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VALIDATING REAL-TIME PCR AND FIELD MANIPULATIONS USING THE CORAL *MONTIPORA CAPITATA*

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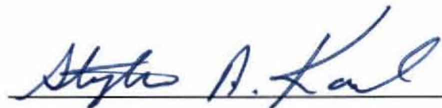

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LIST OF ABBREVIATIONS

Abbreviation

1. hsp70: heat shock protein 70
2. RNA: ribonucleic acid
3. rRNA: ribosomal RNA
4. tRNA: transfer RNA
5. mRNA: messenger RNA
6. 28s: eukaryotic 28 Svedberg units of the large subunit of the ribosomal RNA
7. 18s eukaryotic 18 Svedberg units of the small subunit of the ribosomal RNA
8. DNA: deoxyribonucleic acid
9. gDNA: genomic DNA
10. cDNA: complementary DNA
11. PCR: polymerase chain reaction
12. qRT-PCR: quantitative Real-Time PCR
13. RT-PCR: reverse transcription PCR
14. EST: expressed sequence tag
15. SL DNA spike: somatolactin exogenous DNA reference gene
16. SL RNA spike: somatolactin exogenous RNA reference gene
17. SMP: symbiont molecular proxy
18. C_T: threshold cycle

Chapter 1.0. Introduction

Scleractinian corals form obligate symbioses with dinoflagellates of the genus *Symbiodinium*, which are located in the endodermal cells of the coral host (Lewin and Chang 1989, Norton *et al.* 1992, Farmer *et al.* 2001, Stat *et al.* 2006). The stability and success of this mutualism is driven by the metabolic exchange of nutrients (nitrogen, phosphorus, and potassium) and photosynthate between symbiotic partners (Muscatine 1967, Pearse and Muscatine 1971, Trench 1979, Muscatine *et al.* 1984). This maintains nutrient levels within the symbiosis despite the lack of ubiquitous nitrogen in the environment (Muscatine and Porter 1977) and also provides a mechanism for the host to control algal growth. Photosynthetic products produced by the symbiotic algae can also provide energy that promotes calcification (Pearse and Muscatine 1971). While many organisms, including calcareous algae, serve to cement the reef together, corals have been primarily responsible for the structure of the reef ecosystem during the last 200 million years (Hoegh-Guldberg 1999).

Unfortunately, human activities have drastically modified marine ecosystems, and as a result many reefs are exposed to more frequent increases in temperature (Hoegh-Guldberg and Smith 1989, Glynn and D'Croz 1990, Lesser *et al.* 1990), light intensity (Lesser 1989, Lesser and Shick 1989), sedimentation (Bak 1978), nutrient input, and pollutants (Guzmán and Jiménez 1992). Cnidarian-dinoflagellate symbioses are vulnerable to all of these stresses, with exposure often triggering a reduction in the population of *Symbiodinium* and/or chlorophyll concentration of photosynthetic pigments— a process commonly referred to as coral bleaching (Glynn 1991).

Despite the importance of the cnidarian-dinoflagellate symbiosis in the marine environment and the interest in reef health, very little is known about the molecular and cellular basis of the intact or stressed symbiosis (Rodriguez-Lanetty *et al.* 2006). Exposure to one or more stressors in concert will change the physiological performance of a coral and its symbionts such that a variety of molecular cascades are activated to repair damage at the molecular and cellular level (van Oppen and Gates 2006). Paling of live coral tissue reflects the inability of subcellular, cellular, and organismal processes to compensate for the stress and restore homeostasis (McCarthy and Shugart 1990, Connell *et al.* 1999, Depledge 1999). Defining the behavior of genes and proteins involved in maintaining homeostasis and ameliorating stress will ultimately contribute to the overall understanding of initiation, regulation, and maintenance of this complex symbiosis (Rodriguez-Lanetty *et al.* 2006).

Ideally, exploring both gene and protein expression provides insight into the transcriptional steps and post-translational modification of proteins involved in the onset of symbiosis or during a stress event. The majority of studies, however, have only examined protein expression of ubiquitin, heat shock protein 70, and superoxide dismutase (Sharp *et al.* 1997, Downs *et al.* 2000, Richer *et al.* 2005). These proteins have been the primary focus because they are involved in cell defense, repair, or detoxification processes in the cell, and therefore, become direct and specific markers of exposure and effect during stress (Bierkens 2000). The focus on protein expression perhaps reflects the "abnormal protein hypothesis of stress-protein induction" theory that damaged proteins in the cell are responsible for the activation of stress genes (Anathan *et al.* 1986). Stress gene expression can be regulated in many ways via messenger RNA, initiation of

translation, or elongation, but the primary form of regulation is at the transcriptional level (Bierkens 2000). Exploring the expression of genes, therefore, is imperative for understanding the underlying molecular processes involved in stress induction or during the onset of symbiosis.

An accurate and rapid method of quantifying gene expression is quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). In comparison to Reverse Transcriptase-PCR (RT-PCR), which quantifies transcripts based on endpoint PCR using densitometry, qRT-PCR is capable of quantifying the initial number of transcripts of a specific gene within the exponential phase of PCR. The qRT-PCR method is highly sensitive and reproducible; it requires very little tissue and yields accurate measurements of transcripts for a specific gene (Bustin 2004). Once primers have been tested and the protocol optimized, it is easy to run several samples quickly and efficiently for the analysis of several genes.

One of the major obstacles for gene expression analysis in the cnidarian-algal symbiosis is the lack of genomic resources. Currently, there is no whole genome sequence for a coral or for *Symbiodinium*, although the genome of the closely related model anemone, *Nematostella vectensis*, has recently been sequenced (Sullivan *et al.* 2006). In addition, several expressed sequence tag (EST) projects have been completed or are underway for corals and/or their symbionts. These include: 1) *Acropora millepora* (Kortschak *et al.* 2003), 2) stress response of *Symbiodinium* (Leggat *et al.* 2007), 3) symbiosis-specific expression in *Acropora palmata*, and 4) *Montastrea faveolata* (Schwartz *et al.* 2008). These projects provide sequence information regarding those genes that are differentially regulated under stress or during a specific symbiotic state.

EST projects are completed by cloning complementary DNAs (cDNAs) generated from RNA transcripts in a given sample representing a specific state of the symbiosis (i.e. aposymbiotic, symbiotic, under stress). Cloned sequences are then compared to sequences in existing databases for identification and can be used in DNA microarrays or for individual analysis of specific genes in qRT-PCR (reviewed in van Oppen and Gates 2006). Both the sequencing of *N. vectensis* and the EST databases provide insight and sequence information regarding the abundance of genes that may be involved in regulation, homeostatis, or stress reduction in the cnidarian-algal symbiosis that can be used in further gene expression or microarray studies (van Oppen and Gates 2006).

In the absence of sequence data for a given species of coral, genes can be identified using degenerate PCR. Here, genes that are highly conserved can be sequenced from an unknown organism such as a coral or *Symbiodinium* (Finnerty 2001). After the target gene has been selected, sequence information for that gene is collected for related organisms using the GenBank databases. These sequences are aligned and used to design degenerate primers (primers with some bases that code for more than a one nucleic acid at a given position). These primers are then used in standard PCR to try to amplify the gene from the unknown organism (coral or symbiont) (Murtha *et al.* 2001). Bands that are amplified in PCR of the correct size can be cloned and sequenced. This sequence data is then compared to the existing sequence information in GenBank to confirm and/or assign identity (for review see Brower *et al.* 1997). Although this process can be time consuming, it is relatively inexpensive and is tractable for a number of highly conserved genes. These sequences can then be used to design qRT-PCR primers in order

to quantify the abundance of a given transcript from isolated RNAs in marine organisms over time, space, or treatment.

Once target genes have been chosen, isolating RNA from marine organisms can be tricky due to RNases, viscous polysaccharides, lipids, and proteins that co-extract during the purification process, as well as difficulties disrupting cellular walls and adapting the method for a specific organism (Ho *et al.* 1996, Santiago-Vasquez *et al.* 2006). There are several commercial kits that can be used to extract RNA including Qiagen's RNeasy kits and Invitrogen's TRIzol®Reagent kit, both of which have been shown to extract high quality RNA from the gorgonian coral *Pseudopterogorgia elisabethae*, which contains *Symbiodinium* (Santiago-Vázquez *et al.* 2006). Additionally, TRIzol®Reagent has a secondary high salt buffer that can be easily made and added during the precipitation step to remove any additional polysaccharides and lipids. At present, a standard effective method for RNA extraction in scleractinians is not available.

