the requirements for an M.Sc. degree by MR at Auburn University. All sampling for this study complies with the current laws of Israel.

Figure Legends

Fig 4.1. Map of northern Gulf of Eilat (Aqaba). The asterisks denote the Interuniversity Institute for Marine Science (IUI) and sampling sites.

Fig. 4.2. Variation in host and zooxanthella parameters with depth among giant sea anemones *Entacmaea quadricolor* at Eilat, northern Red Sea. Data are presented as X ± SD.

Fig. 4.3. Variation in number of resident anemonefish *Amphiprion bicinctus* per anemone among giant sea anemones *Entacmaea quadricolor* on coral reefs at Eilat, northern Red Sea.

Fig. 4.4. Variation in zooxanthella abundance within tentacles of giant sea anemones *Entacmaea quadricolor* among microhabitats with different irradiance levels on coral reefs at Eilat, northern Red Sea.. Data are presented as X ± SD. Letter values denote significance at the 0.05 level. See text for details

Fig. 4.5. Variation in rates of ammonia excretion with time of day by adult anemonefish *Amphiprion bicinctus* in the field at Eilat, northern Red Sea. N= 5 anemonefish tested per time period. Data are presented as X ± SD. Letter values denote significance at the 0.05 level.

Fig. 4.6. Variation in dissolved ammonia concentration with time of day (morning versus evening) and location (water among anemone tentacles inhabited by adult anemonefish *Amphiprion bicinctus* [black bars] versus that in the surrounding water column [5-7m away, white bars]). N=12 anemones examined during each time period. Data are presented as X ± SD. Letter values denote significance at the 0.05 level.

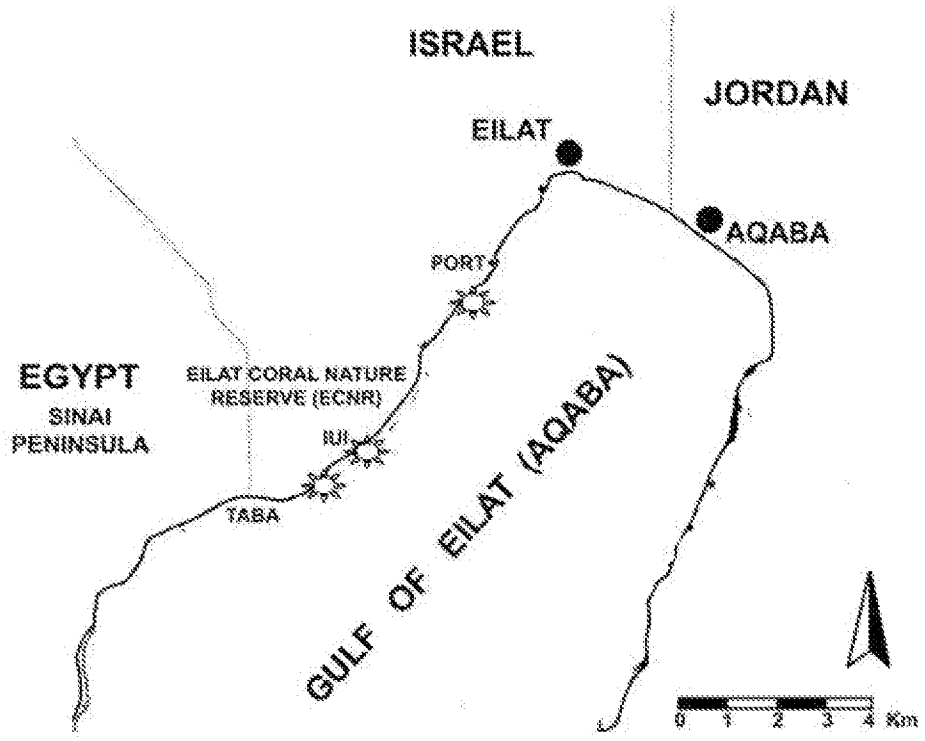
ISRAEL
JORDAN
EILAT
AQABA
PORT
EGYPT
SINAI
PENINSULA
EILAT CORAL NATURE
RESERVE (ECNR)
RIF
TABA
GULF OF EILAT (AQABA)
0 1 2 3 4 Km

Fig 4.1

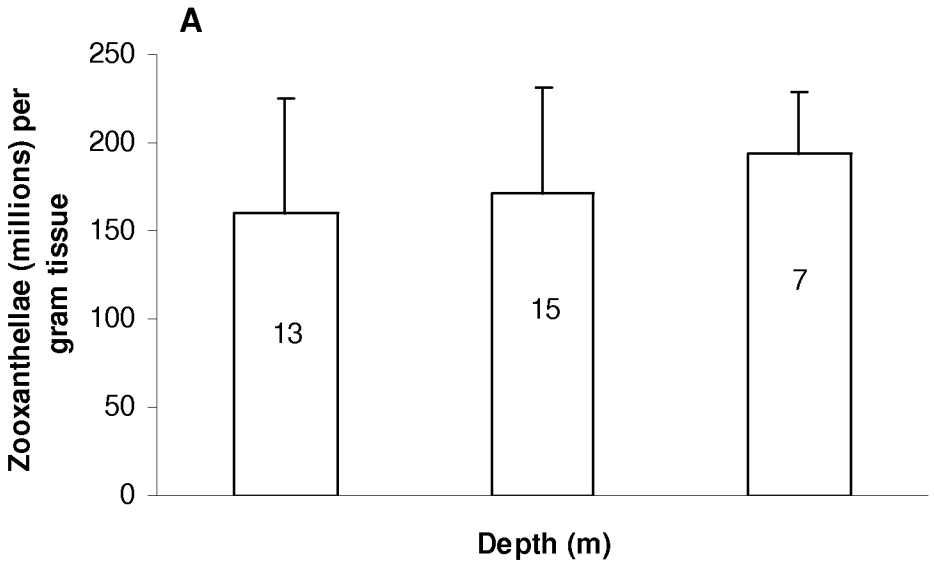

Fig. 4.2A

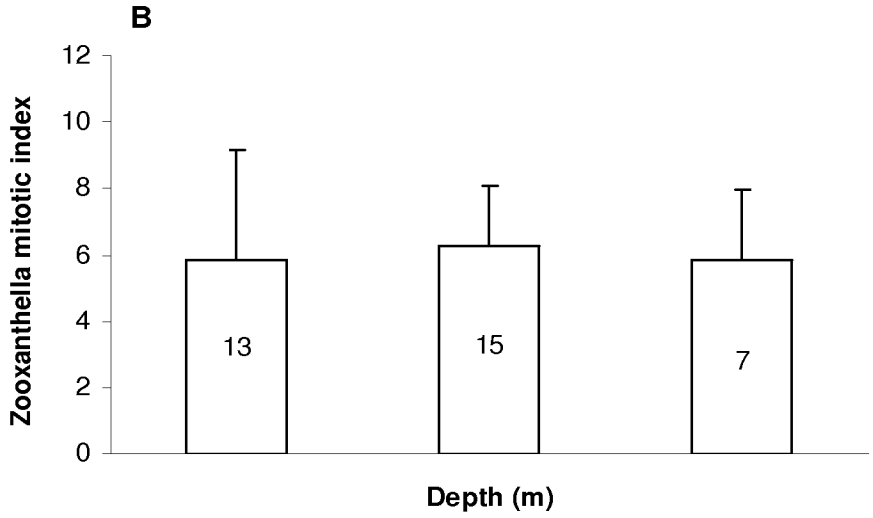

Fig. 4.2B

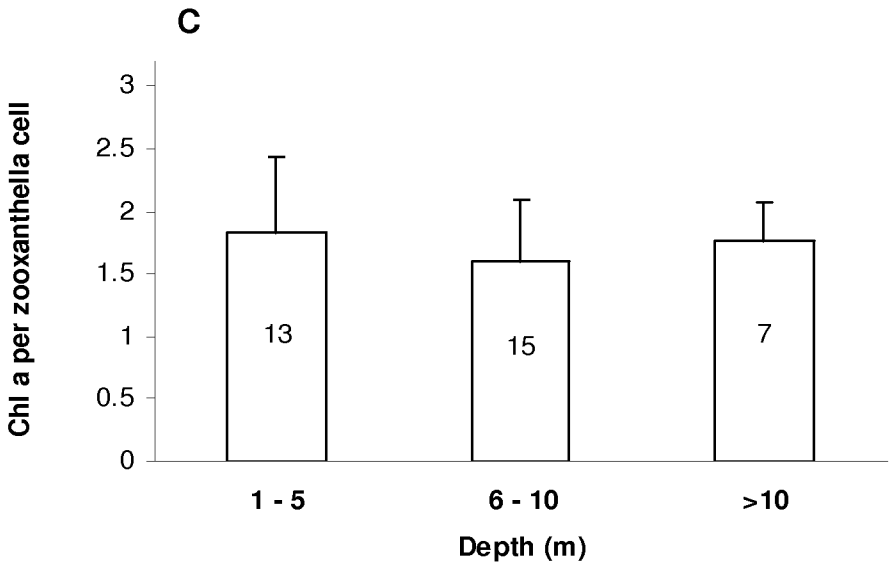

Fig. 4.2C

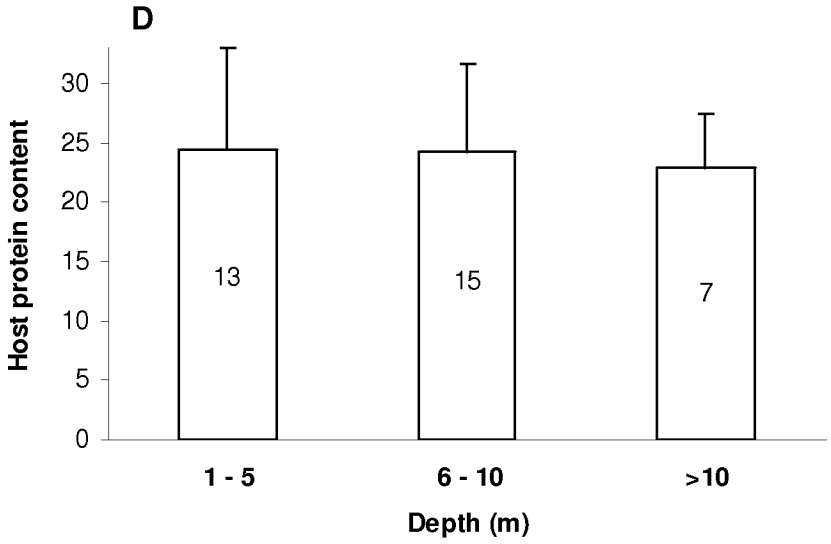

Fig. 4.2D

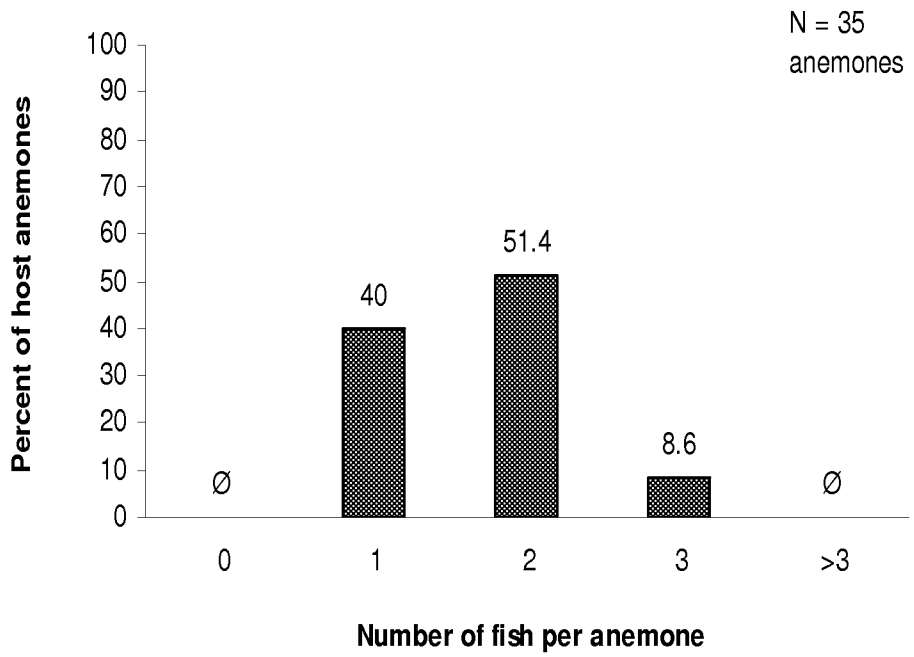
N = 35
anemones
Percent of host anemones
100
90
80
70
60
50
40
30
20
10
0
Ø
40
51.4
8.6
Ø
0
1
2
3
>3
Number of fish per anemone

