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Virginia M. Weis

Intracellular symbioses between cnidarians and dinoflagellates from the
genus *Symbiodinium* are widespread throughout the marine environment. These
associations are ecologically significant, especially in tropical waters where
symbiotic interactions between corals and *Symbiodinium* culminate in the
formation of limestone reefs. This thesis focuses on cellular and molecular aspects
of the symbiosis, specifically the initiation of the symbiosis and characterization of
a host gene, sym32, that is believed to function in the symbiosis. Sym32 was
originally identified as a differentially expressed protein in symbiotic vs.
aposymbiotic individuals of the sea anemone, *Anthopleura elegantissima*. Based
on its deduced amino acid sequence, sym32 belongs to a family of cell adhesion
proteins that play roles in cell recognition in a diverse array of organisms.

Chapter 2 examines the process by which a new cnidarian host acquires its
first symbionts. Larvae of the scleractinian coral *Fungia scutaria,* which are
initially aposymbiotic, acquired symbionts while feeding. Symbionts that entered
the larval gastric cavity with food were subsequently taken into host gastrodermal
cells by phagocytosis. Chapter 3 describes immunolocalization of sym32 in *A.
elegantissima* tentacles. In aposymbiotic tentacles, sym32 was localized to vesicles

within the host gastrodermal cells. Symbiotic tentacles lacked sym32-containing vesicles. Instead, sym32 was present among the membranes that enclose the symbionts within host cells. Western blots of proteins from *Symbiodinium* revealed a 45/48kD doublet that cross-reacts with anti-sym32 antiserum. This suggests that homologous proteins are expressed in both host (32kD) and symbiont (45/48 kD). Chapter 4 describes the effects of environmental factors on expression of host sym32. Aposymbiotic and symbiotic anemones maintained in continual darkness for 3 weeks experienced a dramatic decline in sym32 protein levels, relative to anemones maintained on a 12:12 h light:dark cycle. This suggests that light plays a major role in regulating sym32. Exposure of anemones to elevated temperatures for 2 days in the dark caused a mild bleaching response (expulsion of symbionts from the host), but did not affect the levels of sym32 protein. Chapter 5 examines the role of sym32 during the infection process, using antibody interference techniques. *F. scutaria* larvae and symbionts incubated in sym32 antiserum during the infection process experienced a decline in infection rates. Further, symbionts that were incorporated into host gastroderm appeared to be degenerating in antiserum treatments, but appeared to be healthy in preimmune controls.

Cellular and Molecular Aspects of Cnidarian-Algal Associations

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Jodi A. Schwarz, Author

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CONTRIBUTION OF AUTHORS

Dr. Dave Krupp assisted with maintenance of corals and larval rearing in Hawaii.

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CHAPTER 1

INTRODUCTION

Animal-microbe associations

Intracellular symbiotic associations between eukaryotic microbes (protozoans) and animals occur throughout all ecosystems and encompass interactions ranging from parasitic to mutualistic (Paracer and Ahmadjian, 2000). The formation and maintenance of these associations results primarily from cellular and molecular interactions between the partners, including modulation of gene expression and protein synthesis in both partners. Until recently, questions regarding the cellular and molecular events that culminate in a mature association were difficult to address, but the development of genomic and proteomic technologies and advances in microscopic techniques over the past two decades has allowed researchers to begin examining the complex interactions between the partners (Barrett et al. 2000, Colley 2000, de Souza et al. 1998)

The most extensively studied animal-protozoan systems are those producing pathogenic symptoms in humans and livestock. Genome and EST sequencing has allowed researchers to examine how some major disease-causing protozoans, such as *Plasmodium, Toxoplasma, Giardia, Trypanosoma, Leishmania*, among others, invade host cells, maintain their metabolic processes within host cells, replicate or differentiate within host cells, and produce or evade a host immune response.

Likewise, researchers are now able to examine host response at a cellular and molecular level.

To date, relatively little attention has been paid to animal-protozoan interactions that result in mutual benefits to host and symbiont, in part because such associations do not exist in humans or livestock, or indeed in any terrestrial environment. However, in the marine environment such associations commonly occur between several taxa of invertebrate animals and various photosynthetic unicellular algae (Paracer and Ahmadjian 2000). By far, the most significant of these is the mutualistic association between cnidarians and photosynthetic dinoflagellates. In these associations, host species include a wide variety of scleractinian corals, sea anemones and octocorals and symbiont species are almost exclusively from genus *Symbiodinium.*

Cnidarian-algal associations are widespread in both temperate and tropical marine environments. The relationship is usually mutually beneficial to the partners; the symbionts provide hosts with reduced organic carbon (Lewis and Smith 1971, Trench 1971, Battey and Patton 1987, Wang and Douglas 1999), while hosts provide symbionts with carbon dioxide (Weis and Reynolds, 1999) a source of nitrogen (reviewed in Wang and Douglas, 1998) and a high light environment. Cnidarians that host *Symbiodinium* are abundant and conspicuous members of the habitats in which they occur and *Symbiodinium* contained within their hosts account for a significant fraction of photosynthetic output in both tropical and temperate systems (Muller-Parker and Davy 2001, Muscatine 1980, Dubinsky 1990). Such tight nutrient cycling between symbiont and host promotes high productivity in otherwise nutrient-poor marine environments (Muscatine and Porter 1977). As a result, gross productivity of algae contained in their hosts makes *Symbiodinium* a major contributor to global carbon fixation (Muscatine 1990, Muscatine and Weis, 1992). The most dramatic manifestation of this symbiotic interaction is the coral reef ecosystem, where coral-algal associations form both a

structural and trophic cornerstone of these highly productive and biologically diverse marine communities (Muscatine, 1990, Dubinsky 1990).

Over the past few decades, coral reefs have experienced increasing rates of decline globally, a result of global warming and various anthropogenic influences including sedimentation and pollution of the marine environment (Glynn 1991, Hoegh-Guldberg 1999, Wilkinson 1999). As a result, it has become clear that in order to preserve and improve the health of remaining reefs, we need a much better understanding of how coral reefs themselves form and are maintained. Because reefs are the culmination of symbiotic interactions between corals and dinoflagellates, there has been increasing interest in understanding coral-algal symbiosis at the level at which they are regulated, that is at the cellular and molecular level.

This thesis describes efforts to understand some of the cellular and molecular events that play roles in the establishment (Chapter 2 and 5), maintenance (Chapter 3), and regulation (Chapter 4) of symbiotic interactions between cnidarians and *Symbiodinium*.

Cnidarian-*Symbiodinium* associations

Cnidarians are the most primitive of the eumetazoan animals. While they possess true tissues, they lack organ systems. Almost all cnidarians have, at some stage in their life cycle, a body plan consisting of a sessile, radially symmetrical polyp with a ring of tentacles encircling a single opening that serves as both a mouth and as a release site for waste products. Many also produce a swimming medusa body plan that is essentially an inverted motile polyp. Cnidarians are constructed from only two thin tissue layers consisting of only about 8 cell types, separated by a collagenous matrix, called the mesoglea (Shick 1991). The

gastrodermis lines the gastric cavity and primarily serves a digestive function. The epidermis faces the external environment and primarily serves a protective function. Some epidermal cells contain specialized organelles, called nematocysts, that are used for capturing food and for defense.

The life cycle of cnidarians typically includes both sexual and asexual cycles. In asexual reproduction, the polyp undergoes budding, fission or pedal laceration to form new polyps or medusae (Shick 1991). In some cases, the new polyps remain attached to form interconnected colonies. In sexual reproduction, cnidarians employ one of two modes of gamete release and fertilization: either gametes are free-spawned and fertilization and development occur in the water column, or eggs are retained within the female, and fertilization and development occur within the maternal polyp. In either case, a planula type of larva develops, a ciliated barrel-shaped larva that may possess a mouth and gastric cavity. Within days to months, depending on species, the larva settles to the substrate and undergoes metamorphosis to form the polyp stage of the life cycle.

The genus *Symbiodinium* consists of exclusively symbiotic dinoflagellates that reside within hosts species from a number of invertebrate taxa. Because they are so similar morphologically, members of this species are often generically referred to as "zooxanthellae," which is loosely defined as the various species of dinoflagellates (*Symbiodinium* and others) that associate with invertebrates. Initially it was thought that only a single species of *Symbiodinium* formed associations with cnidarians, but biochemical and DNA evidence has firmly established that there are many species within the genus *Symbiodinium* and that their patterns of distribution within and between host species is complex (for example, see Baker and Rowan 1997 and Goodson et al. 2001). Even within a single host, two or more species from different clades may co-occur, living in distinct microhabitats created by the topography of the host (Rowan and Knowlton 1995, Rowan et al. 1997).

Members of *Symbiodinium* live in a symbiotic association with a variety of
invertebrate animals, but also live freely within the water column. Many strains or
species have also been successfully brought into culture in enriched seawater.
When maintained in culture, they develop into two distinct morphologies; a
flagellated and motile gymnodinium-like form and an "encysted" coccoid
nonmotile form. The development of these two morphologies often occurs on a
diurnal cycle, with flagellated and coccoid stages alternately predominating during
a portion of the cycle.

The larger taxon to which *Symbiodinium* belongs, Dinoflagellata, comprises an
extremely diverse group of marine and freshwater organisms, possessing members
that are either or both photosynthetic or heterotrophic, as well as members that are
free living or form either parasitic or mutualistic associations with animals or other
eukaryotic phytoplankton (Taylor 1987). Dinoflagellates also possess unusual
cellular features for eukaryotes. Their nuclear DNA is permanently condensed, the
nucleus lacks histone proteins, mitosis is unusual, and they possess a plastid that
may have originated from a secondary symbiosis with a red alga (Taylor 1987, Fast
et al. 2001). Interestingly, dinoflagellates are a member of the Alveolata, a group
which includes human apicomplexan parasites, such as *Plasmodium* and
Toxoplasma, although dinoflagellates diverged from these taxa hundreds of
millions of years ago. Nonetheless, it is noteworthy that dinoflagellates, the
primary mutualistic symbiont of animal cells shares an ancestry with the
apicomplexan parasites, the largest group of parasitic eukaryotes that inhabit the
intracellular environment of animal cells.

Morphology and physiology of cnidarian-*Symbiodinium* symbiosis

Cnidarians house their symbiotic dinoflagellates primarily within gastrodermal cells of the gastrodermis. The dinoflagellates reside within a vacuole, a distinct compartment delineated by multiple layers of membranes (for example, see Wakefield and Kempf 2000). Very little is known about the origin or biochemistry of these membranes. Recent evidence supports the idea that only the outermost membrane layer is of host origin, while the multiple layers of inner membranes are of algal origin (Wakefield and Kempf 2001). However, nothing is known about the biochemical components of the membranes, or about where the vacuole fits into endocytic pathways of the host.

Within host cells, the dinoflagellates continue to asexually replicate (sexual reproduction in this genus has never been observed). However, their growth rates in host cells are suppressed, relative to those measured in culture (Falkowski et al., 1993) even though their photosynthetic rates remain similar. The suppression of symbiont growth is the primary reason why stable and persistent associations are able to form between the partners. Furthermore, because rates of photosynthesis remain high, the symbionts pass excess photosynthate to their hosts, which use it for daily respiration requirements (Falkowski et al., 1993).

Onset of symbiosis

The transmission of symbionts from one generation of hosts to the next can occur either by maternal transmission of symbionts to oocytes or brooded offspring, or by acquisition of symbionts from the environment. Although the

majority of host species utilize the latter mechanisms for infection, very little is known about when or how hosts acquire their first complement of symbionts from the environment (Fadlallah 1983, Babcock and Heyward 1986, Harrison and Wallace 1990, Richmond and Hunter 1990, Richmond 1997).

The initial infection of a host cell by a *Symbiodinium* cell has been experimentally examined in a number of host species, including Chapter 2 of this thesis. From these studies, it is clear that in the vast majority of cases *Symbiodinium* is taken into host cells as a result of phagocytic uptake by gastrodermal cells of the host (Fitt and Trench, 1983, Schwarz et al. 1999, Schwarz et al. 2002). The specific steps by which this occurs, such as the various molecules and biochemical processes that allow *Symbiodinium*, but not other algae, to be taken into and reside within host cells, are entirely unknown. This subject is addressed in Chapter 5, which describes an experimental attempt to interfere with the onset of symbiosis by selectively inhibiting a protein, sym32, that we suspect plays a role in regulating interactions between the partners. The discovery of this "symbiosis protein" is described below, and my efforts to characterize the sym32 gene/protein are described in Chapters 3 - 5 of this thesis.

Symbiosis genes

Efforts to understand the regulation and maintenance of cnidarian-algal associations have included the identification of host genes from the temperate sea anemone, *Anthopleura elegantissima*, that likely play roles in facilitating interactions between the partners. This has been accomplished by comparing protein profiles or cDNA libraries of symbiotic anemones with those from aposymbiotic anemones (which are naturally free of symbionts), and identifying proteins or genes with enhanced expression in the symbiotic hosts (Weis and

Levine 1996 and unpubl. data). By these methods, numerous proteins and genes have been identified, including carbonic anhydrase, which supplies CO_2 to symbionts (Weis and Reynolds 1999), sym17, a novel gene that is homologous to calcium-binding proteins in other organisms, and several that are yet to be characterized and described.

One of the most highly expressed "symbiosis" genes in *A. elegantissima* is a 32 kiloDalton, 7.9 pI protein, sym32 (Weis and Levine 1996). In symbiotic anemones both mRNA transcripts and sym32 protein are significantly upregulated, compared with aposymbiotic anemones (Reynolds et al. 2000). A BLAST search placed sym32 within a family of cell adhesion proteins, called Fasciclin I proteins (Reynolds et al. 2000). Members of this family have been identified in organisms as diverse as mycobacteria (Terasaka et al. 1989), *Volvox* (Huber and Sumper 1994), insects (Zinn et al. 1988), sea urchins (Brennand and Robinson 1994), and humans (Skonier et al. 1992, Takeshita et al. 1993). All share 1 to 4 repeats of 3 different Fas-I domains (Hu 1998 et al., Reynolds et al. 2000). All members of this family appear to play roles in cell adhesion and/or cell-cell recognition. Several function in mutualistic or parasitic interactions, such as the plant-rhizobial bacteria mutualism (the nex-18 gene from *Sinorhizobium meliloti*; Oke and Long 1999), the fungal-cyanobacterial lichen mutualism (protein encoded by contig 543: gene 11, from the *Nostoc* genome; Paulsrud and Lindblad 2002), and the animal pathogen *Mycobacterium bovis* (the immunogenic protein MPB-70; Harboe and Nagai 1984, Harboe et al. 1995).

Based on this information, we hypothesized that sym32 functions in the symbiosis as a cell-cell adhesion molecule with a potential role in host-symbiont recognition. This thesis describes efforts to further characterize the sym32 gene and to determine its role in the symbiosis. This work was performed using two different model systems that we have developed, which are described below.

Model systems in cnidarian-algal research

The *Anthopleura elegantissima – Symbiodinium spp.* association

One of the models that has been used extensively to examine the physiology of cnidarian-algal symbiosis is the association between the Pacific anemone *Anthopleura elegantissima*, and *Symbiodinium spp.* This species lives along the mid-intertidal zone of the eastern Pacific, from Mexico through Alaska (Hand 1955, Francis 1979, McFadden et al. 1997). This species has also, recently, been developed as a model for identifying and characterizing cnidarian host genes that play roles in mediating symbiotic interactions with *Symbiodinium.* One of the distinct advantages of using *A. elegantissima* for identifying potential "symbiosis genes" is that it is facultatively symbiotic, unlike most other symbiotic cnidarians. This allows for the identification of genes and protein that are differentially regulated based on whether or not the anemone contains symbionts. The facultative nature of the symbiosis is a product of the microenvironments inhabited by the host anemones. Because *Symbiodinium* is photosynthetic, anemones residing in very low light environments, such as deep rock overhangs or caves, are aposymbiotic (they lack symbionts; Buchsbaum 1968, Secord and Augustine 2000). Symbiotic specimens are abundant in light-exposed rocky mid intertidal regions. The facultative nature of the symbiosis has permitted the identification of proteins and genes that appear to be regulated by the presence or absence of symbionts (Weis and Levine 1996).

The *Fungia scutaria – Symbiodinium sp.* association

The sub-tropical solitary scleractinian coral *Fungia scutaria* is obligately symbiotic with *Symbiodinium.* Despite the obligate nature of its association with *Symbiodinium,* sexual reproduction in this species produces offspring that lack symbionts and must acquire them from the environment. This feature of its biology has allowed us to use *F. scutaria* as a model for examining questions regarding the cellular and molecular events associated with the onset of symbiosis (Schwarz et al. 1999, Weis et al. 2001). This species has a distinct advantage over using *A. elegantissima* for studying early onset of symbiosis: unlike *A. elegantissima* its gametogenic and spawning cycle is extremely predictable such that we are able to repeatedly obtain and rear the larval stage for examination (Weis et al. 2002).

Thesis chapters

The research described in this thesis focuses on 1) the initial infection event and early onset of symbiosis in cnidarian-algal associations (Chapters 2 and 5) and 2) characterization of sym32 in symbiotic cnidarians (Chapters 3, 4 and 5). Chapter 2 describes the process by which a new cnidarian host acquires its first complement of symbiotic dinoflagellates. Chapter 3 examines, at a cellular and ultrastructural level, the location of the sym32 protein within tentacles of symbiotic and aposymbiotic specimens of *A. elegantissima.* Chapter 3 also describes the identification of a potential sym32 homolog, p45/38 that is produced by the symbionts. Chapter 4 examines the effect of environmental factors, specifically elevated temperature and light, on patterns of expression of the host sym32 gene

and protein. Chapter 5 describes experimental attempts to uncover the function of the sym32 gene during the early stages of the association.

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CHAPTER 2

LATE LARVAL DEVELOPMENT AND ONSET OF SYMBIOSIS IN THE SCLERACTINIAN CORAL, *FUNGIA SCUTARIA*

Jodi A. Schwarz, Dave A. Krupp, and Virginia M. Weis

ABSTRACT

Many corals which harbor symbiotic algae (zooxanthellae), produce offspring that initially lack zooxanthellae. This study examined late larval development and the acquisition of zooxanthellae in *Fungia scutaria,* a scleractinian coral which produces planula larvae that lack zooxanthellae. Larvae reared under laboratory conditions developed the ability to feed three days after fertilization; feeding behavior was stimulated by ground *Artemia.* Larvae began to settle and metamorphose five days after fertilization. In laboratory experiments, larvae acquired experimentally added zooxanthellae by ingesting them as they fed. Zooxanthellae entered the gastric cavity and were phagocytosed by endodermal cells. As early as one hour after feeding, zooxanthellae were observed in both endodermal and ectodermal cells. Larvae were able to form an association with three genetically distinct strains of zooxanthellae. Both zooxanthellate and azooxanthellate larvae underwent metamorphosis, and azooxanthellate polyps were able to acquire zooxanthellae from the environment. Preliminary evidence suggests that the onset of symbiosis may influence larval development; in one study symbiotic larvae settled earlier than aposymbiotic larvae. Protein profiles of eggs and larvae throughout development did not show gross differences in proteins synthesized by zooxanthellate *vs.* azooxanthellate larvae, but did show a putative yolk protein doublet that was abundant in eggs and one-day-old larvae and declined over time.

INTRODUCTION

The life history of symbiotic associations between organisms necessarily includes a stage during which a new generation of hosts first acquires its symbionts (Douglas 1994). Symbionts may be acquired either vertically, whereby the

symbiont is transmitted directly from parent to offspring, or horizontally, whereby the offspring must acquire symbionts from the environment. Vertical transmission ensures that offspring are provided with a complement of symbionts, while horizontal transmission is more uncertain; environmental variability may prevent contact between symbiont and host, resulting in the failure of the host to become infected by its symbiont.

Many members of the phylum Cnidaria (such as corals, sea anemones, and jellyfish) harbor intracellular photosynthetic dinoflagellates (*Symbiodinium* spp.) in a mutually beneficial symbiotic association. The dinoflagellates, also known as zooxanthellae, contribute to host nutrition by translocating photosynthetically fixed carbon, while hosts provide zooxanthellae with nutrients and a protected, high light environment. Many cnidarian host species are obligately symbiotic with zooxanthellae, thus vertical transmission would be predicted to be the dominant mode of symbiont transmission. However, this is not the case, at least in scleractinian corals. The majority of scleractinian coral species spawn gametes that are azooxanthellate (i.e., lack zooxanthellae) (Fadlallah 1983, Babcock and Heyward 1986, Harrison and Wallace 1990, Richmond and Hunter 1990, Richmond 1997). The gametes are fertilized within the water column and develop into azooxanthellate planula larvae that must acquire zooxanthellae at some stage of their development.

There are very few studies of horizontal transmission of zooxanthellae. Observations of zooxanthellae suddenly appearing in previously azooxanthellate hosts have been reported for both planulae (Krupp 1983) and newly settled polyps (Babcock and Heyward 1986, Benayahu *et al.* 1989), but few studies have explicitly addressed the infection process in species which produce azooxanthellate offspring. Kinzie (1974) observed that newly settled polyps of the octocoral *Pseudopterogorgia bipinnata* became infected after he added cultured zooxanthellae and that motile zooxanthellae appeared to be attracted to the polyps;

however, he observed only one zooxanthella enter the mouth of a polyp. Schwarz (1996) found that planulae of the temperate sea anemone *Anthopleura elegantissima* acquired zooxanthellae as a result of feeding on animal tissue which contained zooxanthellae recently isolated from a previous host.

