Symbiosis and Nitrogen Cycling:
Physiological Effects of Anemone Shrimps on Host Sea Anemones in the Caribbean Sea

by

Channing Cantrell

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Approved by:

Jack Feminella, Chair & Professor of Biological Sciences
Nanette Chadwick, Associate Professor of Biological Sciences
Raymond Henry, Professor of Biological Sciences
Holly Ellis, Associate Professor of Biochemistry

Abstract

Symbioses are characteristic of coral reef communities and can involve networks of species interactions as well as specialized adaptations to reef conditions. Because coral reefs are surrounded by nutrient-poor water, mutualisms provide an opportunity for tight nutrient cycling among reef organisms. However, little is known about how mobile ectosymbionts, such as crustaceans, benefit their hosts, including how they contribute to nutrient cycling in reef cnidarians. I examined the benefits of ammonium contributions by 2 species of obligate ectosymbionts, the spotted cleaner shrimp *Periclimenes yucatanicus,* and the Pederson's cleaner shrimp *Ancylomenes pedersoni,* to selected physiological processes in host Caribbean sea anemones *Bartholomea annulata*. Rates of ammonia excretion from 3 client fish species were also measured to estimate the potential nitrogen contributions from client fishes which pose and are cleaned by shrimps near host sea anemones. Starved sea anemones were subjected to 1of 3 laboratory treatments for 6 weeks: 1) shrimps present, 2) ammonium present, or 3) neither shrimps nor ammonium present (control). Changes in anemone body size and protein content were measured along with the density, mitotic index, chlorophyll a content and mycosporine-like amino acids of their endosymbiotic microalgae *Symbiodinium.* Shrimps excreted ammonium at whole-organismal rates that were much lower than those of reef fishes, while sea anemones absorbed ammonium at relatively high rates, indicating that shrimp presence alone cannot meet the needs of host nutrient budgets. The only clear impact of experimental nutrient supplements was enhancement of the mitotic index of *Symbiodinium.* Shrimp presence also caused a small but

statistically insignificant increase in the oral disc size of sea anemones. Future experiments may demonstrate whether shrimp presence causes significant increase in anemone size, resulting in more obvious visual cues for client fishes to locate cleaner shrimp stations on the reef. The ammonia excretion rates of client fishes reveal that they theoretically are able to meet the nitrogen needs of host sea anemones. Thus, a nutrient-driven positive feedback loop may occur among among cleaner shrimps, host sea anemones, their endosymbiotic microalgae, and the client fishes attracted by the shrimps to these cleaning stations. The ectosymbiotic shrimp alone do not appear to provide enough ammonium to support the physiological needs of microalgae in host sea anemones, but the potential client fishes may. These mutualistic interactions may facilitate tight nutrient cycling among multiple levels of species belonging to different phyla in this symbiotic system. Further research on the impacts of ammonia and other nutrients provided by client fishes is necessary to determine benefits from this mutualistic interaction to host sea anemones.

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Chapter One

General Introduction

Coral reefs are some of the most productive ecosystems in the world and supply tropical coastal nations with food and other resources that contribute to economic development. Reef systems are also a foundation for many tourist industries worldwide, often in countries with struggling economies (Sinclair 1998). These ecosystems have levels of biodiversity comparable to tropical rainforests (Hubbell 1997), but questions remain about how they achieve this in a nutrient-poor environment with low available nitrogen in the surrounding water column (<1 μM/L, Muscatine and Porter, 1977). The intricate cycling of nitrogen in these vast communities is a key to understanding their productivity, as are many of the adaptations, symbioses and behaviors of reef organisms.

On coral reefs, many benthic organisms form intimate symbioses, partly in response to a highly competitive environment where space and nutrients are limiting, particularly nitrogen (Trench, 1979). A major example of this relationship is the association between corals, the organisms that provide the physical structure of reef environments, and their dinoflagellate endosymbionts *Symbiodinium.* These dinoflagellates are symbiotic with corals and other benthic invertebrates such as sea anemones, clams and nudibranchs (Muscatine and Porter 1977, Trench 1979, Kempf 1984). The algae benefit from the association by obtaining protection from predation and from the host providing a physically and chemically stable environment inside another animal. In return, the animal host receives organic carbon skeletons produced from photosynthesis by the algal cells (Cates and McLaughlin 1976, Muscatine 1980, Battey and Patton 1987, Cleveland et al. 2011). When bleaching occurs, in which symbiotic corals and

anemones lose their microalgae, the hosts cannot survive for more than a few weeks on the particulate matter they capture from the water column. If the *Symbiodinium* algae are unable to recolonize the host, the result is a mass die-off of corals and other bleached cnidarians, threatening the structure and survival of entire reefs and the organisms which depend on them (Baker et al. 2008). Although one could argue that this specific symbiosis is the most integral to coral reef communities, it is certainly not the only symbiosis found in reef systems. The complexity and frequency of organismal interactions, including symbioses, are bioindicators of community-wide reef health, and can serve as proxies to estimate levels of biodiversity on regional and local scales (Linton and Warner 2003). Examination of various symbioses in reef communities helps to tease apart the factors that allow such nutrient-barren environments to support high levels of biodiversity. Central to nutrient conservation is the formation of mutualistic symbioses that allow tight and efficient nutrient cycling between organisms, and contribute to the high productivity of reef ecosystems.

Research on the flow of nutrients in these complex ecosystems is necessary to help us understand changes in organismal adaptations (like symbiosis) on reefs in response to environmental change, how these adaptations vary, and how they impact ecological function. In a reef system, each type of resource is often utilized by more than one species for a number of services. For example, a benthic invertebrate such as a sea anemone can be used by many organisms including fishes, crabs and shrimps for shelter and habitat (Chadwick et al. 2008). In the Red Sea, the bulb-tentacle sea anemone *Entacmea quadricolor* is used by the two-banded anemonefish *Amphiprion bicinctus* as a refuge and nesting site (Huebner et al. 2012). These fish use the stinging qualities of the anemone as a means of protection from predation as well as for provision of a suitable long-term habitat for a mating pair to spawn (Mariscal 1970). Benefits to

the anemone from the fish symbionts include nutrient enrichment (specifically nitrogen, Roopin and Chadwick 2008) and a reduction of hypoxic areas nearest the host due to ventilation behaviors of the fish (Szczebak et al. 2013). A positive feedback loop of benefits to both partners perpetuates the existence of the symbiosis, in which anemones hosting fish survive longer and grow larger than those without fish symbionts, thus allowing more and larger fish to inhabit them (Porat and Chadwick-Furman 2004). Other types of symbioses between motile ectosymbionts and cnidarian hosts include the association between trapeziid crabs and reef building corals of the genera *Acropora* and *Pocillopora*. The crabs cause increases in the growth and survival rates of these coral colonies, in part because they remove sediment from among the coral branches (Stewart et al. 2006), an invaluable asset with increasing sediment loads being deposited on coral colonies worldwide. Trapeziid crabs also protect their branching coral hosts from predation by crown of thorns sea stars (Pratchett 2001).

On Caribbean Sea coral reefs, the ubiquitous corkscrew sea anemone *Bartholomea annulata* hosts several species of small crustaceans (Knowlton and Keller 1985, Briones-Forzan et al. 2012). The shrimp *Alpheus armatus* is an obligate symbiont of these anemones and protects the host anemone from predatory bristle worms (Smith 1977, McCammon 2010). This alpheid shrimp also appears to prevent burial of the anemone in sand, by actively removing sand from around the host (S. Ratchford and N. Chadwick, unpublished data). Other crustacean associates of this anemone include two species of paleomonid shrimps – the spotted shrimp *Periclimenes yucatanicus,* and Pederson's cleaner shrimp *Ancylomenes pedersoni*. The Pederson's shrimp provides an ecosystem service which is imperative for reef fish populations – ectoparasite control. Cleaner organisms occur on reefs worldwide, and they are often either crustacean or fish species. They function as organisms which keep ectoparasite loads in check on reef fish

populations, by consuming parasitic isopods and gnathiids (Bunkley-Williams and Williams 1998, Sikkel et al. 2004). Although no experimental studies of the benefits of long-term cleaning over the lifetime of a fish are available, in situ work in the Caribbean on the longfin damselfish *Stegastes diencaeus* found that diel variation in gnathiid loads on fish positively correlated with how long clients spent at cleaning stations (Sikkel et al. 2004). The Pederson's cleaner shrimp attracts client fishes from at least 16 families (Wicksten 1998, Hubebner and Chadwick 2012a), indicating that their cleaning stations provide services with widespread effects throughout the reef community. By increasing the health and well-being of resident fish populations, overall biodiversity of the reef system may be increased. Effect of cleaners on client populations have been documented in Egypt and Australia, where the removal of cleaners results in a decline in fish biodiversity over the long-term (Bshary 2003, Grutter et al. 2003, Waldie et al. 2011).

Without healthy populations of fish in reef systems, the trophic flow of energy can become easily disrupted by keeping most nutrients locked in primary and lower trophic levels. A loss of higher trophic levels of fishes may lead to decreasing efficiency and productivity yields on reefs, resulting in a decrease in species diversity and richness, and a decrease in overall ecosystem function (Roberts 1995). This has been observed in the Caribbean as the reefs of Jamaica and other nations have seen decades of degradation, pollution, nutrient loading and overfishing. The reef fish populations around Jamaica have steadily decreased over generations, resulting in a loss of total fish biomass (Hughes 1994, Holmlund and Hammer 1999). Other areas in the Caribbean are facing similar degradation of their reef systems, which is jeopardizing the economic stability of many developing countries. With nearly two-thirds of all human populations living within 100 miles of a coastline, these countries often depend on the reefs as a constant resource for food and other materials necessary for sustaining their growing human

populations (Cesar et al. 2003). Overfishing is an increasing problem in marine ecosystems, with populations of once numerous fish species declining. The economic importance of fisheries in these regions of the world cannot be understated. As a human resource, in 2009 the Caribbean and South American region captured a total of 17.1 million tons of live fishes (FAO 2009), with most of the catch being kept and sold within the country.

Fish populations serve many ecological functions, such as linking different ecosystems (benthic vs. pelagic), providing services for other species (Roopin et al. 2008), preserving ecological memory and genetic diversity, and serving as major vehicles that drive nutrient cycles between communities worldwide, while providing ecosystem resilience (Holmund and Hammer 1999, Allgier et al. 2014). In the Caribbean Sea, determination of physiological processes in marine organisms, such as their rates of photosynthesis, primary productivity, and growth, is often an overlooked component of coastal management, and cannot remain unaccounted for if integrated management strategies are to be effective (Linton and Warner 2003).

Client fishes in the Caribbean Sea use the corkscrew sea anemone *B. annulata* as a visual cue to find shrimp cleaning stations on reefs (Huebner and Chadwick 2012b), highlighting the importance of the sea anemone host to cleaning interactions in this region. If anemone abundances decline, interspecies aggression can occur on sea anemones shared by more than one species of shrimp, indicating that cleaner dynamics may be susceptible to host anemone population declines (Silbiger and Childress 2008). The same study also observed that densities of anemone and cleaner shrimp populations appear to be higher in areas frequented by potential fish clients, indicating a relationship between abundance of cleaners and clients. Focusing on potential interactions between these partners on a physiological level can help us understand the

underpinnings of how this relationship evolved, a process which remains unclear in many interspecies cleaning interactions (Poulin and Grutter 1996).

Other studies on the potential benefits to cnidarian hosts from their association with ectosymbionts have concluded, for example, that the close association of anemonefishes increases sea anemone fitness by increasing host surface area, oxygenation/respiration, and *Symbiodinium* densities, as well as chlorophyll a levels of the endoalgal symbionts (reviewed in Roopin et al. 2008, Szczeback et al. 2013). Nitrogen cycling from the excretion products of these fishes explains in part how such symbioses can evolve in an environment that is a nutrient desert. In symbiotic clams, an increase in available nitrogen in the form of ammonium under light conditions stimulates photosynthesis in *in situ* algal populations (Summons et al. 1986). Similar results were found in the scleractinian coral *Pocillopora damicornis* and the hydroid *Myrionema amboinense* (Fitt and Cook 2001, Hoegh-Guldberg 2006). Other studies have demonstrated that association with an ectosymbiont can provide phosphate and nitrogen nutrient enrichment which is utilized for sea anemone growth, reproduction, regeneration, biosynthesis of metabolites, and stimulation of symbiotic algal growth (Muller-Parker et al. 1994, Godinot and Chadwick 2009, Cleveland et al. 2011). While ammonium enrichment coupled with phosphate enrichment yields greater densities of *Symbiodinium*, nitrate and ammonium enrichment alone has shown lesser effects on algal populations, and phosphate enrichment alone showed no significant effects (Fitt and Cook 2001). These data are consistent with the results of nutrient enrichment in Red Sea coral populations, which indicate that corals and related species are nitrogen limited (Muscatine et al. 1989) and other work that indicates that this pattern is evident in other symbiotic marine organisms (Lee 1994). While some research suggests that nitrogen could be supplied to hosts via shrimp symbionts (Spotte 1996), no definitive evidence is available concerning the physiological

benefits that cleaner shrimp may deliver to host anemones. In the Indo-Pacific, anemonefish provide excess nutrients in the form of nitrogenous wastes that enhance host anemone fitness, but on Caribbean reefs it remains unclear how the symbiosis between sea anemones *B. annulata* and the shrimps *P. yucatanicus* and *P. pedersoni* may increase anemone fitness. Inorganic nitrogen can be utilized by the anemone's symbiotic algae as a fertilizer, causing an increase in abundance and in organic compounds transferred to the host (Muscatine et al. 1984). These nutrients can include sugars, amino acids, and other nitrogenous compounds such as mycosporine-like amino acids (MAAs). The idea that shrimp excretions may have positive effects on host anemones is a largely unexplored aspect of this symbiosis (Spotte 1996). The transfer of nutrients from shrimps to host anemones could have implications for the dynamics and evolution of their populations, as well as the conservation and management of these species.

The objective of this thesis is to investigate potential physiological benefits of shrimp nitrogenous wastes to host sea anemones, by assessing how host body size, protein content, *Symbiodinium* populations, chlorophyll a and MAA levels are affected when shrimp presence and nutrient supplies are manipulated.

Chapter Two

Effects of Symbiotic Cleaner Shrimp on Caribbean Sea Anemone Physiology: Nitrogen Cycling in a Coral Reef Mutualism

Introduction

The metabolism of proteins and amino acid complexes by animals results in the formation of nitrogenous waste products in the body. In animal tissues, these occur primarily as forms of ammonia, which may be separated into dissolved gaseous ammonia (NH_3) or the positively charged ion ammonium (NH_4^+). Many marine organisms excrete these products directly into the surrounding water column (Weihrauch et al. 2004). Mechanisms of total ammonia excretion have been a topic of debate, and often vary among environments and species. Rates of ammonia excretion among crustaceans vary up to 10x among species within genera (Dall and Smith 1986), and may be linked to specific activity levels and dietary factors. Excretion rates also varies with external conditions such as temperature, salinity, and time of day, as well as individual physiological state (starved vs. well-fed, healthy vs. injured), with fed organisms normally excreting more total ammonia than starved conspecifics (Rychly and Marina 1977). Whether the organism is primarily aquatic or terrestrial can also have an effect on excretion rates and mechanisms, with terrestrial crustaceans often converting ammonia to less volatile compounds such as urea or purines, and then excreting them via specialized excretory organs. In contrast, amphibious crustaceans often retain their nitrogenous waste in the form of ammonia until they reach a body of water, and then release their wastes at high rates in a manner similar to fully aquatic species (Greenaway 1991, Weihrauch et al. 2004). External environmental levels of ammonia dissolved in seawater can exceed physiological concentrations,

thus impeding the process of passive diffusion of ammonia. This occurs, for example, when high rates of organic matter decomposition take place in low water flow or, in the case of lungfish, during drought season when external ammonia levels are concentrated in small volumes of surrounding water (Lohnse et al. 1993). This fact highlights the importance of ammonia excretion and mechanisms which work against potential and partial gradients under such conditions.

Because ammonia quickly becomes protonated in the hemolymph or blood to form the lipid-insoluble ion ammonium, most early research on aquatic organism excretion focused on the discovery and explanation of mechanisms by which ammonium ions are expelled from the body in fish species (Evans 1977, Maetz 1973, Maetz and Garcia-Romeu 1964). Later though, it was realized that the proper measurements to determine acid/base balance in these experiments were not accounted for, and that Na^+ uptake that appeared to be stimulated by ammonium injection could also be explained by H^+ exchange. Once these experiments were repeated it was noted that a dramatic drop in pH occurred when NH_4Cl was injected into catfish and trout species. The drop in pH in response to ammonium salt addition could only be explained when the permeability of gaseous ammonia and ionic ammonium were taken into account. This suggests that there is a cellular equilibrium of NH_3 and NH_4^+ within the gill epithelium that allows for the passive diffusion of NH_3. This is also supported by a small (~65 µtorr) partial pressure gradient which favors the passive diffusion of NH_3 from the gills to the external aquatic environment. In order for this dramatic pH drop to occur, the permeability ratios must be greatly in favor of NH_3 diffusion, with over 50% of excreted excess total ammonia occurring in the form of NH_3 (Cameron and Heisler 1983). The ionic exchange of NH_4^+ with Na^+ or H^+ occurs in the gills of various crustaceans and invertebrates (Mangum 1978, Claiborne et al. 1982, Greenaway 1991).

In contrast, this mechanism in fishes may serve to maintain acid-base equilibrium and potential gradients which favor NH_3 diffusion, and not direct NH_4^+ excretion.

Another pathway by which NH_4^+ can enter the gill epithelium is by utilizing Na^+/K^+-ATPase. In blue crabs, excretion rates correlated with Na^+ uptake rates and depend on ATP availability. NH_4^+ can replace K^+ ions due to their similar ionic properties, supporting the hypothesis that a Na^+/K^+- ATPase may facilitate excretion by allowing substitution of NH_4^+ in ion exchange (Pressley et al. 1981, Towle and Holleland 1987). Other methods of ammonium excretion have been found in other marine species. In green shore crabs, H^+- ATPase acidified vesicles collect gaseous ammonia and transform it into ammonium, trapping it until microtubules transport the vesicles to the apical cell membrane of the gill epithelium and release the ammonium via exocytosis (Weihrauch et al. 2002).

Other potential mechanisms or facilitators of total ammonia excretion in crustaceans include $Na^+/K^+/2Cl^-$ co-transporters and carbonic anhydrase (CA) activity (Henry 1988, Rankin and Jensen 1993). CA may maintain concentration gradients in favor of ammonium release in freshwater organisms, by creating an acidic environment at the gill epithelia which traps the ion ammonium in the water column, keeping the partial pressure gradient in favor of NH_3 diffusion to the outside. This has yet to be demonstrated for marine species due to the confounding effects of ionic differences between fresh and saltwater and the higher buffering capacity of seawater. More recently, Rhesus (Rh) proteins are glycoproteins have been found in the gill tissue of some species of fish. Rh protein gene sequences have similarities with another group of proteins demonstrated to function as ammonia transporters in yeast and plants (Huang and Peng 2005) and their function in fish and other marine species, is speculated to transport ammonia. They are speculated to function as ammonia transporters in some animals, because gene expression of this

class of glycoproteins occurs in various mammals and fishes (Weihrauch 2004, Nakada et al. 2007, Nawata et al. 2007). They are thought to be a mechanism embedded in the gill epithelium for the transport of either NH_3 or NH_4^+ through the gill epithelial layer to the external environment. This mechanism may function one of three ways; direct facilitated diffusion of NH_3, transport of NH_4^+, or by coupling with Na^+/H^+ exchange, but this has yet to be determined. Gene expression of Rh proteins increased in response to elevated external ammonia levels (Wood and Wright 2009) supporting the theory that they function in ammonia excretion for these organisms.

