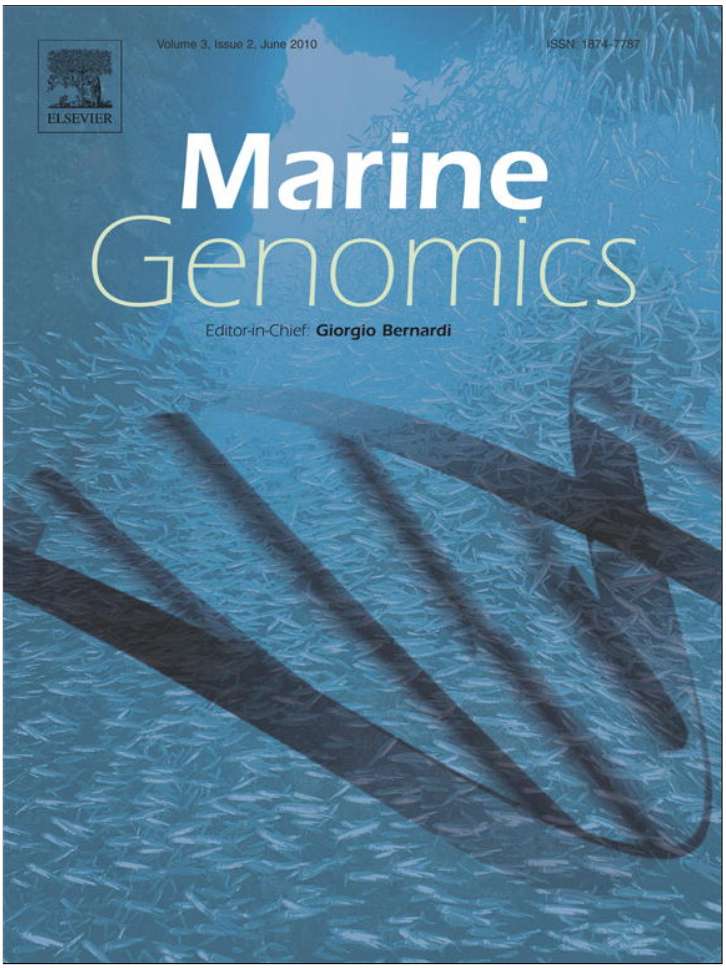

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Coral larvae exhibit few measurable transcriptional changes during the onset of coral-dinoflagellate endosymbiosis

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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 endosymbiosis due to the predictable availability of gametes, the ability to raise non-symbiotic larvae and establish the symbiosis experimentally, and the ability to precisely quantify infection success. The goal of this study was to identify genes differentially expressed in *F. scutaria* larvae during the initiation of endosymbiosis with *Symbiodinium* sp. C1f. Newly symbiotic larvae were compared to non-symbiotic larvae using a custom cDNA microarray. The 5184-feature array was constructed with cDNA libraries from newly symbiotic and non-symbiotic *F. scutaria* larvae, including 3072 features (60%) that were enriched for either state by subtractive hybridization. Our analyses revealed very few changes in the *F. scutaria* transcriptome as a result of infection with *Symbiodinium* sp. C1f, similar to other studies focused on the early stages of this symbiotic interaction. We suggest that these results may be due, in part, to an inability to detect the transcriptional signal from the small percentage of infected cells compared to uninfected cells. We discuss several other potential explanations for this result, including suggesting that certain types of *Symbiodinium* sp. may have evolved mechanisms to suppress or circumvent cnidarian host responses to infection.

1. 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 endosymbiosis forms the structural and trophic basis of biodiverse coral reef ecosystems. The coral-dinoflagellate endosymbiotic relationship is a mutualistic 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 (Richmond and Hunter, 1990; Harrison and Wallace, 1990; Fadlallah, 1983).

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 (McFall-Ngai et al., 2010; Hooper, 2009; Silver et al., 2007; Feldhaar and Gross, 2008).