Equally essential for gene quantification in qRT-PCR is a highly efficient reverse transcriptase to convert RNA to cDNA. PCR cannot amplify target genes from an RNA template because RNA is not the proper starting template for DNA polymerase (the enzyme used to catalyze the PCR reaction). It is imperative, therefore, that the cDNA produced in the reverse transcriptase (RT) step accurately reflects the amount of mRNA in the original RNA extraction (Kubista 2006). Several other factors can affect the efficiency of cDNA synthesis including the type of primers used in the reaction and the temperature of cDNA synthesis step. Following the synthesis step, cDNA can readily degrade over time and or when freeze-thawed. When comparing the technical

reproducibility of the RT step and qRT-PCR, the RT reaction contributes the most variation in the experimental determination of mRNA quantities (Ståhlberg *et al.* 2004).

In order to be useful in a comparative framework, qRT-PCR data must be normalized. A common approach is to normalize to the total mass of RNA extracted, however, this method is constrained by the inability to accurately quantify RNA (Bustin 2000) and variations in the relative ratios of rRNA, tRNA, and mRNA in the total (Nomura *et al.* 1984). RNA is normally quantified using the traditional method of absorbance at 260 nm in a spectrophotometer. Other methods have been developed such as the RiboGreen RNA quantification assay, which relies on a dye (proprietary) that fluoresces when bound to nucleic acids and can be detected in a spectrofluorometer (Jones *et al.* 1998). Both of these methods for quantifying RNA yield similar results for total RNA quantification as long as the RNA concentration is not below a certain threshold (100 ng/μL), but provides no information about the quality of RNA (Bustin 2002). Additionally, because starting material is variable between samples and is often derived from different individuals exposed to experimental treatments, misinterpretation of expression in target genes can be amplified by normalizing to total RNA (Bustin 2000).

Normalizing to RNA does not control for the reverse transcription (RT) step or any inhibitors that may be present in the extraction taken through to qRT-PCR (Bustin 2002). In order to account for these two factors, many gene expression studies use an endogenous reference to normalize. The internal reference gene controls for the efficiency of the RT reaction and the presence of inhibitors because it is co-extracted and amplified in qRT-PCR along with the target gene. Gene expression is subsequently

normalized to the internal reference gene. Unfortunately, for every new experimental system, reference genes have to be identified and tested for stability to determine their value as a housekeeping gene (Dheda *et al.* 2004). Often, there is no stable reference gene suitable under experimental conditions, making normalization impossible. In fact, the housekeeping gene, glyeraldehyde-3-phosphate dehydrogenase (GAPDH), continues to be used, despite the well-documented reports that GAPDH mRNA levels are not constant (Zhu *et al.* 2001).

To account for the difficulties of normalizing to total RNA and/or housekeeping genes as well as account for extraction integrity and efficiency between samples, researchers have incorporated exogenous RNA spikes as reference genes (Temeles *et al.* 1994, Davis *et al.* 1996, De Sousa *et al.* 1998). In several studies, the RNA spike was added during the cDNA synthesis step, which does not account for extraction efficiency or integrity of starting RNA and relies solely on accurate quantification of RNA (Bower *et al.* 2007). An exogenous RNA spike added prior to extraction and taken through all steps (RNA extraction, DNase treatment, cDNA synthesis), will take into account the quality of the RNA sample and the reliability of technical processing (Johnson *et al.* 2005, Bower *et al.* 2007) as well as simultaneously be used as a reference gene. After sample processing, recovered quantities of the exogenous spike and the target genes are independently measured in qRT-PCR. The sample-specific fractional recovery of the exogenous spike is used as a normalization factor to control for the loss of mRNA during RNA extraction, DNase treatment, and cDNA synthesis. The measurements of the spike are independent of the cell state during experimentation and the growth stage, allowing for comparison across different treatments (Johnson *et al.* 2005).

In most gene expression studies, quantification of target genes is often restricted to a single tissue type or more generally within a single organism. Symbiotic systems such as the cnidarian-dinoflagellate symbiosis, however, represent a very challenging scenario. The host and algal tissue are extremely difficult to separate because *Symbiodinium* cells reside in the symbiosome within the endodermal cells of the coral host (Karako *et al.* 2002). Often it is in a researcher's interest to keep the symbiosis intact to understand the regulation of genes *in vivo*. Many experiments in the laboratory manipulate temperature, salinity, light intensity, sedimentation, and heavy metals in order to explore the response of several stress genes. These stressors can cause coral bleaching and the ensuing loss of *Symbiodinium* from the intact symbiosis. Because symbionts are expelled during the bleaching process, RNA extractions taken from bleached corals will by definition have more host tissue than controls. In the final analysis, if there is an upregulation in a host gene, for example, it is impossible to distinguish whether this is due to a true upregulation of the gene or simply because more host tissue was extracted due to the loss of symbionts relative to the control. In some cases, such as high light intensity, stress does not cause the loss of symbionts but a massive reduction in chlorophyll a (Hoegh-Guldberg and Smith 1989). Even if *Symbiodinium* cells are not expelled, it is still more accurate to normalize gene expression to relative host and symbiont tissue because this will reduce sample-to-sample variability of quantification measurements in qRT-PCR.

In a recent study, *Symbiodinium* cells were isolated from symbiotic *Aiptasia pulchella* and *M. capitata* and counted using a hemacytometer (Mayfield *et al.* accepted). RNA and DNA were extracted from a known number of *Symbiodinium* cells based on the

hemacytometer counts (1.25×10^5, 1.50×10^5, 2.5×10^5, 5.0×10^5, 1.0×10^6, 2.0×10^6, and 2.5×10^6 algal cells) using TRIzol®Reagent. A DNA spike (somatolactin, SL, a pituitary gene found in fish) was added to each *Symbiodinium* cell extraction sample and both heat shock protein 70 (hsp70) in the symbiont and the SL DNA spike were quantified using qRT-PCR from genomic DNA (gDNA). From qRT-PCR, the genomic copy number of hsp70 was calculated for each sample normalized to the sample specific fractional recovery of the exogenous DNA spike (SL) (referred to as *Symbiodinium* molecular proxy [SMP]). A standard curve was then generated between SMP and the original number of *Symbiodinium* cells extracted (n = 5 for each cell dilution run in triplicate reactions). A positive linear relationship existed between SMP and *Symbiodinium* cell number up to one million cells (1.73×10^{-5}, $r^2 = 0.97$, $p < 0.01$). The results from this study reveal that genomic copy number of hsp70 in the symbiont (SMP) is directly related to the number of algal cells extracted from each sample and will allow for normalization of each coral sample relative to *Symbiodinium* cell number. This can be further normalized to a measure that takes into account total tissue extracted from each sample, making normalization of gene expression relative to host and symbiont contribution of the original extraction (Mayfield *et al.* accepted).

In addition to troubleshooting molecular protocols for use in qRT-PCR, several aspects of experimental design must be controlled prior to manipulation. Corals can be extremely sensitive and easily stressed, when moved around in the field and into the laboratory (Brown 1997). Many researchers utilize fragments taken from branches of parent colonies or cores drilled from corals and allow them to acclimate to tanks or

acclimatize[a] in the field for a set period of time before experimentation. The sensitivity of corals to touching or moving as observed by retracted polyps, fingerprints, or mucus sheets has been noted, but no studies have explicitly tested how long it takes fragments or cores to recuperate. It is essential that coral fragments, cores, or colonies are not undergoing stress related to movement or handling during an experiment as this will confound the results of the experiment and make it impossible to differentiate changes in gene expression based on the manipulation or due to prior stress. For example, in a study conducted by Brown *et al.* (2002), control coral fragments showed significant increases in hsp70 expression in the coral host over a two-day period during experimentation in laboratory tanks. This suggests that a stress existed within control and experimental coral fragments during experimentation, making it impossible to distinguish effects caused by the experiment, prior stress, or the set-up. An understanding of the time it takes coral fragments, cores, or colonies to acclimate in the laboratory or acclimatize in the natural environment will reduce confounding results associated with either the handling of the coral or the experimental set-up.

In addition, many coral studies have been completed in the laboratory setting, which is practical for exploring the explicit response of corals or their symbionts after exposure to a singular manipulation (Heinz Center 2002) or to identify a potential stressor. Experiments conducted within the natural environment, however, will provide more ecologically relevant information as they more accurately reflect the environmental conditions found on reefs (Lejeusne *et al.* 2006). When conducting experiments in the field, it is critical to document the environment and define whether the experimental

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[a] Acclimatization is the process of an organism adjusting to changes in its environment, often related to temperature or climate. In laboratory conditions the same process is referred to as acclimation.

treatments are significantly different from controls. For example, a treatment that results in a difference of 0.5°C may be statistically significant and different when measured every 15 minutes for 30 days, but biologically, may be within the range of temperatures that a coral will be exposed to in a single day. A 0.5°C shift in temperature over the course of a day may not be enough to elicit a physiological stress response, while a 1°C change over the course of a day causes a measurable response. Although intuitive, it is necessary to ensure that experiments designed in the field maintain control conditions that are different from treatment conditions and that the treatment is biologically relevant.