Fig. 4.3

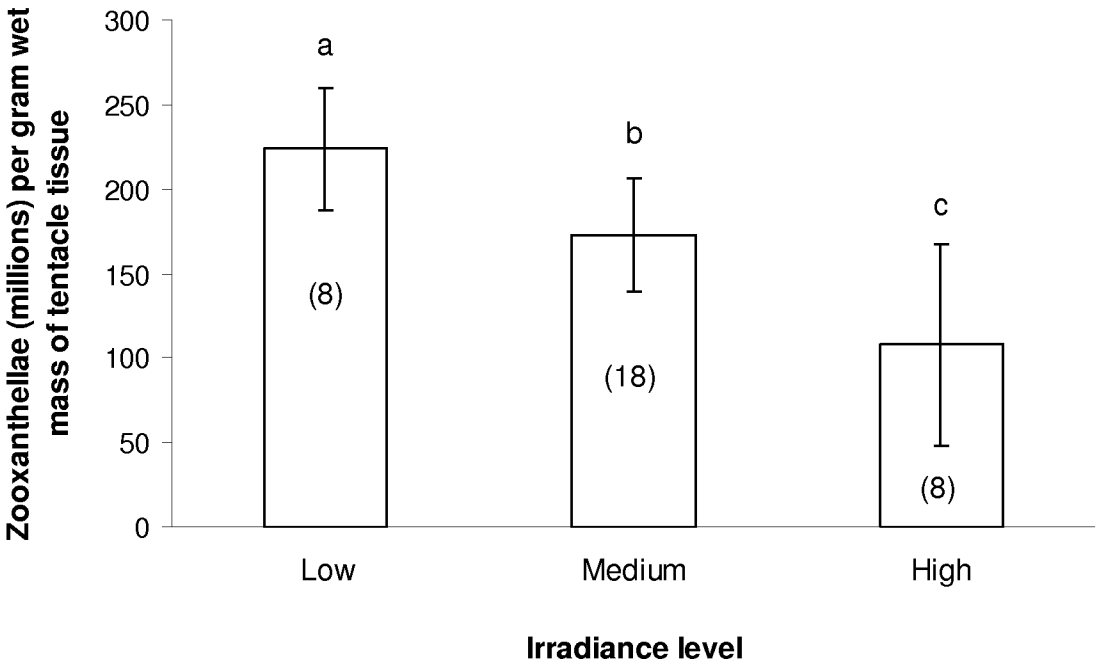
Zooxanthellae (millions) per gram wet mass of tentacle tissue
300
250
200
150
100
50
0
a
b
c
(8)
(18)
(8)
Low
Medium
High
Irradiance level

Fig. 4.4

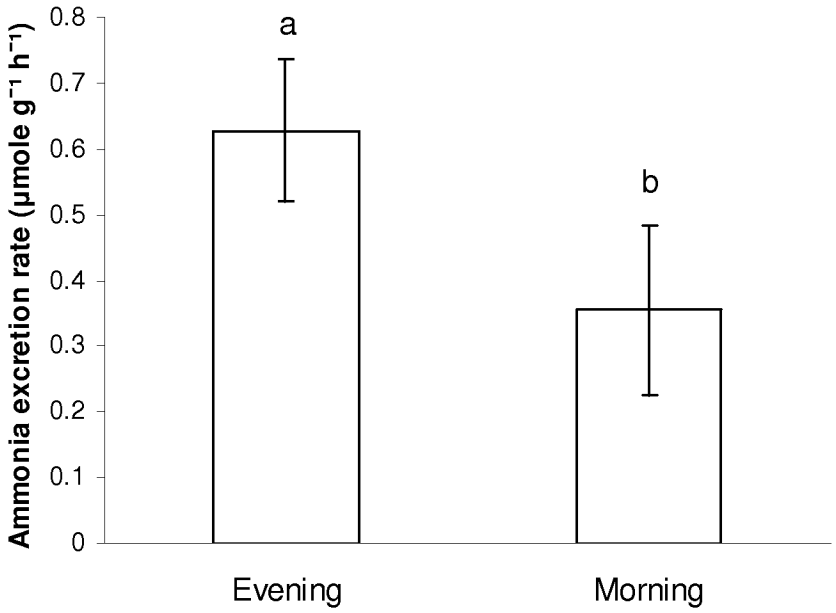
Ammonia excretion rate (µmole g⁻¹ h⁻¹)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
Evening
Morning

Fig. 4.5

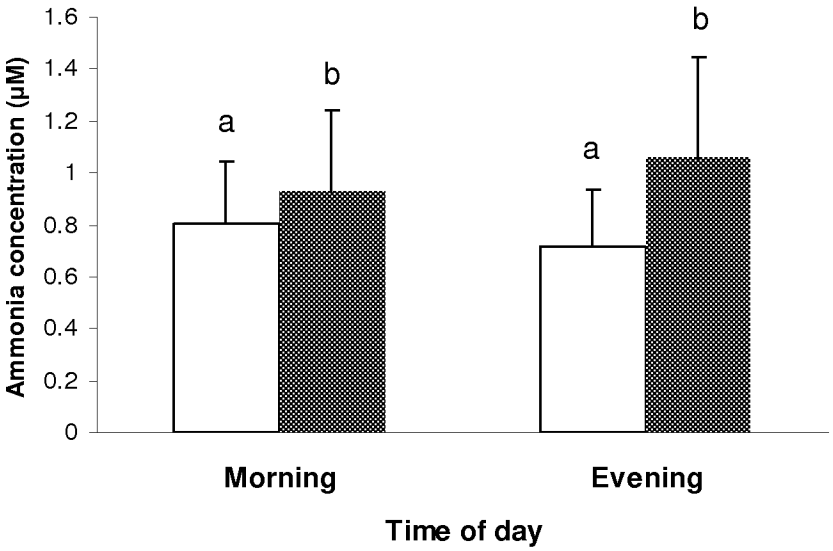
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ammonia concentration (µM)
a
b
a
b
Morning
Evening
Time of day

Fig. 4.6

CHAPTER V

PATTERNS OF ASSOCIATION BETWEEN THE BUBBLE-TIP ANEMONE (*Entacmaea quadricolor*) AND ENDOSYMBIOTIC DINOFLAGELLATES (*Symbiodinium* spp.) ACROSS A DEPTH GRADIENT IN THE NORTHERN RED SEA

Abstract

Most efforts to elucidate host-zooxanthella interactions have concentrated on corals, and as such the diversity of *Symbiodinium* within many other reef cnidarians remains unknown. Some cnidarian species vary with depth the algal types they host and thus potentially enhance their ability to respond to environmental disturbances by altering their symbionts. At Eilat in the northern Red Sea, I examined the bathymetric diversity of zooxanthella populations in the giant sea anemone *Entacmaea quadricolor*. This sea anemone is an important reef component due to its symbiosis with obligate anemonefishes and anemoneshrimps that contribute to interactions among several trophic levels. Based on 18S small subunit ribosomal DNA, the zooxanthellae within tentacle tissues of *E. quadricolor* showed two distinct RFLP patterns, one corresponding to clade C and the other an undescribed novel pattern. Both patterns were present in sea anemones

from all depths examined (1-23m). Further analysis of the variable ITS region and the chloroplast large subunit (23S) molecule revealed that the novel RFLP pattern represents a closely-related clade C *Symbiodinium*. These zooxanthellae likely are similar to those of clade C in their physiological responses to environmental conditions. It is not clear why this pattern has not been reported for the many other cnidarian species previously surveyed at the same sites. It is possible that this novel RFLP pattern may be restricted to zooxanthellae symbiotic with sea anemones in general, or even specific to *E. quadricolor*. It is concluded that at these study sites in the Red Sea, the giant sea anemone *E. quadricolor* exhibits a stable and highly specific interaction with clade C *Symbiodinium*, some of which have a unique RFLP pattern unreported in the literature. The lack of flexibility in this interaction may contribute to potential susceptibility of this important sea anemone to the impacts of global climate change.

Introduction

Reef cnidarians host diverse dinoflagellate symbionts in the genus *Symbiodinium* Freudenthal (Taylor 1974). The association between host cnidarians and these dinoflagellates, commonly known as zooxanthellae, persists in part due to efficient nutrient cycling. This partnership reduces the overall loss of key nutrients from the symbiotic system (Muscatine and Porter 1977; Falkowski et al. 1993) and allows cnidarians to thrive in low nutrient waters (Crossland 1983). Coral reef giant sea anemones that host anemonefish also form symbiotic associations with *Symbiodinium* sp. (Dunn 1981; Fautin 1991). The zooxanthellae supply energy-rich photosynthate

compounds that are utilized by the sea anemone hosts for respiration, growth and reproduction (Shick and Dykens 1984; Steen 1988; Achituv and Dubinsky 1990; Davies 1997; Cook et al. 1998; Whitehead and Douglas 2003). Investigations of *Symbiodinium* diversity and specificity have focused mainly on scleractinian corals, the dominant reef-building taxa (Rowan et al. 1997; Van-Oppen et al. 2001; LaJeunesse 2002). Additional examination of zooxanthellate diversity from other reef inhabitants such as coral reef giant sea anemones will broaden our perspective and may contribute to a more general understanding of the mechanisms of specificity involved in these important host-symbiont associations (reviewed in Muller-Parker and Davy 2001).

Members of *Symbiodinium* belong to one of eight distinct clades (A through H; reviewed in Pochon et al. 2006) based on sequence diversity in their small subunit (SSU) or large subunit (LSU) ribosomal DNA, internal transcribed spacers (ITS1 and ITS2), 5.8S regions, and/or large subunit chloroplast genes (reviewed in Baker 2003; Coffroth and Santos 2005; Stat et al. 2006). Within some clades, numerous taxonomically-specific "types", based on analysis of ITS regions, have been documented (LaJeunesse 2001; van Oppen 2001). Various studies have revealed that most species of scleractinian corals examined (77%), host a single symbiont type throughout their geographic and depth range, while other coral species (23%) have more flexible zooxanthellae-host partnerships (Goulet 2006, 2007). In this context, the occurrence of certain *Symbiodinium* types sometime correlates with the geographic distributions of host cnidarians, or their local light or temperature environments (Rowan and Knowlton 1995; Rowan et al. 1997; Toller et al. 2001; LaJeunesse 2002; Ulstrup and van Oppen 2003; LaJeunesse et al. 2003, 2004 a,b, 2005; Baker et al. 2004; Fabricius et al. 2004; Rowan 2004; Thornhill et

al. 2006). Effects of the external physical environment on certain partner combinations could cause the depth zonation patterns observed for some zooxanthellae types (Rowan and Knowlton 1995; Kuguru et al. 2007). Thus, under different environmental conditions, some symbionts may be able to provide greater physiological benefits to the host than others. Depth zonation also may provide an alterative ecological mechanism to photoadaptation in some species (Falkowski and Dubinsky 1981; Dustan 1982; Iglesias-Prieto and Trench 1994).