Some fishes also convert internal ammonia to urea via uricolysis and excrete less toxic nitrogenous products (Smith 1929, Gregory 1977, Randall and Wright 1987), however this process is not common with most marine fishes due to the disadvantages of excreting urea as nitrogenous waste in seawater compared to using passive diffusion and partial pressure gradients. Some fish species, mainly elasmobranchs, synthesize and store urea, primarily as an osmolyte in order to maintain osmotic homeostasis in response to external osmotic changes (Ip and Chew 2010).

In coral reef environments, symbiotic interactions facilitate tight nutrient exchange among organisms (Muscatine and Porter 1977, Sorokin 1993, Roopin and Chadwick 2009) and help organisms to cope with low nutrient levels in the surrounding water column. Nutrient cycling thus is likely to occur between mobile ectosymbionts (fishes and crustaceans) and their coral reef cnidarian hosts that harbor endosymbiotic microalgae. Fish ectosymbionts are known to provide essential nutrients to giant sea anemones in clownfish symbioses on coral reefs. Host anemones become larger, absorb more nitrogen, and withstand starvation longer when associated with symbiotic pomacentrid fishes (Holbrook and Schmitt 2005, Porat and Chadwick-Furman

2005, Roopin and Chadwick 2009). Anemonefishes also enhance oxygen exchange in host anemones (Szczebak et al. 2013), and supply other nutrients such as carbon and phosphate that may be used for host growth and metabolism (Godinot and Chadwick 2009, Cleveland et al. 2011). In turn, anemonefishes are larger, older and have higher reproductive success when associated with larger anemones (Fautin 1992), thus creating a positive feedback loop of nutritional and other benefits among partners in this mutualism (Roopin et al. 2011). Likewise, in the Caribbean Sea, excess nitrogen from crustacean ectosymbionts such as Pederson's cleaner shrimp, *Ancylomenes pedersoni* and spotted cleaner shrimp, *Periclimenes yucantanicus*, could provide valuable nitrogen and other nutrients for endosymbiotic mircoalgae, *Symbiodinium* spp. (also known as zooxanthellae) within the tentacles of host corkscrew sea anemones, *Bartholomea annulata*. Benefits from this process are expected to cause physiological changes in both the host and symbiotic microalgal populations.

Zooxanthella cells occur inside vacuoles within host cnidarian endothelial cells, the cytoplasm of which harbors high concentrations of nitrogen (5-50µM for ammonium) and phosphorous compared to the surrounding water column outside the host (<1µM, Wilkerson and Muscatine 1984). This relatively nutrient rich environment provides a suitable habitat for microalgal growth (Hoegh-Guldberg 2006). However, excessive nutrient levels have been shown to lower growth rates in the stony coral *Stylophora pistillata*, presumably due to inhibition of calcification reactions by excess available phosphorous, and decreased photosynthesis (Ferrier-Pagés et al. 2000). Nitrogen supplement experiments in *B. annulata*, reveal that nitrogen is stored in host anemone tissues surrounding the microalgal cells, as well as in low molecular weight molecules (LMWM) pools in both host and algal cell bodies (Lipshultz and Cook 2000). In the sea anemone *Aiptasia pallida*, exposure to high nitrogen levels (20-50µM) results in enhanced

rates of dark C fixation in microalgal cells, with light C fixation rates similar to those of well-fed individuals. In contrast, individual anemones starved for 30 days rapidly increase their uptake of carbon over time when incubated in nitrogen rich water (Cook et al. 1992). Other studies on the hermatypic coral *Pocillopora damicornis* and the hydroid *Myrionema ambionense* concluded that an increase in cell densities and mitotic indices occurs when available levels of ammonium in solution are increased (Fitt and Cook 2001, Hoegh-Guldberg 2006).

Generally, fertilizing endosymbiotic microalgal cells with inorganic nutrients leads to an abundance of sugars and nutrients in the algal cell body that are translocated to the host. Microalgal transfer of fixed carbon to their hosts primarily in the form of glycerol (Muscatine 1967) and LMWMs that form amino acids. These molecules are then transformed and stored in host cells as lipids, wax esters and protein. All are used for anabolic activity and maintenance as well as cellular functions like osmoregulation (Battey and Patton 1987, Mayfield and Gates 2007, Dubinsky and Stambler 2011). Research conducted with *B. annulata* using ^{15}N tracers in NH_4^+ revealed a rapid accumulation of label in internal host ammonium concentrations, with the amount of labeling increasing in zooxanthella LMWM pools over time. This continued even after host ammonium concentrations began to decline and turnover rates slowed (Lipshultz and Cook 2002). The data suggest that symbiotic algae growth and metabolism is controlled by the amount of ammonium made available within host cnidarian tissues. Host feeding rates are directly linked with continual algal growth and division also supports the theory that nitrogen obtained by the host regulates algal populations (Fitt and Cook 2001). Laboratory experiments on giant sea anemones have revealed that ammonium supplements significantly enhanced the abundance and mitotic index of their microalgae, resulting in prevention of shrinkage in starved anemones (Roopin and Chadwick 2009).

Coral reef habitats are considered oceanic oases in a desert of clear, tropical waters that contain very little suspended organic matter or dissolved nutrients. Corals and other reef invertebrates are able to absorb these limited nutrients directly from the water column. Microalgal cells facilitate host absorption of dissolved ammonium, and assimilate ammonium via the glutamine synthetase/2-oxoglutamate animotranferase (GS/GOGAT) pathway (Anderson and Burris 1987, Wilkerson and Muscatine 1984), as first revealed in 1970 (Meers et al.) with the discovery of the enzyme glutamate synthase (GOGAT). Discovery of this new enzyme coupled with glutamine synthetase (GS) solved the question of which alternate pathway allowed ammonium uptake without utilizing glutamate dehydrogenase (GDH), when K_m values for GDH in cnidarians exceeded intracellular NH_4^+ levels by an order of magnitude or more (Wilkerson and Muscatine 1987). Other data have demonstrated that the uptake and assimilation of NH_4^+ is concentrated in the tentacles of sea anemones, which suggests that algal populations are a strong influence on host metabolism and nutrient uptake (Lipshultz and Cook 2002).

The regulation of *Symbiodinium* populations within host tissues is of great importance when considering host metabolism and dietary needs. These algal cells provide a source of energy for the surrounding animal cells, and can supplement up to 90% of host dietary needs, an especially important fact in an oceanic desert such as the tropical waters around coral reefs (Muscatine et al. 1984, Rahav et al 1989). Other major molecules absorbed and utilized by host cnidarian cells include amino acids and mycosporine-like amino acids (MAAs), which can only be found in various plants, fungi and protozoans (Haslam 1974) with many animals obtaining them from their diet. These molecules have photoprotective properties, with an absorbency spectrum of ~310-360 nm (Bandaranayake 1998, Stochaj et al. 1994). This class of molecules can confer advantages to cnidarian species living in the upper photic zone with daily exposure to

high levels of UVR (ultraviolet radiation) by combating the potentially detrimental effects of the UVR extremes that are characteristic of coral reef environments. These molecules have been shown to increase in macroalgae when they are subjected to high levels of nutrients (Korbee et al. 2004, Korbee et al. 2005), and thus also may be enhanced in endosymbiotic microalgae by nutrient enrichment to host sea anemones.

This study quantifies rates of ammonia excretion by anemoneshrimps and client fishes of host sea anemones, as well as ammonia uptake rates of the anemones. It also determines whether nitrogen excreted by ectosymbiotic shrimps leads to enhanced levels of fitness-related characteristics in host sea anemones and their associated microalgae. The fitness characters examined here are host body size and protein content, and microalgal *Symbiodinium* abundance, mitotic index, growth rate, chlorophyll a and MAA content. Changes in these parameters are examined in response to experimental manipulation of shrimp presence and nutrient exposure.

Methods

Animal Collection and Maintenance

Individuals of the Corkscrew sea anemone (*Bartholomea annulata),* the spotted anemoneshrimp *Periclimenes yucatanicus*, and Pederson's cleaner shrimp *Ancylomenes pedersoni* were collected by hand from hard bottom and rubble habitats at 0-3m depth on the bayside of the upper and middle Florida Keys. They were placed in plastic bags of seawater,

transferred to coolers with aerated seawater changed approximately every 12 hours, and transported to Auburn University within 1-3 days of collection. Individuals of bicolor damselfish *Stegastes partitus*, yellowtail damselfish *Microspathodon chrysurus*, and longfin damselfish *Stegastes dienceaous* were ordered from a commercial collector (Dynasty Marine, Marathon, FL) and shipped live to Auburn University. These 3 fishes were selected for study due to their small size (5-15 cm length) and omnivorous diet (Humann and Deloach 1989), which make them easy to culture in closed-system aquaria, and because damselfishes are common clients of Pederson's cleaner shrimp (Cheney and Coté 2001, Huebner and Chadwick 2012b)

For at least one month prior to laboratory experiments, all organisms were maintained in 40 gallon saltwater aquarium tanks (77cm x 33cm x 32cm) at Auburn University under controlled conditions. Lights were kept on 12:12 hour timers and salinity (34-36 ppt) and temperature (25-26°C) were monitored daily and kept stable. Tanks were illuminated with 400W Radium Metal Halide Lamps (Ocean Light 250, AquaMedic). PAR (photosynthetically active radiation) levels in all tanks averaged 66-73 $\mu E/m^2/sec$; within the range of recorded irradiance levels in natural reef habitats occupied by these anemones (Nelson 2008), as measured using a QSL-2001 Scalar PAR Sensor (Biospherical Instruments, San Diego, CA, USA; for details on culture regime see Roopin and Chadwick 2009, Huebner et al. 2012). Nutrient levels were monitored throughout the experimental period, and levels of NH_4^+, NO_2^- and NO_3^{-2} were maintained at low levels to mimic coral reef conditions. All animals were fed regularly a diet of frozen shrimps (San Francisco Bay Brand Newark, CA, USA) and a commercial pellet food (Ocean Nutrition San Diego, CA, USA). Fish and shrimps were fed daily, while anemones were fed weekly. All organisms were measured regularly, with most maintaining their original body sizes or growing during regular laboratory culture.

Nitrogen excretion and uptake rates

To quantify ammonium excretion rates by anemoneshrimps, a phenolhypochlorite method and methods adjusted from Solorzano (1969) and Spotte (1996) were used. Glass beakers (Fischer Scientific) for all analyses were rinsed first with 10% HCl solution, and then rinsed again with microfiltered saltwater (MFSW- 0.45µm). Beakers of MFSW were placed in a polystyrene tub with heated water (25-26°C) and a water circulation pump. To measure shrimp excretion rates, each individual shrimp (*A. pedersoni:* 0.06-0.25 grams wet mass, N = 12; *P. yucatanicus*: 0.12-0.36 grams wet mass N = 10) was placed in 250 mL of MFSW water for excretion studies. All shrimp excretions are daytime rates at 2 hours after feeding. All experiments included an empty vessel of MFSW as a control, in which the change in ammonium level was <1.0% of maximum ammonium levels recorded in experimental vessels thus adjustments were not made to compensate for atmospheric loss or bacteria utilization of ammonium. Light levels in sea anemone ammonium uptake experiments were maintained using halogen lamps, at natural levels described above. Water samples (5mL) were taken from the beakers every 40 minutes for 2 hours, and immediately prepared for ammonium analysis (for methods see Solorzano 1969, Roopin et al. 2009).

To quantify rates of ammonium excreted by potential client fishes of these anemoneshrimps, individuals of *S. partitus* (5.8-7.2 grams wet mass, N = 8), *M. chrysurus* (4.3-5.9 grams, N = 4), and *S. dienceaous* (4.2 – 5.7 grams, N = 3) each were incubated separately in 750 mL of MFSW, with water samples taken every 20 minutes for 1 hour. Sampling intervals for

fishes were more frequent than for shrimps because the fish are larger vertebrates, so they produced more ammonium per unit time compared to shrimps. Beakers containing fishes received airstone bubblers to ensure high dissolved oxygen levels during excretion measurements of ammonium. Excretion rates were measured for fishes both when starved (24 and 48 hours without food) and fed (2 hours after feeding, after Roopin et al. 2008).

Daytime rates of ammonium uptake were measured for host sea anemones *B. annulata* because they contain *Symbiodinium* microalgae, and thus were able to absorb inorganic forms of nitrogen from the surrounding water column during the daytime (Muscatine and D'Elia 1978, Wilkerson and Muscatine 1984, Lipschultz and Cook 2002). Each anemone used in treatment groups for laboratory experiments (see details below, N = 39) also was examined for variation in ammonium uptake rates among individuals, within 1 week after the experiments ended. Sea anemones were placed in 1L of MFSW in beakers under florescent light bulbs at the above natural irradiance levels. Due to their sessile nature, the anemones were allowed 20-30 minutes for acclimation from the disturbance of moving to the beakers, so they could attach their basal discs to the beaker and expand their tentacles. 5mL water samples then were taken from each beaker every 40 minutes for 2 hours, and immediately processed for quantification of NH_4^+ (Solorzano 1969).

Effects of shrimp nitrogen excretion on host anemones

To determine potential impacts of nitrogen excretion by anemoneshrimps on host sea anemones, a preliminary laboratory experiment was conducted, in which 4-5 sea anemones were

randomly assigned to each of 9 tanks (N = 32 sea anemones total), and each tank then was randomly assigned to 1 of 3 treatments for a period of 6 weeks: (1) shrimp present: at least 3 individuals of *A. pedersoni* and 1 *P. yucatanicus,* (2) nutrient supplement of NH_4Cl spiked seawater (see details below), or (3) neither shrimps nor added nutrients (control, modified after Roopin and Chadwick 2009). Thus, 3-4 anemones per tank x 3 tanks per treatment = 10-11 anemones per treatment x 3 treatments, with 32 anemones total. Due to their symbiotic relationship with dinoflagellate *Symbiodinium,* these anemones are able to withstand periods of starvation (Battey and Patton 1987). Thus, to ensure that the anemones began the experiment without large energy or nutrient reserves, and were in a similar basal metabolic state so they potentially would respond to nutrient supplements, all anemones were subject to a month of starvation prior to the beginning of treatments. This starvation period was based on previous growth experiments on symbiotic anemone species in the same culture system (Godinot and Chadwick 2009, Roopin and Chadwick 2009).

The treatment of NH_4^+ enrichment (~30μM) was administered 3x per week (every other day) for the duration for the experiment (6 weeks), with each enrichment period lasting 1.5-2 hours. This differs from methods in Roopin and Chadwick (2009), in which individuals of the coral reef sea anemone *Entacmea quadricolor* were placed in nutrient treatments (~10μM) every day, because individuals of *B. annulata* are more delicate in body form, with more slender, longer columns and delicate tentacles. Thus, nutrient treatments were reduced in frequency to every other day, to avoid damage to the delicate body structure of this anemones through frequent moving and disturbance of individuals. Ammonium supplement concentration was increased in the present study, to compensate for the infrequency of nutrient baths compared to previous studies (Roopin et al. 2008). To control for effects of the physical disturbance of

moving anemones to and from the nutrient enrichment containers, every other day all sea anemones in all treatments were carefully extracted from their tanks and each placed a 1L plastic container which fit inside the experimental tank. Sea anemones in shrimp and control treatments were exposed to micro filtered saltwater (MFSW, 0.45μ) with no added nutrients, while nutrient enrichment anemones were placed in MFSW spiked to a concentration of 30 μM/L. MFSW was used to ensure that no microorganisms utilized the inorganic nitrogen in the nutrient treatment water.

The above preliminary experiment was conducted during June to August 2012 (N = 32) with all anemone parameters measured only at the end of the experimental treatments. Then a more complete experiment was run during March to May 2013 (N = 39), with all anemone parameters measured both before the start of treatments, and after they had ended. In both experiments, the following parameters were measured for each anemone: body size, protein content, chlorophyll a content, *Symbiodinium* density and mitotic index (after Wilkerson et al. 1983, Kuguru et al. 2007, Godinot and Chadwick 2009, Roopin and Chadwick 2009). Mycosporine-like amino acid (MAA) content was analyzed only in the second experiment

Measurement of physiological parameters in sea anemones

To assess changes in anemone body size, tentacle crown surface area (TCSA) and oral disc surface area (ODSA) were measured. Calipers were used for all measurements, and calculations were made using the equation for the elliptical shape of the oral disc of sea anemones (Hattori 1991, Mitchell and Dill 2005, Huebner et al. 2012).

$$\text{TCSA} = \left(\frac{L}{2} \cdot \frac{W}{2} \cdot \pi \right)$$

To determine physiological parameters in tentacles of sea anemones, 5 tentacle clippings were obtained from each individual sea anemone, each 1-2 cm in length and weighing 0.025-0.0120 grams. Clippings were made from long tentacles attached to the inner oral disc, avoiding the smaller outer tentacles, with no single tentacle sampled twice. Tentacle selection was based on length of tentacle, ease of collection and minimization of harm to the anemone. Clippings were taken at midday, both before and after 6 weeks of treatments, in the case of the second experiment. Each tentacles sample was homogenized in 1mL of MFSW and centrifuged at 5g for 5 minutes at ~24°C, following a standard protocol for cnidarian tissue and algal separation. Supernatant containing homogenized animal tissue from samples was saved for immediate protein analysis. The remaining pellet of microalgal cells was vortexed thoroughly before being diluted for microalgal cell counts, mitotic index and chlorophyll analysis (after Porat and Chadwick-Furman 2005, Roopin et al. 2011).

Analyses of protein content in the animal fraction of tentacle samples were performed using the Bradford Method (BioRAD Quick start, after Roopin et al. 2011). Serial dilutions were made for the standard curve according to instructions provided with the BioRAD kit. Protein samples were analyzed immediately after tentacle homogenization (5000rpm for 5 minutes) at 590nm using a Genesys 5 spectrophotometer.

To assess microalgal cell abundances in anemone tentacles, five subsamples of each diluted algal slurry were taken and *Symbiodinium* cells counted using a haemocytometer under 400x magnification with a confocal microscope. Preliminary 24 hour studies of *B. annulata* revealed no discernible diel pattern of microcell division in this species, similar to many

Caribbean corals (Wilkerson et al. 1983, Wilkerson et al. 1988), but unlike *E.quadricolor* and some other sea anemone species previously investigated (Wilkerson et al. 1988, Roopin et al. 2011). Environmental influences, habitat or climate could be a possible explanation for this deviation from other documented anemone species. Therefore, tentacles clippings were taken haphazardly between the hours of 10:00 am and 4:00 pm. Mitotic index was calculated as a percentage of doubling cells per 1000 cells. Doubling times and growth rates were calculated using methods and equations described in Wilkerson et al. (1983).