Despite the uncertainty of infection via horizontal transmission, the benefit to acquiring symbionts from the environment is that it might allow the host to form an association with genetically distinct symbionts that are adapted to local conditions. Conventionally, cnidarian/algal host-symbiont specificity was assumed to be static and unchanging, but recent evidence suggests that associations between host and symbiont are more dynamic. Rowan and Knowlton (1995) found that the corals *Montastraea faveolata* and *M. annularis* can associate with several different species of *Symbiodinium* which occur along an environmental gradient, and that hosts can contain up to two species at one time. Davy *et al.* (1997) found that under laboratory conditions, the sea anemone *Cereus pedunculatus* was able to form long term associations at equivalent densities with both homologous zooxanthellae (i.e., isolated from conspecifics) and heterologous zooxanthellae (i.e., isolated from different host species). Thus while vertical transmission ensures that offspring are provided with zooxanthellae, horizontal transmission may allow for the acquisition of symbionts that are adapted to the differing environments in which offspring ultimately settle.

Although little is known about the mechanisms by which larval stages acquire symbionts from the environment, the infection process has been investigated in polyp stages of the scyphozoan *Cassiopeia xamachana* and the freshwater hydrozoan *Hydra viridis*, which hosts a green alga. In both species, symbionts enter through the mouth of the host and are phagocytosed by endodermal cells lining the gastric cavity of the host (Cook *et al.* 1978, Fitt and Trench 1983). This mechanism is likely to be the same for larvae that acquire zooxanthellae from the environment, but since it requires that larvae possess a mouth and gastric cavity,

non-feeding larvae may not be competent for infection until after a mouth develops during metamorphosis.

Symbiotic state can influence host gene expression. Weis and Levine (1996) found that zooxanthellate polyps of the sea anemone *Anthopleura elegantissima* contained numerous proteins that were absent in azooxanthellate polyps. One of these proteins, carbonic anhydrase, functions in the symbiosis to facilitate inorganic carbon transport to symbionts (Weis *et al.* 1989, Weis 1991, 1993). Although nothing is known about when such symbiosis-specific genes are induced in initially azooxanthellate hosts, they likely begin to be expressed soon after the incorporation of symbionts into host tissue.

In this study we examined the process of symbiont acquisition in *Fungia scutaria*. This solitary coral is gonochoric and females spawn azooxanthellate eggs that are fertilized within the water column and develop into azooxanthellate larvae (Krupp 1983). Krupp reported on early development in this species and observed that larvae reared in aquaria that contained adult corals, acquired zooxanthellae four to five days following spawning. He observed a "mouth opening" response to the addition of zooxanthellae obtained from homogenized tissues of adult *F. scutaria*, but did not observe zooxanthellae entering the mouths of larvae. This paper describes late larval development and the process of symbiont acquisition (infection) in *F. scutaria*, including developmental stages at which the host is competent to become infected by zooxanthellae, the mechanism of zooxanthella acquisition by the host, the mechanism of zooxanthella incorporation into host tissue, the effect of feeding behavior on the infection rate, host-symbiont specificity, and comparisons between protein profiles of azooxanthellate and zooxanthellate larvae through development.

MATERIALS AND METHODS

Gamete collection and larval cultures

Approximately 75 adult specimens of *Fungia scutaria* are maintained year-round in running seawater tables at the Hawaii Institute of Marine Biology on Coconut Island, Kaneohe Bay, Hawaii. Prior to spawning, the corals were rinsed with seawater and placed in standing seawater in individual glass fingerbowls. Spawning in this species generally occurs between 5 and 7 pm 2 - 4 days following the full moon during June through August. In August 1995, August 1996 and June and July 1997, eggs were collected by removing the adults from the finger bowls and leaving the eggs in the bowl into which they were spawned. If the egg density was greater than a single layer of eggs at the bottom of the dish, some of the eggs were collected with a turkey baster and transferred to a new fingerbowl. Within 30 min after spawning, water from the dishes of all spawning males was combined and a small volume was gently pipetted into the dishes containing eggs. The dishes were left in a seawater table overnight for fertilization and early larval development. The following day, the water was changed (0.45μm filtered seawater was used throughout). Larvae from all parental crosses were combined and the larvae were maintained in large glass finger bowls in filtered seawater, which was changed every day.

Preparation of zooxanthella isolates

Zooxanthellae were isolated from adult specimens of *F. scutaria* using a waterpik to remove and homogenize coral tissue and were subsequently concentrated by centrifugation using a tabletop centrifuge at 2000 g. The

zooxanthella pellet was partially cleaned of animal tissue by twice rinsing in filtered seawater and was again concentrated by centrifugation. Zooxanthella isolates were used within 2 h of preparation. Zooxanthellae were isolated from the sea anemone *Aiptasia pallida* using the same methods, except that whole animals were homogenized in a ground glass tissue grinder.

Preparation of homogenized *Artemia* sp.

To stimulate feeding behavior in larvae, homogenized *Artemia* sp. (brine shrimp) was added to larval cultures. A small pinch of frozen *Artemia* was homogenized in a ground glass tissue grinder in approximately 1 ml seawater and filtered through a 60 µm mesh to remove large particulate matter. The resulting slurry was used within 15 min of preparation.

Acquisition of zooxanthellae

To identify a) the developmental stages at which *F. scutaria* is competent to become infected and b) the mechanisms of zooxanthella acquisition, larvae from 4 different stages of development (Table 1) were exposed to zooxanthellae from different sources, with or without homogenized *Artemia*, a feeding stimulant. Homologous algae were freshly isolated from adult *F. scutaria,* and heterologous algae were either freshly isolated from the sea anemone *Aiptasia pallida* or taken from algal cultures originating from the jellyfish *Cassiopeia xamachana.* Three replicates were established for all treatments. Larvae were concentrated in glass finger bowls ($>10^4$ larvae per bowl) and zooxanthellae were added by pipetting an even layer of zooxanthellae along the bottom of the bowls. Several drops of homogenized *Artemia* were added to the appropriate treatments. Zooxanthellae and

Artemia slurry were removed either 4 or 24 h later (Table 1) by concentrating larvae on a filter and placing them into clean filtered seawater. Some larvae from each treatment were observed under a compound microscope, either immediately after zooxanthellae were removed or 24 h later, to determine if they had become infected with zooxanthellae.

Table 1. Experimental treatments of *Fungia scutaria* larvae.

Treatment: Developmental Stage	Source of Algae	*Artemia* added?	Exposure Duration	Infection determined
A: embryo-early planula (0-12 h old)	*F. scutaria*	no	overnight	immediately
B: early planula (1-2 days old)	*F. scutaria*	no	overnight	immediately
C1: fully developed planula (3 days old)*	*F. scutaria*	no	4 h	after 24 h
C2: fully developed planula (3 days old)*		yes	4 h	after 24 h
D1: fully developed planula (3 days old)*	*A. pallida*	no	4 h	after 24 h
D2: fully developed planula (3 days old)*		yes	4 h	after 24 h
E1: fully developed planula (3 days old)*	*C. xamachana*	no	4 h	after 24 h
E2: fully developed planula (3 days old)*		yes	4 h	after 24 h
F: polyp (after metamorphosis)	*F. scutaria*	no	overnight	after 24 h

* planulae were considered fully developed once they had acquired the ability to feed

For treatments C1 and C2, we used the following method to determine the fraction of larvae that became infected. Twenty-four h after larvae were exposed to zooxanthellae, water in the larval cultures was swirled and 1 aliquot per replicate was removed. Between 25 and 56 larvae per aliquot were observed under a compound microscope and the number of larvae that contained zooxanthellae was quantified.

Larval development

To observe and quantify the developmental progression of both azooxanthellate and zooxanthellate larvae, 6 replicate cultures of each were maintained in plastic 6-well culture dishes (300-500 larvae per well in 5 ml filtered sea water). Water was changed approximately once a day. Larval development was monitored for approximately 2 weeks. Each replicate well was placed haphazardly under a dissecting microscope, and within the field of view, the number of larvae at each developmental stage was counted.

Electron microscopy

To follow the process of zooxanthella incorporation into host tissue, larvae from treatment C2 were sampled and fixed for electron microscopy 1 and 24 h after zooxanthellae were added to larval cultures. Larvae were placed in sampling cups, prepared by cutting off the bottoms of microfuge tubes and affixing 50 μm mesh across the bottom. The cups were placed in 1% glutaraldehyde in a phosphate-buffered saline (PBS, 0.1M sodium phosphate, 0.45M sodium chloride, pH 7.2) for 1 h, rinsed 3 x 10 min in PBS, postfixed for 1 h in 1% osmium tetroxide in PBS, rinsed 3 x 10 min in PBS, and dehydrated for 15 min each in 30%, 50%, and 70% ethanol, and then 1 h each in 80%, 95%, and 3 x 100% ethanol. Samples for scanning electron microscopy were dried for 15 min in hexamethyldisilane, mounted on stubs, coated with 60:40 Au:Pd, and viewed on a Amray 3300FE scanning electron microscope. Samples for transmission electron microscopy were infiltrated with Spurr's resin in 1:1 ethanol:resin for 2.5 h, 1:3 mix for 2.5 h, 2 x 100% resin for 1 h, and 100% resin overnight at 60°C. Thin sections were prepared on an ultra-microtome, stained with uranyl acetate and lead citrate, and viewed on a Philips CM12 transmission electron microscope.

Polyacrylamide gel electrophoresis

To determine whether symbiotic state affects protein synthesis in larvae, we prepared one-dimensional SDS-PAGE protein profiles of both azooxanthellate and zooxanthellate larvae through development (eggs through seven-day-old larvae). For each sample, approximately 1,000 larvae were counted, collected by centrifugation, and frozen at -80°C. Protein extracts were prepared by homogenizing frozen larvae over ice in a ground glass grinder in 100 μl homogenization buffer (40mM Tris-HCl, 10mM EDTA, protease inhibitor cocktail (Sigma), pH 7.4). Homogenates were centrifuged for 10 min at 14,000g to pellet zooxanthellae and animal debris. Protein concentration of the supernatant was determined spectrophotometrically (Bradford, 1976); larvae contained approximately 50-100 ng protein/larva. Larval proteins were resolved on 12.5% SDS-PAGE gels under reducing conditions (methods modified from Laemmli, 1970). Gels were silver stained (methods modified from Heukeshoven and Dernick, 1986) and scanned on an Imagemaster desktop scanner (Pharmacia) and analyzed using Imagemaster software (Pharmacia).

RESULTS

Larval development

Larval development was observed over three summers (1995, 1996, 1997). Larvae from all years followed the same progression of developmental stages, as illustrated in Figure 2.2.1a and detailed below in Figure 2.2, progressing from swimming to creeping to settled. The duration of each developmental stage,

however, was variable and for the later stages, differed by up to several days both within and among replicates. Figure 2.2.1b shows the time course of developmental events for zooxanthellate larvae from 1996.

All larvae progressed through the following series of stages. Within 12 h after fertilization, slowly moving, ciliated spherical planulae developed, and within 24 h, barrel-shaped planulae, approximately 100μm in length (shown in Figure 2.2a), had developed and were actively swimming at all depths in the culture dishes. By day three, larvae had fully formed mouths and functional gastric cavities, and were capable of feeding. Upon addition of food (homogenized *Artemia*), larvae ceased swimming and dropped to the bottom of the dish. They extruded mucus, their oral ends expanded, and they ingested whatever they landed on, including experimentally added zooxanthellae. As they fed, their gastric cavities became filled with particulate matter, as shown in Figure 2.2b. Some larvae resumed swimming while trailing a strand of mucus; the mucus trapped particulate matter that slowly entered the mouth. Larvae continued to feed for several hours, and then resumed swimming. Except for zooxanthellae, all ingested particulate matter was digested or expelled by the following day. When larvae were approximately four days old, they assumed a ball shape, ceased active swimming, and began creeping slowly over the substrate. Starting on day five, the ball-shaped larvae began to settle. They spread out over the substrate and metamorphosed into volcano-shaped polyps, which began to develop tentacle buds several days after metamorphosis, as shown in Figure 2.2c.
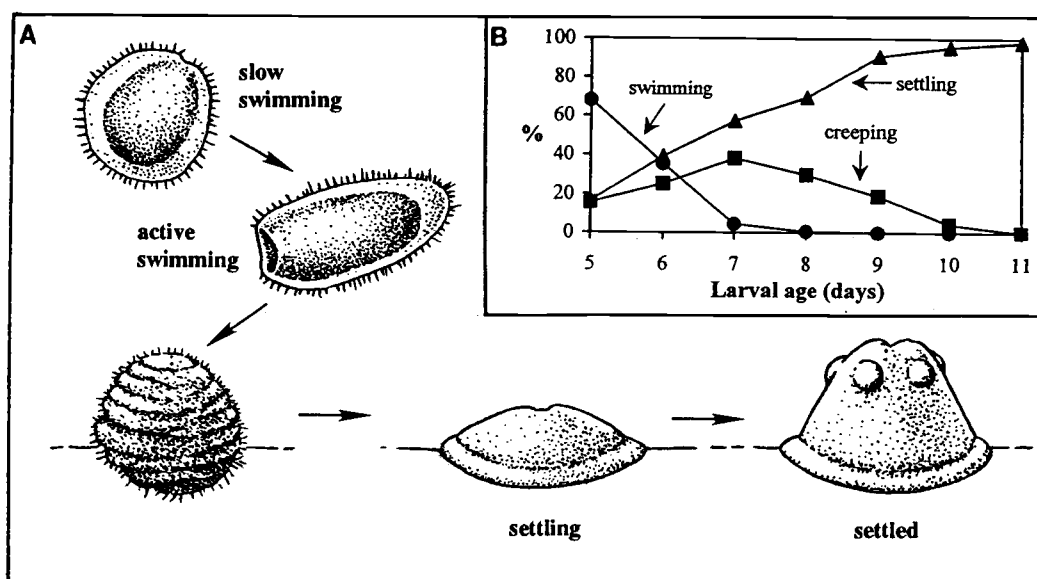
Figure 2.1. Progression of developmental events in larvae of *Fungia scutaria* larvae. A) Schematic representation of developmental stages from the early planula through metamorphosed polyp. B) Example of the time course of developmental events. Data shown are from zooxanthellate larvae in 1996. Larvae were infected with zooxanthellae on day-three and then divided into six replicate dishes which were monitored daily. Each point represents data pooled from the six replicates. Larvae progressed from swimming to creeping to settled.
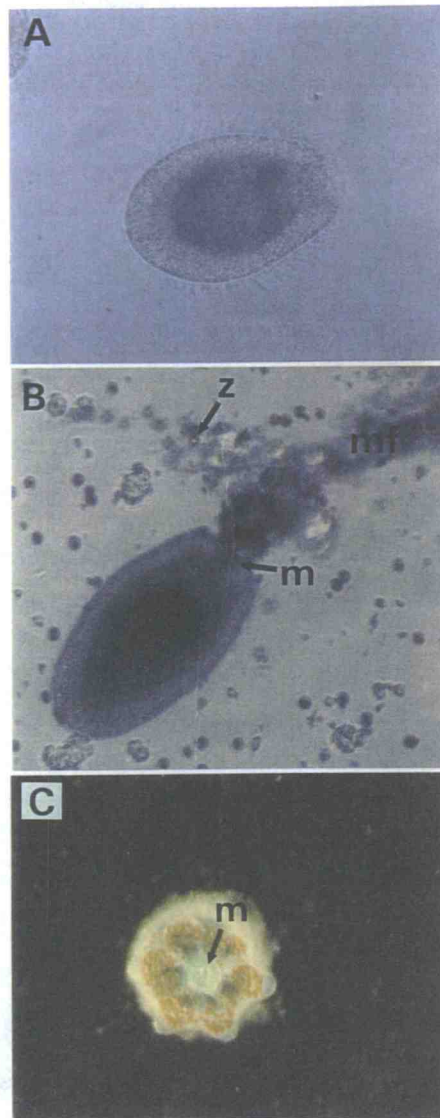
Figure 2.2. Light micrographs of developmental stages in *Fungia scutaria* larvae. A) Two-day-old planula larva, prior to development of a mouth. B) Three-day-old feeding planula (m = mouth, mf = mucus strand with food particles attached, z = zooxanthella). C) Polyp with tentacles, 6 days after settling. Zooxanthellae visible as golden spheres. Planula length and polyp diameter, approximately 100 μm.

Acquisition of zooxanthellae and onset of symbiosis

Prior to the development of a functional mouth on day three, planulae of *F. scutaria* did not become infected by experimentally added zooxanthellae. Once a functional mouth had developed, however, planulae were able to acquire zooxanthellae. When stimulated to feed, larvae indiscriminately ingested any particulate matter, including experimentally-added zooxanthellae. Zooxanthellae were either ingested as part of a larger mass that was fully engulfed by the mouth, or adhered to mucus strands that were ingested by the larvae. Figure 2.3a shows a zooxanthella adhered to a larval mucus strand and Figure 2.3b shows several zooxanthellae surrounding and contained within the oral cavity of a larva. One hour after zooxanthellae were added, larvae were sampled and fixed for transmission electron microscopy. Figure 2.4 shows a representative planula one hour post-feeding, in longitudinal section, with several algae resident in endodermal cells. Micrographs suggest that zooxanthellae are phagocytosed by endodermal cells lining the coelenteron (Figure 2.5a & b) and appear in both endodermal (Figure 2.5c) and ectodermal tissue (Fig 5d). Although zooxanthellae were still present in ectoderm 24 hours later, we did not determine how long zooxanthellae remained within the ectoderm or whether they eventually migrated into the endoderm or were digested or expelled from the host.

