AN ABSTRACT OF THE DISSERTATION OF

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Title: Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis

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The intracellular mutualism between cnidarians and photosynthetic dinoflagellates (genus *Symbiodinium*) is responsible for the physical and trophic structure of diverse coral reef ecosystems. This relationship, based on nutrient exchange, allows for high productivity in tropical waters, which are generally nutrient-poor environments. Numerous environmental stressors currently threaten the health of corals, most notably elevated seawater temperatures due to global climate change, many of which can cause coral bleaching, or symbiosis collapse. Despite this, relatively little is known about the mechanisms underpinning the onset and maintenance of the association. In this dissertation, I studied the onset of cnidarian-dinoflagellate symbiosis using ecological, molecular, and genomic approaches.

First, I examined effects of elevated seawater temperature on coral larvae (*Fungia scutaria*) during the period of symbiosis establishment (Chapter 2). I found that larvae exposed to a 2-4°C increase in temperature were significantly impaired in their ability to form the symbiosis. These results are the first to quantify the effect of elevated temperature on coral symbiosis onset and are important in light of projected increases in seawater temperatures.

Next, I created a cDNA microarray from non-symbiotic and newly symbiotic *F.*

scutaria larvae to identify host transcripts that were differentially expressed in response to symbiosis onset (Chapter 3). Analyses revealed very few changes in the larval transcriptome as a result of infection with its homologous symbiont. I hypothesize that *Symbiodinium* sp. has evolved mechanisms to suppress or circumvent cnidarian host responses to colonization similar to those seen in the invasion of animal cells by protozoan parasites.

Finally, I explored a family of genes (tumor necrosis factor receptor associated factors, or TRAFs), which are key signal transducers in pro-inflammatory innate immune pathways, in cnidarian genomes (Chapter 4). Phylogenetic analyses identified 8 major lineages of TRAFs, including 3 new subfamilies, each with cnidarian TRAF sequences, indicating that the TRAF gene family was fully diversified prior to the divergence between cnidarians and bilaterians. I also cloned TRAF6-like genes from two model symbiotic cnidarians, *Aiptasia pallida* and *F. scutaria*, laying the groundwork for future functional studies that can examine the role of TRAF6 in cnidarian immunity, and a possible role for TRAF6 in regulating cnidarian-dinoflagellate mutualisms.

Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis

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APPROVED:

Major Professor, representing Zoology

Chair of the Department of Zoology

Dean of the Graduate School

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

Christine E. Schnitzler, Author

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ABBREVIATIONS

AP-1 – activator protein-1

ApTRAF6 – *Aiptasia pallida* TRAF6

CART – C-rich motif associated with RING and TRAF domains

CYLD – cylindromatosis

DUBs – deubiquitinating enzymes

EST – expressed sequence tag

FsTRAF6 – *Fungia scutaria* TRAF6

FSW – filter-sterilized seawater

IL-1R – Interleukin-1 receptor

IRAKs – interleukin-1 receptor-associated kinases

MATH – meprin and TRAF-C homology

NF-κB – nuclear factor- κB

NO – nitric oxide

NvTRAF – *Nematostella vectensis* TRAF

RANK – receptor activator of nuclear factor κB

RANKL – RANK ligand

RNS – reactive nitrogen species

ROS – reactive oxygen species

SOD – superoxide dismutase

SSTs – sea surface temperatures

TLR – Toll-like receptor

TNF – tumor necrosis factor

TNFR – TNF receptor

TRAF – TNF receptor associated factor

TRGs – taxonomically restricted genes

Ub – ubiquitin

Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis

Chapter 1: Introduction

Symbiosis is among the most widespread and enduring biological themes in the history of our planet. Mutualisms, where both partners benefit from the association, can provide a selective advantage to organisms responding to changing environments, as the physiological capabilities of the two organisms together can often surpass that of each individual. Photosymbioses, where photosynthetic microorganisms (symbionts) live inside an animal (host), are found among calcifying foraminifera, giant clams and sponges, but are best exemplified in cnidarians, especially reef-building corals (Venn et al. 2008). In cnidarians, the partnership is formed with photosynthetic algae, specifically dinoflagellates (unicellular eukaryotes) in the genus *Symbiodinium*. Photosynthetic products are translocated from living *Symbiodinium* cells to the cnidarian host at considerable rates (Venn et al. 2008). Housing photosymbiotic dinoflagellates intracellularly can also pose risks to the host because environmental stressors, especially elevated temperature, can lead to the overproduction of reactive oxygen species (ROS) by the symbiont, resulting in symbiont and host cell membrane and protein damage, and even coral bleaching, which is a breakdown of the symbiosis (Weis 2008).

Symbionts can be transmitted vertically from parent to offspring or horizontally when they are acquired from the surrounding environment with each host generation. Most coral species acquire their photosymbiotic partners horizontally, although little is

known about the cellular recognition and signaling mechanisms that occur between cnidarian cells and symbiont cells during symbiosis establishment. Inter-partner signaling mechanisms between animal cells and unicellular eukaryotes have been far better studied in other symbiotic systems, especially in the invasion of animal cells by parasites (Schwarz 2008). Investigators studying the cnidarian-dinoflagellate relationship are beginning to frame their investigations in this context. Key research areas that will undoubtedly benefit from this perspective include understanding initial recognition events, mechanisms of symbiont invasion of a host cell, symbiont manipulation of the host cell response to invasion, and the host's immune response to invasion under both ambient and stressful environmental conditions.

Background

Overview

The aim of this background section is to describe the biological systems and major topics addressed in this dissertation. In section A, I provide information on the basic biology of cnidarian-dinoflagellate mutualisms. In section B, I explain the advantages of the *Fungia scutaria*-*Symbiodinium* sp. C1f model system for studying symbiosis onset in corals. In section C, I describe what is known about how elevated seawater temperatures impact cnidarian-dinoflagellate symbiosis. In section D, I discuss the current state of knowledge regarding host genes involved in the onset of cnidarian-dinoflagellate symbiosis. In section E, I give an overview of pathways and molecules present in the host cnidarian immune system that may be involved in regulating the onset of symbiosis, obtained through analogies to pathogenic associations. In section F, I discuss the genomic resources that are currently available for studying cnidarian-dinoflagellate symbiosis.

A. Biology of the cnidarian-dinoflagellate association

The Phylum Cnidaria includes jellyfish (Classes Scyphozoa and Cubozoa), hydroids (Class Hydrozoa), and anemones and corals (Class Anthozoa). Cnidarians are basal

metazoans and are often regarded as the closest outgroup to the Bilateria. They are not generally considered to be the most early diverging metazoan phylum – the evolutionary relationships among the Porifora (sponges), the Placazoa (*Trichoplax*), and the Ctenophora (comb jellies) are contentiously debated and, once the dust finally settles, the designation of the most basal extant metazoan will presumably be bestowed upon one of these groups. Cnidarians are amongst the simplest animals at the tissue grade of organization, having only two identifiable tissue layers – an outer epidermis and an inner gastrodermis, separated by an acellular gelatinous mesoglea (Fig. 1.1). Cnidarians have two body forms, the sessile polyp and the actively swimming medusa. Some cnidarians exhibit both body forms over the course of their life cycle, while others omit one or the other form. Anthozoans are exclusively of the polyp form, consisting of a basal disc at the base of the body column that attaches to the substrate, an oral disc on top of the body column, and numerous stinging tentacles that surround the oral disc. There is a single opening to the body, through the mouth, that leads to a gastrovascular cavity that is the site of digestion, circulation and reproduction. The gastrodermal tissue layer is where symbiotic dinoflagellate cells are housed intracellularly inside host vacuoles (Fig 1.1).

Dinoflagellates are unicellular eukaryotes classified in the Kingdom Alveolata and are the sister taxon to apicomplexan parasites such as *Plasmodium* and *Toxoplasma*. Cnidarian endosymbionts are in the extraordinarily diverse genus *Symbiodinium*, which has been divided into 8 clades (A-H) and numerous subclades (Pochon et al. 2006). The clades exhibit order-level sequence diversity, and the current classification does not reflect the true magnitude of diversity that exists. Dinoflagellate nuclear genomes are unusually large; their size is estimated to be 1-77 times that of the human genome (Hou and Lin 2009). *Symbiodinium*'s nuclear genome is estimated to be around 3000 Mb (equal to the size of the haploid human genome) with 38,188 protein-coding genes (Hou and Lin 2009). This is the smallest estimated dinoflagellate nuclear genome among examined dinoflagellate taxa (Hou and Lin 2009). Genome reduction

among endosymbionts is a common theme (Bapteste and Gribaldo 2003) and is exemplified at yet another level – in the mitochondrial genomes of dinoflagellates, which have also been massively reduced (Waller and Jackson 2009). Due to their prohibitively large size, no dinoflagellate nuclear genomes have been sequenced to date, so it is unclear why dinoflagellates have such enormous nuclear genomes.

Cnidarians obtain their dinoflagellate symbionts in one of two ways. In some cases, symbionts are passed from parent to offspring via vertical transmission. In most cases, however, S*ymbiodinium* is acquired from the environment through horizontal transmission (Fadlallah 1983; Harrison and Wallace 1990). Little is known about free-living *Symbiodinium*, including where they occur in the marine environment or how they are dispersed. One recent study found little overlap between types of *Symbiodinium* found in the coral communities of one Hawaiian reef and the types of *Symbiodinium* isolated from environmental samples (water column and sediments) taken adjacent to the reefs, suggesting that perhaps endosymbionts of some corals have evolved such high levels of integration with their hosts that they cannot survive independently (Pochon et al. 2010). Another study found several types of free-living *Symbiodinium* within macroalgal beds and viable *Symbiodinium* cells in the feces of a type of parrotfish in Caribbean waters (Porto et al. 2008). Regardless of how they are obtained, once ingested, host gastrodermal cells, in the form of nutritive phagocytes, take up dinoflagellate cells via phagocytosis and host them intracellularly inside host-derived vacuoles (Colley and Trench 1983). Once the association is established, host cnidarians gain organic nutrients that are produced by the dinoflagellates during photosynthesis (Venn et al. 2008), while symbiotic dinoflagellates receive a protected, high light environment, and nutrients such as dissolved inorganic carbon and nitrogen excreted by the coral (Yellowlees et al. 2008). This arrangement of a "producer inside a consumer" increases nutrient availability for both partners, as fewer nutrients are lost to the external environment (Yellowlees et al. 2008). Together the cnidarian host and dinoflagellate symbiont is known as the "holobiont." This term encompasses the

complex web of interactions between the two partners, the holobiont interaction with the surrounding environment, and the complex microbial communities that have recently been found on the surface of symbiotic cnidarians (Yokouchi et al. 2006; Wegley et al. 2007; Dinsdale et al. 2008; Vega Thurber et al. 2009).

The association between cnidarians and *Symbiodinium* first appeared at least 225 million years ago (Stanley 2003; Kiessling 2010). The holobiont relationship is the basis of coral reef ecosystems, which are remarkably diverse, productive, and valuable global marine resources, providing a global annual net economic benefit of $30 billion (Cesar et al. 2003). Coral reefs maintain an incredible level of productivity considering the oligotrophic (nutrient-poor) waters in which they are found. Most coral species are obligate hosts, and without their symbionts, they exhibit decreased growth and increased mortality, resulting in reef degradation. Currently, reefs are facing multiple environmental threats that can lead to severe degradation. Chief among these threats are elevated seawater temperatures and ocean acidification; both are the result of global climate change from increasing atmospheric levels of CO_2 (Hoegh-Guldberg et al. 2007; Carpenter et al. 2008). Many corals live at temperatures near the upper limits of their thermal tolerance (Glynn 1993; Coles and Brown 2003) so that even minimal increases in seawater temperatures can have devastating effects on coral health. Coral bleaching is a loss of symbionts culminating in the collapse of the symbiosis. This phenomenon results from a fundamental breakdown in the cellular mechanisms that allow the symbiont to reside within the host cell (Weis 2008). Bleaching has been primarily attributed to elevated seawater temperatures in combination with high light levels (Weis 2008). Although the recent increase in bleaching events worldwide has driven much research on corals, we still know very little about fundamental aspects of coral-dinoflagellate symbiotic associations, including how they are established on a molecular or cellular scale (Edmunds and Gates 2003).

B. F. scutaria-Symbiodinium sp. C1f as a model system for symbiosis onset

The planula larva of the scleractinian coral *F. scutaria* and its dinoflagellate symbiont *Symbiodinium* type C1f is an ideal model for studying the onset of coral-dinoflagelate symbiosis due to the predictable availability of gametes and the ability to raise non-symbiotic (aposymbiotic) larvae and establish the symbiosis experimentally. Adult *F. scutaria* are large, solitary polyps that do not attach to the substrate, allowing for the non-invasive collection of adults, which can be maintained in running seawater tables (Fig. 1.2). For more than a decade, this association has been developed as a model for studying symbiosis onset and precisely quantifying infection success. The Weis Lab performs studies using this system in collaboration with Dr. Dave Krupp and Dr. Ruth Gates at the Hawaii Institute of Marine Biology (HIMB), University of Hawaii's marine lab. *F. scutaria* is a sexually dimorphic coral species that broadcast spawns between 17:00 and 19:00 h, two to four days following the full moon during summer months (Krupp 1983). Fertilization occurs in the water column and zygotes develop into aposymbiotic planula larvae that actively swim by 1- 2 days of age, develop a mouth by day 3, and are competent to settle by day 7 (Schwarz et al. 1999). The larvae are able to acquire symbionts through their mouth as soon as they are able to feed. Since each *F. scutaria* adult spawns thousands of gametes during one spawning event, this system provides sufficient material for multiple experimental manipulations and samplings. Symbionts can be freshly isolated from the tissue of adult *F. scutaria*, homogenized to remove residual host tissue, and used to infect the naturally aposymbiotic larvae. Infection conditions can be manipulated by altering the type of *Symbiodinium* used to infect and/or the environmental parameters. During the infection process, the larvae release mucus strands and use them to trap symbiont cells, which they subsequently ingest through their mouth (Schwarz et al. 1999). Populations of larvae can be sampled at any point throughout the infection process and/or in the hours and days following infection. Infection success (% infection and density of infection) can be quantified by examining preserved larval samples under a compound microscope.

F. scutaria associates with a single homologous *Symbiodinium* type in Hawaii - clade C, subclade 1f (LaJeunesse et al. 2004; Rodriguez-Lanetty et al. 2004). Studies have demonstrated that this high level of specificity can be seen during the larval stage starting in the first few hours following the infection period (Weis et al. 2001; Rodriguez-Lanetty et al. 2006b). Infections with non-homologous symbiont types within clade C result in significantly lower infection levels (Weis et al. 2001; Rodriguez-Lanetty et al. 2004; Rodriguez-Lanetty et al. 2006b).

C. Effects of elevated temperature on coral-dinoflagellate symbiosis

How do elevated temperatures result in coral bleaching?

Hosting photosymbiotic dinoflagellates can prove to be a risky strategy for corals that become exposed to elevated temperatures because these conditions can result in oxidative stress. Oxidative stress is defined as the production and accumulation of reduced oxygen intermediates such as superoxide (O_2^-) and singlet oxygen ($^1O_2$), collectively called reactive oxygen species (ROS). Oxidative stress has been proposed as a general mechanism that leads to coral bleaching (Lesser 1997; Lesser and Farrell 2004; Lesser 2006) via degradation of dinoflagellates, programmed cell death of host cells and/or dinoflagellates, exocytosis of symbionts from host coral cells, or via host cell detachment (Gates et al. 1992; Dunn et al. 2004; Lesser and Farrell 2004). Exposure to elevated temperatures can result in photoinhibition of photosynthesis in dinoflagellate symbionts (Lesser 1996). During normal temperatures and irradiances, antioxidant enzymes (e.g. SOD) in the chloroplasts of dinoflagellate cells degrade excess ROS (Matta and Trench 1991). During heat stress, the production of ROS increases (Lesser 2006). The additional ROS overwhelm available antioxidants, and some species are exported from the chloroplast and are permeable through the cell wall (Smith et al. 2005), or can be produced by host cells directly via host mitochondrial damage (Dykens et al. 1992; Nii and Muscatine 1997). If the host cell detects excess ROS, antioxidant enzymatic defenses will be initiated, but these can be

quickly overwhelmed allowing ROS to accumulate and damage to occur, ultimately leading to bleaching (Richier et al. 2005). There is also evidence for a dramatic increase in reactive nitrogen species (RNS), such as nitric oxide (NO), in host tissues in response to elevated temperature (Perez and Weis 2006). Furthermore, the addition of NO at ambient temperature can lead to bleaching (Perez and Weis 2006). Very little, however, has been established experimentally regarding how host coral cells react to excess ROS and RNS (Weis 2008).

Effects of elevated temperature on coral larvae

Rising temperatures are predicted to have detrimental effects on tropical marine larvae by negatively impacting development and increasing mortality while also reducing recruitment potential and decreasing the exchange of individuals between populations (Munday et al. 2009). Coral larvae are no exception to these predictions as previous studies have shown increased larval mortality (Bassim and Sammarco 2003; Nozawa and Harrison 2007; Randall and Szmant 2009b,a), decreased fertilization and impaired development (Krupp et al. 2006; Negri et al. 2007) in response to higher temperature. Less clear is how elevated temperatures affect the process of symbiosis establishment and very few studies have examined the larval holobiont response to elevated temperature. These studies have reported conflicting results in terms of larval survivorship (Baird et al. 2006; Yakovleva et al. 2009). No studies have quantified the impact of elevated temperature on the ability of the partners to form the association.

D. Identifying host genes involved in cnidarian-dinoflagellate symbiosis

There are few data regarding the precise recognition and signaling mechanisms that mediate inter-partner contact in cnidarian-dinoflagellate symbiosis. There is evidence that this process is initiated through lectin-glycan interactions, which act as pre-phagocytic recognition mechanisms (Lin et al. 2000; Koike et al. 2004; Wood-Charlson et al. 2006; Vidal-Dupiol et al. 2009). Functional genomic studies suggest that host corals possess innate immune pathways (Miller et al. 2007) that

dinoflagellate symbionts may evade or mediate during symbiosis onset (Schwarz 2008). Dinoflagellates also may prevent the fusion of host phagosomes containing live symbionts to lysosomes by controlling the trafficking of the vesicles inside host cells using Rab GTPases (Fitt and Trench 1983; Chen et al. 2003; Chen et al. 2004; Chen et al. 2005). Much remains to be discovered about the cascade of events that occurs as the holobiont association proceeds from initial contact through stable establishment.

The first studies to identify host molecules involved in cnidarian-dinoflagellate symbiosis used a gene-by-gene (or protein-by-protein) approach (Weis and Levine 1996; deBoer et al. 2007). More recent studies have applied higher-throughput approaches to identify relevant transcripts or proteins (e.g. Rodriguez-Lanetty et al. 2006a). The most popular high-throughput method is the microarray, and arrays have been created to perform transcriptional profiling of many aspects of cnidarian biology, from larval development (Grasso et al. 2008) to coral bleaching (Desalvo et al. 2008). A cDNA microarray experiment retrieves transcripts from a sample of interest through hybridization to cDNAs bound to a solid surface, such as a glass microscope slide. Fluorescence signal intensity from the resulting hybrids indicates the relative amount of each transcript in the original sample. The advantage of this method is that you can profile thousands of genes at once. Disadvantages are that you can miss transcripts that are expressed at low levels in a sample, cross-hybridization to genes similar to the cDNA probes can occur, and results are limited to both the genes that are represented and the length and quality of the cDNAs that are printed on the array. Most recently, next generation sequencing methods, such as 454 GS FLX have been used to sequence the aposymbiotic larval coral transcriptome (Meyer et al. 2009). As next generation sequencing and genome assembly and analysis methods improve, and costs decrease, there will be many biological questions that can be addressed with this technology.

E. Pathways and molecules in the host immune system that may be involved in regulating the onset of symbiosis

Cnidarians, like all metazoan organisms, must manage interactions with beneficial and harmful microbes. Cnidarian immunity therefore must include strategies for both resisting and accepting microbes that attempt to invade their tissues. Microbial invaders of coral tissues include dinoflagellate symbionts, which can be either beneficial or harmful depending on the *Symbiodinium* type and the circumstances of the invasion. The immune system of cnidarians is largely unexplored to date. Specialized immune cells have not been found in cnidarians, but some species display highly specific allorecognition characteristics to distinguish self from non-self (Bosch and David 1986; Grosberg 1988; Chadwick-Furman and Rinkevich 1994; Cadavid et al. 2004; Rinkevich 2004).

Interactions between animal cells and unicellular eukaryotes have been far better studied in animal-parasite systems. Mechanisms employed by cnidarians during colonization by dinoflagellates may be similar to some of those seen in the invasion of animal cells by protozoan parasites (Schwarz 2008). Analyses of cnidarian genomic resources have revealed that they possess many of the key innate immune pathway components found in the genomes of higher animals (Miller et al. 2007). These pathways include immune-stimulating pathways, such as the Interleukin-1 receptor (IL-1R)/Toll-receptor (TLR) pathway and the complement pathway. The ultimate step in the IL-1R/TLR pathway is translocation of transcription factor NF-κB into the nucleus, where it stimulates transcription of appropriate immune response genes (Fig.1.3). An activated complement system targets invading pathogens for phagocytosis. Our group also has evidence that the TGFβ pathway, an immunosuppressive pathway well-characterized in vertebrates (Dennler et al. 2002) and often manipulated by microbes to promote host tolerance (Ndungu et al. 2005; Waghabi et al. 2005), is present in anemones and functions in the tolerance of homologous *Symbiodinium* types (Detournay, Schnitzler and Weis, unpublished manuscript). Detailed study of the genes and gene families involved in these host

immune pathways and functional studies of how these pathways operate in cnidarians challenged with beneficial and/or detrimental microbes, however, are largely absent.

Cnidarian immune systems may be evaded or suppressed by dinoflagellates. Apicomplexan parasites, a sister taxon to dinoflagellates within the Alveolata (Adl et al. 2005), employ numerous strategies to manipulate the host cells that they invade. *Toxoplasma gondii* generates its own vacuolar membrane to avoid host detection and prevent fusion with host lysosomes (Blader and Saeij 2009), and inhibits host inflammatory cytokine production (Leng et al. 2009). *Plasmodium falciparum* promotes tolerance of its presence by suppressing or disrupting host immunity (D'Ombrain et al. 2007), even going as far as exporting proteins that induce substantial changes in the morphology, physiology and function of the host cell (Maier et al. 2009). Future studies on the regulation of cnidarian-dinfolagellate mutualisms will undoubtedly find many more parallels with well-characterized symbiotic systems, especially animal-parasite interactions.

F. Genomic resources available for studying cnidarian-dinoflagellate symbiosis

At this time, two cnidarians have fully sequenced, assembled and annotated genomes. The non-symbiotic anthozoan *Nematostella vectensis* (Putnam et al. 2007) and the non-symbiotic freshwater hydrozoan *Hydra magnipapillata* (Chapman et al. 2010). In addition to these resources, there are several EST databases available from several cnidarians, including both non-symbiotic and symbiotic representatives (Table 1.1). The EST databases vary in size from 373 from the anemone *Aiptasia pulchella* to 99,091 from the coral *Acropora millepora*. Genome sequencing is currently underway for two species of *Acropora*, and the first genome from a symbiotic cnidarian will soon be available. There are also two very small EST databases for two types of *Symbiodinium* (Table 1.1). As genomic and transcriptomic resources for symbiotic cnidarians and their dinoflagellate symbionts increase and improve, our ability to explore the cnidarian-dinoflagellate association will be vastly expanded.

Summary

In this dissertation, I focus on the onset of symbiosis between cnidarians and their dinoflagellate symbionts by (1) examining how an environmental stressor (elevated seawater temperature) affects the ability of coral larvae to establish symbiosis (Chapter 2), (2) using a discovery-based method to identify host transcripts that are differentially regulated during symbiosis onset (Chapter 3), and (3) performing an in-depth exploration of a gene family (TRAF) involved in mammalian innate immune systems in the genomes of cnidarians and by cloning and sequencing TRAF6 homologs (a key TLR pathway component) from two model symbiotic cnidarians, which paves the way for functional studies of this gene in regulating cnidarian-dinoflagellate symbiosis (Chapter 4). I conclude this work with a discussion that puts Chapters 2-4 in a broader context related to global climate change and genomics and I present some thoughts on future experiments that will further explore the molecules and mechanisms involved in the onset of cnidarian-dinoflagellate symbiosis (Chapter 5).

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Figures and Tables

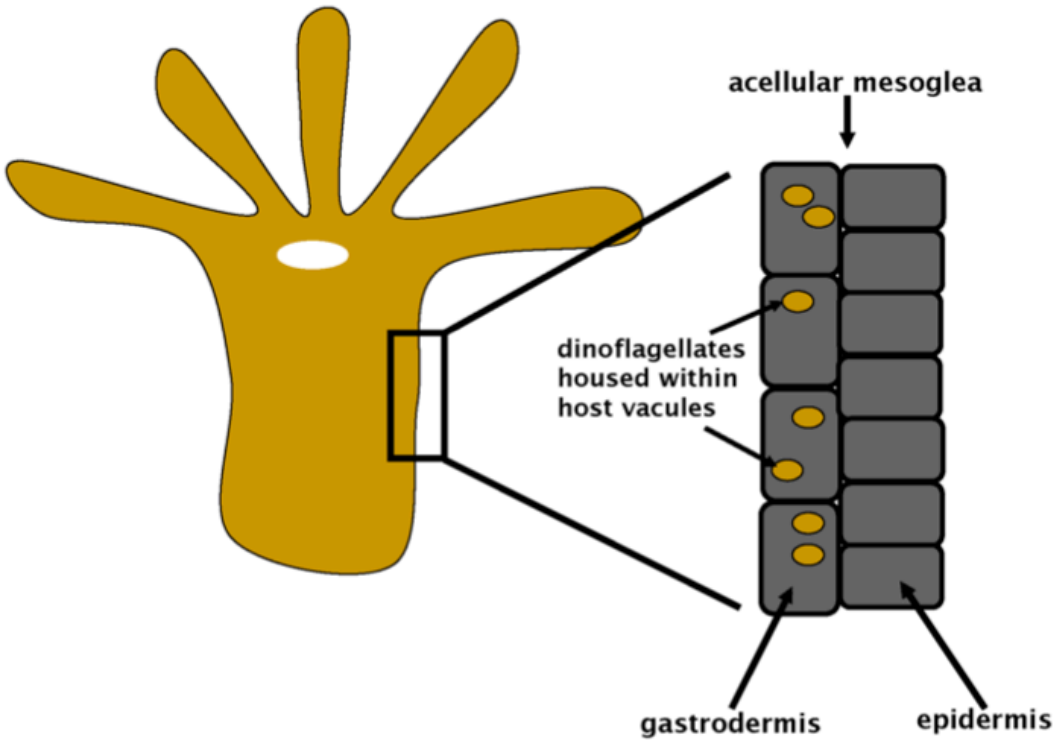

Fig. 1.1. Schematic of a cnidarian polyp demonstrating the organization of the two tissue layers. Dinoflagellates are housed inside host vacuoles in the gastrodermis.

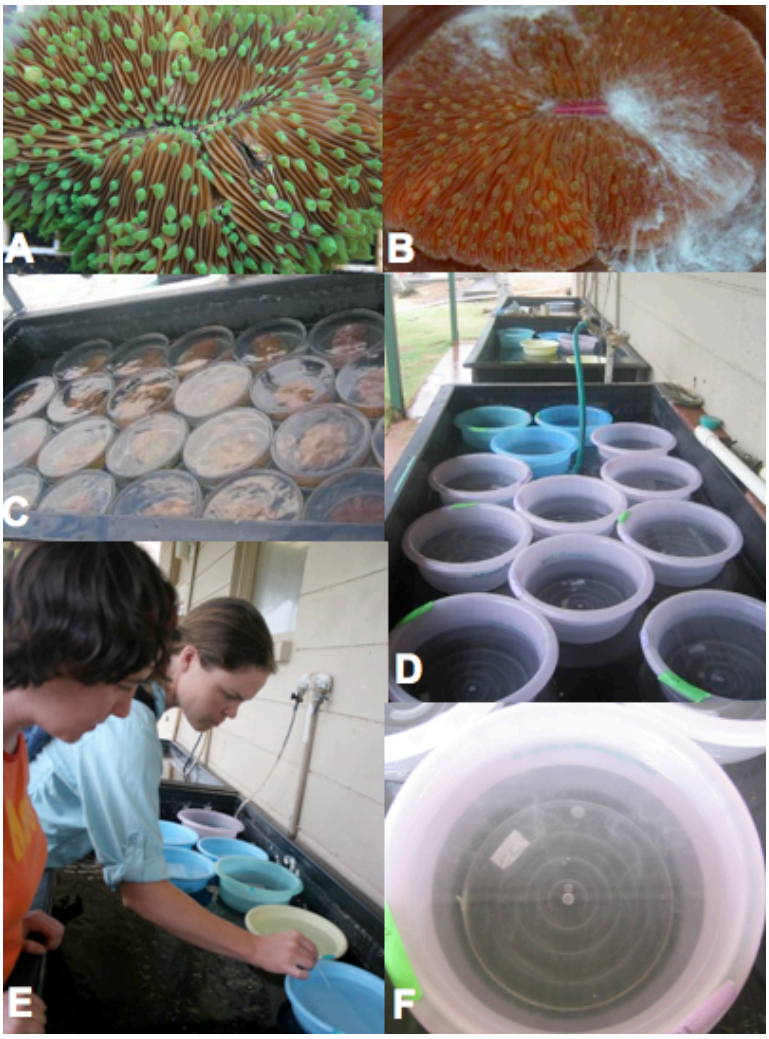

Fig. 1.2. *Fungia scutaria-Symbiodinium* sp. C1f as a model system to study coral larvae. A) Adult *F. scutaria* occur as individual polyps on the reef. B) Adults spawn 1-2 days after the full moon during summer months. This individual is spawning eggs. C) Adult corals are maintained in running seawater tables year-round. D) Eggs and sperm are fertilized artificially and kept in plastic bowls in running seawater tables. E) Larvae are cleaned daily with filtered seawater to maintain an environment free of symbionts. F) Larvae are often seen swimming together, forming spiral columns in the bowls.