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 (Vidal-Dupiol et al., 2009; Lin et al., 2000; Wood-Charlson et al., 2006; Koike et al., 2004). 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 *Symbiodinium* sp. (Rodriguez-Lanetty et al., 2006a). 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, 2004, 2005). Host cell apoptosis has been reported as a

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post-phagocytic mechanism that removes *Symbiodinium* sp. types that do not successfully colonize hosts (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.

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 (Moya et al., 2008; Weis and Reynolds, 1999); 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. (2006b) used a 10,368 feature cDNA microarray with the same system to assess adult anemone gene expression profiles. This study resulted in 79 host unigenes with significantly different expression as a function of the endosymbiosis, 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 recently increased in number. 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 (Rodriguez-Lanetty et al., 2009; Desalvo et al., 2008; Grasso et al., 2008; Richier et al., 2008; Reyes-Bermudez et al., 2009; C. Voolstra et al., 2009a; Portune et al., 2010). 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). The majority of these studies, however, have not taken the endosymbiotic nature of corals into consideration. Comparative EST analyses have revealed some potential genes involved in established symbiosis (Sabourault et al., 2009; Kuo et al., 2004).

Transcriptomic and proteomic studies relating directly to the initial onset of cnidarian-dinoflagellate endosymbiosis 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 protein was consistently differentially expressed in symbiotic larvae and its identity was not determined. Dot blots were used in membrane hybridization with either a symbiosis-enriched or an aposymbiosis-enriched PCR probe. Fifty-one of 176 cDNAs exhibited at least two-fold higher expression with the symbiosis-enriched probe than the aposymbiosis-enriched probe. ESTs corresponding to six of these cDNAs were sequenced, although bioinformatic searches found significant homology to a known sequence for only one — a hypothetical protein from the parasitic flatworm, *Schistosoma japonicum* (GenBank Accession ID: AAX30965.2). 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). A recent study, however, used a new technique called high coverage gene expression profiling (HiCEP) and found 765 genes that exhibited differential expression between aposymbiotic *Acropora tenuis* juveniles and juveniles infected *Symbiodinium* (20d post-infection) (Yuyama et al., 2010). Only 33 of these genes could be annotated.

Only one other study has used array hybridization to examine expression differences between aposymbiotic and newly endosymbiotic coral larvae (Voolstra et al., 2009b). Larvae of *Acropora palmata* and *Montastraea faveolata* were infected with a cultured *Symbiodinium* sp. type previously determined to successfully establish symbioses in larvae of that coral species (competent) or a type that had previously failed to establish symbiosis in coral larvae (incompetent). Samples were taken 30 min and 6 d post-infection. Very few changes were seen in either coral's transcriptome upon infection with the competent *Symbiodinium* sp. type at both time points. In contrast, dramatic differences were found in both species at 6 d post-infection with an incompetent *Symbiodinium* sp. type (Voolstra et al., 2009b). Unfortunately, infection dynamics were not quantified in that study and relatively little is known about the onset of endosymbiosis in either of the coral species examined. Further, the rationale for choosing each of the competent and incompetent *Symbiodinium* types used in the infections and the ecological relevance of these types to the coral species in the study was not clear.

We chose to examine host transcriptional profiles during the initiation of endosymbiosis 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 endosymbiosis for more than a decade and methods for precisely quantifying infection success in this system have been established (Wood-Charlson et al., 2006; Rodriguez-Lanetty et al., 2006a; Weis et al., 2001; Schwarz et al., 1999). In Hawaii, *F. scutaria* harbors a single dinoflagellate symbiont type, *Symbiodinium* sp. C1f (Rodriguez-Lanetty et al., 2004; LaJeunesse et al., 2004). 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 endosymbiosis experimentally. We focused on changes in expression levels in the larval coral transcriptome 48 h after the initial onset of endosymbiosis with *Symbiodinium* sp. C1f. 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 *Symbiodinium* sp. C1f. Since we quantified the infection level in each sample, we can make inferences about the relative contributions from the transcriptomes of each partner in the endosymbiotic relationship.