On coral reefs in the Red Sea, individuals of the sea anemone *Entacmaea quadricolor* are common across wide depth range, and form a three-way symbiosis with zooxanthellae and obligate ectosymbiotic anemonefish (*Amphiprion bicinctus*) (Chadwick and Arvedlund 2005). Environmental conditions such as food availability and irradiance vary among depths and could potentially result in associations with several different *Symbiodinium* types, as has been observed in other cnidarians (e.g., Rowan et al. 1997; LaJeunesse et al. 2002; Iglesias-Prieto et al. 2004; Thornhill et al. 2006; Kuguru et al. 2007). Here, I describe the bathymetric distribution of *Symbiodinium* populations within the sea anemone *Entacmaea quadricolor* in the northern Red Sea. *Symbiodinium* diversity was investigated using three types of molecular markers: 18S small subunit ribosomal DNA, internal transcribed spacer (ITS), and chloroplast large subunit (cp23S)-rDNA. This work represents the first description of *Symbiodinium* diversity associated with giant sea anemones in the Red Sea, and reveals a novel RFLP pattern.

Methods

Study site and collection of samples

Field samples were collected during May to July 2006 on coral reefs adjacent to the Interuniversity Institute for Marine Science (IUI) near Eilat, Israel (Fig. 5.1) at the northern tip of the Gulf of Aqaba, Red Sea (29°30N, 34°55E). Individuals (N=32) of the giant sea anemone *Entacmaea quadricolor* were selected haphazardly and tagged for future reference, then several tentacle tips (2-3 cm) were removed from each anemone for molecular analysis. Small and unequal numbers of anemones were sampled at each depth, due to the limited numbers of individuals available, and restrictions on my collecting permit in the protected nature reserve that extends along the entire Red Sea cost of Israel.

Determination of Symbiodinium diversity

Tissue samples were preserved in 100% acetone (Fukatsu 1999) immediately following collection. Total nucleic acids from the preserved tissue were extracted and quantified on 0.7% tris-acetate (TAE) agarose gels according to the methods of Coffroth et al. (1992).

Symbiodinium 18S-rDNA was amplified by PCR using the dinoflagellate-biased primer set ss5 (5' GGTTGATCCTGCCAGTAGTCATATGCTTG-3') and ss3z (5'-AGC ACTGCGTCAGTCCGAATAATTCACCGG-3'), as described in Rowan and Powers (1991b). PCR products were digested with the restriction enzyme *TaqI* to generate restriction fragment length polymorphisms (RFLPs) (Rowan and Powers 1991b). RFLP

analysis of 18S-rDNA PCR products separates *Symbiodinium* into several large clades [i.e., *Symbiodinium* A, B, C (Rowan and Powers 1991a, b), D (Carlos et al. 1999), and E (*Symbiodinium californium*; LaJeunesse and Trench 2000; LaJeunesse 2001], and allows simple and rapid symbiont clade identification. Digested products were separated on 2% sodium-borate (SB) agarose gels and visualized with ethidium bromide under UV light. Cladal identity was established by comparing the RFLPs obtained from the anemones to known standards or to the literature to assign each sample to one of these established *Symbiodinium* 18S-rDNA RFLP clades.

Cladal identity of a novel 18S-rDNA RFLP pattern

To determine the cladal identity of a novel 18S-rDNA RFLP *Symbiodinium* pattern that was observed in some of the samples, Domain V of the chloroplast large subunit cp23S-rDNA, and the entire internal transcribed spacer (ITS)-rDNA region, were amplified and sequenced directly from PCR products. An approximately 0.7-kb conserved region of cp23S-rDNA, corresponding to domain V (Harris et al. 1994), was PCR amplified from samples that exhibited the clade "C" and the novel RFLP patterns at my deep (20-23m) and shallow (1-5m) sites. The primer pair 23S1 (5'-GGCTGTAA CTATAACGGTCC-3') and 23S2 (5'-CCATCGTATTGAACCCAGC-3') (Zhang et al. 2000) were used following the procedures outlined in Santos et al. (2002a). Likewise, the entire ribosomal ITS-rDNA region was PCR amplified from the same four samples as those above using the primers ZITSUP (5'-CCGGTGAATTATTCGGACTG ACGCAGTGCT-3') and ZITSDN2 (5'-CTGTTTAG TTCCTTTTCCTCCGC-3')

following the procedures outlined in Santos et al. (2001). Amplified products were purified, sequenced and aligned as described below.

Amplified products were purified with Montage™ PCR Filter Units (Millipore) according to the manufacturer's recommendations, cycle-sequenced in both directions using BigDye Terminators and read on a PRISM 3100 Genetic Analyzer (Applied Biosystems). Ambiguities in the chromatograms were corrected by comparison to the complement DNA strand in Sequencher version 4.6 (Gene Codes Corporation). Corrected sequences were aligned manually against each other and against other available zooxanthellae sequences using Se-Al version 2.0a11 (available at http://evolve.zoo.ox.ac.uk/). Comparisons among sequences were made by visual inspection.

For (ITS)-rDNA sequences that presented a high level of "noise" and as a result reduced reliability, the internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse 2001, 2002). This region was amplified from the DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" (5'-CGCCCGCCGCGCCCCGCG CCCGTCCCGCCGCCCCCGCCCGGGATCCATATGCTTAAGTTCAGCGGGT-3') and "ITSintfor 2" (5'GAATTGCAGAACTCCGTG-3'). PCR amplification followed the "touchdown" thermal cycle protocol of LaJeunesse (2002). Products of these PCR reactions were checked by electrophoresis on 2% sodium-borate (SB) agarose gels and visualized with ethidium bromide under UV light. Then they were electrophoresed for 1500 volt-hours at 60°C on 8% acrylamide denaturing gradient gels (45-80%) using a CBScientific system (Del Mar, CA). The diagnostic bands in each profile were excised, reamplified, and directly sequenced using Big-Dye Terminator 3.1 reagents and read on a

PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the protocol of LaJeunesse (2002).

Results

Two distinct profiles were revealed by 18S-rDNA RFLP analysis of zooxanthellae in the sea anemone *Entacmaea quadricolor* in the Red Sea. One group of zooxanthellae exhibited the pattern of *Symbiodinium* clade C, as described by Rowan and Powers (1991a,b). The second group exhibited a novel RFLP profile with 3 fragments at approximately 800-, 620-, and 260-bp, respectively (Fig. 5.2a, lanes 1,2 and 5). To the best of my knowledge, this pattern has not been reported previously in the literature. At all three sample depths, the majority of host anemones contained zooxanthellae with this novel RFLP profile (Fig. 5.3), but the proportion of anemones with each pattern did not vary significantly among depths (chi-square, $\chi^2(2, N=32) = 1.335$, $p > 0.5$). Each sampled anemone contained zooxanthellae of only one of the two *Symbiodinium* genotypes. PCR amplification of the entire (ITS)-rDNA region and cp23S-rDNA domain V from representative sea anemones exhibiting each of the two RFLP patterns resulted in DNA fragments of approximately 800bp and 600bp respectively (on 2% agarose gels), regardless of collection depth.

DNA sequences generated from (ITS)-rDNA fragments contained a high level of sequence ambiguity. However, in a BLAST search, all sequences aligned very closely (99%) with (ITS)-rDNA sequences of clade C *Symbiodinium* from giant clams (accession #AF195144). All DNA sequences generated from the cp23S-rDNA were identical and

were identified as clade C *Symbiodinium* (100% match to "UnAneinhospitePCR", accession #AF427470, when aligned against known *Symbiodinium* sequences, available at: http://www. auburn.edu/ %7Esant osr/sequencedatasets). Previous phylogenetic analyses characterized this exact sequence as *Symbiodinium* clade C (refer to the individual named "unknown anemone [Ua#31, Japan]" on the phylogenetic tree in Santos et al. 2002a). A BLAST search of all cp23S-rDNA sequences revealed a high degree of similarity (99%) with *Symbiodinium* sp. type C1 (accession #EF140804). PCR-DGGE fingerprinting of representative *Symbiodinium* for each one of the two distinct RFLP profiles from the shallow and deep sites (N=4) yielded 2 distinct profiles both exhibiting a characteristic dominant DGGE band, representing type C1 *Symbiodinium* (Fig. 5.4; LaJeunesse 2001, 2002).

Discussion

It is shown here that clades of zooxanthellae associated with the giant sea anemone *Entacmaea quadricolor* do not appear to vary over a wide depth range on coral reefs in the northern Red Sea. My findings also indicate that this association may be restricted to two closely related *Symbiodinium* populations that cluster within clade C, possibly reflecting a specific and stable interaction between this sea anemone and this zooxanthella clade.

Although the cladal identities of zooxanthellae in most sea anemone species remain unknown, in the few that have been examined the level of host-algal specificity varies widely among anemone species. For example, *Aiptasia* sp. anemones were found

to be considerably more specific to their original clade B symbiont than to a variety of other *Symbiodinium* isolates (Schoenberg and Trench 1980; Belda-Baillie et al. 2002). *Heteractis* sp. anemones also were able to establish successful association with only clade C *Symbiodinium* (Rodriguez-Lanetty et al. 2003). In contrast, other anemone hosts such as *Cereus pedunculatus*, *Anthopleura elegantissima*, and *Aiptasia pallida* were able to establish symbiotic associations with more than one zooxanthella clade, and thus demonstrated a flexibility that may increase their chances of survival should they lose all of their zooxanthellae in a bleaching event (Davy et al. 1997; LaJeunesse and Trench 2000; Goulet et al. 2005).

A recent study also conducted at Eilat showed that zooxanthellae clades associated with the corallimorpharian *Rhodactis rhodostoma* changed from clade C in shallow water to clade D at depth (Kuguru et al. 2007). Giant sea anemone *Entacmaea quadricolor* populations from the Ryukyu Islands, Japan, also have been shown to possess zooxanthellae of both clade C and clade D (Santos et al. 2003). My results reveal that at my sites in the Red Sea, *E. quadricolor* does not harbor clade D zooxanthellae at any depth, and exhibits high specificity at all depths to only closely-related clade C *Symbiodinium* populations, one of which has a previously-unreported novel RFLP pattern.