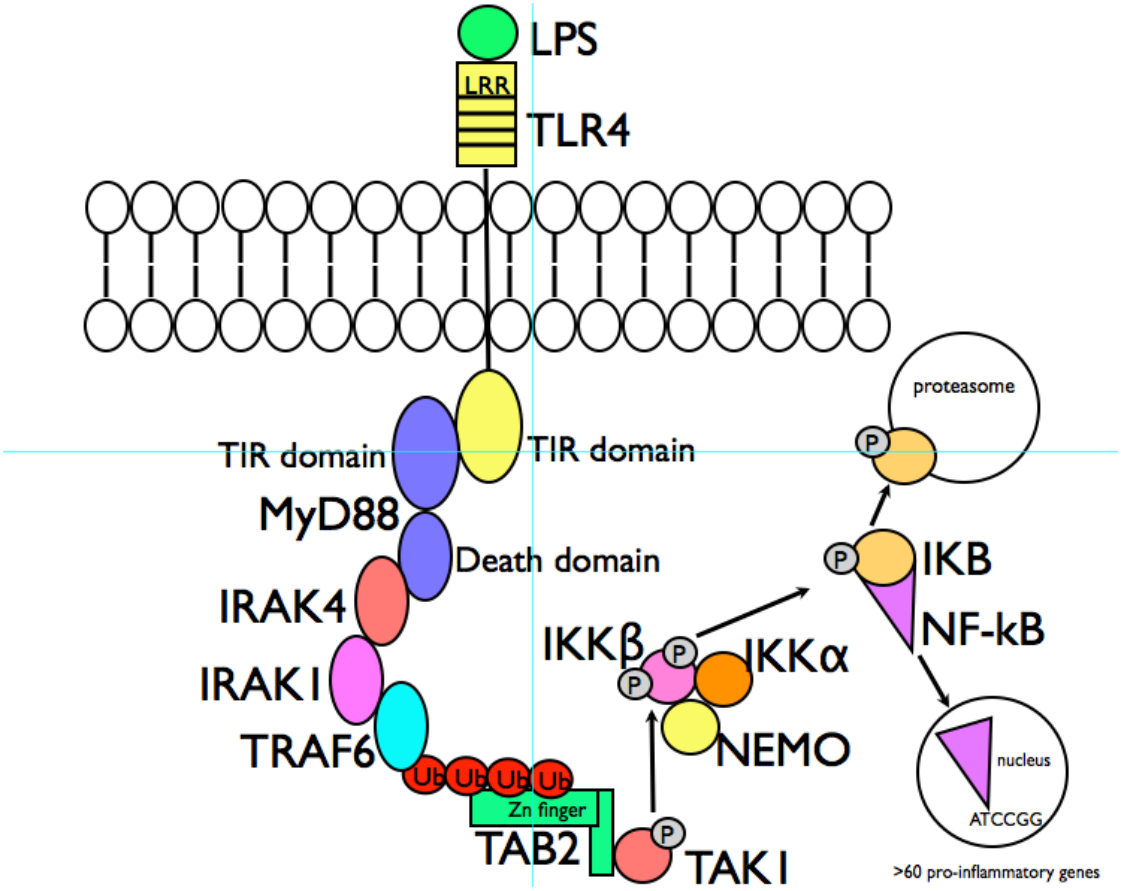

Fig. 1.3. The TLR4 innate immune signaling pathway, which has been well-characterized in vertebrates. The pathway starts with lipopolysaccharide (LPS) binding to a receptor (TLR4). This initiates signaling through the intracellular TIR domain of TLR4, which then recruits downstream signals, such as MyD88, IRAKs and TRAF6. TRAF6 is activated through polyubiquitination, which forms a molecular scaffold for other downstream factors. Ultimately, IKB is phosphorylated, causing a protein conformational change that releases bound NF-kB. NF-kB is then translocated to the nucleus, where can activate transcription of numerous pro-inflammatory genes.

Table 1.1. Publicly available draft genome and EST sequences from symbiotic (S) and non-symbiotic (NS) cnidarians and their symbionts. U denotes that this information is unknown.

Species	# genes/unique ESTs	Database*	S or NS	Developmental stage or exposure used for material sequenced
Acropora millepora	99,091 ESTs	SymBioSys, COMPAGEN	S	prawn chip (NS), planula larvae (NS), post-settlement crown stage (NS)
Acropora palmata	14,647 ESTs	SymBioSys, COMPAGEN	S	unfertilized eggs (NS), planula larvae (NS & S), adult (S)
Aiptasia pallida	4,698 ESTs	AiptasiaBase, SymBioSys, COMPAGEN	S	adult (S)
Aiptasia pulchella	373 ESTs	GenBank	S	adult (NS and S)
Anemonia viridis	14,504 ESTs	GenBank	S	U
Clytia hemisphaerica	85,991 ESTs	GenBank, COMPAGEN	NS	U
Hydra magnipapillata Draft genome assembled	163,221 ESTs ~19,845 genes	COMPAGEN, Hydrazome	NS	adult with stage 1-5 buds
Hydra vulgaris	8,993 ESTs	COMPAGEN	NS	U
Hydractinia echinata	9,460 ESTs	COMPAGEN	NS	U
Metridium senile	29,412 ESTs	COMPAGEN	NS	U
Montastraea annularis	2,388 ESTs	SymBioSys	S	U
Montastraea faveolata	11,611 ESTs	SymBioSys, COMPAGEN	S	unfertilized eggs (NS), planula larvae (NS & S), adult (S)
Nematostella vectensis Draft genome assembled	166,595 ESTs ~18,000 genes	JGI, StellaBase	NS	adult
Podocoryne carnea	3,466 ESTs	GenBank	NS	embryo and adult
Porites astreoides	6,681 ESTs	SymBioSys, COMPAGEN	S	adult
Porites lobata Genome shotgun reads	11,450	COMPAGEN	S	U
Symbiodinium sp. clade C3 from *Acropora aspera*	2,632 ESTs	SymBioSys	N/A	exposed to a variety of stressors
Symbiodinium sp. clade A KB8 from *Cassiopea* sp.	1, 220 ESTs	SymBioSys	N/A	cultured symbiont

*Databases can be found at the following websites:
SymBioSys: http://sequoia.ucmerced.edu/SymBioSys/index.php
COMPAGEN: http://compagen.zoologie.uni-kiel.de/
AiptasiaBase: http://aiptasia.cs.vassar.edu/AiptasiaBase/index.php
GenBank ESTs: http://www.ncbi.nlm.nih.gov/nucest/
Hydrazome: http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra/
JGI: http://genome.jgi-psf.org/Nemve1/Nemve1.home.html
StellaBase: http://stellabase.com/

Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis

Chapter 2: Elevated temperature impairs onset of symbiosis and reduces survivorship in larvae of the Hawaiian coral, *Fungia scutaria*

Christine E. Schnitzler
Lea L. Hollingsworth
Dave A. Krupp
Virginia M. Weis

To be submitted to:

Coral Reefs

Springer

Rolf P.M. Bak

Royal Netherlands Institute for Sea Research

PO Box 59

1790 AB Den Burg, The Netherlands

Abstract

Many species of coral obtain their obligate intracellular symbionts horizontally from the environment as larvae or juvenile polyps, however, the early life history of most coral species remains largely unexplored in terms of symbiosis onset, especially under stress conditions. This study addresses the ability of *Fungia scutaria* (Lamarck 1801) larvae to establish symbiosis with *Symbiodinium* sp. C1f while exposed to elevated temperature; the survivorship of larvae under these conditions; and the effect of symbiotic state on survival at increased temperature. Larvae and dinoflagellates were exposed to 27°C, 29°C, or 31°C for one hour prior to infection, throughout the 3-hour infection period, and up to 72 h following infection. Elevated temperature significantly impacted symbiosis establishment. By 5 h post-infection, the infection level of larvae at 31°C was 19% lower than larvae at 27°C; by 36 h, the infection level of larvae at 29°C was 18% lower than larvae at 27°C. Temperature also strongly impacted larval survivorship. By 72 h post-infection, larvae at the highest temperature had 33.5% lower survivorship than larvae at 27°C. Symbiotic state had a measurable, but non-significant impact on survival at high temperature. By 72 h, larvae that were infected with symbionts at 31°C had a 13% lower survival rate than larvae without symbionts at the same temperature. These results indicate that exposure to elevated temperatures impairs the ability of coral larvae to establish symbiosis and reduces larval survivorship. As sea surface temperatures rise, the potential costs of forming this mutualism may be amplified and the benefits may decrease, which in turn may negatively impact future generations of corals.

Introduction

Symbiosis occurs when unrelated organisms come together to form a stable association. Mutualistic symbioses are traditionally defined as beneficial associations that enhance the physiological capabilities of both organisms (Paracer and Ahmadjian 2000). This definition is changing, however, to reflect the existence of unequal partnerships, the potential for conflict between partners, and costs associated with

mutualisms (Ferriere et al. 2002; Douglas 2008). For many symbiotic systems, it remains uncertain how environmental variation, genotype, and condition of the partners influence the cost-benefit balance for the individuals in the association (Stachowicz 2001; Douglas 2008; Oliver et al. 2009). In particular, little is known about how stress affects the establishment of mutualistic symbioses or if changing environmental conditions augment potential costs and/or diminish benefits.

The mutualism between scleractinian corals and unicellular dinoflagellates (genus *Symbiodinium*), known together as the holobiont, forms the foundation of coral reef ecosystems (Dubinsky 1990). Both partners have numerous adaptations to the obligate phototrophic endosymbiotic condition that maximize mutual benefits (Furla et al. 2005). Host corals gain organic nutrients that are produced by the dinoflagellates during photosynthesis (Venn et al. 2008). Symbiotic dinoflagellates are given shelter, access to sunlight, and nutrients such as dissolved inorganic carbon and nitrogen excreted by the coral (Yellowlees et al. 2008).

Before mutualists can reap the benefits of their partnership, they must first find one another and mutually agree to their living arrangement. The majority of scleractinian coral species broadcast spawn gametes free of dinoflagellates. Horizontally transmitted symbionts are taken up from the environment by each host generation (Fadlallah 1983; Harrison and Wallace 1990). Symbiosis is typically established when dinoflagellates enter through the mouth of a coral larva or polyp, and are subsequently phagocytosed by endodermal cells lining the gastrovascular cavity (Colley and Trench 1983; Fitt and Trench 1983; Schwarz et al. 1999), although other mechanisms of incorporation exist (Marlow and Martindale 2007). Dinoflagellates ultimately reside in vacuoles of host gastrodermal cells (Wakefield et al. 2000).

Potential costs of the partnership are regulated through several mechanisms. Under stable circumstances, both partners manage dangerous reactive oxygen species (ROS)

produced as a byproduct of photosynthesis by employing antioxidant enzymes such as superoxide dismutase (SOD) and catalase (Richier et al. 2005; Lesser 2006). When stressful circumstances such as increased temperature lead to excess ROS, the stable association can rapidly deteriorate as symbionts and hosts become overwhelmed by damaging oxygen radicals as their ROS-scavenging enzymes fail to keep up (Lesser 2006). This oxidative stress can eventually lead to downstream coral bleaching, or collapse of the symbiosis, through one of several cellular mechanisms (Douglas 2003; Weis 2008).

The effects of global climate change, including elevated sea surface temperatures and ocean acidification resulting from increased atmospheric levels of CO_2 (Hoegh-Guldberg et al. 2007; Carpenter et al. 2008) threaten the health of corals; many of which live at temperatures near the upper limits of their thermal tolerance (Glynn 1993; Coles and Brown 2003). Bleaching in adult corals can be induced by short-term exposure (1–2 days) to temperatures 3-4°C above the normal summer ambient temperature or by long-term exposure (several weeks) to temperatures 1-2°C above ambient (Jokiel and Coles 1977; Jokiel and Coles 1990). Recent bleaching events, primarily initiated through elevated seawater temperatures, have already greatly reduced coral populations (Coles and Brown 2003). Bleaching is the ultimate downstream consequence of amplified costs that result when the coral-dinoflagellate partnership responds to stress conditions.

Since average sea surface temperatures (SSTs) are predicted to rise 1.8-4°C by 2100 with increasing atmospheric CO_2 emissions (Meehl et al. 2007), there will likely be more frequent bleaching-related mortality of corals, unless corals can undergo rapid thermal acclimation (Hoegh-Guldberg 1999; Jones 2008). Less clear is how an increase in temperature will affect early life history stages of corals that are undergoing the process of symbiosis establishment. Rising temperatures are predicted to have detrimental effects on tropical marine larvae by reducing recruitment potential

and connectivity between populations, as well as having negative impacts on development and survivorship (Munday et al. 2009).

The importance of considering the holobiont and early life history stages when predicting coral adaptation to climate change is now recognized (Coles and Brown 2003; Day et al. 2008). Previous research on coral larvae and stress, however, focused almost exclusively on host responses, such as settlement and survivorship, in isolation. Results of these efforts showed a general pattern of increased larval settlement at higher temperatures with short term (minutes to hours) exposure (Edmondson 1929; Coles 1985; Nozawa and Harrison 2007), and decreased settlement at higher temperatures with longer durations (days to months) of exposure (Jokiel and Guinther 1978; Wilson and Harrison 1997; Bassim and Sammarco 2003; Nozawa and Harrison 2007; Randall and Szmant 2009a). Many experiments have found increased larval mortality in response to higher temperature (Bassim and Sammarco 2003; Nozawa and Harrison 2007; Randall and Szmant 2009a,b). Others found decreased fertilization and embryogenesis with increasing temperature (Krupp et al. 2006; Negri et al. 2007). Overall, these results indicate that exposure to increased temperatures ranging from 30-33°C negatively impact coral larvae.

A few investigations of coral larvae have considered the holobiont response to elevated temperature. Two of these used the larvae of *Porites asteroides,* a species that vertically transmits symbionts from parent to offspring, to consider changes in the density of their symbionts over 24 h in response to increased temperature. One experiment found no difference in symbiont density in response to high temperature (Edmunds et al. 2001); the other found that larvae had the highest dinoflagellate densities at intermediate temperatures (Edmunds et al. 2005).

Recent studies of *Acropora* sp. attempted to quantify the effect of the symbiotic state on the larval response to temperature (Baird et al. 2006; Yakovleva et al. 2009). Baird

et al. (2006) found no significant difference in survivorship between symbiotic and aposymbiotic (without symbionts) larvae exposed to elevated temperatures for 7 days. Yakovleva et al. (2009) found significantly decreased survivorship, and increased SOD and malondialdehyde levels (a measure of oxidative damage) in symbiotic larvae compared to aposymbiotic larvae after 3 days of high temperature exposure. Infections of the *Acropora* larvae were performed at ambient temperatures before temperature exposure began, and the change in density of the infection in response to elevated temperature was not quantified. These studies demonstrate our limited understanding of how thermal stress conditions affect the coral-dinoflagellate relationship during the earliest stages of the association.

In this investigation, the goals were: (1) to quantify the effects of elevated temperatures on the establishment of symbiosis (2) to quantify the effects of elevated temperatures on larval survivorship and (3) to examine the interaction of symbiotic state and elevated temperatures on larval survivorship. Laboratory experiments were designed to examine effects of elevated temperature using larvae of the solitary Hawaiian coral, *Fungia scutaria*, a broadcast spawning species that lives in shallow reef flat environments where large fluctuations in temperature can occur. *F. scutaria* obtains its symbionts via horizontal transmission and has been used as a model for studies of symbiosis onset for more than a decade (Schwarz et al. 1999; Weis et al. 2001; Rodriguez-Lanetty et al. 2006; Wood-Charlson et al. 2006; deBoer et al. 2007; Dunn and Weis 2009). This study represents the first examination of how thermal stress affects the process of symbiosis establishment and whether larvae exposed to thermal stress have greater mortality when they are also in the process of symbiosis establishment.

Materials and methods

Collection and maintenance of coral larvae

Adult *F. scutaria* were collected over several years from multiple reefs in Kaneohe Bay (Oahu, Hawaii) and maintained year-round in seawater tables at the Hawaii Institute of Marine Biology, University of Hawaii's marine laboratory. The corals broadcast spawn aposymbiotic gametes between 17:00 and 19:00 h, 2-4 d following the full moon in the summer months (Krupp 1983). Eggs from several females were kept in bowls with 1l of 0.22 µm filter-sterilized seawater (FSW). Sperm from multiple males (>3) were mixed prior to fertilizing each bowl. Bowls were placed in outdoor tables with a continuous flow of seawater at ambient temperature (27°C). Larvae that developed in each bowl were cleaned daily with 0.22 µm FSW and checked for the development of a mouth using a compound microscope.

Preparation of dinoflagellates

In Hawaii, *F. scutaria* associates with *Symbiodinium* sp. clade C, subclade 1f (Rodriguez-Lanetty et al. 2004). Since *F. scutaria* symbionts have not been cultured, dinoflagellates are extracted from adults. Coral tissue containing C1f symbionts was removed from the skeleton of adult *F. scutaria* using an oral hygiene device (Water Pik). The mixture of host tissue and dinoflagellates was further separated by homogenization with a glass tissue homogenizer and centrifugation at 6000 x *g*. Homogenization of the dinoflagellate pellet in FSW was repeated several times to remove most host tissue. The isolated dinoflagellate cells were checked for host tissue debris and quantified using a hemocytometer. Cleaned dinoflagellates were used within 2 h of preparation.

Infection of larvae with dinoflagellates

Infections were performed in 6-well culture dishes (2008 preliminary experiment), square dishes with dimensions of 4.5 x 4.5 x 1.5 cm (2009 infection success experiment), or 24-well culture dishes (2009 survivorship experiment). Four days after

fertilization, larvae from 7 bowls were combined, concentrated and quantified using a Sedgewick-Rafter Counting Cell. To each well or dish, 200 larvae ml^{-1} (2008) or 97 larvae ml^{-1} were added (2009) (n = 4 wells per temperature bath) (Table 2.1). Larvae and dinoflagellates were incubated separately in their treatment temperature for 1 h prior to the infection. Following the pre-incubation period, dinoflagellates were mixed with homogenized *Artemia* sp. to stimulate a feeding response in the larvae (Schwarz et al. 1999) and added to each well of larvae at a concentration of 75 dinoflagellates per larva (2008) or 26 dinoflagellates per larva (2009) (Table 2.1). After 3 h, the larvae were washed to remove any remaining dinoflagellates by concentrating larvae onto a 50 µm mesh filter, rinsing them with FSW at treatment temperature, and placing them into clean dishes. Once the washing was complete, this was considered to be time = 0 h post-infection.

Experimental design

2008 experiment

In July 2008, a preliminary exposure of *F. scutaria* larvae to elevated temperature was conducted to determine the best design for the study. Larvae and dinoflagellates were exposed to 27°C or 32°C for 1 h prior to infection, throughout the 3 h infection period, and up to 48 h post-infection. This duration of temperature exposure was chosen to focus on how temperature stress affects the initial stages of symbiosis formation and to examine the larval response to infection with a potentially compromised symbiont.

2009 experiment

Based on results from the preliminary experiment, the temperature range was narrowed to 27-31°C, and lower concentrations of larvae (97 larvae ml^{-1}) and dinoflagellates (26 dinoflagellates per larva) were chosen (Table 2.1). Water was changed once a day and oxygen levels in individual replicates were measured prior to each sampling point over the course of the experiment using a YSI 55 dissolved oxygen meter. Five constant temperature waterbaths (PolyScience digital waterbath),

plus one outdoor seawater table with a continuous flow of water at ambient temperature were used to maintain experimental temperatures of 27°C, 29°C and 31°C. Larvae and dinoflagellates were exposed to their treatment temperature for 1 h prior to infection, throughout the 3 h infection period, and up to 72 h post-infection. Waterbaths and the seawater table were equipped with HOBO Temperature/Light Pendant data loggers (Onset) to record temperature and light levels every 10 min. Waterbaths were located inside a laboratory with standard overhead fluorescent lights with a 12:12 h light:dark photoperiod.

Infection success

Samples were collected from 4 replicate square dishes per temperature at each of 5 time points (t=0, 5, 13, 21.5, 36 h post-infection) using plastic pipettes, and placed into 15 ml tubes. Larvae were gently pelleted by centrifugation for 1 min at 1000 x *g*, FSW supernatant was removed, and larvae were fixed in 750µl Z-fix (Anatech Ltd). To quantify infection success, larvae were rinsed in 1X PBS and visualized with a compound microscope. For each treatment replicate [*n* (dishes) = 4], infection success was determined as percent of larvae infected [*n* (larvae) = 100] and average density of dinoflagellates in larvae [*n* (larvae) = 30].

Survivorship

To follow survivorship of a cohort of larvae through time, at each time point (t=2, 7, 14, 24, 36, 48, 72 h post-infection) the entire 1 ml volume from each treatment replicate [n (wells) = 4] was pipetted onto a Sedgewick-Rafter Counting Cell and photographed with a Macrofire digital camera attached to an Olympus light microscope using PictureFrame for Mac 2.0 software. Larvae were then pipetted back into their treatment well and culture dishes were placed back in their treatment temperature. At the final time point (t=72 h), after larvae were photographed, they were fixed in Z-fix to determine infection success as described above. Survivorship was measured by counting the number of larvae in each photograph, setting the

number of larvae at t=2 h post-infection in each replicate to 100% and calculating the percent larval survival for each replicate compared to t=2 h post-infection.

Statistical analysis

Statistical comparisons among temperature treatments over time for the infection success data (response variables = percent infection success and density) were computed using two-way analysis of variance (ANOVA) with temperature (3 levels) and duration of exposure (5 levels) as fixed factors. Statistical comparisons for the survivorship data (response variable = percent larval survival) were computed by fitting a linear mixed-effects model to the data to test how larval survival was affected by exposure duration (within-subjects/repeated measure factor, 7 levels, fixed), temperature (between-subjects factor, 3 levels, fixed) and symbiotic status (between-subjects factor, 2 levels, fixed). A test of autocorrelation of the data (since samples were repeated on the same individuals through time) was included in the model testing. A two-way ANOVA (temperature, symbiotic status) was also performed for the final time point (t=72 h). An additional one-way ANOVA (temperature, 3 levels, fixed) was performed for the infection success data from the survivorship experiment (t=72 h). The statistical program R version 2.10.1 (http://cran.r-project.org) was used for all analyses. Percent data for the survivorship study were arcsine square root transformed to meet the assumptions of normality. All data were tested through graphical analyses and had acceptable normality and heteroscedasticity to meet the assumptions of ANOVA and the linear mixed-effects model. Post hoc analyses using Tukey's honestly significant difference (HSD) test were performed to determine which treatments differed when the ANOVA was significant at $p<0.05$. For the linear mixed-effects model, the function lme in the package nlme was used to test the model. The function glht in the package multcomp was used to perform post hoc Tukey HSD analysis of the linear mixed-effects model.

Results

Environmental Parameters

Parameters measured for each experiment, including the average temperatures and dissolved oxygen levels maintained, are summarized in Table 2.2. Dissolved oxygen levels ranged from 5.56 mg l^{-1} (88%) to 6.08 mg l^{-1} (92.8%) for larvae at 27°C and from 4.98 mg l^{-1} (81.3%) to 5.58 mg l^{-1} (86.2%) for larvae at 31°C (Table 2.2) for the 2009 experiment.

Infection success

2008 Experiment

Infection success, represented by percent infection and density of infection, was dramatically reduced in larvae incubated at 32°C compared to 27°C starting immediately post-infection in the preliminary experiment (Fig. 2.1a and b). Larvae in the control temperature maintained an average infection level of 67%, whereas larvae at the high temperature were never infected more than 7% (Fig. 2.1a). Larvae at 27°C had a density of infection that started around 13 dinoflagellates per larva, decreased slightly to 9 dinoflagellates per larva and leveled out at about 10 dinoflagellates per larva, while larvae at 32°C had very low overall densities throughout the experimental period (Fig. 2.1b). Since there were so few larvae infected at the high temperature, average density was calculated as the overall density in both infected and non-infected larvae for both treatments.

2009 Experiment

Trends in infection due to duration of exposure

Larvae at the control temperature maintained an average percent infection of 69% that did not change throughout the duration of the experiment (Fig. 2.2a). In contrast, larvae in the two higher temperatures exhibited a downward trend in infection through time and percent infection varied significantly with duration of exposure (Two-way ANOVA, $F_{4,45}=6.67$, $p<0.001$) (Fig. 2.2a). Larvae at the intermediate temperature

started out with percent infection similar to larvae at the control temperature, but their level declined progressively after t=5 h post-infection. These larvae had significantly lower percent infection at t=21.5 h (post hoc p=0.016) and t=36 h (post hoc p=0.006) than at t=5 h. Larvae incubated at 31°C began with a slightly lower percent infection of 60%, but this rapidly decreased to 52% by t=5 h, before dropping again and leveling off around 43% for the final three time points. Larvae incubated at high temperature had significantly lower infection success by t=21.5 and 36 h (post hoc p=0.033 and p=0.043 respectively) compared to t=0 h.

Trends in infection due to temperature

At all time points, except immediately following infection, exposure to high temperature resulted in a significantly decreased percent infection compared to control temperature (Fig. 2.2b). By 36 h, larvae exposed to the intermediate temperature also were significantly less infected than larvae at the control temperature (Two-way ANOVA, $F_{2,45}$=45.20, p<0.001). By t=5 h post-infection, the percent infection of larvae at 31°C was already significantly lower than that of larvae at 27° and 29°C (post hoc p=0.012 and p=0.014 respectively). Percent infection of larvae at 31°C remained significantly lower than larvae at 27°C at t=13, t=21.5, and t=36 h (all post hoc p≤0.001). By 36 h, the percent infection of larvae at 29°C was significantly lower than larvae at 27°C (post hoc p=0.020). Overall, it took 40 h of temperature exposure (t=36 h post-infection) before larvae at the intermediate temperature had an infection level that was lower than larvae at the control temperature, but only 9 h (t=5 h post-infection) before larvae at the high temperature had a lower infection level than larvae at 27°C.

Density of infection

The average number of dinoflagellates per larva was consistently higher in larvae exposed to control and intermediate temperatures compared with larvae at high temperature throughout the experimental period (Fig. 2.2c). In contrast to the 2008

density levels, all density averages for 2009 were determined from infected larvae only. The density of infection varied significantly with temperature (Two-way ANOVA, $F_{2,45}$=11.87, p<0.001) and duration of exposure ($F_{4,45}$ =3.65, p=0.012), however, post hoc tests revealed no significant comparisons of interest among temperatures within individual time points or within temperatures over time. Significant comparisons were found between larvae at 29°C at t=0 h and larvae at 31°C at each of the other time points.

Survivorship

Larval survival depended on duration of exposure as well as an interaction between exposure duration and temperature, according to the linear mixed model. Symbiotic status did not significantly impact survivorship. Thus, data from symbiotic and aposymbiotic treatments were combined for each temperature to better visualize these trends (Fig. 2.3a). Larvae in the control temperature had a high percent survival that did not decrease significantly over time, leveling off around 78%. Survival of larvae at 29°C remained slightly lower than controls over time following a slight decrease after t=24 h, and leveled off at 68% by 72 h. Larvae exposed to 31°C followed a similar trend as larvae in the other temperatures for the first 24 h (post infection) before their survivorship decreased precipitously, ending with 45% survivorship after 72 h. Survivorship of larvae exposed to 27°C was significantly higher than larvae exposed to 31°C at t=48 h [linear mixed model, t(108)=2.3035, p=0.023] and t=72 h [t(108)=2.6573, p=0.009]. A likelihood-ratio test indicated there was no statistical significance of autocorrelation, so an autocorrelation term was excluded from the final model.

There were some apparent differences in survivorship between symbiotic and aposymbiotic larvae by t=48 h post-infection, although the linear model did not detect a significant effect due to symbiotic status (Fig. 2.3b). Within each temperature, aposymbiotic *F. scutaria* larvae exhibited higher survivorship than symbiotic larvae

by t=48 h (Fig. 2.3b). By 72 h, there was a 7% difference in percent survival between aposymbiotic and symbiotic larvae maintained at 27°C; at 29°C, the difference was 5%; and at 31°C there was a 13% difference (Fig. 2.3b and c).

Temperature had a significant effect on percent survivorship at 72 h (Two-way ANOVA, $F_{2,18}$=18.0576, $p<0.001$) (Fig. 2.3c). Again, a significant effect on survivorship due to symbiotic status could not be detected, yet post hoc comparisons hinted at differences due to both temperature and symbiotic status (Fig. 2.3c). Symbiotic larvae at the high temperature had significantly lower survivorship than aposymbiotic larvae at 27°C (post hoc $p<0.001$), symbiotic larvae at 27°C (post hoc $p=0.003$), and aposymbiotic larvae at 29°C (post hoc $p=0.014$). This was in contrast to aposymbiotic larvae at 31°C, which only had significantly lower survivorship than aposymbiotic larvae at 27°C by this time point (post hoc $p=0.012$).

Infection success (percent infection) was also measured using larvae from the survivorship experiment (at t=72 h) and results indicated a significant difference in infection success among temperatures (One-way ANOVA, $F_{2,9}$= 7.23, p= 0.013). Larvae at 27°C had a 66% infection level, larvae at 28°C had a 57.5% infection level, and larvae at 31°C had a 42% infection level (data not shown). The 24% difference in infection success between larvae at 27°C and larvae at 31°C was significant (post hoc $p=0.011$). These levels mirrored the results of the infection success experiment (Fig. 2.2b), making comparisons between the two experiments possible.

Observations of larval behavior and settlement

Microscopic observations of larvae from the survivorship experiment revealed clear effects of elevated temperature on the development, appearance, and behavior of the larvae, as well as on their settlement patterns. At the start of the experiment, all larvae were approximately 100µm long, ciliated, barrel-shaped and swimming actively. Larvae in the control temperature followed a normal progression of developmental

stages through the course of the experiment from swimming actively to creeping along the substrate to settling (Schwarz et al. 1999). There was a difference in settlement between aposymbiotic and symbiotic larvae at ambient temperature. Most symbiotic larvae in control and intermediate temperatures had begun to settle by t=72 h post-infection (7 days post-fertilization), whereas fewer than half of the aposymbiotic larvae had settled at this point. *F. scutaria* larvae are known to settle in ambient temperatures starting on day 5 post-fertilization to approximately 2 weeks post-fertilization (Schwarz et al. 1999) and differences in settlement between larvae with and without symbionts has been observed previously (Schwarz et al. 1999).

After 76 h of exposure to elevated temperature, most of the larvae in the 31°C treatment were small and spherical or irregularly shaped and exhibited clear developmental abnormalities. Aposymbiotic and symbiotic larvae in the high temperature had similar abnormalities and patterns of behavior. All larvae exposed to 31°C exhibited increasingly limited movement as the experiment progressed, and by t=72 h, although they were resting on the bottom of the culture well, most had not settled. This observation was reinforced when larvae were pipetted onto the Sedgewick-Rafter Counting Cell for t=72 h survivorship photos as pipetting them from the culture well required little effort to remove them from the bottom of the well. This was in contrast to the gentle pressure required to remove settled larvae at control and intermediate temperature from their culture wells.