In this study, qRT-PCR amplification of the gene encoding hsp70 from *Montipora capitata* and *Symbiodinium* were tested and normalized using the SMP method (Mayfield *et al.* accepted). The goal of this work was to confirm the utility of the exogenous spike as a reference gene for calculating SMP and as a housekeeping gene in qRT-PCR amplification of cDNA as well as to validate the general methods utilized for qRT-PCR using corals (the RNA extraction, cDNA synthesis, and amplification steps). Two field experiments were also designed to explore how the *Symbiodinium* residing in coral hosts responded to 1) fragmentation and recuperation and 2) transplantation by tracking symbiont photophysiological data and changes in *Symbiodinium* cell densities using qRT-PCR. Temperature and light intensity data collected from the transplantation experiment were used to determine whether control depth and experimental depth were statistically different. Coral fragments used in the field experiments were also used to test qRT-PCR methods.

Chapter 2.0. Materials and Methods

Molecular protocols

RNA extractions and quantification

Approximately 200 mg of live tissue was removed from a fragment of *M. capitata* with sterile bone cutters and the RNA extracted using the Weis protocol (Weis and Reynolds 1999, Reynolds *et al.* 2000). Briefly, the live tissue was placed in a sterile mortar wiped down with RNase Away® Reagent (Invitrogen), and grinding solution was added. The grinding solution was made by diluting 1 volume of solution D (4.2 M guanidine thiocyanate, 17 mM N-lauryl sarcosine, 2.5 mM sodium citrate pH = 7.0 and 0.69% [vol/vol] 2-mercaptoethanol [added just before use]) with 2 volumes of 1X phosphate buffered saline (PBS). The tissue was ground down to a fine liquid/paste and placed in a 15 mL conical bottomed centrifuge tube. Additional grinding solution was used to rinse residual tissue from the mortar, and this was also added to the centrifuge tube. The sample was spun at 10,000 RPM for 6 minutes at 4°C (Jouan C3i Tabletop Centrifuge). The supernatant was removed, placed in a fresh tube, and two volumes of Solution D added. RNA was purified by adding 3 M sodium acetate, pH = 5.2, to the supernatant (1/10 the volume after Solution D was added) and an equal volume of saturated phenol, pH = 4.3 and chloroform (1/3 of the volume after solution D was added). The solution was vortexed, kept on ice for 15 minutes, and then spun at 10,000 RPM for 15 minutes at 4°C. The supernatant was then removed, placed in a new tube, and an equal volume of chloroform added. The tube was mixed vigorously and spun at 10,000 RPM for 20 minutes at 4°C. The supernatant was once again removed, placed in

a new tube, and equal volume of isopropanol added. RNA was precipitated overnight at -80°C.

The following day, the sample was thawed and spun at 10,000 RPM for 30 minutes at 4°C. The supernatant was poured off and the pellet (RNA + DNA) washed by adding 6 mL of 70% ethanol. The sample was mixed and spun at 10,000 RPM for 10 minutes at room temperature. The supernatant was removed, and the pellet dried for at least 45 minutes. The RNA pellet was resuspended in 50 μl of DEPC treated water and incubation at room temperature for 30 minutes. The RNA concentration and quality was measured at an absorbance of 260 nm using the Nanodrop® ND-1000 UV-Vis Spectrophotometer. The RNA was then converted to cDNA synthesis (see *cDNA synthesis*), which served as the template for isolation of hsp70 from the coral host.

DNA extractions and quantification

An air gun, covered with a 1000 μL plastic pipette tip, was attached to a regulator on a SCUBA tank, and coral tissue was gently air-stripped from a one-inch fragment of *M. capitata*. Tissue was re-suspended in 2 mL of 22 μm filtered seawater, transferred into a 2 mL microcentrifuge tube, and spun at 13,200 RPM for 5 minutes.

After removing and disposing the supernatant, DNA was extracted from the cell pellet using a CTAB (cetyltrimethylammonium bromide) protocol modified from Dempster et al. (1999). The pellet was resuspended in 500 μL CTAB buffer (100 mM tris-hydrochloride, 1.4 M sodium chloride, 20 mM EDTA, 2% [wt/vol] CTAB, 1% [wt/vol] PVP (polyvinylpyrrolidone) and 0.4% [vol/vol] 2-mercaptoethanol [added just before use]) by vortexing, and the mixture incubated at 65°C for 30 minutes in a water bath. Following incubation, 500 μL phenol/chloroform/isoamyl alcohol (24:25:1) was

added to the sample in the hood, the tubes vortexed, and then placed on a rocker at room temperature for 20 minutes. Samples were then centrifuged at 13,200 RPM for 15 minutes in a benchtop centrifuge (Eppendorf 5415D). An aliquot of aqueous phase was removed from the sample and placed in a new 1.5 mL microcentrifuge tube. Five hundred microliters of chloroform/isoamyl alcohol (24:1) was added, the mixture was vortexed briefly, and centrifuged for 15 minutes at 13,200 RPM. An aliquot of this aqueous phase was removed and placed into a new 1.5 mL microcentrifuge tube. DNA was precipitated by adding an equal volume of cold isopropanol and half the volume of 5 M NaCl and incubating the mixture overnight at -80°C. The sample was removed from the -80°C, thawed, and the DNA pelleted by centrifugation at 13,200 RPM for 30 minutes. The aqueous phase was removed with a pipette, the DNA pellet washed by suspension in 200 µL of 70% ethanol, and the sample centrifuged at 13,200 RPM for 5 minutes (this was repeated twice). After the last ethanol wash was removed, the DNA pellet was re-suspended in 30 µL of UV-treated, Milli-Q water. The concentration and quality of DNA was measured by absorbance at 260 nm using a Nanodrop ND-1000 UV-Vis Spectrophotometer. The DNA isolated using this method served as the template for isolation of hsp70 in the symbiont.

TRIzol® Reagent extraction of RNA and DNA from the same sample

RNA extractions

RNA and DNA were extracted from coral fragments used in the fragmentation experiment and transplant experiments using TRIzol®Reagent. Isolating both nucleic acid fractions from the same sample was required to validate the downstream normalization of gene expression data described in *Validating and normalizing qRT-*

PCR. Small pieces (50 - 300 mg) of coral were removed 1 cm below the tip of coral fragments that had previously been snap frozen in liquid nitrogen. All coral pieces were kept in liquid nitrogen or on dry ice until Trizol®Reagent was added. RNA was extracted from these pieces using the manufacturer's protocol with the following minor modifications. Coral fragments were ground to a liquid paste with plastic homogenizers that had been soaking in RNase® Away. During homogenization, 5 μL of both the exogenous RNA and DNA spike (5 pg μL^{-1}) were added to each sample (the protocol for making spikes is described in *Synthesis of exogenous DNA and RNA spikes*). The red organic phase was set aside and utilized for the DNA extraction described below.

Precipitation, washing, and re-suspension of RNA

As recommended by the Trizol®Reagent protocol, samples were precipitated with isopropanol with 250 μL of high salt buffer (0.8 M Sodium Citrate and 1.2 M NaCl) to remove lipids and polysaccharides. Samples were spun at 13,200 RPM for 10 minutes at 4°C, the supernatant removed and discarded, and 1 mL of 75% ethanol (diluted with DEPC water) added. Samples were vortexed, spun at 9,200 RPM for 5 minutes at 4°C, and the supernatant removed (repeated twice). Extracted RNA pellets were re-suspended in 30 μL of DEPC treated water by pipetting and heating at 60°C for 10 minutes. The concentration and quality of RNA was assessed at 260 nm using the Nanodrop® ND-1000 UV-Vis Spectrophotometer.

DNA extractions

DNA was extracted fro the red organic lower phase saved from the RNA extraction described above. Five hundred microliters of back extraction buffer (4 M Guanidinium Thiocyanate, 50 mM sodium citrate, and 1 M Tris) were added to the

organic phase, the contents mixed by inversion (15 times), and placed on a rotator for 10 minutes. Following a spin at 13,200 RPM for 10 minutes at 4°C, the supernatant was removed and placed into a new 1.5 mL microcentrifuge tube with 2 μL of Pellet Paint® NF Co-Precipitant, which allows visualization of the DNA pellet. The contents of the tube were mixed by inversion, the DNA precipitated, and the precipitated DNA pellet washed as described above in *Precipitation, washing, and re-suspension of RNA* with the following modifications. DNA was precipitated with 50 μL of 3M sodium acetate (pH= 5.2) and 900 μL of isopropanol, DNA pellets were inverted, and precipitated DNA was re-suspended in 50 μL of 0.1X Tris EDTA (1 mM Tris and 0.1 mM Ethylene diamine tetra acetic acid), mixed by flicking the tubes containing DNA and heating tubes at 55°C for 4 minutes. The tubes were flicked again and kept at 4°C overnight. The following day, the tubes were spun at 13,200 RPM for 10 minutes at 4°C, and the supernatant containing the DNA placed in a new 1.5 mL microcentrifuge tube. The DNA concentration and quality was assessed at an absorbance of 260 nm using the Nanodrop® ND-1000 UV Vis Spectrophotometer.