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

To analyze anemone tentacles for MAA content, a different set of 5 tentacles was clipped from each anemone, in order to accurately quantify MAA amounts and normalize them to host tissue protein content. Due to retraction of tentacles immediately after clipping, it is unlikely that any tentacles were sampled twice in this study. Tentacle clippings were blotted dry and weighed before being manually homogenized using a Potter-Elvehjem tissue grinder at room temperature. Samples were centrifuged for 5 minutes at 5000rpm. Protein portions were set aside for immediate analysis (see above to normalize MAA content to protein content. Algal pellets were concentrated using an Eppendorf Vacufuge for 1 hour or until dry. Pellets were frozen for

later analysis at -10°C for up to 2 weeks. Frozen pellets were thawed and later suspended in 25% (v:v) methanol and sonicated using a Branson Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT) at 30W for 60 seconds, after which samples were heated at 45°C for 2 hrs. This extraction method was chosen due to the high extraction rate reported in Tartarotti and Sommaruga (2002). The solution was centrifuged at 10000rpm for 10 minutes. The resulting pellet was discarded and the supernatant filtered through a Millipore (Millex GP) 0.22µm filter. This supernatant was then stored at -80°C until LC/MS and spectrophotometric analysis.

Liquid Chromatography /Mass Spectrometry

Extraction samples for MAA content were analyzed using an Ultra Performance LC Systems (ACQUITY, Waters Corp., Milford, MA, USA) coupled with a quadruple time-of-flight mass spectrometer (Q-Tof Premier, Waters) with electrospray ionization (ESI) in ESI^+-MS mode operated by the Masslynx software (V4.1). Each sample, in aqueous solution, was injected into a C18 column (ACQUITY UPLC® BEH C18, 1.7 µm, 2.1 x 50 mm, Waters) with a 150 µL/min flow rate of mobile phase of solution A (95% H_2O, 5% acetonitrile, 0.1% formic acid) and solution B (95% acetonitrile, 5% H_2O, 0.1% formic acid) in a 10 min gradient starting at 95% A to 5% A in 6 min and back to 95% in 8 min.

The ion source voltages were set at 3 KV, sampling cone at 37 V and the extraction cone at 3 V. The TOF MS scan was from 200 to 800 *m*/*z* at 1 s with 0.1 s inter-scan delay. For s calibration, sodium formate solution (10% formic acid/0.1M NaOH/isopropanol at a ratio of 1:1:8) at 1 sec/10 sec to ion source at 1µL/min was used.

LC/MS conditions were tuned so that metabolites were separated from each other in a linear gradient while maintaining optimal sensitivity. The instrument was calibrated at the time of data acquisition in addition to real time calibration by the lockmass. Mass accuracy at 5 ppm or less was the key for assuring the presence of target molecules from abundant noise molecules from the biological samples that could increase false positive results. Ion source parameters such as the source temperature (gas and sample cone), mobile phase flow rate, and cone voltage were fixed throughout analysis.

Ions of interest were analyzed for mass accuracy, elemental composition (using accurate mass measurement of less than 5 ppm error) and isotope modeling to identify the formula. Identification of unknowns were confirmed by having the same retention times and molecular weight.

Extracted MAAs were taken to the Mass Spectrometry Laboratory at Auburn University for acquisition and data analysis. Correspondence with published experts on MAA analysis in cnidarians revealed a lack of either individually prepared or commercially produced standards for MAA assessment in corals and sea anemones (personal communication, J.I. Carreto [South America], W.C. Dunlap [Australia], U. Karsten [Europe], and J.M. Shick [North America]). As such, after identification via LC/MS, samples were quantified using published maximum UV absorbencies and extinction coefficients (Bandaranayake 1998) and the Beer-Lambert equation. This method has been used recently as an accepted means to quantify MAAs in a Caribbean coral species (Torres-Perez and Armstrong 2012).

Statistical Analyses

All statistical analyses were conducted using R 3.0.2 For Statistical Computing. Variation in excretion rates between the two shrinp species examined was analyzed using nonparametric procedures (Mann-Whitney-Wilcoxon tests), while fish excretion rates were analyzed using repeated measures ANOVA with time as a repeated variable, because they were assessed at several times since feeding. Data from the preliminary experiment were analyzed using nonparametric methods (Kruskal-Wallis tests), while results from the second experiment were analyzed using repeated measures ANOVA with time as a repeated variable for all treatments. Tukey post-hoc testing was utilized to identify which groups differed from each other. Differences among groups were considered significant at $p < 0.05$ for all statistical tests. All results are presented as means +/- one standard error unless otherwise noted.

Results

Ammonium excretion by shrimps and fishes

Fed individuals of the spotted anemone shrimp *Periclimenes yucatanicus* (N =10) excreted ammonium at a rate of 3.44 ± 0.61 μM g^{-1} hr^{-1}, while fed individuals of Pederson's cleaner shrimp *Ancylomenes pedersoni* (N = 12) excreted ammonium at 1.16 ± 0.12 μM g^{-1} hr^{-1} (Figure 1), significantly less than *P. yucatanicus* (Mann-Whitney U-test, U = 8.81, $p < 0.01$, Figure 2). Excretion rates for *P. yucatanicus* ranged from 1.03 to 5.96 μM NH_4^+ g^{-1} hr^{-1}, while those for *A. pedersoni* ranged from only 0.74 to 1.72 μM g^{-1} hr^{-1}. Rates of mass specific excretion for *A. pedersoni* increased with body mass, while no clear pattern was visible for *P.*

yucatanicus (Figure 3). Thus, whole-individual rates of excretion by *P. yucatanicus* (mean body mass 0.27 grams) and *A. pedersoni* (0.13 grams) were approximately 0.87 μM NH_4^+ hr^{-1} (0.014 μM NH_4^+ min^{-1}) and 0.17 μM NH_4^+ hr^{-1} (0.003 μM NH_4^+ min^{-1}), respectively.

Rates of excretion by bicolor damselfish *Stegastes partitus* (N = 8) at 2 hours after feeding were 3.75 ± 0.17 μM NH_4^+ g^{-1} hr^{-1}, at 24 hours after feeding 2.42 ± 0.21 μM NH_4^+ g^{-1} hr^{-1}, and at 48 hours after feeding 3.66 ± 0.31 μM NH_4^+ g^{-1} hr^{-1}(Figures 4 & 5). These rates did not vary significantly with time since feeding (repeated measures ANOVA, $F = 0.012$, $p = 0.916$). Mass-specific excretion rates decreased with increasing body mass (Figure 6). The total amount of ammonium excreted per individual was approximately 0.21 μM NH_4^+ min^{-1} for individuals below 5 grams body mass, to about 0.41 μM NH_4^+ min^{-1} for individuals above 5 grams (using basal excretion rate after 48 hours starvation).

Rates of excretion by the yellowtail damselfish *Microspathodon chrysurus* (N = 4) at 2 hours after feeding were 4.85 ± 0.42 μM NH_4^+ g^{-1} hr^{-1}. Starved rates of excretion were 2.35 ± 0.16 μM NH_4^+ g^{-1} hr^{-1} after 24 hours of fasting, and 1.65 ± 0.1 μM NH_4^+ g^{-1} hr^{-1} after 48 hours of fasting (Figure 7). Excretion rate varied significantly with time since feeding (Repeated measures ANOVA, $F = 40.67$, $p < 0.001$, Figure 8), with all pair-wise comparisons being significantly different (Tukey post-hoc tests, $p < 0.05$), except between 24 and 48 hours since feeding ($p = 0.20$). Rates of excretion in the fed state resulted in 2x to 4x higher rates of excretion when compared to 24 and 48 hours starved, respectively. Whole-animal excretion rates ranged from about 0.14 μM NH_4^+ min^{-1} (for individuals < 5.1 grams) to 0.44 μM NH_4^+ min^{-1} for individuals > 5.5 grams.

Rates of excretion in the longfin damselfish (N = 3) *Stegastes diencaeus* were 3.89 ± 0.13 μM NH_4^+ g^{-1} hr^{-1} at 2 hours after feeding and 1.58 ± 0.26 μM NH_4^+ g^{-1} hr^{-1} at 24 hours after feeding, and 1.60 ± 0.36 μM NH_4^+ g^{-1} hr^{-1} at 48 hours (Figure 9). They varied significantly with time since feeding (repeated measures ANOVA, $F = 12.62$, $p < 0.05$), except for between the 2 starved states (Tukey post-hoc test, $p = 0.99$, Figure 10). Whole-animal excretion for these juvenile fish was about 0.13 μM NH_4^+ min^{-1} (<4.9 grams body mass) to 0.33 μM NH_4^+ min^{-1} (>5.1 grams).

Ammonium uptake by sea anemones

The wet mass of individuals of *B. annulata* correlated positively with tentacle crown surface area (TCSA, Figure 13). Thus, all uptake rates were converted to mass-specific values based on TCSA–body mass conversions. Sea anemones that had been subjected to experimental treatments (shrimp, nutrients or neither, see Methods) had similar nitrogen uptake rates, with no significant difference among groups (ANOVA, $F = 0.098$, $p = 0.907$). Rate of ammonium uptake by *B.annulata* in all treatments pooled together (N = 32) was 3.47 ± 0.03 μM $L^{-1}hr^{-1}$. Sea anemone ammonium uptake rates declined significantly with increasing mass (Figure 12). Mass specific rates were calculated via linear regression based upon individuals weighed and measured in lab (Figure 13). Ammonium uptake rates for individuals that had been cultured with anemoneshrimps (0.40 ± 0.07, μM g^{-1} hr^1), ammonium supplements (0.37 ± 0.12 μM g^{-1} hr^{-1}), or neither (0.23 ± 0.05 μM g^{-1} hr^{-1}) for the preceding 6 weeks were similar to those for the whole group together (Figure 12). The whole-animal nitrogen uptake rate for individuals of *B. annulata*

was around 5.05 μM NH_4^+ hr^{-1} or 0.08 μM NH_4^+ min^{-1} , based on a mean uptake rate of 0.34 μM NH_4^+ g^{-1} hr^{-1} and 14.9 grams body mass on average.

Effects of shrimp and nutrient treatments on sea anemones

In the preliminary experiment, none of the examined parameters varied significantly among anemones after exposure to the 3 types of 6-week treatment groups. Anemone body sizes did not exhibit a clear pattern of overall increase or decrease in any treatment group. The mitotic indices of endosymbiotic microalgae also did not vary among the 3 treatment groups (Kruskal-Wallis test, $X^2 = 3.47$, df = 2, p = 0.18) with indices for the shrimp, nutrient, and control treatments being 3.57 ± 0.75 %, 5.78 ± 0.93 % and 4.67 ± 0.71 %, respectively (Figure 15 B). Thus, at any given time, 3-6% of the microalgae cells in all of the sea anemones were undergoing mitotic division (Figure 15B). The T, or doubling time of groups also did not differ significantly between the three treatments (Kruskal Wallis test, $X^2 = 3.31$, p = 0.19) at 15.94 ± 3.65 days for sea anemones exposed to symbiotic shrimps, 9.76 ± 3.56 days for sea anemones exposed to nutrient -rich water, and 8.25±0.98 days for control anemones exposed to neither treatment.

The growth rate (day^{-1}) of *Symbiodinium*, or μ, was 0.08 ± 0.02 in the shrimp treatment, 0.12 ± 0.02 in the nutrient treatment, and 0.10 ± 0.01 in the control treatment, and did not vary significantly among the groups (Kruskal Wallis test, $X^2 = 3.31$, $p = 0.19$).

The abundance of *Symbiodinium* cells varied widely among individual anemones and did not very significantly among treatments (Kruskal-Wallis test, $X^2 = 1.37$ $p = 0.50$); measured levels were $1.38 \times 10^8 \pm 8.65 \times 10^6$, $1.53 \times 10^8 \pm 1.36 \times 10^7$, and $1.99 \times 10^8 \pm 3.43 \times 10^7$ cells gwm^{-1} in the shrimp, nutrient and control groups, respectively (Figure 15A).

Chlorophyll a level per *Symbiodinium* cell likewise did not vary significantly with treatment (Kruskal-Wallis test, $X^2 = 5.23$, $p = 0.07$), and was 1.26 ± 0.43, 0.35 ± 0.11, and 0.71 ± 0.45 pg $cell^{-1}$ in the shrimp, nutrient and control treatments, respectively (Figure 16A). However, the p-value for this comparison was almost significant, and the mean values above show that chlorophyll in the shrimp treatment was almost 3x that in the nutrient treatment and almost twice that in the control.

Finally, the protein content of host anemone tissues did not vary significantly (Kruskal-Wallis test, $X^2 = 0.63$ $p = 0.73$) among groups (Figure 16B). Anemone tissue in each group had a narrow range of protein content between 30.0 -35.00 mg g^{-1}. Shrimp treatment protein levels were 34.56 ± 3.26 mg g^{-1}, nutrient treatment levels were slightly lower at 32.45 ± 2.80 mg g^{-1}, and control sea anemones had the lowest protein content of 31.35 ± 3.30 mg g^{-1} (Figure 8B).

In the second experiment, similar but slightly different trends were observed. Anemone body size measurements (TCSA) varied widely among individuals, but did not vary significantly with treatment (repeated measures ANOVA, $F = 1.14$, $p = 0.33$). The average change in TCSA for the nutrient group averaging -0.5%, -9.7% in the control group, and +17% in the shrimp

group TCSA declined in all 3 groups, from 1003.01 ± 272.59 cm^2 to 968.37 ± 241.49 cm^2 when cultured with shrimps . from 970.61 ± 148.18 cm^2 to 689.00 ± 90.80cm^2 when exposed to nutrients, and from 1031.66 ± 146.24 cm^2 to 610.18 ± 88.49cm^2 when exposed to neither (Figure 14A).

Oral disc surface area (ODSA) also did not vary significantly with treatment (repeated measures ANOVA, $F = 0.73$, $p = 0.49$), but did not decline in all groups.. Sea anemones subjected to nutrient treatments declined from 70.82 ± 18.00 cm^2 to 46.97 ± 6.07 cm^2 and control sea anemones declined from 69.96 ± 13.02 cm^2 to 53.36 ± 11.77 cm^2, while anemones cultured with shrimps increased slightly (but not significantly) in OSDA from 60.42±12.33 cm^2 to 76.67±14.78 cm^2 (Figure 14B).

Mitotic indices of *Symbiodinium* were significantly different among treatment groups (repeated measures ANOVA, $F = 7.47$ $p \leq 0.001$), with a significant difference between anemones given nutrient supplements and control anemones (Tukey post-hoc tests, $p < 0.001$). The shrimp treatment group did not vary significantly from those given nutrient supplements ($p>0.05$). The algal mitotic indices for nutrient treatments almost doubled from 6.14 ± 0.86% to 11.8 ± 0.77% after 6 weeks of experimental treatment. Anemoneshrimp treatment indices also increased slightly from 5.97 ± 0.56% to 7.90 ± 1.38%. In contrast, for the control anemones exposed to neither anemoneshrimp nor nutrients, indices remained stable averaging 5.16 ± 0.64% at the beginning of the experimental period to 5.80 ± 1.0% at the end of experimental period (Figure 18). Thus, algal division rates almost doubled with exposure to increased levels of nutrient treatments and became significantly higher than the control treatments.

The doubling time, or T, of each group also varied significantly among treatments over time (repeated measures ANOVA, $F = 5.35$, $p \leq 0.01$), with a significant difference between only the nutrient and control groups (Tukey post-hoc, $p < 0.05$). Doubling times at the beginning of the experiment were 6.00±0.48 , 6.18±0.89 and 7.73±1.10 days for shrimp, nutrient and control treatment groups, respectively. After 6 weeks of treatments the values for the shrimp group rose to 12.48±2.80 days, while nutrient treatments stayed stable with an average of 6.17±0.43 days. Control sea anemones saw the highest increase in doubling time with an average of 19.79±4.58 days at the end of the experimental period.

The growth rate (day^{-1}) of *Symbiodinium*, or μ, before the experiment was 0.13±0.01 for the shrimp treatments, 0.13±0.02 for the nutrient treatments, and 0.11±0.01 for the control sea anemones. After the experimental treatments, microalgal growth rates of anemones exposed to anemoneshrimp rose to 0.16±0.03, while nutrient treatments saw the highest growth rate of 0.24±0.02. Control sea anemones also saw a slight increase in growth rates with an average of 0.122±0.02. These data reveal statistically significant variation in microalgal growth rates among the three groups over time (repeated measures ANOVA, $F = 7.40$, $p \leq 0.001$), with differences only between the nutrient and control groups (Tukey post-hoc, $p<0.05$).

Abundance of *Symbiodinium* cells in host tentacles did not vary significantly with treatments (repeated measures ANOVA, $F = 0.202$, $p = 0.82$, Figure 17). The abundance of microalgae in shrimp treatments increased slightly from $1.41x10^8 \pm 0.15x10^8$ cells gwm^{-1} to $2.0 x10^8 \pm 0.24x10^8$ cells gwm^{-1}. Nutrient treatments also saw a slight increase in density from $1.60x10^8 \pm 0.16x10^8$ to $1.87x10^8 \pm 0.16x10^8$ cells gwm^{-1}. Those anemones which were neither subject to anemoneshrimp nor nutrient treatments also saw a slight increase from their beginning density of $1.28x10^8 \pm 0.14x10^8$ to $2.0x10^8 \pm 0.23x108$ cells gwm^{-1} at the end.

Chlorophyll a levels additionally did not vary significantly between experimental groups (repeated Measures ANOVA, $F = 1.54$, $p = 0.22$, Figure 19). Average chlorophyll a levels in the shrimp treatment groups were 1.01 ± 0.22 pg cell^{-1} before the experiment and decreased to 0.22 ± 0.11 pg cell^{-1}. Anemones which had received dissolved nutrients also saw a decline in chlorophyll per algal cell from 0.71 ± 0.16 pg cell^{-1} to 0.20 ± 0.07 pg cell^{-1}. Anemones which received neither treatment saw a decline in chlorophyll a from 0.78 ± 0.17 to 0.31 ± 0.14 pg cell^{-1}.

Animal protein levels in all groups remained stable with no significant difference over time among treatment groups (repeated measures ANOVA, $F = 0.08$ $p = 0.93$, Figure 20). Anemones exposed to shrimp had concentrations of 36.45 ± 2.3 mg protein mg^{-1} in the beginning and 36.51 ± 3.8 mg protein g^{-1} at the end. Protein content in the nutrient treatments declined slightly from 37.20 ± 1.74 mg protein g^{-1} to 35.60 ± 3.10 mg protein g^{-1}. Anemones which received neither anemoneshrimp nor nutrient treatments averaged 36.80 ± 1.78 mg protein g^{-1} in the beginning of the experiment and 36.67 ± 2.92 mg protein g^{-1} at the end.