Discussion

This study focused on the ability of coral larvae to form a symbiotic relationship with *Symbiodinium* sp. when exposed to elevated temperature using *F. scutaria* as a model. The preliminary experiment showed dramatic differences in infection success between larvae at 27°C and 32°C starting immediately post-infection (Fig. 2.1a and b). It is possible that the inability of larvae to establish symbiosis could have resulted from low oxygen levels in treatment wells due to increased larval and dinoflagellate

respiration at high temperature (Edmunds 2005; Karako-Lampert et al. 2005). This possibility was taken into consideration for the subsequent experimental design. Dissolved oxygen levels never fell below 4.98 mg l^{-1} (81.3%) in the seawater of the 31°C treatments for the duration of the 2009 experiment (Table 2.2), indicating that oxygen was not depleted in the high temperature treatment due to increased metabolic rates of larvae.

The temperatures used in this investigation were chosen to reflect the local environment, since variation in normal summer temperature maxima that exists across regions leads to geographic disparity in coral responses to temperatures (Coles et al. 1976; Jokiel and Coles 1990). In Hawaii there has been a warming trend in SSTs over the past several decades (Jokiel and Coles 1990; Jokiel and Brown 2004) and bleaching events in 1996 and 2002 have been attributed to temperature anomalies exceeding +1°C (Jokiel and Brown 2004). In Kaneohe Bay, the average summer monthly temperature is 27 ± 1°C (Jokiel and Coles 1977), and it regularly reaches temperatures of 29°C during mid-day on reef flats (Krupp et al. 2006). As the temperature of Hawaiian waters rises, larvae of the corals that spawn during summer months in this region, including *F. scutaria* larvae, will be subject to temperatures similar to those examined here.

The potential for variation in larval response to temperature due to genotype was incorporated into the design of this study. Meyer et al. (2009) used aposymbiotic *Acropora millepora* larvae to examine the influence of genotype on host response to temperature and found between-family differences in several physiological traits including enzyme activity and changes in protein content. In the present investigation, a genetically heterogeneous population of larvae was used. By pooling larvae that originated from a range of parent corals collected from diverse locations in Kaneohe Bay, any between-family differences should not have influenced results.

Elevated temperature had a strong negative impact on symbiosis establishment, as *F. scutaria* larvae exposed to intermediate and high temperatures were significantly impaired in their ability to form a stable symbiosis. A modest 2°C increase in temperature for a day and a half significantly impacted symbiosis onset (Fig. 2.2a and b). Furthermore, a more dramatic 4°C increase above ambient for periods of only a few hours significantly reduced the ability of larvae to form a stable symbiotic association (Fig. 2.2a and b). The period of initial uptake of symbionts and the hours immediately following seem particularly sensitive for larvae exposed to elevated temperature, but the mechanisms involved in determining if the association will persist are unknown. The onset of symbiosis in corals likely involves a complex series of events including pre- and post-phagocytic host-symbiont recognition events, and cellular cross-talk between the partners (Fitt and Trench 1983; Chen et al. 2004; Rodriguez-Lanetty et al. 2006; Wood-Charlson et al. 2006; Dunn and Weis 2009). It is unclear how elevated temperature affects the ability of coral larvae to progress through these steps and form a stable association. It is possible that corals recognize and reject compromised (e.g. heat stressed) dinoflagellates during these early stages.

A proportion of larvae remained infected with the homologous symbiont type C1f at the highest temperature treatment even up to 72 h post-infection (Fig. 2.2). This result contrasts with those of previous studies of *F. scutaria* infected with heterologous types of *Symbiodinium* spp., which are completely eliminated from larvae at ambient temperature by 14 h post-infection (Rodriguez-Lanetty et al. 2006; Dunn and Weis 2009). This elimination may have been due, in part, to the activation of post-phagocytic apoptosis (Dunn and Weis 2009). There may be differences in the timing of cellular events leading to symbiont loss in response to infection with a heterologous symbiont compared to a heat stressed homologous symbiont.

Average density of infection for larvae in the control temperature in the 2009 experiment was relatively low (3-4 symbionts/larva) (Fig. 2.2c) compared with the

preliminary experiment (10-11 symbionts/larva) (Fig. 2.1b) and previous *F. scutaria* infection experiments with C1f (8-25 symbionts/larva) (Weis et al. 2001; Rodriguez-Lanetty et al. 2006). The difference in density among experiments was likely due to the number of *Symbiodinium* sp. available per larva during the infection, as seen in other cnidarian species (Montgomery and Kremer 1995). This number was decreased from 75 dinoflagellates per larva in the preliminary experiment to 26 dinoflagellates per larva in the 2009 experiment in an attempt to prevent a decrease in oxygen levels in the replicate wells due to increased *Symbiodinium* respiration at high temperature (Karako-Lampert et al. 2005). Correspondingly, the density of infection in larvae at control temperatures in the preliminary experiment was approximately 3 times higher than in the 2009 experiment (Fig. 2.1b vs. Fig. 2.2c). If more dinoflagellates were added during the infection period in 2009, it is possible that a higher density in controls and greater differences in density among temperatures may have resulted.

Elevated temperature had a clear impact on larval survival, regardless of symbiotic state (Fig. 2.3). Larval survivorship was significantly reduced in response to the high temperature within 2 d of exposure. In addition to impairing the ability to establish symbiosis and decreasing survivorship, microscopic observations of larvae indicated that exposure to high temperature resulted in numerous sub-lethal effects that impacted behavior, development, and settlement. These sub-lethal effects were similar to those found for larvae of other coral species (Randall and Szmant 2009a,b) and *F. scutaria* embryos (Krupp et al. 2006) and indicate that increased temperature impairs normal physiological processes and larval morphogenesis. It is highly unlikely that heat stressed larvae would survive to settlement and metamorphosis and grow into healthy adult corals.

Less clear was how the interaction between symbiotic state and temperature impacted larval survivorship. Aposymbiotic larvae at high temperature had a 13% higher survival rate than symbiotic larvae exposed to 31°C for 3 d (Fig. 2.3c). An even

greater difference in larval survival between these two groups might have occurred if symbiotic larvae exposed to high temperature had a higher initial infection level and density of infection. Only 42% of the larvae exposed to 31°C had symbionts after 3 d (data not shown) and of those larvae that were infected, density of symbionts in larvae was likely very low. Since fewer than half of the "symbiotic" larvae harbored symbionts by the end of the exposure duration, it is difficult to determine to what extent the presence of stressed symbionts affected larval survival. A 30% difference in survival between aposymbiotic and symbiotic *Acropora* larvae was detected by Yakovleva et al. (2009) after 3 d of exposure to 32°C. In that experiment, however, infections were carried out at ambient temperatures, resulting in initial infection levels of 100% and average density of 17.3 symbiont cells per larva. The higher infection level and density of infection obtained by that approach could help explain the greater difference in survival due to the interaction of symbiotic state and high temperature found in that study.

Whether or not acquiring symbionts at high temperature results in significantly decreased larval survivorship, therefore, remains an open question. Results presented here provide evidence that as coral larvae attempt to respond to elevated temperature, the extra burden of hosting endosymbionts may lead not only to collapse of the symbiosis, but also to increased mortality. The timing of symbiosis onset may be a crucial factor in the larval response to temperature. If the onset of symbiosis coincides with an increase in seawater temperature, larvae may have to contend with costs associated with forming symbiosis at a higher temperature that could include increased ROS production and oxidative damage, and decreased benefits such as less carbon being translocated from the symbiont to the host due to decreased photosynthesis rates (Iglesias-Prieto et al. 1992; Edmunds et al. 2001; Ferrier-Pages et al. 2007; Yakovleva et al. 2009). In reality, the cost of harboring intracellular symbionts at elevated temperature may be inconsequential if the larvae are unable to successfully establish the obligate symbiotic relationship in the first place.

Results from this study indicate that exposure to elevated temperatures during the initiation of symbiosis impairs the ability of coral larvae to maintain an endosymbiotic relationship with newly acquired symbionts, suggesting that the burden of hosting symbionts at high temperature can outweigh the benefits of coral-dinoflagellate symbiosis at this life stage. The cost of establishing symbiosis may prove too great if temperatures are elevated beyond the ability of larvae to cope with increased levels of oxidative stress or to proceed through normal cellular development. If SSTs continue to increase over the next several decades as predicted, the potential for coral larvae to form stable symbiotic relationships and survive to establish the next generation of corals may be greatly diminished.

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Figures and Tables

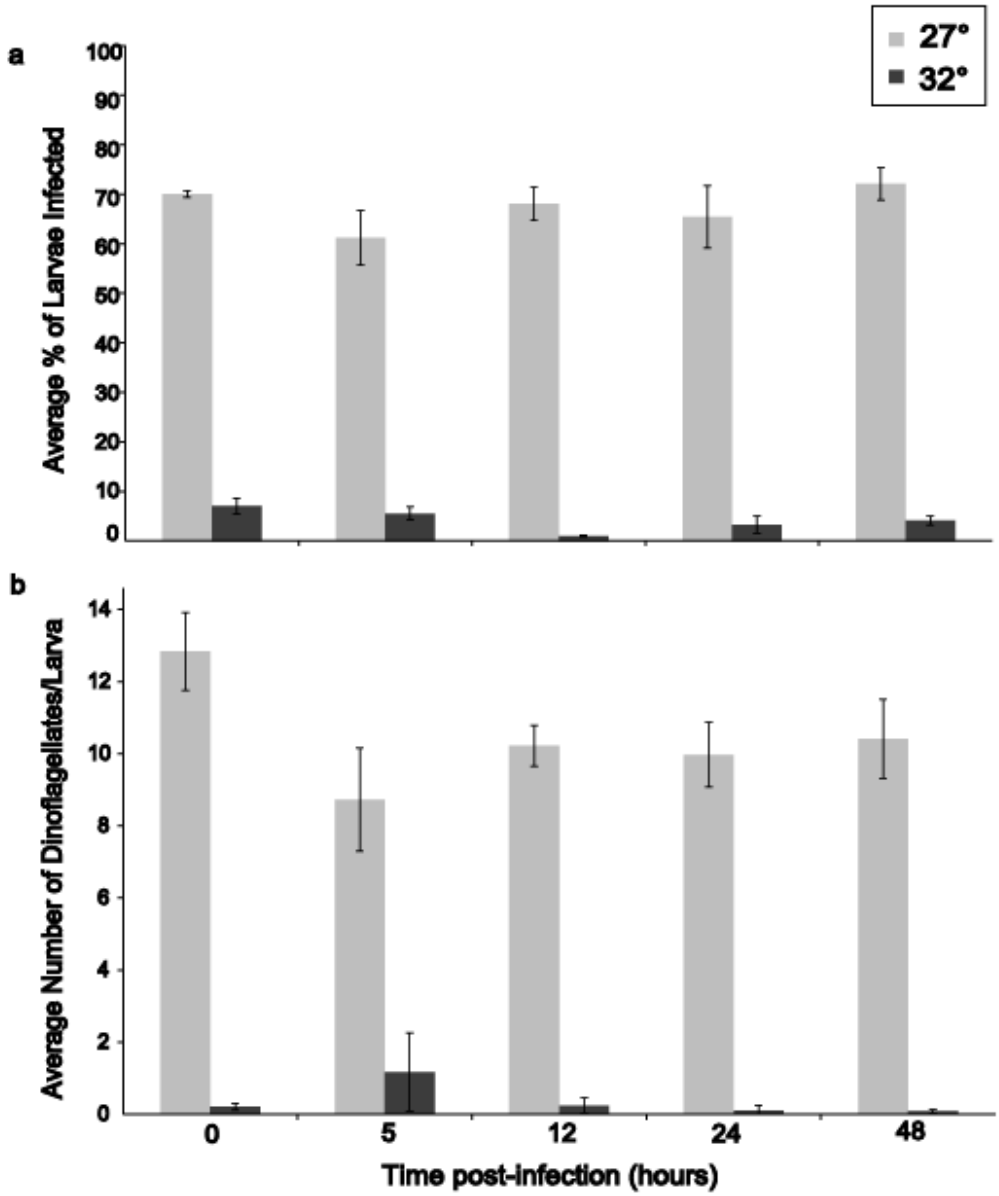

Figure 2.1. Infection success from a preliminary exposure of *Fungia scutaria* larvae to experimental temperatures for up to 52 h (48 h post-infection) in July 2008. (a) Average percent infection at two temperatures. Values are mean ± SE [n(dishes per temperature)=4, n(larvae per dish)=variable]. (b) Overall density at two temperatures. Values are mean ± SE [n(dishes per temperature)=4, n(larvae per dish)=variable].

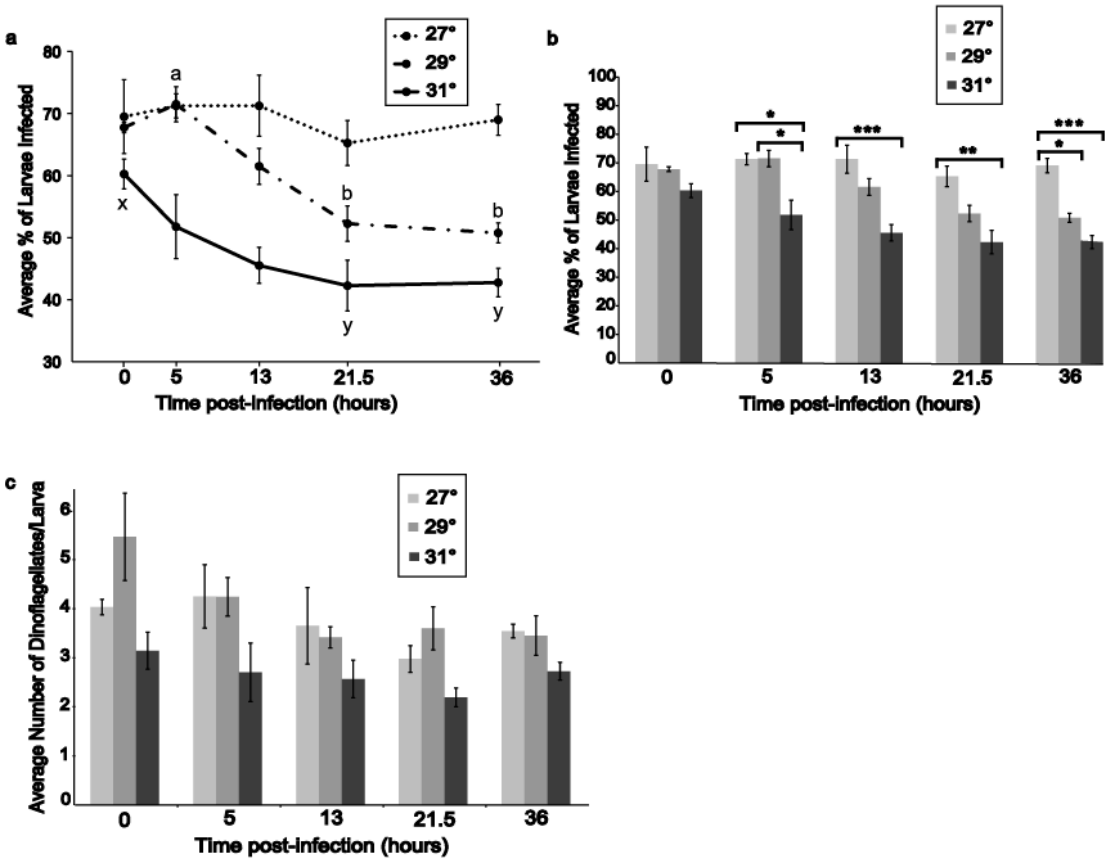

Figure 2.2. Infection success of *Fungia scutaria* larvae that were incubated at experimental temperatures for up to 40 h (36 h post-infection) in July 2009. (a) Trends in infection due to duration of exposure at three temperatures. Values are mean ± SE [n(dishes per temperature)=4, n(larvae per dish)=100]. The letters a, b and x, y denote a significant difference in percent infection within a temperature over time (Two-way ANOVA post-hoc $p<0.05$). (b) Trends in infection due to three different temperatures. Values are mean ± SE [n(dishes per temperature)=4, n(larvae per dish)=100]. Lines with asterisks denote significant comparisons among the three temperatures within a time point (Two-way ANOVA post-hoc *** $p<0.001$, ** $p<0.01$, * $p<0.05$). (c) Average density of dinoflagellates in larvae at three temperatures. Values are mean ± SE [n(dishes per temperature)=4, n(larvae per dish)=30].

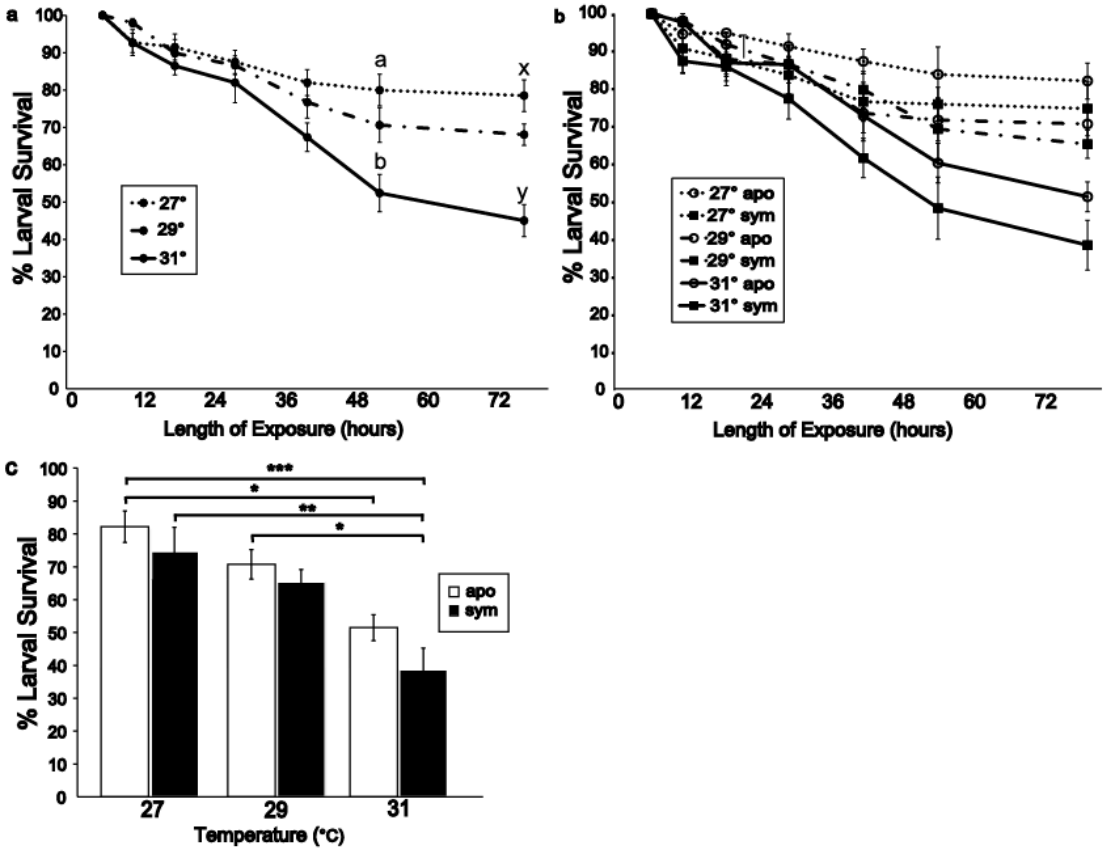

Figure 2.3. Survivorship of *Fungia scutaria* larvae incubated at three experimental temperatures for up to 76 h (72 h post-infection) in July 2009. (a) Percent survival of larvae at three temperatures through time (values for apo and sym larvae were combined). Values are mean ± SE [n(dishes per temperature)=4]. The letters a, b and x, y denote a significant difference in % survival between temperatures within a time point (linear mixed model, $p<0.05$). (b) Percent larval survival for aposymbotic (apo) and symbiotic (sym) larvae at three different temperatures through time. Values are mean ± SE [n(dishes per temperature)=4]. (c) Percent larval survival of apo and sym larvae at three temperatures at t=72 h post-infection. Values are mean ± SE [n(dishes per temperature)=4]. Lines with asterisks denote significant comparisons (Two-way ANOVA post-hoc *** $p<0.001$, ** $p<0.01$, * $p<0.05$).

Table 2.1. Summary of experimental design used to study the effects of elevated temperature on infection and survivorship of *Fungia scutaria* larvae. Rep.= replicate, temp.= temperature, avg.= average, exp.=experimental

Year	Response measured	Type of vessel; total volume per rep.	Number of reps/ temp.	Number of larvae measured/ rep.	Avg. density of larvae ml^{-1}	Avg. number of symbionts available per larva during infection period	Exp. duration (h)
2008	% Infection (Density)	6-well plate; 10ml	4	variable (variable)	200	75	48
2009	% Infection (Density)	Square dish; 21ml	4[a]	100 (30)	97	26	36
2009	Survivorship (% Infection)	24-well plate; 1 ml	4 sym, 4 apo	97 (30)	97	26	76

[a]Larvae were divided among 8 dishes per temperature, but 4 dishes were sampled at each time point

Table 2.2. Summary of environmental parameters measured throughout the 2008 and 2009 experiments. DO = dissolved oxygen.

Year	**2009**						**2008**	
Experimental temp. (°C)	27		29		31		27	32
Waterbath	A	B	A	B	A	B		
Mean	26.30	27.16	29.13	29.09	30.87	31.58	27.38	31.73
SD	0.40	0.11	0.33	0.15	0.53	0.41	0.31	0.68
Min.	25.53	26.78	27.86	28.16	30.15	30.56	26.49	30.25
Max.	27.18	27.47	29.55	29.55	32.29	32.29	27.96	33.12
DO (mg l^{-1}; %)								
Mean	ND	5.83; 90.2	ND	ND	5.33; 83.7	5.31; 84.2	ND	ND
SD	ND	0.22; 1.99	ND	ND	0.24; 1.92	0.24; 2.09	ND	ND
Min.	ND	5.56; 88.0	ND	ND	5.01; 81.0	4.98; 81.3	ND	ND
Max.	ND	6.08; 92.8	ND	ND	5.53; 85.3	5.58; 86.2	ND	ND

Temperatures were logged every 10 minutes by Onset HOBO UA-002 Temperature/Light Pendant data loggers placed in each temperature bath for the entire experimental duration for a total of 464 temperature readings per temperature bath. There were two replicate baths per temperature, designated A and B. In 2009, 27°C A was a water table, not a waterbath. DO levels were measured with a YSI 55 handheld meter at each sampling point. ND = no data.

Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis

Chapter 3: Coral larvae exhibit few transcriptional changes during the onset of coral-dinoflagellate symbiosis

Christine E. Schnitzler
Virginia M. Weis

To be submitted to:
Marine Genomics
Elsevier
Giorgio Bernardi
Stazione Zoologica Anton Dohrn,
Villa Comunale,
80121 Napoli, Italy

Abstract

The cellular mechanisms controlling the successful establishment of a stable mutualism between cnidarians and their dinoflagellate partners are largely unknown. The planula larva of the solitary Hawaiian scleractinian coral *Fungia scutaria* and its dinoflagellate symbiont *Symbiodinium* sp. type C1f represents an ideal model for studying the onset of cnidarian-dinoflagellate symbiosis due to the predictable availability of gametes, and the ability to raise non-symbiotic larvae and establish the symbiosis experimentally. The goal of this study was to identify genes differentially expressed in *F. scutaria* larvae during the initiation of symbiosis with its dinoflagellate symbiont using a high-throughput technique. Newly symbiotic larvae were compared to non-symbiotic larvae using a custom cDNA microarray. The 5,184-feature array was constructed with cDNA libraries from newly symbiotic and non-symbiotic larvae, including 3,072 features (60%) that were enriched for either state by subtracted hybridization. Our analyses revealed very few changes in the *F. scutaria* transcriptome as a result of infection with its homologous symbiont, similar to other studies focused on the early stages of this symbiotic interaction. We suggest that *Symbiodinium* sp. has evolved mechanisms to suppress or circumvent cnidarian host responses to infection that, in the case of non-homologous symbiont types, result in elimination. We discuss how these mechanisms may be similar to some of those seen in the invasion of animal cells by protozoan parasites.

Introduction

The mutualism between corals and photosynthetic dinoflagellates in the genus *Symbiodinium* represents a partnership of two eukaryotic organisms that first appeared at least 225 million years ago (Stanley 2003; Kiessling 2010). This symbiosis forms the structural and trophic basis of diverse coral reef ecosystems. The coral-dinoflagellate endosymbiotic relationship is a give-and-take partnership that includes an exchange of nutrients benefiting both host and symbiont and access to a protective and productive intracellular environment for the symbiont (Venn et al. 2008).

Typically, initial symbiont acquisition by larval or juvenile corals occurs via horizontal transmission from the environment (Fadlallah 1983; Harrison and Wallace 1990; Richmond and Hunter 1990).

Little is known about the molecular signaling pathways involved in recognition and in establishing specific, stable relationships between these two partners. It is unclear, for example, if corals utilize the same defense and immune pathways to respond to uptake of dinoflagellates that other organisms use to respond to pathogens or bacterial symbionts. Host responses to pathogens, subject to continuous diversifying selection pressure imposed by co-evolution (Woolhouse et al. 2002) can include a marked change in host transcriptome expression, including pathogen-controlled induction or suppression of host genes (Jenner and Young 2005). In mutualisms, there is often a complex series of processes involved in partner recognition and in establishment of the relationship - all are necessary, but none is individually sufficient to result in a stable partnership. Frequently, the host immune system is involved in the initiation, development and maintenance of the mutualism (Silver et al. 2007; Feldhaar and Gross 2008; Hooper 2009; McFall-Ngai et al. 2010).

A similar series of steps likely occurs during the onset of cnidarian-dinoflagellate symbioses (Fitt and Trench 1983). There is evidence that this process is initiated through lectin-glycan interactions, which act as pre-phagocytic recognition mechanisms (Lin et al. 2000; Koike et al. 2004; Wood-Charlson et al. 2006; Vidal-Dupiol et al. 2009). Functional genomic studies suggest that host corals possess innate immune pathways (Miller et al. 2007) that dinoflagellate symbionts may evade or modify during symbiosis onset (Schwarz 2008). There is also spatial and temporal evidence for pre- and post-phagocytic mechanisms during the uptake of symbionts (Rodriguez-Lanetty et al. 2006b). Other studies indicate that dinoflagellates may prevent the fusion of host phagosomes containing live symbionts to lysosomes by controlling the trafficking of the vesicles inside host cells (Fitt and Trench 1983; Chen

et al. 2003; Chen et al. 2004; Chen et al. 2005). Host cell apoptosis has been reported as a post-phagocytic mechanism that removes heterologous symbionts (Dunn and Weis 2009). It remains to be seen exactly how all of these pieces fit together during the initiation of cnidarian-dinoflagellate mutualisms and how the cascade of events, most of which are yet to be discovered, proceeds from initial contact through symbiosis establishment.

In the pre-genomic/pre-proteomic era, the search for genes and proteins involved in cnidarian-dinoflagellate symbiosis typically involved discovery-based screening of a small number of potential targets at a time. The first study that isolated symbiosis-specific molecules in this system resulted in protein profiles that showed differential production of a number of proteins between aposymbiotic and symbiotic adults of the anemone *Anthopleura elegantissima* (Weis and Levine 1996). This led to the characterization of two molecules differentially produced by symbiotic anemones: carbonic anhydrase, an enzyme known to transport inorganic carbon to dinoflagellates for photosynthesis (Weis and Reynolds 1999; Moya et al. 2008); and sym32, a protein that is homologous to the fasciclin I family of cell adhesion proteins that may function in cell signaling (Reynolds et al. 2000; Schwarz and Weis 2003). More recently, Rodriguez-Lanetty et al. (2006a) used the same model system to examine adult anemone gene expression profiles, but scaled up by using hybridization to 10,368-feature cDNA microarray. This study resulted in 79 host unigenes with significantly different expression as a function of the symbiosis, 28 of which had similarity to genes in publicly available databases. The functions of the known genes fell in several broad categories including cell growth and maintenance, metabolism, cell signaling and transport.

Investigations of the transcriptome of cnidarians have begun to increase in number recently. A growing number of studies of cnidarians have utilized custom cDNA microarrays and various hybridization techniques to identify genes differentially

expressed during different life stages or upon exposure to various conditions (Desalvo et al. 2008; Grasso et al. 2008; Richier et al. 2008; Reyes-Bermudez et al. 2009; Rodriguez-Lanetty et al. 2009; Voolstra et al. 2009a). Comparative EST analyses have revealed potential genes involved in established symbiosis (Kuo et al. 2004; Sabourault et al. 2009). There has also been one whole transcriptome sequencing analysis of aposymbiotic coral larvae undergoing a range of conditions including thermal stress and settlement induction (Meyer et al. 2009).

Transcriptomic and proteomic studies relating directly to the initial onset of cnidarian-dinoflagellate symbiosis are fewer in number. deBoer et al. (2007) examined changes in both the transcriptome and the proteome of larvae from the Hawaiian solitary coral *Fungia scutaria* during the onset of symbiosis. Two-dimensional PAGE was used to resolve proteins from aposymbiotic and symbiotic larvae, but surprisingly few differences were found; only one spot was consistently differentially expressed in symbiotic larvae and its identity was not obtained. Dot blots were used in membrane hybridization with either a symbiosis-enriched or an aposymbiosis-enriched PCR probe. Fifty-one of 176 spots exhibited at least two-fold higher expression with the symbiosis-enriched probe than the aposymbiosis-enriched probe. ESTs corresponding to six of these spots were sequenced, although bioinformatic searches found significant homology to a known sequence for only one - a hypothetical protein from the parasitic flatworm, *Schistosoma japonicum* (SJCHGC09657 protein). Authors of another proteomic analysis of aposymbiotic and newly symbiotic juveniles of a Red Sea soft coral, *Heteroxenia fuscescens,* also found few differences between the two states (Barneah et al. 2006). Protein profiles from symbiotic and aposymbiotic polyps were obtained and compared each week for six weeks in that study.

Only one other study has used array hybridization to examine expression differences between aposymbiotic and newly symbiotic coral larvae (Voolstra et al. 2009b). Larvae of *Acropora palmata* and *Montastraea faveolata* were infected with either a

homologous or heterologous symbiont type and samples were taken 30 min and 6 d post-infection. Very few changes were seen in either coral's transcriptome upon infection with the homologous symbiont type at both time points. In contrast, dramatic differences were found in both species at 6 d post-infection with a non-homologous symbiont type (Voolstra et al. 2009b). Infection dynamics were not quantified in that study, however, and relatively little is known about the onset of symbiosis in either of the coral species examined.