Analysis of the internal transcribed spacer (ITS) region, consisting of ITS1, 5.8S, and ITS2, typically provides phylogenetic resolution at or below the species level (Gonzalez et al. 1990; Lee and Taylor 1992; Goff et al. 1994; Coleman et al. 1994; Baldwin et al. 1995; Hunter et al. 1997; LaJeunesse 2001; van Oppen et al. 2001). Potentially conflicting results were obtained regarding the zooxanthellae associated with

E. quadricolor in the Red Sea, in which 18S-rDNA RFLP analysis produced two distinct profiles, but sequence analysis of cp23S-rDNA consistently aligned with clade C1 *Symbiodinium* sp. (99%) for all samples, suggesting only one type of zooxanthella population. This conflict was partly resolved by PCR-DGGE fingerprinting of the ITS 2 region (LaJeunesse 2001), in which samples produced two profiles but in both the dominant band was distinctive of a symbiont type C1 fingerprint profile (Fig. 5.4). Thus, the analysis of two molecules with high phylogenetic resolution indicated that the two distinct RFLP profiles produced by zooxanthellae in this sea anemone likely represent closely-related type C1 zooxanthellae populations. The presence of *Symbiodinium* with this previously-unreported novel RFLP pattern at all sampled depths is intriguing, and since it does not correlate with the variation observed in DGGE profiles it may be explained by mutation in the relatively conserved 18S-rDNA gene. Since there is no strong correlation with depth, and the proportion of anemones with each pattern did not significantly vary among depths, this mutation likely does not affect the functionality of these *Symbiodinium* populations, but this inference should be verified with larger sample sizes. It remains unclear why this novel RFLP profile occurs at all sampled depths in this sea anemone, and why it has not been reported for other cnidarian host species in the Red Sea.

Members of clade C *Symbiodinium* are the most abundant and ecologically important group of zooxanthellae in the tropical Indo-Pacific region (Baker 1999, Rodriguez-Lanetty et al. 2002, Savage et al. 2002; Baker 2003). Thus, it is not surprising that I observed zooxanthellae of this clade to be dominant in sampled sea anemones in the northern Red Sea. In addition, clade C zooxanthellae are known to be particularly

bleaching-susceptible (Glynn et al. 2001), and so are expected to be common in this region where no serious bleaching events have occurred. The lack of flexibility in host-algal associations observed here for *E. quadricolor* may increase the susceptibility of this host cnidarian to the effects of future climate change.

Although extensive recent research has occurred on of zooxanthellae-cnidarian associations, it is not clear yet if specificity of these interactions is a feature dictated by the host, the symbiont, the environmental conditions or a summation of all these factors. To determine whether the unique clade C1 zooxanthella populations discovered here exhibit high partner specificity to sea anemones, and the extent to which they occur in other geographical regions and hosts, additional sampling will be required.

Acknowledgements

I thank Baraka Kuguru of the Interuniversity Institute for Marine Science for assistance with field sampling during this project. I also thank Mark Liles for his help with the DGGE analysis. This work was supported by start-up funds to NEC and a graduate research award to MR, both from Auburn University. This research is submitted in partial fulfillment of the requirements for an M.Sc. degree by MR at Auburn University. All sampling for this study complies with the current laws of Israel.

Figure Legends

Fig. 5.1. Map of northern Gulf of Eilat (Aqaba), Red Sea. The asterisks denote the Interuniversity Institute for Marine Science (IUI) and sampling sites.

Fig. 5.2. Variation in zooxanthella clades among host individuals of the giant sea anemone *Entacmaea quadricolor* at Eilat, northern Red Sea. Representative individuals are shown from (a) shallow (lanes 1–5), (b) intermediate (lanes 1–6), and (c) deep (lanes 1–6) sites on the coral reef. *Symbiodinium* cladal identity was determined by digesting PCR-generated algal small subunit ribosomal DNA with the restriction enzyme *TaqI* to generate restriction fragment length polymorphism patterns. L = DNA 100bp size ladder, A - E = *Symbiodinium* clade standards.

Fig. 5.3. Variation in RFLP patterns of zooxanthellae associated with the giant sea anemone *Entacmaea quadricolor* among depths at Eilat, northern Red Sea. The proportion of anemones with each zooxanthellae genotype does not vary significantly among depths (chi-square, $\chi^2(2, N = 32) = 1.335,\ p > .05$). Numbers in parentheses represent the number of sea anemones sampled per depth.

Fig. 5.4. PCR-DGGE profiles of zooxanthellae from the giant sea anemone *Entacmaea quadricolor.* Representative individuals are shown from 2 locations: The northern Red Sea (clade C RFLP pattern 55, 4 and novel RFLP pattern 27). And cultured anemones originally from Palau in the western Pacific Ocean (clade C RFLP pattern 53, U1). The

anemones from Palau were collected in the 1980's, cultured at Waikiki Aquarium, and then transported to Auburn University in 2006. MKR = cloned standard exhibiting the C1 type pattern. The dominant band distinctive to C1 symbiont type is indicated by an arrow; *capital letter* indicates lineage (clade), the *number* represents ITS type.

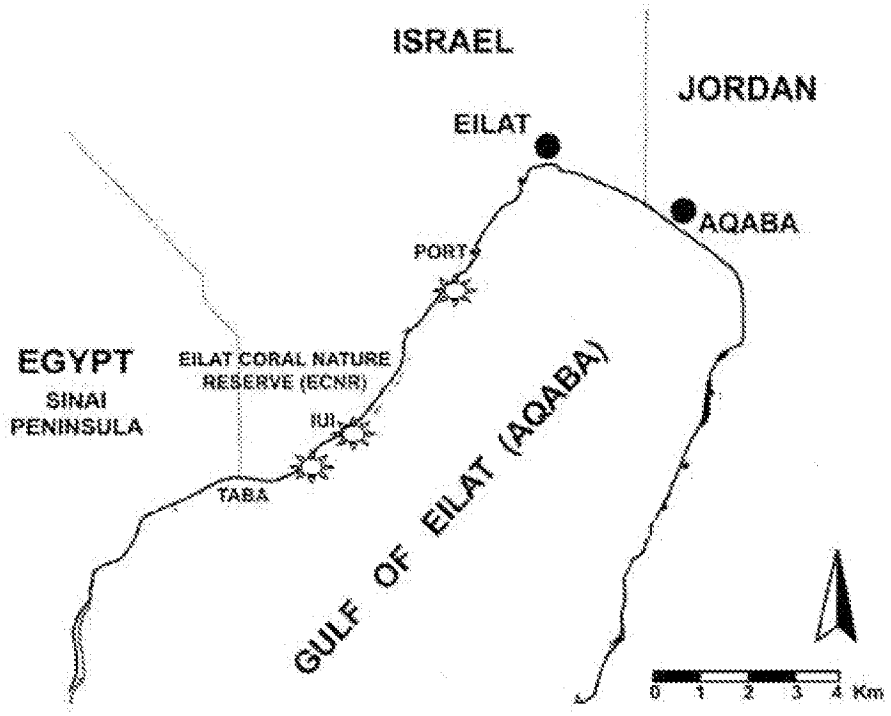
ISRAEL
JORDAN
EILAT
AQABA
PORT
EGYPT
SINAI
PENINSULA
EILAT CORAL NATURE
RESERVE (ECNR)
IUI
TABA
GULF OF EILAT (AQABA)
0 1 2 3 4 Km

Fig 5.1

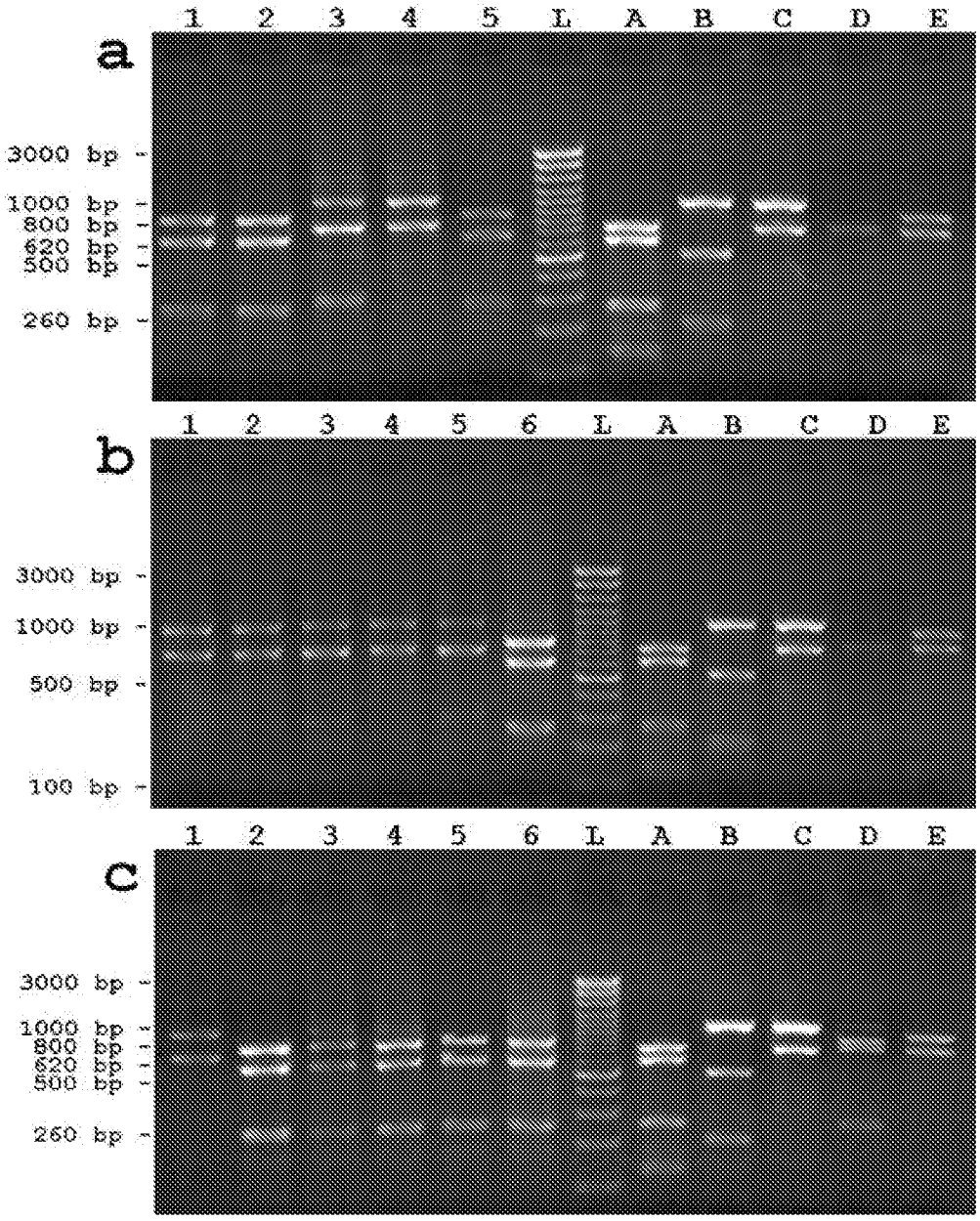
a
1 2 3 4 5 L A B C D E
3000 bp
1000 bp
800 bp
620 bp
500 bp
260 bp
b
1 2 3 4 5 6 L A B C D E
3000 bp
1000 bp
500 bp
100 bp
c
1 2 3 4 5 6 L A B C D E
3000 bp
1000 bp
800 bp
620 bp
500 bp
260 bp