2. Materials and methods

2.1. Collection and maintenance of coral larvae

Adult *F. scutaria* corals were collected from patch reefs in Kaneohe Bay (Oahu, Hawaii) and maintained in seawater tables at the Hawaii Institute of Marine Biology (Schwarz et al., 1999). In Hawaii, the corals broadcast spawn gametes without *Symbiodinium* sp. between 17:00 and 19:00 h, 2–4 d following the full moon during June through August (Krupp, 1983). Samples for array hybridization were collected from the July 2006 spawning event. To ensure proper biological replication, eggs from six individual females were kept in separate 3 l bowls containing 1.5 l 0.22 μm filter-sterilized seawater (FSW). Sperm from multiple males were mixed prior to fertilizing each bowl, so that eggs from each female were fertilized with sperm from multiple males. Bowls were placed in outdoor seawater 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 with a compound microscope. Four days after fertilization, larvae from each bowl were concentrated on a 50 um mesh filter, divided into two 400 ml bowls, and randomly assigned a treatment, either endosymbiotic or aposymbiotic.

2.2. Preparation of Symbiodinium sp. C1f

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 homogenized with a glass homogenizer and the mixture of host tissue and dinoflagellate cells was separated by centrifugation at 6000×*g* for 3 min. 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.

2.3. Infection of larvae with Symbiodinium

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 (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. Time = 0 h post-infection began at the end of the washing period.

2.4. 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) and stored at 4 °C for use in determining infection success. Forty-eight h after infection (t = 48 h), all six replicate samples (representing ~10^5 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×*g*, flash frozen in liquid nitrogen, stored at −80 °C, shipped to Oregon State University (OSU) on dry ice and stored at −80 °C prior to RNA isolation.

Fixed samples were rinsed 3 times in 1× PBS before examination by light microscopy. Infection success was determined by both calculating the percentage of larvae infected and quantifying the *Symbiodinium* density across 100 infected larvae (Rodriguez-Lanetty et al., 2004). Two-way ANOVA (R, ver. 2.10.1) was used to test the effects of sample and time on infection success after confirming normality and heteroscedasticity of data.

2.5. 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, 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 6 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 15 μl printing buffer (3× SSC, 1.5 M betaine). After resuspension, samples were transferred to 384-well (Genetix) plates using a Rapidplate robot (Qiagen). These samples were used to print the 5184 feature array, which included 1536 random clones from each of the subtracted cDNA libraries and 768 random clones from each of the non-subtracted cDNA libraries (Supplemental Table 1). Commercial alien DNAs (Lucidea Universal ScoreCard from Amersham Biosciences/GE Healthcare and SpotReport Alien cDNA Array Validation System from Stratagene) were spotted throughout the array for use in setting scanning parameters and normalization after hybridization. An 18×18 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.

2.6. RNA isolation and hybridization of arrays

To construct probes, total RNA was isolated from all larval samples collected in July 2006 using Trizol (Invitrogen) according to the manufacturer's instruction with slight modification. Protocol modification included the addition of the optional centrifugation step at 12,000×*g* for 10 min at 4 °C to remove polysaccharides and other contaminants. RNA samples were subsequently loaded into an RNeasy column (Qiagen) and washed to remove residual salts (Dumur et al., 2004). RNA was eluted with RNAse-free water and stored at −80 °C until use. Contamination of total RNA with *Symbiodinium* RNA in symbiotic samples is expected to be low since the average number of *Symbiodinium* cells in each infected larva was low (20 ± 6) compared to the total number of host cells in 6 day old larvae (see Results).