Total MAA levels did not vary significantly with treatment over time (repeated measures ANOVA, $F = 0.27$, $p = 0.77$, Figure 21). MAAs found in *B. annulata* were Mycosporine-glycine (λ_{max}= 310 nm), Asterina-330 (λ_{max} = 300 nm), Palythinol (λ_{max} = 332nm), Palythene(λ_{max} = 360), and Palythine-threonine-sulfate (λ_{max} = 320nm, Figures 22-26). Total MAA levels declined in all treatments by almost half. Shrimp treatments declined from 822.50 ± 49.82 to 476.00 ± 21.23 nmole mg protein $^{-1}$, while nutrients treated anemones declined from 960.26 ± 35.76 to 448.92 ± 22.17 nmols mg protein^{-1}. Control anemones saw a similar decline from 620.48 ± 35.01 in the beginning of the experiment to 397.01 ± 20.17 nmols mg protein^{-1} (Figure 21).

Discussion

The whole-organism nitrogen budget for *B. annulata* on a per minute basis reveals a demand for nitrogen at a rate that outstrips the ammonia supplied by ectosymbiotic cleaner shrimps. With each shrimp weighing on average less than 0.5 grams, their contribution is small. In contrast, potential client fishes for the cleaner shrimps on this anemone had much higher nitrogen contributions. The damselfishes used in the present study were small juveniles, but large adult reef fishes such as groupers can contribute much greater amounts of ammonia at an average rate of ~8.75 μM NH_4+ g^{-1} hr^{-1} or 0.146 μM NH_4+ g^{-1} min^{-1} (Leung et al. 1999). As such, large client fishes could be selected by cleaner shrimps over smaller ones due to their potential for greater parasite loads, which is a direct benefit to the shrimp, but also possibly because they provide indirect benefits through nitrogen excretion to host sea anemones. The trophic levels of client fishes can also have varying impacts on host sea anemones. Larger fishes such as those in the family Serranidae (groupers) and could impact microalgal population patterns in host sea anemones through providing different N:P ratios, with piscivorous species potentially delivering higher phosphorous loads (Allgiers et al. 2014). The combination of N and P contributions impacts *Symbiodinium* populations, as well as potentially providing cleaner shrimp with a major food source, more so than do the nutrient ratios excreted by smaller-bodied herbivorous fishes (Poulin and Grutter 1996). Cleaner stations could be especially negatively impacted by population declines in large client fishes, which also tend to be overfished due to their economic value. In general, fish biomass and population dynamics contribute significantly to N:P ratios

that sustain coral reefs and their adjacent environments (Allgiers et al. 2014). Thus, overfishing of large client fishes populations could have unforeseen impacts on coral reef health, and should be of special concern to coral reef managers in the Caribbean Sea.

Results from the preliminary laboratory experiment on benefits to host anemones from cleaner shrimps and nutrients were inconclusive. However, to accurately compare group changes, information from both before and after the treatments was needed. While the preliminary data did not support the idea that symbiotic shrimps contribute to anemone metabolism, they provided useful information on ranges of physiological parameters for this sea anemone, which is lacking for many tropical actiniarians. This is valuable information due to the abundance of *B. annulata* in the Florida Keys, with a recent census (Miller et al. 2009) revealing *B. annulata* as 90% of the total anemone populations surveyed from the Upper Keys to Key West.

Symbiodinium counts were similar to those reported in previous publications on sea anemones that used equivalent methodology (Stambler and Dubinsky 1987, Spotte 1996, Roopin and Chadwick 2009), as were mitotic indices (Wilkerson et al. 1983, Table 1). Additionally, specific growth rate (μ) and doubling times (T) were all within the ranges found in previous publications on other species of actiniarian anemones (Wilkerson et al. 1983, Porat and Chadwick 2005, Roopin and Chadwick 2009). Animal protein content was slightly higher in *B. annulata* compared to some previous publications (Muller-Parker 1987), but still within the range of known protein content for sea anemones (Table 1). The information in Table 1 underlines the necessity for the use of uniform methodologies by future researchers, to provide accurate species to species comparisons for sea anemones.

In the second experiment, clearer patterns emerged, the most obvious being increased microalgal mitotic indices with the addition of nitrogen supplements to the water column, resulting in different patterns of algal replication and growth. Although no apparent effects of nutrient enrichment were seen in sea anemone size or protein content, the effect on *in situ Symbiodinium* populations was significant. This indicates that while excess nutrients do not make an observable effect on the sea anemone exclusively, the impacts on microalgal populations within anemone tentacles are clear. This pattern is seen in other cnidarian microalgal populations subjected to nutrient supplements (Fitt and Cook 2001, Hoegh-Guldburg 2006, Roopin and Chadwick 2009). This, along with the fact that no significant change in algal density was detected in the present study, could suggest a high turnover rate for microalgal populations in this anemone species, with a potential to expel unwanted microalgae. Expulsion rates of microalgae have been demonstrated in some species of sea anemones, but not for corals. Rapid expulsion rates have been linked to high temperatures, with bleaching being the extreme end of microalgal regulation. Various cnidarian species have exhibited a positive linear relationship between division rates and expulsion rates of *Symbiodinium.* The physiological mechanism of this expulsion is still unknown, but it is believed that some algal cells are selected for expulsion in order to keep the holobiont at a "steady state" equilibrium. Expelled algal cells also retain higher levels of ^{3}H-Thymidine, a chemical involved in the regulation of cell cycles, than algal cells kept in the host. This also supports the hypothesis that certain cells are selected and preferred for expulsion, with cell cycle phase as an indicator (Baghdasarian and Muscatine 2000). Algal density patterns seen in this study could be an effect of this sea anemone species regulating in situ algal populations. Microalgae in starved *Aiptasia* exhibited a significant

decrease in chlorophyll and growth rates, and an increase in the carbon content of algal cells, consistent with the findings of the present study.

MAAs found here in *B. annulata* are also consistent with previous publications that have reported MAA content of cnidarians. MAAs in the stony coral *Porites furcata* that were also detected here in *B. annulata* include Mycosporine-glycine and asterina-330 (Torres-Perez and Armstrong 2012), with asterina-330 also found in the macroalga *Porphyra*. Other research has revealed mycosporine-glycine and palythinol as primary MAAs found in the stony coral *Acropora formosa,* similar to MAAs found in this study (Dunlap and Chalker 1986). Although MAAs in another actiniarian sea anemone species, *Anthopleura elegantissima,* were not found in *B. annulata,* this could be due to differences in environment, as *A. elegantissima* is a temperate sea anemone in nutrient rich waters of the Pacific Ocean along the western coast of North America (Stochaj et al. 1994).

Although, the decrease seen sea anemone size was unexpected and the results indicate that host anemones were not significantly impacted by shrimp presence alone, the data show a pattern of increasing mean oral disc size in anemones cultured with shrimps, compared to a mean decrease in the size of the oral disc in sea anemones cultured without shrimp or nutrients and those supplemented with nutrients, but no shrimps. This supports the hypothesis that sea anemones which are inhabited by shrimp may be more visible to client fishes (Huebner and Chadwick 2012b). The results of this experiment indicate that shrimp presence alone may not provide enough nutrients to significantly alter the aspects of host anemone physiology or growth measured here. It is probable that the behaviors of obligate cleaner shrimps that draw client fishes close to host anemones also are of major importance in nutrient cycling among the symbiotic partners.

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Table 1

Characteristics of sea anemone hosts and endosymbiotic microalgae

	Algal density (cells/gwm)	Algal chl a (pg/cell)	MI %	Algal mitotic regularity	μ (cell day^{-1})	T (days)	Host protein (mg/gwm)	Host nitrogen uptake rate	Source
Actiniaria									
Aiptasia pallida	ND	ND	ND	ND	ND	ND	5.0	ND	Muller-Parker 1987
A. pulchella	0.2-0.3x10^{7}	1.7-3.0	ND	ND	ND	ND	4.3-14.9	ND	Muller-Parker 1987
A. pulchella	ND	ND	<1	R	0.02	42	ND	ND	Wilkerson et al. 1983
Anemonia sulcata	4.5-6.0x10^{7}	ND	ND	ND	ND	ND	ND	ND	Stambler &Dubinsky 1987
Anthopleura elegantissima	ND	ND	3.0-5.0	R	0.06-0.10	6.9-11.2	ND	ND	Wilkerson et al. 1983
Aulactinia stelloides	ND	ND	ND	ND	ND	ND	20.0	ND	Smith 1986
Bartholomea annulata	13.0-16.0x10^{7}	0.4-1.0	5.0-6.0	R	0.11-0.24	6.0-7.7	35.1-37.2	0.05 μmol/L/min	Present study
Bunodeopsis globerifera	ND	ND	2.0-7.0	ND	0.05	ND	ND	ND	Day 1994
Condylactis gigantea	13.0-16.0x10^{7}	ND	ND	ND	ND	ND	ND	0.03-0.04 μmol/L/min	Spotte 1996
Entacmaea quadricolor	2.0-4.0x10^{7}	2.0-3.0	3.0-6.0	P [when?]	ND	ND		0.6-0.12 μmol/g/hr	Porat and Chadwick 2005
E. quadricolor	10.0-30.0x10^{7}	ND	1.0-5.0	P (08:00)	ND	ND	22.2-27.4	0.12 μmol/g/hr	Roopin and Chadwick 2009
Corallimorpharia									
Rhodactis rhodostoma	1.0-2.0x10^{7}	0.5	1.0-5.0	P [when?]	ND	ND	ND	ND	Kuguru et al. 2007

[gwm = grams of wet mass, MI = mitotic index]

Note: All data are from individuals in shallow marine habitats (<10m depth) or under laboratory conditions.

Thus, data from any deep individuals in each study have been excluded.

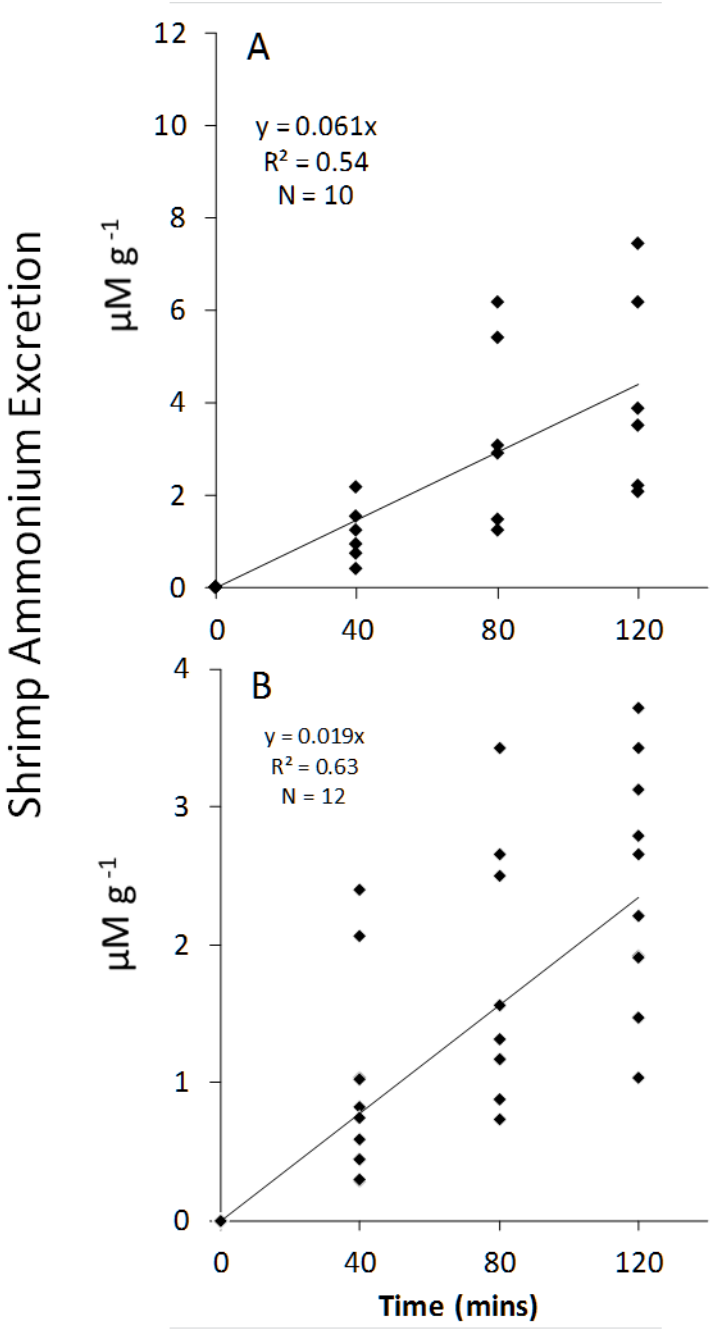
Shrimp Ammonium Excretion
A
y = 0.061x
R² = 0.54
N = 10
µM g-1
12
10
8
6
4
2
0
0
40
80
120
B
y = 0.019x
R² = 0.63
N = 12
µM g-1
4
3
2
1
0
0
40
80
120
Time (mins)

Figure 1

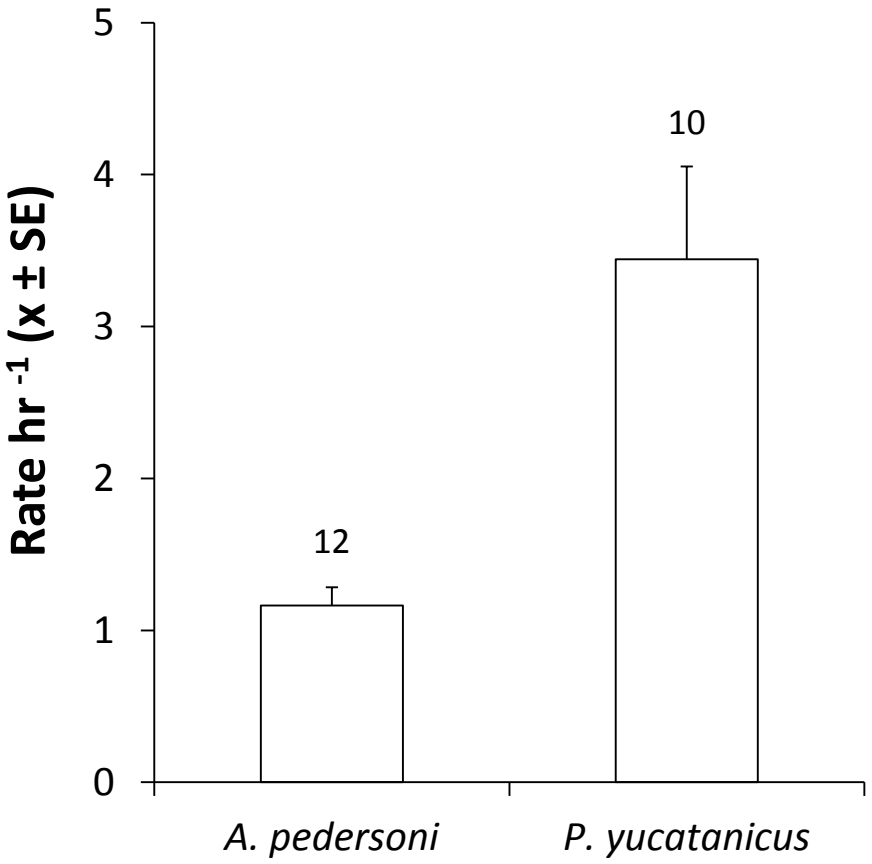

Figure 2

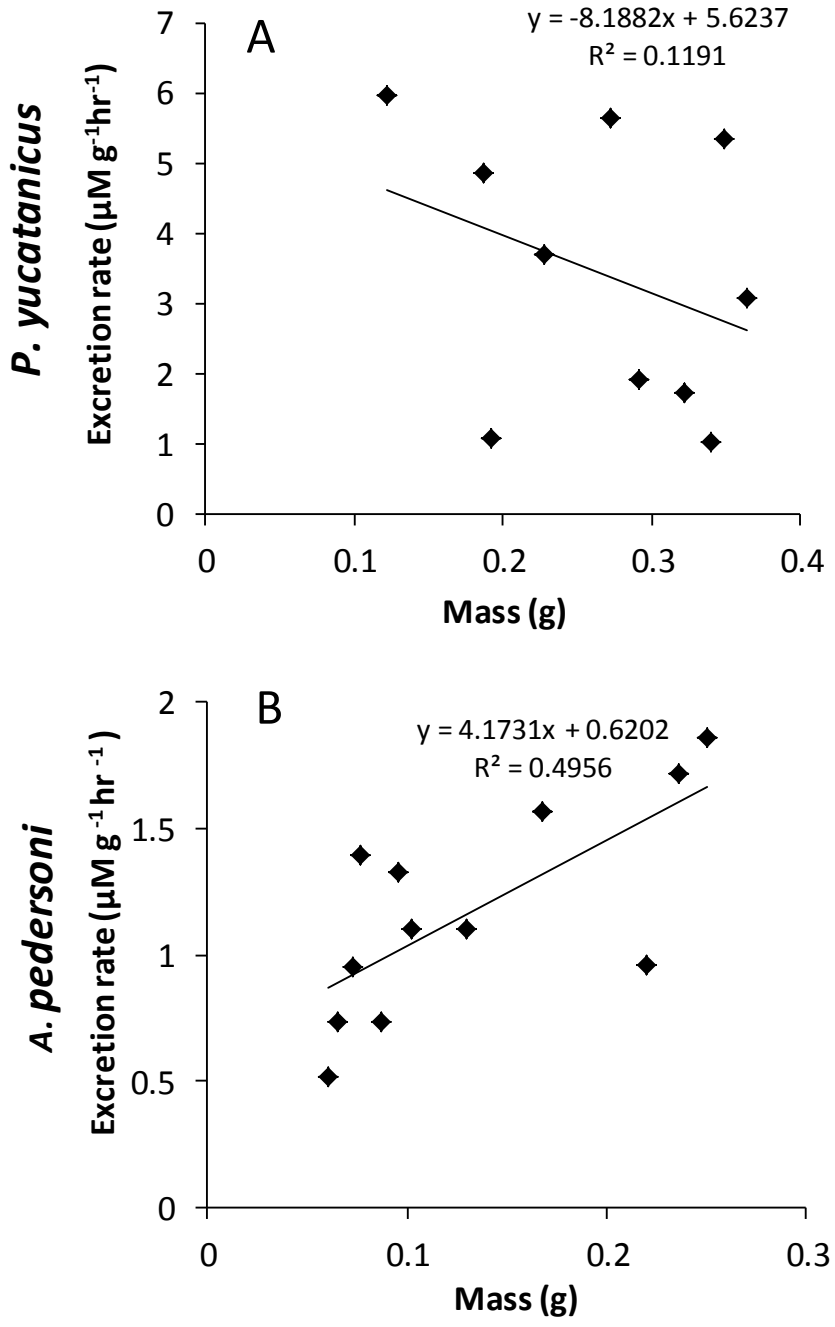
A
y = -8.1882x + 5.6237
R² = 0.1191
P. yucatanicus
Excretion rate (µM g-1hr-1)
Mass (g)
B
y = 4.1731x + 0.6202
R² = 0.4956
A. pedersoni
Excretion rate (µM g -1 hr -1)
Mass (g)