DNase treatment of RNA

RNAs extracted using the Trizol®Reagent were DNase treated prior to cDNA synthesis. An aliquot of RNA from each sample was diluted between 150 - 250 ng μL^{-1} with DEPC treated water and the dilution factor noted. Fifteen microliters of diluted RNA was added to 1X RQ1 RNase free Buffer (Promega), 3 U RQ1 RNase free DNase (Promega), and DEPC treated water and the mixture heated at 37°C for 30 minutes.

Samples were precipitated, washed, and re-suspended as describe in *Precipitation, washing, and re-suspension of RNA* with the following modifications: 1) RNA in DEPC

water was precipitated with an equal volume of isopropanol and 190 mM Sodium Acetate (pH=5.2) at -80°C, and 2) precipitated samples were thawed and incubated at room temperature for 20 minutes prior to washing and re-suspension.

cDNA synthesis

RNA was diluted two fold (for gene discovery) and added to 10 nM oligo-dT primer (16-mer, Operon) and DEPC water. The tubes were heated at 65°C for 5 minutes, cooled and maintained at room temperature for 10 minutes. The cDNA was synthesized using the cDNA High Capacity cDNA synthesis kit (Applied Biosystems) according to the manufacturers protocol. The cDNAs were stored at -80°C.

Primer design and standard PCR

A degenerate PCR strategy was employed to isolate hsp70 gene fragments from both the host and symbiont. To design the primers, protein and nucleic acid sequences were recovered form the National Center for Biotechnology Information (NCBI) database. Two protein alignments were constructed using MacVector® 8.1.1 software (Accelrys Inc. division of Symantec Corp.). The first contained animal hsp70 sequences and the second, plants/algae/alveolate hsp70 protein sequences. Regions with 100% conservation at the amino acid level were then aligned at the nucleic acid level and these alignments were used to design degenerate primer sets for the host (hsp70A) and symbiont (hsp70Z). The sequences for these and all other primer sets described in this thesis are presented in Table 1. Two rounds of PCR were required to amplify hsp70 from *M. capitata*. For the first round of PCR, an annealing temperature of 50°C was employed with a cDNA template synthesized as described above. For the second round of PCR, 1

μL from the first PCR amplification served as the template and a more stringent annealing temperature of 61°C was used (see Table 2 for details regarding the reaction setup and other cycling conditions). The hsp70 gene fragment was amplified from the symbiont using a DNA template (described above in *DNA extraction and quantification*) and a single round of PCR (Table 2).

The host and symbiont hsp70 gene fragments isolated above were aligned in ClustalW (European Bioinformatics Institute) and the alignments used to design compartment specific qRT-PCR analysis primers (see *Validating and normalizing qRT-PCR*) (Table 1 and 2).

An intron region of the beta subunit of ATP synthase was used to genotype the coral host and amplified using the primer set ATPSβ (Table 1 and 2). Greg Concepcion designed and provided the ATPSβ primer set (unpublished).

Visualization and isolation of PCR products

For the primer sets hsp70A, hsp70Z, and ATPSβ, PCR products were separated on 1.4% agarose gels and visualized using ethidium bromide and a UV transilluminator (BioRad). The hsp70A PCR amplification produced several bands, and the 330 bp band was excised with an autoclaved scalpel. The hsp70Z PCR amplification produced a single band that was also gel isolated. The single PCR product from ATPSβ was not gel excised.

For the qRT-PCR primer sets hsp7016a and hsp70Z1, PCR products were separated on 2.5% agarose gels and visualized with ethidium bromide and a UV transilluminator. Both the hsp70Z1 and hsp7016a PCR amplifications produced single bands, but hsp70Z1 was gel isolated. The single PCR product from hsp7016a PCR was

sequenced directly and clean reads were obtained (no mixed template) so further gel excising and cloning was unnecessary.

The ethidium and agarose were removed and the DNA bands isolated using the Qiaex II Gel Extraction Kit (Qiagen) according to the manufacturers protocol.

Cloning and sequencing

Gel isolated fragments were cloned into the pGEM®-T Easy Vector (Promega) following instructions in the pGEM®-T Easy Vector Systems Technical Manual with slight modifications. Half reactions were used for all ligations and transformations, and α-Select-Gold Competent Cells (Bioline) were used. Following recuperation after heat shock, only 125 μL of SOC medium {2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose} was added to each transformation.

The following day, three to five white colonies were checked for inserts in PCR using the M13 forward and reverse primers (see Table 1 and 2). Amplifications were visualized as previously described and colonies containing inserts grown up in 5 mL of LB media + Ampicillin (25 mg mL^{-1}) overnight at 37°C in the shaking incubator.

Bacterial cells (2 mL) were pelleted by centrifugation at 8,000 RPM for 2 minutes. Plasmid DNAs were isolated from these cell pellets using the GeneJET™ Plasmid Miniprep Kit (Fermentas) as described in the handbook. The plasmids containing PCR amplified hsp70A, hsp70Z, and hsp70Z1 fragments were then sequenced on the 3130XL Genetic Analyzer (Applied Biosystems) through the Core Facility at the Hawaii Institute of Marine Biology.

For hsp7016a and ATPSβ, PCR amplifications were not cloned. The hsp7016a

Table 1. PCR primer sets, primer sequences (F= Forward Primer, R= Reverse primer), type of primer set (STD= standard PCR primer sets, or qPCR= quantitative Real-Time PCR primer sets) and length (in base pairs).

Primer Set	Primer Sequence	Type	Length (bp)
[A] hsp70A	F- 5′- AACCCYGACGAGGCYGTG -3′ R- 5′- TGGNGCWGGRGGRATTCC -3′	STD	309
[B] hsp70Z	F- 5′- GTNGGTGGTTCNACCCGTATT -3′ R- 5′- CCCTTNTCGTTGGTGATGGT -3′	STD	515
[C] hsp7016a	F- 5′- GTGCTTCTGGATGTTAATCCTCTT ACT -3′ R- 5′- AGTGTTCTGGTTGTCAGCAGTTG -3′	qPCR	138
[D] hsp70Z1	F- 5′- CTGTCCATGGGCCTGGAGACT -3′ R- 5′- GTGAACGTCTGTGCCTTCTTGGTT -3′	qPCR	92
[E] tilSLα[a]	F- 5′- GGCTGGCTTTGCATGTATCA -3′ R- 5′-AGTGGAGCAACCATTTATCAGATA TCT -3′	qPCR	88
[F] ATPSβ	F- 5′- CGTGAGGGAAATGATTTCTACCA TGA -3′ R- 5′- CGGGCACGGGCGCCGGGGGTTC GT -3′	STD	272
[G] M13[b]	F- 5′- GTAAAACGACGGCCAGT -3′ R- 5′- GTTTTCCCAGTCACGAC -3′	STD	-

[a]Moriyama and Uchida, unpublished; [b]Operon Biotechnologies, Inc.

products were directly sequenced because only a single band was produced in PCR and there were no mixed template sequence reads. For ATPSβ, cloning would have revealed alleles for each genotype, but enough information was obtained from directly sequencing PCR products. Twenty microliters each of the hsp7016a and ATPSβ PCR products were treated with 8 μL Exonuclease 1 –shrimp alkaline phosphatase (ExoSap) and heated at 37°C for 30 minutes and then 85°C for 15 minutes. Following ExoSap treatment, samples were submitted directly for sequencing on the 3130XL Genetic Analyzer (Applied Biosystems) through the Core Facility at the Hawaii Institute of Marine Biology.

BLASTn analyses

Sequences obtained for hsp70A, hsp70Z, hsp70Z1, and hsp7016a were trimmed to remove primers in 4Peaks v. 1.7 (mekentosj.com©). All sequences recovered were compared with all sequences in GenBank using BLASTn (NCBI). Sequences obtained for DNA amplified with the ATPSβ primer set were trimmed to remove sequence stutter at the beginning and end of the sequence, submitted to BLASTn, and the exon-intron boundaries identified. Sequences were then aligned in Sequencher™ 4.8 (Gene Codes Corporation) to identify variable sites.

Validating and normalizing qRT-PCR

DNA and RNA (converted to cDNA) extracted from TRIzol®Reagent was diluted (four-fold for DNA, twenty-fold for cDNA) and used as the template for qRT- PCR. The cDNA (and RNA) was stored at -80°C for several weeks before use. Reactions were conducted in a 96-well plate format that consisted of 25 coral samples, 5 dilutions of the

Table 2. PCR primer sets, reaction components, and thermocycling conditions. For the hsp70A primer set, two rounds of PCR were completed, the first with annealing temperature at 50°C, the second with an annealing temperature of 61°C.