Fig. 5.2

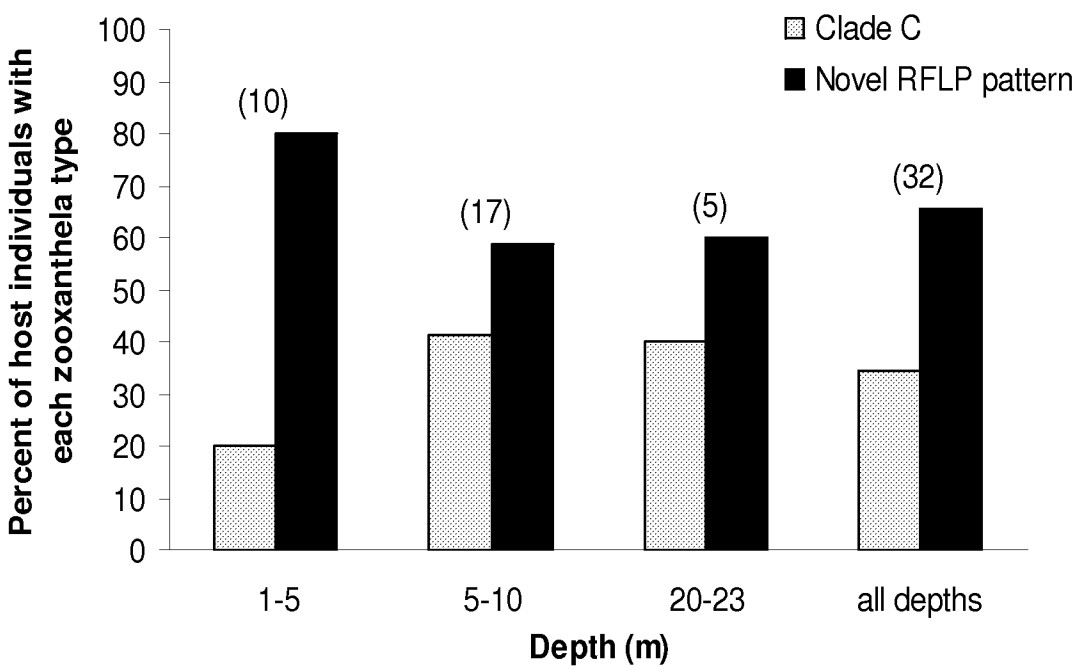
Percent of host individuals with each zooxanthela type
100
90
80
70
60
50
40
30
20
10
0
(10)
(17)
(5)
(32)
Clade C
Novel RFLP pattern
1-5
5-10
20-23
all depths
Depth (m)

Fig. 5.3

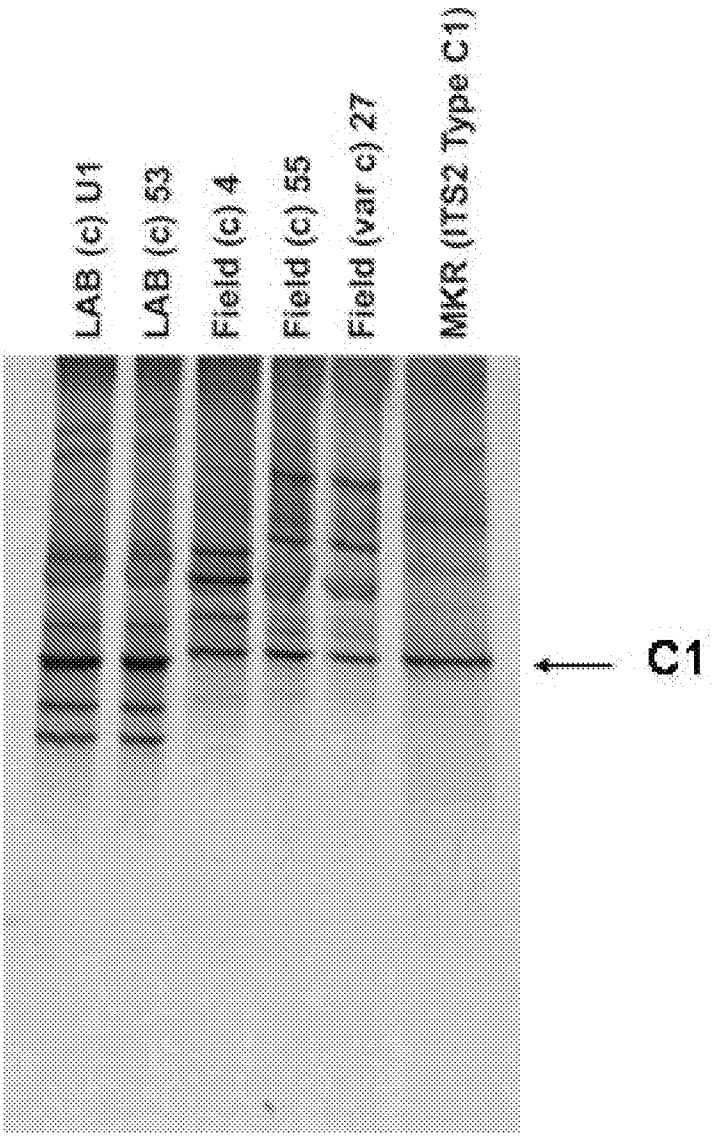
LAB (c) U1
LAB (c) 53
Field (c) 4
Field (c) 55
Field (var c) 27
MKR (ITS2 Type C1)
C1

Fig. 5.4

CUMULATIVE BIBLIOGRAPHY

Achituv Y, Dubinsky Z (1990) Carbon budgets in marine, mutualistic association between microalgae and cnidarians. Comp Physiol 5:36-48.

Achituv Y, Mizrahi L (1996) Recycling of ammonium within a hydrocoral (*Millepora dichotoma*)-Zooxanthellae cirripede (*Savignium milleporum*) symbiotic association. Bull Mar Sci 58:856-860.

Allen GR (1972) The anemonefishes: their Classification and Biology. T F H Publications, Inc, Neptune City, New Jersey, 288 pages.

Allen GR (1991) Damselfishes of the World. Mergus Publishers, Melle, Germany 271 pp.

Baker AC (1999) The symbiosis ecology of reef building corals. PhD dissertation, Univ Miami 120 pp.

Baker AC (2003) Flexibility and specificity in coral–algal symbioses: diversity, ecology, and biogeography of *Symbiodinium*. Annual Rev of Ecol and System 34:661-689.

Baker AC, Starger CJ, McClanahan TR, Glynn PW (2004) Corals' adaptive response to climate change. Nature 430:741.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Miss Bot Gard 82:247-277.

Beaver R (1996) Regulation of the population of symbionts in *Anemonia viridis*. Ph.D. dissertation, University of Glasgow, Glasgow, 168 pp.

Becker J, Grutter A (2004) Cleaner shrimp do clean. Coral Reefs 23:515-520.

Belda-Baillie CA, Baillie BK, Maruyama T (2002) Specificity of a model cnidarian–dinoflagellate symbiosis. Biol Bull 202:74-85.

Bhagooli R, Hidaka M (2003) Comparison of stress susceptibility of in hospite and isolated zooxanthellae among five coral species. J Exp Mar Biol Ecol 291:181-197.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bray RN, Purcell LJ, Miller AC (1986) Ammonium excretion in a temperate-reef community by a planktivorous fish, *Chromis punctipinnis* (Pomacentridae), and potential uptake by young giant kelp, *Macrocystis pyrifera* (Laminariales). Mar Biol 90:327-334.

Brett JR, Zala CA (1975) Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled condition. J Fish Res Bd Canada 32:2479-2486.

Bronstein JL (1994) Our current understanding of mutualism. Q Rev Biol 69:31-51.

Bruno JF, Stachowicz JJ, Bertness MD (2003) Inclusion of facilitation into ecological theory. Trends Ecol Evol 18:119-125.

Burris RH (1983) Uptake and assimilation of NH_4^+ by a variety of corals. Mar Biol 75:151-155.

Carlos AA, Baillie BK, Kawachi M, Maruyama T (1999) Phylogenetic position of *Symbiodinium* (Dinophyceae) isolates from Tridacnids (Bivalvia), Cardiids (Bivalvia), a sponge (Porifera), a soft coral (Anthozoa), and a free-living strain. J Phycol 35:1054-1062.

Chadwick NE, Arvedlund M (2005) Abundance of giant sea anemones and patterns of association with anemonefish in the northen Red Sea. J Mar Biol Assoc UK 85:1287-1292.

Chang SS, Prezelin BB, Trench RK (1983) Mechanisms of photoadaptation in three strains of the symbiotic dinoflagellate *Symbiodinium microadriaticum*. Mar Biol 76:219-229.

Cikala M, Wilm B, Hobmayer E, Bottger A, David CN (1999) Identification of caspases and apoptosis in the simple metazoan Hydra. Curr Biol 9:959-962.

Clayton WS Jr, Lasker HR (1984) Host feeding regime and zooxanthellal photosynthesis in the anemone, *Aiptasia pallida* (Vcrrill). Biol Bull mar biol Lab Woods Hole 167:590-600.

Cleveland A, Verde EA, Lee R (2003) Investigating the physiological basis for the clownfish/host anemone symbiosis: Do resident fish provide their host with nitrogen? Abstract, 4th Internat Symbiosis Soc Conf Halifax, Nova Scotia, Canada.

Cleveland A, Verde EA, Lee R (2006) Nutritional exchange in clownfish/host anemone symbiosis: Stable isotope analysis demonstrates nutrient exchange is bi-directional. Abstract, 5th Internat Symbiosis Congr Vienna, Austria.

Coffroth MA, Lasker HR, Diamond ME, Bruenn JA, Bermingham E (1992) DNA fingerprints of a gorgonian coral: a method for detecting clonal structure in a vegetative species. Mar Biol 114:317-325.

Coffroth MA, Santos SR (2005) Genetic diversity of symbiotic dinoflagellates in the genus *Symbiodinium*. Protist 156:19-34.

Coleman AW, Suarez A, Goff LJ (1994) Molecular delineation of species and syngens in volvocacean green algae (Chlorophyta). J Phycol 30:80-90.

Cook CB (1985) Equilibrium populations and long-term stability of mutualistic algae and invertebrate hosts. In: Boucher D (ed) The ecology of mutualism: ecology and evolution Croon-Helm, London, pp.171-191.

Cook CB, D'Elia CF (1987) Are natural populations of zooxanthellae ever nutrient-limited? Symbiosis 4:199-212.

Cook CB, D'Elia CF, Muller-Parker C (1988) Host feeding and nutrient sufficiency for zooxanthellae in the sea anemone *Aiptasia pallida.* Mar Biol 98:253-262.

Cook CB, Muller-Parker G, D'Elia CF (1992) Ammonium enhancement of dark carbon fixation and nitrogen limitation in symbiotic zooxanthellae: effects of feeding and starvation of the sea anemone *Aiptasia pallida*. Limnol Oceanogr 37:131-139.

Cook CB, Muller-Parker G, Orlandini CD (1994) Ammonium enhancement of dark carbon fixation and nitrogen limitation in zooxanthellae symbiotic with the reef corals *Madracis mirabilis* and *Montastrea annularis*. Mar Biol 118:157-165.

Crossland CJ (1983) Dissolved nutrients in coral reef waters. In: Bames DJ (ed) Perspectives on coral reefs Publ 200 Aust Inst Mar Sci, Townsville, pp.56-68.

Davies PS (1997) Anthozoan endosymbiosis. Proc 6th Int Conf Coelent Biol, Leiden, pp.123-134.

Davy SK, Lucas IAN, Turner JR (1997) Uptake and persistence of homologous and heterologous zooxanthellae in the temperate sea anemone *Cereus pedunculatus* (Pennant). Biol Bull 192:208-216.

D'Elia CF, Cook CB (1988) Methylamine uptake by zooxanthellae-invertebrate symbioses: Insights into host ammonium environment and nutrition. Limnol Oceanogr 33:1153-1165.