Figure 3

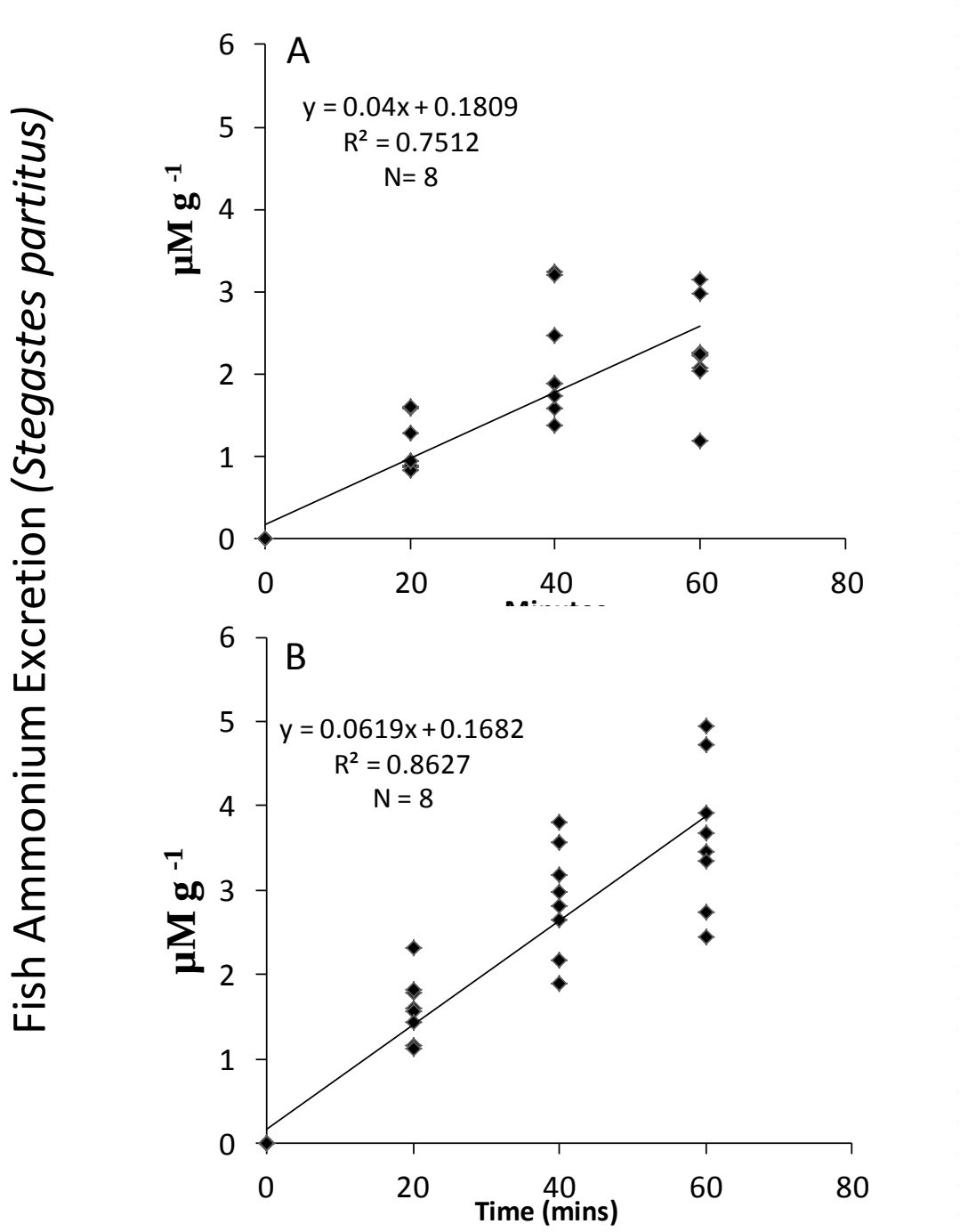
Fish Ammonium Excretion (Stegastes partitus)
A
y = 0.04x + 0.1809
R² = 0.7512
N= 8
µM g -1
B
y = 0.0619x + 0.1682
R² = 0.8627
N = 8
µM g -1
Time (mins)

Figure 4

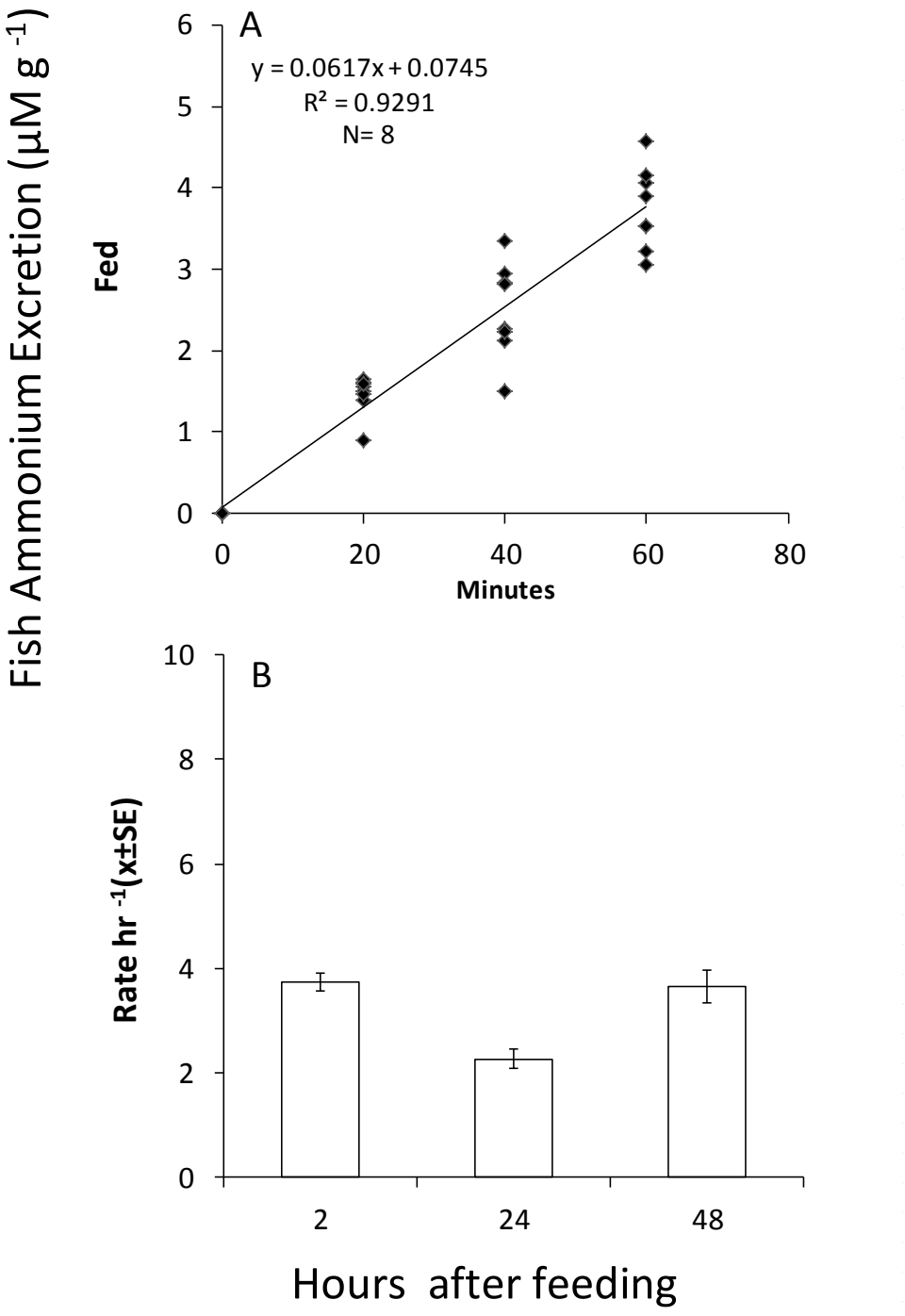
Fish Ammonium Excretion (µM g -1)
A
y = 0.0617x + 0.0745
R² = 0.9291
N= 8
Fed
Minutes
B
Rate hr -1(x±SE)
Hours after feeding

Figure 5

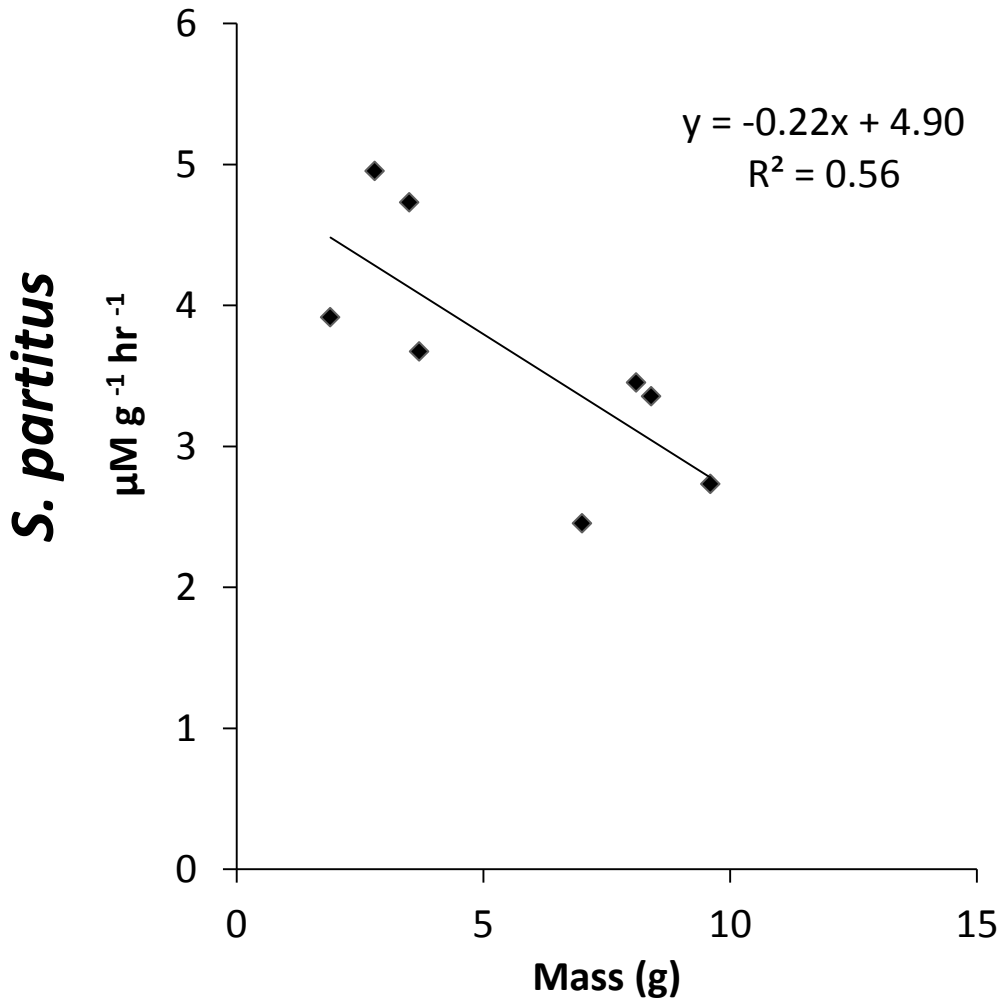

Figure 6

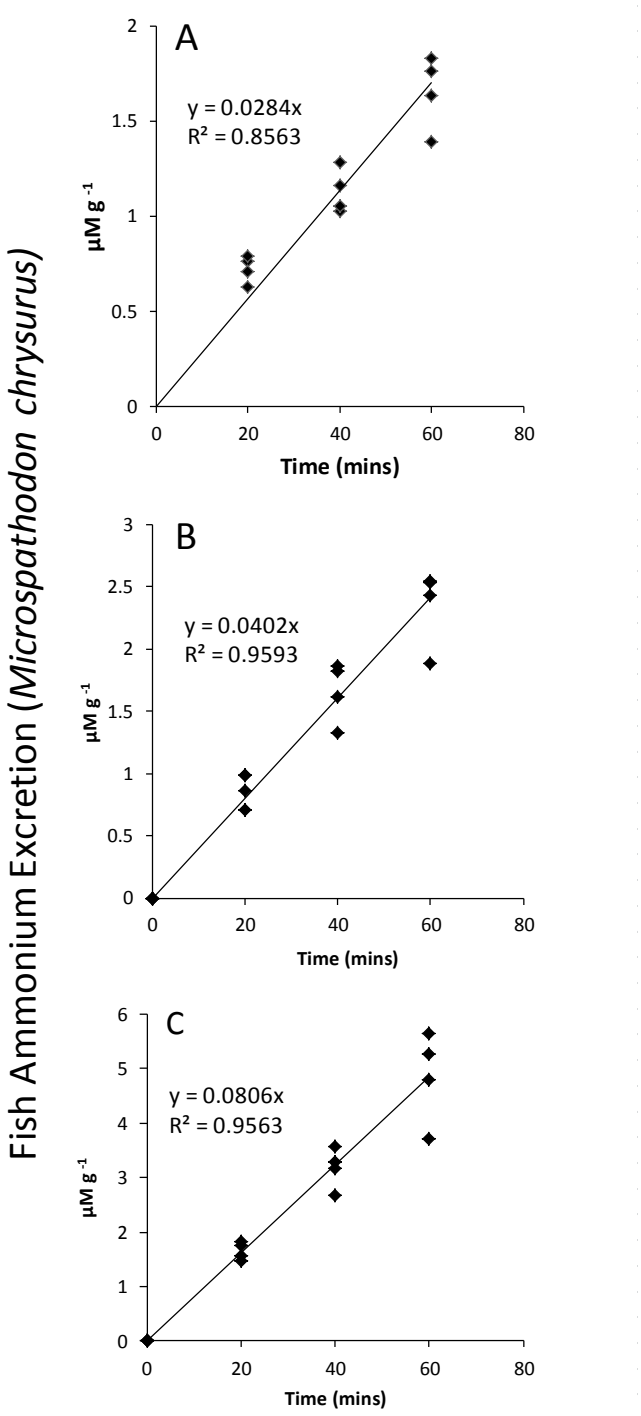
Fish Ammonium Excretion (*Microspathodon chrysurus*)
A
y = 0.0284x
R² = 0.8563
µM g -1
Time (mins)
B
y = 0.0402x
R² = 0.9593
µM g -1
Time (mins)
C
y = 0.0806x
R² = 0.9563
µM g -1
Time (mins)

Figure 7

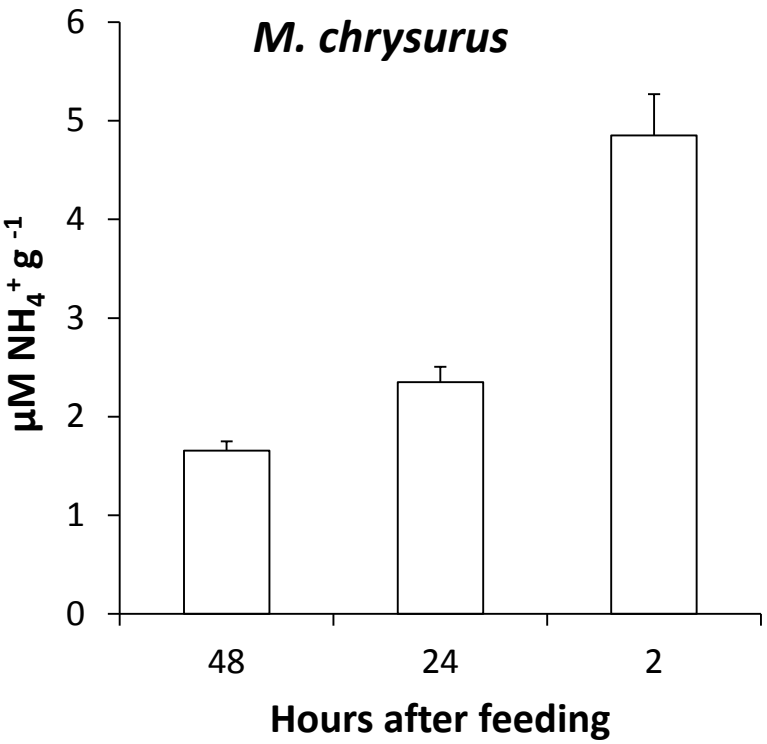

Figure 8

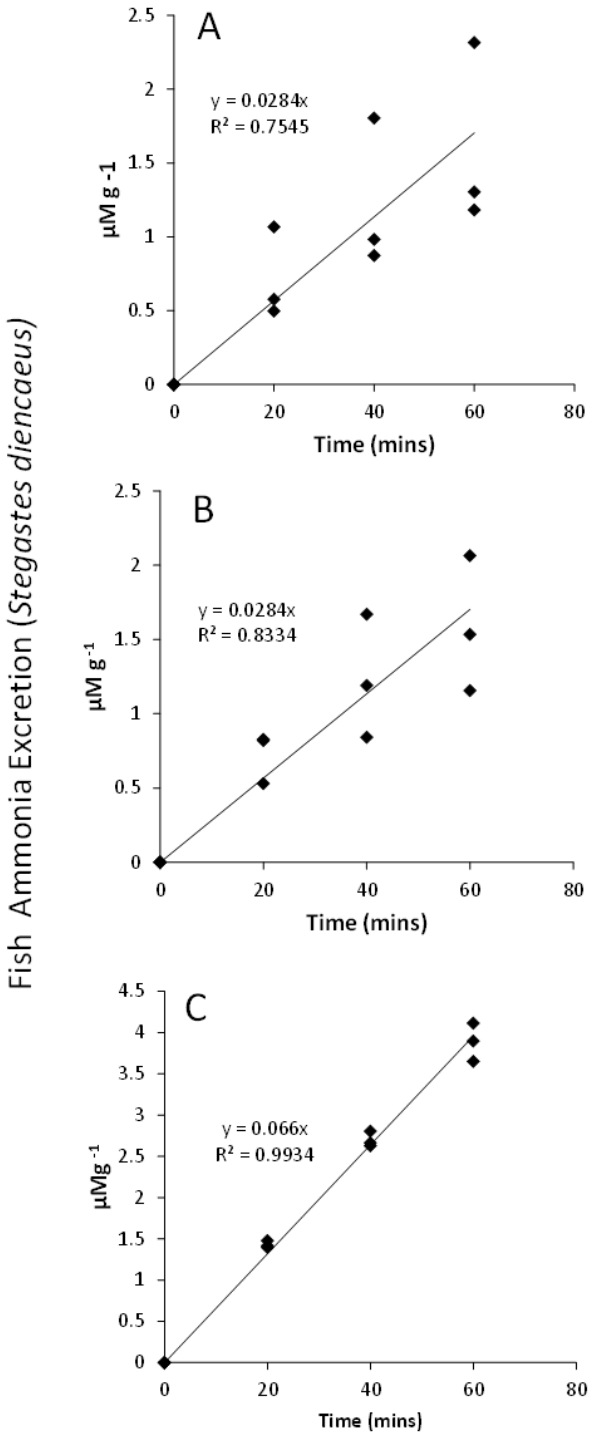
Fish Ammonia Excretion (Stegastes diencaeus)
A
y = 0.0284x
R² = 0.7545
µM g -1
Time (mins)
B
y = 0.0284x
R² = 0.8334
µM g-1
Time (mins)
C
y = 0.066x
R² = 0.9934
µMg-1
Time (mins)