Primer Set	Reaction Components	Thermocycling Conditions
[A] hsp70A	1X Immuno™ Buffer[c] 25 ng μL^{-1} BSA[d] 2 mM $MgCl_2$ [c] 0.2 mM dNTPs [c] 0.5 µM Hsp70A-F, R 0.02 units μL^{-1} Immolase™ [c] 1 µL 10-fold diluted cDNA	94°C, 7 min X1 94°C, 30 sec 50°C (61°C), 45 sec 72°C, 60 sec X33 72°C, 5 min X1 4°C, ∞
[B] hsp70Z	Same as [A], except: No BSA Hsp70Z-F, R primers 1 µL DNA (15 ng μL^{-1})	Same as [A], annealing temperature at 45°C
[C] hsp7016a	Same as [A], except: No BSA 1.5 mM $MgCl_2$ [c] 100 nM Hsp7016a-F, R 1 µL of 20-fold diluted cDNA	Same as [A], except: Annealing: 61°C, 60 sec 40 cycles
[D] hsp70Z1	Same as [A], except: Hsp70Z1-F, R primers 1 µL 10-fold diluted cDNA	Same as [C], except: Annealing: 62°C
[E] tilSLα	Same as [A], except: No BSA 500 nM tilSLα-F, R primers 1 µL 10-fold diluted cDNA	Same as [C], except: Annealing: 57°C
[F] ATPSβ	Same as [A], except: No BSA 3 mM $MgCl_2$[c] 130 nM ATPSβ-F, R primers 1 µL DNA (20 ng μL^{-1})	95°C, 10 min X1 95°C, 30 sec 53°C, 30 sec 72°C, 30 sec X35 72°C, 10 min X1 15°C, ∞
[G] M13	Same as [A], except: No BSA 1X KCl Buffer [c] 0.5 mM $MgCl_2$ [c] 500 nM M13-F, R[e] Taq Polymerase[a] Spot of E. coli	Same as [A], except: Denaturation step: 2 min Annealing: 55°C, 60 sec 35 cycles Final Extension: 10 min

[c]Bioline; [d]New England Biolabs® Inc.; [e]Operon Biotechnologies, Inc.

standard curve, and a no template control. All samples, standards, and no template controls were run in triplicate to control for pipetting error and to accurately calculate C_T values. A separate standard curve was run for each gene on every plate to account for differences in reaction component mixtures and to calculate primer efficiency as follows:

$$(1)\ E = (10^{-1/slope} - 1),$$

E = efficiency of the primer set

slope = the slope of the line calculated from plotting threshold values of the diluted standards vs. the $\log_{10}$(concentration diluted standards). Standard curves used in DNA amplifications were made by serially diluting TRIzol®Reagent extracted DNA from one of the coral samples. Standard curves used in cDNA amplifications were made from serially diluting PCR products of the specific gene product. A melting curve was also run for every sample, standard, and no template reaction to ensure that the proper product was being amplified. All primer sets, reactions components, and thermocycling conditions can be found in Table 3. Two machines were used: 1) the Chromo4® (MJ Research) for gDNA and 2) the StepOnePlus® for cDNA (Applied Biosystems). Samples with different amplification kinetics, multiple peaks in the melting curve analysis, or delayed amplification were removed from further analysis.

In order to quantify the symbiont molecular proxy (SMP) and to normalize gene expression relative to host/symbiont tissue, the primer sets hsp70Z1 and tilSLα were amplified separately for each sample. The averaged C_T value calculated from triplicate reactions for each sample and gene and the efficiency for each primer set was then used to calculate SMP of hsp70 in the symbiont as follows:

$$(2)\ SMP = E_{SL}^{(SLC_T)} / E_{hsp70Z1}^{(hsp70Z1\ C_T)},$$

E_{SL} = the PCR efficiency of the SL (tilSLα) primer set

${}^{SLC}{}_{T}$ = averaged C_T value of SL from the sample

$E_{hsp70Z1}$ = the PCR efficiency of the hsp70 symbiont (or hsp70Z1) primer set

${}^{hsp70Z1\ C}{}_{T}$ = averaged C_T value of hsp70 in the symbiont from the sample.

The *Symbiodinium* cell number was then calculated using the SMP according to the following linear equation taken from Mayfield *et al.* (accepted):

$$(3)\ \textit{Symbiodinium}\ \text{cell number} = \text{SMP} / 1.73 \times 10^{-5},$$

which approximately corresponds to the number of cells that were used for the initial extraction. The *Symbiodinium* cell number was further normalized to account for differences in the total of tissue used from each coral sample by the following equation:

$$(4)\ \textit{Symbiodinium}\ \text{cell number / Total RNA (ng)},$$

Total RNA (ng) = nanograms of RNA extracted from a coral sample after DNase treatment and precipitation.

Total RNA (ng) was used because it was positively correlated with the mass of the fragment used for the RNA and DNA extraction with TRIzol®Reagent (a 50-300 mg piece of coral tissue from the original coral fragment) ($m = 91688$, $r^2 = 0.3179$, p-value < 0.0001, not shown). Normalized expression values for hsp70 in the symbiont and host were not computed due to the low reproducibility, high CT values, and low efficiency of primer sets.

<u>*Synthesis of exogenous DNA and RNA spikes*</u>

<u>DNA spike</u>

The primer set tilSLα (Table 1 and 2) was used to amplify a fragment of somatolactin (SL) from cDNA isolated from the pituitary gland of *Oreochromis*

mossambicus provided by Dr. Andrew Pierce (Moriyama and Uchida unpublished). This produced a PCR product of a single band, and the PCR reaction was diluted to 5 pg μL^{-1} in aliquots and stored in the -20°C. A new tube was thawed for each new day of extractions.

RNA spike

The PCR product described above (in ***DNA spike***), was visualized, excised, cloned, and sequenced as described above (***Visualization and isolation of PCR products*** and ***Cloning and sequencing***). The plasmid containing the 88-bp SL fragment was linearized with Nsi I (FastDigest™ Restriction Enzyme) (Fermentas) with 1X FastDigest™ Buffer, 7.5 units of Nsi I, ~750 ng μL^{-1} DNA (vector + insert), and DEPC water. The digests were incubated at 37°C for 10 minutes and 85°C for 5 minutes. To remove any excess buffer and enzyme, the DNA was cleaned with the QIAquick PCR Purification kit (Qiagen) according to the manufacturers instructions. The only adjustment to the protocol was that DNA was eluted in 17 μL of DEPC treated water.

RNA was synthesized from the linearized, clean plasmid using T7 RNA Polymerase (New England Biolabs). Each reaction contained 1X RNA Polymerase Reaction Buffer, 2 mM rNTPs, 1 unit of T7 RNA Polymerase, ~750 ng μL^{-1} of linearized DNA (vector + insert), and DEPC water. The reaction was incubated at 37°C for 1 hour. DNA was removed from the newly synthesized RNA and precipitated, washed, and re-suspended as described in section ***DNase treatments***. The only modification was that samples were precipitated at room temperature.

A RNA was tailed using the Poly(A) Tailing Kit (Ambion, Inc.) as described in Ambion's Protocol. Following tailing, the RNA was precipitated, washed, and

Table 3. Real-Time PCR primer sets, reaction components, and thermocycling conditions. Under primer set, samples are identified as (1) RNA or (2) gDNA. This is to separate Real-Time PCR conditions for primer sets amplified using RNA converted to cDNA (RNA) or primers sets amplified using genomic DNA (gDNA). Reaction volumes were 20 μL for (1) RNA and were 19 μL for (2) gDNA.

Primer Set	Reaction Components	Thermocycling Conditions
[C] hsp7016a		
(1) RNA	(1) 1X SensiMix[f] 1X SYBR® Green solution[f] 100 nM hsp7016a-F, R 1 μL 20-fold diluted cDNA	(1) 95°C, 10 min X1 95°C, 15 sec 61°C, 60 sec X40 Melt Curve: 65°C-90°C (0.5°C, 15 sec)
[D] hsp70Z1		
(1) RNA	(1) Same as [C] except: 50 nM hsp70Z1-F, R primers	(1) Same as [C] except: 62°C for annealing temperature
(2) gDNA	(2) Same as [C] except: 1X TaqMan® Universal PCR Master Mix[g] 0.053 mg mL^{-1} BSA[h] 526 nM hsp70Z1-F, R 2 μL 4-fold diluted DNA	(2) Same as [C] except: 62°C, 45 sec for annealing temperature and time
[E] tilSLα		
(1) RNA	(1) Same as [C] except: tilSLα-F, R primers	(1) Same as [C] except: 57°C for annealing temperature
(2) gDNA	(2) Same as [D], (2) except: 1X of iQ™ SuperMix[i] 526 nM tilSLα-F, R	(2) Same as [C] except: 62°C, 45 sec for annealing temperature and time

[f]2X SensiMix DNA Kit (Quantace Ltd.); [g]Applied Biosystems; [h]New England Biolabs®, Inc.; [i]BioRad Laboratories, Inc.

resuspended as described above.