D'Elia CF, Domotor SL, Webb KL (1983) Nutrient uptake kinetics of freshly isolated zooxanthellae. Mar Biol 75:157-167.

D'Elia CF, Webb KL (1977) The dissolved nitrogen flux of reef corals. Proc 3rd Int Coral Reef Syrnp Miami 1:326-330.

Dennison WC, Barnes DJ (1988) Effect of water motion on coral photosynthesis and calcification. J Exp Mar Biol Ecol 115:67-77.

Domotor SL, D'Elia CF (1984) Nutrient uptake kinetics and growth of zooxanthellae maintained in laboratory culture. Mar Biol 80:93-101.

Douglas AE, Smith DC (1984) The green hydra symbiosis. VIII. Mechanisms in symbiont regulation. Proc R Soc Lond Series B Biol Sci 221:291-319.

Drew EA (1972) The biology and physiology of alga-invertebrate symbioses. ii The denslty of symbiotic algal cells in a number of hermatypic hard corals and alcyanarians from various depths. J Exp Mar Biol Ecol 9:71-75.

Dubinsky Z, Falkowski PG, Porter JW, Muscatine L (1984) Absorption and utilization of radiant energy by light- and shade-adapted colonies of the hermatypic coral *Stylophora pistillata*. Proc R Soc Lond Series B Biol Sci 222:203-214.

Dubinsky Z, Stambler N, Ben-Zion M, McCloskey LR, Muscatine L, Falkowski PG (1990) The effect of external nutrient resources on the optical properties and photosynthetic efficiency of *Stylophora pistillata*. Proc R Soc Lond Series B Biol Sci 239:231-246.

Dunn DF (1981) The clownfish sea anemones: Stichodactylidae (Coelenterata: Actinaria) and other sea anemones symbiotic with pomacentrid fishes. Trans Amer Phil Soc 71:1-115.

Durbin EG, Durbln AG (1981) Assimilation efficiency and nitrogen excrehon of a filter-feeding planktivore, the Atlantic menhaden, *Brevoortia tyrannus* (Pisces: Clupeidae). Fish Bull US 79:601-616.

Durbin AG, Nixon SW, Oviatt CA (1979) Effects of the spawning migration of the alewife, *Alosa pseudoharengus*, on freshwater ecosystems. Ecology 60:8-17.

Dustan P (1982) Depth dependent photoadaption by zooxanthellae of reef coral *Montastrea annularis*. Mar Biol 68:253-264.

Entsch BK, Boto G, Sim RG, Wellington TJ (1983) Phosphorus and nitrogen in coral reef sediments. Limnol Oceanogr 28:465-476.

Fabricius KE, Mieog JC, Colin PL, Idip D, van Oppen MJH (2004) Identity and diversity of coral endosymbionts (zooxanthellae) from three Palauan reefs with contrasting bleaching, temperature and shading histories. Mol Ecol 13:2445-2458.

Falkowski PG, Dubinsky Z (1981) Light-shade adaptation of *Stylophora pistillata*, a hermatypic coral from the Gulf of Eilat. Nature 289:172-175.

Falkowski PG, Dubinsky Z, Muscatine L, McCloskey L (1993) Population control in symbiotic corals: Ammonium ions and organic materials maintain the density of zooxanthellae. Bioscience 43:606-611.

Falkowski PG, Dubinsky Z, Muscatine L, Porter JW (1984) Light and the bioenergetics of a symbiotic coral. BioScience 34:705-709.

Falkowski PG, Jokiel PL, Kinzie RA (1990) Irradiance and corals. In Dubinsky Z (ed) Coral Reefs Ecosystems of the World Amsterdam: Elsevier Science Publishers, pp.89-107.

Fautin DG (1985) Competition by anemone fishes for host actinians. in: Gabrie C and Vivien MH (eds.) Proc of the Fifth Intern Coral Reef Congr, Tahiti Vol 5 Antenne Museum-Ephe, Moorea, French Polynesia, pp.373–377.

Fautin DG (1986) Why do anemonefishes inhabit only some host actinians? Environ Biol Fishes 15:171-180.

Fautin DG (1991) The anemonefish symbiosis: What is known and what is not. Symbiosis 10:23-46.

Fautin DG, Allen GR (1992) Field guide to anemonefishes and their host sea anemones. Perth: Western Australian Museum, p. 60.

Fautin DG, Allen GR (1997) Anemone Fishes and their Host Sea Anemones: A Guide for Aquarists and Divers. Revised edition Western Australian Museum:160 pp.

Fautin DG, Guo CC, Hwang JS (1995) Costs and benefits of the symbiosis between the anemoneshrimp *Periclimenes brevicarpalis* and its host *Entacmaea quadricolor*. Mar Ecol Prog Ser 129:77-84.

Fitt WK, Cook CB (2001) The effects of feeding or addition of dissolved inorganic nutrients in maintaining the symbiosis between dinoflagellates and tropical marine cnidarian. Mar Biol 139:507-517.

Fitt WK, Heslinga GA, Watson TC (1993a) Utilization of dissolved inorganic nutrients in growth and mariculture of the tridacnid clam *Tridacna derasa*. Aquaculture 109:27-38.

Fitt WK, Pardy RL (1981) Effects of starvation, and light and dark on the energy metabolism of symbiotic and aposymbiotic sea anemones, *Anthopleura elegantissima*. Mar Biol 61:199-205.

Fitt WK, Pardy RL, Littler MM (1982) Photosynthesis, respiration, and contribution to community productivity of the symbiotic sea anemone *Anthopleeura elegantissima*. J Exp Mar Biol Ecol 61:213-232.

Fitt WK, Trench RK (1983) Endocytosis of the symbiotic dinoflagellate *Symbiodinium microadriaticum* (Freudenthal) by endodermal cells of the scyphistomae of *Cassiopea xamancha* and resistance of the algae to host digestion. J Cell Sci 64:195-212.

Forster RP, Goldstein L (1969) Formation of excretory products. In: Hoar WS, Randall DJ (ed) Fish physiology, Vol 1 Excretion, ion regulation and metabolism New York: Academic Press, pp.313-350.

Freudenthal HD (1962) *Symbiodinium* gen. nov. and *Symbiodinium microadnaticurn* sp. nov., a zooxanthella: Taxonomy, life cycle and morphology. J Protozool 9:45-52.

Fricke HW (1974) Oko-ethologie des monogamen anemonefisches *Amphiprion bicinctus* (Freiwasseruntersuchung aus dem Roten Meer). Zeits Tierpsychol 36:492-512.

Fricke VHW (1975) Selective enemy recognition in the anemonefish *Amphiprion bicinctus*. J Exp Mar Biol Ecol 19:1-7.

Fricke HW (1979) Mating system, resource defense and sex change in the anemonefish *Amphiprion akallopisos*. Zeits Tierpsychol 50:313-326.

Fukatsu T (1999) Acetone preservation: a practical technique for molecular analysis. Mol Ecol 8:1935-1945.

Gattuso JP, Jaubert J (1990) Effect of light on oxygen and carbon dioxide fluxes and on metabolic quotients measured in situ in a zooxanthellate coral. Limnol Oceanogr 35:1796-1804.

Glaholt SPJr, Vanni MJ (2005) Ecological responses to simulated benthic-derived nutrient subsidies mediated by omnivorous fish. Freshw Biol 50:1864-1881.

Glynn PW, Mat´e JL, Baker AC, Calder´on MO (2001) Coral bleaching and mortality in Panam´a and Ecuador during the 1997–1998 El Ni˜no-Southern Oscillation event: spatial/temporal patterns and comparisons with the 1982–1983 event. Bull Mar Sci 69:79-109.

Godinot C, Chadwick NE (2007) Effects of phosphate excretion by anemonefish on host sea anemones. Manuscript in preparation, 19 pp.

Godwin J, Fautin DG (1992) Defense of host actinians by anemonefishes. Copeia 3:902-908.

Goff LJ, Moon DA, Coleman AW (1994) Molecular delineation of species and species relationships in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales). J Phycol 30:521-537.

Gonzalez IL, Sylvester JE, Smith TF, Stambolian D, Schmickel RD (1990) Ribisomal RNA gene sequences and hominoid phylogeny. Mol Biol Evol 7:203-219.

Gotto RV (1969) Marine animals, Partnerships and other Associations. Eng Univer Pres Lond., 96 pp.

Goulet TL (2006) Most corals may not change their symbionts. Mar Ecol Prog Ser 321:1-7.

Goulet TL (2007) Most scleractinian corals and octocorals host a single symbiotic zooxanthella clade. Mar Ecol Prog Ser 335:243-248.

Goulet TL, Cook CB, Goulet D (2005) Effects of short-term exposure to elevated temperatures and light levels on photosynthesis of different host-symbiont combinations in the *Aiptasia pallida/Symbiodinium* symbiosis. Limnol Oceanogr 50:1490-1498.

Grover R, Maguer JF, Reynaud-Vaganay S, Ferrier-Pagès C (2002) Uptake of ammonium by the scleractininan coral *Stylophora pistillata*: Effect of feeding, light, and ammonium concentrations. Limno Oceanogr 47:782-790.

Gunnersen J, Yellowlees D, Miller DJ (1988) The ammonium/methylammonium uptake system of *Symbiodinium microadriaticum*. Mar Biol 97:593-596.

Haines KC, Wheeler PA (1978) Ammonium and nitrate uptake by the marine macrophytes *Hypnea musciformis* (Rhodophyta) and *Macrocystis pyrifera* (Phaeophyta). J Phycol 14:319-324.

Harris EH, Boyton IE, Gillham NW (1994) Chloroplast ribosomes and protein synthesis. Microbiol Rev 58:700-754.

Hattori A (2006) Vertical and horizontal distribution patterns of the giant sea anemone *Heteractis crispa* with symbiotic anemonefish on a fringing coral reef. J Ethol 24:51-57.

Hirose Y (1985) Habitat, distribution and abundance of coral reef sea anemones (Actiniidae and Stichodactylidae) in Sesoko Island, Okinawa, with notes of expansion and contraction behavior. Galaxea 4:113-127.

Hoegh-Guldberg O (1994) Population dynamics of symbiotic zooxanthellae in the coral *Pocillopora damicornis* exposed to elevated ammonium [$(NH_4)_2SO_4$] concentrations. Pac Sci 48:263-272.

Hoegh-Guldberg O, Hinde R, Muscatine L (1986) Studies on a nudibranch that contains zooxanthellae, II. Contribution to animal respiration (CZAR) in *Pteracolidia ianthina* with high and low levels of zooxanthellae. Proc R Soc Lond Series B Biol Sci 228:511-521.

Hoegh-Guldberg O, McCloskey LR, Muscatine L (1987) Expulsion of zooxanthellae by symbiotic cnidarians from the Red Sea. Coral Reefs 5:201-204.

Hoegh-Guldberg O, Smith GJ (1989b) Influence of the population density of zooxanthellae and supply of ammonium ions on the biomass and metabolic characteristics of the reef corals *Seriatopora hystrix* and *Stylphora pistillata*. Mar Ecol Prog Ser 57:173-186.

Hoegh-Guldberg GO, Williamson J (1999) Availability of two forms of dissolved nitrogen to the coral *Pocillopora damicornis* and its symbiotic zooxanthellae. Mar Biol 133:561-570.

Hoeksema JD, Schwartz MW (2001) Modelling interspecific mutualisms as biological markets. In: Economics in Nature: Social Dilemma, Mate Choice and Biological Markets. Cambridge University Press, pp.173-183.