Total RNA purity and concentration was determined with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed by running an aliquot of each preparation on a non-denaturing 1% agarose gel and staining the gel with ethidium bromide and by running 1 μl of each RNA sample 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 reagents from the Genisphere 3DNA-900 microarray kit, according to the manufacturers' instructions. One μl of each commercial alien RNA spike mix (Lucidea Universal ScoreCard and SpotReport Alien cDNA Array Validation System) 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 h 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. Final washes were performed following hybridization of capture reagents. All hybridizations took place in a formamide-based hybridization buffer under LifterSlips in a sealed humidified chamber. All washes were as follows: 2× SSC, 0.2% SDS for 10 min at 65 °C, 2× SSC for 10 min at RT, and 0.2× SSC for 10 min at

RT. 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).

2.7. 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. Analysis of GenePix result files was accomplished using functions in the limma library of the Bioconductor package (Gentleman et al., 2004; Dudoit, 2003) within the R 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 features 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 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).

2.8. 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 for all sequences that had no significant BLASTx results. Homologs with significant E-values from the BLASTn search 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.

3. Results and discussion

3.1. 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 of the randomly sequenced 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 were most similar to proteins of undetermined function (Table 1). Not surprisingly, many of the genes were most similar to putative orthologs from the anthozoan cnidarian *Nematostella vectensis*, the closest taxon to *F. scutaria* with a complete genome sequence (Table 1). Seventy-eight of the 166 clones (47%) did not have a significant BLASTx hit and were considered unknown sequences (discussed in Section 3.4).

3.2. 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 $87.3\pm6.2\%$) and ending 48 h later (average $83.7\pm8.9\%$) (Two-way ANOVA, $p>0.05$) (Fig. 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 *Symbiodinium* sp. per larva) to $t=48$ h (average 20.3 ± 6.3 *Symbiodinium* sp. per larva) (Two-way ANOVA, $p>0.05$) (Fig. 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 (Rodriguez-Lanetty et al., 2006a; Weis et al., 2001).

3.3. 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 subtracted features on the array. 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., 2006b). The (log)fold change ranged from 0.75–2.41 (Fig. 2a), which also resembled results from the *A. elegantissima* array, where most expression ratios ranged from 1–2 (Rodriguez-Lanetty et al., 2006b). Thirty-three cDNAs were upregulated as a function of the endosymbiosis and 9 cDNAs were downregulated (Fig. 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-value $<10^{-4}$) with homologs to known genes (Table 2, Fig. 2b). Ten had a significant BLASTn hit to the EST database (Table 3). The top EST sequence from the BLASTn search was then used as a query in a BLASTx search. 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.

3.4. 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 (Tables 1–3). Nearly half of the sequences from the redundancy sequencing and over half (10/17) from the expression study (Fig. 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% (Rodriguez-Lanetty et al., 2006b; Richier et al., 2008; Sabourault et al., 2009; Kuo et al., 2004). 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

Table 1
Functional annotation of redundancy check sequences with significant hits (E-value$<10^{-04}$) from BLASTx searches using categories from GO and UniProtKB. All *Fungia scutaria* GenBank Accession IDs are from the dbEST database.