Larvae were not limited to forming an association with a specific strain of zooxanthellae; planulae were capable of becoming infected by zooxanthellae isolated from *F. scutaria* (Treatment C2) and *Aiptasia pallida* (Treatment D2), as well as by cultured zooxanthellae from *Cassiopeia xamachana* (Treatment E2). To determine whether the host had retained zooxanthellae, larvae from Treatments C2 and D2 were observed over a period of 10 to 14 days. Larvae that had acquired zooxanthellae on day three remained infected as they progressed through development and metamorphosis into polyps.
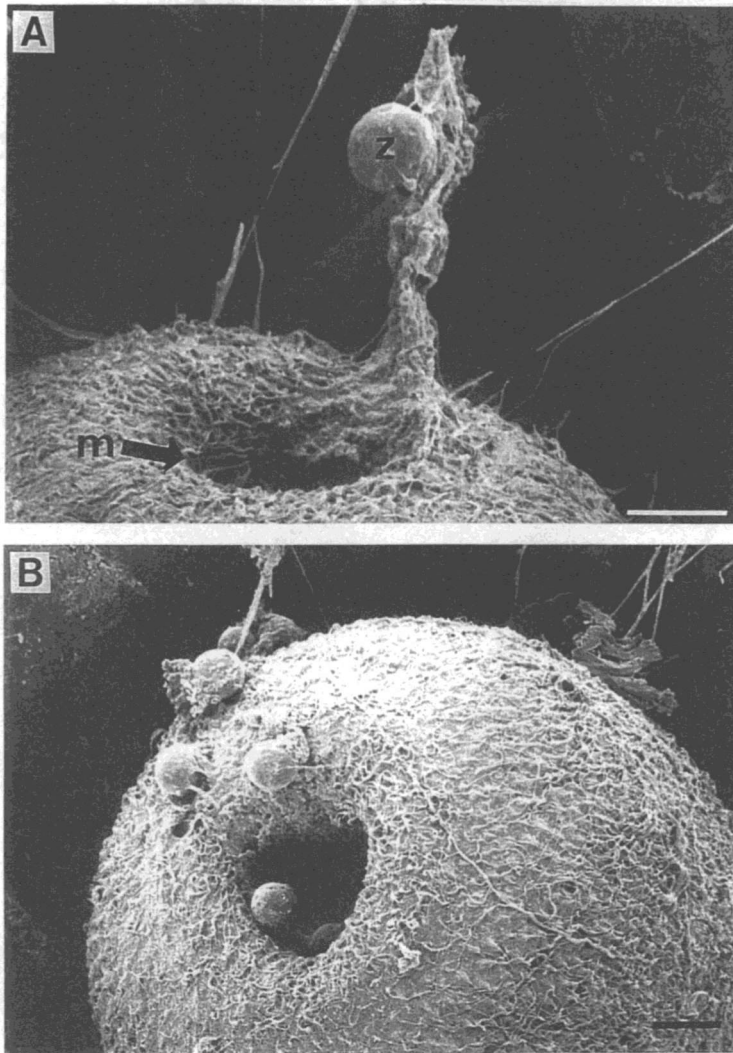
Figure 2.3. Scanning electron micrographs detailing zooxanthella acquisition by three-day-old *Fungia scutaria* planulae. A) Feeding planula with zooxanthella adhered to mucus strand (m = mouth, z =zooxanthella). B) Feeding planula, with multiple zooxanthellae entering the mouth. Larvae were fixed for electron microscopy one hour after exposure to zooxanthella isolates and homogenized *Artemia* (see Methods). Bars = 10μm.
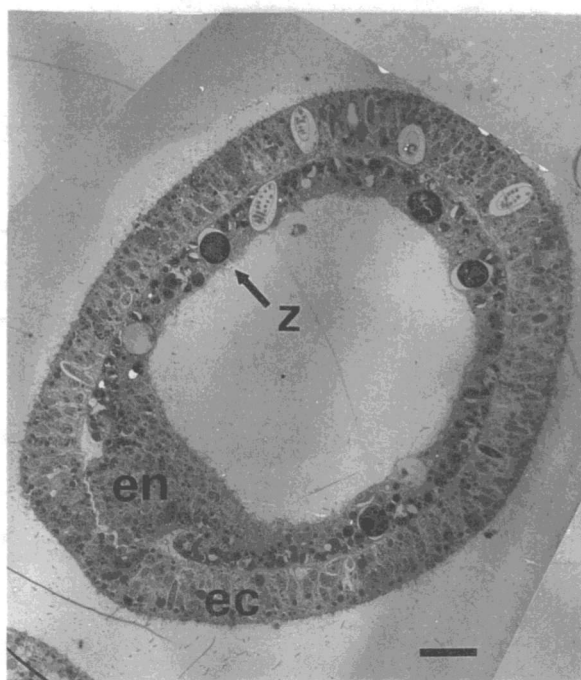
Figure 2.4. Transmission electron micrograph of a longitudinal section through a
Fungia scutaria larva infected with zooxanthellae. Thickened oral end at lower
left. Zooxanthellae appear in the endoderm as dark spheres. Light ellipses, mostly
in the ectoderm, are poorly preserved nematocysts. ec = ectoderm, en = endoderm,
z = zooxanthella. Bar = 20 μm.
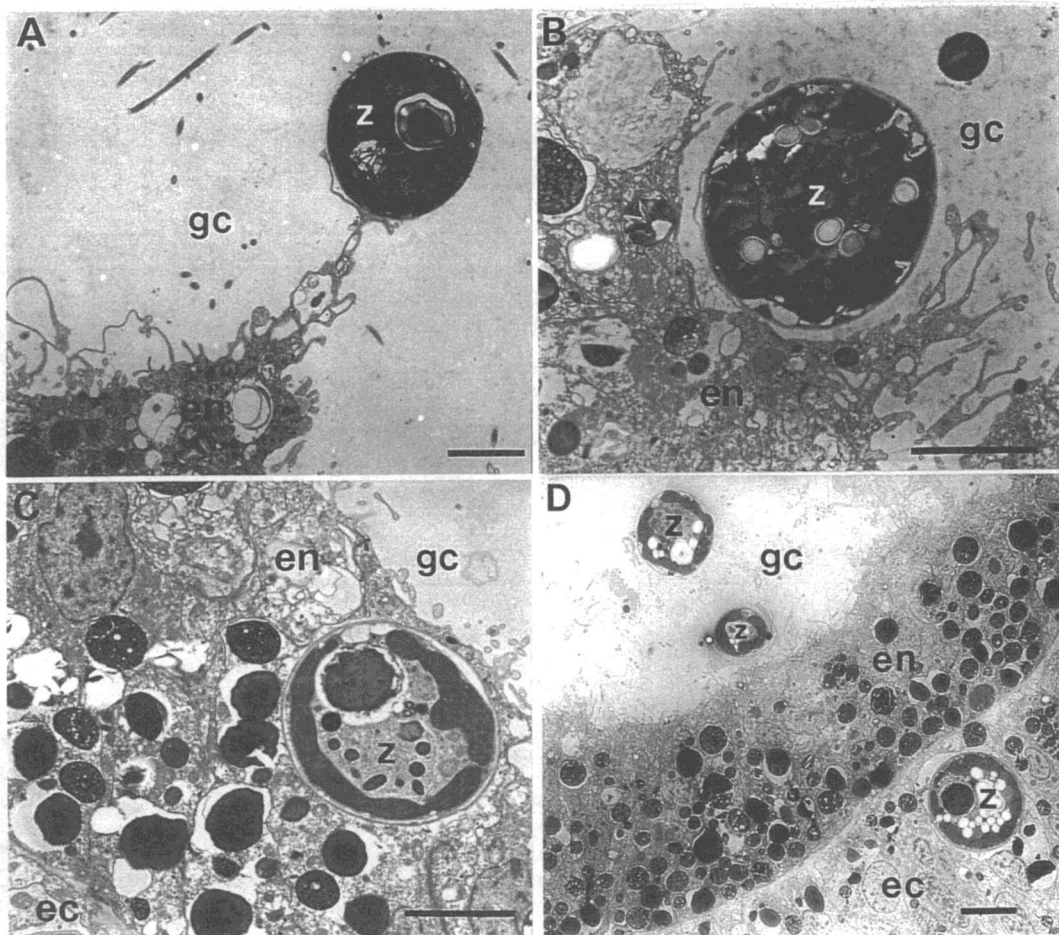
Figure 2.5. Transmission electron micrographs of onset of symbiosis between *Fungia scutaria* planulae and zooxanthellae. A) Section through endoderm and gastric cavity of a planula showing initial contact between an endodermal cell and a zooxanthella. Host endodermal membranes are very closely associated with the alga. B) Endodermal cell partially surrounding a zooxanthella, suggesting that the alga is being ingested by the host cell. C) Zooxanthella resident within a vacuole in an endodermal cell. Gastric cavity is on upper right. D) Zooxanthella resident within a vacuole in an ectodermal cell. ec = ectoderm, en = endoderm, z = zooxanthella. Bars = 5 μm.

Infection by zooxanthellae was not required for metamorphosis. As shown in Figure 2.6, both zooxanthellate and azooxanthellate larvae successfully settled and metamorphosed into polyps. Larvae infected with zooxanthellae from *F. scutaria* (Treatment C2) and *A. pallida* (Treatment D2) both underwent metamorphosis (we did not monitor settlement for larvae infected with zooxanthellae cultured from *C. xamachana*). Aposymbiotic polyps were able to ingest zooxanthellae via ciliary currents produced by the polyps that swept particles, including zooxanthellae, over and into their mouths. Observations over the six days following showed that the zooxanthellae were retained within the polyps throughout this period.

The proportion of larvae that became infected by zooxanthellae isolated from adult *F. scutaria* (Treatment C) depended on the strength of the feeding response. Feeding was observed to be strongly stimulated (i.e., virtually all larvae began to feed) by the addition of homogenized *Artemia*, but was also stimulated to a lesser extent (i.e., some larvae began to feed) simply by the addition of zooxanthella-isolates, which contained residual animal host tissue. We quantified the effect of larval feeding strength on zooxanthella acquisition for treatments C1 (zooxanthellae alone) and C2 (zooxanthellae and *Artemia*). In the zooxanthellae alone treatment, 25.0% ± .02 (n=2) of larvae acquired zooxanthellae, whereas 96.8% ± .01 (n=2) became infected when exposed to both zooxanthellae and *Artemia*. It was clear that larvae in Treatments D and E also became infected at a higher rate when exposed to both zooxanthellae and homogenized *Artemia* than to zooxanthellae alone, although the results were not quantified.

An experiment in 1996 provided preliminary evidence that symbiotic state may influence development in *F. scutaria*. Zooxanthellate larvae settled and metamorphosed earlier than azooxanthellate larvae, most of which became arrested in the "ball stage" and then eventually died (Figure 2.7). However, the same experiment repeated in 1997 showed low rates of metamorphosis for both

zooxanthellate and azooxanthellate larvae and no difference in the timing of metamorphosis.
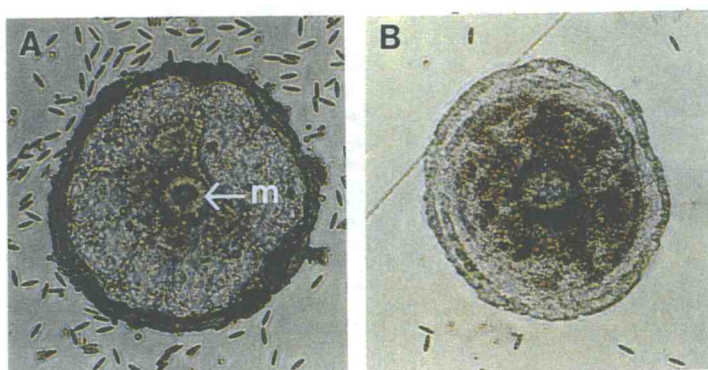
Figure 2.6. Light micrographs of newly settled polyps of *Fungia scutaria*. A) Azooxanthellate polyp (m = mouth). B) Zooxanthellate polyp. Zooxanthellae appear as brown spheres in the polyp. The two polyps shown in this figure were settled in the same dish, adjacent to one another. Contaminating diatoms appear as small ellipses around the polyps. Polyp diameter = 100μm.
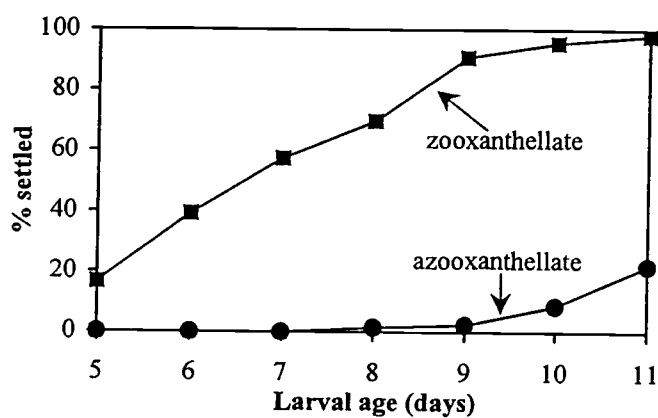
Figure 2.7. Effect of symbiotic state on larval settlement. Results from 1996 experiment. Nearly 100% of zooxanthellate planulae underwent settlement and metamorphosis by day ten, whereas most azooxanthellate planulae failed to settle. Each point represents data pooled from six replicate dishes for each treatment.

Larval protein profiles

Protein profiles of larvae through development showed changes in profiles with age of larvae, but no gross differences between zooxanthellate and azooxanthellate larvae at day six, as shown in Figure 2.8a. Two bands, at 84 and 79 kiloDaltons (kDa), were abundant in eggs and one-day-old larvae. As shown in Figure 2.8b, this protein doublet comprised a significant proportion (36%) of total protein in one-day-old larvae, but was almost absent by day six. The apparent depletion of this protein corresponds to the onset of settlement and metamorphosis.

DISCUSSION

Larval development and acquisition of zooxanthellae

Larval development has been observed in many coral species, and development in *Fungia scutaria* progressed similarly to that reported in other broadcast spawning species (review in Harrison and Wallace 1990). Within 24 hours after fertilization, fully developed planula larvae had developed, which is within the range of one to several days reported for other species. Larvae of *F. scutaria* were approximately 100μm in length, ciliated, had a barrel-shaped appearance, and exhibited active swimming behavior until they settled at the age of five days to approximately two weeks, which is typical for the appearance and behavior of externally-developed planula larvae.
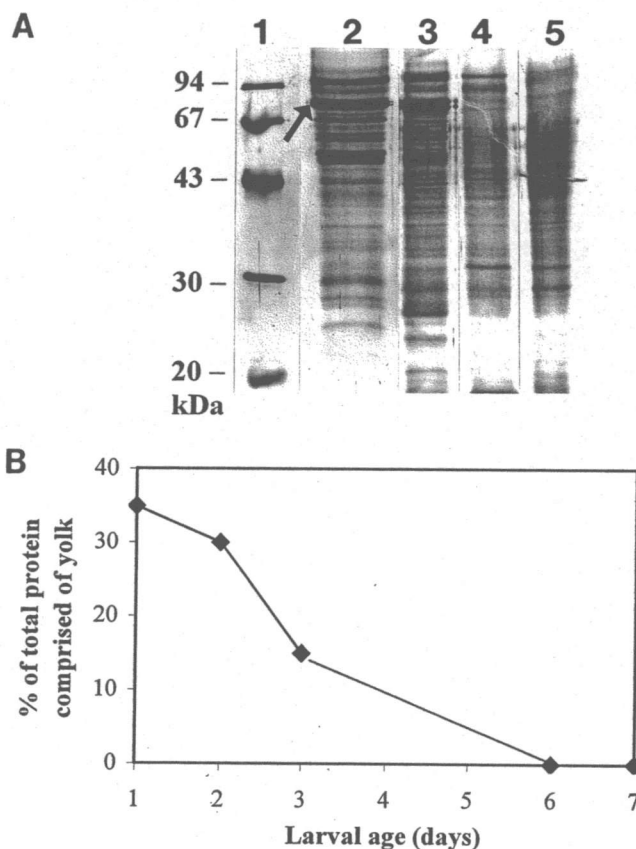
Figure 2.8. Protein profiles of *Fungia scutaria* larvae. A) Silver-stained 1D SDS-polyacrylamide gel of total protein extracted from eggs (lane 2), one-day-old larvae (lane 3), six-day-old azooxanthellate larvae (lane 4), and six-day-old zooxanthellate larvae (lane 5). Each lane contained 1.2 µg protein. Molecular weight standards in lane 1. Arrow highlights a putative yolk protein doublet (84 and 79 kDa) which is abundant in eggs and one-day larvae but absent by day six. B) Decline in putative yolk protein abundance through larval development. The depletion of putative yolk protein corresponded with the onset of settlement on day seven. There was no difference in putative yolk protein abundance in azooxanthellate *vs.* zooxanthellate larvae (days five and six); data shown represent the average of the two treatments.

Very little is known about the feeding ability or behavior of coral planulae, as most reports of larval development do not address this aspect of larval behavior. While it appears that many species, particularly brooding species, produce a non-feeding larva, it is likely that the ability to feed has not been recognized in some species, due to larval rearing techniques, which generally include maintenance of larvae in clean, filtered seawater. We found that feeding behavior in *F. scutaria* was very similar to that reported for the temperate coral *Caryophyllia smithi* (Tranter et. al. 1982) and for the temperate sea anemones *Anthopleura elegantissima* and *A. xanthogrammica* (Siebert 1974, Schwarz 1996). Feeding consisted of a mouth-opening response to the addition of ground animal tissue, as well as secretion of mucus strands which trapped particulate matter that was ingested.

Although the majority of scleractinian coral species spawn azooxanthellate gametes that develop into azooxanthellate planulae (review in Richmond 1997), little is known about how such offspring might acquire zooxanthellae from the environment. Results of this study indicate that competency for infection by zooxanthellae may depend on the development of a functional mouth. Prior to the development of a mouth, larvae of *F. scutaria* did not become infected by experimentally-added zooxanthellae, but after the development of a mouth, all developmental stages were competent to become infected. Reports of infection events in other species support this hypothesis. Both *F. scutaria* and *A. elegantissima* planulae exhibited feeding behavior that led to the ingestion of zooxanthellae (Schwarz, 1996), while species that were infected as polyps appeared to have a non-feeding planula that did not develop a mouth until after metamorphosis (Babcock and Heyward 1986, Benayahu *et al.* 1989). It will be interesting to examine other species that produce a feeding planula larva to see

whether zooxanthella acquisition occurs in the same manner as that shown for *F. scutaria* and *A. elegantissima.*

Both endodermal and ectodermal cells incorporated zooxanthellae within one hour after larvae were exposed to zooxanthellae. The appearance of zooxanthellae in ectodermal tissue was unexpected, since anthozoans generally contain zooxanthellae exclusively in the endoderm. We did not determine the mechanism by which zooxanthellae entered the ectoderm nor the fate of ectodermal zooxanthellae. It is possible that the movement of zooxanthellae into the ectoderm provides an escape from endodermal lysosomes. It is also possible that larval endoderm has a limited volume. Zooxanthellae could be temporarily stored in the ectoderm until growth of the host provides sufficient volume for them to be moved back into the endoderm. Future work will include long term sampling of newly infected larvae to investigate the fate of ectodermal zooxanthellae.

Horizontal transmission of symbionts would appear to be disadvantageous for obligately symbiotic species because of the possibility that infection may not occur. However, it is possible that for larvae dispersed to an area with different environmental conditions, the ability to acquire zooxanthellae from the environment might confer a greater advantage to the host than directly inheriting maternal zooxanthellae. This study found that larvae were capable of forming an association with members from three different clades of zooxanthellae classified by Rowan and Powers (1991a,b); zooxanthellae from *C. xamachana* are in group A, those from *A. pallida* are in group B, and those from *F. scutaria* are in group C. The degree to which zooxanthellae from different clades persist in *F. scutaria* remains to be investigated, but our results point to the potential for considerable flexibility in host-symbiont specificity in this species. This is in contrast to the finding that *A. elegantissima* planulae, although able to form an association with zooxanthellae recently isolated from a conspecific adult, were unable to form an

association with cultured *S. californium*, which is the species reported to occur in *A. elegantissima* (Banaszak *et al.* 1993, Schwarz 1996).

The relationship between larval feeding behavior and rate of infection, whereby a stronger larval feeding response resulted in higher rates of infection, indicates that larval feeding behavior may play an important role in acquiring zooxanthellae from the ambient environment. Because so little is known about the distribution and abundance of zooxanthellae in the natural environment, it is difficult to speculate on potential sources of zooxanthellae. However, one source of zooxanthellae that is likely to occur in abundance is mucus expelled by corals. Cnidarian hosts regularly expel mucus which contains high concentrations of zooxanthellae (Steele 1975, McCloskey *et al.* 1996, Schwarz, pers. obs.), and it has been noted that spawning is accompanied by increased rates of mucus expulsion (Montgomery and Kremer 1995, D. Krupp, pers. obs.). Although this study did not examine whether larvae will feed on coral mucus, larvae of the sea anemone, *Anthopleura elegantissima*, did feed on mucus expelled by adults and became infected by zooxanthellae contained within the mucus (Schwarz 1996). The acquisition of zooxanthellae as an incidental consequence of feeding behavior indicates that larvae may be able to acquire zooxanthellae which are present both where spawning occurred and where larvae ultimately settle, allowing larvae to acquire zooxanthellae adapted to different environments.

Effect of symbiont acquisition on larval development and protein profiles

It is well known that zooxanthellae affect the physiology of adult hosts, and it is likely that the acquisition of zooxanthellae by larval hosts influences larval development. For example, it is possible that the acquisition of symbionts acts as a settlement cue. An experiment in 1996 demonstrated that zooxanthellate larvae settled earlier than azooxanthellate larvae (Figure 2.6), indeed most azooxanthellate

larvae failed to settle. However, the same experiment repeated the following year showed no differences in settlement (data not shown). It is possible that symbiotic state does influence developmental events, but that it either acts in concert with, or is overridden by, environmental variables, such as temperature. The 1996 experiment was conducted during a period of anomalously warm water temperatures that induced a bleaching event on the reef flat in Kaneohe Bay, while the 1997 experiment was conducted during a period of normal water temperatures. Thus water temperature may have influenced larval development more strongly than symbiotic state. Experimental manipulation of environmental parameters will allow us to examine this question in more detail.

Presence of zooxanthellae has been shown to affect protein profiles of adult *A. elegantissima* (Weis and Levine 1996). There are both gross and subtle differences between zooxanthellate and azooxanthellate anemones as revealed by comparing two-dimensional (2D) gel profiles. We detected no gross differences in one-dimensional (1D) protein profiles between zooxanthellate and azooxanthellate *F. scutaria*. It is likely that we need to use the higher resolution 2D gel analysis and [35][S]-methionine labeling that detects newly synthesized proteins (Weis and Levine 1996) to reveal subtle changes in host protein production that are occurring during the onset of symbiosis.

Potential effect of symbiont acquisition on larval energetic strategies and dispersal

The larval stage serves as a means for dispersal in the life histories of sessile marine invertebrates. The length of the larval stage depends in part on the amount of energy available for metabolism, (Boidron-Métairon 1995, Levin and Bridges 1995). Larvae of *F. scutaria* have several potential sources of energy that may allow them to extend the larval stage sufficiently to explain their widespread occurrence throughout the Pacific. First, larvae may initially obtain nutrition from

yolk protein supplied through the egg. Second, once the mouth has developed, larvae may obtain energy through feeding. Third, larvae that have acquired zooxanthellae may receive nutrition in the form of organic carbon translocated by zooxanthellae. Each of these modes of nutrition may operate at different times through development.

Initially, larvae of *F. scutaria* may obtain energy through yolk reserves supplied in the egg. Eggs and one-day-old larvae of *F. scutaria* contained a protein doublet that was absent by day six. The pattern of its decline and the correlation between its depletion and the onset of settlement suggests that larvae may metabolize this protein over the course of their development. Immunoblot analysis showed no cross-reactivity with antibodies to a known yolk protein from the sea urchin *Strongylocentrotus purpuratus*, (*S. purpuratus* antibodies from William Lennarz, SUNY Stonybrook; data not shown). This result was not surprising, since yolk proteins are not highly conserved. Scott and Lennarz (1989) demonstrated that while the yolk protein antibody from *S. purpuratus* cross reacts with yolk proteins from other echinoids, it does not cross react with yolk proteins from members of other echinoderm classes, nor with those from other animal phyla.

From day three on, larvae were able to feed on homogenized animal tissue. If the length of the larval stage is a function of the amount of energy available to larvae, then the ability to feed would allow larvae to postpone metamorphosis for an extended period of time. However, feeding would be restricted to environments that contain a source of food, and if larvae were dispersed away from the reef into open-ocean water masses, they may no longer be able to obtain energy through feeding.

Larvae may also benefit from reduced organic carbon translocated from zooxanthellae. Richmond (1981, 1987) demonstrated that symbiotic planulae of the coral *Pocillopora damicornis* received about 13 - 27% of the carbon fixed by zooxanthellae, an amount that he considered sufficient to potentially extend the

larval stage. If a similar benefit occurs in *F. scutaria*, the input of energy from zooxanthellae may be sufficient for larvae to maintain their metabolism after being dispersed away from the reef and thus allow them to postpone settlement until they are transported to an appropriate settlement site. However, virtually nothing is known about how the onset of symbiosis might influence development, and it could be argued that the presence of zooxanthellae might either lengthen or shorten the larval stage. While translocation of organic carbon by zooxanthellae might provide sufficient energy for larvae to postpone metamorphosis and thus extend the dispersal stage, the onset of symbiosis might instead serve as a settlement cue and thus shorten the larval stage.