Holbrook SJ, Schmitt RJ (2002) Competition for shelter space causes density-dependent predation mortality in damselfishes. Ecology 83:2855-2868.

Holbrook SJ, Schmitt RJ (2004) Population dynamics of a damselfish: effects of a competitor that also is an indirect mutualist. Ecology 85:979-985.

Holbrook SJ, Schmitt RJ (2005) Growth, reproduction and survival of a tropical sea anemone (Actiniaria): benefits of hosting anemonefish. Coral Reefs 24:67-73.

Hunter CL, Morden CW, Smith CM (1997) The utility of ITS sequences in assessing relationships among zooxanthellae and corals. Proc 8th Int Coral Reef Symp 2:1599-1602.

Iglesias-Prieto R, Beltran VH, LaJeunesse TC, Reyes-Bonilla H, Thome PE (2004) Different algal symbionts explain the vertical distribution of dominant reef corals in the eastern Pacifc. Proc R Soc Lond B Biol Sci 271:1757-1763.

Iglesias-Prieto R, Trench RK (1994) Acclimation and adaptation to irradiance in symbiotic dinoflagellates. I. Responses of the photosynthetic unit to changes in photon flux density. Mar Ecol Prog Ser 113:163-175.

Jeffrey SW, Humphrey GF (1975) New spectrometric equation for determining chlorophyll a, b and c2 on higher plants, algae, and natural phytoplankton. Biochem Physiol Pflanz 167:191-194.

Jobling M (1981) The influence of feeding on the metabolic rates of fishes. A short review J Fish Biol 18:385-400.

Kawaguti S (1953) Ammonium metabolism of the reef corals. Biol J Okayama Univ 1:171-176.

Kevin KM, Hudson RCL (1979) The role of zooxanthellae in the hermatypic coral *Piesiastra urvillei* (Milne Edwards and Haime) from cold waters. J exp Mar Biol Ecol 36:157-170.

Kuguru B, Winters G, Beer S, Santos SR, Chadwick NE (2007) Adaptation strategies of the corallimorpharian Rhodactis rhodostoma to irradiance and temperature. Mar Biol 151:1287-1298.

LaJeunesse TC (2001) Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the its region: In search of a "species" level marker. J Phycol 35:866-880.

LaJeunesse TC (2002) Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. Mar Biol 141:387-400.

LaJeunesse TC, Bhagooli R (2004a) Closely related Symbiodinium spp. differ in relative dominance in coral reef host communities across environmental, latitudinal and biogeographic gradients. Mar Eco Prog Ser 284:147-161.

LaJeunesse TC, Lee S, Bush S, Bruno JF (2005) Persistence of non-Caribbean algal symbionts in Indo-Pacific mushroom corals released to Jamaica 35 years ago. Coral Reefs 24:157-159.

LaJeunesse TC, Loh WK, van Woesik R, Hoegh-Guldberg O, Schmidt GW, Fitt WK (2003) Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean. Limnol Oceanogr 48:2046-2054.

LaJeunesse TC, Thornhill DJ (2004b) High diversity and host specificity observed among symbiotic dinoflagellates in reef coral communities from Hawaii. Coral Reefs 23:596-603.

LaJeunesse TC, Trench RK (2000) Biogeography of two species of *Symbiodinium* (Freudenthal) inhabiting the intertidal sea anemone *Anthopleura elegantissima* (Brandt). Biol Bull 199:126-134.

Lee SB, Taylor JW (1992) Phylogeny of five fungus-like protoctistan Phytophthora species, inferred from the internal transcribed spacers of ribosomal DNA. Mol Biol Evol 9:636-653.

Liberman T, Genin A, Loya Y (1995) Effects on growth and reproduction of the coral *Stylophora pistillata* by the mutualistic damselfish *Dascyllus marginatus*. Mar Biol 121:741-746.

Loya Y, Sakai K, Yamazato K, Nakano Y, Sambali H, Van Woesik R (2001) Coral bleaching: the winners and the losers. Ecol Lett 4:122-131.

Marsical RN (1970a) A field and laboratory study of the symbiotic behavior of fishes and sea anemones from the tropical Indo-Pacific. U Calif Publ Zool 91:1-43.

Marsical RN (1970b) The nature of the symbiosis between Indo-Pacific anemone fishes and the sea anemones. Mar Biol 6:58-65.

Marubini F, Davies PS (1996) Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals. Mar Biol 127:319-328.

McAuley PJ, Cook CB (1994) Effects of host feeding and dissolved ammonium on cell division and nitrogen status of zooxanthellae in the hydroid *Myrionema boinense*. Mar Biol 121:343-348.

McCarthy JJ, Whitledge TE (1972) Nitrogen excretion by anchovy (*Engraulis mordax* and *E. ringens*) and jack mackerel (*Trachurus symmetricus*). Fish Bull US 70:395-401.

McCloskey LR, Cove TG, Verde EA (1996) Symbiont expulsion from the anemone *Anthopleura elegantissima* (Brandt)(Cnidaria; Anthozoa). J Exp Mar Biol Ecol 195:173-186.

McDuff RE, Chisholm SW (1982) The calculation of in situ growth rates of phytoplankton populations from fractions of cells undergoing mitosis: a clarification. Limnol Oceanogr 27:783-788.

Meroz A, Fishelson L (1997) Juvenile production of *Amphiprion bicinctus* (Pomacentridae, Teleostei) and rehabilitation of impoverished habitats. Mar Ecol Prog Ser 151:295-297.

Meyer JL, Schultz ET (1985a) Migrating haemulid fishes as a source of nutrients and organic matter on coral reefs. Limnol Oceanogr 30:146-156.

Meyer JL, Schultz ET (1985b) Tissue condition and growth rate of corals associated with schooling fish. Limnol Oceanogr 30:157-166.

Meyer JL, Schultz ET, Helfman GS (1983) Fish schools: an asset to corals. Science 220:1047-1049.

Miller DJ, Yellowless D (1989) Inorganic nitrogen uptake by symbiotic marine cnidarians: a critical review. Proc R Soc Lond Series B Biol Sci 237:109-125.

Mokady O, Loya Y, Lazar B (1998) Ammonium contribution from boring bivalves to their coral host – a mutualistic symbiosis? Mar Ecol Prog Ser 169:295-301.

Moyer JT (1980) Influence of temperate waters on the behavior of the tropical anemonefish *Amphiprion clarkii* at Miyake-jima, Japan. Bull Mar Sci 30:261-272.

Muller-Parker G (1985) Effect of feeding regime and irradiance on the photophysiology of the sea anemone *Aiptasia pulchella*. Mar Biol 90:65-74.

Muller-Parker G, Cook CB, D'Elia CF (1990) Feeding affects phosphate fluxes in the symbiotic sea anemone *Aiptasia pallida*. Mar Ecol Prog Ser 60:283-290.

Muller-Parker G, Cook CB, D'Elia CF (1994a) Elemental composition of the coral *Pocillopora damicornis* exposed to elevated seawater ammonium. Pac Sci 48:234-246.

Muller-Parker G, Davy SK (2001) Temperate and tropical algal-sea anemone symbioses. Invert Biol 120:104-123.

Muller-Parker G, McCloskey LR, Hùegh-Guldberg O, McAuley PJ (1994b) Effect of ammonium enrichment on animal biomass of the coral *Pocillopora damicornis*. Pac Sci 48:273-283.

Muscatine L (1967) Glycerol excretion by symbiotic algae from corals and *Tridacna* and its control by the host. Science 156:516-519.

Muscatine L (1980) Productivity of zooxanthellae. In: Falkowski PG (ed) Primary productivity in the sea Plenum Publ Corp, New York, pp.381-402.

Muscatine L, D'Elia CF (1978) The uptake, retention, and release of ammonium by reef corals. Limnol Oceanogr 23:725-734.

Muscatine L, Falkowski PG, Dubinsky Z, Cook PA, McCloskey LR (1989) The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc R Soc Lond Ser B Biol Sci 236:311-324.

Muscatine L, Falkowski P, Porter J, Dubinsky Z (1984) Fate of photosynthetically-fixed carbon in light and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. Proc R Soc Lond Series B Biol Sci 222:181-202.

Muscatine L, Marian RE (1982) Dissolved inorganic nitrogen flux in symbiotic and nonsymbiotic meduase. Limnol Oceanogr 27:910-918.

Muscatine L, Pool RR (1979) Regulation of numbers of intracellular algae. Proc R Soc Lond Ser B Biol Sci 204:131-139.

Muscatine L, Porter JW (1977) Reef corals mutualistic symbioses adapted to nutrient-poor environments. BioScience 27:454-460.

Noe R (2001) Biological markets: partner choice as the driving force behind the evolution of mutualisms. In: Economics in Nature: Social Dilemmas, Mate Choice, and Biological Markets. Cambridge University Press, New York, pp.93-118.

Noe R, Hammerstein P (1995) Biological markets. Trends Ecol Evol 10:336-339.

Patzner RA (2004) Associations with sea anemones in the Mediterranean Sea: a review. Ophelia 58:1-11.

Pochon X, Montoya-Burgos JI, Stadelmann B, Pawlowski J (2006) Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*. Mol Phylogenet Evol 38:20-30.

Pool RR, (1976) Symbiosis of *Chlorella* and *Chlorohydra viridissima*. PhD dissertation, University of California, Los Angeles, 122pp.

Porat D, Chadwick-Furman NE (2004) Effects of anemonefish on giant sea anemones: expansion behavior, growth, and survival. Hydrobiologia 530/531:513-520.

Porat D, Chadwick-Furman NE (2005) Effects of anemonefish on giant sea anemones: Ammonium uptake, zooxanthella content and tissue regeneration. Mar Freshw Behav Physiol 38:43-51.

Porter JW (1980) Primary productivity in the sea: reef corals in situ. . In: Falkowski PG (ed) Primary productivity in the sea Plenum, New York, pp.403.

Porter JW, Muscatme L, Dubinsky Z, Falkowski PG (1984) Primary production and photoadaptation in light and shade-adapted colonies of the symbiotic coral, *Stylophora pistdlata*. Proc R Soc Lond Ser B Biol Sci 222:161-180.

Rahav O, Dubinsky Z, Achituv Y, Falkowski PG (1989) Ammonium metabolism in the zooxanthellate coral, *Stylophora pistillata*. Proc R Soc Lond Series B Biol Sci 236:325-337.

Randall JE, Fautin DG (2002) Fishes other than anemonefishes that associate with sea anemones. Coral Reefs 21:188-190.

Redjale R (1976) Light adaptation strategies of hermatypic corals. Pac Sci 30:212.

Rees TAV, Ellard FM (1989) Nitrogen conservation and the green hydra symbiosis. Proc R Soc Lond Ser B Biol Sci 236:203-212.

Roberts JM, Davies PS, Fixter LM (1999a) Symbiotic anemones can grow when starved: nitrogen budget for *Anemonia viridis* in ammonium-supplemented seawater. Mar Biol 133:29-35.

Roberts JM, Davies PS, Fixter LM, Preston T (1999b) Primary site and initial products of ammonium assimilation in the symbiotic sea anemone *Anemonia viridis*. Mar Biol 135:223-236.

Roberts JM, Fixter LM, Davies PS (2001) Ammonium metabolism in the symbiotic sea anemone *Anemonia viridis*. Hydrobiologia 461:25-35.