Functional category	GenBank Accession ID (dbEST)	E-value of closest homolog from BLASTx search	Species of closest homolog from BLASTx search	GenBank Accession ID of closest homolog from BLASTx search
Metabolic process				
S-adenosylmethionine synthetase	HO054896; HO054907	2e−79; 3e−76	*Nematostella vectensis*	XP_001629913
S-adenosyl-L-homocysteine hydrolase	HO054914; HO054918	2e−105; 9e−55	*N. vectensis*	XP_001639319
Cytosolic malate dehydrogenase	HO054917	3e−08	*Strongylocentrotus*	XP_796283
GCN5-related N-acetyltransferase (GNAT)	HO054916	4e−05	*N. vectensis*	XP_001628360
Cholesterol and lipid metabolism				
Vigilin/High density lipoprotein binding protein	HO054952	5e−77	*Saccoglossus kowalevskii*	XP_002735964
Nucleic acid metabolism				
Histone H3	HO054906	4e−60	*Oncorhynchus mykiss*	ACO08255
Protein metabolism				
Ribosomal protein L10	HO054934	8e−51	*Haliotis discus discus*	ABO26700
Ribosomal protein L5e	HO054901	2e−20	*N. vectensis*	XP_001634531
Ribosomal protein S8e	HO054948	7e−82	*N. vectensis*	XP_001622696
Ribosomal protein S27e	HO054905	3e−20	*N. vectensis*	XP_001633500
Translation initiation factor eIF-5A	HO054922	2e−07	*Xenopus laevis*	NP_001080536
Translation elongation factor EFTu/EF1A, domain 2	HO054923	8e−48	*N. vectensis*	XP_001631027
Elongation factor 1 alpha	HO054953	1e−107	*Microciona prolifera*	AAZ30676
Purine salvage				
Adenosine kinase	HO054941	9e−80	*N. vectensis*	XP_001635774
RNA binding				
Polyadenylate-binding protein 1	HO054930	4e−48	*N. vectensis*	XP_001625306
RNA-dependent DNA replication				
Endonuclease/reverse transcriptase	HO054936	8e−14	*Sus scrofa*	ABR01162
Pol-like protein	HO054938	4e−09	*Ciona intestinalis*	BAC82623
Cell cycle				
Putative senescence-associated protein	HO054946	4e−31	*Pisum sativum*	BAB33421
Cell growth and maintenance				
Beta tubulin	HO054929	1e−35	*Tribolium castaneum*	XP_969993
Cell growth regulation				
Granulin protein	HO054913	2e−55	*S. kowalevskii*	XP_002739994
Cell signaling				
Guanine nucleotide binding protein (GTPase)	HO054928	9e−34	*N. vectensis*	XP_001631784
Cytoskeleton structure and reorganization				
Actin	HO054949	3e−84	*Favites chinensis*	BAC44869
Intracellular protein transport				
Coatomer protein	HO054924	1e−30	*N. vectensis*	XP_001631811
Tpr (translocated promoter region) protein	HO054945	5e−52	*S. purpuratus*	XP_784812
Transport/transmembrane transport				
ADP/ATP transporter on adenylate translocase	HO054910	1e−50	*N. vectensis*	XP_001639131
Proteolysis and peptidolysis				
Peptidase M16	HO054902	7e−21	*N. vectensis*	XP_001632139
Immune response				
Complement C3	HO054915	1e−45	*Acropora millepora*	ABK78771.2
Undetermined				
Hypothetical protein	HO054931	1e−07	*Pan troglodytes*	XP_001135501
Conserved hypothetical protein	HO054908	7e−28	*Pediculus humanus corporis*	XP_002426744
Hypothetical protein ACLA_028940	HO054935	3e−11	*Aspergillus clavatus*	XP_001269594
Hypothetical protein SORBIDRAFT_05g016450	HO054926	1e−17	*Sorghum bicolor*	XP_002449484
NEMVEDRAFT_v1g155763	HO054942	3e−22	*N. vectensis*	XP_001618098
NEMVEDRAFT_v1g157418	HO054951	3e−23	*N. vectensis*	XP_001617522
NEMVEDRAFT_v1g68210	HO054911	2e−25	*N. vectensis*	XP_001622139
NEMVEDRAFT_v1g248425	HO054920	1e−05	*N. vectensis*	XP_001622460
NEMVEDRAFT_v1g247499	HO054944	2e−22	*N. vectensis*	XP_001624711
NEMVEDRAFT_v1g217095	HO054937	6e−11	*N. vectensis*	XP_001625221
NEMVEDRAFT_v1g245535	HO054925	5e−36	*N. vectensis*	XP_001628457
NEMVEDRAFT_v1g114138	HO054947	2e−43	*N. vectensis*	XP_001630346

(*continued on next page*)

Table 1 (*continued*)