ACKNOWLEDGEMENTS

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CHAPTER 3

IMMUNOLOCALIZATION OF HOST SYM32 AND AN
UNDESCRIBED PROTEIN, p45/48, IN THE SEA ANEMONE-
DINOFLAGELLATE ASSOCIATION *ANTHOPLEURA ELEGANTISSIMA-
SYMBIODINIUM MUSCATINEI*

INTRODUCTION

Intracellular associations between eukaryotic microorganisms and animal
hosts encompass a wide range of interactions ranging from parasitic/pathogenic to
mutualistic. The majority of eukaryotic microbes that infect animals reside within
a membrane-bound vacuole within the cytoplasm of host cells (Hackstadt 2000).
The formation of the vacuole is a dynamic process that is initiated either by active
invasion of the host cell by the microbe, for example the parasite *Toxoplasma*
(Dobrowolski and Sibley 1996), or by phagocytic uptake of the microbe by the host
cell, for example the parasite *Leishmania* (Courret et al 2002) and the symbiont
Symbiodinium (Fitt and Trench 1983).

Studies on eukaryotic microbial parasites show that, in the vast majority of
cases, the nascent vacuole that contains the microbe usually fails to continue
through the complete endocytic pathway that culminates in acidification of the
vacuole and fusion with lysosomes. Instead, the microbial inhabitant actively
interferes with one or more steps in the normal process of fusion between the
vacuole and endocytic organelles that direct the vacuole through the phagocytic
process (Mellman 1996). The result is that the microbe successfully transforms
what would have been a phagolysosome into a compartment that is hospitable for
growth, replication, and/or differentiation into various life history stages (Hackstadt
2000, and for examples see Mukkada 1985, Sinai and Joiner 1997).

In the marine environment, associations between animals and eukaryotic microbes include mutualistic as well as parasitic interactions. The most prevalent mutualistic association occurs between cnidarians (most commonly sea anemones and corals) and photosynthetic dinoflagellates (usually *Symbiodinium* spp.). Cnidarian-algal associations are, overall, considered to be mutualistic associations, characterized primarily by reciprocal nutritional interactions between host and symbiont (Falkowski et al. 1984, Muller-Parker and D'Elia 1998). The photosynthetic symbionts contribute glycerol and other organic compounds to host metabolism (Lewis and Smith 1971, Battey and Patton 1987, Muscatine 1990), and the host contributes nitrogen (Wang and Douglas 1998, 1999) and carbon dioxide (Weis 1993) for algal photosynthesis. These associations occur in the photic zones of both temperate and tropical benthic habitats, and they thrive in nutrient-poor tropical marine environments due to their ability to conserve and recycle nutrients.

In their associations with dinoflagellates, the host cnidarian most commonly houses symbionts within gastrodermal cells, in a vacuole of phagosomal origin. The initial infection event during which the dinoflagellate symbionts are internalized typically occurs when dinoflagellates that enter the host's mouth are taken into phagocytic gastrodermal cells that line the gastric cavity (Colley and Trench 1983, Fitt and Trench 1983, Schwarz et al. 1999, Schwarz et al. 2002). The phagosome, through unknown mechanisms, fails to fuse with lysosomes (Fitt and Trench 1983) and the dinoflagellates remain undigested within the vacuole. Ultimately, the dinoflagellates reside within a compartment delineated by multiple membranes (Taylor 1968, Taylor 1987, Wakefield 2000). There has historically been uncertainty over the origin of the multiple membranes that surround the symbiont. Recently, immunolocalization studies using host-specific and dinoflagellate-specific monoclonal antibodies suggest that only the outermost membrane originates from the host and all of the inner membranes originate from the dinoflagellates, possibly the accumulation of membranes from repeated cycles of ecdysis within the host vacuole (Wakefield and Kempf 2001).

We have been interested in identifying host genes that play roles in symbiotic interactions in cnidarian-dinoflagellate symbioses (Weis and Levine 1996, Reynolds et al. 2000, Weis and Reynolds 1999). We have used, as a model system, the temperate symbiotic sea anemone, *Anthopleura elegantissima* which is abundant in the intertidal region of the eastern Pacific, from Mexico through Alaska. This species is able to form associations with three different symbiotic algae, two species of dinoflagellates from the genus *Symbiodinium* and a green alga (*Chlorella*). The presence of one or more of the symbionts within a particular host depends upon microhabitat differences that are created along temperature and light gradients that occur along latitudinal and intertidal ranges. Along the Oregon coast, the majority of hosts contain only a single symbiont, *Symbiodinium muscatinei* (LaJeunesse and Trench 2000).

We have previously described a host gene, sym32, whose expression and protein synthesis is dramatically upregulated in symbiotic specimens of *A. elegantissima*, relative to aposymbiotic (lacking symbiotic algae) specimens (Reynolds et al. 2000). Homology searches of the full cDNA sequence of host sym32 revealed that sym32 belongs to a class of cell adhesion proteins, called Fasciclin I proteins (Fas I) (Reynolds et al. 2000). Fas I proteins share low overall sequence identity, but all possess between 1 and 4 repeats of three highly conserved "Fasciclin" domains (Hu et al. 1998). Members of this family have been identified in organisms as diverse as mycobacteria (Terasaka et al. 1989), *Volvox* (Huber and Sumper 1994), insects (Zinn et al. 1988), sea urchins (Brennan and Robinson 1994), and humans (Skonier et al. 1992, Takeshita et al. 1993). All Fas I proteins characterized to date are either secreted or membrane proteins that typically function in cell-cell or cell-matrix adhesion or as antigenic proteins.

The recent identification of Fas I homologs in diverse symbiotic associations: cnidarian-algal (sym32, a host gene), plant-rhizobacterial (nex-18, a symbiont gene), and fungal-cyanobacterial (543:11, a symbiont gene) suggests a role for Fas-I proteins in symbioses between taxonomically diverse associations

(Oke and Long 1999, Reynolds et al. 2000, Paulsrud and Lindblad 2002). In the association between the nitrogen fixing bacterium, *Rhizobium meliloti* and leguminous plants, deletion of the rhizobial nex-18 gene reduced the nitrogen-fixing capabilities of nitrogen-fixing nodules that form as a result of symbiotic interactions between bacteria and plant (Oke and Long 1999). In the fungal-cyanobacterial lichen association, cyanobacterial symbionts (*Nostoc sp.*) produce high levels of an unnamed fasciclin I homolog (gene 11 from contig 543 of the Nostoc genome) when they are associated with the fungal host, and produce only low levels when cultured outside of their host (Paulsrud and Lindblad 2002).

To further investigate the role played by sym32 in symbiotic interactions between cnidarian hosts and dinoflagellate symbionts, we examined the distribution of the sym32 protein within the *A. elegantissima – S. muscatinei* association, using immunocytochemical techniques. In this paper we demonstrate that sym32 protein is differentially distributed in symbiotic vs. aposymbiotic hosts both at the cellular and subcellular level. In particular, sym32 antiserum labels the multiple layers of membrane that surround the symbiont within the host cell. We also show that sym32 antiserum specifically labels the accumulation body of the dinoflagellates. Western blots of soluble proteins isolated from two *Symbiodinium* species revealed a 45kD-48kD doublet protein that cross reacts with the sym32 antiserum. Thus both host and symbiont appear to synthesize homologous proteins, one or both of which are localized to the host-symbiont interface.

METHODS

Animal maintenance

Symbiotic and aposymbiotic specimens of *A. elegantissima* were collected at low tide from the intertidal zone at Seal Rock, Oregon. Aposymbiotic anemones were taken from under rock overhangs or crevices where there was little or no light to support the growth of symbiotic dinoflagellates. Symbiotic anemones were taken from the open rock benches that are exposed to light. Anemones were transported to the lab where they were maintained in an 11° recirculating seawater aquarium on a 12:12h light:dark cycle. Anemones were fed previously frozen adult brine shrimp approximately once a week.

Light-level immunocytochemistry

Tentacles from both symbiotic and aposymbiotic specimens of *A. elegantissima* were clipped and immediately transferred to tissue freezing medium (Triangle Biomedical Sciences) and frozen at -80°C. Tentacles were cryosectioned at -20°C on a Reichert-Jung cryostat (50μm sections) and placed on polylysine slides, then immersed in 4% paraformaldehyde fixative in phosphate buffered saline (PBS: 10mM phosphate buffer, pH 7.2 + 150mM NaCl) for 1.5 hours. Sections on slides were rinsed 3 X 5 min in PBS with 0.5% BSA, dehydrated in a methanol series (25%, 50%, 75%, 100%, 75%, 50%, 25%), and rinsed again in PBS/BSA. Sections were incubated in a 1:200 dilution of goat serum:PBS/BSA for 30 min at room temperature and then rinsed 3 X 5 min in PBS/BSA. Slides were incubated for 1 h in either a 1:2,000 dilution of sym32 antiserum from rabbit (antibody development described in Reynolds et al. 2000) in PBS/BSA or in a

1:2,000 dilution of preimmune serum from the same rabbit. Sections were rinsed 3 x 5 min in PBS/BSA and then incubated for 1 h in a 1:200 dilution of goat anti-rabbit IgG – 5 nm colloidal gold conjugate (Ted Pella). Slides were rinsed as above. Gold particle labeling was silver enhanced using a silver enhancement kit (Ted Pella). To stop color development, slides were washed in ePure water. Coverslips were affixed with a glycerol mount and sealed with fingernail polish.

EM-Level Immunocytochemistry

Immunocytochemistry was performed on 3 different occasions using symbiotic anemones collected at different times and on 1 aposymbiotic anemone. Tentacles from aposymbiotic and symbiotic anemones were clipped and immersed in 1% paraformaldehyde, 1% glutaraldehyde fixative in PBS for 1.5 h. Tentacles were rinsed 3 x 10 min in PBS and then dehydrated 15 min each concentration in a methanol series (15%, 30%, 50%, 85%, 95%, 100%, 100%). Tentacles were infiltrated with LR White resin on a rotating table in a series of MeOH dilutions (1:3 LR White:MeOH overnight, 1:1 overnight, 100% LR White for 3 hours), and then placed in gelatin capsules in fresh LR White. LR White was allowed to polymerized at 52° C for 2 days.

Ultra-thin, gold-silver sections were cut with a diamond knife and placed onto formvar-coated nickel grids. Grids were processed for immunocytochemistry as follows: immersed in blocking solution (PBS + 5%BSA) for 15 min, incubated in a 1:1000 dilution of sym32 antiserum in PBS for 1.5 h, rinsed 3 X 10 min in PBS/BSA + 0.1% Tween, incubated in a 1:75 dilution of EM grade goat anti-rabbit IgG – 15 nm colloidal gold (Ted Pella) in PBS for 1 h, rinsed as above, rinsed in deionized water for 5 min, and then allowed to dry. Grids were stained in 2% uranyl acetate for 5 min, rinsed by dipping into water 3 x 10 times, then immediately stained in 0.4% lead acetate for 3 min, with water rinses as above, and

then air dried. Between 5 and 10 grids of each type of anemone were viewed under 60kV using a CM-12 Phillips transmission electron microscope.

Preparation of anemone and dinoflagellate proteins, 1D and 2D SDS-PAGE and Western analysis

Proteins were isolated from the host as follows: a host anemone was removed from an 11°C recirculating aquarium and flash frozen in liquid nitrogen. The anemone was minced with a razor blade and placed into a glass grinder with a teflon pestle driven by a hand drill in 4x volume (of anemone's weight) ice-cold grinding buffer (100mM Tris, 100 mM NaCl, 10mM EDTA) with protease inhibitors (Sigma: 5μl per 10ml buffer). The homogenate was placed into a centrifuge tube and the grinder was rinsed with 2X volume buffer, which was added to the tube and mixed well. The rest of the homogenate was centrifuged at 16,000g for 10 minutes at 4°C to remove algal cells and host cell debris and membranes from the homogenized anemone tissue. The supernatant was removed to a new tube and centrifuged again. Protein concentration was determined on this cleared homogenate using the Bradford Assay (Pierce Coomassie Reagent). It has been previously demonstrated that host proteins prepared using this protocol are free from contamination by symbiont proteins (Weis and Levin 1996).

Proteins were isolated from symbionts that had been A) continuously maintained in culture with no host contact for many generations or B) freshly isolated from an *A. elegantissima* host. Many species of *Symbiodinium* can be isolated from their hosts and brought into culture in nutrient-supplemented seawater. These symbionts are therefore free from any host cell contact. We obtained frozen pelleted symbionts from cultures of *S. bermudense*, which was originally isolated from the tropical sea anemone *Aiptasia pallida* (generously donated by Wayne Stochaj). A chunk of the pelleted symbionts (approximately

100μl in volume) was briefly ground in a glass tissue grinder with a teflon pestle in an equal volume of grinding buffer with protease inhibitor cocktail. Examination under a light microscope revealed that the vast majority of symbionts were still intact after this step. An equal volume of acid-rinsed glass beads (Sigma: 425-600μm) were added and the mixture alternately vortexed for 15-30 sec and placed on ice approximately 30 sec, for a total of 20 times. With repeated vortexing, the homogenate became intensely orange, likely indicating the release of the major water soluble accessory pigment, peridinin-chlorophyll-protein. By this means, at least 75% of symbionts were broken open, as determined by light microscopy. Using a syringe and a 24 gauge needle, the homogenate was removed from the glass beads and centrifuged at 16,000g for 10 minutes at 4°C to remove cellular debris. The supernatant was placed into a new tube and again centrifuged. This cleared supernatant fraction was then assayed for protein concentration, as described above, and then prepared for 1D or 2D SDS-PAGE, as described below.

Proteins from freshly isolated symbionts were prepared by isolating symbionts from a host and then extracting proteins using glass beads to fracture the symbiont cell wall and release contents of the cytoplasm. A host anemone weighing approximately 2 grams was removed from an 11°C recirculating aquarium and flash frozen in liquid nitrogen. The anemone was minced with a razor blade and placed into a glass grinder with 3 ml of PBS plus protease inhibitor. All subsequent steps were performed on ice. The anemone was ground with a teflon pestle instead of a ground glass pestle to homogenize the animal tissues without shearing the cell walls of the symbionts. This homogenate was centrifuged at 2,000 g for 10 minutes to pellet the symbionts. The pellet, approximately 300μl in volume, was partially cleaned of anemone debris by regrinding the pellet in filtered sea water using a glass tissue grinder with a teflon pestle (this regrinding step was sufficient to break up the pellet, but not break open the symbionts) and re-concentrating the symbionts by centrifugation. The partially-cleaned pellet was ground a final time before adding 500 μl buffer with protease inhibitor cocktail and

an equal volume of pre-rinsed glass beads. This resuspended pellet contained a significant amount of host tissue, as revealed by the presence of numerous nematocysts. We then followed the same vortexing and centrifugation protocol as described above. The cleared supernatant fraction (intensely orange in color) was used for 1D SDS-PAGE, as described below.

One dimensional SDS-PAGE was performed on host, freshly isolated, and cultured symbiont proteins using 10% Nu-PAGE Bis-Tris gels (Invitrogen). Samples were denatured and prepared for electrophoresis using 4X LDS buffer + DTT (Invitrogen) according to manufacturer's instructions. Ten μg of protein was loaded for each sample. Electrophoresis was performed in MOPS buffer according to the manufacturer's instructions. Gels were transferred to 12.5mM Tris, 100mM glycine, 10% MeOH for 20 min, and proteins were electrophoretically transferred onto nitrocellulose membrane for 1.25 h at 100 Volts in a BioRad chamber.

Two dimensional SDS-PAGE was performed using proteins extracted from freshly isolated symbionts. Proteins were extracted as described above, except that no salt was used in the buffer, as salt interferes with isoelectric focusing. 2D SDS-PAGE was carried out on a Multiphor II system (Amersham Pharmacia), according to the manufacturer's instructions and as described in Reynolds et al. (2000). Thirty microliters of symbiont homogenate containing 60 μg of protein was used for isoelectric focusing on an 180mm IPG strip, pH 3-10. After isoelectric focusing, the IPG strip was equilibrated and placed on a 12% ExcelGel Electrophoresis was performed according to manufacturer's instructions. After electrophoresis, the gel was placed into transfer buffer (50mM Tris, 40mM glycine, .03% SDS, 20% methanol) for 20 minutes. The gel was removed from the plastic backing and proteins were transferred to a nitrocellulose membrane under a discontinuous buffer system (Multiphor II system from Amersham).

For Western analysis, The membranes from 1D and 2D SDS-PAGE were incubated at 4°C overnight in blocking buffer (TBS: 20mM Tris, 500mM NaCl, ph7.5 + 5% powdered milk + 0.01% Tween-20). The following morning, the

membrane was washed 15 min in TBS + 0.5% Tween-20 (TBST), incubated for 45 minutes in a 1:1500 dilution of sym32 antiserum:block buffer, rinsed 10 min each in TBS, TBST, TBS, incubated 45 minutes in a 1:5000 dilution of HRP-antirabbit IgG (Amersham Pharmacia), and washed as before. Sym32 protein was detected via chemiluminescence using ECL detection reagents (Amersham Pharmacia) and exposing membranes to film for 1 minute.

RESULTS

Light microscopy

Cryosectioned tentacles of aposymbiotic and symbiotic anemones were incubated with 1) preimmune serum as a negative control for endogenous staining or 2) sym32 antiserum. As shown in Figure 3.1, there was differential staining for sym32 in aposymbiotic vs. symbiotic tentacles, relative to preimmune controls. In both symbiotic and aposymbiotic tentacles, preimmune controls showed light brown staining in gastrodermal and epidermal tissues, and no staining in the mesoglea. Aposymbiotic tentacles incubated in sym32 antiserum showed slightly darker staining in both epidermal and gastrodermal layers, relative to preimmune controls. Symbiotic tentacles also showed slightly higher levels of staining in the epidermis, relative to preimmune controls, and significantly higher levels of staining in the gastrodermis, where dinoflagellates are housed. The mesoglea layer of both aposymbiotic and symbiotic tentacles remained unstained.
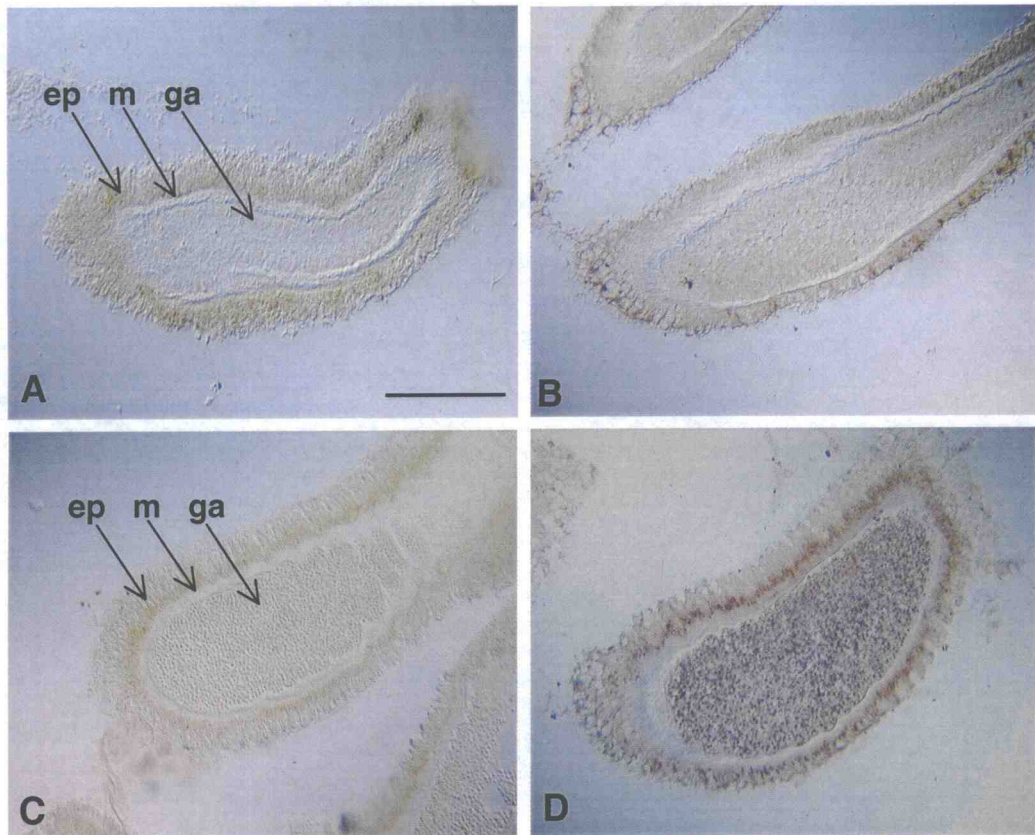
Figure 3.1 Immunocytochemical localization of sym32 protein within cross sections of tentacles from aposymbiotic (panels A and B) and symbiotic (panels C and D) *Anthopleura elegantissima*. Symbiotic dinoflagellates are visible in the symbiotic tentacles as brownish spheres within gastrodermal tissue. Sections were incubated in either preimmune serum (panels A and C) or sym32 anti-serum (panels B and D) and sym32 was visualized using silver enhancement of colloidal gold labeling. Relative to preimmune controls, aposymbiotic tentacles show light labeling in both epidermal and gastrodermal cells, and no labeling at all in the mesoglea. Similarly, symbiotic tentacles show light labeling of the epidermis and no labeling in mesoglea. In contrast to aposymbiotic tentacles, the gastrodermal tissue of symbiotic tentacles (which houses symbionts) shows strong labeling for sym32 . Host tissue layers are marked as ep = epidermis, ga = gastrodermis, m = mesoglea. Scale bar = 300mm for all panels.