Rodriguez-Lanetty M, Cha HR, Song JI (2002) Genetic diversity of symbiotic dinoflagellates associated with anthozoans from Korean waters. Proc Int 9th Coral Reef Symp, Bali 1:163-166.

Rodriguez-Lanetty M, Chang SJ, Song JI (2003) Specificity of two temperate dinoflagellate-anthozoan associations from the northwestern Pacific Ocean. Mar Biol 143:1193-1199.

Roughgarden J (1975) Evolution of marine symbiosis - a simple cost-benefit model. Ecology 56:1201-1208.

Rowan R (2004) Coral bleaching—thermal adaptation in reef coral symbionts. Nature 430:742.

Rowan R, Knowlton N (1995) Intraspecific diversity and ecological zonation in coral algal symbiosis. Proc Natl Acad Sci USA 92:2850-2853.

Rowan R, Knowlton N, Baker AC, Jara J (1997) Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. Nature 388:265-269.

Rowan R, Powers DA (1991a) A molecular genetic identification of zooxanthellae and the evolution of animal–algal symbioses. Science 251:1348-1351.

Rowan R, Powers DA (1991b) Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). Mar Ecol Prog Ser 71:65-73.

Sachs JL, Mueller UG, Wilcox TP, Bull JJ (2004) The evolution of cooperation. Quart Rev Biol 79:135-160.

Santos SR, Taylor DJ, Coffroth MA (2001) Genetic comparisons of freshly isolated vs. cultured symbiotic dinoflagellates: implications for extrapolating to the intact symbiosis. J Phycol 37:900-912.

Santos SR, Taylor DJ, Kinzie RAIII, Hidaka M, Sakai K, Coffroth MA (2002a) Molecular phylogeny of symbiotic dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. Mol Phylogenet Evol 23:97-111.

Santos SR KRI, Sakai K, Coffroth MA (2003) Molecular characterization of nuclear small subunit (18S)-rDNA pseudogenes in a symbiotic dinoflagellate (*Symbiodinium*, *Dinophyta*). J Eukaryot Microbiol 50:417-421.

Savage A, Trapido-Rosenthal MH, Douglas AE (2002) On the functional significance of molecular variation in *Symbiodinium*, the symbiotic algae of cnidaria: photosynthetic response to irradiance. Mar Ecol Prog Ser 244:27-37.

Schaus MH, Vanni MJ, Wissing TE, Bremigan MT, Garvey JE, Stein RA (1997) Nitrogen and phosphorus excretion by detrivorous gizzard shad in a reservoir ecosytem. Limnol Oceanogr 42:1386-1397.

Schoenberg DA, Trench RK (1980) Genetic variation in *Symbiodinium* (*_Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. III. Specificity and infectivity of *Symbiodinium microadriaticum.* Proc R Soc Lond B Biol Sci 207:445-460.

Sebens KP (1976) The ecology of Caribbean sea anemones in Panama: Utilization of space on a coral reef. In Mackie GO (ed), Coelenterate ecology and behavior Plenum Press, New York, pp.67–77.

Sebens KP, DeRiemer K (1977) Diel cycles of expansion and contraction in coral reef anthozoans. Mar Biol 43:247-256.

Sheppard CRC (1981) Illumination and the coral community beneath tabular *Acropora* species. Mar Biol 64:53-58.

Shick JM, Dykens JA (1984) Photobiology of the symbiotic sea anemone *Anthopleura elegantissima:* Photosynthesis, respiration, and behavior under intertidal conditions. Biol Bull 166:608-619.

Smith GJ, Muscatine L (1999) Cell cycle of symbiotic dinoflagellates: variation in G1 phase-duration with anemone nutritional status and macronutrient supply in the *Aiptasia pulchella–Symbiodinium pulchrorum* symbiosis. Mar Biol 134:405-418.

Snidvongs A, Kinzie RA III (1994) Effects of nitrogen and phosphorus enrichment on in vivo symbiotic zooxanthellae of *Pocillopora damicornis*. Mar Biol 118:705-711.

Solorzano L (1969) Determination of ammonium in natural waters by the phenolhypochlorite method. Limnol Oceanogr 14:799-801.

Sournia A (1977) Notes on primary productivity of coastal waters in the Gulf of Eilat (Red Sea). Int Revue ges Hydrobiol 62:813-819.

Spotte S (1996) Supply of regenerated nitrogen to sea anemones by their symbiotic shrimp. J Exp Mar Biol Ecol 198:27-36.

Stambler N, Dubinsky Z (1987) Energy relationships between *Anemonia sulcata* and its endosymbiotic zooxanthellae. Symbiosis 3:233-247.

Stambler N, Dubinsky Z (2005) Corals as light collectors: an integrating sphere approach. Coral Reefs 24:1-9.

Stambler N, Popper N, Dubinsky Z, Stimson J (1991) Effects of nutrient enrichment and water motion on the coral *Pocillopora damicornis*. Pac Sci 45:299-307.

Stat M, Carter D, Hoegh-Guldberg O (2006) The evolutionary history of *Symbiodinium* and scleractinian hosts – Symbiosis, diversity, and the effect of climate change. Persp Plant Ecol Evol System 8:23-43.

Steen G (1988) The bioenergetics of symbiotic sea anemones (Anthozoa: Actiniaria). Symbiosis 5:103-142.

Steen RG (1986) Evidence for heterotrophy by zooxanthellae in symbiosis with *Aiptasia pulchella*. Biol Bull 170:267-278.

Stimson JS (1988) The rate and diel pattern of release of zooxanthellae by undisturbed colonies of *Pocillopora damicornis* at two levels of dissolved nitrogen. Abstract Proc 6th Int Coral Reef Symp, pp. 96.

Stimson J, Kinzie RA (1991) The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* (Linnaeus) under nitrogen-enrichment and control conditions. J Exp Mar Biol Ecol 153:63-74.

Summons RE, Boag TS, Osmond CB (1986) The effect of ammonium on photosynthesis and the pathway of ammonium assimilation in *Gymnodinium microadriaticum* in vitro and in symbiosis with tridacnid clams and corals. Proc R Soc Lond Ser B Biol Sci 227:147-159.

Svoboda A, Porrmann T (1980) Oxygen production and uptake by symbiotic *Aiptasia diaphana* adapted to different light intensities. In: Smith DC, Tiffon Y (eds.) Nutrition in the lower Metazoa Pergamon Press, Oxford, pp.82-99.

Syrett PJ (1981) Nitrogen metabolism of microalgae. In:Platt T (ed) Physiological basis of phytoplankton ecology Can Bull Fish Aquat Sci 210:182-210.

Szmant AM, Ferrer LM, FitzGerald LM (1990) Nitrogen excretion and O:N ratios in reef corals: evidence for conservation of nitrogen. Mar Biol 104:119-127.

Szmant-Froelich A, Pilson MEQ (1980) The effects of feeding frequency and symbiosis with zooxanthellae on the biochemical composition of *Astrangia danae* Milne Edwards and Haime 1848. J Exp Mar Biol Ecol 48:85-97.

Szamant-Froelich AM, Pilson MEQ (1984) Effects of feeding frequency and symbiosis with zooxanthellae on nitrogen metabolism and respiration of the coral *Astrangia danae*. Mar Biol 81:153-162.

Tatrai I (1981) The nitrogen metabolism of bream, *Abramis brama*. L Compar Biochem and Physiol 68A:119-121.

Taylor DL (1969) On the regulation and maintenance of algal numbers in zooxanthellae-coelenterate symbiosis, with a note on the nutritional relationship in *Ammonia sulcata*. J Mar Biol Ass UK 49:1057-1065.

Taylor DL (1974) Symbiotic marine algae: Taxonomy and biological fitness. In: Vernberg WB (ed) Symbiosis in the sea Univ of South Carolina Press, Columbia, pp. 245-262.

Thornhill DJ, Fitt WK (2006) Highly stable symbioses among western Atlantic brooding corals. Coral Reefs 25:515-519.

Titlyanov EA, Leletkin VA, Dubinsky Z (2000) Autotrophy and predation in the hermatypic coral *Stylophora pistillata* in different light habitats. Symbiosis 29:263-281.

Titlyanov EA, Titlyanova TV, Leletkin VA, Tsukahara J, van Woesik R, Yamazato K (1996) Degradation of zooxanthellae and regulation of their density in hermatypic corals. Mar Ecol Prog Ser 139:167-178.

Toller WW, Rowan R, Knowlton N (2001) Zooxanthellae of the *Montastraea annularis* species complex: patterns of distribution of four taxa of *Symbiodinium* on different reefs and across depths. Biol Bull 201:348-359.

Tytler EM, Davies PS (1986) The budget of photosynthetically derived energy in the *Anemonia sulcata* (Pennant) symbiosis. J Exp Mar Biol Ecol 99:257-269.

Ulstrup KE, van Oppen MJH (2003) Geographic and habitat partitioning of genetically distinct zooxanthellae (*Symbiodinium*) in *Acropora* corals on the Great Barrier Reef. Mol Ecol 12:3477-3484.

Van Oppen MJH, Palstra FP, Piquet AMT, Miller DJ (2001) Patterns of coral-dinoflagellate associations in *Acropora*: significance of local availability and physiology of *Symbiodinium* strains and host-symbiont selectivity. Proc R Soc Lond B Biol Sci 268:1759-1767.

Vanni MJ (2002) Nutrient cycling by animals in freshwater ecosystems. Annual Review of Ecol and System 33:341-370.

Wang JT, Douglas AE (1998) Nitrogen recycling or nitrogen conservation in an alga–invertebrate symbiosis? J Exp Biol 201:2445-2453.

Warner ME, Fitt WK, Schmidt GW (1999) Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. Proc Natl Acad Sci USA 96:8007-80012.

Webb KL, DuPaul WD, Wiebe W, Sottile W, Johannes RE (1975) Enewetak (Eniwetok) atoll: aspects of the nitrogen cycle on a coral reef. Limnol Oceanogr 20:198-210.

Whitehead LF, Douglas AE (2003) Metabolite comparisons and the identity of nutrients translocated from symbiotic algae to an animal host. J Exp Biol 206:3149-3157.

Whitledge TE (1982) Regeneration of nitrogen by the nekton and its significance in the northwest Africa upwelling ecosystem. Fish Bull 80:327-335.

Wilkerson FP, Muller-Parker G, Muscatine L (1983) Temporal patterns of cell division in natural populations of endosymbiotic algae. Limnol Oceanogr 28:1009-1014.

Wilkerson FP, Muscatine L (1984) Uptake and assimilation of dissolved inorganic nitrogen by a symbiotic sea anemone. Proc R Soc Lond Series B Biol Sci 221:71-86.

Wilkerson FP, Trench RK (1986) Uptake of dissolved inorganic nitrogen by the symbiotic clam *Tridacna gigas* and the coral *Acropora* sp. Mar Biol 93:237-246.

Winters G, Loya Y, Rottgers R, Beer S (2003) Photoinhibition in shallow-water colonies of the coral *Stylophora pistillata* as measured in situ. Limnol Oceanogr 48:1388-1393.

Zamer WE, Shick JM (1989) Physiological energetics of the intertidal sea anemone *Anthopleura elegantissima* III. Biochemical composition of body tissues, substrate-specific absorption, and carbon and nitrogen budgets. Oecologia 79:117-127.

Zhang Z, Green BR, Cavalier-Smith T (2000) Phylogeny of ultrarapidly evolving dinoflagellate chloroplast genes: A possible common origin for sporozoan and dinoflagellate plastids. J Mol Evol 51:26-41.