Functional category	GenBank Accession ID (dbEST)	E-value of closest homolog from BLASTx search	Species of closest homolog from BLASTx search	GenBank Accession ID of closest homolog from BLASTx search
Undetermined				
NEMVEDRAFT_v1g142320	HO054900	2e−11	*N. vectensis*	XP_001622138
NEMVEDRAFT_v1g211211	HO054940	5e−96	*N. vectensis*	XP_001629792
NEMVEDRAFT_v1g95124	HO054943	2e−22	*N. vectensis*	XP_001636478
NEMVEDRAFT_v1g178205	HO054909	3e−07	*N. vectensis*	XP_001641154
Predicted protein	HO054897	2e−22	*Trichoplax adhaerens*	XP_002118239
SJCHGC09657	HO054939	1e−14	*Schistosoma japonicum*	AAX30965.2
Unknown protein	HO054912	6e−19	*Picea sitchensis*	ABR16542
Hypothetical protein LOC100382981	HO054899	6e−23	*Zea mays*	XP_002426744

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 over 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.

3.5. Genes affected by symbiosis onset

Only 17 unigenes were identified as differentially expressed as a function of the onset of symbiosis with *Symbiodinium* sp. C1f. Of these, only a few sequences had significant hits to known sequences in public databases (Tables 2 and 3). Identified genes from the BLASTx searches were in one of three functional categories (Table 2). Beta tubulin was the only identified gene that was downregulated as a function of the endosymbiosis (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. (2006b) also found that genes involved in this category, including beta tubulin, were down-regulated as a function of the established symbiotic state. Conversely, Voolstra et al. (Voolstra et al., 2009b) found that beta tubulin was upregulated in coral larvae as a result of infection with a heterologous *Symbiodinium* sp. type. Due to their large size (~10 μm diameter), *Symbiodinium* cells take up most of the cytoplasmic space inside host

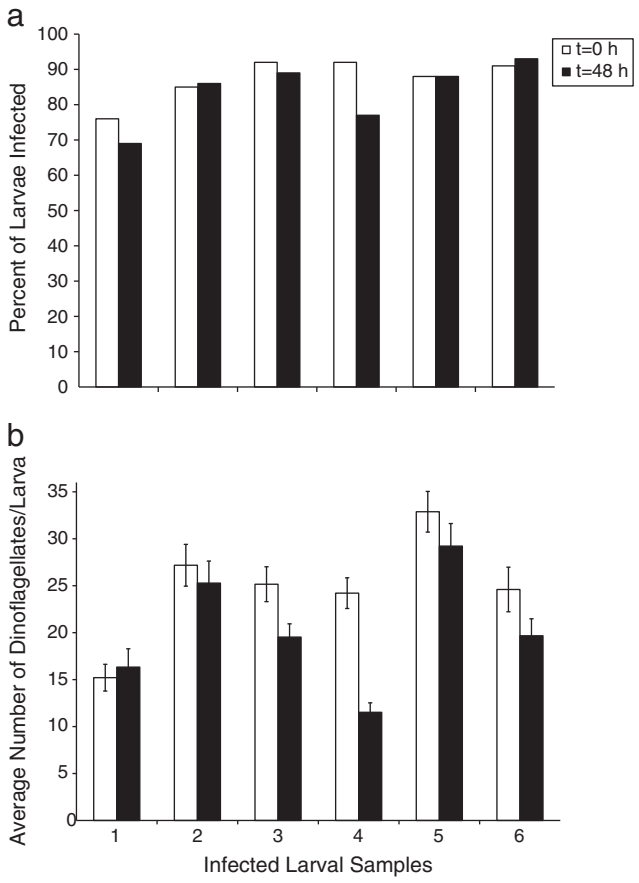

Fig. 1. Infection success by *Symbiodinium* sp. C1f in *Fungia scutaria* larvae. (a) Percent of larvae infected (n [larvae per sample] = 100), (b) density of dinoflagellates in larvae. Error bars represent standard error of the mean (n [infected larvae per sample] = 100).