To ensure that the poly-A-tailing worked, the *in-vitro* synthesized RNA with the poly-A-tail was reverse transcribed to cDNA as described in section *cDNA synthesis*. The cDNA was diluted 30-fold with UV treated dH_2O and PCR amplified with the tilSLα primer set (Table 1 and 2). Amplifications were visualized on a 2.5% agarose gel with ethidium bromide using a UV transilluminator. The *in-vitro* synthesized RNA with the poly-A-tail was then diluted to 5 pg μL^{-1} and stored in 100 μl aliquots at -80°C.

Field Experiments: Exploring fragmentation and transplantation

Site selection

The field experiments were conducted in a channel on the leeward side of Coconut Island (N 21°26.083', W 157° 47.397') (see Figure 1). This site was chosen because it is a sheltered, low wind area close to molecular facilities. This made it convenient to set up the experiment, collect, process, and store samples. To prepare for the experiments, 13 colonies of *M. capitata* were selected at approximately the same depth (~2.44 m at high tide) and tagged A-M.

Fragmentation and recuperation experiment

An acrylic sheet was punctured to make an "X" at 60 evenly spaced locations. Each X was labeled with a randomly assigned colony (A-E) and fragment number (1 - 12). A HOBO® Pendant data logger (temperature and light intensity) was attached to the acrylic sheet so that it was lying horizontally and environmental data was logged every 15 minutes (Figure 2). The acrylic sheet was attached to concrete blocks (10.16 cm height) sitting at the bottom of the channel in the study site (~2.44 m depth).

Three branches were removed from colonies A-E were and immediately frozen in liquid nitrogen with all five colonies being sampled within 15 minutes of one another. Another 12 branches were removed from each of these same colonies and placed in their pre-labeled positions in the acrylic sheet by gently pushing the base of the coral branch into the center of the pre-cut X (Figure 2). Three fragments of each colony were then collected once a week for one month. These fragments were used to 1) assess symbiont cell number over time using qRT-PCR and 2) genotype the coral host.

Transplantation experiment

In the same location as described above, ten split-faced concrete blocks (20.32 cm x 10.16 cm x 40.64 cm) were placed haphazardly in the channel. A 20.32 cm x 40.64 cm acrylic sheet with 8 X punctures, prepared as described above, was attached to each concrete block and the X punctures randomly labeled G-M representing coral colony and 1 – 10 representing replicate fragment numbers within a colony (Figure 3). A HOBO® Pendant data logger was attached horizontally to the middle of each acrylic sheet. Ten fragments were removed from each of the colonies G – M and one fragment from each colony placed in an X puncture in each block. Corals in the blocks were left undisturbed for one month prior to transplantation.

After one month, five blocks were randomly designated as the treatment and raised in the water column approximately 0.91 m by placing them on platforms consisting of 5 concrete blocks. This manipulation was completed by 7:30 am on August 21, 2007, and the first coral fragments collected immediately afterwards (t = 0 h). The first coral fragments collected were taken from the block farthest from Coconut Island (Figure 4), and in subsequent collections, coral fragments were always collected in the same order.

All fragments were collected within 20 minutes of each other and immediately frozen in liquid nitrogen. Branches from colonies F, H, and I were pale in color so these colonies were excluded from the analysis. A fragment from each colony and block was collected at t = 0 h, 6 h, 30 h, 78 h, 174 h. These fragments were used to 1) assess symbiont cell number over time and 2) genotype the coral host. Measurements of temperature and light intensity collected from the HOBO® Pendant data logger were compared between lifted blocks and control blocks.

Analysis of photophysiological performance

During the course of both field experiments, minimal and maximal fluorescence measurements were taken just below the tip of each fragment using a diving Pulse Amplitude Modulation (PAM) Fluorometer (Heinz Waltz GmbH, Effeltrich, Germany) at approximately 6:30 pm. The capacity of photosystem II (PSII) to absorb light photons is maximized in the dark (Fitt *et al.* 2001). After dark acclimation, all PSII reactions centers are in the "open state" to accept light photons and the minimum fluorescence of the sample can be measured (F_o). The PAM Fluorometer produces a flash of light that is applied to the sample, and the reaction centers saturate (PSII reaction centers are full of photons and are now in a "closed" state) so that the maximum amount of fluorescence can be measured (F_m). Measurements of F_o and F_m can then be used to calculate variable fluorescence (F_v) by:

$$(5)\ F_v = F_m - F_o.$$

Subsequently, maximum quantum yield values can be calculated by:

$$(6)\ F_v / F_m = 1 - (F_o / F_m).$$

Maximum quantum yield is a measurement used to assess the condition of the photosynthetic apparatus (i.e. are all reactions centers functioning properly). Changes in F_v/F_m occur rapidly under stress, which is why this parameter was used as an indicator of physiological stress in these studies. For the fragmentation and recuperation experiment, F_o and F_m measurements were taken from three randomly selected fragments from each of the five colonies two to three times a week for the duration of the study to calculate F_v/F_m.

For the transplant experiment, F_o and F_m measurements were taken for all fragments the night before the blocks were lifted to calculate F_v/F_m. After transplantation, F_o and F_m measurements were taken on all remaining fragments the evenings prior to collection time points (t = -13 h [prior to lifting blocks], 11 h, 59 h, 155 h).

Statistical analysis

All statistical analyses were completed using JMP version 6.0. To determine whether maximum quantum yield or *Symbiodinium* cell densities changed by week for the fragmentation and recuperation experiment, matched pairs t-tests were used to compare each time point with sequential Bonferroni adjustments to significance levels (Rice 1989). Variance component estimates were completed to determine variability between and within colonies for each time point as well as for the overall experiment using the estimated means squares (EMS) method. The variance component analysis was used because the subject, colony, was treated as a random factor. Maximum quantum yield values were transformed by taking the original values to the fourth power to ensure

homoscedasticity. *Symbiodinium* cell number values were also transformed by taking the fourth root of the original values.

To resolve whether temperature and light intensity differed between lifted blocks versus control blocks during the entire transplantation experiment, the following tests were completed: 1) matched pairs t-test for temperature and 2) Wilcoxon signed-rank test for light intensity because the distribution was highly skewed. Wilcoxon signed-rank tests were also completed for midday values (between 11 am – 2 pm) for both temperature and light intensity because distributions for both were non-normal. Values for temperature and light intensity were averaged over the five lifted blocks or the five control blocks before analysis.

To determine whether maximum quantum yield changed by date, treatment, or the interaction of date x treatment in the transplantation experiment, repeated measures ANOVA was used (univariate split-plot method). A conservative F-test (the degrees of freedom were adjusted) was used to account for violations of sphericity. Variance component estimates were computed to determine variability between and within colonies for each time point, separated by treatment, as well as for the overall experiment. Maximum quantum yield values were transformed by taking the original values to the fourth power to ensure homoscedasticity.

Chapter 3.0. Results

Gene identification

The 273 bp sequence isolated from *M. capitata* (with primer removed; Accession Number EU476881) was most closely related to the coral *Pocillopora damicornis* mRNA for hsp70 (complete CDS, Accession Number AB201748.1, e-value of 5×10^{-80}). The 474 bp sequence isolated from *Symbiodinium* contained in *M. capitata* (with primer removed; Accession Number EU476880) was identical to *Symbiodinium sp.* C3 mRNA for hsp70 (partial CDS, Accession Number DQ144979, e-value of 0).

The 70 bp sequence isolated from *M. capitata* using the hsp7016a primer set (with primer removed) was most closely related to the coral *P. damicornis* mRNA for hsp70 (complete CDS, e-value of 1×10^{-5}). The 50 bp isolated from *Symbiodinium* contained within *M, capitata* using the hsp70Z1 primer set (with primer removed) was most closely related to *Symbiodinium sp.* C3 mRNA for hsp70 (partial CDS, e-value of 2×10^{-15}). The 42 bp sequence isolated from *O. mossambicus* (with primer removed) was most closely related to the cichlid fish, *Cichlasoma dimerus* mRNA for somatolactin (partial CDS, Accession Number EF192603.1, e-value of 3×10^{-8}).