Figure 9

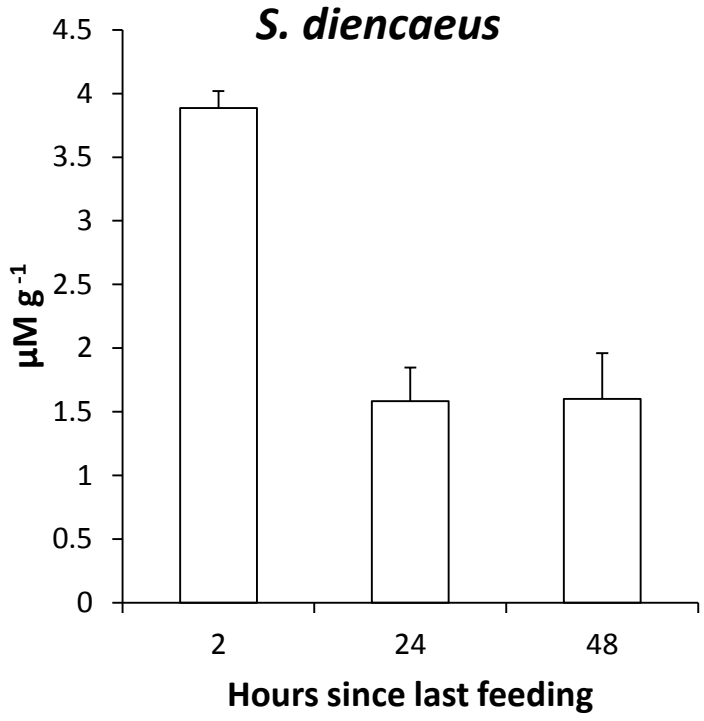
S. diencaeus
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
µM g-1
2
24
48
Hours since last feeding

Figure 10

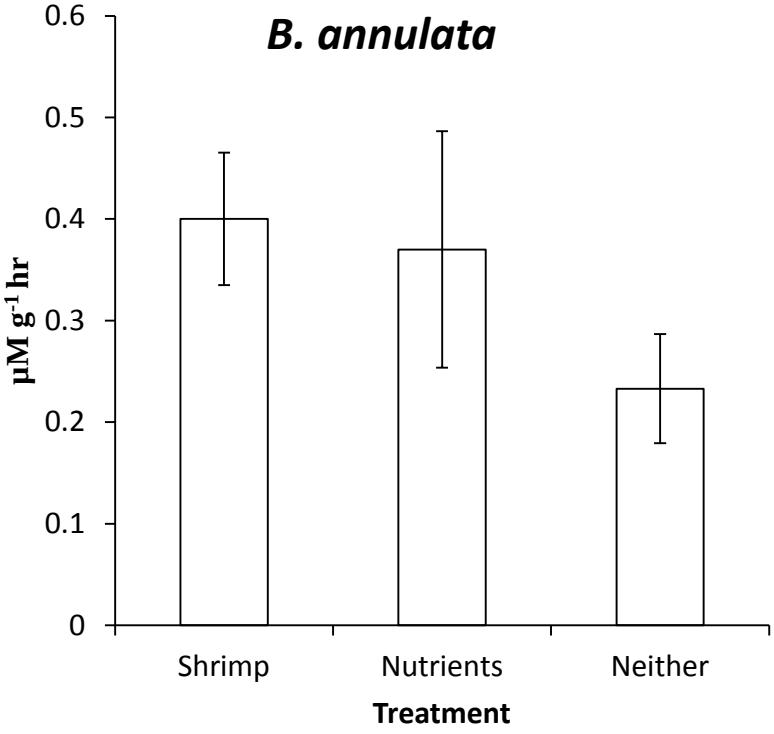
B. annulata
0.6
0.5
0.4
0.3
0.2
0.1
0
µM g-1 hr
Shrimp
Nutrients
Neither
Treatment

Figure 11

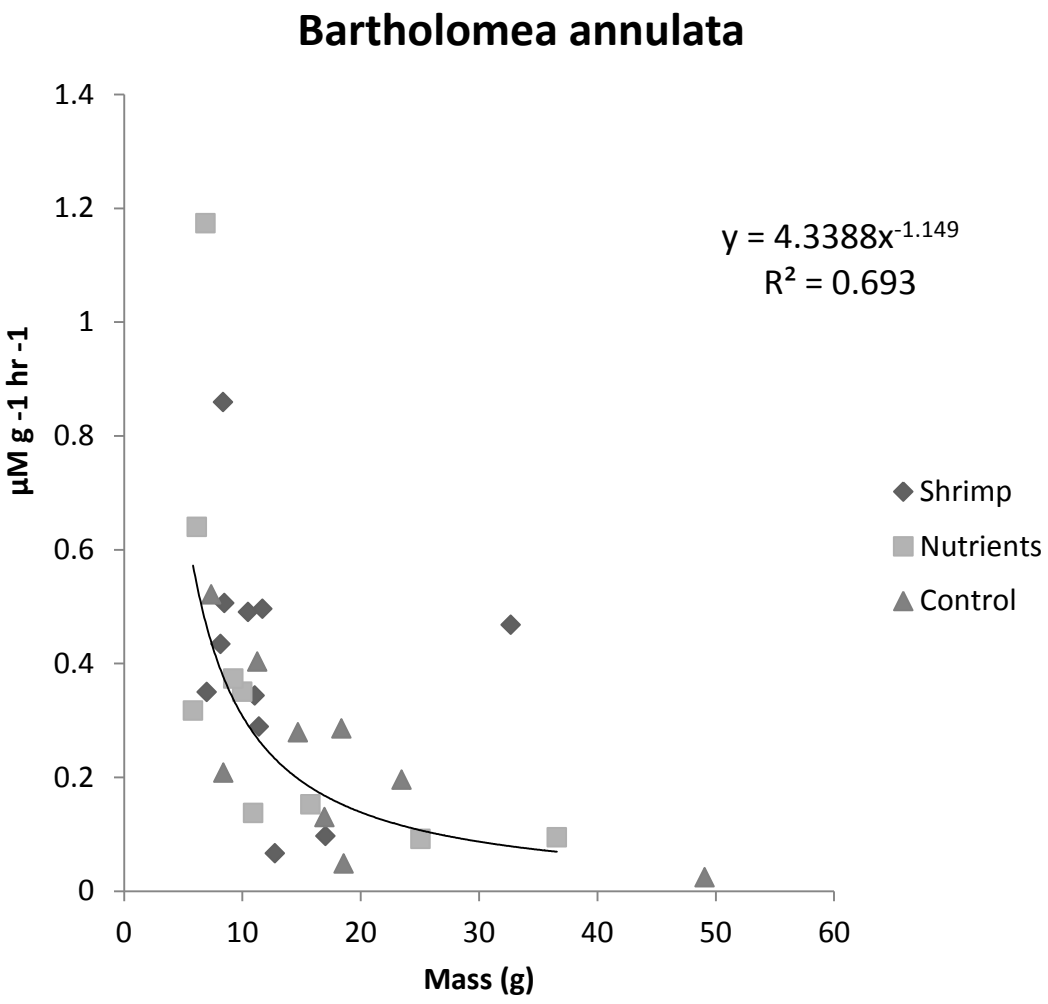
Bartholomea annulata
y = 4.3388x-1.149
R² = 0.693
µM g -1 hr -1
Mass (g)
Shrimp
Nutrients
Control
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
10
20
30
40
50
60

Figure 12

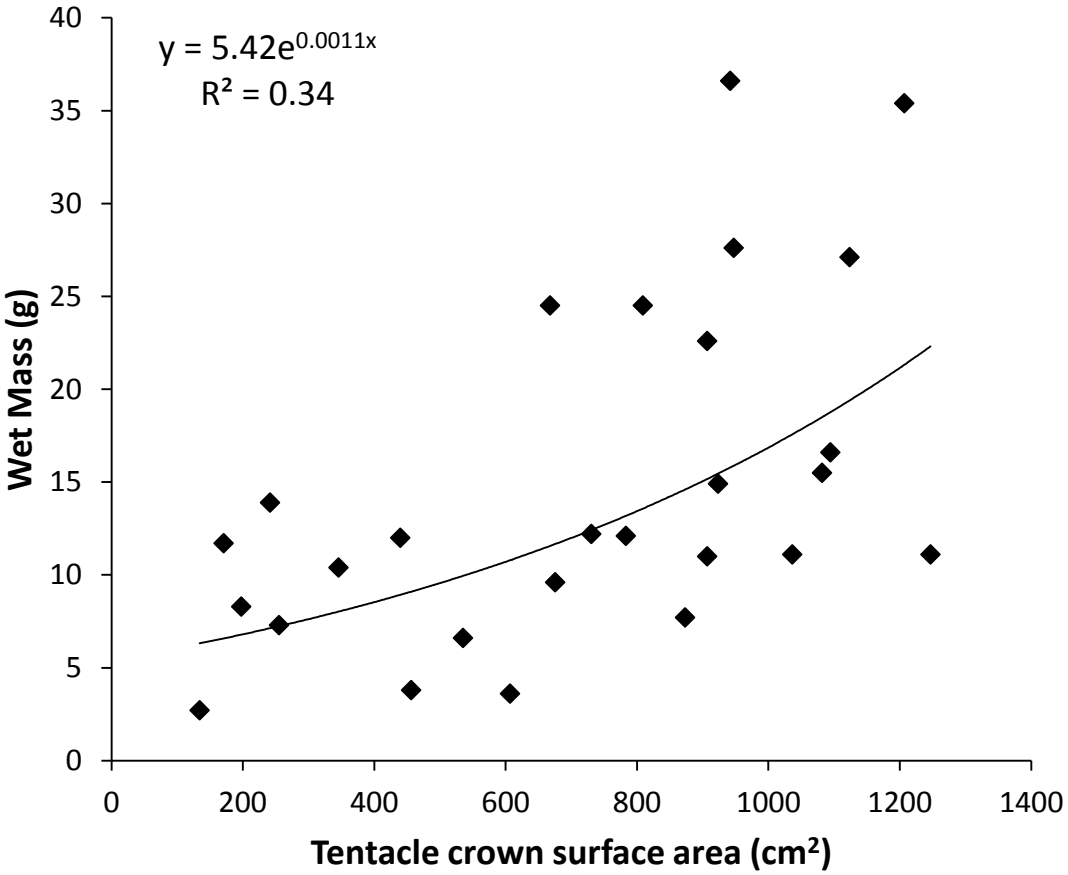
y = 5.42e0.0011x
R² = 0.34
Wet Mass (g)
Tentacle crown surface area (cm²)
0
5
10
15
20
25
30
35
40
0
200
400
600
800
1000
1200
1400

Figure 13

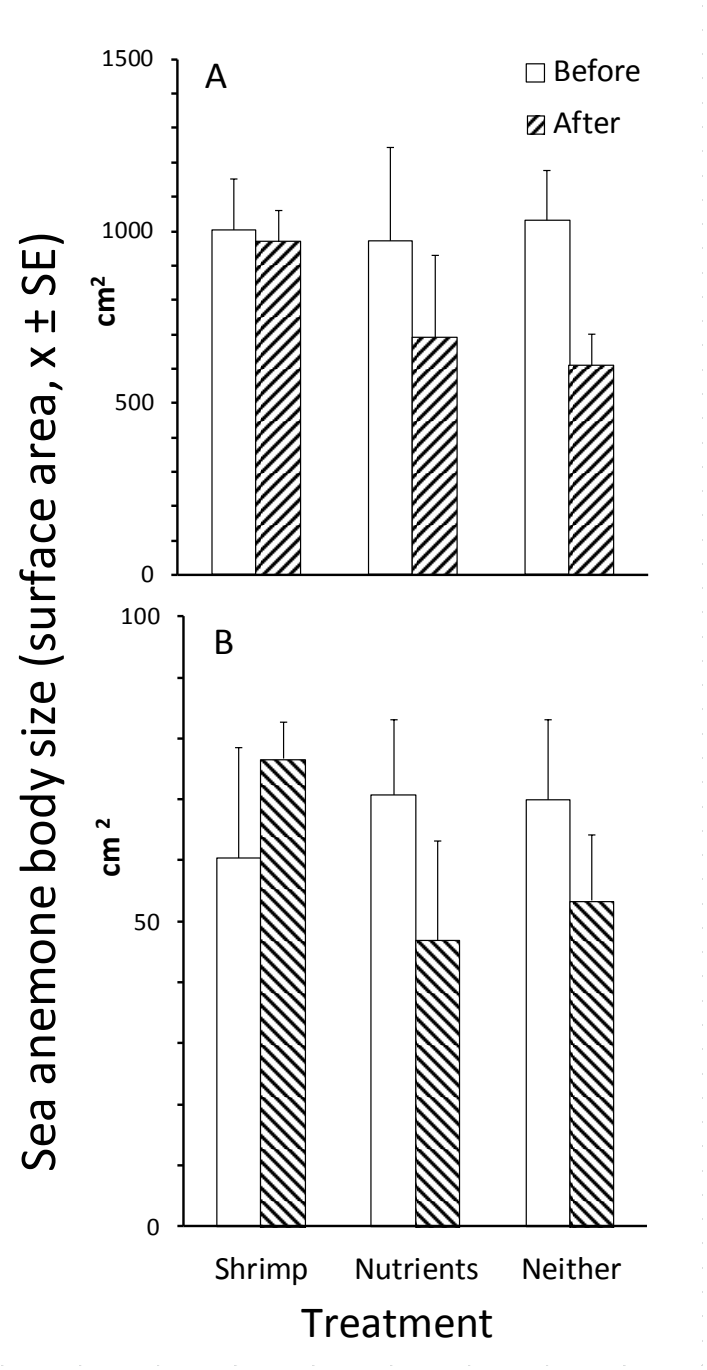
Sea anemone body size (surface area, x ± SE)
A
Before
After
1500
1000
500
0
cm²
B
100
50
0
cm ²
Shrimp
Nutrients
Neither
Treatment

Figure 14

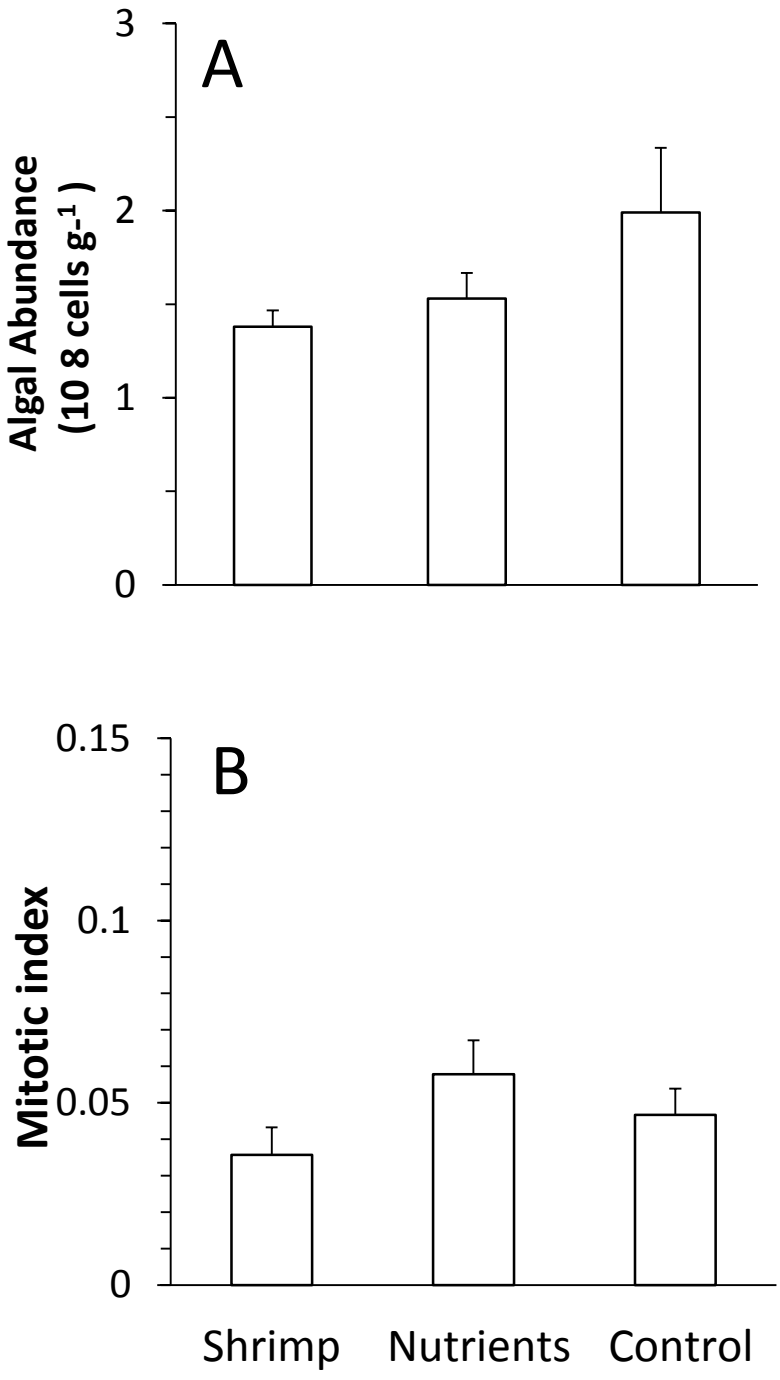
A
Algal Abundance
(10 8 cells g-1)
3
2
1
0
B
Mitotic index
0.15
0.1
0.05
0
Shrimp
Nutrients
Control