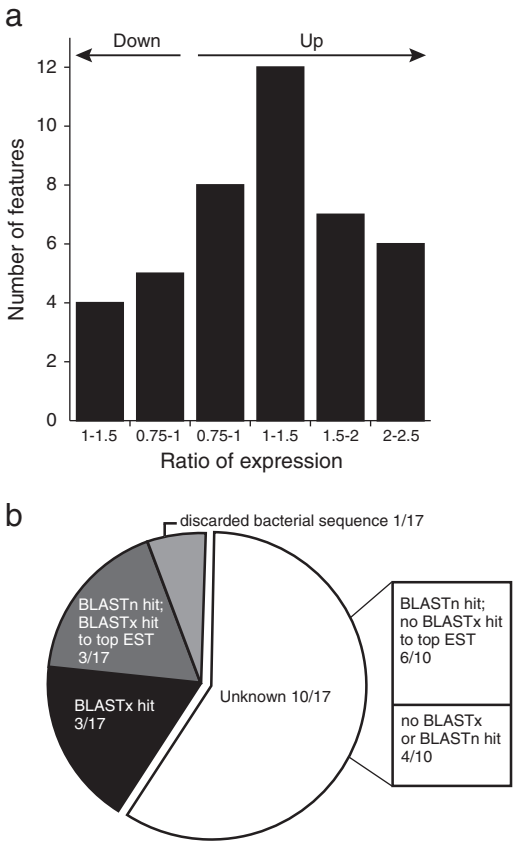

Fig. 2. Fold changes of differentially expressed features found in coral larvae as a function of symbiosis onset and distribution of BLAST hits from resulting unigenes. (a) Distribution of the fold changes of the 42 differentially expressed features. Categories are fold changes of expression of newly 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 2
Functional annotation of differentially expressed sequences with significant BLASTx hits (E-value $< 10^{-04}$) 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. All *Fungia scutaria* GenBank Accession IDs are from the dbEST database.

Functional category	GenBank Accession ID (dbEST)	Fold change	GenBank Accession ID of closest homolog
Cell growth and maintenance			
Beta tubulin	HO054921	↓ 0.79	NP_954525.1*
Protein metabolism			
Ribosomal protein L14	HO054932	↑ 0.82	XP_001631282**
Undetermined			
NEMVEDRAFT_v1g245639	HO054927	↑ 1.09	XP_001628265**

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 endosymbiosis (Rodriguez-Lanetty et al., 2006b) 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 endosymbiosis 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 endosymbiosis. Several features from the array were most similar to this protein (Tables 1 and 2). One feature (GenBank Accession ID: HO054927) 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 endosymbiosis 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.

3.6. 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 $83.7 \pm 8.9\%$ at the time of sampling. This indicates that most, but not all, larvae in the symbiotic treatment were infected and that there was a sub-population of larvae in each replicate that harbored no *Symbiodinium* sp. cells at the time of sampling. These uninfected larvae could dilute the signal from the infected larvae. Similarly, whole 6-day old larvae were sampled and each larva had an average of only 20.3 ± 6.3 *Symbiodinium* sp. cells per larva at the time of sampling. Symbiosis-related transcripts should be highest in the gastrodermal cells that host *Symbiodinium* cells. The thousands of host

Table 3
Annotation of differentially expressed sequences with significant BLASTn hits to the est_others (non-human, non-mouse EST) database. The top BLASTn hit was then used as a query in a BLASTx search. All *Fungia scutaria* GenBank Accession IDs are from the dbEST database. ~ indicates that the fold change is an average taken from multiple features on the array that had the same sequence.