Electron Microscopy

To further examine the location of sym32 within the host-symbiont association, we performed EM-level colloidal gold immunocytochemistry using sym32 antiserum to label the sym32 protein within thin sections of resin-embedded tentacles from 1 aposymbiotic and 3 symbiotic anemones (Figures 3.2 – 3.4). We examined between 15 and 25 sections from each anemone. We also performed negative controls using preimmune serum to check for non-specific labeling of the tissues. In all cases preimmune controls were almost completely free of gold sphere labeling (data not shown).

Aposymbiotic tentacles

In aposymbiotic tentacles, sym32 gold sphere labeling was associated exclusively with medium-density vesicles located within both epidermal and gastrodermal cells, as illustrated in Figure 3.2. There was no evidence of any sym32 label within the mesoglea layer. The pattern of distribution of the sym32-containing vesicles differed between the epidermal and gastrodermal tissues in aposymbiotic tentacles. In the epidermis, the sym32-containing vesicles were relatively uncommon, and appeared to be located primarily in association with nematocysts, which occur along the apical edges of the epidermal cells (Fig. 3.2 A, B). In the gastrodermis, sym32-containing vesicles were more abundant than in the epidermis and were concentrated along the apical end of the gastroderm, near the interface between the gastroderm and the gastric cavity (Fig 3.2 C, D).
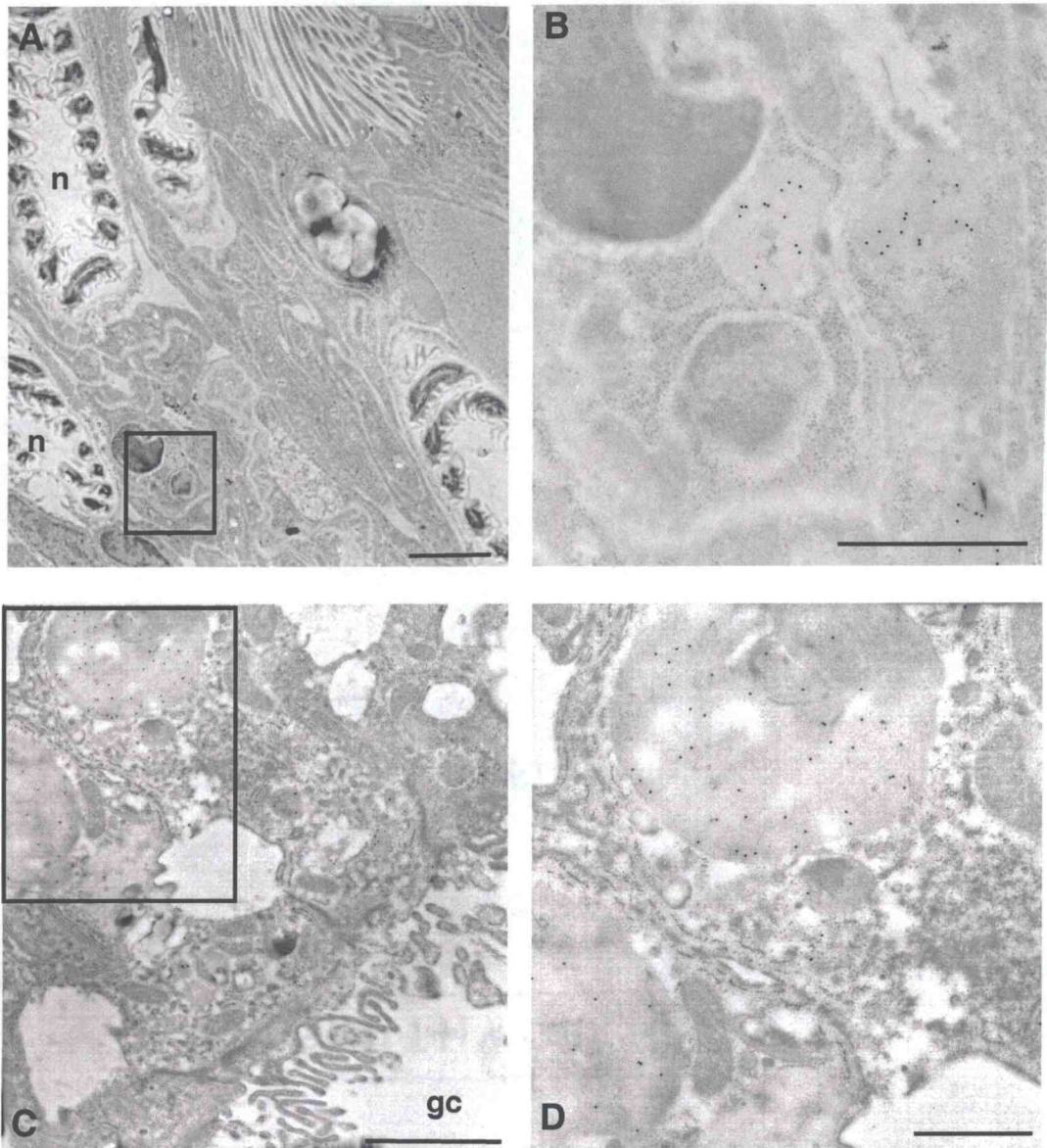
Figure 3.2 Transmission electron micrographs of immunogold labeled sections from tentacles of aposymbiotic anemones. Gold spheres, visible as black dots, indicate the presence of sym32. Panel A shows epidermal cells with nematocysts (n), and panel B is an enlargement of the boxed section of Panel A, with gold spheres labeling vesicles located near nematocysts. Panel C shows ciliated gastrodermal cells adjacent to the gastric cavity (gc), and Panel D is an enlargement of the boxed section of Panel C, showing gold spheres within vesicles in the gastrodermal cells. Scale bars = 2μm (A,C) and 1μm (B,D).

Symbiotic tentacles

In the epidermis of symbiotic tentacles, the pattern of distribution was the same as that found in aposymbiotic tentacles; sym32 gold sphere labeling was contained within vesicles that were relatively sparsely distributed, most commonly occurring near nematocysts. In contrast, the distribution of sym32 within the gastrodermis was dramatically different. The sym32-containing vesicles that were so abundant in aposymbiotic gastroderm were not present in symbiotic gastroderm. Instead, sym32 label was associated with the multiple membranes that enclose the dinoflagellate symbiont within the host cell (Fig. 3.3 A, B). Gold spheres were diffusely arranged within these membranes, not clearly associated with any single membrane layer. To confirm that this labeling was specific to the membraneous layers, we quantified the staining relative to areas outside the membranes. The membraneous layers contained an average of 12.2 gold spheres ± 4.65, n = 19, while equivalent areas outside the membraneous layers contained an average of 1.0 ± 1.1, n = 19).

Symbiodinium within host cells

In addition to sym32 label occurring around the periphery of the symbionts, there was a significant amount of labeling within the symbionts themselves (Fig 3.4). Specifically, sym32 gold spheres were located within the accumulation body, a poorly described organelle that is believed to function in endocytic pathways in many dinoflagellates. The density of labeling within the accumulation bodies was highly variable. Some accumulation bodies contained only a few gold spheres, while others contained hundreds (average = 56.4 ± 83.2 gold spheres /μm^2, n = 18).
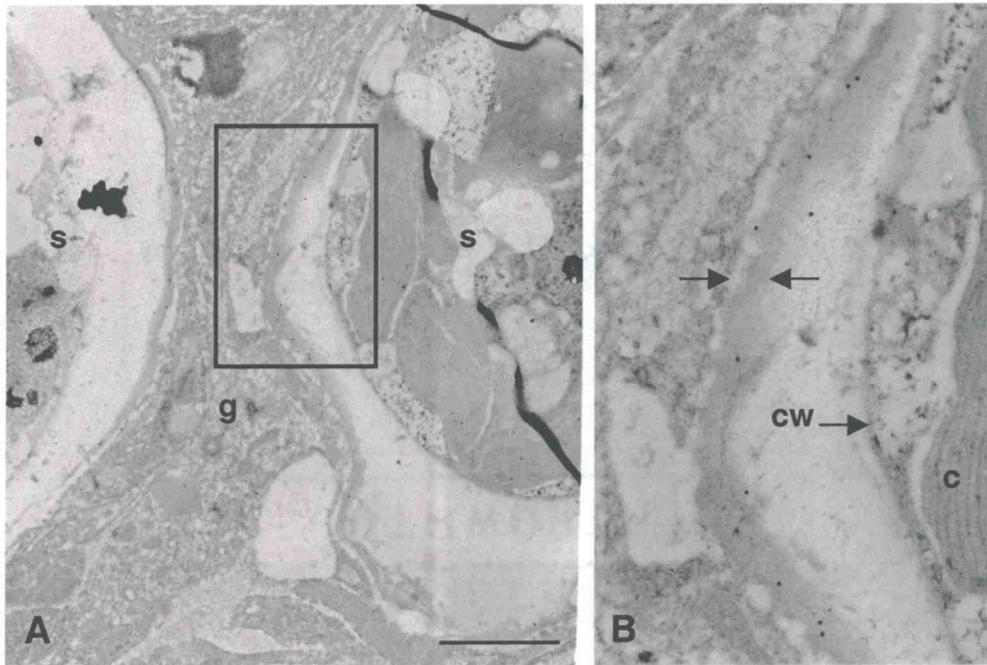
Fig 3.3. Transmission electron micrographs of immunogold labeled sections of tentacles from symbiotic *A. elegantissima*. Panel A illustrates the presence of dinoflagellates within host gastrodermal cells. Panel B is an enlargement of box from panel A, showing gold spheres associated with the multiple layers of membrane that surround the dinoflagellates. The double arrows delineate the margins of the multiple membranes surrounding the dinoflagellate. Preimmune controls showed virtually no gold sphere labeling (data not shown). c = symbiont chloroplast, cw = symbiont cell wall, g = gastrodermal cell of the host, s = dinoflagellate symbiont. Scale bar = 2μm.
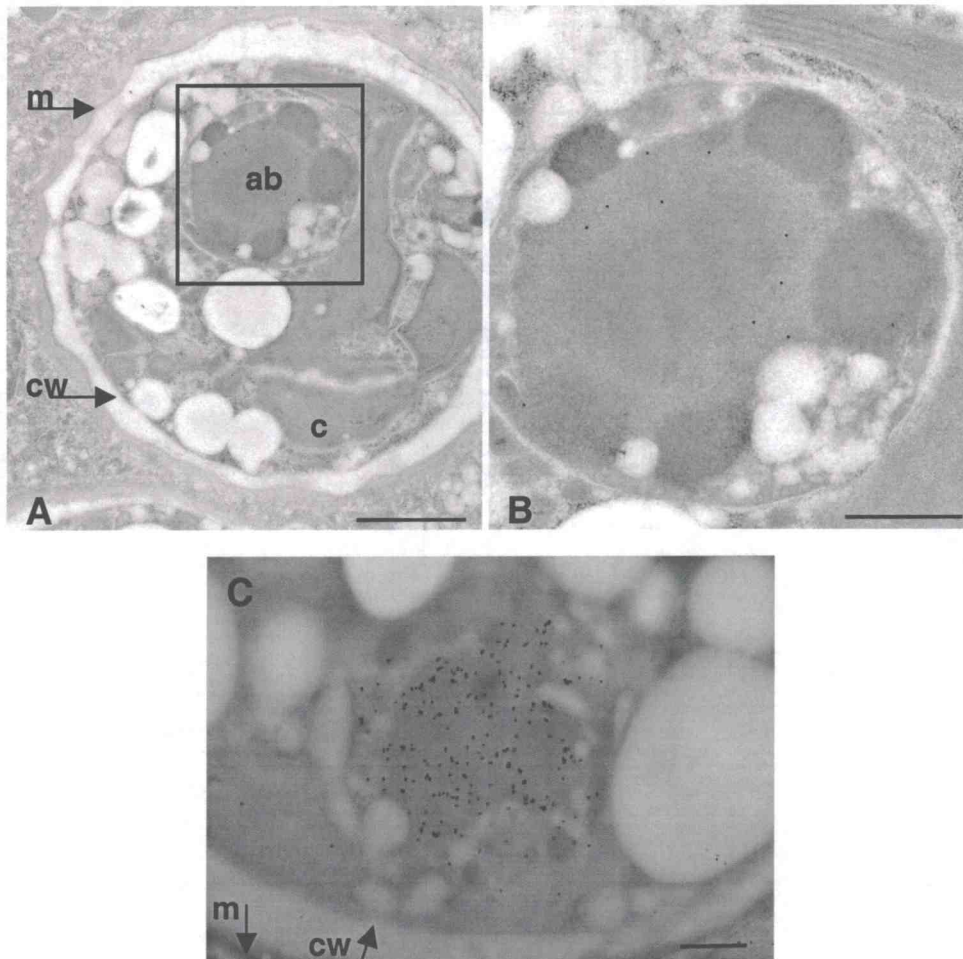
Figure 3.4 Transmission electron micrographs of immunogold labeled sections of dinoflagellate symbionts contained within host gastrodermal cells. A: dinoflagellate contained within a host gastrodermal cell. B: enlargement of the accumulation body, shown boxed in panel A, illustrating light gold labeling of the dinoflagellate accumulation body. C: region around the accumulation body of a another symbiont (section not counterstained) showing intense labeling specific to the accumulation body. The cell wall (cw) and membrane layers (m) are visible as concentric grey rings around the symbiont. Sections incubated with preimmune serum showed virtually no gold sphere labeling (data not shown). m = membranes surrounding the dinoflagellate, cw = dinoflagellate cell wall, c = dinoflagellate chloroplast, ab = accumulation body of the dinoflagellate. Scale bar = 2.5μm (A), 1.0μm (B,C).

Western blots

       The presence of sym32 gold sphere label within the accumulation bodies of the symbionts suggested that the symbiont might be producing a sym32 homolog. We used sym32 antiserum to look for a cross-reactive protein in homogenates from symbionts freshly removed from a host anemone and cultured symbionts that were not in contact with host cells. Both 1D and 2D PAGE Western blots illustrate the presence of 2 proteins, a 32kD and a 45/48kD doublet protein that cross react with the sym32 antibody (Figure 3.5). In 1D gels, the host lane (Figure 3.5A, lane 1) contains the 32kD sym32 band, and also contains a faint 48kD band. Lane 2 (symbionts that had been freshly isolated from a host anemone) contains a 45/48 kD protein doublet, suggesting a cross-reactive protein that is produced by the symbionts. Lane 2 also contains a strong 32kD band, which was expected, as protein preps of the symbionts are are invariably contaminated by host proteins (Weis et al. 1998). Lane 3 (cultured symbionts that had no contact with host cells) contains only the 45/48 kD doublet band, and completely lacks the 32kD band. The 45/48 kD protein doublet that is present in lanes of cultured *Symbiodinium bermudense* and in *S. muscatinei* freshly harvested from a host, and also faintly visible in the host lane, therefore represents a protein produced by *Symbiodinium* both when it is in symbiosis with a host and when it is free-living.
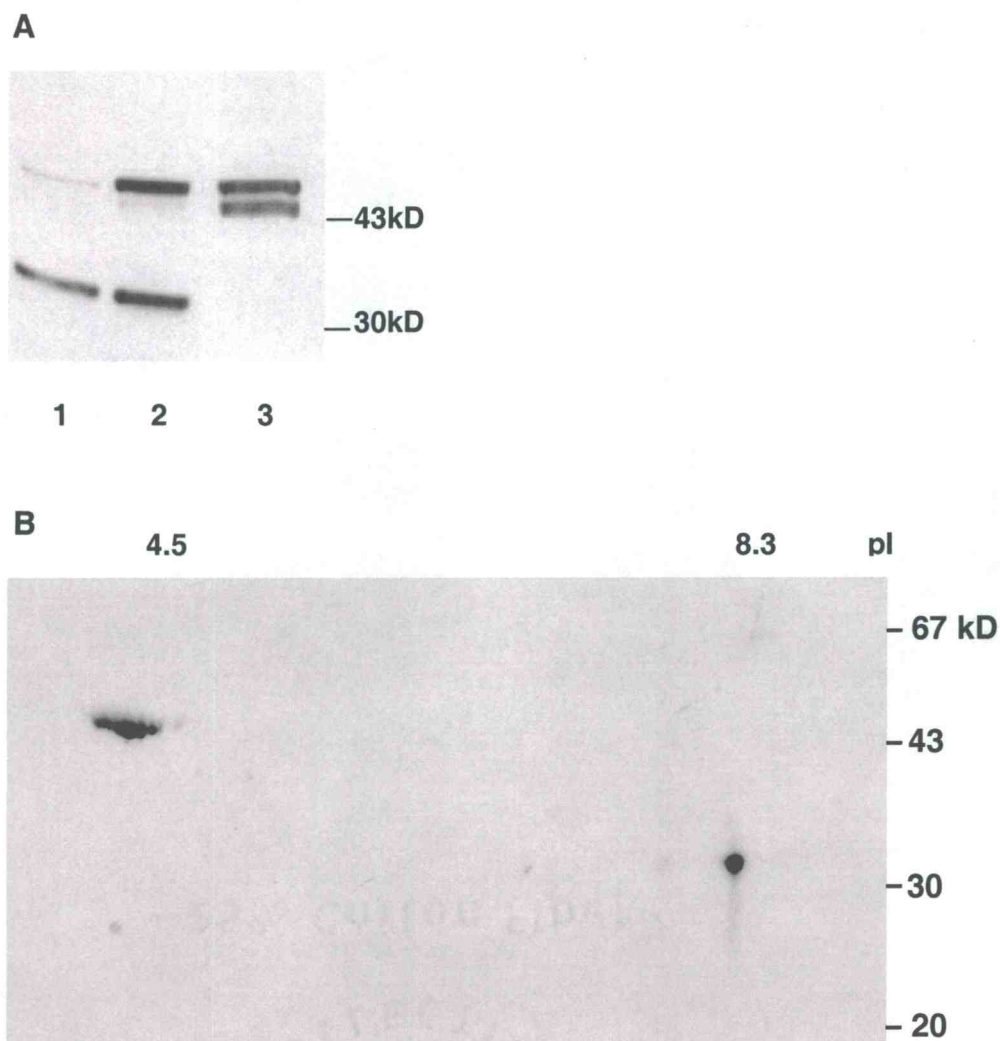
Figure 3.5. Identification of proteins from *Symbiodinium* that cross-react with sym32 antiserum. A: Western blot of 1D SDS-PAGE of 10μg soluble proteins. Lane1, host proteins, shows a 32kD band and a faint 48kD band. Lane 2, *Symbiodinium muscatinei* freshly harvested from a host anemone (and therefore contaminated with host tissues), shows 3 bands: a strongly staining 32kD band, a strongly staining 48kD band, and a faint 45kD band. Lane 3, cultured *Symbiodinium bermudense,* contains two equal intensity bands at 45kD and 48kD. B: Western blot from 2D SDS-PAGE of 60μg soluble protein from *Symbiodinium muscatinei* freshly harvested from an anemone host. Two spots are present; a 32kD, pI 8.0 spot, identifiable as sym32, presumably from contaminating host tissue, and a 48kD spot, pI range 4.3-4.5.

DISCUSSION

The sym32 protein is distributed among different subcellular compartments in symbiotic vs. aposymbiotic anemones. Most significantly, the presence of sym32-containing vesicles along the apical edges of gastrodermal cells in aposymbiotic hosts and the absence of those vesicles in symbiotic hosts concomitant with the presence of sym32 in the membranes surrounding the symbiont suggests that sym32 is relocated from vesicles in the host's gastrodermal cells to the vacuolar membranes surrounding the dinoflagellates during the internalization process, possibly as part of the endocytic pathways of the host cells. This is the first evidence that a putatively described "symbiosis" gene (Reynolds et al. 2000) in fact encodes a protein that, in addition to serving an unknown function in aposymbiotic hosts, can be traced to the host-symbiont interface. This suggests that the role of the sym32 protein is to mediate host-symbiont contact.

The presence of sym32 within the dinoflagellates themselves complicates the picture and adds a new dimension to our studies of sym32. The accumulation body in dinoflagellates is postulated to function as a lysosome although this organelle has not been studied in many species. In the dinoflagellate *Procentrum,* there are multiple accumulation bodies that contain electron-dense material, fibrous material, and membranous material and which possess acid phosphatase activity, react positively with the periodic acid/Schiff reagent, and stain with acridine orange (Zhou and Fritz 1994). These features are characteristic of eukaryotic lysosomes. In *Symbiodinium*, there is a single accumulation body that varies in size (Taylor 1987, Wakefield and Kempf 2000). I observed that the accumulation body is invariably located adjacent to the nucleus, often appearing to displace the edge of the nucleus. If it is the case that the accumulation body is a lysosome, it is possible that sym32 contained within the accumulation body originates from the host. If so, host sym32 must somehow be transported from the vacuolar membranes, across the

dinoflagellate cell wall, to enter a degratory pathway in the dinoflagellate. The ability to transport molecules from the host cell cytoplasm, across the vacuolar membrane, into the cytosol or organelles of an intracellular inhabitant is common in parasitic protozoans and there are many different mechanisms by which this occurs. These include the formation of pores in the vacuolar membrane (Schwab et al. 1994, Raibaud et al. 2001), the presence of specialized parasite organelles that transport host derived molecules to a food vacuole (Goodyer et al. 1997), and "parasitophorous ducts" through the membrane layers that surround the parasite (Goodyer et al. 1997). If it is the case that *Symbiodinium* is transporting sym32 from the vacuole membrane into its accumulation body, this would provide strong evidence that the symbionts play an active role in modifying the host membrane(s) that surround them.