Figure 15

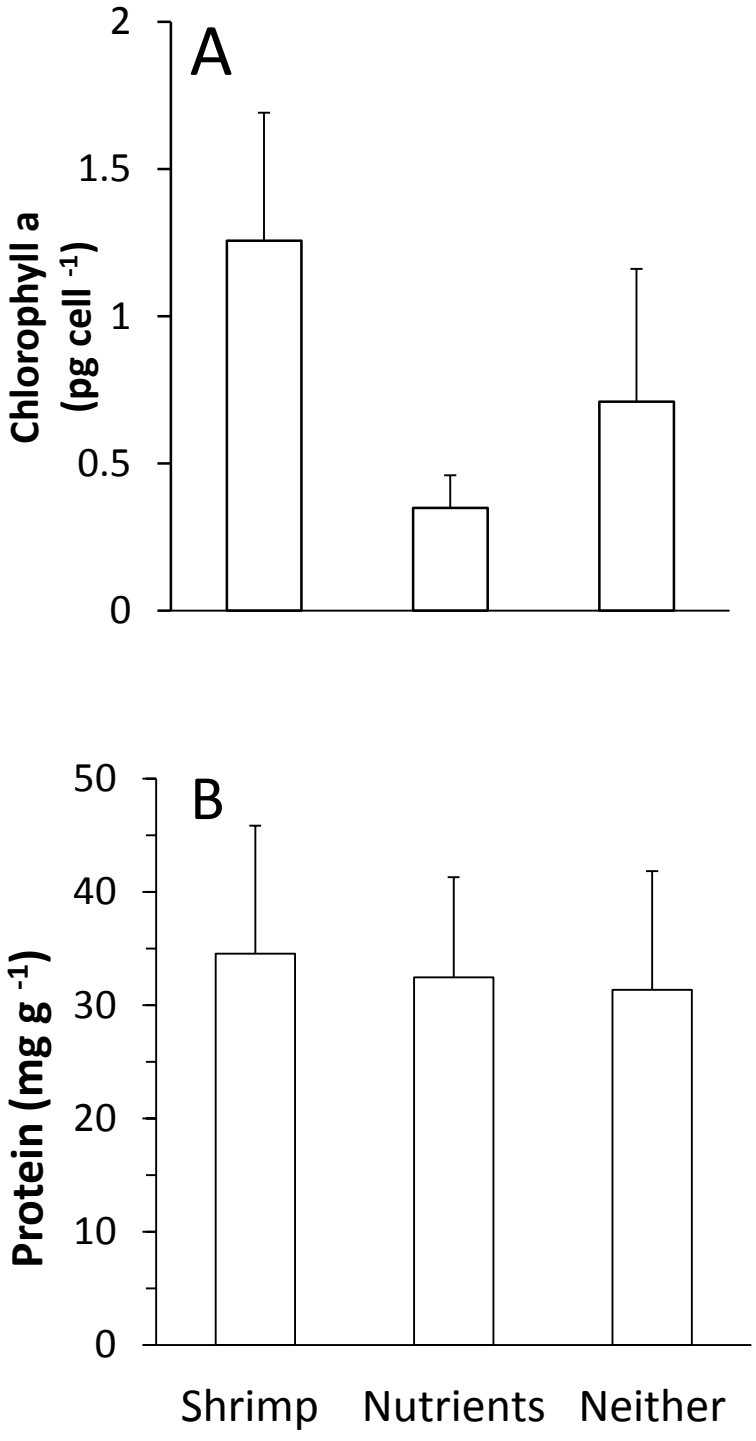
A
Chlorophyll a (pg cell -1)
2
1.5
1
0.5
0
B
Protein (mg g -1)
50
40
30
20
10
0
Shrimp
Nutrients
Neither

Figure 16

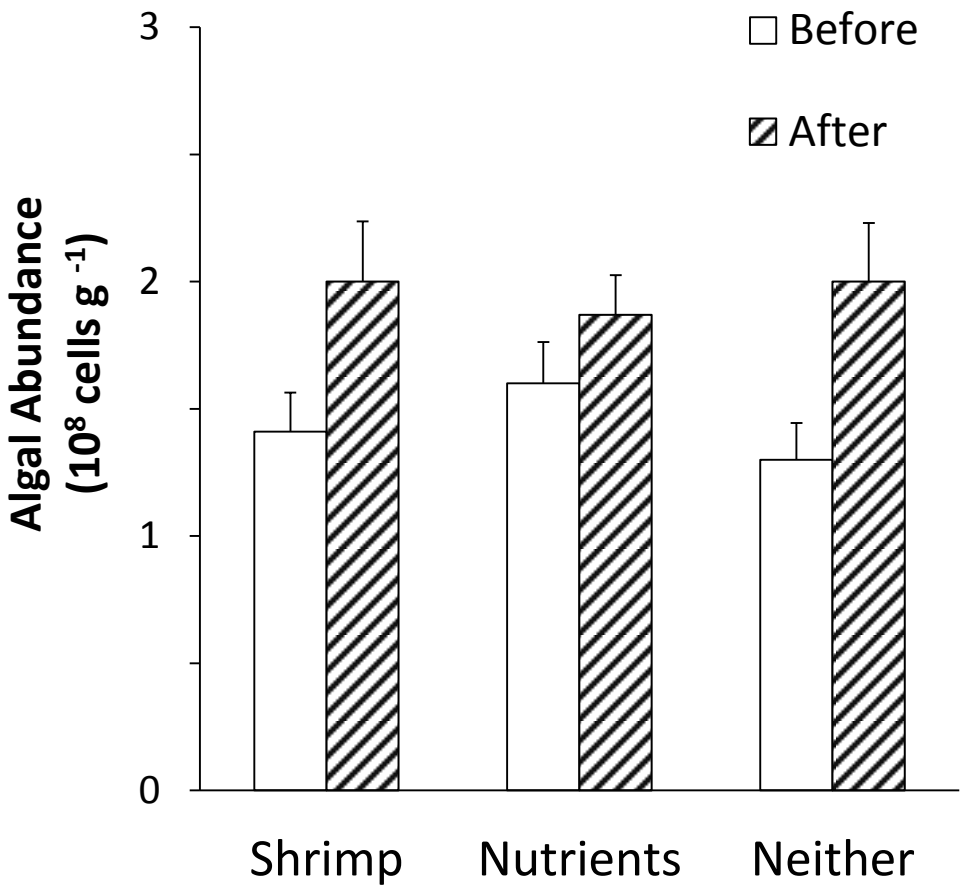
Before
After
Algal Abundance
(10^8 cells g^{-1})
3
2
1
0
Shrimp
Nutrients
Neither

Figure 17

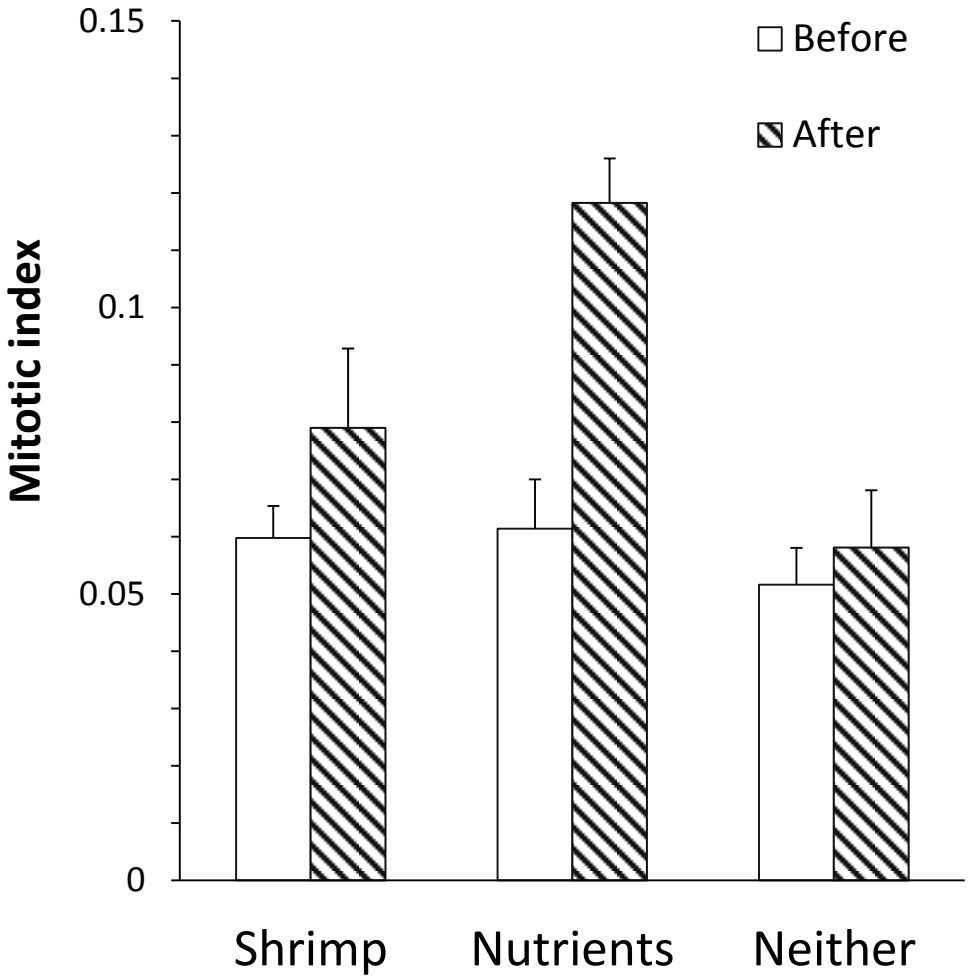
0.15
0.1
0.05
0
Mitotic index
Before
After
Shrimp
Nutrients
Neither

Figure 18

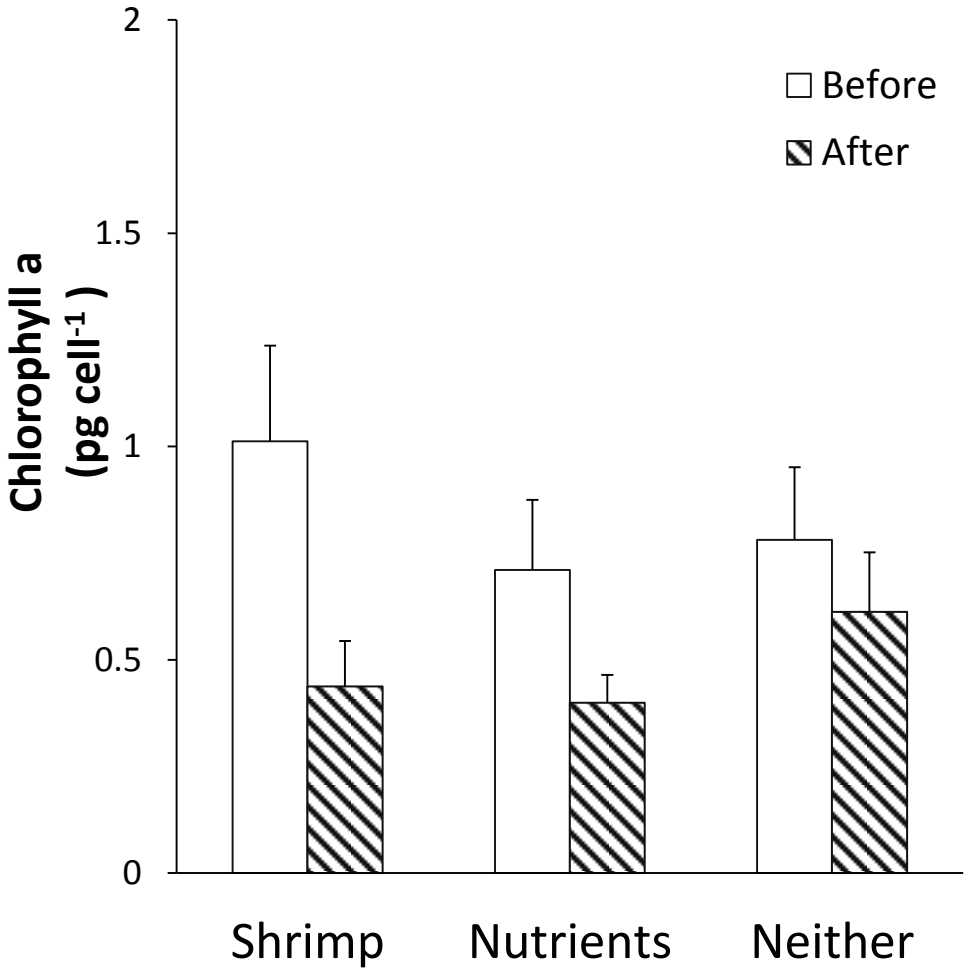
2
1.5
1
0.5
0
Chlorophyll a
(pg cell-1)
Before
After
Shrimp
Nutrients
Neither

Figure 19

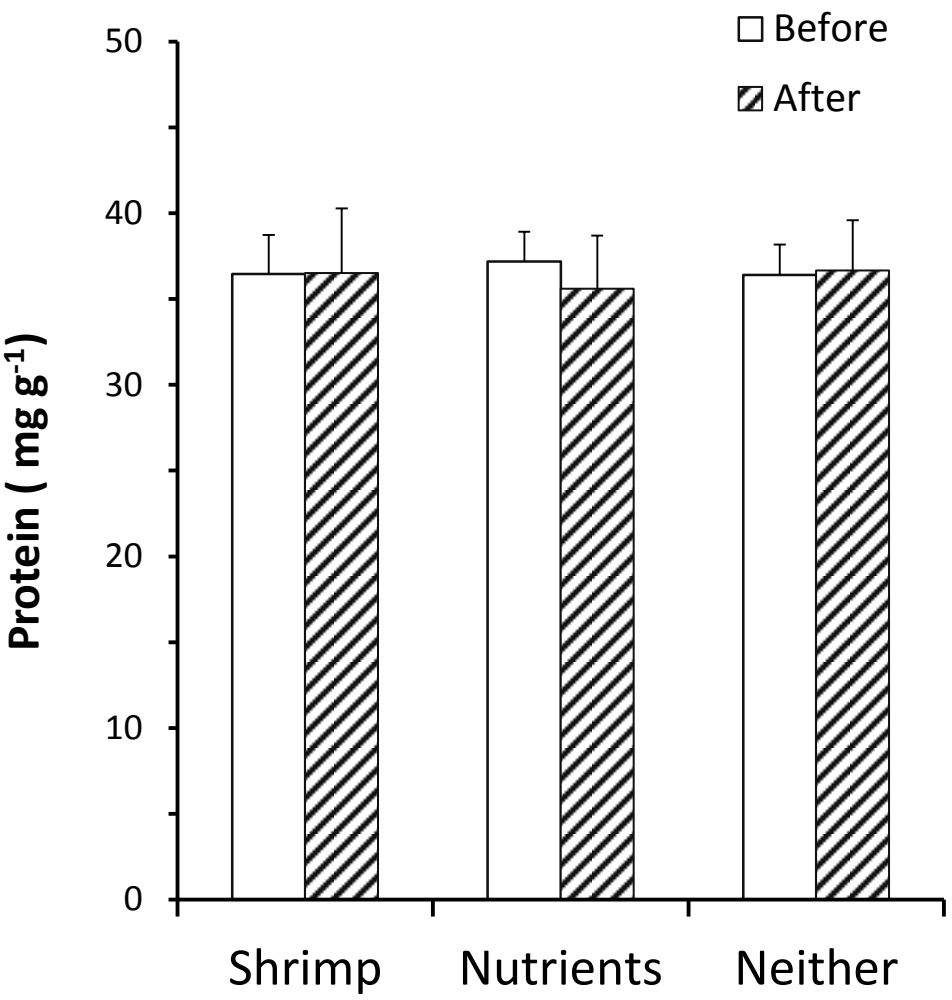
Before
After
50
40
30
20
10
0
Protein (mg g-1)
Shrimp
Nutrients
Neither

Figure 20

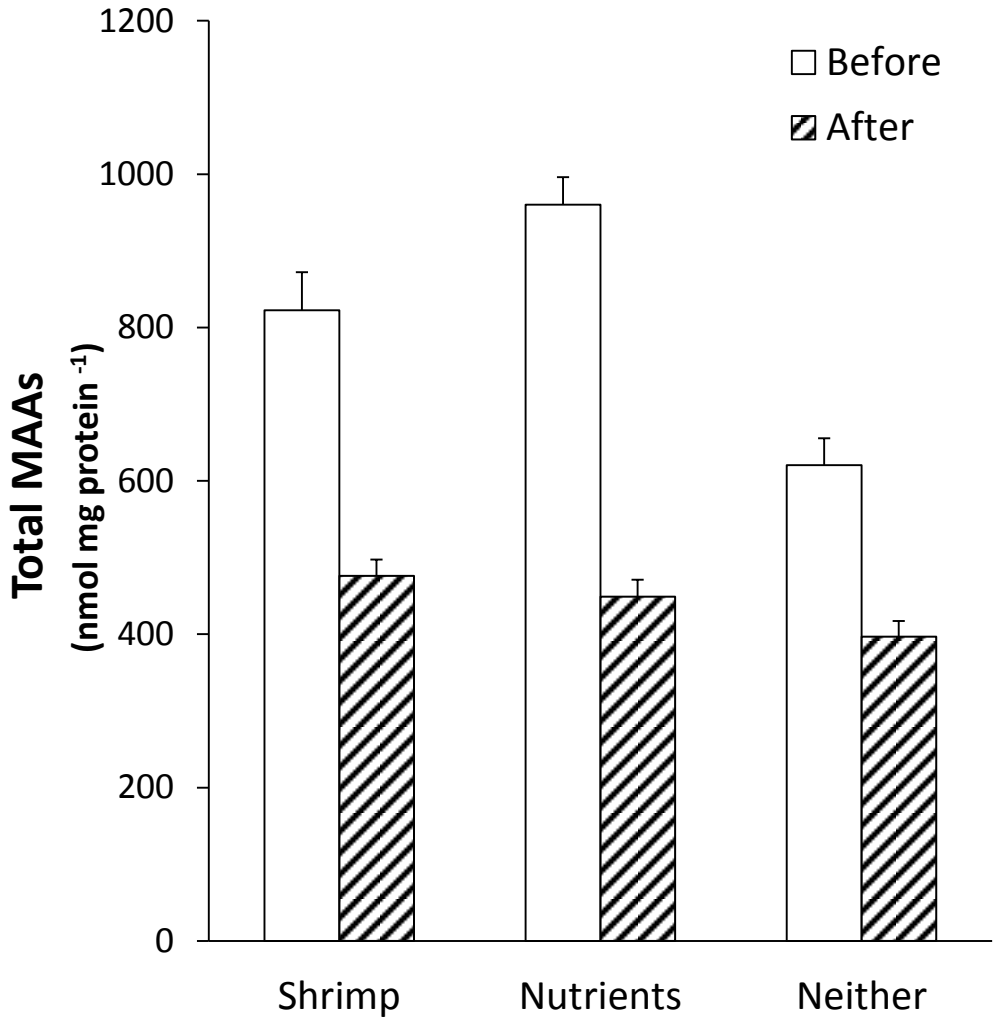
1200
1000
800
600
400
200
0
Total MAAs
(nmol mg protein -1)
Before
After
Shrimp
Nutrients
Neither