GenBank Accession ID (dbEST)	Fold Change	E-value of closest homolog from BLASTn search	Species of closest homolog from BLASTn search	GenBank Accession ID of closest homolog from BLASTn search	Species of closest homolog from BLASTx search (protein name)	GenBank Accession ID of closest homolog from BLASTx search
HO054904	~↑ 1.39	2e−51	*Acropora palmata*	GW214694	*Nematostella vectensis* (NEMVEDRAFT_v1g245639)	XP_001628265
HO054950	~↑ 1.08	3e−22	*Montastraea faveolata*	GW264218	*N. vectensis* (NEMVEDRAFT_v1g242270)	XP_001634377
HO054933	↓ 0.96	4e−46	*Schistosoma japonicum*	BU779826	*S. japonicum* (SJCHGC09657)	AAX30965.2
HO054898	~↑ 1.70	8e−13	*A. palmata*	GW214695	No hit	
HO054903	~↓ 1.37	1e−23	*Symbiodinium sp.* C3	FE865106	No hit	
HO054919	↓ 1.20	3e−25	*M. faveolata*	GW272230	No hit	
HO054968	↑ 0.84	2e−62	*M. faveolata*	GW267181	No hit	
HO054967	↑ 0.82	1e−74	*M. faveolata*	GW264217	No hit	
HO054969	↓ 0.77	1e−13	*N. vectensis*	FC298242	No hit	

cells in each sample that contained no *Symbiodinium* cells may diminish any signal from the relatively few symbiotic host cells.

This signal-to-noise ratio problem is a technical issue that must be addressed in future studies. One group has made some progress along these lines by separating gastrodermal from ectodermal tissue using the reducing agent *N*-acetylcysteine (NAC) (Peng et al., 2008). The NAC treatment was able to separate tissue layers without inducing protein degradation, however, it is not clear if the RNA was intact following this treatment. If the infected gastrodermal cells could be further separated from non-infected gastrodermal cells and sampled without compromising the RNA, the transcriptional signal from infected cells would be greatly enhanced. Although we considered this issue when designing the present study, we were unable to devise a way to separate this material adequately and to get enough material for our array hybridizations. If the separation could be done, RNA amplification could be performed to generate enough material for a microarray experiment.

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. Voolstra et al. (2009b) examined expression levels 30 min and 6 d post-infection, but did not find many differences between aposymbiotic larvae and larvae infected with a *Symbiodinium* sp. type previously known to successfully colonize those larvae. It is highly possible that symbiosis-related transcripts are expressed at some other point in time that has not yet been examined.

A third possibility is that highly expressed "housekeeping" genes may be overrepresented on the array and genes expressed at relatively low levels may be underrepresented. In this scenario, changes in expression from relatively rare transcripts would not be detected since they are not represented adequately on the array. Since we did not fully sequence the array, we cannot rule out this possibility. Another possible reason for the low number of differential genes is that there may have been high genetic variation among the six biological replicates, which could have canceled out any signal coming from one or a few of the individuals.

A final hypothesis for the lack of change in gene expression by host larvae in the early stages of symbiosis establishment is that when *Symbiodinium* cells are phagocytosed by gastrodermal cells, they avoid recognition and evade rejection by the host (Voolstra et al., 2009b). Specific *Symbiodinium* types may accomplish this by actively circumventing, modifying or suppressing host responses. Alternately, *Symbiodinium* 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, 2004, 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).

4. Conclusions

Results presented here, combined with previous investigations (deBoer et al., 2007; Barneah et al., 2006; Voolstra et al., 2009b) indicate that the transcriptome and the proteome of cnidarians undergoing early stages of endosymbiosis establishment may be largely unaltered. The lack of detectable differential expression between aposymbiotic and newly symbiotic coral larvae found in this study may be due technical constraints of the study system such as (1) the inability to pick up a 'symbiosis signal' from a handful of symbiotic cells in the midst of hundreds to thousands of non-symbiotic cells within a larva, (2) the inability to discern specific differences in microarrays performed on populations of thousands of genetically different individuals, (3) the choice of sampling time may have missed the window of transient differential expression. It is also possible, however, that lack of differences between symbiotic and aposymbiotic reflect real biological information. Cnidarian responses to colonization by dinoflagellate symbionts may be kept in check by successfully invading types of *Symbiodinium*, 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. A 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 diverse types of *Symbiodinium* and investigations exploring potential mechanisms of *Symbiodinium*-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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