Validating and normalizing qRT-PCR

To calculate the SMP, the SL DNA spike and hsp70 in the symbiont were amplified from DNA extracted using TRIzol®Reagent. The SL DNA spike was extremely robust and amplified within the 10 - 30 cycle range for both the fragmentation and recuperation and transplantation experiments. The average range of amplification for the standards was between 16.12 – 28.75 with an efficiency of 108% (corresponds to a slope of -3.14, $r^2 = 0.983$). The range of C_T values of samples from the fragmentation

and recuperation and transplantation experiment were 12.65 – 18.1 and 12.97 – 20.61, with some samples amplifying below the range of the lowest C_T values from the standard curve (Appendices A and E). Only 2 samples out of 75 amplified from the fragmentation and recuperation experiment had standard deviations above 0.300 (with one of these having a standard deviation of 0.304) and only 1 sample out of 50 from the transplantation experiment. Even though some of the sample C_T values were below the lowest C_T values from the standard curve, standard deviations of all but 3 samples (from both experiments) were above 0.300. In addition, amplification kinetics were similar for all samples except those with high standard deviations. This suggests that those samples with C_T values below 16.12 are still quantifiable as long as the standard deviation of the replicates is below 0.300. In future experiments, however, the amount of DNA spike added to each sample should be reduced so that C_T values from samples are within the range of C_T values from the standard curves.

SL DNA spike samples from both field experiments had C_T values with tight replication among at least two replicates, with the majority of samples having standard deviations under 0.300 for all three replicates (Appendices A and E). Melting curve analysis from samples amplifying the SL DNA spike consistently revealed single peaks with a melting temperature of 75.25°C. Those samples that revealed more than one peak were removed from any further analysis (3/75 and 4/50 samples had one replicate out of three with a bad melt curve in the fragmentation and transplantation experiments respectively).

Amplification of hsp70 in the symbiont from DNA samples was less robust than the SL DNA spike. There were several samples from both the fragmentation and

recuperation and transplantation experiments with C_T values above 30 cycles (20/75 samples or 26.7% and 22/50 samples or 44% respectively). Standard curves had a range of C_T values between 15.25 – 29.53 with tight replication for all dilution replicates and an average efficiency of 95% (corresponding to a slope of -3.458, $r^2 = 0.982$). The range of C_T values for samples from the fragmentation and recuperation experiment were 21.26 – 32.88 and for the transplantation experiment 19.92 – 35.96 (Appendices B and F). The highest value of the C_T ranges is well above the highest dilution of the standard curve and within the range that binding probabilities are greatly reduced. However, of those samples with C_T values over 30, only 2 samples had standard deviations above 0.300 from the fragmentation and recuperation experiment (2.7% of total samples) and 5 samples had standard deviations above 0.300 from the transplantation experiment (10% of total samples). Samples with standard deviations above 0.300 were removed from any further analysis. To ensure that those samples with high C_T values are in fact quantifiable, it is important to run out a standard curve that includes the range of unknown samples. DNA was diluted four-fold prior to qRT-PCR, and it may be better to dilute the samples only two-fold. A test run that includes different dilutions of DNA would identify the appropriate range of dilution for amplification of hsp70Z1 from DNA within the 10 - 30 cycle range as well as identify any potential PCR inhibitors.

Samples of hsp70 in the symbiont amplified with tight replication among at least two samples, with over half having standard deviation below 0.300 over three sample replicates (Appendices B and F). Melting curve analysis from samples amplifying hsp70 from the symbiont consistently revealed single peaks with a melting temperature of 81.5°C. Those samples that revealed more than one peak were removed from any further

analysis (4/75 and 7/50 had samples with one or more replicates with bad melt curves in the fragmentation and transplantation experiments respectively). In the transplantation experiment, there were 4 samples that revealed only a single C_T value with a good melting curve. These samples were removed from further analysis because only one replicate out of three amplified, making quantification unreliable and irreproducible.

The majority of C_T values of the SL DNA spike and hsp70 in the symbiont were quantifiable by qRT-PCR in the fragmentation and recuperation experiment so that SMP (Eq. 2) could be calculated from each sample. From the values of SMP, *Symbiodinium* cell number could be calculated based on the standard curve generated in Mayfield *et al.* (accepted) (Eq. 3) and further normalized to total RNA to account for tissue extraction differences from each coral sample. For the transplantation experiment, amplification of hsp70 in the symbiont was less robust and many samples had C_T values over 30 with standard deviations over 0.300 among replicates. In the transplantation experiment, there were only two representatives of each colony for each time point, one on a lifted block and one on a control block. Several samples were removed from analysis because they had standard deviations above 0.300 or the melting peaks were poor, which made it impossible to complete any further analysis of *Symbiodinium* cell number.

Amplification of the SL RNA spike from cDNA was much less robust then with genomic DNA (gDNA). More than half of the SL RNA spike samples amplified over 30 cycles for the fragmentation and recuperation and transplantation experiments (41/75 or 54.7% and 28/47 or 59.6% respectively). The standard curve amplified dilutions with C_T values from 20.94 – 33.44 with tight replication for all dilution replicates and an average efficiency of 107% (corresponding to a slope of -3.16, $r^2 = 0.991$). The range of C_T

values from the fragmentation and recuperation were 23.66 – 33.78 and 23.38 – 33.21 for the transplantation experiment (Appendices D and G). These ranges are approximately within the range of the standard curve, but the high C_T values are in the arena where binding probabilities are reduced and reproducibility among replicates is reduced. Of the samples with C_T values above 30 cycles, 5 had standard deviations above 0.300 for the fragmentation and recuperation experiment (6.7% or 5/75 samples) and 3 had standard deviations above 0.300 for the transplantation experiment (6.4% or 3/47 samples). Melting curve analysis from samples amplifying the SL RNA spike revealed single peaks with a melting temperature of ~76.85°C except for 5/75 and 9/47 from the fragmentation and transplantation experiments respectively that had samples with at least one replicate with a bad melt curve. There were some samples with only a single C_T value with a solid melting curve (3 in both experiments) amplifying over 30 cycles.

Lastly, amplification of hsp70 in the host and symbiont separately from cDNA was not quantifiable. All samples either did not amplify or amplified far above the 30-cycle maximum (35 and above out of 40 cycles). At this range of the amplification cycle, quantification and reproducibility are low due to extremely low binding probabilities and low template concentrations. Of those samples that did amplify, only one replicate amplified whereas the other two did not amplify or had multiple peaks or extremely different amplification kinetics (different exponential, linear, and plateau phases). Several samples had multiple peaks in the melting curve analysis and the efficiency of the primer set with cDNA was extremely low (average 82.4% for the host and 77.6% for the symbiont). This made it impossible to quantify gene expression of hsp70 in the host or

symbiont. Melting curve analysis for standards for hsp70 in the host and symbiont were 79.09°C and 82.82°C respectively.

There are several possible explanations for the problems amplifying hsp70 in the host and symbiont from cDNA including: 1) poor RNA template, 2) poor efficiency of cDNA synthesis, and 3) PCR inhibitors. A subset of the RNA extracted from each sample was DNase treated, precipitated, and washed prior to cDNA synthesis. The 260 / 280 ratios for the RNA after precipitation were all between 1.71 – 2.01 with the exception of 3 samples in the fragmentation and recuperation experiment that had low yields of RNA (260 / 280 ratio around 1.60). The 260 / 230 ratios, which are indicative of purity of RNA, were all above 1 suggesting high quality and pure RNA. Figure 5 shows total RNA extractions from five coral samples prior to DNase treatment (5A) as compared to the same five coral samples post-DNase treatment and precipitation (5B). The images show the typical 28s and 18s rRNA bands as well as a smear of several different size classes of RNA (rRNA, tRNA, mRNA) and DNA. There is also a high molecular weight band (5A) that disappears after DNase treatment. DNase treatment removed the high molecular weight DNA and likely degraded small DNAs. Still visible are the 28s and 18s large and small ribosomal subunit bands as well as a light smear of other RNAs. This suggests that quality and quantity of RNA was still good prior to cDNA synthesis. These results imply that that the RNA template was not the source of poor amplification in qRT-PCR, but rather that cDNA synthesis reactions were not efficient or that PCR inhibitors were present in coral samples.

Field experiments: Exploring fragmentation and transplantation

Fragmentation and recuperation experiment

Symbiodinium cell number

To identify whether *Symbiodinium* cell number normalized to total RNA (ng) differed significantly over time after fragmentation and during recuperation, matched pairs t-test were completed by week. Transformed *Symbiodinium* cell number did not significantly differ for any matched pair comparisons by week (Figure 6 and Table 4). Using the sequential Bonferroni correction, $\alpha = 0.005$ ($\alpha = 0.05 / 10$, where 0.05 is the overall α for all tests and 10 is the number of matched pairs comparisons). None of the tests were significant at this alpha level. The raw data for *Symbiodinium* cell densities can also be found in Appendix C.

Table 4. Matched pairs t-test comparisons of *Symbiodinium* cell densities by week for the fragmentation and recuperation experiment, where N = sample size for each comparison and DF = degrees of freedom.