Figure 21

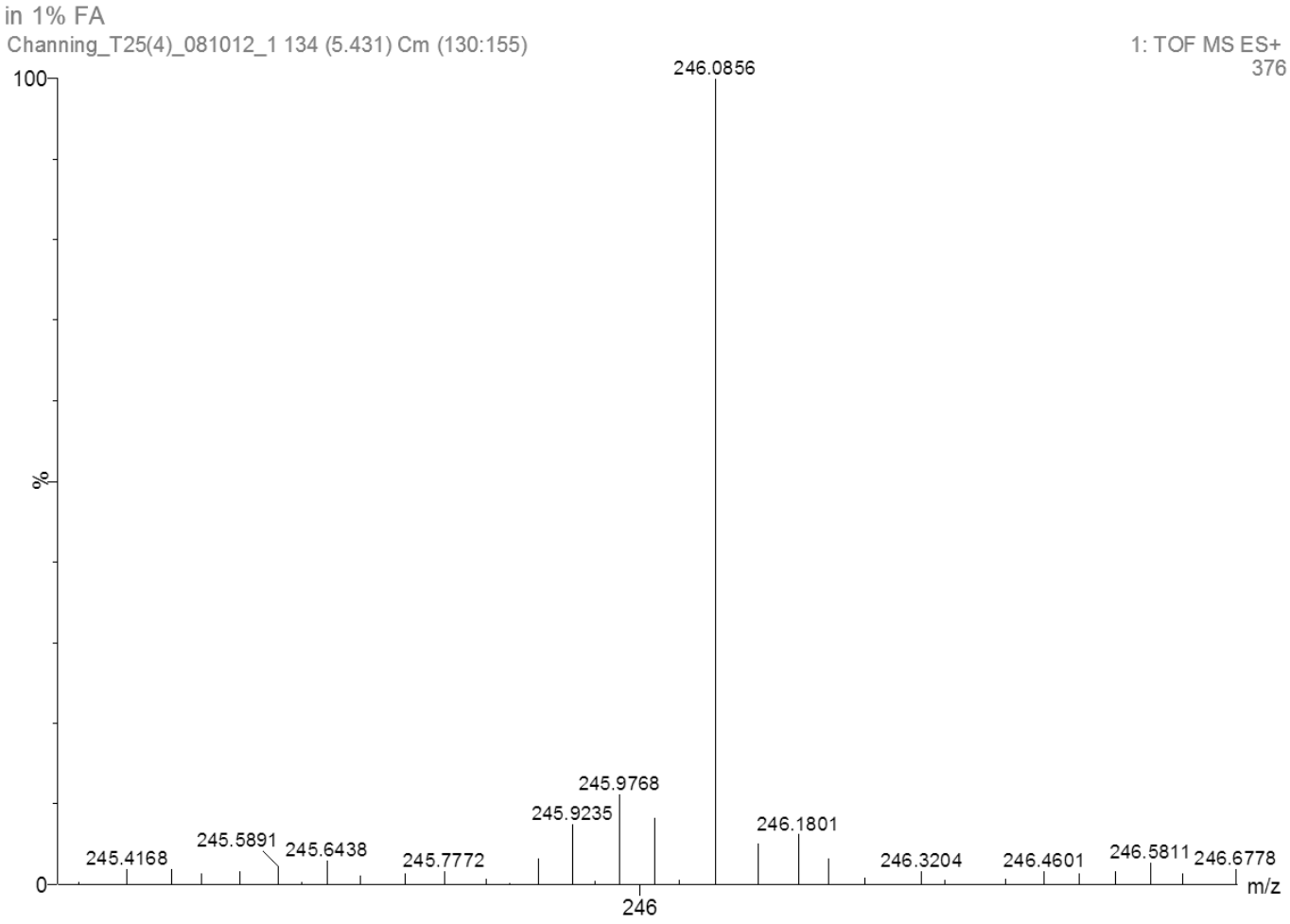
in 1% FA
Channing_T25(4)_081012_1 134 (5.431) Cm (130:155)
1: TOF MS ES+
376
100
%
0
246.0856
245.9768
245.9235
246.1801
245.4168
245.5891
245.6438
245.7772
246.3204
246.4601
246.5811
246.6778
246
m/z

Figure 22

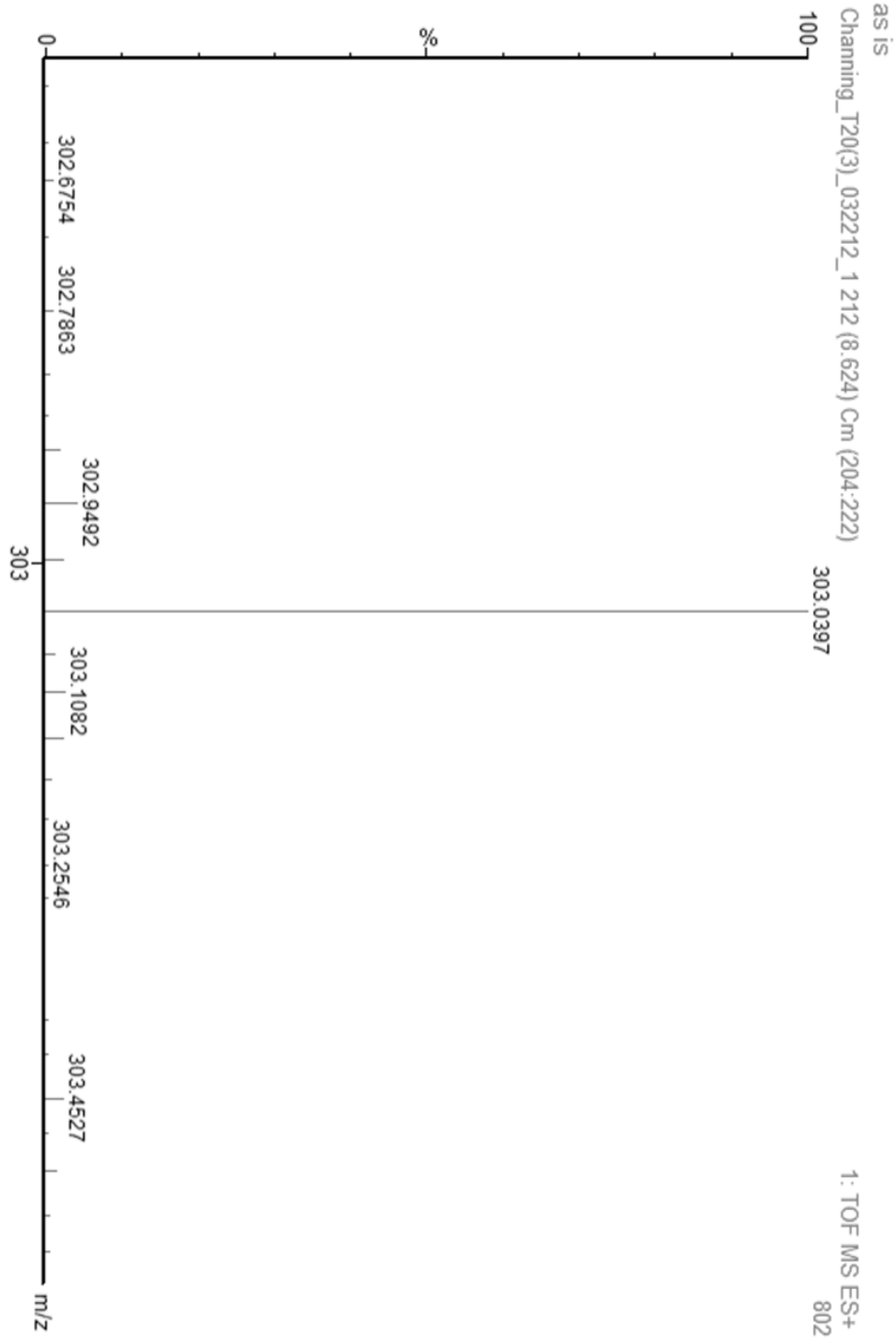
as is
Channing_T20(3)_032212_1 212 (8.624) Cm (204:222)
1: TOF MS ES+
802
100
%
0
303.0397
302.6754
302.7863
302.9492
303
303.1082
303.2546
303.4527
m/z

Figure 23

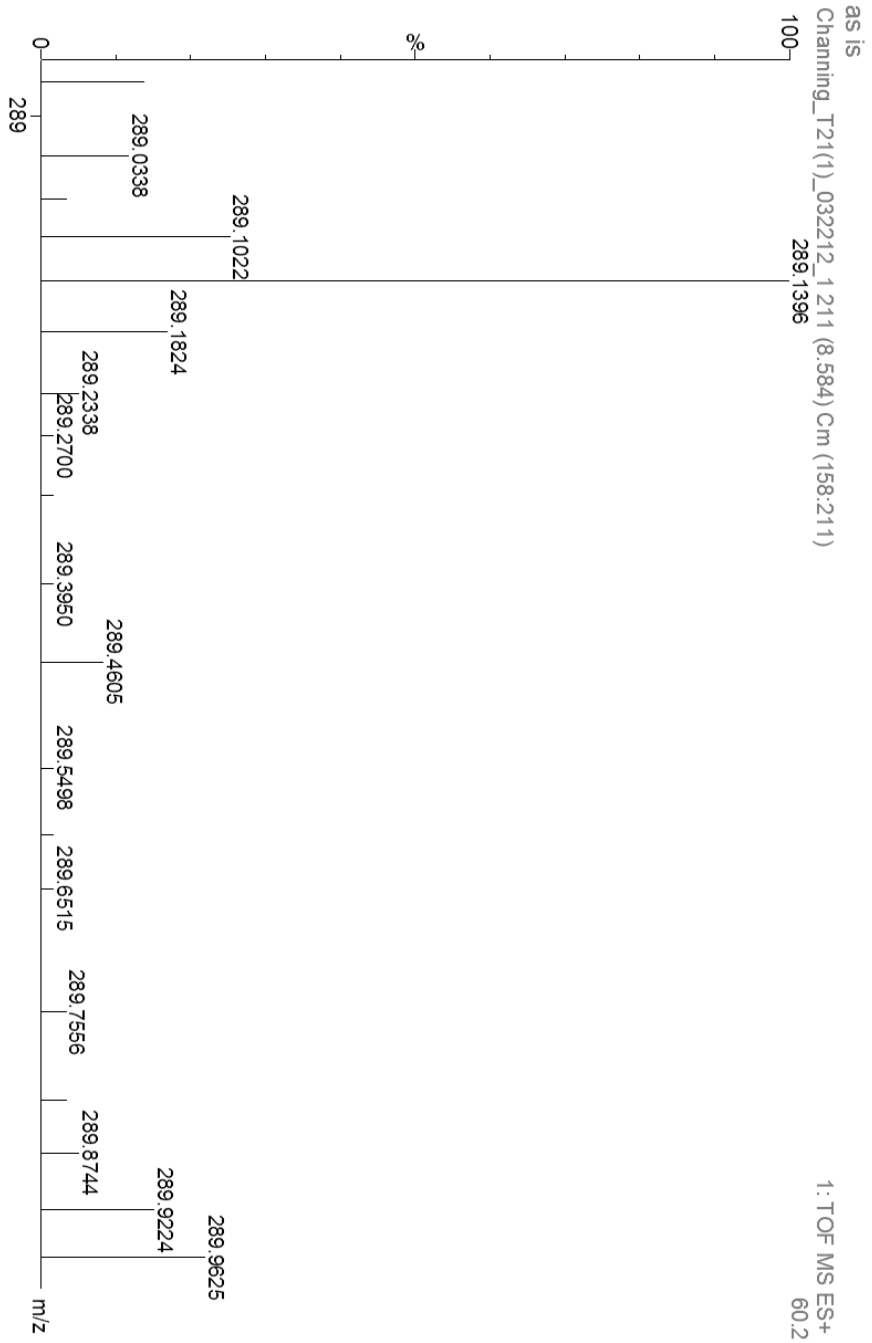
as is
Channing_T21(1)_032212_1 211 (8.584) Cm (158:211)
1: TOF MS ES+
60.2
100
%
0
289
289.0338
289.1022
289.1396
289.1824
289.2338
289.2700
289.3950
289.4605
289.5498
289.6515
289.7556
289.8744
289.9224
289.9625
m/z

Figure 24

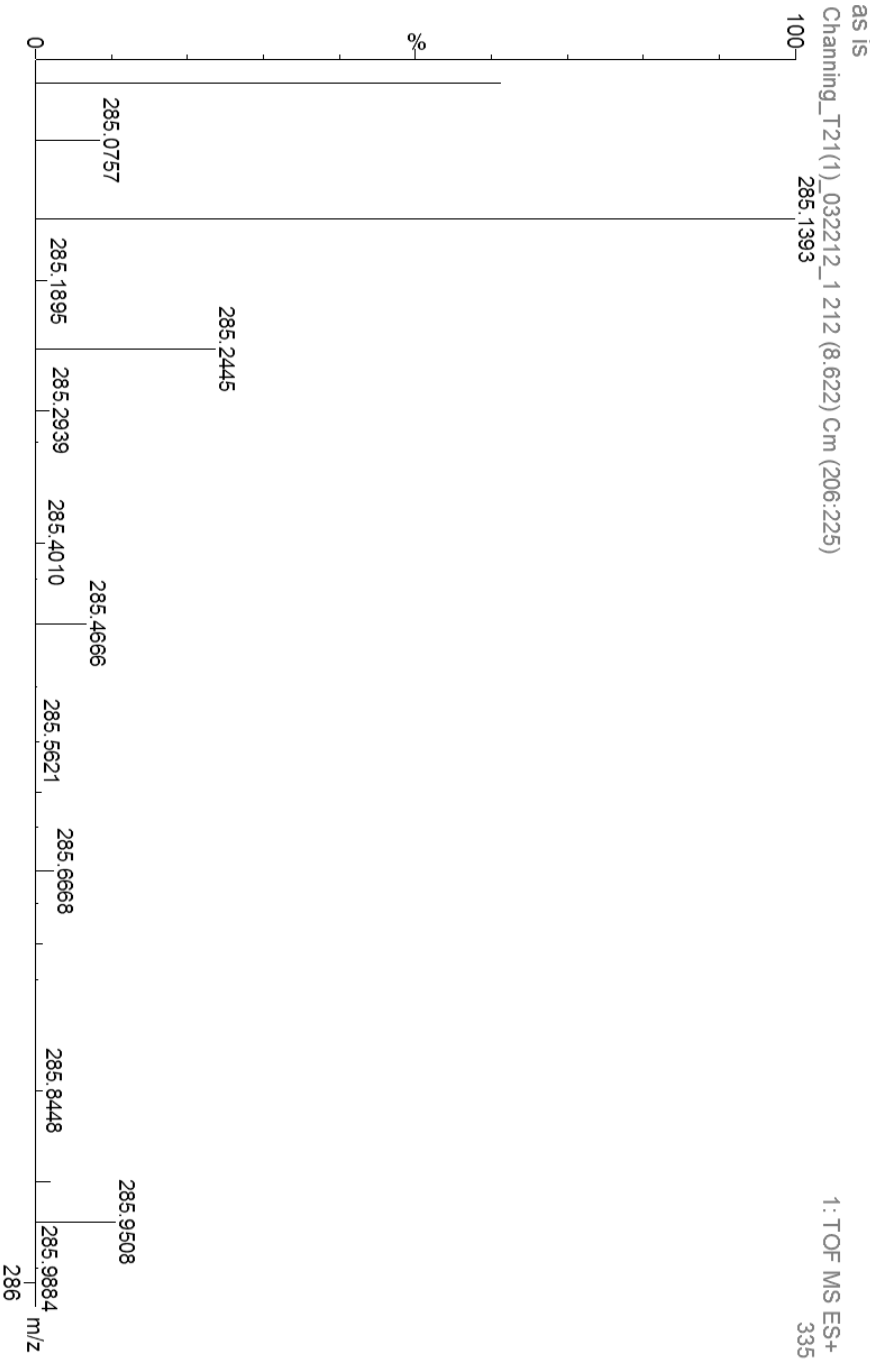
as is
Channing_T21(1)_032212_1 212 (8.622) Cm (206:225)
1: TOF MS ES+
335
100
%
0
285.0757
285.1393
285.1895
285.2445
285.2939
285.4010
285.4666
285.5621
285.6668
285.8448
285.9508
285.9884
286
m/z

Figure 25

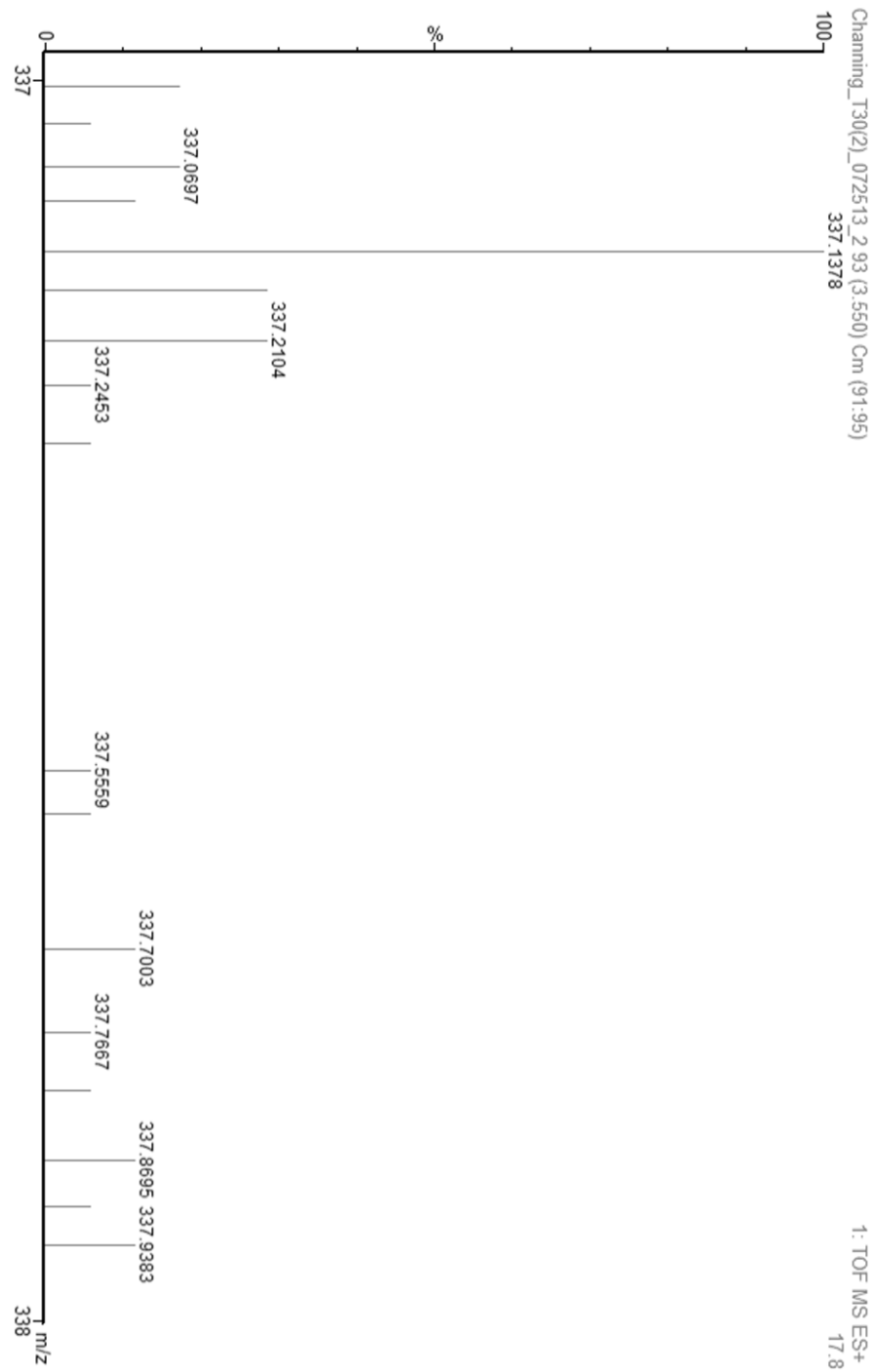
as is
Channing_T30(2)_072513_2 93 (3.550) Cm (91:95)
1: TOF MS ES+
17.8
100
%
0
337
338
m/z
337.1378
337.0697
337.2104
337.2453
337.5559
337.7003
337.7667
337.8695
337.9383

Figure 26

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