It is also possible, however, that the labeling of the accumulation body represents a protein that originated from the symbiont itself. We show evidence that there is a 45/48 kD cross-reactive protein doublet that is produced both by cultured *Symbiodinium* that had not had contact with host cells for many generations, and by *Symbiodinium* that had just been removed from host cells (Figure 3.5). It is highly likely that this 45/48 kD cross-reactive protein is a sym32 homolog. Fas I proteins have been found in bacteria (Paulsrud and Lindblad 2002, Terasaka et al. 1989) photosynthetic algae (Huber and Sumper 1994), invertebrate animals (Bastiani et al. 1987, Zinn et al. 1988, Brennan and Robinson 1994, Bostic and Strand 1996, Reynolds et al. 2000), and humans (Skonier et al. 1992). Furthermore, its size is consistent with the Fas I proteins, which consist of between 1 and 4 repeats of an approximately 15kD domain. Efforts to sequence the 45/48 kD protein from 2D PAGE gels are underway.

If indeed the dinoflagellate symbionts produce a sym32 homolog, then it is possible that the vacuolar membranes contain both host and algal homologs and that they directly interact. Fas-I proteins are known to act as homophilic adhesion proteins in insects, where two cells expressing the protein adhere to each other

(Elkins et al. 1990) and it has been postulated that the highly conserved motifs that characterize the Fas I family are directly involved in mediating interactions with other Fas-I proteins or with the extracellular matrix (Hu et al. 1998). Further investigation of the relationship and interaction of host sym32 and the putative algal homolog requires the development of host-specific and symbiont-specific monoclonal antibodies to distinguish between the two in immunocytochemical studies.

The recent evidence that fasciclin I type proteins function in mediating symbiotic interactions in other associations is worthy of note. In both the rhizobium-plant association and the cyanobacterial-fungal lichen association, the identification of Fas-I homologs is from the symbiont genome and both symbionts provide the ability to fix nitrogen (Paulsrud and Lindblad 2002, Oke and Long 1999). Our data suggest that Fas-I homologs occur in both host and symbiont and that either or both occur within the membranes that comprise the interface between the two partners.

The sym32 story is complex. The sym32 protein apparently has functions in multiple biological processes within the host; both in symbiotic interactions with dinoflagellates (as evidenced by the presence of sym32 within the membranes surrounding the dinoflagellates) as well as in other non-symbiosis-related processes (as evidenced by the presence of sym32 in the epidermis of both aposymbiotic and symbiotic anemones). Furthermore, the presence of a cross-reactive protein in both free-living *Symbiodinium* as well as *Symbiodinium* freshly isolated from a host, suggests that the symbionts possess a sym32 homolog. The degree to which the host and symbiont proteins interact and the roles that each play in the biology of each partner separately and the partners in symbiosis, remain to be elucidated.
In conclusion, the evidence presented in this paper strongly suggests that host sym32 as well as a putative symbiont homolog function in mediating host-symbiont recognition and/or specificity and suggests that the process of symbiont

incorporation into a vacuole and host-symbiont recognition processes are a dynamic interplay between the genomes of the partners.

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CHAPTER 4

EFFECT OF LIGHT EXPOSURE AND THERMAL STRESS ON EXPRESSION
OF A HOST SYMBIOSIS GENE, SYM32, IN THE *ANTHOPLEURA
ELEGANTISSIMA-SYMBIODINIUM MUSCANTINEI* ASSOCIATION

INTRODUCTION

Efforts to identify and characterize genes that play roles in mediating
interactions between two partners in a symbiosis must consider environmental or
other factors that influence expression patterns or functions of putative "symbiosis"
genes. Many genes that play roles in mediating interactions between the partners
are co-opted from the pre-existing, pre-symbiotic, genome of each partner and
these genes are likely to continue to function in non-symbiosis-related biological
processes (for example, see Laplaze et al. 2002, Weis and Reynolds 1999). Thus
regulation of many "symbiosis" genes might include both symbiosis-specific and
non-symbiosis related factors.

Among the most significant marine symbioses are cnidarian-dinoflagellate
associations. Cnidarian-algal associations are widespread in both temperate and
tropical marine environments. The relationship is usually mutually beneficial to
the partners; the symbionts provide hosts with reduced organic carbon (Lewis and
Smith 1971, Trench 1971, Battey and Patton 1987, Wang and Douglas 1999), while
hosts provide symbionts with carbon dioxide (Weis and Reynolds, 1999) a source
of nitrogen (reviewed in Wang and Douglas, 1998) and a high light environment.
Cnidarians that host *Symbiodinium* are abundant and conspicuous members of the
habitats in which they occur and *Symbiodinium* contained within their hosts
account for a significant fraction of photosynthetic output in both tropical and
temperate systems (Dubinsky, 1990; Muller-Parker and Davy, 2001; Muscatine

1980). Such tight nutrient cycling between symbiont and host promotes high productivity in otherwise nutrient-poor marine environments (Muscatine and Porter 1977). As a result, gross productivity of algae contained in their hosts makes *Symbiodinium* a major contributor to global carbon fixation (Muscatine and Weis, 1992). The most dramatic manifestation of this symbiotic interaction is the coral reef ecosystem, where coral-algal associations form both a structural and trophic cornerstone of these highly productive and biologically diverse marine communities (Muscatine, 1990, Dubinsky 1990).

Although there is a fairly robust understanding of the significance of cnidarian-algal associations at the global, ecological, and physiological scales, there is a very poor understanding of how the partners interact at the molecular and biochemical levels. Efforts to identify host genes that play roles in regulating interactions between the partners include the identification of a host gene, sym32, from the symbiotic sea anemone *Anthopleura elegantissima*. In symbiotic specimens, the sym32 protein was identified as a heavily stained 32kD, 7.9 pI spot in 2D PAGE gels of soluble proteins. In specimens that naturally lack symbionts (aposymbiotic), the protein was almost absent from 2D profiles (Weis and Levine 1996). Based on this information, the sym32 protein was classified as a candidate symbiosis gene and was selected for further study.

BLAST results of the sym32 cDNA sequence place the protein within a class of adhesion proteins, called Fasciclin I proteins (Reynolds et al. 2000). Several other members of this protein family also function in parasitic or mutualistic associations, such as the plant-rhizobial bacteria mutualism (Oke and Long 1999), the fungal-cyanobacterial lichen mutualism (Paulsrud and Lindblad 2002), and the pathogenic *Mycobacterium bovis* (Harboe and Nagai 1984, Harboe et al. 1995). Further suggestion that the sym32 protein functions in mediating cnidarian-*Symbiodinium* interactions come from immunocytochemical studies in *Anthopleura elegantissima* that localize the protein to the membrane layers of the vacuole that separate host cell cytoplasm from the symbiont (Chapter 3 of this

thesis). These studies also revealed a putative homolog, p45/48, produced by the symbiont (Chapter 3 of this thesis). Sym32 also appears to play a role in facilitating the onset of the symbiotic association (Chapter 5 of this thesis). In the scleractinian coral, *Fungia scutaria*, antibody inhibition of sym32 function during the process of host infection resulted in a lower rate of infection and appeared to cause degradation of zooxanthellae within host lysosomes. This suggests that sym32 functions as a host-symbiont recognition molecule that prevents the symbiont from being degraded by host lysosomal attack.

There is also evidence, however, that sym32 functions in other processes in addition to symbiosis. For example, both symbiotic and aposymbiotic anemones possess a significant amount of sym32 protein within their epidermal cells (which do not contain symbionts), and aposymbiotic anemones also contain sym32 within vesicles in gastrodermal cells (Chapter 3 of this thesis). Because the sym32 gene is expressed in both aposymbiotic and symbiotic anemones, we were interested in examining some of the factors that influence its expression patterns. In their natural habitats, symbiotic and aposymbiotic anemones typically reside in different microenvironments and thus expression of the sym32 gene may be influenced by environmental aspects of those differing environments.

The most dramatic differences between the two types of environments inhabited by symbiotic vs. aposymbiotic *A. elegantissima* are levels of exposure to light and heat. Light levels are dramatically different: aposymbiotic anemones reside in areas that receive little or no light for long periods of time, such as caves, deep rock overhangs, and areas that experience prolonged periods of burial by sand (Buchsbaum 1968, Secord and Augustine 2000). Symbiotic specimens are abundant in light-exposed rocky mid-intertidal regions, where the intensity of the light is modulated by the depth of water (Turner 1988) and, when anemones are emerged during low tides, to behavioral adaptations that minimize direct exposure to light (Pearse 1974a,b, Dykens and Shick 1984, Shick and Dykens 1984) . Aposymbiotic anemones reside in areas that stay cool, even during summer low

tides, while symbiotic anemones reside on rocks and in tidepools that heat up dramatically during summer low tides (Jensen and Muller-Parker 1994).

The effect of elevated temperatures deserves special consideration because exposure of symbiotic cnidarians to elevated temperatures for 1 to several days commonly causes destabilization of the host-symbiont association. This results in expulsion of symbionts from host tissues and/or expulsion of the host's own gastrodermal cells (which contain symbionts) (Gates et al. 1992, Brown et al. 1995). In *Anthopleura elegantissima*, it has been demonstrated that exposure to 30°C for 2 days in complete darkness causes sufficient stress to produce a complete or partial bleaching response (Buchsbaum 1968, Pearse 1974a). For many symbiotic cnidarians, particularly scleractinian corals, this bleaching response may kill the host outright, or may have detrimental effects on host physiology, fecundity, and ability to resist disease, all of which produce severe ecological consequences (Glynn 1990, Glynn 1991, Glynn 1993, Hoegh-Guldberg, 1999).

Other environmental factors that cause the host-symbiont association to destabilize and produce a "bleaching" response, include high levels of UV light, heavy metal pollution, sedimentation of the marine environment, and outbreaks of microbial diseases. We have shown, previously, that exposure to cadmium resulted in a decline in sym32 gene expression in *A. elegantissima* (Mitchelmore et al. 2002) and in the studies reported here, we were interested in determining whether heat stress would also cause a decline in expression.

In this chapter I discuss two experiments that examined the effect of light and heat on expression of host sym32. In the first experiment, both symbiotic and aposymbiotic *A. elegantissima* were placed in either complete darkness or in a 12 hour light:12 hour dark (12L:12D) light regime for 3 weeks and sym32 protein levels were determined after the exposure period. In the second experiment, symbiotic anemones were subjected to a transient, 2-day heat shock of either 20°C or 30° and levels of sym32 protein and transcript were monitored before, during and after the heat shock and compared to controls (anemones maintained at 11°C).

The population density of the symbionts within host tissues was also monitored to determine whether hosts responded to the heat treatments by bleaching.

METHODS

Light exposure experiment

Anemones were collected in May 1999 from Neptune Beach, Oregon. Symbiotic anemones from 3 clearly distinct clones were collected from open rocky benches, and aposymbiotic anemones were collected from low light environments created by overhangs and rock crevices. Anemones were placed in a 100 gallon recirculating seawater aquarium at 11° C set on a 12 hour light: 12 hour dark (12L:12D) cycle. Irradiance levels in the light were approximately 150 μmoles quanta/m^2/sec. Animals were placed onto floating plexiglass platforms, maintained for 3 weeks prior to the start of the experiment, and fed previously frozen brine shrimp twice a week. The platforms were then distributed between 2 light regimes; either complete darkness, or a 12L:12D cycle. Anemones remained at these light regimes for 3 weeks, and were fed twice a week for the duration of the experiment. At the end of the experiment, anemones were frozen in liquid nitrogen and then processed for Western analysis (see below).

Transient Heat Shock Experiment

Several hundred small anemones (less than 1 g) were collected from 5 different rocky bench areas at Seal Rock Beach, Oregon in late July, 2000 and

placed in ziploc bags filled with seawater for transport to Oregon State University (Corvallis, Oregon) where they were randomly placed into 6 "Critter Keeper" containers that were submerged just below the surface of a 100 gallon recirculating, seawater tank at 11°C on a 12L:12D cycle. Irradiance levels in the light were approximately 150 μmoles quanta/m^2/sec. The containers were slotted, allowing water to flow through to keep the water oxygenated. Approximately 50 anemones were placed into each container. Anemones were allowed to acclimate for 2 weeks and were fed thawed adult brine shrimp every 4 – 5 days. The containers were cleaned the day following feeding. The containers were rotated through the 6 positions in the recirculating tank every 2 to 3 days to reduce effects due to placement within the tank.

The influence of heat on sym32 expression and symbiont population was examined by exposing symbiotic anemones to ambient or elevated temperature for 2 days. The 2 elevated temperatures used in this experiment were chosen based on observations of temperatures that are routinely experienced by anemones residing in tidepools during summer months (i.e., 20° C) and temperatures that are higher than those routinely experienced during the summer (i.e., 30° C), and which are known to produce partial or complete bleaching in *A. elegantissima* from the central California coast (Buchsbaum 1968).

The 6 containers of anemones were randomly assigned to 1 of 3 treatments for a total of 2 containers per treatment: control (11°C), 20°C, and 30°C, all in complete darkness for 48 h and then all placed back at 11°C on a 12L:12D h light cycle for the duration of the experiment. The control group was placed in the recirculating aquarium in a chamber made with black walls and a black cover. For the elevated temperature treatments, large plastic tubs equipped with aquarium heaters and aquarium air pumps were filled with seawater and allowed to stabilize to the appropriate temperatures (20°C or 30°C). The anemone containers were submerged in the tubs and the lids fastened, to create a dark chamber. After 24 hours incubation, the water was replaced with pre-heated, freshly oxygenated,

water. After 48 hours, all the anemone containers were placed back into the recirculating tank on a 12L:12D h light regime, where the anemones continued to be fed once per week and the containers cleaned and rotated on the same schedule.

For all time points that anemones were sampled, 3 anemones from each container were randomly selected, cleaned and blotted dry, and then flash frozen in liquid nitrogen at the following time points: just prior to the start of heat exposure (Day 0), at the end of heat exposure (Day 2), 5 days after the end of heat exposure (=Day 7 of the experiment), and 33 days after the end of heat exposure (Day 35). Because there were 2 containers per treatment, a total of 6 anemones per treatment were frozen at each time point. Frozen anemones were then cut into halves and 1 half each was used for Western and Northern analyses, as described below.

Preparation of anemone protein, SDS-PAGE and Western analysis

Soluble proteins were isolated from the host as follows: frozen anemones were weighed, minced with a razor blade, and homogenized in a glass grinder with a teflon pestle driven by a hand drill in 4x volume (of anemone's weight) ice-cold buffer (100mM Tris, 100 mM NaCl, 10mM EDTA, and protease inhibitors). The homogenate was placed into a centrifuge tube and the grinder was rinsed with 2X volume buffer, which was added to the tube and mixed well. For most samples, 200μl of this homogenate was removed and used to determine algal population (see below). The rest of the homogenate was centrifuged at 16,000g for 10 minutes at 4°C to remove algal cells and host cell debris and membranes from the homogenized anemone tissue. Protein concentration was determined on this cleared homogenate using the Bradford Assay (Pierce Coomassie Reagent).

SDS-PAGE was performed on host soluble proteins using 10% Nu-PAGE Bis-Tris gels (Invitrogen). Samples were denatured and prepared for electrophoresis using 4X LDS buffer + DTT (Invitrogen) according to

manufacturers instructions. Ten μg of protein was loaded for each sample. The same positive control was run on all gels to standardize band intensity between gels. Electrophoresis was performed at 200V for 60 minutes in MOPS buffer,. Gels were transferred to 12.5mM Tris, 100mM glycine, 10% MeOH for 20 minutes, and proteins transferred onto Nitrocellulose membrane for 1.25 hour under 100 Volts in the same buffer. The membranes were pooled into a single tub and were incubated at 4°C overnight in blocking buffer (TBS + 5% milk + 0.01% Tween-20). The following morning, the membranes were washed 15 min in TBS + 0.5% Tween-20 (TBST), incubated for 45 minutes in a 1:1500 dilution of sym32 antiserum:block buffer, rinsed 10 min each in TBS, TBST, TBS, incubated 45 minutes in a 1:5000 dilution of HRP-antirabbit IgG (Amersham Pharmacia), and washed as before. Sym32 protein was detected via chemiluminescence using ECL detection reagents (Amersham Pharmacia) and exposing membranes to film for 1 minute. Band intensity was determined using the PhosphoImager Densitometry system and ImageQuant software.

Determination of algal population

Using aliquots taken prior to preparation of host proteins, algal cells were counted on a hemacytometer, after diluting the samples. Cell counts were then standardized to anemone wet weight.

Anemone RNA extraction and Northern analysis

Total RNA was isolated from frozen anemones using a methods modified from Chomczynski and Sacchi (1987). Frozen anemones were minced with a razor blade and then homogenized in a glass grinder in a 1:2 dilution of denaturing

solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH7, 0.5% N-lauryl sarcosine, and 100 mM ß-mercaptoethanol) and 100mM phosphate buffer, pH 7.4. with 450mM NaCl and 10mM vanadyl ribonuclease complexes (Life Technologies). This initial grinding step using diluted denaturing solution was performed because it leaves the algal cells intact so that they can be removed by centrifugation before continuing with the RNA isolation. Homogenates were centrifuged at 7,000 g for 5 min to pellet the algal cells, and the supernatant was removed. A 2X volume of denaturing solution was added to the supernatant and the samples processed as described in Chomczynski and Sacchi (1987).

Six μg total RNA was resolved on a denaturing 1% agarose, 2.2M formaldehyde gel in 1X MOPS buffer. RNA was blotted onto a nylon membrane and then hybridized to a sym32 DIG-labelled RNA antisense probe that was synthesized in a T7 RNA DIG-labeling reaction from a vector template containing a 376 bp fragment of the sym32 sequence. The hybridization took place overnight at 68°C in DIG Easy Hyb solution (Roche) with 100μg/ml salmon sperm DNA as a blocking agent. The membrane was washed 2X at room temperature in 2X SSC, 0.1% SDS and then 2X at 68°C with 0.2% SSC, 0.1% SDS. Immunodetection was performed using an anti-DIG antibody conjugated to alkaline phosphatase (Roche) and visualized using the chemiluminescent substrate CSPD. Densitometry was performed as described above.

Statistical analysis

The statistical package *SAS v. 8.1* was used to analyze the data.

RESULTS

Light exposure experiments

This experiment examined the effect of light regime on sym32 protein expression in both symbiotic states of the host, symbiotic and aposymbiotic. Anti-sym32 Western blots demonstrate that light levels dramatically influence sym32 levels when anemones are maintained under continual darkness or in a 12L:12D h light regime for 3 weeks (Figure 4.1). In both symbiotic and aposymbiotic anemones, sym32 levels were higher after exposure to light than after exposure to continual darkness (Figure 4.1AB). However, the effect of light was more consistent in symbiotic compared to aposymbiotic hosts; variability in the intensity of the signal was consistently greater in aposymbitoic anemones than in symbiotic anemones (Figure 4.1A,B). Densitometry of the Western blot bands showed that the effect of the two light regimes on sym32 protein levels was significantly different (2-Way ANOVA, p<0.01), and that these differences were independent of the symbiotic state of the host (2-Way ANOVA, p = 0.22).

Figure 4.1C shows the effect of light regime on the symbiont population contained within host tissues. Aposymbiotic anemones contained no symbionts after the 3 week incubation period, regardless of the light regime. However, symbiotic anemones maintained in the dark showed a threefold decline in their symbiont population relative to those maintained under a 12L:12D h light regime. This was a significant decline (ANOVA p<0.01).
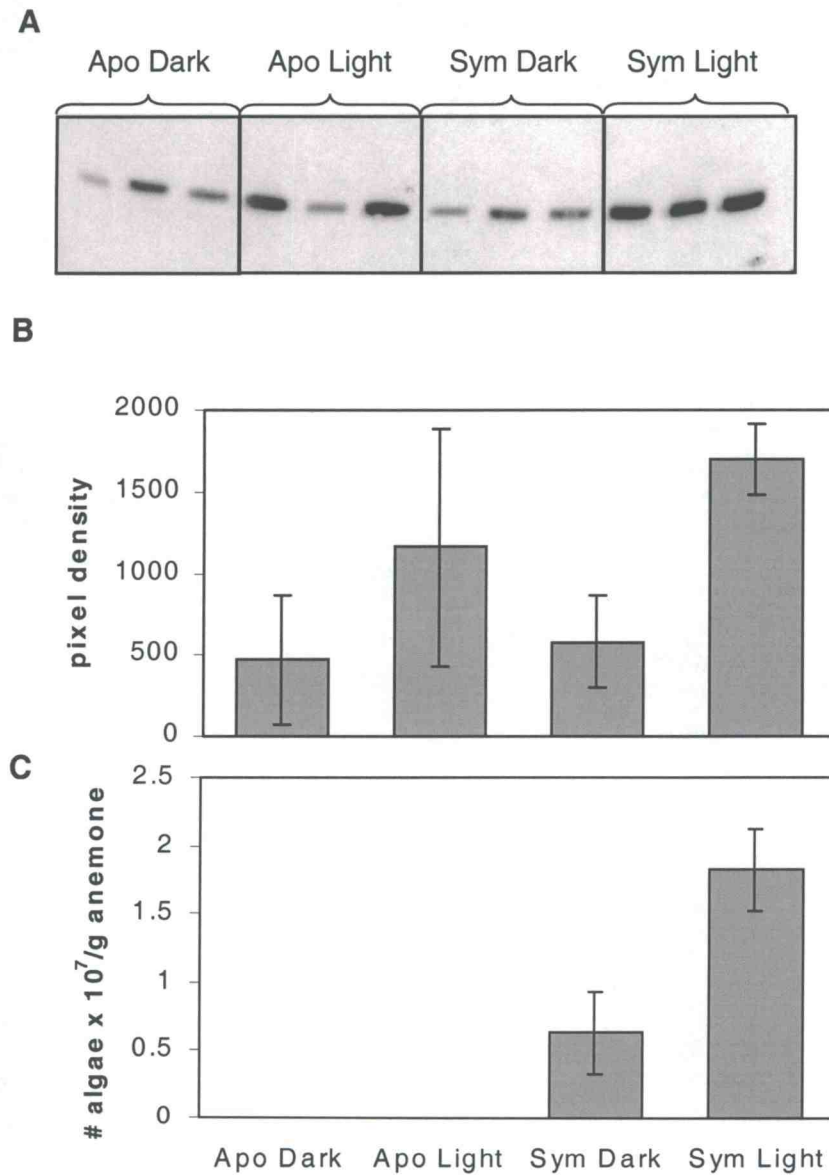
Figure 4.1. Effect of light and symbiotic state on host expression of sym32 protein. A: Anti-sym32 Western blots of host homogenates from 3 anemones per treatment. B: Digital quantification (densitometry) of the bands from panel A, showing relative levels of sym32. The effect of light had a significant effect on sym32 levels, regardless of symbiotic state (2-Way ANOVA, p<0.01). C: Symbiont densities within host tissues. Aposymbiotic anemones were free of symbionts, regardless of light regime, while symbiotic anemones maintained in light contained significantly greater number of symbionts than those maintained in the dark. (ANOVA, p<.0.01).