We chose to examine host transcriptional profiles during the initiation of symbiosis to identify differentially regulated host genes using the *F. scutaria*-*Symbiodinium* sp. C1f model system. This system has been used as a model for studying the onset of coral-dinoflagellate symbiosis for more than a decade and methods for precisely quantifying infection success in this system have been established (Schwarz et al. 1999; Weis et al. 2001; Rodriguez-Lanetty et al. 2006b; Wood-Charlson et al. 2006). In Hawaii, *F. scutaria* harbors a single homologous symbiont type (LaJeunesse et al. 2004; Rodriguez-Lanetty et al. 2004) and the system is amenable to manipulation due to the predictable availability of gametes (Krupp 1983), and the ability to raise non-symbiotic larvae and establish the symbiosis experimentally. We focused on changes in expression levels in the larval coral transcriptome 48 h after the initial onset of symbiosis with its homologous symbiont. We created a 5,184-feature array that included transcripts from both aposymbiotic- and symbiotic-subtracted libraries and employed a multiple dye-swap design to directly compare expression patterns between populations of larvae with and without symbionts. Very few changes in expression were found as a result of homologous infection. Our results suggest that homologous *Symbiodinium* sp. types have evolved mechanisms to modify coral host responses to symbiont colonization to promote their tolerance and avoid elimination.

Materials and Methods

Collection and maintenance of coral larvae

Adult *F. scutaria* corals were collected over several years from patch reefs in Kaneohe Bay (Oahu, Hawaii) and maintained year-round in seawater tables at the Hawaii Institute of Marine Biology, University of Hawaii's marine laboratory. The corals broadcast spawn gametes without symbionts between 17:00 and 19:00 h, 2-4 d following the full moon in the summer months (Krupp 1983). Samples for array hybridization were collected from the July 2006 spawning event. To ensure proper biological replication, eggs from six individual females (mothers) were kept in separate 3 l bowls containing 1.5 l of 0.22 μm filter-sterilized seawater (FSW). Sperm from multiple males was mixed prior to fertilizing each bowl. Bowls were placed in outdoor tables with a continuous flow of seawater at ambient temperature (27°C). Larvae were cleaned daily with 0.22 μm FSW and checked for the development of a mouth using a compound microscope. Four days post-fertilization, each bowl of larvae was concentrated on a 50 μm mesh filter, placed into a smaller (800 ml) bowl and split into two equal bowls of 400 ml each and labeled according to the identification of the mother coral and the treatment they would receive (symbiotic or aposymbiotic).

Preparation of dinoflagellate symbionts

Since *Symbiodinium* sp. C1f has not yet been cultured, freshly isolated dinoflagellates were prepared by using an oral hygiene device (Water pik) to remove symbiotic host tissue from an adult *F. scutaria* coral skeleton. The tissue was transferred to a glass homogenizer and the mixture of host tissue and dinoflagellate cells was separated by homogenization and centrifugation at 6000 x g for 3 minutes. Homogenization of the resulting dinoflagellate pellet in FSW was repeated several times until host tissue debris was absent. The isolated dinoflagellate cells were checked for host tissue debris and quantified using a hemocytometer. Cleaned dinoflagellates were used within 2 h of preparation.

Infection of larvae with symbionts

Larvae in the symbiotic treatment bowls were inoculated with 20 ml of 1.55×10^6 cells

ml^{-1} of freshly isolated dinoflagellates mixed with homogenized and filtered *Artemia* sp. to induce feeding behavior in the larvae (Schwarz et al. 1999). Larvae in the aposymbiotic treatment bowls were provided with only the *Artemia* sp. preparation. After an infection period of 3 h, larvae were cleaned to remove remaining dinoflagellates. Washing involved concentrating larvae onto a 50 µm mesh filter, rinsing them with FSW, and transferring them back into 3 l bowls. Once the washing was complete, this was considered to be time = 0 h post-infection.

Sampling and determination of infection success

At t=0 h, 1 ml of each sample was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS: 2 mM NaH_2PO_4, 7.7 mM Na_2HPO_4, 0.14 M NaCl) and stored at 4°C for use in determining infection success. Forty-eight h after the infection period ended (t=48 h), all six replicate samples (representing thousands of larvae) for each treatment were taken. Larvae in each sample were concentrated into a 200 ml volume. One ml of each sample was fixed and stored as described above for use in determining infection success. The remaining larvae in each sample were spun for 1 min at 1000 x g, flash frozen in liquid nitrogen, stored at -80°C, shipped to Oregon State University (OSU) on dry ice and stored at -80°C until RNA isolation.

Fixed samples were rinsed 3 times in 1X PBS before examination by light microscopy. Infection success was determined by calculating the number of infected larvae per 100 larvae and average density of dinoflagellates in 100 infected larvae (Rodriguez-Lanetty et al. 2004). Statistical comparisons among samples for the infection success data (response variables = percent infection success and density) were computed using two-way analysis of variance (ANOVA) with time and sample as factors. All data were tested through graphical analyses and had acceptable normality and heteroscedasticity to meet the assumptions of ANOVA. The statistical program R version 2.10.1 was used for all analyses.

Microarray construction

Unsequenced, or anonymous, cDNA microarrays were constructed with transcripts from cDNA libraries from symbiotic and aposymbiotic *F. scutaria* larvae, including transcripts that were enriched for either state by suppression subtractive hybridization (SSH). The process of SSH removes sequences common to two samples and enhances rare transcripts that are specific to one sample or the other (Diatchenko et al. 1996; Diatchenko et al. 1999; Lukyanov et al. 2007). The larvae that provided the material for the cDNA libraries were sampled during the August 1998 *F. scutaria* spawning event (deBoer et al. 2007). Samples were taken at 5 d post-fertilization (48 h post-infection) and all samples had infection rates >78% at 24 h post-infection (deBoer et al. 2007). These libraries were constructed with a PCR-Select cDNA subtraction kit (Clontech) (deBoer et al. 2007). Separate libraries were also created using non-subtracted material from both symbiotic and aposymbiotic larvae sampled in August 1998.

Each library was ligated into either the pCR2.1 vector (Invitrogen) or the pGEM-T Easy vector (Promega), transformed into Max Efficiency DH5α cells (Invitrogen), and plated on LB plates. Multiple clones were picked from each library and grown in LB overnight in 384-well plates. The cDNA inserts were PCR amplified in 96-well plates using M13 forward and reverse vector primers. Random clones were sequenced to check the redundancy of each library (see "Sequencing and gene identity" below for methods).

All PCR products were dried using a vacuum dessicator and resuspended in 15µl printing buffer (3X SSC, 1.5M betaine). After resuspension, samples were transferred to 384-well (Genetix) plates using a Rapidplate robot (Qiagen). These samples were used to print the 5,184 feature array, which included 1,536 random clones from each of the subtracted cDNA libraries and 768 random clones from each of the non-subtracted cDNA libraries. Commercial alien DNAs (Lucidea Universal ScoreCard,

Amersham Biosciences/GE Healthcare and SpotReport Alien cDNA Array Validation System, Stratagene) were spotted throughout the array for use in setting scanning parameters after hybridization. An 18x18 design of 16 blocks was used and all features were randomized among all blocks. Arrays were printed on Corning ULTRAGaps coated slides using a BioRobotics Microgrid II robot (Genomics Solutions) at OSU's Center for Genome Research and Biocomputing (CGRB). Slides were held in a vacuum desiccator for 24 h after printing and then UV cross-linked at 300 mJ before being stored in a desiccator until hybridization. Test slides were subjected to quality control by hybridizing a Cy3 random 9-mer on spotted cDNA.

RNA isolation and hybridization of arrays

To construct probes, total RNA was isolated from larval samples collected in July 2006 using Trizol (Invitrogen) according to the manufacturer's instruction with modification. Contamination of total RNA with *Symbiodinium* RNA in symbiotic samples is expected to be low since the average number of dinoflagellate cells in each infected larva was low (20±6) compared to the total number of host cells in 6 day old larvae (see ahead to Results) and microscopic inspection of homogenized tissue revealed intact *Symbiodinium* cells. Protocol modification included the addition of the optional centrifugation step at 12,000 x g for 10 minutes at 4°C to remove polysaccharides and other contaminants. Since it has been reported that RNA isolated from frozen tissue using Trizol may be refractory to cDNA synthesis, and additional purification using glass fiber filter columns restores its ability to be reverse transcribed (Dumur et al. 2004), samples were subsequently loaded into an RNeasy column (Qiagen) and washed as recommended by the manufacturer's instructions. RNA was eluted with RNAse-free water and stored at -80°C until use.

Total RNA purity and concentration was determined with a Nanodrop 1000 (Thermo Fisher Scientific). RNA integrity was assessed by running an aliquot of each preparation on a non-denaturing 1% agarose gel and by running 1 µl of each RNA

prep in the RNA 6000 Nano LabChip on the 2100 Bioanalyzer (Agilent) following the manufacturer's protocol. The RNA Integrity Number (RIN, range 0-10), provided by the Agilent 2100 Expert Software, is a measure of RNA integrity determined using the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products. Only intact RNA with a RIN above 8.0 was used to synthesize cDNA for hybridization.

cDNA synthesis was performed from 1 µg total RNA using Superscript II Reverse Transcriptase (Invitrogen) and the Genisphere 3DNA-900 microarray kit, according to the manufacturers' instructions. One µl of each alien RNA was added to each cDNA synthesis reaction. Slides for hybridization were chosen randomly from the batch of high quality printed arrays. The slides were pre-hybridized for one hour at 50°C with 5 µg sheared salmon sperm DNA (Gibco-BRL) to reduce background. Following pre-hybridization washes, the slides were spun dry and pre-warmed before the experimental cDNAs were added and left to hybridize overnight at 50°C. Following post-hybridization washes, Cy3 and Cy5 capture reagents were hybridized to the array for 4 h at 50°C. All hybridizations took place in a formamide based hybridization buffer under LifterSlips in a sealed humidified chamber. Arrays were scanned with an Axon GenePix Pro 4200A dual-color confocal laser scanner and photomultiplier tube (PMT) settings were manually balanced based on alien features. Image acquisition, quality control, and signal quantitation were performed using GenePix Pro 5 software (Axon Instruments).

Experimental design and statistical analysis of microarray data

A multiple dye-swap experimental design was used with six biological replicates for each condition (aposymbiotic or symbiotic) for a total of 12 microarrays. All analyses were performed on GenePix result files using functions in the limma library of the Bioconductor package (Dudoit 2003; Gentleman et al. 2004) within the R v 2.8.0 programming environment. Pre-processing included background correction via the

normexp method (Ritchie et al. 2007), print-tip loess normalization within arrays (Smyth and Speed 2003), and quantile normalization between arrays (Yang and Paquet 2005). Following normalization, a linear model incorporating the dye-swap was fitted to the log-ratio data (Smyth 2005) to estimate differences in transcript expression between aposymbiotic and newly symbiotic larvae. All control and alien spots were removed from the analysis before fitting the linear model. Fold difference was calculated from the estimated log ratio. Moderated t-statistics (Smyth 2004), log-odds ratios of differential expression [based on empirical Bayesian shrinkage of the standard errors towards a common value (Lonnstedt and Speed 2002)], and adjusted p-values [obtained using Benjamini and Hochberg's false discovery rate (FDR) at $p < 0.05$ to account for type I errors associated with multiple testing] were computed using functions in the limma library of Bioconductor (Dudoit 2003).

Sequencing and gene identity

PCR products generated from cDNA inserts isolated from clones were sequenced from both directions with sequencing primers specific to either the pGEM-T Easy or pCR2.1 vector depending on the library of origin. Vector and low quality sequences were removed and sequence fragments were assembled into contigs using Sequencher v 4.7 (GeneCodes Corp). BLASTx searches of the NCBI non-redundant protein database were performed for all processed sequences (contigs and singletons) and hits with Expect values (E values) of less than 1×10^{-4} were considered to be homologs to the query sequence. For the differentially expressed sequences, BLASTn searches of the non-human, non-mouse EST (est_others) database were performed. Any hits with significant E values were then used as a query in a BLASTx search. For each gene, the hit with the lowest E value to a known gene is reported. Gene Ontology (GO) classifications were assigned to all sequenced clones via BLAST searches through AmiGO (Carbon et al. 2009) and UniProtKB.

Results and discussion

Details of the Fungia cDNA array

The size of cDNA inserts from all libraries varied between 0.2 and 2.0 Kb. The average length of the symbiotic subtracted library (302 bp) was shorter than the aposymbiotic subtracted library (594 bp). Sequencing random clones revealed that within 81 symbiotic clones (subtracted and unsubtracted libraries), the redundancy was 8.6%; within 85 aposymbiotic clones (subtracted and unsubtracted libraries), the redundancy was 22%; and within all 166 clones, the redundancy was 15.6%.

Homology searches revealed that the genes on the array fall in several distinct functional categories involved in many cellular processes including metabolism, protein biosynthesis, protein transport, cell signaling, cell cycle, and cell growth and maintenance, and that several had top hits to proteins of undetermined function (Table 3.1). Not surprisingly, most of the genes had their top hit or a significant hit ($E < 10^{-4}$) to a gene from the anthozoan cnidarian *Nematostella vectensis*, the closest taxon to *F. scutaria* with a complete genome sequence (Table 3.1). Seventy-eight of the 166 clones (47%) did not have a significant BLASTx hit and were considered unknown sequences (discussed below).

Infection success of larvae for hybridization

Larvae from all replicate bowls had high infection levels that did not change significantly through time starting immediately post-infection (average 87.3 ± 6.2%) and ending 48 h later (average 83.7 ± 8.9%) (Two-way ANOVA, $p>0.05$) (Fig. 3.1a). Percent infection levels did not vary significantly among replicates (Two-way ANOVA, $p>0.05$) confirming their comparable symbiotic status. Similarly, the density of infection did not vary significantly from t=0 h (average 24.9 ± 5.7 symbionts per larva) to t=48 h (average 20.3 ± 6.3 symbionts per larva) (Two-way ANOVA, $p>0.05$) (Fig. 3.1b). Density also did not vary significantly among samples (Two-way ANOVA, $p>0.05$). The density level was comparable with other infection studies

using this system, where density has ranged from 8-25 dinoflagellates per larva (Weis et al. 2001; Rodriguez-Lanetty et al. 2006b).

Differentially expressed genes

Forty-two features were significantly differentially expressed at Benjamini and Hochberg's adjusted p-value cutoff of $p<0.05$. This represents 1.37% of the 3072 features from the aposymbiotic and symbiotic subtracted *F. scutaria* libraries. This percentage is similar to the *A. elegantissima* array that compared gene expression between aposymbiotic and symbiotic adult anemones and found 1.82% features were differentially expressed (Rodriguez-Lanetty et al. 2006a). The (log)fold change ranged from 0.75-2.41 (Fig. 3.2a), which also resembled results from the *A. elegantissima* array, where most expression ratios ranged from 1-2 (Rodriguez-Lanetty et al. 2006a). Thirty-three spots were upregulated as a function of the symbiosis and 9 spots were downregulated (Fig. 3.2a). After DNA sequencing and sequence analyses, the 42 features resolved into 4 contigs and 13 singletons for a total of 17 unigenes. From these 17 identified host unigenes, only 3 had a significant BLASTx hit ($E < 1.0 \times 10^{-4}$) with homologs to known genes (Table 3.2, Fig. 3.2b). Ten had a significant BLASTn hit to the EST database (Table 3.3). The top EST sequence was then used as a query in a BLASTx search and 4 of the 10 searches resulted in a significant hit with homologs to known genes. One returned hits to genes of bacterial origin and was discarded. Finally, 3 of the identified unigenes had no significant hit in any database.

Unknown sequences

We were unable to identify homologs for many of the genes we sequenced from the array. In many cases, when homologs were identified, they were hypothetical or predicted proteins with undetermined function (see all Tables). Nearly half of the sequences from the redundancy sequencing and over half (10/17) from the expression study (Fig. 3.2b) did not return any significant hit that could be identified with a homolog. These percentages are comparable to similar studies, which have not found

significant BLAST hits in publicly available databases for a large proportion of sequences, ranging from 55-65% (Kuo et al. 2004; Rodriguez-Lanetty et al. 2006a; Richier et al. 2008; Sabourault et al. 2009). As annotated sequence data from more whole genome and large EST projects become available, this number of unknown sequences will decrease, but a proportion of unknowns will continue to persist. Cnidarians *Nematostella*, *Hydra*, *Clytia* and *Acropora* have all individually retained a proportion of ancestral genes that are not present in other cnidarians (Forêt et al. 2010). These taxonomically restricted genes (TRGs) have been linked to new traits in *Hydra* (Khalturin et al. 2009). It is possible that some of the unknown sequences differentially expressed during the onset of symbiosis in *F. scutaria* may be TRGs with, cnidarian-, coral-, or species-specific functions. Future studies may reveal the function of some of the unknown genes identified with the *F. scutaria* array. An alternative possibility is that some or all of the unknown sequences cross coding region boundaries and represent 3′ UTR regions of cDNA that often result from 3′ EST library construction (Aaronson et al. 1996). These regions would not return a hit from a BLASTx search as they are not part of the coding sequence.

Genes affected by symbiosis onset

Only 17 unigenes were identified as differentially expressed as a function of the onset of symbiosis with a homologous symbiont. Of these, only a few sequences had significant hits to known sequences in public databases (Tables 3.2 and 3.3). Identified genes from the BLASTx searches were in one of three functional categories (Table 3.2). Beta tubulin was the only identified gene that was downregulated as a function of the symbiosis (0.79 fold). Beta tubulins, together with alpha tubulins, are the proteins that make up microtubules, structural components that form the cytoskeleton of cells (Westermann and Weber 2003). Microtubules are involved in many cellular processes including mitosis, cytokinesis and vesicular transport (Desai and Mitchison 2003). Rodriguez-Lanetty et al. (2006a) also found that genes involved in this category, including beta tubulin, were downregulated as a function of the established symbiotic

state. Conversely, Voolstra et al. (2009b) found that beta tubulin was upregulated in coral larvae as a result of infection with a heterologous symbiont. Due to their large size (~10 µm diameter), *Symbiodinium* cells take up most of the cytoplasmic space inside host cells (Wakefield et al. 2000), leaving little room for intracellular movement, such as the transport of organelles. It is possible that in symbiotic cells, cytoskeletal assembly and cell cycle function is inhibited by the physical presence of dinoflagellate endosymbionts.

Ribosomal protein L14, one of the proteins forming the 60S large ribosomal subunit, was upregulated at the onset of symbiosis (0.82 fold). Ribosomal proteins are involved in protein biosynthesis (Maguire and Zimmermann 2001), specifically in promoting the folding and stabilization of ribosomal RNA. Ribosomal protein L14 belongs to the L14E family of ribosomal proteins. It contains a basic region-leucine zipper (bZIP)-like domain. bZIP domains are found in a family of eukaryotic transcription factors that bind to sequence-specific double-stranded DNA to either activate or repress gene transcription (Hurst 1995). 60S ribosomal protein L26 was strongly upregulated in the *A. elegantissima* array as a function of symbiosis (Rodriguez-Lanetty et al. 2006a) and three ribosomal proteins, including L26, were downregulated in the same array when anemones were subjected to temperature stress (Richier et al. 2008). The differential expression of these cnidarian ribosomal proteins under various conditions suggest that protein synthesis is regulated under normal, unperturbed conditions of symbiosis but disrupted during times of stress.

A homolog to a hypothetical *N. vectensis* protein (NEMVEDRAFT_v1g245639, 681 aa) with unknown function was also upregulated at the onset of symbiosis. Several features from the array had top hits to this protein (Tables 3.2 and 3.3). One feature (APO4J16) resulted in a significant BLASTx hit. A contig of four sequences resulted in a significant BLASTn hit to a non-overlapping downstream portion of the same protein. All features were significantly upregulated as a function of symbiosis onset

(average 1.33 fold). Further analysis of this hypothetical protein, including domain prediction using PfamA (http://pfam.sanger.ac.uk/), ScanProsite (http://www.expasy.ch/tools/scanprosite/), InterProScan http://www.ebi.ac.uk/Tools/InterProScan/) and a CD-Search of NCBI's conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) resulted in no conserved domains. We also used GenThreader and pGenThreader to perform sequence profile based fold recognition through the PSIPRED Structure Prediction Server (http://bioinf4.cs.ucl.ac.uk:3000/psipred/), but these methods returned no significant results.

BLASTn searches of the EST database, followed by BLASTx searches using the top EST hit resulted in two additional gene identifications. The first, a hypothetical protein from *N. vectensis* (NEMVEDRAFT_v1g242270, 552 aa) with undetermined function, was upregulated as a function of symbiosis (average 1.08 fold). Domain searches of this sequence resulted in a predicted bZIP transcription factor motif (63 aa in length) located at the C-terminus of the hypothetical protein. GenThreader returned no significant hits, but pGenThreader (which identifies more distant homologs) returned three significant hits ($p \leq 0.005$), all to the bZIP domains of different proteins, including two CCAAT/enhancer-binding proteins (C/EBPα and C/EBPβ), which function to regulate genes involved in immune and inflammatory responses (Akira et al. 1990; Ramji and Foka 2002) and a cyclic-AMP-dependent transcription factor (ATF-2) that is activated by stress-activated protein kinases, such as p38, via TGFβ and BMP stimulation (Sano et al. 1999).

The second gene identification was the SJCHGC09657 protein from *S. japonicum* (72 aa) that was identified by a previous study of *F. scutaria* larvae (deBoer et al. 2007). This gene was downregulated (0.96 fold) on the array. Domain searches and fold recognition methods returned no significant hits for this short amino acid sequence.

The host transcriptome is largely unaltered during symbiosis onset

Very few transcripts were differentially expressed between aposymbiotic and newly symbiotic *F. scutaria* larvae. There are several potential reasons for this result. First, the signal from cells and larvae not undergoing symbiosis in the symbiotic treatment larvae may have diluted any transcriptional signal from symbiotic cells. Each replicate sample consisted of thousands of individual larvae and each bowl inoculated with *Symbiodinium* C1f had an average infection level of 83.7 ± 8.9%. This indicates that most, but not all, larvae in the symbiotic treatment were infected and there was a sub-population of larvae in each replicate that harbored no symbionts at the time of sampling. These uninfected larvae could dilute the signal from the infected larvae. Similarly, whole larvae were sampled and each larva had an average of 20.3 ± 6.3 symbiont cells per larva at the time of sampling. Symbiosis-related transcripts should be highest in the gastrodermal cells that host symbionts. The thousands of host cells that were sampled but contained no symbionts may diminish any signal from the symbiotic host cells.

This explanation, however, is not supported by a previous study that examined two species of coral larvae exposed to different symbiont types (Voolstra et al. 2009b). Although the authors of that study did not quantify infection success for their system, they did confirm that larvae exposed to a homologous symbiont type ("symbiosis-competent" larvae) were successfully infected over the course of the experiment using fluorescence microscopy. They found that larvae infected with a homologous symbiont type had very few transcriptional changes compared to aposymbiotic larvae, while larvae infected with a heterologous symbiont type ("symbiosis-incompetent" larvae) had differential expression in hundreds of transcripts by 6 d following infection. At that point, the larvae infected with a heterologous symbiont type harbored no symbiont cells but the effect of recognizing and eliminating the "incompetent" *Symbiodinium* type on their transcriptome was strongly evident.

A second explanation is that symbiosis-related transcripts could be expressed transiently through time. Our sampling point of 48 h post-infection may miss the window of when transcripts related to symbiosis establishment are highly expressed. Again, this explanation is not supported by the results of Voolstra et al. (2009b). They sampled 30 min and 6 d post-infection and found very few differences in expression at either time point between aposymbiotic larvae and larvae infected with a homologous symbiont. In contrast, many differences were found in larvae infected with a heterologous symbiont type at the 6 d time point. The sampling point in our study falls between the two time points used by Voolstra et al. (2009b), so that taken together, the results suggest that starting from the first hour through several days following infection with a homologous symbiont, very few coral genes are up- or down-regulated in response. It is possible that some genes are differentially expressed between the 30 min and 48 h post-infection sampling points. It is also possible that highly expressed "housekeeping" genes may be overrepresented on the array and genes expressed at relatively low levels may be underrepresented.

A third hypothesis for the lack of change in gene expression by host larvae in the early stages of symbiosis establishment is that when homologous symbionts are phagocytosed by gastrodermal cells, they avoid recognition and evade rejection by the host (Voolstra et al. 2009b). Symbionts may accomplish this by actively circumventing, modifying or suppressing host responses. Alternately, symbionts might avoid detection, and preclude changes to the host transcriptome, by masking their presence somehow. Examples of these manipulations of the host can be seen in other symbiotic systems, including the invasion of animal cells by parasites (Schwarz 2008).

Many microbes that are phagocytosed by host cells employ a variety of mechanisms for either circumventing or overcoming the maturation of the phagosome into an acidic phagolysosome, and thus avoid proteolytic cleavage and degradation. *Mycobacterium tuberculosis*, for example, accomplishes this, in part, by interfering

with Rab GTPases and associated proteins that mediate vesicular trafficking (Chua et al. 2004). This symbiont-controlled arrest of phagosome maturation is the presumed mechanism by which *Symbiodinium* resides inside the "symbiosome" of host cnidarian cells without being digested (Fitt and Trench 1983). Studies of the symbiotic anemone *Aiptasia pulchella* have provided evidence for a similar symbiont manipulation of host Rab proteins (Chen et al. 2003; Chen et al. 2004; Chen et al. 2005). There is no evidence from either of these systems that the manipulation of host molecules occurs at the transcriptional level. In cells invaded by *Mycobacterium*, one specific host target is phosphatidylinositol 3-phosphate (PI3P), which is important in phagolysosome biogenesis. Two parasite-derived products that interfere with PI3P have been identified as key manipulators of host phagosome maturation. A lipid (LAM) blocks the generation of PI3P, and a phosphatase (SapM) hydrolyzes any PI3P that is generated (Deretic et al. 2006). It is possible that similar post-transcriptional mechanisms preventing phagosome maturation are occurring inside cnidarian cells hosting dinoflagellate symbionts. Such mechanisms would not be detected in a transcriptional study.

Apicomplexan parasites, a sister taxon to dinoflagellates within the Alveolata (Adl et al. 2005), employ a different set of mechanisms to manipulate the host cells that they invade. *Toxoplasma gondii* generates its own vacuolar membrane to avoid host detection and prevent fusion with host lysosomes (Blader and Saeij 2009) and inhibits host tumor necrosis factor-alpha (TNF-α) transcription, and thus proinflammatory cytokine production (Leng et al. 2009). *Plasmodium falciparum*, another apicomplexan parasite, promotes tolerance of its presence by affecting several innate immune pathways of its host (D'Ombrain et al. 2007; Maier et al. 2009). While dinoflagellate and apicomplexan lineages diverged many hundreds of millions of years ago (Berney and Pawlowski 2006), it is possible that they developed similar mechanisms to invade their hosts, and prevent elimination by suppressing or disrupting host innate immunity (Schwarz 2008).

Conclusions

Results presented here, combined with previous investigations (Barneah et al. 2006; deBoer et al. 2007; Voolstra et al. 2009b) indicate that the transcriptome and the proteome of cnidarians undergoing early stages of symbiosis establishment is largely unaltered. As is often the case with parasitic invasion of eukaryotic hosts, cnidarian responses to colonization by dinoflagellate symbionts may be kept in check by homologous symbionts, and this manipulation may occur via suppression of host innate immune response genes, post-translational modifications to host proteins, protein-protein interactions, or epigenetic modifications, none of which would be detected by a cDNA microarray or with two-dimensional PAGE. The fact that Voolstra et al. (2009b) found marked differences in the transcriptome of larvae that were challenged with non-homologous symbionts lends additional support for these ideas. Another recent study found that host coral transcriptional profiles were linked more to *Symbiodinium* sp. types than exposure to experimental treatments (DeSalvo et al. 2010), further illustrating the extent to which symbionts influence host transcription. Future studies comparing host coral responses to both homologous and non-homologous symbionts and investigations exploring potential mechanisms of symbiont-mediated host cell manipulation will provide further understanding of the cellular mechanisms underlying the establishment and maintenance of this important mutualism.

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Figures and Tables

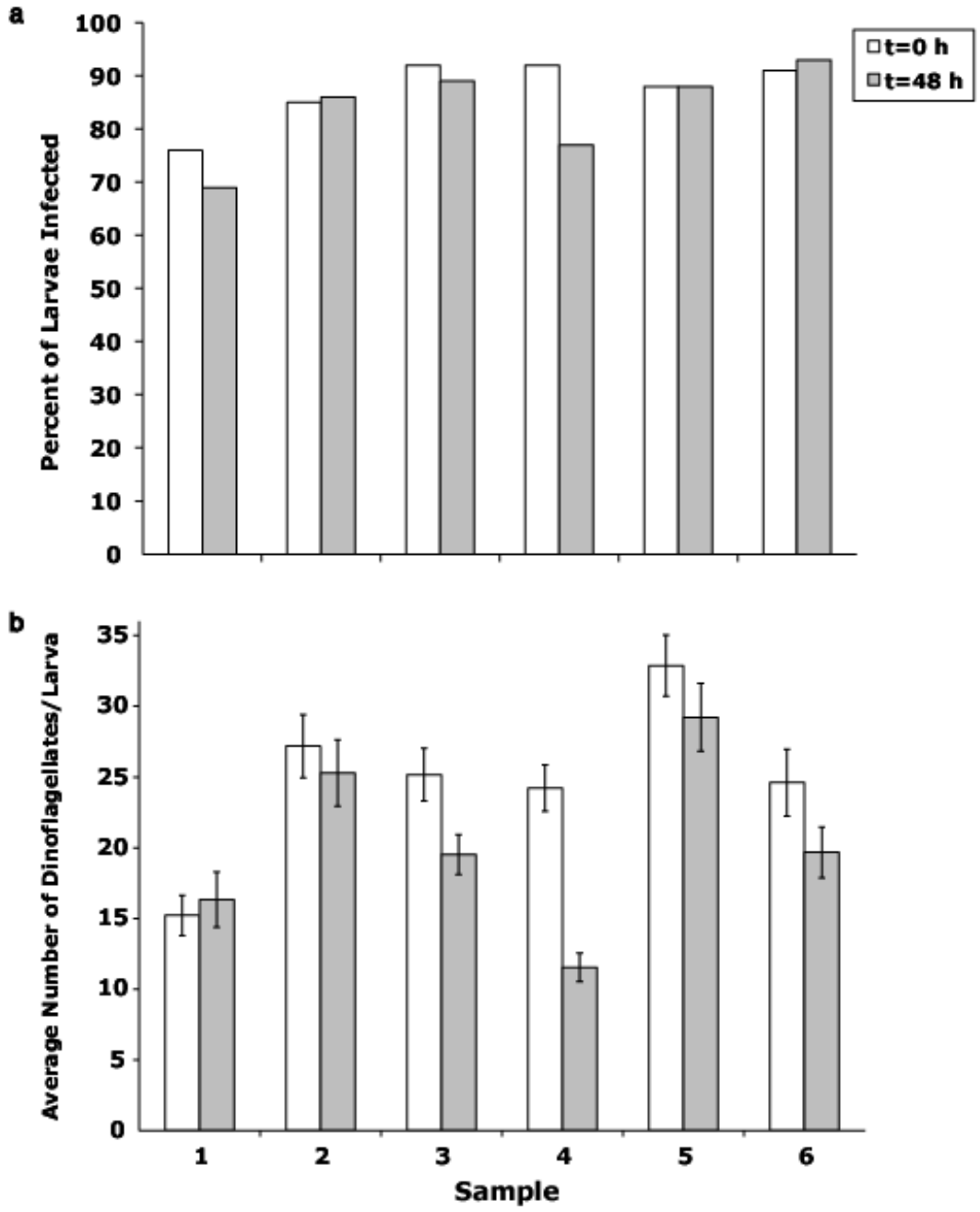

Figure 3.1. Infection success by *Symbiodinium* sp. C1f in *Fungia scutaria* larvae. (a) Percent of larvae infected (n [larvae per sample]=100), (b) Density of dinoflagellates in larvae. Bars represent means ± SE (n [infected larvae per sample] = 100).

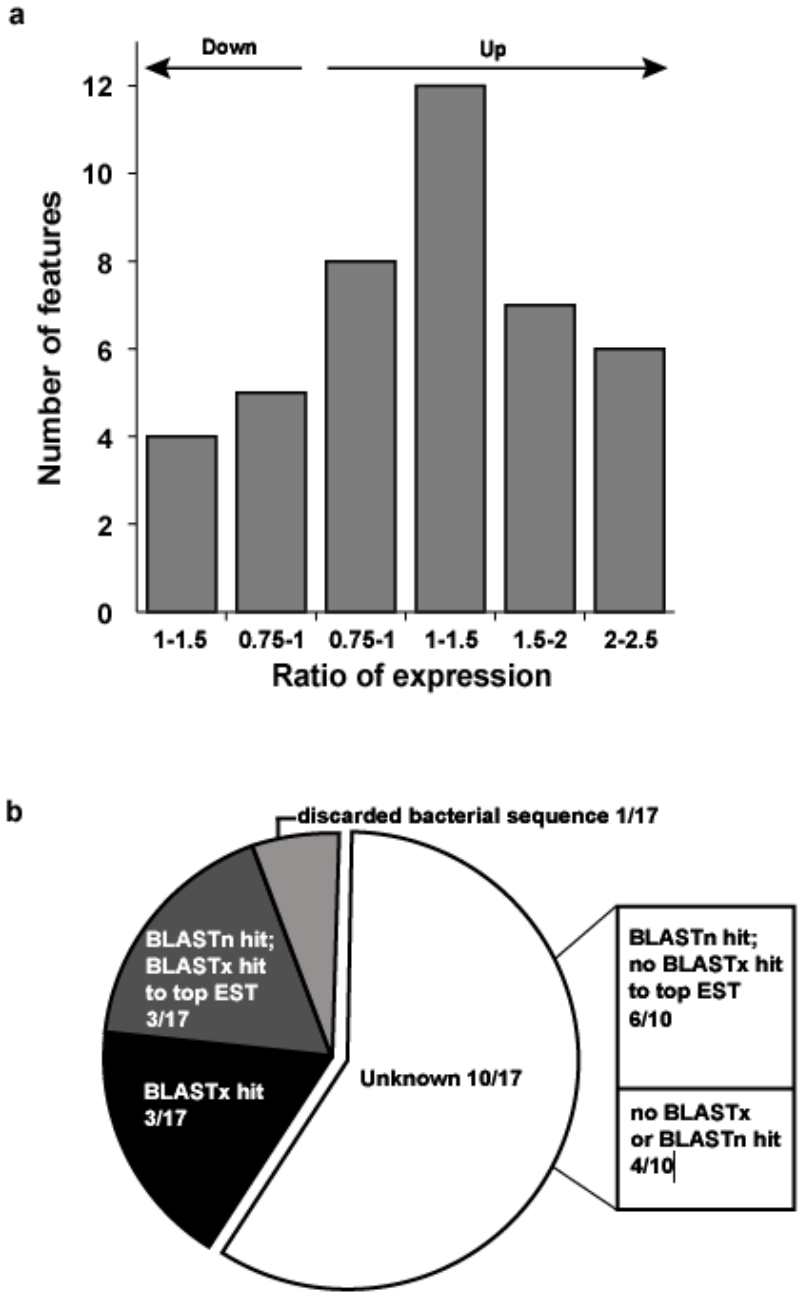

Figure 3.2. Fold changes of differentially expressed features found in coral larvae as a function of symbiosis onset and distribution of BLAST hits from resulting unigenes. (a) Distribution of the fold changes of the 42 differentially expressed features. Categories are fold changes of expression of newly symbiotic larvae compared to aposymbotic larvae. Arrows indicate down- and up-regulated features. (b) Distribution of BLAST results of the 17 identified unigenes that were differentially expressed.

Table 3.1. Functional annotation of redundancy check sequences with significant hits (E<1.00E-04) using categories from GO and UniProtKB. ** denotes sequences whose top hit was to *Nematostella vectensis*. * denotes sequences that had a significant hit to a protein from *N. vectensis*, but not the top hit.

Functional category	OSU_CGRB ID	E values	Accession # of homologs
Metabolic process			
S-adenosylmethionine synthetase	APO1A1; APO2J1	1.00E-78	XP_001629913**
S-adenosyl-L-homocysteine hydrolase	APO3F1; APO3M1	3.00E-80	XP_001639319**
Cytosolic malate dehydrogenase	APO3K1	7.00E-08	XP_796283*
GCN5-related N-acetyltransferase (GNAT)	APO3I1	2.00E-05	XP_001628360**
Cholesterol and lipid metabolism			
Vigilin/High density lipoprotein binding protein	UNSYM2I1	9.00E-47	XP_002610887*
Nucleic acid metabolism			
Histone H3.2	APO2H1	4.00E-61	NP_835734*
Protein metabolism			
Ribosomal protein L10	SYM2C1	4.00E-43	ABO26700
Ribosomal protein L18/L5e	APO2B1	1.00E-20	XP_001634531**
Ribosomal protein S8e	UNSYM1E1	3.00E-82	XP_001622696**
Ribosomal protein S27e	APO2F1	4.00E-20	XP_001633500**
Translation initiation factor eIF-5A	APO4C1	2.00E-07	NP_001080536*
Translation elongation factor EFTu/EF1A, domain 2	APO4D1	2.00E-61	XP_001631027**
Elongation factor 1 alpha	UNSYM2J1	2.00E-67	AAZ30676

Table 3.1. (Continued)

Functional category	OSU_CGRB ID	E values	Accession # of homologs
Purine salvage			
Adenosine kinase	UNAPO1H1	6.00E-48	XP_001635774**
RNA binding			
Polyadenylate-binding protein 1	APO4P1	2.00E-38	XP_001625306**
RNA-dependent DNA replication			
Endonuclease/reverse transcriptase	SYM3A1	2.00E-05	ABR01162
Pol-like protein	SYM4A1	3.00E-09	BAC82623*
Cell cycle			
Putative senescence-associated protein	APO3A1;UNAPO1J1; UNAPO1N1;APO2N1; UNAPO2H1	4.00E-43	BAB33421*
Cell growth and maintenance			
Beta tubulin	APO4O1	2.00E-35	XP_969993*
Cell growth regulation			
Granulin protein	APO3C1	2.00E-55	XP_002110228*
Cell signaling			
Guanine nucleotide binding protein (GTPase)	APO4N1; APO4K1	2.00E-34	XP_001631784**

Table 3.1. (Continued)

Functional category	OSU_CGRB ID	E values	Accession # of homologs
Cytoskeleton structure and reorganization			
Actin	UNSYM1H1	7.00E-72	XP_001637076**
Intracellular protein transport			
Coatomer protein	APO4E1	2.00E-30	XP_001631811**
Tpr (translocated promoter region) protein	UNAPO2B1	5.00E-05	XP_784812
Transport/transmembrane transport			
Mitochondrial substrate carrier protein	APO2P1	1.00E-51	XP_001627436**
Proteolysis and peptidolysis			
Peptidase M16	APO2C1	5.00E-21	XP_001632139**
Immune response			
Complement component C3 precursor	APO3G1	2.00E-25	BAH22724**
Oxidation/reduction			
Cytochrome P450-like protein	APO3B1; APO2K1; APO1D1; UNAPO2E1; APO2O1; APO3P1; UNAPO2C1; APO1J1l SYM2P1; UNSYM1I1	4.00E-31	BAA10929*

Table 3.1. (Continued)

Functional category	OSU_CGRB ID	E values	Accession # of homologs
Undetermined			
Hypothetical protein (*Pan troglodytes*)	SYM1G1	1.00E-07	XP_001135501
Hypothetical protein (*Rattus norvegicus*)	UNAPO2D1	9.00E-05	XP_001054782
NEMVEDRAFT_v1g155763	UNAPO1I1	3.00E-22	XP_001618098**
NEMVEDRAFT_v1g157418	UNAPO1D1; UNSYM2B1	2.00E-23	XP_001617522**
NEMVEDRAFT_v1g248425	APO3O1	1.00E-05	XP_001622460**
NEMVEDRAFT_v1g133140	SYM2O1	2.00E-04	XP_001624580**
NEMVEDRAFT_v1g247499	UNAPO2A1	7.00E-16	XP_001624711**
NEMVEDRAFT_v1g217095	SYM3G1	6.00E-11	XP_001625221**
NEMVEDRAFT_v1g245535	APO4G1	1.00E-35	XP_001628457**
NEMVEDRAFT_v1g114138	UNSYM1A1	2.00E-43	XP_001630346**
NEMVEDRAFT_v1g142320	APO1O1	6.00E-09	XP_001622138**
NEMVEDRAFT_v1g211211	UNAPO1B1	7.00E-96	XP_001629792**
NEMVEDRAFT_v1g95124	UNAPO1K1	2.00E-22	XP_001636478**
Predicted protein (*Trichoplax adhaerens*)	APO1B1; APO4J1	8.00E-23	XP_002118239*
SJCHGC09657 protein (*Schistosoma japonicum*)	SYM4B1	5.00E-05	AAX30965.2
Unknown protein (*Zea mays*)	APO1N1	2.00E-17	CAN31742*

Table 3.2. Functional annotation of differentially expressed sequences with significant BLASTx hits (E<1.00E-04) using categories from GO and UniProtKB. ** denotes sequences whose top hit was to *Nematostella vectensis*. * denotes sequences that had a significant hit to a protein from *N. vectensis* but not the top hit.

Functional category	OSU_CGRB ID	Fold change	Accession # of homologs
Cell growth and maintenance			
Beta tubulin	APO3O23	↓ 0.79	NP_954525.1*
Protein metabolism			
Ribosomal protein L14	SYM2A23	↑ 0.82	XP_001631282**
Undetermined			
NEMVEDRAFT_v1g245639	APO4J16	↑ 1.09	XP_001628265**

Table 3.3. Annotation of differentially expressed sequences with significant BLASTn hits to the est_others (non-human, non-mouse EST) database (E<1.00E-04). The top BLASTn hit was used as a query in a BLASTx search. Any significant results from the BLASTx search are reported below each BLASTn result.

Species of top hit from BLAST search (protein name)	OSU_CGRB ID	Fold Change	Accession # of homologs
BLASTn: *Acropora palmata*	APO2G22; APO2H10;	↑ 1.79; ↑ 1.37;	GW214694
BLASTx: *Nematostella vectensis*	APO1E16; APO2E18	↑ 1.34; ↑ 1.04	
(NEMVEDRAFT_v1g245639)			XP_001628265
BLASTn: *Montastraea faveolata*	UNSYM1M12;	↑ 1.30;	GW264218
BLASTx: *N. vectensis*	APO4L11; APO4G21	↑ 1.04; ↑ 0.91	
(NEMVEDRAFT_v1g242270)			XP_001634377
BLASTn: *Schistosoma japonicum*	SYM2A3	↓ 0.96	BU779826
BLASTx: *S. japonicum*			
(SJCHGC09657 protein)			AAX30965.2
BLASTn: *A. palmata*	APO1G4; APO2E19;	↑ 2.41; ↑ 2.26;	GW214695
	APO1I17; APO3P22;	↑ 2.17; ↑ 2.15;	
	APO3L7; APO3M9;	↑ 2.13; ↑ 1.95;	
	APO3O19; APO4E23;	↑ 1.93; ↑ 1.85;	
	APO4F9; APO3K19;	↑ 1.78; ↑ 1.65;	
	APO3K8; APO2H12;	↑ 1.60; ↑ 1.38;	
	APO1K6; APO4I10;	↑ 1.30; ↑ 1.04;	
	APO2H23; APO1I16	↑ 0.84; ↑ 0.83;	
BLASTn: *Hydra magnipapillata*	APO2C17; APO2B7	↓ 1.41; ↓ 1.32	CX832194

Table 3.3. (Continued)

Species of top hit from BLAST search	OSU_CGRB ID	Fold Change	Accession # of homologs
BLASTn: *M. faveolata*	APO3N17	↓ 1.20	GW272230
BLASTn: *M. faveolata*	APO2G23	↑ 0.84	GW267181
BLASTn: *M. faveolata*	APO1F14	↑ 0.82	GW264217
BLASTn: *N. vectensis*	SYM2N7	↓ 0.77	FC298242

Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis

Chapter 4: Diversity of the TRAF immune gene family in cnidarians

Christine E. Schnitzler
Virginia M. Weis

To be submitted to:

Genome Biology

BioMed Central Ltd.

Floor 6, 236 Gray's Inn Road

London WC1X 8HL

United Kingdom

Abstract

Tumor necrosis factor receptor associated factors (TRAFs) are key signal transducers in the tumor necrosis factor receptor (TNFR) and Interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) innate immune pathways of mammals (which have 7 TRAFs) but nothing is known about TRAFs in basal metazoans. Whole genome sequence assemblies have recently been published for cnidarians *Nematostella vectensis* and *Hydra magnipapillata*, allowing us to explore the TRAF superfamily in the common ancestor to the Bilateria. We identified 16 putative TRAFs (at least 15 loci) in the *N. vectensis* genome, including at least one in each of the five major mammalian TRAF lineages. We found 14 putative TRAFs (at least 12 loci) in the *H. magnipapillata* genome, although these only fell in one of the five mammalian TRAF lineages, suggesting that *H. magnipapillata* has undergone substantial gene loss or sequence divergence within this family. Phylogenetic analyses identified 8 major lineages of TRAFs, including 3 new subfamilies, each containing at least one cnidarian TRAF sequence, indicating that the TRAF gene family was fully diversified prior to the divergence between cnidarians and bilaterians. We also cloned and sequenced novel full-length TRAF6-like genes from two symbiotic cnidarians, the anemone *Aiptasia pallida* and the coral *Fungia scutaria*. This study provides the groundwork for future functional studies that can examine the role of TRAFs in cnidarian innate immunity, and the possible role of TRAF6 in regulating cnidarian-dinoflagellate mutualisms.

Introduction

Tumor necrosis factor (TNF) receptor associated factors (TRAFs) are critical signaling mediators for the TNF receptor (TNFR) superfamily, as well as the Interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily (referred to as TLR henceforth) and the T-cell receptors (Chung et al. 2002). TRAFs are known to play important roles in the cellular biology of animals including during embryonic development, in regulating the innate and adaptive immune systems and in maintaining cellular homeostasis (Chung et al. 2002). Humans and other mammals contain seven TRAFs

(hTRAF1 to 7), while model invertebrates have many fewer TRAFs; the fruit fly (*Drosophila melanogaster*) has 3 TRAF orthologs (dTRAF1 to 3) (Liu et al. 1999; Grech et al. 2000) and the nematode (*Caenorhabditis elegans*) has a single TRAF (ceTRAF) (Wajant et al. 1998).

TRAF proteins have multiple characteristic domains. Most TRAFs have an N-terminal RING finger domain (RINGd). RING domains are one of the top 10 most promiscuous domains found among animal, fungi and plant proteins (Basu et al. 2008). In TRAF proteins, the RINGd mediates protein-protein interactions (Wajant et al. 2001). Most TRAFs have multiple TRAF-type zinc finger domains in the middle of the protein that are important for downstream signaling (Wajant et al. 2001). Finally, most TRAFs contain a C-terminal meprin and TRAF-C homology (MATH) domain, also known as a TRAF domain (TRAFd). The TRAFd of TRAF family proteins consists of an N-terminal coiled-coil portion and a C-terminal TRAF-C portion that mediates self-association and upstream signaling (Rothe et al. 1994). TRAF domains are somewhat widespread as they occur in at least 8 protein families (Zapata et al. 2007). The only mammalian TRAF without a TRAFd is TRAF7, which has a WD40 repeat domain in place of the TRAFd, where the repeating WD40 units serve as a scaffold for protein interactions (Xu et al. 2004).

Most TRAFs exhibit E3 ubiquitin (Ub) ligase activity through their RINGd. Only TRAF1 is missing the RINGd (Wajant et al. 2001). TRAF E3 Ub ligase activity leads to the synthesis of lysine 63 (K63)-linked polyUb chains on target signaling proteins, including themselves (Pineda et al. 2007). In contrast to K48-linked polyUb chains, which mark substrate proteins for proteasomal degradation, K63-linked Ub chains facilitate various non-degradative biological processes, such as protein trafficking and signal transduction, including providing scaffolds to which downstream signals are recruited (Pineda et al. 2007).

Deubiquitinating enzymes (DUBs) such as A20 and the tumor suppressor cylindromatosis (CYLD) counteract TRAF E3 Ub ligase activity by dismantling the polyUb chains formed on target proteins (Simonson et al. 2007; Shembade et al. 2010). DUBs are cysteine proteases classified by their deubiquitinase domain, which can be in one of five subclasses (Amerik and Hochstrasser 2004). A20 contains both an N-terminal deubiquitinase domain and seven zinc-finger domains with E3 Ub ligase activity (Opipari et al. 1990). A20 is thought to function as a dual ubiquitin-editing enzyme, alternating between adding polyUb chains and removing them from target proteins (Heyninck and Beyaert 2005). In contrast to A20, whose expression is inducible, CYLD is constitutively active and may function as a general downregulator of K63 ubiquitination events; however, CYLD activity can be inhibited via phosphorylation (Sun 2009).

TRAF6 is a unique member of the TRAF family as it is the only TRAF that participates in TLR signaling, as well as TNFR signaling (Chung et al. 2007; Inoue et al. 2007). TNFRs and TLRs transduce signals leading to the activation of transcription factors of the nuclear factor- κB (NF-κB) and activator protein-1 (AP-1) families by the initial formation of upstream protein complexes that include TRAFs (O'Neill and Dinarello 2000). NF-κB activation leads to the transcription of over 150 target genes, most of which are involved in the immune response, including inflammatory cytokines such as TNF-α (Pahl 1999). TRAF6 binds to different sites on TNFRs like CD40 and receptor activator of nuclear factor κB (RANK) compared to other TRAFs, and is the only TRAF that interacts with serine/threonine interleukin-1 receptor-associated kinases **(**IRAKs) (Wajant et al. 2001). As a consequence of these differences, loss of TRAF6 function cannot be compensated for by other TRAFs (Lomaga et al. 1999). A recent study presented evidence that the ubiquitination of TRAF6 is dispensable for its signaling function, by demonstrating that a lysine-deficient TRAF6 mutant can transduce IL-1 and RANK ligand (RANKL) signals (Walsh et al. 2008). Other studies have found that the RINGd of TRAF6 is critical for signaling initiated by IL-1, LPS

and RANKL (Lamothe et al. 2008), implying that the ubiquitination by TRAF6 of certain targets is required for downstream signaling.

The full diversity of TRAF sequences in cnidarians has not been explored yet, most likely because draft genomes for the sea anemone *Nematostella vectensis* (Putnam et al. 2007) and the freshwater hydroid *Hydra magnipapillata* (Chapman et al. 2010) have only recently become available. There is genomic evidence for the presence of nearly all basic components of the TLR signaling pathway, including TRAF6, in *N. vectensis*, *H. magnipapillata*, and in the coral *Acropora millepora*, for which a large EST dataset is available (Miller et al. 2007). While much is known about the number of TRAFs and the many cellular roles that they play in mammals, zebrafish (Kedinger et al. 2005) and even *Drosophila* (Grech et al. 2000; Wajant and Scheurich 2004), their identification and potential roles in the immune system of basal chordates such as amphioxus (Huang et al. 2008; Yuan et al. 2009) (which possesses an expanded number of at least 24 putative TRAFs), and higher invertebrates such as the sea urchin *Strongylocentrotus purpuratus* (Hibino et al. 2006; Robertson et al. 2006), the sea scallop *Chlamys farreri* (Qiu et al. 2009), and the squid *Euprymna scolopes* (Goodson et al. 2005) are only beginning to be investigated. In basal metazoans such as placazoans (*Trichoplax adhearens*), sponges (*Suberites domuncula*), and cnidarians (*N. vectensis* and *H. magnipapillata*), TRAFs are only starting to be identified and virtually nothing is known of the specific functional roles TRAF orthologs play. There is no evidence for TRAF family orthologs in prokaryotes, or within non-metazoan eukaryotes, including fungi, plants, alveolates, and euglenoids (Zapata et al. 2007).

We searched the genomes of *N. vectensis* and *H. magnipapillata* for the presence of TRAF homologs to gain insight into the evolution and diversity of this protein superfamily in early metazoan evolution. Prior to this study, TRAFs 1, 2 and 5 had only been identified in vertebrate genomes (Zapata et al. 2007). It has been suggested

that a much larger representation of TRAF sequences from early metazoans would be necessary to obtain a clearer picture of the phylogeny of TRAFs (Zapata et al. 2007).

Our interest in the innate immune system of cnidarians and its potential role in regulating cnidarian-dinoflagellate mutualisms (Schwarz 2008; Weis 2008) led us to target TRAF6 as a signaling molecule central to the TLR signaling pathway. Although no functional studies have examined the role of this pathway in cnidarians, two recent microarray studies have found differential expression of CYLD, a negative regulator of TRAF6, in corals. CYLD was one of the most upregulated genes in a study of the coral *Montastrea faveolata* exposed to heat stress (Desalvo et al. 2008). The heat stress experiments were designed to simulate conditions leading to coral bleaching, or symbiosis collapse (Douglas 2003). CYLD expression was also upregulated in larvae of the coral *Acropora palmata* shortly after infection with their homologous symbiont (Voolstra et al. 2009).

Here, we describe two TRAF6 homologs that we cloned and sequenced from model symbiotic cnidarians *Aiptasia pallida* and *Fungia scutaria*. We used bioinformatic and phylogenetic approaches to analyze and place these sequences in context with well-characterized TRAFs and with the diverse complement of cnidarian TRAF orthologs discovered during our bioinformatic searches. Our discovery and analysis of TRAF6 orthologs from *A. pallida* and *F. scutaria* will pave the way for functional studies that explore the role of this gene in the innate immune system of cnidarians and potentially in the regulation of cnidarian-dinoflagellate mutualisms, which form the basis of diverse coral reef ecosystems.

Materials and Methods

Database searching for TRAFs

Non-cnidarian TRAFs

Vertebrate, urochordate, cephalochordate, sea urchin, mollusc, arthropod, nematode, and sponge TRAF gene sequences used in multiple sequence alignments and phylogenetic tree construction were obtained from NCBI's GenBank database (Table 4.1). Placazoan sequences were obtained using tBLASTn searches of the draft *T. adhaerens* genome assembly (http://genome.jgi-psf.org/Triad1/Triad1.home.html) available from the Joint Genome Institute (JGI) using human TRAF sequences as queries. Meprin sequences were obtained from GenBank for outgroup analyses.

Cnidarian TRAFs

For the hydrozoan *Hydractinia echinata*, the HyTRAF1 sequence was obtained from GenBank. The HyTRAF1a sequence (a splice variant of HyTRAF1) is not available in GenBank, so the sequence was taken from Mali and Frank (2004). Dr. Uri Frank provided the unpublished HyTRAF2 sequence (personal communication).

TRAF sequences from *H. magnipapillata* were first identified through tBLASTn searches of COMPAGEN (http://www.compagen.org), a repository for EST and whole genome assemblies from several basal metazoans, using human TRAFs as queries. Putative TRAFs from *N. vectensis* were first identified through tBLASTn searches of the draft genome assembly of *N. vectensis* version 1.0 (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). Resulting *N. vectensis* and *H. magnipapillata* sequences were BLASTx searched against GenBank. Details of the genomic structure of full-length *N. vectensis* TRAFs, including their scaffold location, genomic length and the number of exons predicted to encode each protein, were also obtained from the draft *N. vectensis* genome assembly available through JGI. Scaffolds are portions of the genome sequence that are reconstructed from end-sequenced whole-genome shotgun clones. The *N. vectensis* draft JGI genome assembly is the result of approximately 7.8X sequence coverage and the JGI assembly has 10,804 scaffolds with a total length of 356 Mbp. Approximately half of the genome is contained in 181 scaffolds, all at least 473 Kb in length.

Putative TRAFs from *A. pallida* were identified through tBLASTn searches of AiptasiaBase, the *A. pallida* EST database consisting of ~5,000 unique sequences (http://aiptasia.cs.vassar.edu/AiptasiaBase/index.php) (Sunagawa et al. 2009) using human TRAF and putative *N. vectensis* TRAF (NvTRAF) sequences as queries. We also searched AiptasiaBase for additional components in the TLR pathway using previously identified sequences from *N. vectensis* and *A. millepora* (Miller et al. 2007) as queries. Resulting *A. pallida* sequences were BLASTx searched against GenBank to identify their top hit. Searches for TRAFs from several other symbiotic cnidarians (*A. millepora, A. palmata, Montastraea annularis, M. faveolata,* and *Porites astreoides*) were performed through tBLASTn searches of the Systems Biology of Symbiosis (SymBioSis) website (http://sequoia.ucmerced.edu/SymBioSys/index.php) which houses EST datasets for these organisms, using human and NvTRAF sequences as queries.

Identification of domains within full-length N. vectensis TRAFs

Protein domains were identified by PfamA (http://pfam.sanger.ac.uk/), ScanProsite (http://www.expasy.ch/tools/scanprosite/), InterProScan http://www.ebi.ac.uk/Tools/InterProScan/) and a CD-Search of NCBI's conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Domains were included if they were predicted by more than one annotation method or if a single annotation method had a significant hit to the domain (E-value <1e-05). Automated searches did not identify all putative zinc fingers, so identification was performed manually by searching for cysteine-rich C_2HC motifs characterized by the following consensus sequence patterns: $CX_6CX_{11}HX_3C$ (found in the first zinc finger of hTRAF3, hTRAF4, ceTRAF), $CX_{2,3}CX_{11,12}HX_3C$ (found in most TRAF zinc fingers), and $CX_6CX_{11}HX_7H$ (the last zinc finger of all TRAFs) as detailed by Wajant et al. (1999). Pattern identification was also aided by the fact that neighboring zinc domains in TRAF proteins are always separated by 6 amino acids (Wajant et al. 1999) and two neighboring zinc fingers form a CART (C-rich motif associated with RING and TRAF domains) domain (Regnier et al. 1995).

Testing the NvTRAFs for evidence of natural selection

Ka/Ks analysis of NvTRAFs

Ka/Ks analyses were used to examine sequence evolution among NvTRAFs for evidence of natural selection. Nucleotide and inferred amino acid sequences for all NvTRAFs were obtained from GenBank. An alignment using amino acid sequences was generated by MUSCLE (Edgar 2004). We created one alignment based on full-length sequences and another based on the TRAFd only. These alignments were manually adjusted and then forced onto the corresponding nucleotide sequences using Mesquite v2.72 (Maddison and Maddison 2009). Ka/Ks analyses were performed in DnaSP v5 (Librado and Rozas 2009), which uses the Nei-Gojobori unweighted pathway method to estimate the numbers of synonymous and nonsynonymous differences between sequences (Nei and Gojobori 1986). Analyses were conducted across the entire gene for all pairwise comparisons among the 16 NvTRAF sequences. A separate set of analyses was performed on the TRAFd alone. Pairs of sequences with Ks values (the number of synonymous substitutions per synonymous site) greater than 1.1 were considered too different from one another and discarded to avoid problems of mutational saturation (Nei and Kumar 2000). Mutational saturation in DNA and protein sequences occurs when individual sites have undergone multiple mutations over time, causing the number of observed differences to no longer accurately reflect the true evolutionary distance, or number of substitutions that have actually occurred since the divergence of the two sequences (Page and Holmes 1998).

Significance testing of results was performed in MEGA v4 (Kumar and Dudley 2007). P values < 0.05 were considered to be significant. These tests included the codon-based z test for positive or negative selection, using the Nei-Gojobori method (Nei and Gojobori 1986) and the codon-based Fisher's exact test of selection using the Nei-Gojobori and modified Nei-Gojobori (assumed transition/transversion bias = 2) (Zhang et al. 1998) methods. The variance of the difference for the codon-based z tests was computed using the bootstrap method (1500 replicates). All positions containing

alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). For the full-length analysis, there were 953 positions in the final dataset. For the TRAFd analysis, there were 357 positions in the final dataset.

Examining similar NvTRAFs for evidence of allelism

The combination of polymorphism and recent tandem duplication may cause the assembly to overestimate the number of TRAFs in the draft *N. vectensis* genome by mistaking different alleles of the same gene for distinct loci (Ryan et al. 2006). Since the draft *N.vectensis* genome assembly may contain such inconsistencies, it is difficult to distinguish between alleles and recent tandem gene duplications. We were interested in exploring this issue with any two sequences (sequence pairs) that occur on the same scaffold and had Ks values less than 1.1. Identified sequence pairs that fit our requirements were NvTRAF9/10 and NvTRAF14/15 (Table 3.2). First, we examined an alternate *N. vectensis* genome assembly created with the Phusion assembler (Mullikin and Ning 2003) available through StellaBase (http://www.stellabase.org) to see if each of the putative TRAFs were also predicted with this assembly. Second, we compared the flanking regions surrounding the coding sequence of each NvTRAF pair. If the flanking region sequence is substantially different between a sequence pair, the possibility of the two sequences being alleles can be eliminated (Ryan et al. 2006). If the flanking regions are highly similar, the possibility of the two predicted TRAFs being alleles of the same gene cannot be ruled out.