Transient heat shock and immersion in dark experiment

Sym32 protein levels and symbiont population density over time

Figure 4.2 illustrates effect of transient heat stress and immersion in dark on sym32 levels and symbiont population density in symbiotic hosts. For each treatment and time point, 6 individual anemones were sampled for both sym32 levels and symbiont density, and the results shown are the averages ± standard deviation. Anemones that were exposed to ambient (11°C), moderately high (20°C), and high temperature (30°C) for 2 days showed no short or long term changes in sym32 abundance, as measured by density of pixelation of Western blot bands (2-Way ANOVA, time: p = 0.16, temperature: p = 0.55). Anemones exposed to the high heat treatment did show a moderate bleaching response (Figure 4.2C), as evidenced by a decline in symbiont density that occurred within the 2 day exposure to heat and dark at Day 2. Thus symbiont densities were affected by temperature (2-Way ANOVA, p<0.01), but only in the 30°C treatment. Symbiont density remained similar in anemones exposed to 11°C and 20°C (Tukey-Kramer, p=0.21), but declined in anemones exposed to 30°C, (Tukey-Kramer, p< 0.01).

Sym32 transcript levels over time

Figure 4.3 illustrates the effect of transient heat stress and immersion in dark on sym32 gene transcript levels over time. Thermal stress did not affect the abundance of sym32 transcripts at any of the time points, relative to controls. Northern blots showed that patterns of sym32 expression were similar for both control and thermally-stressed anemones. However, immersion in dark produced a dramatic, short-term, decline in sym32 transcripts to undetectable levels in both

control anemones and thermally-stressed anemones. After 5 days back in a 12L:12D h light regime (=Day 7), transcript levels were again equivalent in both the control and thermally-stressed anemones. This suggests that light, but not thermal stress, plays a dramatic role in regulating sym32 gene expression.
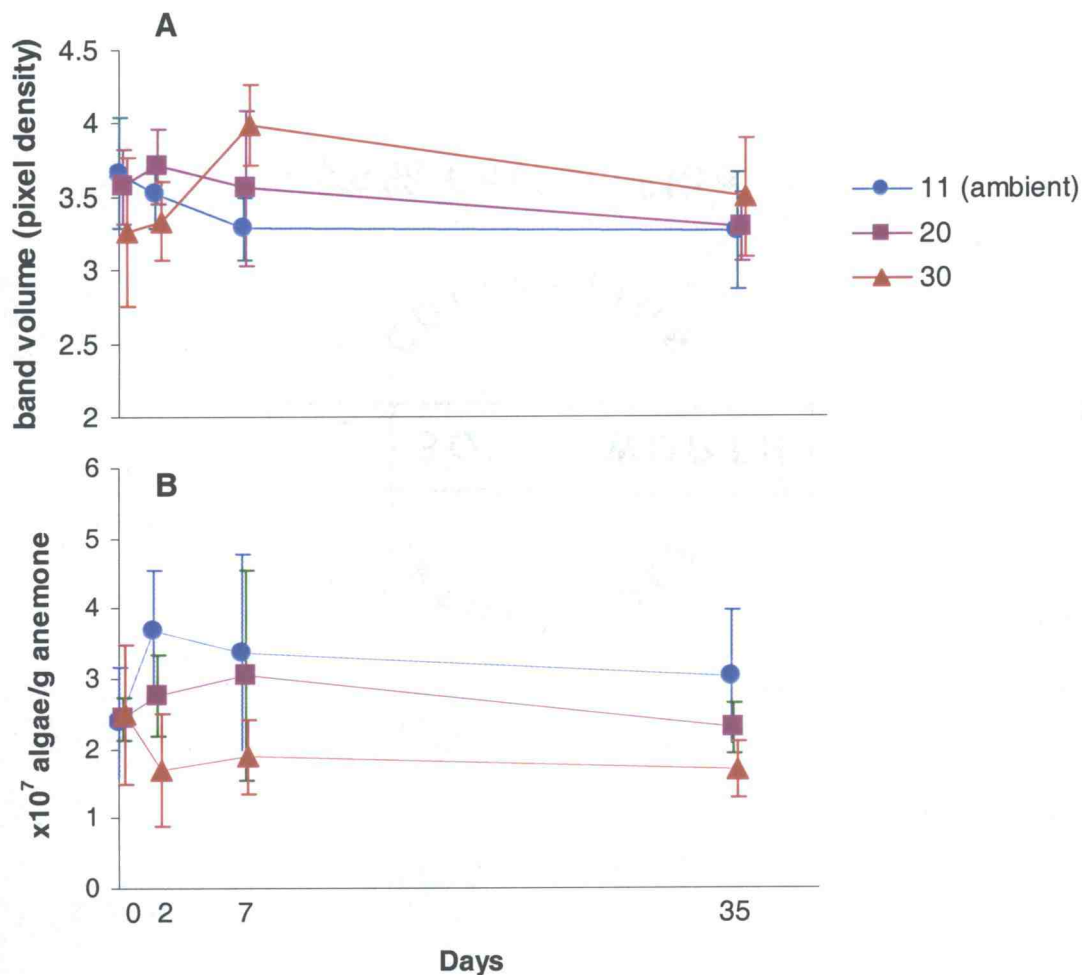
Figure 4.2. Effect of transient heat stress and dark on sym32 protein levels (A) and symbiont density (B) in *Anthopleura elegantissima*. Data points are slightly offset for clarity. A: relative levels of sym32 protein within host tissue (digitally measured as band intensities from Western blots) at 4 time points. There was no significant effect of either heat treatment (p = 0.55) or time (p = 0.16) on sym32 levels (2-Way ANOVA). B: Density of symbionts within host tissue over time. Symbiont densities were affected by temperature (2-Way ANOVA, p<0.01). Symbiont density remained similar in anemones exposed to 11ºC and 20ºC (Tukey-Kramer, p=0.21), but declined in anemones exposed to 30ºC, (Tukey-Kramer, p< 0.01).

Figure 4.3. Effect of transient heat stress and dark on expression of host sym32 gene. Anemones maintained at ambient conditions (11° on a 12L:12D light cycle) were placed into 2 temperature treatments (11° or 30°) in the dark for 48 hours, and then placed back into ambient conditions for the duration of the experiment (35 days). Northern analysis of host sym32 transcript levels in 3 anemones per treatment before, during, and after a 2 day exposure to 2 different temperatures in the dark for 2 days. When maintained on a 12L:12D light cycle, sym32 transcript is typically present (detectable in 12 of 15 anemones), while in dark conditions the sym32 transcript is not detectable in any of the samples, regardless of temperature.

DISCUSSION

Sym32 was originally identified from 2D PAGE gels of symbiotic *Anthopleura elegantissima* (Weis and Levine 1996). Its expression, at both the protein and genetic level appeared to be a function of the symbiotic state of the host; hosts that contained symbionts expressed sym32 at much higher levels than those that lacked symbionts (Reynolds et al. 2000). Immunocytochemical studies demonstrated that the sym32 protein resided within the membrane-bound compartment that physically separates host from symbiont (Chapter 2 of this thesis). Antibody inhibition studies suggest that sym32 plays a role in facilitating the uptake and retention of symbionts within the host (Chapter 5 of this thesis).

The experiments in this chapter demonstrate that light level, and not just the presence of symbionts, plays a major role in regulating the expression of sym32 in *Anthopleura elegantissima*. Long term exposure to complete darkness compared to a 12L:12D light regime resulted in dramatically different patterns in sym32 expression in both symbiotic and aposymbiotic anemones (Figure 4.1). This pattern re-emerged in an experiment that was designed to examine the effects of thermal stress; although heat did not appear to affect sym32 levels (Figure 4.2), immersion in dark produced a dramatic decline in sym32 transcript abundance (Figure 4.3) to undetectable levels in all anemones that were exposed to the dark. Thus the dramatically different levels of protein and gene expression in symbiotic vs. aposymbiotic anemones that are freshly collected are most likely due to the different light levels that each type of anemone experiences in their natural habitat. Symbiotic anemones occur in sun-exposed areas of the intertidal, while aposymbiotic anemones are restricted to areas with very low or undetectable light levels.

A 2 day exposure to 30°C water temperature, in the dark, has been reported to induce a complete, or near complete, bleaching response in *Anthopleura elegantissima* (Buchsbaum 1968) and previous attempts to bleach *A. elegantissima*

using this protocol was very successful, using sea anemones in Monterey Bay, California (unpubl. obs.). Thus it was a surprise to find that anemones collected from Oregon failed to undergo a significant bleaching response when maintained under these conditions. Although a slight decline in symbiont population was observed (Figure 4.2), I would have expected a much higher degree of bleaching. These results suggest that symbiotic anemones along the Pacific coast differ in their susceptibility to bleaching. This difference in susceptibility to bleaching may arise from the different symbiont populations that reside within hosts from Central California vs. Oregon. LaJeunesse and Trench (2000) report that *A. elegantissima* from Central and southern California contain one or both of two species, *Symbiodinium californium* and *S. muscantinei*, whereas anemones from Northern California and Oregon contain only *S. muscatinei*. Perhaps *S. muscatinei* is more tolerant of heat stress, and thus anemones from the northern ranges do not bleach as readily as those from Central or Southern California.

The global increase in frequency and severity of coral bleaching events has prompted the development of tools and techniques to detect and monitor environmental stress on coral reefs. For the past decade, we have been developing *A. elegantissima* as a model system for the discovery and examination of genes that play roles in regulating symbiotic interactions with dinoflagellates (Reynolds and Weis 1999, Reynolds et al. 2000, Weis and Levine 1996). This approach has been successful in identifying several host genes that are present in coral species as well as in *Anthopleura* (unpub. data). Thus we had hoped to also develop *A. elegantissima* as a model for coral bleaching. Our early attempts to use expression levels of symbiosis genes in *A. elegantissima* to detect environmental stress proved successful: sym32 expression in *A. elegantissima* was downregulated in response to heavy metal exposure (Mitchelmore et al, 2002). However, the results of this study indicate that the sym32 gene may not prove useful as a general indicator of stress in these associations, because thermal stress, the most common cause of coral bleaching, did not affect its expression. Furthermore, it appears that sym32

expression is regulated by factors that act independently of symbiosis. Specifically, its regulation by light suggests alternative functions of this gene that may not be related to symbiosis

This study also suggests that *A. elegantissima* is not an appropriate model for examining coral-algal bleaching. *A. elegantissima*, in stark contrast to corals, are very hardy and resistant to bleaching. They routinely live under conditions that vary in temperature upwards of 10°C a day (Jensen and Muller-Parker 1994), whereas corals experience physiological stress when temperature rises by only a few degrees. While *A. elegantissima* has been used successfully to identify "symbiosis" genes in cnidarians, generally, its use as a model for understanding coral bleaching is limited.

Clearly, the sym32 story is complex. Although the sym32 protein is present within the host-symbiont interface, and appears to play a role in the onset of infection in larvae, the regulation of this gene appears to be heavily influenced by light levels. Thus the sym32 gene should be viewed not solely as a "symbiosis" gene, but as a gene that perhaps functions in multiple processes, including symbiosis.

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CHAPTER 5

INHIBITION OF HOST SYM32 and/or SYMBIONT p45/48 PROTEIN
REDUCES INFECTIVITY OF SYMBIOTIC DINOFLAGELLATES IN THE
FUNGIA SCUTARIA-SYMBIODINIUM SP. ASSOCIATION

INTRODUCTION

The identification and characterization of genes that play roles in mediating
interactions between host and symbiont in cnidarian-*Symbiodinium* associations are
hampered by difficulties in assessing the functional significance of these genes. To
date there are no well developed methods of knocking out gene function in either
host or symbiont, either by direct genetic techniques, or by reverse genetics, such
as RNA interference (Ueda 2001). One method that may be possible to employ,
however, is the use of antibodies to interfere with the function of a target protein
(for example, see Nishikawa et al. 2000 and Mosqueda, et al. 2002) This technique
utilizes the highly specific binding of antibodies to epitopes on target proteins to
create steric hindrance or conformational modifications that render the target
protein partially or completely non-functional (List et al. 1999).

We were interested in using this approach to examine the function of a
candidate symbiosis gene, sym32. This gene was originally identified from the
symbiotic sea anemone *Anthopleura elegantissima*. In symbiotic specimens, the
sym32 protein was identified as a heavily stained 32kD, 7.9 pI spot in 2D PAGE
gels of soluble proteins. In specimens that naturally lack symbionts
(aposymbiotic), the protein was almost absent from 2D profiles (Weis and Levine
1996). Based on this information, the sym32 protein was classified as a candidate
symbiosis gene and was selected for further study. BLAST results of the sym32
cDNA sequence place the protein within a class of adhesion proteins, called

Fasciclin I proteins (Reynolds et al. 2000), which mediate cell-cell and cell-matrix interactions (Hu 1998). Several other members of this protein family also function in symbioses, such as the plant-rhizobial bacteria mutualism (Oke and Long 1999), the fungal-cyanobacterial lichen mutualism (Paulsrud and Lindblad 2002), and the pathogenic *Mycobacterium bovis* (Harboe and Nagai 1984, Harboe et al. 1995). Further suggestion that the sym32 protein functions in mediating cnidarian-*Symbiodinium* interactions come from immunocytochemical studies that localize the protein to the membrane layers that separate host from symbiont (Chapter 3 of this thesis). These studies also revealed a putative homolog, p45/48, produced by the symbiont (Chapter 3 of this thesis).

There is evidence, however, that regulation of the sym32 gene is not specifically a function of symbiotic state of the host. For example, aposymbiotic anemones, which lack symbionts entirely, nonetheless possess a significant amount of sym32 protein within vesicles in both the gastrodermis and epidermis (Chapter 3 of this thesis). Also, regulation of sym32 gene expression in both symbiotic and aposymbiotic anemone is heavily influenced by light levels, whereas regulation of gene expression due to presence of symbionts appears to be minimal (Chapter 4 of this thesis).

Further characterization of sym32 within the symbiotic assocation between cnidarians and *Symbiodinium* requires a method of assessing gene function. The only method currently amenable to studying this question in cnidarian-*Symbiodinium* associations is antibody inhibition of protein function. However, its use is limited because it requires that the protein of interest be exposed directly to antibodies so that they can physically interact. This cannot be done in the intact association as there is no method for introducing antibodies into the cytoplasm or membranes of host cells where they might directly interact with the sym32 protein. Thus the only stage at which antibody inhibition might be utilized effectively is during the process of symbiont acquisition by the larval stage of the host. By exposing uninfected larvae and fresh isolates of *Symbiodinium* to antibodies during

the infection process, we hoped to interfere with sym32 during the very earliest stages of the symbiotic association. This approach has proved successful in other systems to investigate mechanisms of host cell invasion by parasites (for example, see Nishikawa 2000 and Mosqueda 2002) and has also been used to examine the function of other Fas I homologs (Romancino et al. 1992, Huber and Sumper 1994).

Many symbiotic cnidarians produced a planula larva that initially lack symbionts. During the larval stage, or after metamorphosis, these uninfected hosts acquire their own symbionts from the environment. We have studied this process in two host species, *Anthopleura elegantissima* and in the Hawaiian scleractinian coral, *Fungia scutaria* (Schwarz et al. 1999, 2002; Weis et al. 2001, 2002). In both species, larvae are competent to acquire symbionts once they have developed a mouth and gastric cavity. While larvae are feeding, they extrude from their mouths mucus that traps particulate matter, and the mucus is pulled into the gastric cavity through the mouth. If *Symbiodinium* is present, they become adhered to the mucus and enter the gastric cavity. Gastrodermal cells lining the cavity internalize the symbionts by phagocytosis and the symbionts take up residence within the host's gastrodermis.

We were originally interested in performing antibody inhibition experiments using our model species, *Anthopleura elegantissima*. The sym32 protein was originally isolated from this species, and the antibodies that were made against this protein react most specifically with the *Anthopleura* sym32 protein, as compared to sym32-like proteins from other cnidarians that we have examined (unpublished data). Unfortunately, our ability to perform antibody inhibition experiments with this species has been fatally hampered by our inability to obtain viable larvae over several years of efforts. Thus we chose instead to conduct these experiments using *Fungia scutaria*. As described in Chapter 2 of this thesis (and Schwarz et al 1999), we have developed a successful system for obtaining gametes, achieving fertilization, rearing larvae, and experimentally infecting the larvae with

Symbiodinium. Although the sym32 antiserum was generated against an *Anthopleura* protein, and not a *Fungia* protein, we had evidence from Western analyses and immunocytochemistry, that there is a cross-reactive protein in *Fungia* (unpublished data), and thus antibody inhibition experiments using *F. scutaria* were initiated.

METHODS

Gamete collection and larval cultures

Approximately 75 adult specimens of *Fungia scutaria* were collected from the reef flats surrounding the Hawaii Institute of Marine Biology on Coconut Island, Kaneohe Bay, Hawaii and placed into seawater tables equipped with unfiltered, running seawater. Prior to spawning, the corals were rinsed with seawater and placed in standing seawater in individual glass fingerbowls. Spawning in this species generally occurs between 5 and 7 pm 2 - 4 days following the full moon during June through August. As the corals spawned, eggs were collected by removing the adults from the finger bowls and leaving the eggs in the bowl into which they were spawned. If the egg density was greater than a single layer of eggs at the bottom of the dish, some of the eggs were collected with a turkey baster and transferred to a new fingerbowl. Within 30 min after spawning, water from the dishes of all spawning males was combined and a small volume was gently pipetted into the dishes containing eggs. The dishes were left in a seawater table overnight for fertilization and early larval development. The following day, the water was changed (0.45μm filtered seawater (FSW) was used throughout). Larvae from all parental crosses were combined and the larvae were maintained in large glass or plastic bowls in filtered seawater, which was changed every day. Materials prepared for infection experiments

Preparation of zooxanthella isolates

Zooxanthellae were isolated from adult specimens of *F. scutaria* using a Waterpik to remove and homogenize coral tissue and were subsequently concentrated by centrifugation using a tabletop centrifuge at 2000 g for 5 min. This centrifugation produced a dark brown pellet that contained zooxanthellae, a cloudy supernatant, and a stiff white foam on the top, containing mucus and zooxanthellae. This foam was removed and ground in a glass grinder, and then diluted with FSW and spun again to produce a second dark brown pellet. The zooxanthella pellets were partially cleaned of animal tissue by twice rinsing in filtered seawater and again concentrated by centrifugation. The resulting pellets were resuspended in FSW to produce a dark brown suspension. Zooxanthella isolates were used within 2 h of preparation.

Preparation of homogenized *Artemia* sp.

To stimulate feeding behavior in larvae, homogenized *Artemia* sp. (brine shrimp) was added to larval cultures. A small pinch of frozen *Artemia* was homogenized in a ground glass tissue grinder in approximately 1 ml seawater and filtered through a 60 μm mesh to remove large particulate matter. The resulting slurry was used within 15 min of preparation.

Infection experiments

These experiments were designed to examine the role played by the sym32 protein during the formation of a symbiotic association between larvae and zooxanthellae. Once larvae were competent for infection when they were 3 days

old (i.e. larvae possessed functional mouths and gastric cavities), infection experiments were conducted using two types of rabbit serum. Preimmune serum lacks antibodies against the protein of interest (in this case, sym32). In contrast, sym32 antiserum contains antibodies that specifically recognize and bind to the sym32 protein (antibody development described in Reynolds et al. 2000).

Larvae were induced to feed during exposure to zooxanthellae and one of the following: 0%, 1% or 10% preimmune serum in FSW or 0%, 0.2%, 1%, 3%, 10% sym32 antiserum in FSW. It has been previously established that, when no serum is present, feeding larvae acquire symbionts readily and achieve close to 100% infection rates (Schwarz et al. 1999). Details of these expoeriments are described below.

Larvae from 7 different mothers were combined, concentrated on a Nitex filter, and added to clean FSW at a density of 500 larvae /ml FSW. From this stock, 5ml each was placed into 24 wells in 6-well culture plates. Three replicate wells were established for each treatment. Preimmune serum and sym32 antiserum were diluted into 1 ml FSW and then added to the 5ml that contained larvae, for a total volume of 6ml. Final concentrations: preimmune serum = 0%, 1 %, 10% and antiserum = 0%, 0.2%, 1%, 3%, 10%.