Cloning ApTRAF6 and FsTRAF6

Collection of material

A. pallida can survive without symbionts in an aposymbiotic state if kept in the dark and fed regularly (Weis et al. 2008). Aposymbiotic anemones were sampled to avoid contamination from *Symbiodinium* sp. RNA. Mass cultures of symbiotic *A. pallida*

were maintained in aquaria of artificial seawater (Instant Ocean) at room temperature on a 12h light:12h dark photoperiod with a light irradiance of 70 μmol quanta m^{-2} s^{-1}. Aposymbiotic anemones were generated from symbiotic individuals by the cold shock technique (Muscatine et al. 1991), then kept in total darkness and fed twice weekly with freshly hatched *Artemia* sp. nauplii for several weeks. Animals were starved for one week prior to sampling to avoid contamination from *Artemia* sp. RNA. For sampling, several aposymbiotic anemones were removed from culture dishes, blotted dry, placed in microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80°C until RNA isolation.

Collection, maintenance and spawning of adult *F. scutaria* from Kaneohe Bay, Oahu, Hawaii were performed as described previously (Schwarz et al. 1999; Weis et al. 2001; Rodriguez-Lanetty et al. 2006) at the Hawaii Institute of Marine Biology in July 2008 and 2009. Five-days post-fertilization, 15 mL conical tubes were filled with aposymbiotic *F. scutaria* larvae suspended in filtered seawater (FSW), spun for 1 min at 6000 x g, and excess seawater was removed. Tubes of pelleted larvae were flash frozen in liquid nitrogen, stored at -80°C, shipped to Oregon State University (OSU) on dry ice and stored at -80°C until RNA isolation.

RNA extraction and cDNA synthesis

Total RNA was isolated from aposymbiotic *A. pallida* adults and *F. scutaria* larvae using Trizol (Invitrogen) according to the manufacturer's instruction with modification. Protocol modification included the addition of the optional centrifugation step at 12,000 x g for 10 minutes at 4°C to remove polysaccharides and other contaminants. Since it has been reported that RNA isolated from frozen tissue using Trizol may be refractory to cDNA synthesis, and that additional purification using glass fiber filter columns restores its ability to be reverse transcribed (Dumur et al. 2004), samples were subsequently loaded into an RNeasy column (Qiagen) and washed as recommended by the manufacturer's instructions. RNA was eluted with

RNAse-free water and stored at -80°C until use. Total RNA purity and concentration was determined with a Nanodrop 1000 (Thermo Fisher Scientific). RNA integrity was assessed by running an aliquot of each preparation on a non-denaturing 1% agarose gel. cDNA synthesis was performed from 0.5 µg total RNA using a Superscript III Reverse Transcriptase kit (Invitrogen). cDNA was stored at -20°C until use.

Primer design

Several sets of degenerate primers were designed based on NvTRAF6 and a putative *A. millepora* TRAF6 sequence fragment (SEQINDEX16187_C_c_s from the *A. millepora* larval transcriptome database on the SymBioSys website). Amino acid sequences were inferred from nucleotide sequences and aligned with MUSCLE (Edgar 2004). This alignment was then forced onto the corresponding nucleotide sequences using Mesquite v2.72 (Maddison and Maddison 2009). Conserved regions from the alignment were targeted for degenerate primer design. Details of successful primer pairs including primer sequences, fold degeneracy of each primer, annealing temperature (Tm) used, and length of fragment that was amplified are provided in Table 4.2. Gene-specific primers, based on the sequenced PCR products generated from the degenerate RT-PCR strategy, were designed to amplify the 5′ and 3′ ends of ApTRAF6 and FsTRAF6 (Table 4.2). Additional gene-specific primers were then designed to amplify full-length ApTRAF6 and FsTRAF6 sequences (Table 4.2).

Degenerate PCR, cloning, sequencing

PCR with degenerate primers was performed in 25µl total volume with 1-2µl cDNA, 2.5 µl 10x buffer, 0.2µl 25µM dNTPs, 1µl 10mM forward and reverse primers, and 0.3µl Paq5000 Hotstart Polymerase (Agilent Technologies). Cycling parameters were: 95°C for 3 min; 35 cycles of 95°C for 35 sec, Tm°C for 30 sec (see Table 4.2 for specific Tm), and 72°C for 2 min; 72°C for 10 minutes. PCR products were visualized on a 1% agarose gel. Products of the correct predicted size (based on the NvTRAF6 sequence) were gel purified with a QIAQuick kit (Qiagen). Purified products were

ligated to the pGEM-T easy vector (Promega), transformed into MAX Efficiency DH5α chemically competent *E. coli* cells (Invitrogen) and plated on LB supplemented with ampicillin. Resulting colonies were visually screened for inserts. Several clones were grown overnight in LB supplemented with ampicillin and plasmids were purified using a mini-prep kit (Qiagen). Resulting clones were screened by EcoRI digest and checked on a 1% agarose gel for the correct insert size. Clones with inserts of the correct size were sequenced using the vector primers T7 and M13R. Sequence fragments were processed (vector and low quality sequence was trimmed) and assembled into contigs using Sequencher v 4.7 (GeneCodes Corp). Sequences were identified as TRAF6 homologs through BLASTx searches of GenBank.

5′ and 3′ RLM-RACE, cloning, sequencing

To amplify the 5′ and 3′ ends of ApTRAF6 and FsTRAF6, RACE reactions were performed with the RLM-RACE kit (Ambion) according to the manufacturer's instructions. Gene-specific primers (Table 4.2) were used with RACE primers provided in the RLM-RACE kit. For *A. pallida*, 2.5 µg total RNA was used for 5′ RACE and 1.0 µg total RNA was used for 3′ RACE. For *F. scutaria*, 0.56 µg total RNA was used for 5′ RACE and 0.35 µg total RNA was used for 3′ RACE. For all RACE reactions, an "outer" PCR was first performed using a gene-specific outer primer (designed upstream from the gene-specific inner primer) with an outer RACE primer and 1 µl RACE-ready cDNA as a template. This was followed by an "inner" PCR using a gene-specific inner primer with an inner RACE primer and 1 µl of the outer PCR product as a template. All RACE PCR reactions were performed with SuperTaq Plus polymerase (Ambion) using a hot start and the following cycling parameters: 94°C for 3 min; 35 cycles of 94°C for 45 sec, Tm°C for 30 sec (see Table 4.2 for specific Tm), and 72°C for 2 min; 72°C for 7 minutes. PCR products were visualized on a 1% agarose gel. Products of the correct predicted size were purified, cloned, sequenced and analyzed as described for degenerate PCR.

Full-length PCR, cloning, sequencing

Gene-specific forward and reverse primers (Table 4.2) were designed to amplify the full-length coding region of ApTRAF6 and FsTRAF6. Methods for full-length PCR, cloning, sequencing, and sequence analysis were identical to those described for degenerate PCR. We sequenced several clones from each product so that we could choose the most common nucleotide at any polymorphic sites.

Sequence analysis of ApTRAF6 and FsTRAF6

Amino acid sequences were inferred from full-length nucleotide sequences. Predicted molecular weights were calculated using the Compute pI/Mw tool on the ExPASy Proteomics Server (http://www.expasy.ch/tools/pi_tool.html). Secondary structures were predicted using the PSIPRED Protein Structure Prediction Server v3.0 (Jones 1999; Bryson et al. 2005) (http://bioinf.cs.ucl.ac.uk/psipred). Amino acid alignments were obtained using MUSCLE (Edgar 2004) and manually adjusted in Mesquite v2.72 (Maddison and Maddison 2009). Domains were predicted using the same methods (automated and manual identification) as described for the NvTRAF sequences. Amino acid identity and similarity comparisons between ApTRAF6, FsTRAF6 and other TRAF6 orthologs were calculated in BioEdit v7.0.5 (Hall 1999) based on amino acid alignments obtained with MUSCLE. For similarity comparisons, a BLOSUM62 matrix was used.

Phylogenetic analysis of cnidarian TRAFs

Phylogeny of the cnidarian TRAF proteins was estimated relative to other TRAFs using maximum likelihood and parsimony methods. Sequences included in these analyses are listed in Table 4.1. First, an amino acid alignment was generated with MUSCLE (Edgar 2004), and manually adjusted in Mesquite v2.72 (Maddison and Maddison 2009). Variable-length tails and any regions that could not be unambiguously aligned were masked from subsequent analyses. Analyses were carried out on a full-length alignment and a TRAFd only alignment.

To choose the best fit model of protein evolution, we used the program ProtTest v2.4 (Abascal et al. 2005) to apply AIC1, AIC2, and BIC2 metrics to a variety of possible substitution matrices and rate assumptions (Akaike 1974). The results from the overall comparison of these metrics indicated the best fit model for the full-length alignment was WAG+G+F (Whelan and Goldman 2001), where G specifies gamma-distributed rates across sites, and F specifies that empirical amino acid frequencies in the dataset are used. The best fit model for the TRAFd alignment was LG+G (Le and Gascuel 2008). The gamma distribution is approximated using a small number of discrete rate categories (Yang 1994). We used 4 rate categories to approximate gamma.

Maximum likelihood (ML) analyses were performed with PhyML v3.0 (Guindon and Gascuel 2003) and RAxML v7.2.6 (Stamatakis 2006). Parsimony analyses were conducted using PAUP* 4.0b10 for MacOSX/Unix (Swofford 2003). For all analyses, we ran 200 bootstraps. PhyML analyses were run on the Montpellier bioinformatics platform online web server (http://www.atgc-montpellier.fr/phyml/) (Guindon et al. 2005). RAxML analyses were run on the ABE teragrid through the CIPRES web portal v2.2 (http://www.phylo.org/sub_sections/portal/) (Miller et al. 2009) using a rapid bootstrapping algorithm (Stamatakis et al. 2008). The most likely trees, displayed with consensus bootstrap support values, were arranged and visualized using FigTree v1.3.1 (Rambaut 2007). Trees were rerooted in FigTree if needed, and then annotated manually using Adobe Illustrator. Nodes with bootstrap support between 50% and 100% support were labeled on the trees.

Results

Database searching for TRAFs

Non-cnidarian TRAFs

We obtained 36 full-length non-cnidarian TRAF protein sequences to be used for creating multiple sequence alignments and gene trees (Table 4.1). We obtained 2 human meprin sequences to be used as outgroups for the analysis. Meprins are a

family of extracellular metalloproteases that have only been identified in vertebrate genomes (Zapata et al. 2007), and are the closest TRAFd-containing proteins to TRAFs (Zapata et al. 2007). tBLASTn searches of the *N. vectensis* draft genome did not identify any putative meprin sequences.

Cnidarian TRAFs

Our searches resulted in 16 full-length putative TRAFs from *N. vectensis* and 14 full-length TRAFs from *H. magnipapillata* (Table 4.1). The *N. vectensis* TRAFs occur on 13 separate scaffolds on the JGI *N. vectensis* draft genome assembly (Table 4.3). There are 3 scaffolds that contain two different *N. vectensis* TRAF sequences: NvTRAF3/11 on scaffold 3, NvTRAF9/10 on scaffold 297, and NvTRAF14/15 on scaffold 374. The NvTRAF proteins are encoded from genomic sequence that varies in length from 3742 bp to 19919 bp and the number of exons for each predicted protein ranges from 3-21 (Table 4.3).

We also found evidence for several short TRAF-like gene fragments in the *N. vectensis* and *H. magnipapillata* genomes. These included 3 TRAF fragments from *N. vectensis* [GenBank: XP_001620870, XP_001630876, and XP_001630964] ranging from 183 to 266 aa in length and 12 TRAF fragments from *H. magnipapillata* [GenBank: XP_002154378, XP_002155932, XP_002156040, XP_002156996, XP_002161248, XP_002163394, XP_002164239, XP_002165877, XP_002167601, XP_002169208, XP_002169572, XP_002171214] ranging from 202 to 327 aa in length. *H. magnipapillata* TRAF fragment XP_002169208 is the sequence that was previously identified as a homolog to hTRAF6 by Miller et al. (Miller et al. 2007), but we found that it does not contain a TRAFd and did not include it in our analyses.

We found sequence evidence for 9 putative TRAF gene fragments in the anemone *A. pallida* from tBLASTn searches of AiptasiaBase (Table 4.4). Several of these ESTs had a top hit to sequences we identified as putative *N. vectensis* TRAFs. We also

found EST evidence in AiptasiaBase for several other components of the TLR pathway, although some sequences were not present (Table 4.4). Since this database contains only ~5,000 unique sequences and is far from exhaustive, the lack of a BLAST hit does not necessarily mean that the sequence does not exist in *A. pallida*.

Additional EST database searches on the SymBioSys website resulted in sequence evidence for putative TRAF gene fragments from corals *A. millepora* (15 unique fragments), *A. palmata* (6 unique fragments), *M. annularis* (2 unique fragments), *M. faveolata* (9 unique fragments), and *P. astreoides* (5 unique fragments). None of these sequences appeared to encode full-length TRAFs in terms of their sequence length or identified domains (data not shown). Since the sequence fragments may not encode functional TRAFs, ultimately we chose to exclude all fragments and focus exclusively on full-length cnidarian TRAFs from the *N. vectensis* and *H. magnipapillata* genome projects, and full-length sequences from *H. echinata* for creating sequence alignments and phylogenetic trees.

Identification of domains within full-length NvTRAFs

All NvTRAFs contain an N-terminal RINGd, and one or more zinc finger domains in the middle of the protein (Fig. 4.1). Fourteen of the sixteen NvTRAFs have a C-terminal TRAFd that is characteristic of the TRAF superfamily. The remaining two NvTRAFs have a WD40 repeat domain in place of the C-terminal TRAFd and closely resemble hTRAF7. We grouped several NvTRAFs with one of the seven hTRAFs or with the *C. elegans* TRAF according to their length, domain arrangement, and grouping on phylogenetic trees (see ahead) and named them accordingly (Fig. 4.1a). Seven NvTRAFs did not group with any hTRAF or with the *C. elegans* TRAF, so these sequences were placed in separate groupings and named sequentially (Fig. 4.1b).

We identified two to three putative zinc fingers in the proteins in the TRAF7 group. A previous report only identified one zinc finger in hTRAF7 (Xu et al. 2004). Two out of

three of the putative zinc finger domains we identified differ from other TRAFs. Instead of the usual C_2HC TRAF zinc finger motif, we identified the following variations: (1) an aspartic acid residue replaces the first cysteine resulting in a DCHC motif, (2) a histidine residue replaces the first cysteine for a HCHC motif. There were also slight variations in the specific sequence patterns of the C_2HC motifs in the hTRAF7 group zinc fingers (data not shown). These variations are found in hTRAF7 and in the two NvTRAFs that group with it. hTRAF7 was only recently discovered (Xu et al. 2004) and detailed functional and structural studies of this protein have not yet been done, so it is possible that the divergent zinc finger domains we identified here are indeed functional.

Testing the NvTRAFs for evidence of natural selection

Ka/Ks analysis of NvTRAFs

The neutral theory of molecular evolution (first proposed in 1968 by M. Kimura (Kimura 1968)) states that most genes and proteins have become so well adapted through previous natural selection that any new mutations will be either deleterious (and selectively removed) or neutral, in which case there is a small chance they will become fixed over time (Page and Holmes 1998). One approach that has been used to test and counter the neutral theory is by finding examples of the action of natural selection at the molecular level. In some cases, molecular adaptation can be inferred through sequence comparisons. Detecting natural selection at the molecular level can be difficult, however, because these events may involve only a few base changes and differentiating between true natural selection and genetic drift can be problematic (Page and Holmes 1998).

Ka/Ks analyses compare the rate of substitutions at synonymous (silent) sites (Ks) to the rate of substitutions at nonsynonymous sites (Ka) between a pair of coding sequences to examine rates of sequence evolution for evidence of natural selection at the molecular level (Nei and Kumar 2000). If the Ka/Ks ratio is < 1, there is evidence

for negative (purifying) selection. The rate of non-synonymous substitution is generally much lower than that of synonymous substitution because changes at coding sites are often constrained since they may alter protein function (Nei and Kumar 2000). If the Ka/Ks ratio is close to 1, the rate of substitution at synonymous and nonsynonymous sites is roughly equal, providing evidence of neutrality (the null hypothesis). If the Ka/Ks ratio is > 1, there is evidence for positive (diversifying) selection since there are more sites in coding positions that are evolving relative to sites in non-coding positions. Positive selection is generally rare among genes (Nei and Kumar 2000).

For the full-length NvTRAF comparisons, a single pair of sequences had a Ks value <1.1. NvTRAF14/15 had a Ka=0.0193 and a Ks=0.0113. Their Ka/Ks ratio was 1.708, indicating positive (diversifying) selection, although the z-test for positive selection and Fisher's exact tests were not significant (Table 4.5). For the TRAFd comparisons, there were 4 pairs of sequences with Ks values < 1.1. Three of the pairs had Ka/Ks values < 1.0, indicating purifying (negative) selection. The z-test for negative selection was significant for all three pairs (Table 4.5). NvTRAF14/15 had a Ka/Ks = 1.563 for the TRAFd. Again, the z-test for positive selection and Fisher's exact tests were not significant for this pair of sequences (Table 4.5). A total of 12 non-synonymous substitutions were found between these two sequences, 10 of which occur in the TRAFd. Only 2 synonymous substitutions were found in this pair of sequences and both of these occur in the TRAFd.

Examining similar NvTRAFs for evidence of allelsism

In contrast to our searches of the JGI assembly, our searches of the StellaBase assembly did not provide evidence that NvTRAF14 and NvTRAF15 are distinct loci. Examination of the flanking regions of these sequences using the JGI *N. vectensis* genome browser showed that these regions are highly similar. This evidence indicates that these two sequences may be alleles of the same gene. It is also possible, however,

that these sequences are distinct loci that have arisen due to recent tandem duplication. Both *N. vectensis* genome assemblies predict that NvTRAF9 and NvTRAF10 encode distinct genes. Examination of the flanking regions of these sequences also showed these regions to be highly dissimilar, giving additional evidence that they are distinct loci.

Cloning ApTRAF6 and FsTRAF6

Our degenerate RT-PCR approach resulted in two fragments from *A. pallida* (802 bp and 1072 bp) and one fragment from *F. scutaria* (662 bp). Similarity to NvTRAF6 and other TRAF6 orthologs was confirmed by BLASTx searches and sequence alignments. Full-length PCR confirmed that ApTRAF6 is 1455 bp in length and FsTRAF6 is 1179 bp (Table 4.6). The inferred amino acid sequence of ApTRAF6 is 484 aa and FsTRAF6 is 392 aa.

Sequence analysis of cnidarian TRAF6 genes

Of all NvTRAFs, NvTRAF6 has the fourth longest total genomic coverage, with 14,018 bp of genomic sequence (Fig. 4.2, Table 4.3). NvTRAF6 includes 6 exons that cover 1446 bp of coding sequence (Fig. 4.2 and Table 4.6). There are also several large introns, including one that is 6475 bp in length. The predicted isoelectric point and molecular weights of the cnidarian TRAFs are listed in Table 4.6. Fig. 4.3 displays the nucleotide and amino acid sequence of ApTRAF6 together with its predicted secondary structure. Fig. 4.4 provides the nucleotide and amino acid sequence of FsTRAF6. The overall predicted secondary structures of NvTRAF6 and FsTRAF6 were similar to ApTRAF6 (data not shown).

Structural studies have been conducted on the RING (PDB: 2JMD) (Mercier et al. 2007) and TRAF domains (PDB: 1LB6) (Ye et al. 2002; Chung et al. 2007) of hTRAF6. The RINGd of hTRAF6 includes a short α–helix and a single β-sheet, which contribute to the hydrophobic core of the protein (Mercier et al. 2007). The TRAFd of hTRAF6 forms an eight-stranded anti-parallel β-sandwich with strands β1, β8, β5 and

β6 in one sheet, and β2, β3, β4 and β7 in the other (Chung et al. 2007). We identified all of these basic secondary structural elements that characterize the RING and TRAF domains of hTRAF6 in ApTRAF6 (Fig. 4.3), NvTRAF6 and FsTRAF6.

The overall domain arrangement of cnidarian TRAF6 proteins closely resembles that of hTRAF6, including the N-terminal RINGd, 5 neighboring zinc fingers with C_2HC motifs, and a C-terminal TRAFd (Fig. 4.5). The amino acid alignment of cnidarian TRAF6 proteins with hTRAF6 also reveals several regions with high sequence conservation, especially in the RING and TRAF domains (Fig. 4.5). TRAF protein RING domains are generally organized in a cysteine-rich C_3HC_4 motif with a $CX_2CX_{11,12}CX_1HX_2CX_2CX_{9,11}CX_2C$ pattern, although hTRAF4, 5, and 6 end with an aspartic acid (D) residue in place of the final cysteine for a C_3HC_3D motif (Wajant et al. 1999). The RING domains of NvTRAF6, ApTRAF6 and FsTRAF6 all contain the C_3HC_3D motif (Fig. 4.5). FsTRAF6 is shorter in total length than other TRAF6 proteins due to several deletions. These deletions are located at the N-terminus before the RINGd, and between the last predicted zinc finger domain and the TRAFd, and thus do not occur in any of the conserved functional domains.

Pairwise amino acid identity (id) and similarity (sim) comparisons reveal that ApTRAF6 has higher sequence homology with NvTRAF6 over the full-length sequence (50% id; 64% sim) and the TRAFd alone (72% id, 85% sim) than with FsTRAF6 (full length: 34% id, 50% sim; TRAFd: 39% id, 66 % sim) (Table 4.7). Although all three species are anthozoan cnidarians, *A. pallida* and *N. vectensis* are both anemones (Order Actiniaria), while *F. scutaria* is a stony coral (O. Scleractinia). Interestingly, ApTRAF6 has relatively high sequence homology with hTRAF6 (full-length: 34% id, 47% sim; TRAFd: 42% id, 54% sim). The level of homology between hTRAF6 and other vertebrate TRAF6 sequences (*X. laevis* and *D. rerio*) was higher than with ApTRAF6, but lower with all other invertebrate TRAF6 sequences (*E.*

scolopes, *C. farreri*, *S. purpuratus, C. intestinalis, D. melanogaster*, *N. vectensis*, and *F. scutaria*) included in the analysis.

Phylogenetic analysis of cnidarian TRAFs

Trees produced by ML methods (PhyML, RAxML) and parsimony methods (PAUP) had similar overall topologies and bootstrap support values. There was strong bootstrap support for all major clade relationships within all trees. All trees were rooted with HmeprinA and HmeprinB. The tree reconstructed from the full-length alignment shows 8 monophyletic clades that group different TRAFs together (Fig. 4.6). The tree reconstructed from the TRAFd alignment has a similar number and composition of monophyletic clades as the tree from the full-length alignment, but there are several distinct differences in the topology of the tree and the taxa included in the groupings (Fig. 4.7). Branch lengths for both trees are from the RAxML analyses (Fig. 4.6 and 4.7).

Phylogeny of NvTRAF sequences

In the full-length tree, the 7 hTRAF sequences form 5 monophyletic clades: hTRAF1/2, hTRAF3/5, hTRAF4, hTRAF6, and hTRAF7 (Fig. 4.6). At least one NvTRAF is located in each of the hTRAF lineages. Another monophyletic clade groups ceTRAF with NvTRAF9 and NvTRAF10 ("ceTRAF" group on the trees). Seven NvTRAFs do not group with other known TRAFs. NvTRAFs 13, 14, and 15 branch next to each other and NvTRAF11, 12, and 17 also fall in this large, exclusively cnidarian clade ("cnidTRAF" on the trees). NvTRAF16 does not form a group or branch with any other TRAFs.

In the tree based on the TRAFd only, some of the NvTRAFs are found in different clades than in the full-length tree (Fig. 4.7). For example, in the TRAFd tree, NvTRAF17 is placed in the ceTRAF clade next to two putative TRAFs from the placazoan *T. adhearens*. Also in the TRAFd tree, NvTRAF5 is found with HmTRAFs

6, 14 and 22 instead of with hTRAF3/5. Finally, NvTRAF16 groups with SpTRAF2 in the TRAFd tree instead of branching alone.

Phylogeny of HmTRAF6 sequences

The *H. magnipapillata* genome contains 14 putative full-length TRAF sequences. The full-length tree reveals that *H. magnipapillata* has homologs in only 1 of the 5 hTRAF lineages. This is not surprising as the *H. magnipapillata* genome exhibits several instances of gene loss and sequence divergence (Miller et al. 2007; Chapman et al. 2010). HmTRAFs 14, 22, 24, 25 are in the hTRAF7 clade (Fig. 4.6). Several of the other HmTRAFs fall in other groups or form their own monophyletic clades. The ceTRAF group includes HmTRAF3, which branches next to NvTRAF9 and 10. HmTRAFs 18, 20 and 21 form an exclusively hydrozoan clade ("hydroTRAF" group on the trees) with HyTRAF2 from *H. echinata*. We did not analyze the HmTRAF sequences for their genomic location or for evidence that they may represent alleles of the same gene, so it is possible that there are fewer than 14 HmTRAFs. From the arrangements and branch lengths on the tree, it appears that the HmTRAF8/16 and HmTRAF20/21 sequence pairs might be allelic variants of the same gene.

Phylogeny of cnidarian TRAF6 sequences

ApTRAF6 and FsTRAF6 are clearly located within the hTRAF6 clade (Fig. 4.6 and 4.7). In the full-length tree, ApTRAF6 is located directly next to NvTRAF6 with FsTRAF6 as their next closest neighbor, forming a highly supported cnidarian TRAF6 group (Fig. 4.6). The hTRAF6 sequence groups with other vertebrate TRAF6 sequences from *X. laevis* and *D. rerio* with strong support. Invertebrate TRAF6 sequences from *S. pupuratus*, *C. farreri*, *E. scolopes*, *C. intestinalis* are found next to the vertebrate TRAF6 group, with the cnidarian TRAF6 sequences sister to this grouping. dTRAF2 (from *D. melanogaster*) falls outside of all of the other TRAF6 sequences with medium bootstrap support.

Discussion

Genomic evidence of TRAFs in cnidarians

Our analysis of the domain arrangements of NvTRAFs supports our observation from the phylogenetic reconstructions that *N. vectensis* has at least one homolog in each of the 5 major hTRAF lineages. Prior to this study, TRAFs 1, 2 and 5 had only been identified in vertebrate genomes (Zapata et al. 2007). It is now clear that there is at least 1 cnidarian TRAF that corresponds to the hTRAF1/2 lineage and two cnidarian TRAFs that correspond to the hTRAF3/5 lineage. We also found sequence evidence for several putative TRAFs in EST databases of symbiotic cnidarians, as well as many of the TLR pathway components in *A. pallida*. These sequences add to the sequence data from *A. millepora* to demonstrate that symbiotic cnidarians possess all of the necessary components of this innate immune pathway.

The discovery of a large number of TRAFs in cnidarians is not surprising because prior analyses of cnidarian genomes have demonstrated that they are far more complex than was predicted for a simple two tissue-layered organism and that other gene families are fully diversified in these common ancestors to the Bilateria (Miller et al. 2005; Miller et al. 2007; Putnam et al. 2007). Recent themes emerging from analyses of cnidarian genomes include (1) ancestral complexity followed by stochastic gene loss within gene families through animal evolution (Miller et al. 2005; Miller et al. 2007; Wood-Charlson and Weis 2009) and (2) the role of selective gene loss in shaping differences within the Cnidaria (Miller et al. 2007; Chapman et al. 2010; Forêt et al. 2010). Our study of TRAF family evolution in cnidarians provides additional evidence for each of these themes. In support of the first theme, protostomes (*D. melanogaster* and *C. elegans*) have apparently lost most of the ancestral complement of TRAF genes and vertebrates have apparently lost half of the ancestral TRAFs. For the second theme, selective gene loss within the Cnidaria can be seen in the divergent phylogenies of TRAFs from *H. magnipapillata* and *N. vectensis*. *H. magnipapillata* has apparently lost some TRAF genes during the course of its evolution since it does

not possess homologs to 4 out of the 5 hTRAF lineages. Apparent gene loss and sequence divergence in *H. magnipapillata* has been previously documented (Miller et al. 2007; Chapman et al. 2010). Hydrozoans and anthozoans (e.g. *N. vectensis*) diverged at least 540 million years ago (Chapman et al. 2010) and many of the *H. magnipapillata* TRAFs may have diverged since that time. In addition, anthozoans are the most basal (ancestral) group within the Cnidaria while hydrozoans are more derived (Schuchert 1993; Bridge et al. 1995).

Evidence for selection and allelism among NvTRAFs

There is a high level of nucleotide sequence divergence within the NvTRAFs, so most sequence pairs within this group could not be examined for evidence of natural selection due to problems of mutational saturation (Nei and Kumar 2000). One very similar pair, NvTRAF14/15, showed evidence for recent positive selection. Upon further analysis, including comparing flanking regions surrounding each gene, and comparing gene predictions from two separate genome assemblies, we found that these two predicted genes may, in fact, represent alleles of the same gene. Despite this, it is interesting that these two sequences demonstrate evidence of recent positive selection and they are good candidates for further study. Other pairs (NvTRAF9/10, NvTRAF13/14, NvTRAF13/15) showed evidence for negative selection. Further analyses of NvTRAF9/10 supported the prediction of these two genes as distinct loci, even though they occur on the same scaffold. As genome assembly methods improve and additional sequence data are added to the *N. vectensis* genome, it will become easier to distinguish the number of distinct loci from allelic variation.

Analysis of cnidarian TRAF6 sequences

Overall, we found very high homology between cnidarian TRAF6 sequences and hTRAF6 in their secondary structure, domain arrangement, sequence motifs within domains, and overall sequence identity and similarity. Conservation of the C_3HC_3D motif in the RINGd of cnidarian TRAF6 sequences and the high sequence homology

of cnidarian TRAF6 RING domains to hTRAF6 provides strong evidence that E3 Ub ligase activity is present in cnidarian TRAF6 proteins. Cnidarian TRAF6 sequences are also similar to hTRAF6 in the number of zinc finger domains, their arrangement, and in specific CH_2C sequence motifs. Thus, zinc finger domains of cnidarian TRAF6 proteins may fill similar functional roles as those found in well-characterized hTRAF6. The TRAF domains of cnidarian TRAF6 sequences exhibit comparatively high sequence homology to hTRAF6. Cnidarian TRAF6 TRAF domains have an average 37% identity and 52% similarity with hTRAF6. Further studies of these proteins will confirm whether cnidarian TRAF6 proteins function in similar ways to their mammalian homologs. Studies exploring the roles of TRAF6, and of the TLR pathway in general, in symbiotic cnidarians such as *A. pallida* and *F. scutaria* will determine if this pathway functions in innate immunity and in the regulation of mutualistic endosymbiotic relationships as we, and others, have hypothesized (Schwarz 2008; Weis 2008).

Phylogenetic analysis of cnidarian TRAFs

Our analysis of TRAF sequences from cnidarians and other metazoans has resulted in a clearer picture of the phylogeny of TRAFs. Our analysis shows that a major radiation of TRAF genes occurred prior to the divergence between cnidarians and bilaterians and that the full complement of TRAFs found in mammals is also present in cnidarians. Our phylogenetic analyses also show that there are at least three monophyletic clades with cnidarian TRAFs that have no mammalian counterpart. One clade includes ceTRAF, 1 *C. intestinalis* TRAF, 1 *S. purpuratus* TRAF and 2 *T. adhaerens* TRAFs together with 2 *N. vectensis* TRAFs and 1 *H. magnipapillata* TRAF (ceTRAF clade on trees). It will be interesting to find out what function(s) genes in this group have and why this lineage was apparently lost in vertebrates. Another clade groups a large number of cnidarian TRAFs including 6 NvTRAFs, 5 HmTRAFs, and HyTRAF1 and its splice variant HyTRAF1a from *H. echinata* together. This group has considerable sequence diversity and it is possible that the TRAFs in this group

have evolved novel functions that may be specific to cnidarians. It is also possible that these TRAFs have redundant functions to other TRAFs, as is often the case with genes involved in innate immunity (Songer et al. 2010). Finally, an exclusively hydrozoan clade (hydroTRAF on trees) has three HmTRAFs plus HyTRAF2 from *H. echinata*. It is likely that these sequences evolved since anthozoans and hydrozoans diverged from one another and possible that they may have functions that are specific to hydrozoans.

Evolution of TRAF sequences from basal metazoans to humans

TRAF family homologs have not been identified from non-metazoan organisms, although TRAFd-containing proteins in other families have been identified in fungi, slime molds, alveolates, euglenoids, and plants (Zapata et al. 2007). It is likely that the first TRAF family proteins evolved in the cnidarian-bilatarian ancestor, and that this ancestor contained a fully diversified complement of TRAFs, as we have found in cnidarians. Basal metazoans with putative TRAF orthologs identified thus far include placazoans (3 TRAFs), sponges (1 TRAF), and cnidarians. Preliminary tBLASTn searches of EST databases from ctenophores *Mnemiopsis leidyi* and *Pleurobrachia pileus* (available on the COMPAGEN website) using the NvTRAFs as queries indicate that TRAFs are also present in the genomes of organisms in this basal metazoan phylum (data not shown). Because it is still unclear which of the metazoan phyla is most basal (Dunn et al. 2008; Philippe et al. 2009; Schierwater et al. 2009), we cannot yet determine how many TRAF sequences were in the common ancestor of the Cnidaria and the Bilateria. This is an area of active research, however, and the addition of a ctenophore genome sequence (*M. leidyi*'s genome is currently being sequenced by the National Human Genome Research Institute), and the release of a sponge genome assembly (*Amphimedon queenslandica*'s genome is currently being assembled by the JGI) will help to resolve this issue.

Within the Bilateria, protostomes appear to have lost many of the ancestral TRAFs (Fig. 4.8). *C. elegans* possesses a single TRAF that does not group with any hTRAF

and *Drosophila* has only 3 TRAFs including homologs to hTRAF4 and hTRAF6. Echinoderms (*S. purpuratus*), which are deuterostomes, retained at least 4 TRAFs, including homologs to hTRAF5, hTRAF6 and ceTRAF (Fig. 4.6). Another report identified 3 additional putative sea urchin TRAFs (Hibino et al. 2006), however, we discarded these sequences from our analysis because they did not fit our criteria of full-length TRAFs with characteristic domain arrangement. TRAFs underwent an expansion in cephalochordates, mainly within a single TRAF lineage. Phylogenetic analyses of the *B. floridae* TRAFs indicate that at least 13 of the 24 putative *B. floridae* TRAFs group in the hTRAF1/2 clade (Huang et al. 2008; Yuan et al. 2009). *B. floridae* also appears to have at least two homologs to hTRAF3 and one homolog each hTRAF4 and hTRAF6 (Yuan et al. 2009). So, although duplications have occurred in cephalochordates, no novel subfamilies of TRAFs evolved. The urochordate *C. intestinalis* has a potentially reduced number of only 3 full-length TRAFs, including homologs to hTRAF4, hTRAF6 and ceTRAF (Fig. 3.6), although other researchers reported 8 CiTRAFs (Terajima et al. 2003). Our searches identified 6 additional putative CiTRAFs, but we discarded these from our analysis because 5 of these sequences did not fit our criteria for full-length TRAFs and one had a divergent domain arrangement. Vertebrates have a higher number of TRAFs, with 8 in zebrafish (*D. rerio*) including homologs to hTRAF1/2, hTRAF3/5, hTRAF4 and hTRAF6 (Stein et al. 2007); at least 4 in frogs (*X. laevis*) including homologs to hTRAF1/2, hTRAF4 and hTRAF6; and 7 TRAFs in humans (and other mammals) in 5 subfamilies, although this represents only half the number of the ancestral TRAFs (Fig. 4.8). This view of TRAF family evolution within the Metazoa is certainly incomplete and will be refined as additional whole genome sequences, and improved genome assemblies become available for more metazoan organisms.

Conclusions

Our identification of multiple TRAF family sequences from the *N. vectensis* and *H. magnipapillata* genomes will be useful for future studies that focus on the functional

role of TRAFs in basal metazoans. These studies may identify TRAFs with functions in immunity similar to their vertebrate counterparts and verify their role in TNFR and TLR pathway signaling, as well as potentially find additional roles for the TRAFs that do not have a clear vertebrate homolog. Our cloning of full-length TRAF6 genes from *A. pallida* and *F. scutaria* and discovery of other TLR pathway components in *A. pallida* supports existing EST sequence evidence (from *A. millepora*) that the components of this innate immune signaling pathway are present in symbiotic cnidarian genomes (Miller et al. 2007). Our discovery and analysis of this gene in these organisms paves the way for the study of TRAF6 regulation and expression in beneficial animal-microbe interactions using the cnidarian-dinoflagellate model.

Although this study substantially expands our understanding of the evolution of the TRAF gene family, we caution that many of the results described here are based on analyses of an incomplete dataset of TRAF sequences. The ongoing annotation of the *N. vectensis* and *H. magnipapillata* genomes will allow us to build on this dataset and improve our understanding. Also, the sequencing of additional bilaterian and basal metazoan genomes, and improved genome assembly, will provide better resources so that our inferences are based on more data points. Finally, the development of more sophisticated computational methods for the analysis of gene family evolution may yield insights that are not attainable with available methods.

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Figures and Tables

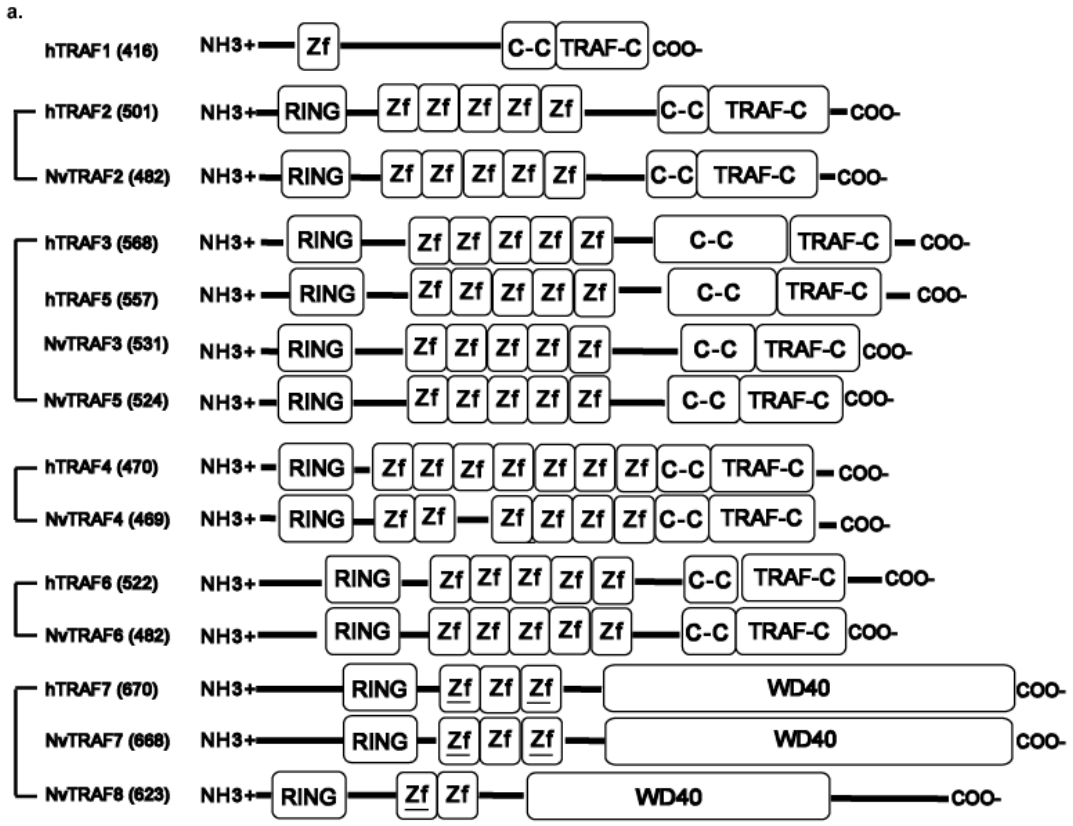

Fig. 4.1. Domain arrangement of NvTRAFs. Amino acid lengths of each protein are listed in parentheses after each gene name. RING= RING finger domain, Zf= zinc finger domain, Zf = divergent zinc finger identified in this study, C-C = coiled-coil domain, TRAF-C= C-terminal TRAF domain. (a) At least one NvTRAF has been grouped with each of the 5 major hTRAF lineages.

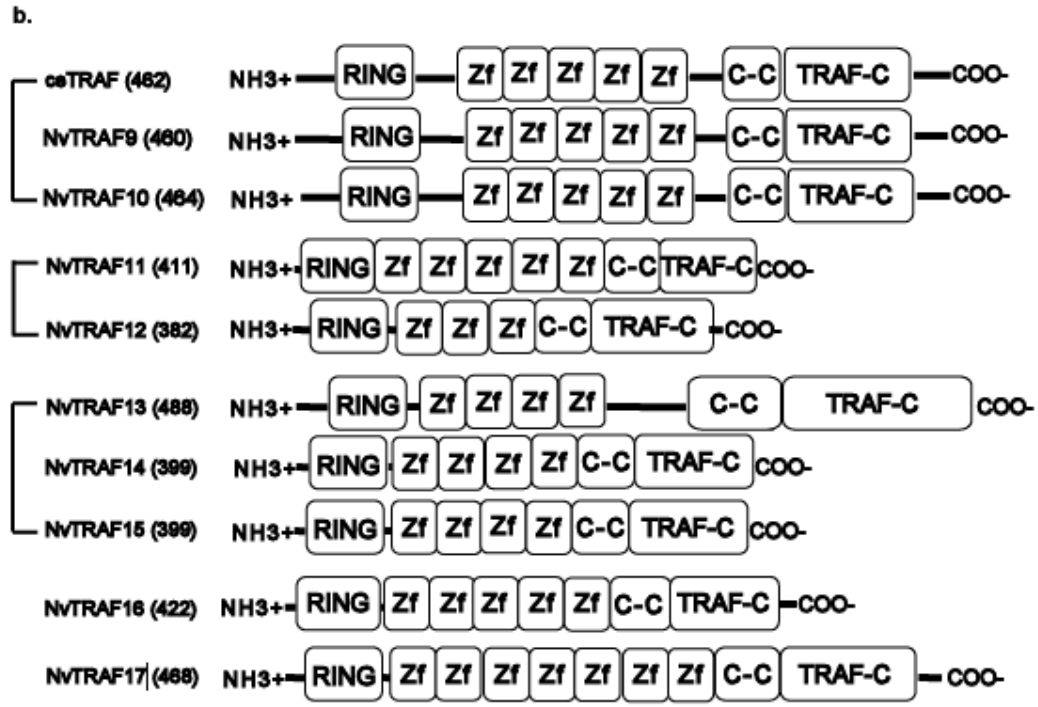

Fig. 4.1. (Continued) (b) Several NvTRAFs do not group with any of the hTRAF lineages. Groupings are based on domain arrangement, length, and placement on phylogenetic trees. Abbreviation as in Fig. 4.1a.

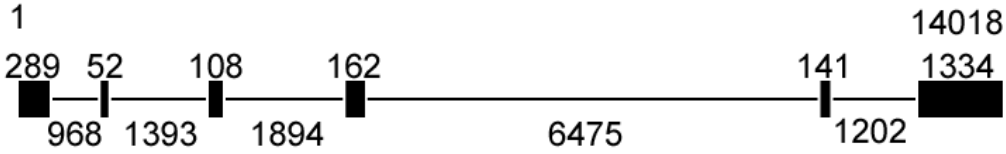

Fig. 4.2. Genomic arrangement of NvTRAF6. Exons and introns are represented by black boxes and lines, respectively. Lengths (in bp) are located above exons and below introns.

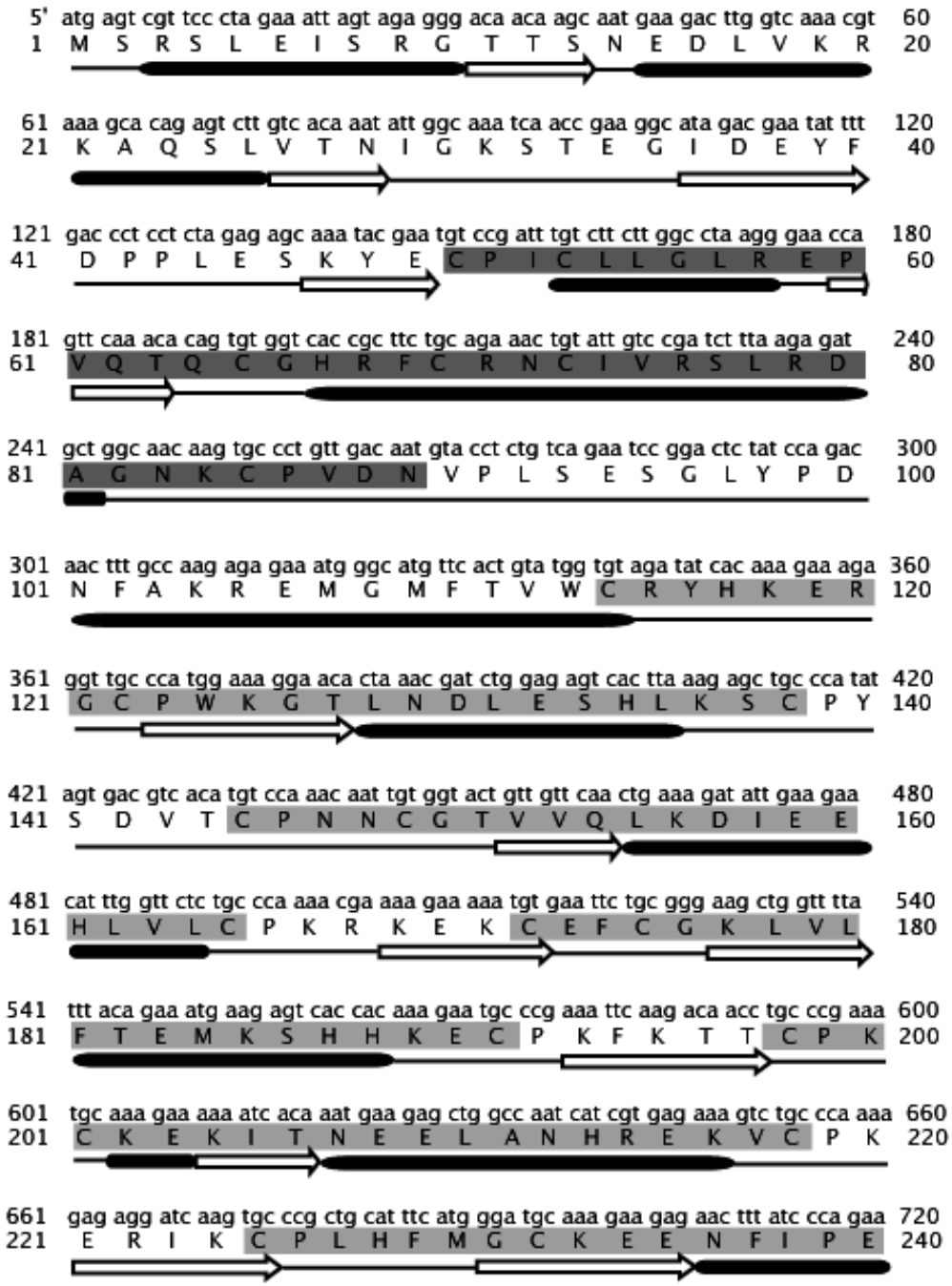

Fig. 4.3. ApTRAF6 nucleotide and amino acid sequence with secondary structure from the anemone *Aiptasia pallida*. Dark grey, medium grey, or light grey boxes over the amino acid sequence highlights the RINGd, zinc finger domains or TRAFd, respectively.

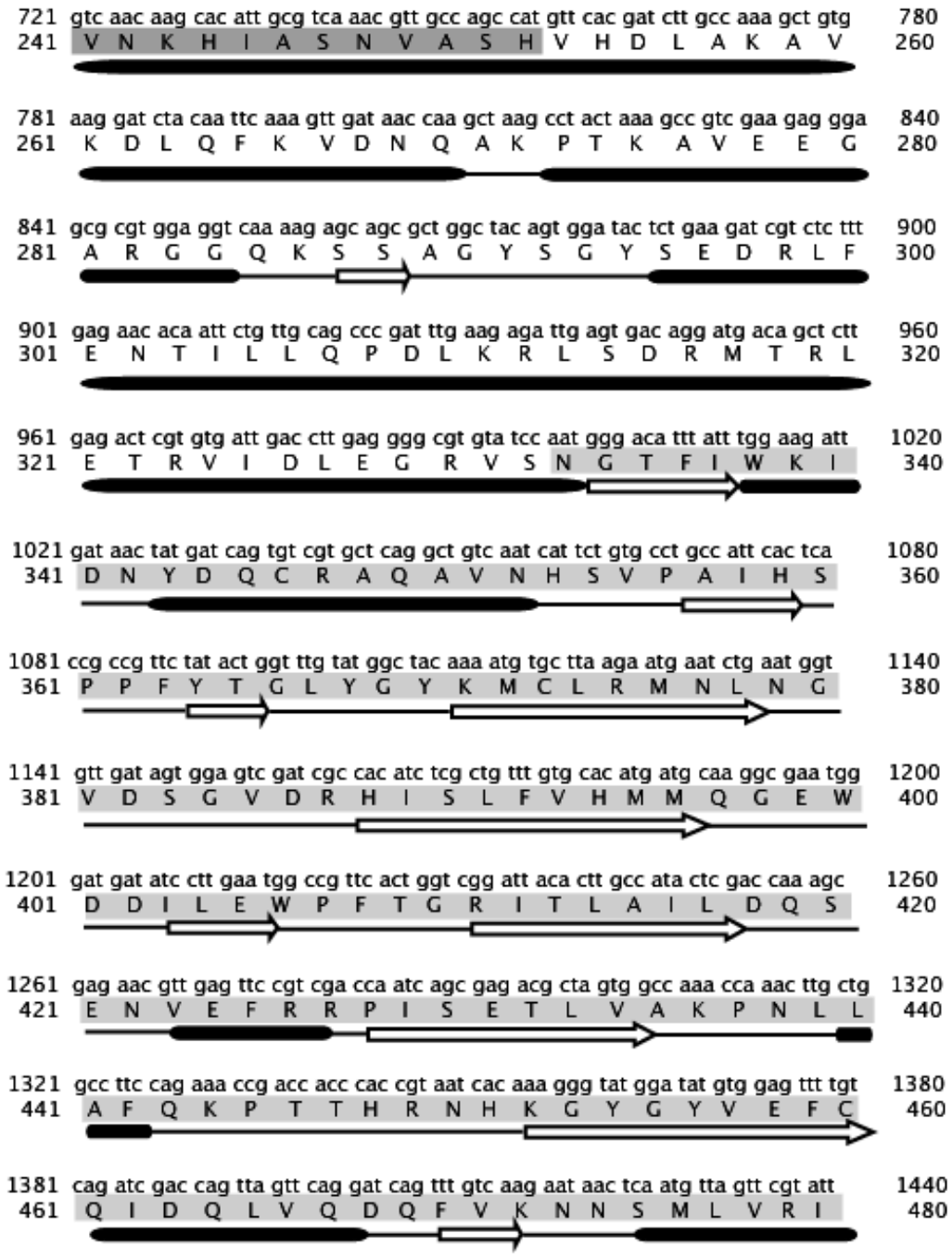

Fig. 4.3. (Continued).

Fig. 4.3. (Continued).

a.

```
   1  ATGAATACTC CACGTGGTTA CGACGCTTGC TTTGTCCCTC CGTTGGACGA TAAGTACGAA
  61  TGTCCAATAT GTTATTTGGG ATTGAAAGAA CCGGTCCAAA CAAACTGTGG TCATCGTTTT
 121  TGCAGAGATT GTATTACGAA ATCTTTGGAG AGTGTCGGAC ACAAATGTCC TATAGATAAC
 181  TGTAAACTTA CAGAACAAAT GATGTTTCCA GACAACTTCA CAAAGCGAGA AATGCTCTCT
 241  TTTACTGTAA AATGTGAAAA AGAGAAGTGT TGTTGGTCGG GGGAATTGAA AAATCTGGAA
 301  TCTCACAAAA ACGAGTGCGG ATTTGGCATA ATCGAATGTA CAAATAGCTG CGGTGTAAAA
 361  ATCGAGCGAC AAGCCCTCGA AGAGCATCTC AAAGARTGCC CACTTCGAAA AATCGAGTGC
 421  CAATTCTGCA AACAGCAAGT TTGTATTACA GTTGATCACT ACCCAGTCTG TACAAAGATG
 481  CCTATCGTTT GTAGTTGTAA AGAGAAAATT CCAAGAGACA AGATGGACGA TCACCTTGAA
 541  AACGATTGCT CCAGGACTAT ATCAGCTTGC CCACTAGCTT GGACAGGTTG TACATTTGAG
 601  GGGTTTAATC CAGAGGTGGC CGCGCATATA GCGAGCGATA TGAGGCATTT GAAATTTATT
 661  TCAAAAACAC TGCAAAATTT AACTGCGGCC TCAGCTCAAA CGTTGCCGCA ATTACCAGAG
 721  CCGTCGAACA ACGATTTTAC TAACAATATT TTTGTTTGGA AGCTTGTAAA CTTTCGAACC
 781  AAGCGCGCAA ATGGAGCCTC TGAACACTCT CCACCGTTTT ATTTATTCGG TTATAAGTTA
 841  TGCTTAAGGG TGAACACCAA CGGTATTGGC TCTGGTTATG GAAAACATCT CGCGGTCTTT
 901  TTACACGCGA TGAAAGGAAA ATATGACAAT ATGTTAACAT GGCCTTTTCA AAGTAAAATT
 961  ACATTGAGCA TTATGGATCA AACTCCAAAT GTAAACTACA AAGAGAACAT AACAGAACAT
1021  TTTAACACGA GCCCATCCAC GTGTTTTCGG CAACCACGAG AGACCAGAAC TACTACAGGA
1081  TATGGGTTTG TTGAATTTTG TACCCTTGAA CAATTGATTC TGCCTGAAAA ATACGTCAGA
1141  GATGACGCTT TGTTTTTTCG CGTTGAGGCA ACTCAGTGA
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b.

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   1  MNTPRGYDAC FVPPLDDKYE CPICYLGLKE PVQTNCGHRF CRDCITKSLE SVGHKCPIDN
  61  CKLTEQMMFP DNFTKREMLS FTVKCEKEKC CWSGELKNLE SHKNECGFGI IECTNSCGVK
 121  IERQALEEHL KECPLRKIEC QFCKQQVCIT VDHYPVCTKM PIVCSCKEKI PRDKMDDHLE
 181  NDCSRTISAC PLAWTGCTFE GFNPEVAAHI ASDMRHLKFI SKTLQNLTAA SAQTLPQLPE
 241  PSNNDFTNNI FVWKLVNFRT KRANGASEHS PPFYLFGYKL CLRVNTNGIG SGYGKHLAVF
 301  LHAMKGKYDN MLTWPFQSKI TLSIMDQTPN VNYKENITEH FNTSPSTCFR QPRETRTTTG
 461  YGFVEFCTLE QLILPEKYVR DDALFFRVEA TQ*
```

Fig. 4.4. FsTRAF6 sequence from the coral *Fungia scutaria*. (a) Nucleotide sequence of FsTRAF6 (1179 base pairs) (b) Inferred amino acid sequence of FsTRAF6 (392 amino acids).

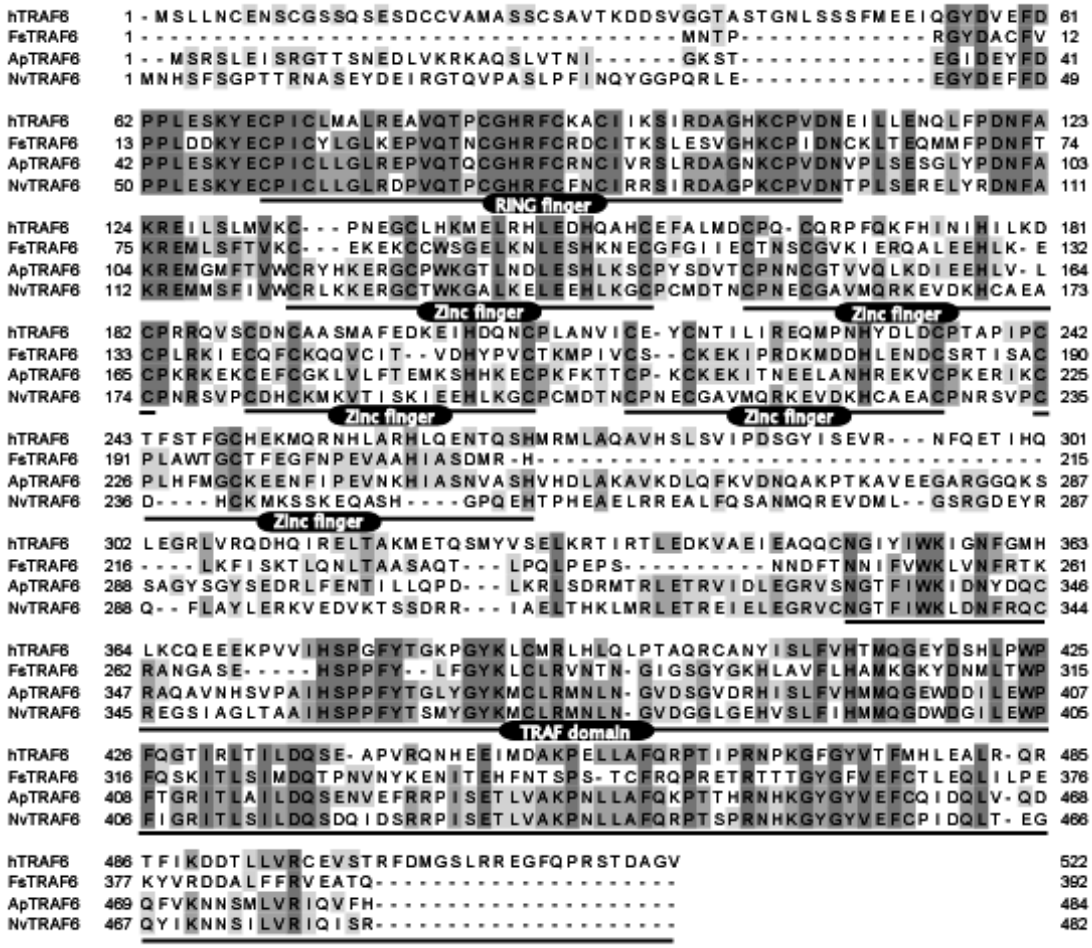

Fig. 4.5. Amino acid alignment of cnidarian TRAF6 sequences with hTRAF6. Positions where 100% of the residues are the same, 75% are the same, or 50% are the same are indicated in dark grey boxes, medium grey boxes or light grey boxes, respectively. Each domain is underlined and the type of domain is indicated.