Immediately after the addition of serum, $400\mu l$ of the symbiont suspension was added to each well along with 2 drops of macerated brine shrimp (which initiates a feeding response in the larvae). Larvae were allowed to feed and acquire symbionts for approximately 3 hours (this 3 hour period is referred to as the "infection period") and were then concentrated on a filter and placed into clean FSW.

Approximately 24 hours after the termination of the infection period, at least 60 live larvae per replicate were examined under a light microscope and scored for either presence or absence of symbionts.

The data was arcsine square root transformed and subjected to a Two-Way ANOVA. Slopes of the regression lines were subjected to t-tests.

Immunocytochemistry

Separate wells were established for a second set of 10% preimmune serum and 10% antiserum treatments. From these wells, larvae were collected during the infection period, exactly 1 hour after the addition of serum and symbionts. Larvae were removed and concentrated on mesh filters made from microcentrifuge tubes from which the tip had been cut off and 50um Nitex mesh affixed. Larvae contained within the microfuge filters were immersed in fixative (1% formadehyde, 1% glutaraldehyde in 100mM phosphate-buffered saline--PBS; 100mM phosphate buffer, ph 7.2 and 450mM NaCl) for 1 hour, with several transfers to fresh fixative during this period. Larvae were then rinsed 3 x 15 min in PBS, and dehydraded in a methanol series for 15 min each in 15%, 30%, 50%, 70%. Larvae were transported back to Oregon State University in 70% methanol, where they were further dehydrated 15 min each in 85%, 90%, 100%, 100% methanol. Larvae were infiltrated with LR White resin in a series of MeOH dilutions (1:3 LR White:MeOH overnight, 1:1 overnight, 100% LR White for 3 hours), and then placed in gelatin capsules in fresh LR White. LR White was allowed to polymerized at 52° C for 2 days.

Ultra-thin, gold-silver sections were cut with a diamond knife and placed onto formvar-coated nickel grids. Grids were stained in 2% uranyl acetate for 5 min, rinsed by dipping into water 3 x 10 times, then immediately stained in 0.4% lead acetate for 3 min, with water rinses as above, and then air dried. Grids were viewed under 40 or 60kV using a CM-12 Phillips transmission electron microscope.

RESULTS

Exposing larvae and zooxanthellae to either preimmune serum or sym32 antiserum during the infection period significantly reduced the number of larvae that successfully acquired zooxanthellae (Figure 5.1). When no serum was added during the infection process, an average of 89% of larvae acquired zooxanthellae, whereas only 34% and 16% of larvae acquired zooxanthellae in the 10% preimmune and antiserum treatments, respectively. The effect of adding antiserum, was greater than that of adding preimmune serum (ANOVA on arcsine square root transformed data, $p<0.01$); in the 10% serum treatments, infection success declined by 50% in the antiserum treatments as compared with the preimmune treatments (16% vs. 34%, respectively). Regression lines drawn through each set of replicates illustrate the differential effect of the two types of serum on infection success; the slopes of the regression lines of the antiserum treatments were significantly different than those of the preimmune serum treatments (t-test $p<0.01$).

In addition to the effect of antiserum on infection success, antiserum also appeared to affect the viability of internalized zooxanthellae. I used transmission electron microscopy to examine the morphology of zooxanthellae that had been internalized by larvae. Between 10 and 15 individual larvae, each containing 2 to > 10 zooxanthellae (or zooxanthella-like structures), were observed. Figures 5.2 and 5.3 illustrate the appearance of zooxanthellae from preimmune serum (Figure 5.2) or antiserum (Figure 5.3) treatments.

Figure 5.2 illustrates the typical appearance of an infected larva from one of the 10% preimmune treatments. The larva contains within its gastrodermal cells two healthy-appearing symbionts, one of which is shown magnified to demonstrate that all the cellular structures are intact, including well-defined chloroplasts and a nucleus with condensed chromosomes (a typical dinoflagellate feature). In contrast none of the antiserum-exposed larvae contained normal-appearing symbionts (Figure 5.3). Instead, they contained 1 or 2 symbionts that appeared to be

degenerating and/or several large lysosomal-like structures that were the same diameter as symbionts (8-10μm in diameter). These were deduced to be highly degraded symbionts, as such structures were never observed in infected larvae that had been incubated in preimmune serum during the infection period.
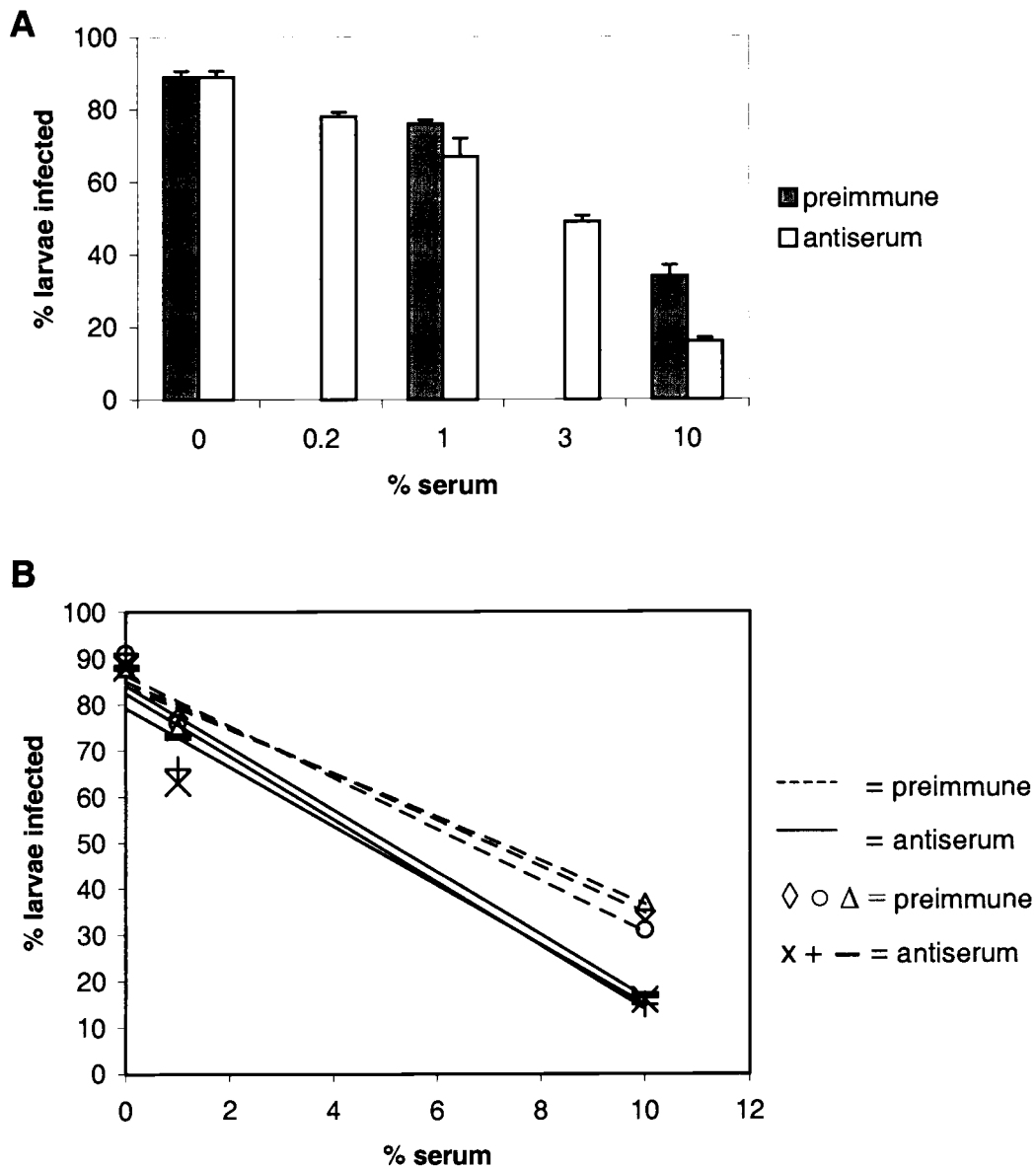
Figure 5.1 Effect of sym32 antiserum and preimmune serum (control) on acquisition of zooxanthellae by larvae of *Fungia scutaria*. Bars represent mean ± standard deviation, n=3. A: As serum levels increased, infection success descreased, regardless of the type of serum added to infection vessels, but the effect of antiserum was greater than that of preimmune serum (ANOVA using arcsine square root transformed data, p< 0.01). B: Regression lines showing decline in infection success. The slopes of the regression lines from the antiserum treatment are significantly greater than those from the preimmune serum treatments (t-test <0.01).
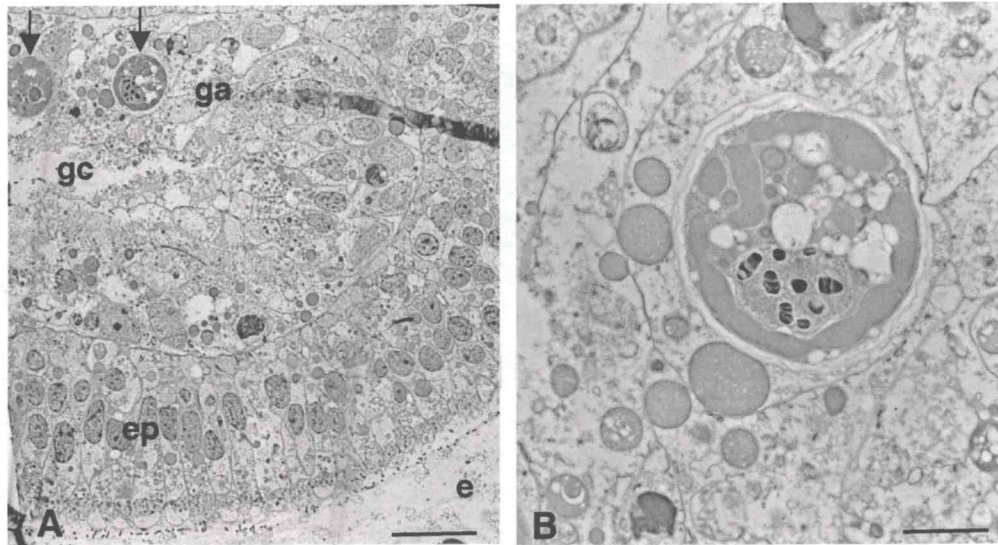
Figure 5.2. Transmission electron micrographs illustrating the typical appearance of a larva newly infected by symbiotic dinoflagellates. A: low magnification micrograph showing two zooxanthellae within the gastrodermis of a larva that had been incubated in 10% preimmune serum during infection by zooxanthellae (ep = epidermis, ga = gastrodermis, gc – gastric cavity, e = area external to the larva. Arrows point to the two symbiotic dinoflagellates.) Scale bar = 12.5μm. B: enlargement of symbiont on right, which displays normal morphology, including distinct membraneous layer around the perimeter of the cell, intact nucleus and chromosomes, and distinct chloroplasts. Scale bar = 2μm.
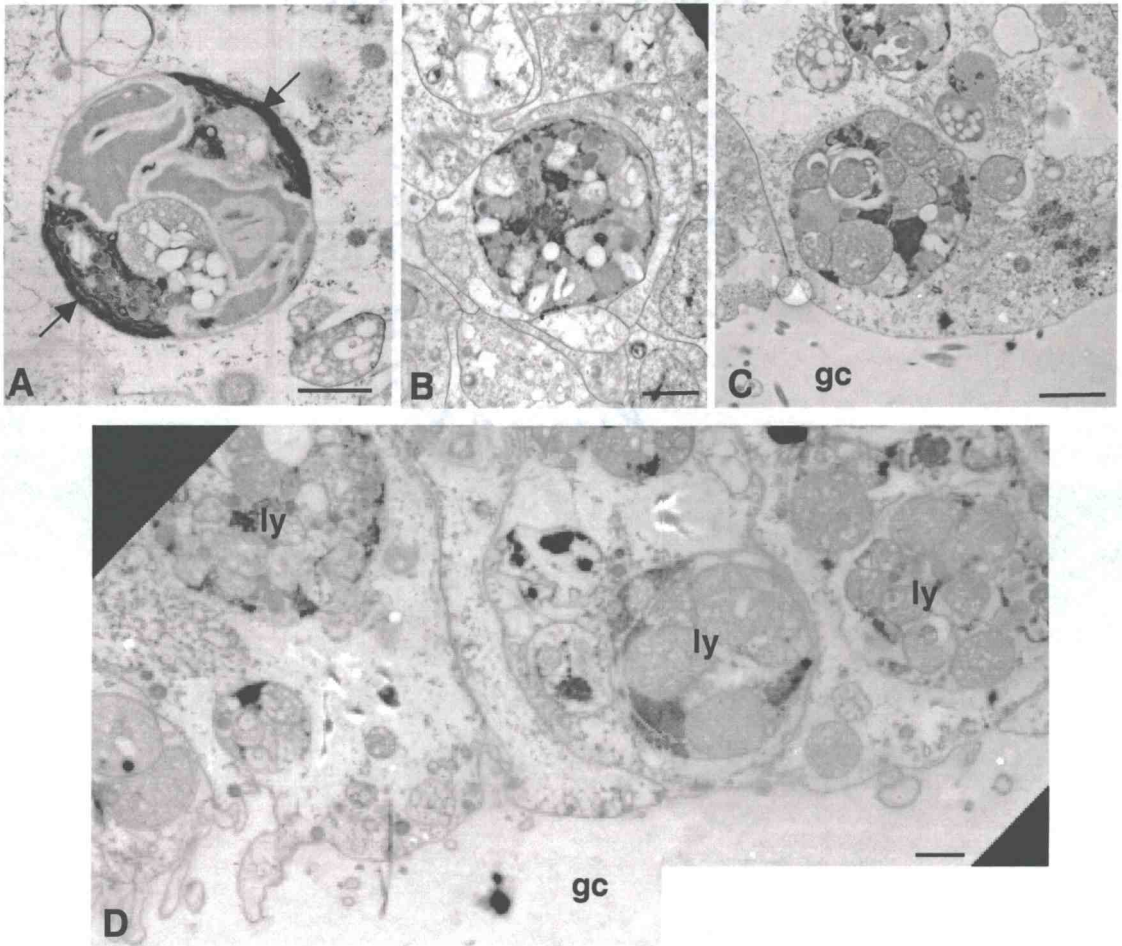
Figure 5.3. Transmission electron micrographs of apparently degenerating symbionts and atypical, large lysosomal-like structures within the gastrodermis of larvae incubated in sym32 antiserum during infection by symbiotic dinoflagellates. A: degenerating symbiont, with identifiable, but degrading chloroplasts (arrows point to chloroplasts). B, C, D: examples of the large lysosomal-like structures that occur in larvae treated with sym32 antiserum, but are absent in larvae incubated in preimmune serum. These structures likely represent degraded symbionts that no longer contain any identifiable cellular structures (gastric cavity = gc). D: photo montage showing a lower magnification view of the gastrodermis, containing multiple lysosome-like structures (l= lysosome-like structures, gastric cavity = gc). For all panels, scale bar = 2μm.

DISCUSSION

These experiments sought to examine the role of sym32 protein in mediating interactions between host and symbiont at the very earliest moments of their association – during the process of infection. In the *Anthopleura elegantissima/Symbiodinium muscatinei* association, there is evidence that both host and symbiont synthesize sym32 homologs (host sym32, and symbiont p45/48) and that one or both reside within the multiple layers of membrane that surround the symbiont (see Chapter 3 of this thesis). Thus the antibody inhibition experiments described here may have involved inhibition of both host and algal homologs.

In typical infection experiments (i.e., larvae and zooxanthellae not exposed to serum during the infection process), the vast majority of larvae readily internalized zooxanthellae to achieve infection rates approaching 100% (Schwarz et al. 1999, Weis et al 2001). However, in the experiments described here, any type of serum, even serum lacking antibodies against sym32, dramatically affected the ability of host and symbiont to form an association (Figure 5.1). The dramatic decline in infection rates due simply to exposure to serum may result from the presence in serum of numerous components that may influence the uptake of symbionts, such as the presence of antibodies that bind to host cell or symbiont surface proteins or proteases that degrade host cell or symbiont proteins that play roles in phagocytosis or host-symbiont recognition.

The precise mechanism of inhibition that serum plays remains to be elucidated. There are two different steps in the infection process that could be affected by components of serum. First, the presence of serum may have inhibited the internalization process whereby host gastrodermal cells incorporate zooxanthellae via phagocytic mechanisms (Ernst 2000). Inhibition of phagocytosis during the infection process has been observed in polyps of *Cassiopeia xamachana*

(Colley and Trench 1983). Exposure of new, uninfected *C. xamachana* polyps to either cultured zooxanthellae or fresh isolates of zooxanthellae that had been treated with reagents to remove contaminating host membranes, were phagocytosed at much lower rates than were fresh, unmanipulated, zooxanthella isolates. This suggests that the distinction between "food" and "symbiont" may begin before symbiont cells enter the host's tissues, when host cell and symbiont cell first make contact in the gastric cavity of the host.

Alternately, serum may not be inhibiting the process of phagocytosis, but rather forcing newly internalized zooxanthellae to appear as "food," perhaps by masking recognition molecules that are present either on host cells or symbiont cells. It has been observed in host polyps from *Cassiopeia xamachana* that when hosts internalize zooxanthellae, the symbiont-containing vacuoles fail to fuse with lysosomes and thus zooxanthellae are not degraded (Fitt and Trench 1983). However, when heat or freeze-killed zooxanthellae or non-zooxanthella particles are internalized, the vacuoles do fuse with lysosomes and the particles are degraded (Fitt and Trench 1983). In this study, the presence of serum may force the vacuoles to behave like those that contain material bound for fusion with lysosomes.

The results from this study do not appear to support the latter scenario. Degradation of zooxanthellae was observed only in larvae that had been exposed to sym32 antiserum, not in larvae exposed to preimmune serum. However, it is possible that serum does target zooxanthellae into degradation pathways, but that it does so at a slower rate than sym32 antiserum. The timeline of sampling is consistent with this interpretation. Samples for TEM were collected during the infection period (at 1 hour after addition of zooxanthellae and serum), while the quantification of infected larvae was done approximately 24 hours later. Zooxanthellae that were observed to be degrading within 1 hour of infection would no longer be identifiable 24 hours later and thus larvae that contained degraded zooxanthellae would be considered not infected. If preimmune serum operated at a slower rate than antiserum, targeting zooxanthellae for degradation after the 1 hour

sampling period, it is likely that many zooxanthellae were degraded by the time infection rates were determined at 24 hours.

Sym32 antiserum has a greater inhibitory effect on the formation of an association to a greater degree than preimmune serum. This provides evidence that the sym32 protein does play a role in mediating contact between the partners from the earliest moments of the association. The appearance of degrading zooxanthellae and large lysosomal-like structures within larvae that had been exposed to antiserum suggests that sym32 functions as a recognition molecule that targets the symbiont-containing vacuole away from the pathways that lead to fusion with lysosomes. Comparison of micrographs from this study with those from the Fitt et al. (1983) study reveal very similar appearance of degenerated zooxanthellae in both studies. This suggests that sym32 and/or p45/48 operate separately or in coordination to render the symbiont-containing vacuole independent from the endocytic pathways leading to fusion with lysosomes. The presence of sym32 and/or p45/48 within the multiple layers of membranes around the symbiont place this protein in the appropriate location for mediating this type of interaction (see Chapter 3 of this thesis).

In other organisms for which the function of Fas-I proteins has been examined, these proteins typically mediate cell-cell and cell-ECM interactions by functioning as adhesion molecules (Zinn et al. 1988, Terasaka et al. 1989, Skonier et al. 1992, Takeshita et al. 1993, Brennand and Robinson 1994, Huber and Sumper 1994). There have been other attempts to use antibodies to inhibit the function of Fas I homologs. In *Volvox*, monoclonal antibodies interfered with cell-cell contacts in the 4-cell embryo, causing a disruption in cell division (Huber and Sumper). In sea urchin development, antibody inhibition of the bep1 and bep4 proteins inhibited reaggregation of embronic cells derived from the blastula stage, indicating that these proteins mediate cell-cell adhesion and interaction (Romancino et al. 1992). In *Drosophila*, normally nonadhesive S2 cell that were transfected with fasciclin I

spontaneously formed aggregates that subsequently disassociated when incubated with antiserum agains fasciclin I (Elkins et al, 1990).

Clearly, Fas-I proteins are involved cell recognition and signaling (and several specifically function in symbiotic interactions). It is therefore highly likely that sym32 and/or p45/48 serve this function. This would be consistent with a role in mediating fusion events between the symbiont-containing vacuole and lysosomes. Future work on the functional significance of sym32 and p45/48 will likely elucidate some of the recognition processes that allow zooxanthellae to take up residence within host cells.

The specific mechanisms by which serum interferes with the ability of larvae to acquire symbionts, and the elucidation of the role of the sym32 protein both require more detailed examination. Due to the nature of my observations, using TEM, it was very difficult to observe sufficient numbers of larvae to make any quantitative conclusions about the effect of preimmune serum vs. antiserum on zooxanthella viability. However, I did observe the same effect in larvae that I sampled from two different "serum" experiments from two different years (2000 and 2001). In addition, over 3 separate years of rearing and infecting larvae I have viewed dozens of larvae using TEM and in all cases where larvae were incubated in the absence of any serum, symbionts within the larvae appeared healthy (see Schwarz et al. 1999 for an example). Thus there does appear to be a repeatable, negative effect of sym32 antiserum on the ability of larvae to acquire and establish an association with zooxanthellae.

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