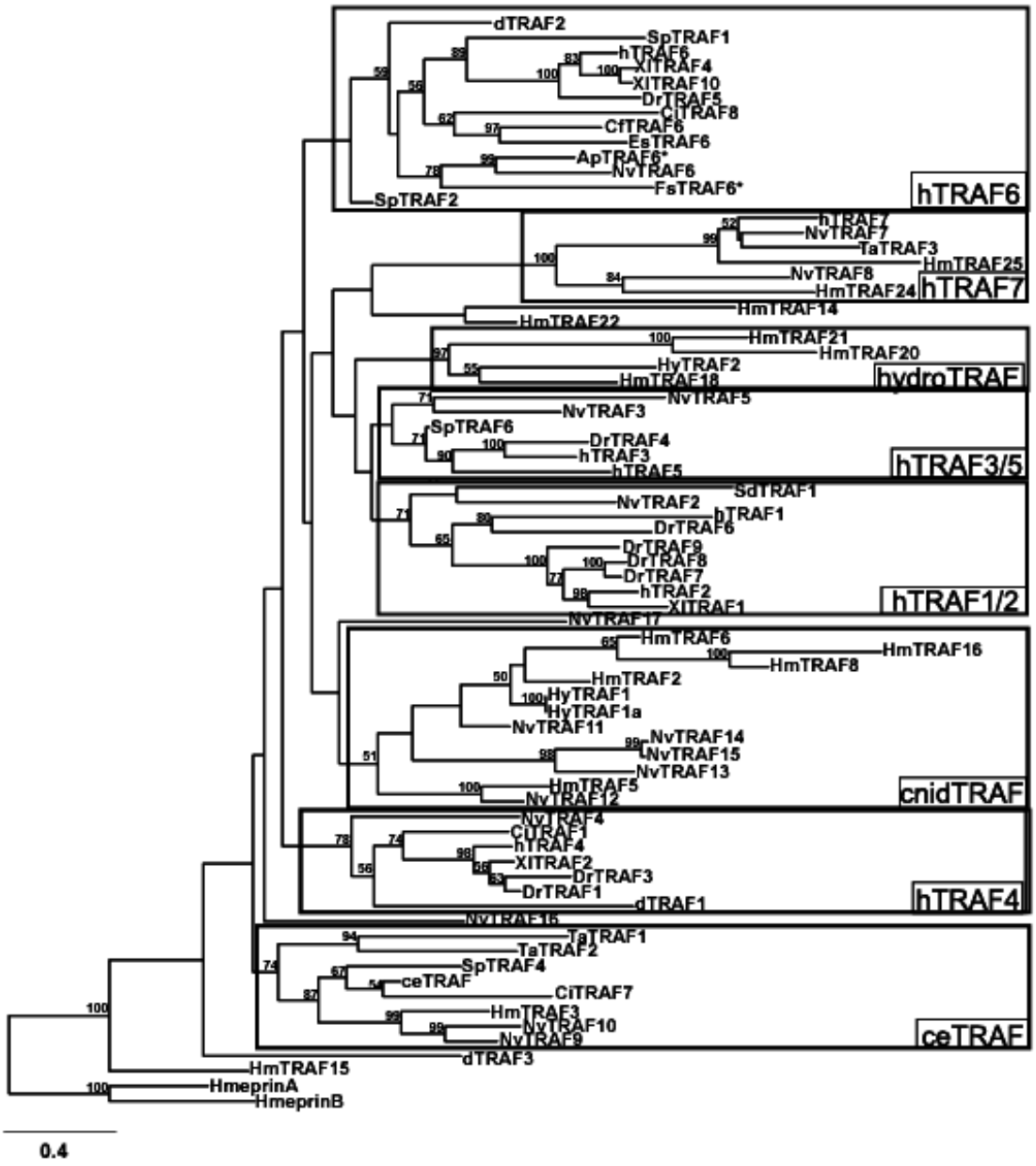

Fig. 4.6. Phylogenetic tree reconstructing relationships among TRAFs based on the full-length amino acid alignment. Bootstrap support values >50% are labeled at nodes. There are 8 major lineages of TRAFs indicated by boxes. HmeprinA and HmeprinB were used to root the tree.

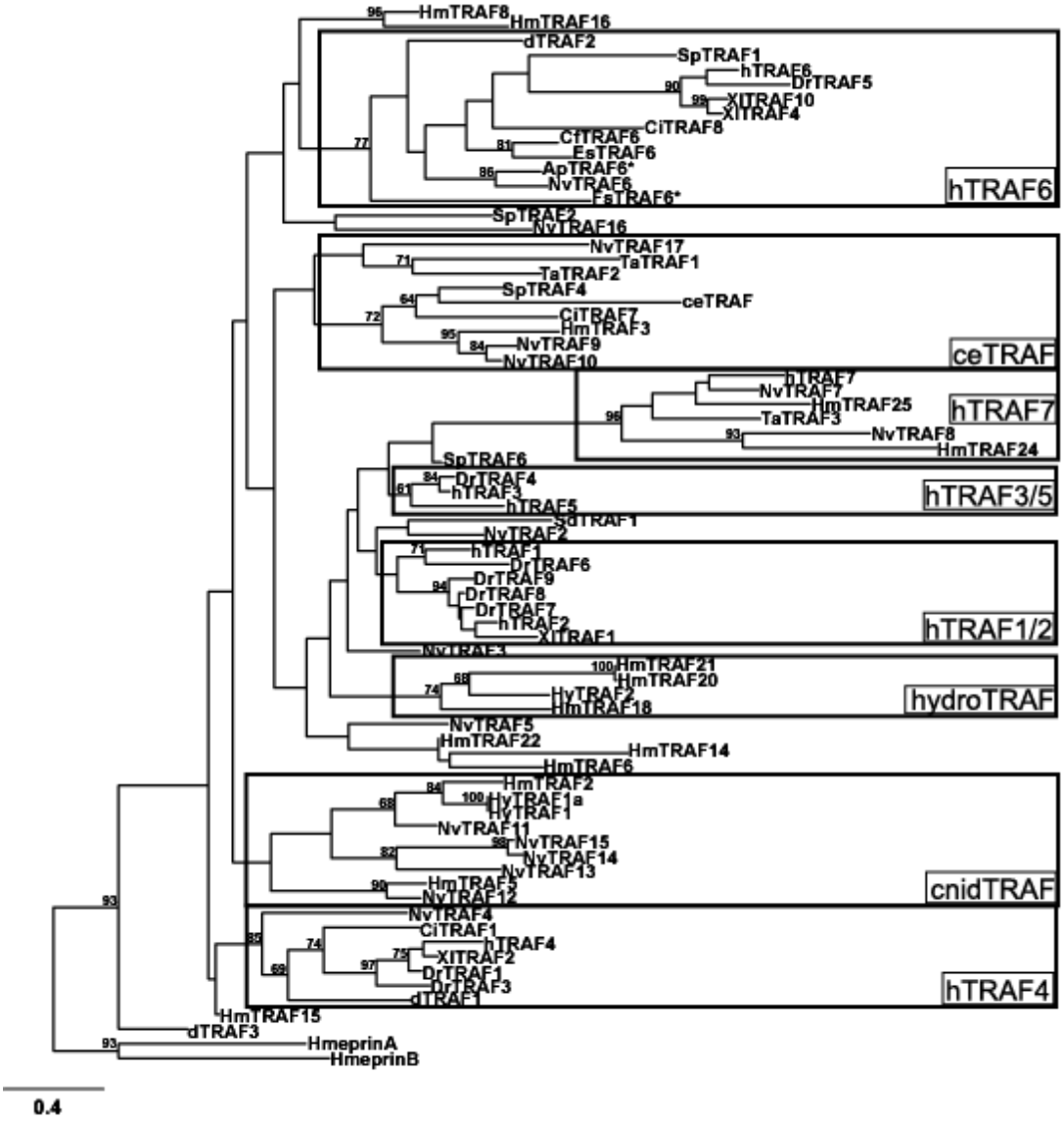

Fig. 4.7. Phylogenetic tree reconstructing relationships among TRAFs based on an amino acid alignment of the TRAFd only. Bootstrap support values >50% are labeled at nodes. There are 8 major lineages of TRAFs indicated by boxes. HmeprinA and HmeprinB were used to root the tree.

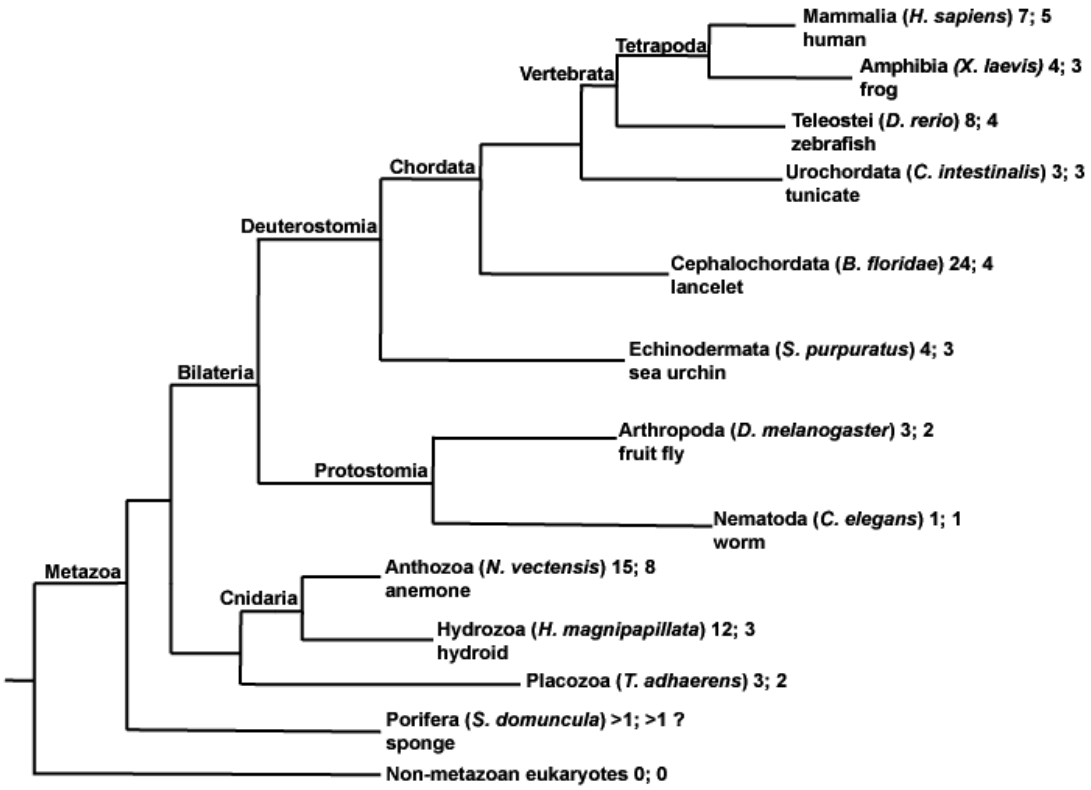

Fig. 4.8. Evolution of metazoan TRAFs. Evolutionary relationships after Dunn et al. (2008) (branch lengths are approximate). The total number of TRAFs is listed after the taxon name followed by the number of TRAF lineages represented by that given taxon. There is a question mark after the number of sponge TRAF sequences because a sponge genome has not yet been released.

Table 4.1. TRAF sequences included in phylogenetic analyses.

Species name	**Sequence name**	**GenBank Accession ID**
Suberites domuncula (sponge)	SdTRAF	CAH04636
Trichoplax adhaerens (placazoan)	TaTRAF1	XP_002113634
	TaTRAF2	XP_002113632
	TaTRAF3	XP_002107658
Hydractinia echinata (hydrozoan cnidarian)	HyTRAF1	CAE88928
	HyTRAF1a	Mali and Frank 2004
	HyTRAF2	U. Frank (pers. comm.)
Hydra magnipapillata (hydrozoan cnidarian)	HmTRAF2	XP_002156351
	HmTRAF3	XP_002170576
	HmTRAF5	XP_002160084
	HmTRAF6	XP_002165691
	HmTRAF8	XP_002156789
	HmTRAF14	XP_002160384
	HmTRAF15	XP_002158239
	HmTRAF16	XP_002164212
	HmTRAF18	XP_002157792
	HmTRAF20	XP_002159365
	HmTRAF21	XP_002169387
	HmTRAF22	XP_002153777
	HmTRAF24	XP_002164816
	HmTRAF25	XP_002157096
Aiptasia pallida (anthozoan cnidarian)	ApTRAF6	
Fungia scutaria (anthozoan cnidarian)	FsTRAF6	
Nematostella vectensis (anthozoan cnidarian)	NvTRAF2	XP_001630936
	NvTRAF3	XP_001641527
	NvTRAF4	XP_001629813
	NvTRAF5	XP_001627337
	NvTRAF6	XP_001640836
	NvTRAF7	XP_001640067
	NvTRAF8	XP_001629152
	NvTRAF9	XP_001624951
	NvTRAF10	XP_001624949
	NvTRAF11	XP_001641608
	NvTRAF12	XP_001632728
	NvTRAF13	XP_001640457
	NvTRAF14	XP_001623792

Table 4.1. (Continued)

Species name	**Sequence name**	**GenBank Accession ID**
Nematostella vectensis (anthozoan cnidarian)	NvTRAF15	XP_001623790
	NvTRAF16	XP_001637978
	NvTRAF17	XP_001633306
Drosophila melanogaster	dTRAF1 (dTRAF4)	AAG21891
	dTRAF2 (dTRAF6)	AAD34345
	dTRAF3	NP_727976
Caenorhabditis elegans	ceTRAF	NP_499773
Chlamys farreri (sea scallop)	CfTRAF6	ABC73694
Euprymna scolopes (squid)	EsTRAF6	AAY27978
Strongylocentrotus purpuratus (sea urchin)	SpTRAF1	XP_001176631
	SpTRAF2	XP_001180056
	SpTRAF4	XP_001192301
	SpTRAF6	XP_001189633
Ciona intestinalis (urochordate)	CiTRAF1	NP_001123338
	CiTRAF2	NP_001071977
	CiTRAF8	NP_001071979
Danio rerio (zebrafish)	DrTRAF1	NP_991325
	DrTRAF3	NP_997982
	DrTRAF4	NP_001003513
	DrTRAF5	NP_001038217
	DrTRAF6	NP_001121853
	DrTRAF7	XP_001344501
	DrTRAF8	XP_001924047
	DrTRAF9	XP_683631
Xenopus laevis (frog)	XlTRAF1	NP_001084921
	XlTRAF2	NP_001087501
	XlTRAF4	NP_001089863
	XlTRAF10	NP_001086348
Homo sapiens	hTRAF1	NP_005649
	hTRAF2	NP_066961
	hTRAF3	NP_003291
	hTRAF4	NP_004286
	hTRAF5	NP_004610
	hTRAF6	NP_004611
	hTRAF7	NP_115647
	HmeprinA	NP_005579
	HmeprinB	Q16820

Table 4.2. Primers used to amplify TRAF6 from *Aiptasia pallida* and *Fungia scutaria*.

Species-Fragment	Primer Name	Primer Sequence (fold degeneracy)	Tm (°C)	Product length (bp)
A. pallida-middle	CnidTRAF6_F CnidTRAF6_R1 CnidTRAF6_R2	5′-GAC AAC TTY RCM AAG CGR GAA ATG-3′ (16) 5′-GYY KGW ATA RAA DGS YGG DSW GTG-3′ (4608) 5′-RTA SCC ATA GCC KTT GTR GTT RCG-3′ (32)	 52 52	 802 1072
A. pallida-5′end	AipTRAF_OUTER5′RACE AipTRAF_INNER5′RACE	5′-GGA CAT GTG ACG TCA CTA TAT GGG C-3′ 5′-CCA GAT CGT TTA GTG TTC CTT TCC ATG GGC AAC CTC-3′	55 60	 453
A. pallida-3′end	AipTRAF_OUTER3′RACE_2 AipTRAF_INNER3′RACE_2	5′-CCC GAT TTG AAG AGA TTG AGT GAC-3′ 5′-GGC GTG TAT CCA ATG GGA CAT TTA TTT GGA A-3′	55 60	 963
A. pallida-full-length	AipTRAF_f AipTRAF_r	5′-ATG AGT CGT TCC CTA GAA ATT AG-3′ 5′-TCA GTG GAA AAC TTG AAT ACG-3′	 50	 1455
F. scutaria-middle	TRAF6_universal_F1 TRAF6_universal_R1	5′-ACR GAR TGY GGH CAY CGK TWB TGC-3′ (576) 5′-VCG VRN GCA CAK YTT GTA VCC ATA-3′ (864)	 55	 662
F. scutaria-5′end	FunTRAF_OUTER5′RACE FunTRAF_INNER5′RACE	5′-ACA TTC GAT TAT GCC AAA TCC GCA C-3′ 5′-CAA TTC CCC CGA CCA ACA ACA CTT CTC TTT TTC ACA-3′	54 60	 342
F. scutaria-3′end	FunTRAF_OUTER3′RACE_2 FunTRAF_INNER3′RACE_2	5′-CAC TGC AAA ATT TAA CTG CGG C-3′ 5′-TTT GGA AGC TTG AAA ACT TTC GAA CCA AGC-3′	55 60	 877
F. scutaria-full-length	FunTRAF_f1 FunTRAF_4	5′-ATG AAT ACT CCA CGT GGT TA-3′ 5′-TCA CTG AGT TGC CTC AAC-3′	 50	 1182

Table 4.3. Genomic location and structure of NvTRAFs according to the JGI draft genome assembly.

Protein name	Scaffold location	# Exons	gDNA length (bp)
NvTRAF2	111: 354498-359258	6	4761
NvTRAF3	3: 46148-52844	6	6697
NvTRAF4	133: 58913-71157	8	12245
NvTRAF5	198: 291081-294822	3	3742
NvTRAF6	6: 266006-280023	6	14018
NvTRAF7	10: 556788-576706	21	19919
NvTRAF8	149: 497249-506567	11	9319
NvTRAF9	297: 14085-22957	10	8873
NvTRAF10	297: 361-9615	9	9255
NvTRAF11	3: 19777779-1987527	7	9749
NvTRAF12	82: 384433-388529	7	4097
NvTRAF13	8: 996203-1005949	6	9747
NvTRAF14	374: 175185-180602	6	5418
NvTRAF15	374: 133861-151977	6	18111
NvTRAF16	25: 149932-156733	9	6802
NvTRAF17	74: 772306-787354	9	15049

Table 4.4. Genomic sequence evidence for TLR pathway components in *Aiptasia pallida* from AiptasiaBase and other sources. ND=no significant BLAST hits in AiptasiaBase.

Gene Name	**UniSeq in AiptasiaBase or other source**	**Species of highest BLAST hit**	**E-value of top BLAST hit**	**GenBank Accession # of top BLAST hit (corresponding NvTRAF)**
LPS-binding protein (LBP)	CCAS5500.g1	*Homo sapiens*	7.0e-06	P18428
TLR	ND	ND	ND	ND
MyD88	ND	ND	ND	ND
IRAK	ND	ND	ND	ND
TRAF	CCAS1367.g1 CCAS1367.b1	*Nematostella vectensis*	1.0e-61 2.0e-39	XP_001624951 (NvTRAF9)
	CCAS2450.g1 CCAS2450.b1	*N. vectensis*	1.0e-29 1.0e-24	XP_001630936 (NvTRAF2)
	CCAS648.b1	*Branchiostoma belcheri*	4.0e-23	ABN04151
	CCAS4496.g1 CCAS4496.b1	*N. vectensis*	1.0e-37	XP_001630936 (NvTRAF2)
	Contig1292	*B. belcheri*	1.0e-26	ABN04151
	CCAS6380.g1	*B. belcheri*	5.0e-13	ABN04151
	CCAS4880.g1	*N. vectensis*	4.0e-20	XP_001641527 (NvTRAF3)
	CCAS6010.g1 CCAS6010.b1	*N. vectensis*	3.0e-35 0.36	XP_001629813 (NvTRAF4)
	CCAS6514.g1	*Sus scrofa*	3.0e-08	CAE51070
CYLD	CCAS6027.g1	*N. vectensis*	2.0e-24	XP_001622527
TAK1	ND	ND	ND	ND
IκB	Contig749	*N. vectensis*	2.0e-70	ABU48531
NF-κB	Sequence cloned (N. Traylor-Knowles, pers. comm.)	ND	ND	ND

Table 4.5. Results of Ka/Ks analysis and significance testing for selection among NvTRAFs.

Sequence pair	Full-length or TRAFd	Ka/Ks	Test used	p value
NvTRAF14-NvTRAF15	Full-length	1.708	z test of positive selection: Nei-Gojobori	0.178
			Fisher's exact test: Nei-Gojobori	0.297
			Fisher's exact test: modified Nei-Gojobori	0.132
	TRAFd	1.563	z test of positive selection: Nei-Gojobori	0.252
			Fisher's exact test: Nei-Gojobori	0.427
			Fisher's exact test: modified Nei-Gojobori	0.277
NvTRAF9-NvTRAF10	TRAFd	0.069	z test of negative selection: Nei-Gojobori	0.00
NvTRAF13-NvTRAF14	TRAFd	0.376	z test of negative selection: Nei-Gojobori	0.00
NvTRAF13-NvTRAF15	TRAFd	0.355	z test of negative selection: Nei-Gojobori	0.00

Table 4.6. Details of TRAF6 sequences from *Aiptasia pallida, Fungia scutaria* and *Nematostella vectensis*. Amino acid lengths do not include the stop codon.

Sequence name	Nucleotide length (bp)			Amino acid length	Predicted isoelectric point	Predicted molecular weight (kDa)
	5′UTR	Coding	3′UTR			
ApTRAF6	20	1455	444	484	8.33	55.41
FsTRAF6	15	1179	404	392	7.40	44.86
NvTRAF6	29	1446	608	482	6.76	55.14

Table 4.7. Pairwise amino acid identity of ApTRAF6, FsTRAF6 and other TRAF6 proteins. Comparisons using full-length sequence alignments are shown in the upper right and comparisons with alignments of the TRAFd only are in the lower left. Percent similarity for comparisons of interest are shown in parentheses. Abbreviations: T= TRAF, Ap= *Aiptasia pallida* Fs= *Fungia scutaria*, Nv= *Nematostella vectensis*, h= human, Es= *Euprymna scolopes*, Cf= *Chlamys farreri*, Sp= *Strongylocentrotus purpuratus*, Ci=*Ciona intestinalis*, Xl=*Xenopus laevis*, Dr=*Danio rerio*, d=*Drosophila*.

TRAF	ApT6	FsT6	NvT6	hT6	EsT6	CfT6	SpT1	CiT8	XlT10	XlT4	DrT5	dT2
ApT6		34 (50)	50 (64)	34 (47)	33	30	23	27	31	30	30	20
FsT6	39 (66)		30 (45)	26 (40)	25	22	20	20	25	25	25	18
NvT6	72 (85)	39 (65)		32 (45)	30	29	20	25	30	30	30	21
hT6	42 (54)	30 (46)	40 (55)		28	29	22	28	63	63	55	20
EsT6	54	36	52	38		42	25	33	28	28	28	16
CfT6	53	35	52	39	71		21	30	27	2	29	17
SpT1	44	34	41	39	43	44		23	22	23	22	14
CiT8	44	28	41	36	48	50	42		26	27	26	15
XlT10	42	29	38	73	40	37	35	34		89	51	20
XlT4	41	28	40	73	38	37	36	34	89		52	20
DrT5	38	29	35	66	38	37	35	34	62	63		19
dT2	31	24	29	27	27	29	30	26	25	26	26	

Temperature stress, gene expression, and innate immunity at the onset of cnidarian-dinoflagellate symbiosis

Chapter 5: Conclusion

The endosymbiosis between cnidarians and their dinoflagellate symbionts represents one of the only examples of a mutualism between an animal cell and a unicellular eukaryote (Edmunds and Gates 2003; Schwarz 2008). As a result, much less is known about the molecular and cellular mechanisms that regulate the cnidarian-dinoflagellate association than those that function in animal-parasite and animal-beneficial bacteria interactions. The field of cnidarian-dinoflagellate symbiosis biology seems poised for a breakthrough (Weis et al. 2008). Investigators are starting to draw parallels with other better-studied host-microbe interactions, and genomic resources for cnidarians are rapidly expanding (reviewed in Chapter 1). One major research area that has exploded lately is the discovery and characterization of genes expressed in host cnidarians exposed to various conditions by performing comprehensive transcriptional profiling (Rodriguez-Lanetty et al. 2006; Forêt et al. 2007; Desalvo et al. 2008; Richier et al. 2008; Schwarz et al. 2008; Meyer et al. 2009; Reyes-Bermudez et al. 2009; Rodriguez-Lanetty et al. 2009; Sunagawa et al. 2009; Vidal-Dupiol et al. 2009; Voolstra et al. 2009a; Voolstra et al. 2009b; DeSalvo et al. 2010).

In this dissertation, I focused on the onset of cnidarian-dinoflagellate symbiosis by exploring three key areas of research identified as fundamental to furthering our understanding of the association (Weis et al. 2008). The first area is the role of symbiont invasion in establishment, regulation and breakdown of the symbiosis. I explored this topic by investigating ability of coral larvae to establish symbiosis while

exposed to elevated seawater temperature, a stressor that is known to overwhelm the photosynthetic apparatus of dinoflagellates and lead to coral bleaching (Chapter 2). Experiments on coral larvae demonstrated that elevated temperature significantly affected their ability to establish a stable symbiosis. The second area I addressed was identifying the host genes and cellular pathways involved in symbiosis onset. I investigated this by using a cDNA microarray to profile transcripts in coral larvae differentially expressed during the early stages of symbiosis (Chapter 3). This approach resulted in few host transcripts changing as a result of homologous infection. I discuss how the symbionts may be suppressing or manipulating host gene expression by drawing parallels with mechanisms employed by parasites invading animal cells. The final topic I explored was the host innate immune response to symbiont colonization. I used genomic resources to explore the TRAF gene family, which functions in mammalian innate immune systems, in cnidarians (Chapter 4). I also cloned, sequenced and analyzed full-length TRAF6 genes (a key immune pathway component) from two model symbiotic cnidarians, paving the way for functional studies that can consider the role of this gene in regulating cnidarian-dinoflagellate symbiosis. The results from this dissertation further our understanding of how cnidarians and their photosynthetic dinoflagellate partners establish and maintain their productive relationship, and highlight how sensitive the holobiont is to environmental stress, which is especially important in the face of global climate change and rising seawater temperatures.

Future studies that could extend the work presented in this dissertation include:

1. Additional experiments that quantify infection levels in coral larvae exposed to various stressors. Experiments that quantify oxidative stress levels (e.g. ROS, RNS) and measure antioxidant enzymatic activity (e.g. SOD, catalase) in aposymbiotic and symbiotic larvae exposed to various stressors. My attempts to quantify antioxidant activity in *F. scutaria* larvae were stymied by my inability to collect enough protein material from larvae exposed to elevated

temperatures to be used with commercial SOD and catalase kits. I also attempted to perform live-cell imaging of oxidative stress in coral larvae exposed to temperature stress by using membrane-diffusible molecular dyes that fluoresce in the presence of either ROS or nitric oxide production. This method proved difficult to use for many reasons, but especially because *F. scutaria* larvae have a natural green autofluorescence that interfered with visualization and quantification of oxidative stress signals. Future studies could use dyes that fluoresce at wavelengths that do not overlap with either the coral larval or dinoflagellate autofluorescence. Also, I used an epifluorescence microscope for my trials and the imaging would be much better visualized with confocal microscopy.

2. Additional transcriptional profiling of the onset of symbiosis. Considering the many disadvantages of microarray methods (discussed in Chapter 1), I would recommend trying an alternative method. RNAseq, or whole transcriptome sequencing, using next generation sequencing techniques, is gaining in popularity. With the release of a coral genome assembly, it will be easier to assemble reads from RNAseq experiments (having an existing genome sequence for assembly is much simpler than *de novo* assembly). Experiments that compare transcriptome expression of coral larvae infected by different *Symbiodinium* sp. types seems especially promising.
3. Functional studies of TRAF6 in regulating cnidarian-dinoflagellate symbiosis:
    a. Q-RT-PCR experiments to quantify TRAF6 gene expression in cnidarians exposed to various conditions including: (1) *F. scutaria* larvae that are uninfected, infected with the homologous symbiont type (*Symbiodinium* sp. C1f), or infected with a heterologous symbiont type (*Symbiodinium* sp. C31). The predicted outcome is that the highest TRAF6 expression will be seen in larvae infected with a heterologous symbiont type because heterologous infection should upregulate the pro-inflammatory TLR pathway. I have already performed this

experiment and am in the process of developing multiple housekeeping genes for *F. scutaria* to carry out the Q-RT-PCR. (2) *A. pallida* adults with and without symbionts. It would be interesting to see if TRAF6 expression differs between these two states.

b. RNAi experiments to knock down TRAF6 expression in cnidarians including: (1) Microinjecting TRAF6 dsRNA or control gene dsRNA into *F. scutaria* embryos and then performing Q-RT-PCR on samples collected at various time points to quantify the knockdown effect. This will determine if RNAi using dsRNA can be successfully employed in this system. A preliminary version of this experiment was completed last summer in collaboration with Mark Martindale at the University of Hawaii's Kewalo Marine Lab. Only a few larvae survived the microinjection treatment and this material awaits Q-RT-PCR.
(2) Microinjecting TRAF6 dsRNA into *F. scutaria* embryos, then infecting the resulting larvae with different symbiont types and comparing the infection success of larvae that have been exposed to TRAF6 dsRNA and larvae that have been exposed to control gene dsRNA. The expectation is that infection levels would increase in all larvae where TRAF6 expression was inhibited. Q-RT-PCR would also be used to quantify the knockdown effect. This will require some technical tinkering as *F. scutaria* embryos seem fairly sensitive to microinjection manipulations.

c. Infection experiments of *A. pallida* or *F. scutaria* with different types of *Symbiodinium* sp. after pre-exposure to an anti-TRAF6 blocking antibody or blocking peptide. The expectation is that if TRAF6 is blocked, infection rates of all symbiont types will increase compared to controls. Initial trials of this experiment with *A. pallida* resulted in no differences in infection between anemones exposed to a blocking antibody or peptide and controls. Infections were performed with

Symbiodinium sp. B1 (Culture ID: CCMP830) and quantified using confocal microscopy. Infection was quantified at a single time point, 72 h post-infection. It is not clear if the antibody and blocking peptides that were used were at an appropriate concentration (3 concentrations were tested for each) or if the antibody or the blocking peptide (which were designed from mammalian TRAF6 sequences) were specific enough to effectively block ApTRAF6. Future experiments could explore these issues and test additional antibodies or other methods of blocking TRAF6 protein expression.

d. Analyses of TRAF6 protein expression and activation in *F. scutaria* and/or *A. pallida* including immunoprecipitation and western blotting of TRAF6 from animals exposed to various conditions, including lipopolysaccharide (LPS) stimulation, infection with various *Symbiodinium* sp. types, or other immune stimulators. My undergraduate research intern, Daryl Khaw, spent last summer trying to get this type of experiment working in *A. pallida*. Ultimately, he found slightly increased TRAF6 expression in LPS-stimulated aposymbiotic *A. pallida* compared to aposymbiotic *A. pallida*, symbiotic *A. pallida*, and LPS-stimulated symbiotic *A. pallida*. However, he was unable to repeat this result in subsequent trials. It is not clear if the antibody used (designed from mammalian TRAF6 sequences) was specific enough to ApTRAF6. Additional future experiments could involve immunoprecipitation using an anti-TRAF6 antibody followed by western blotting with an anti-ubiquitin antibody to see if the TRAF6 protein the sample was activated via polyubiquitination, as has been seen in other systems.

4. Further genomic exploration of cnidarian innate immune pathways (e.g. TLR, complement, TGFβ) and the numerous gene families involved in those pathways. As genomic resources expand, the ability to identify genes that may

function in regulating cnidarian-dinoflagellate associations will certainly increase. A comparative genomics approach, such as the one I took with the TRAF gene family, will be useful in highlighting genes that are homologs to those found in vertebrates, for which there are substantially more functional data.

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