Time Point Comparisons	N	DF	t-Ratio	p-value
Difference 1 - 0	13	12	1.469	0.168
Difference 2 - 0	12	11	0.906	0.385
Difference 3 - 0	11	10	0.775	0.457
Difference 4 - 0	14	13	1.855	0.086
Difference 2 - 1	12	11	-1.342	0.207
Difference 3 - 1	11	10	-0.250	0.808
Difference 4 - 1	13	12	0.376	0.713
Difference 3 - 2	12	11	0.456	0.657
Difference 4 - 2	12	11	1.244	0.239
Difference 4 - 3	11	10	0.797	0.444

Variance component estimates at each of the time points revealed that for weeks 0, 3, and 4, 100% of the variability in *Symbiodinium* cell number was due to within colony variability (Table 5). At weeks 1 and 2, variability was attributed to both variation between and within colonies. Overall variability between and within colonies for the duration of the experiment was 10.4% and 89.6% respectively, which suggests

that the majority of the variability in *Symbiodinium* cell densities was due to variability of within colony measurements.

Table 5. Variance component estimates and percentages for between and within colony measurements of *Symbiodinium* cell densities by week for the fragmentation and recuperation experiment.

	Time Point (Weeks)				
	0	1	2	3	4
Colony	0.000	0.425	0.383	0.000	0.000
Within	0.115	0.581	0.135	1.094	0.933
Total	0.115	1.006	0.518	1.094	0.933
Percentage					
Colony	0.0	42.2	74.0	0.0	0.0
Within	100.0	57.8	26.0	100.0	100.0
Total	100	100	100	100	100

Maximum quantum yield

To observe whether maximum quantum yield differed significantly over time after fragmentation and during recuperation, matched pairs t-tests were completed by date. Transformed maximum quantum yield was significantly different and higher for July 24, 2007 compared with August 2, 2007 (df = 14, t-ratio = -6.234, p-value = 0.000) (Figure 7 and Table 6). Other matched pairs were nearly significant including: 1) July 24th vs. August 7th, 2) August 2nd vs. August 14th, and 3) August 2nd vs. August 16th (Figure 7 and Table 6).

Variance component analysis revealed that variability in maximum quantum yield was 100% due to variability within each colony for the dates July 26, August 1, 9, and 16 (Table 7). For the remaining dates, within colony variability represented the majority of variability in maximum quantum yield measurements, with between colony variation

representing less than 50% of the variability for all dates except July 24th (Table 7). Overall, 15.8% of the variability in maximum quantum yield can be attributed to variability between colonies, while 84.2% of the variability is due to within colony measurements of maximum quantum yield.

Transplantation experiment

Temperature and light intensity differences: Lifted versus control blocks

To determine whether there were significant differences in temperature between lifted blocks and control blocks, a matched pairs t-test was completed. The distributions of temperature for lifted blocks and control blocks were both normally distributed with standard deviations of 0.870 and 0.814 respectively. The mean temperature for lifted blocks was 27.78°C and for control blocks it was 27.70°C (see Figure 8 for temperature data throughout the experiment). Lifted blocks were warmer and significantly different from control blocks (mean difference of ~ 0.07, degrees of freedom = 699, t = 13.695, p-value < 0.0001 [two-tailed]). The temperature resolution of the HOBO®Pendant Data Logger for temperature is to 0.1°C, which suggests that this difference though statistically significant, is not larger than this resolution.

In addition to determining whether temperature was significantly different for lifted versus control blocks for the entire duration of the experiment, comparisons of temperature were made during midday (11 am – 2 pm) for lifted versus control blocks. The distribution of temperatures for both lifted and control blocks were not normally distributed, therefore, a non-parametric Wilcoxon Sign-Rank test was used to compare temperatures during midday for lifted versus control blocks. The median midday

Table 6. Matched pairs t-test comparisons of maximum quantum yield by date where N = sample size for each comparison and DF = degrees of freedom for the fragmentation and recuperation experiment. The * denotes a significant p-value.

Time Point Comparisons	N	DF	t-Ratio	p-value
7/25 vs. 7/24	15	14	-1.878	0.081
7/26 vs. 7/24	15	14	-2.221	0.043
7/31 vs. 7/24	15	14	-2.844	0.013
8/1 vs. 7/24	15	14	-2.273	0.039
8/2 vs. 7/24*	15	14	-6.234	0.000
8/7 vs. 7/24	15	14	-3.212	0.006
8/9 vs. 7/24	15	14	-2.927	0.011
8/14 vs. 7/24	15	14	-0.868	0.400
8/16 vs. 7/24	15	14	-0.911	0.378
7/26 vs. 7/25	15	14	-0.308	0.762
7/31 vs. 7/25	15	14	-0.538	0.600
8/1 vs. 7/25	15	14	-0.410	0.688
8/2 vs. 7/25	15	14	-2.959	0.010
8/7 vs. 7/25	15	14	-1.099	0.290
8/9 vs. 7/25	15	14	1.116	0.283
8/14 vs. 7/25	15	14	1.116	0.283
8/16 vs. 7/25	15	14	0.881	0.393
7/31 vs. 7/26	15	14	-0.352	0.730
8/1 vs. 7/26	15	14	-0.146	0.886
8/2 vs. 7/26	15	14	-2.406	0.031
8/7 vs. 7/26	15	14	-1.198	0.251
8/9 vs. 7/26	15	14	-0.525	0.608
8/14 vs. 7/26	15	14	1.437	0.173
8/16 vs. 7/26	15	14	1.336	0.203
8/1 vs. 7/31	15	14	0.189	0.853
8/2 vs. 7/31	15	14	-1.610	0.130
8/7 vs. 7/31	15	14	-0.528	0.606
8/9 vs. 7/31	15	14	-0.089	0.930
8/14 vs. 7/31	15	14	1.568	0.139
8/16 vs. 7/31	15	14	1.446	0.170
8/2 vs. 8/1	15	14	-1.421	0.177
8/7 vs. 8/1	15	14	-0.664	0.517
8/9 vs. 8/1	15	14	-0.382	0.708
8/14 vs. 8/1	15	14	2.020	0.063
8/16 vs. 8/1	15	14	2.048	0.060
8/7 vs. 8/2	15	14	1.068	0.304

Table 6. (Continued) Matched pairs t-test comparisons of maximum quantum yield by date where N = sample size for each comparison and DF = degrees of freedom for the fragmentation and recuperation experiment. The * denotes a significant p-value.

Time Point Comparisons	N	DF	t-Ratio	p-value
8/9 vs. 8/2	15	14	1.443	0.171
8/14 vs. 8/2	15	14	3.570	0.003
8/16 vs. 8/2	15	14	3.070	0.008
8/9 vs. 8/7	15	14	0.462	0.651
8/14 vs. 8/7	15	14	2.357	0.034
8/16 vs. 8/7	15	14	2.130	0.051
8/14 vs. 8/9	15	14	2.777	0.015
8/16 vs. 8/9	15	14	2.514	0.025
8/16 vs. 8/14	15	14	-0.071	0.945

temperature for lifted blocks was 28.8°C with an interquartile range of 27.7 – 29.2°C whereas the median midday temperature for control blocks was 28.6°C with an interquartile range of 27.4 – 28.9°C. The Wilcoxon Sign-Rank test showed that midday temperature medians were significantly higher and different for lifted versus control blocks ($z = 2084$, p-value < 0.000 [two-tailed]).

To determine whether there were significant differences in light intensity between lifted versus control blocks, a Wilcoxon Sign-Rank test was completed. The distributions of light intensity for both lifted and control blocks were skewed, with several light intensity values of 0 (corresponding to darkness) and the majority of light intensity values at or below 214 μmol quanta m^{-2} s^{-1} for lifted blocks and at or below 128 μmol quanta m^{-2} s^{-1} for control blocks (see Figure 8 for light intensity values throughout the experiment). The median light intensity for lifted blocks was 16 μmol quanta m^{-2} s^{-1} with an interquartile range of 0 - 214 μmol quanta m^{-2} s^{-1}. The median light intensity for control

Table 7. Variance component estimates and percentages for between and within colony values of maximum quantum yield by date for the fragmentation and recuperation experiment.

	7/24/07	**7/25/07**	**7/26/07**	**7/31/07**	**8/1/07**	**8/2/07**	**8/7/07**	**8/9/07**	**8/14/07**	**8/16/07**
Colony	0.0005	0.0004	0.0000	0.0005	0.0000	0.0001	0.0000	0.0000	0.0002	0.0000
Within	0.0005	0.0010	0.0008	0.0008	0.0006	0.0007	0.0006	0.0004	0.0004	0.0005
Total	0.0010	0.0014	0.0008	0.0013	0.0006	0.0008	0.0006	0.0004	0.0006	0.0005
Percentage										
Colony	51.3	29.7	0.0	35.6	0.0	10.2	2.3	0.0	35.8	0.0
Within	48.7	70.3	100.0	64.4	100.0	89.8	97.7	100.0	64.2	100.0
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

blocks was 12 with an interquartile range of 0 – 129 μmol quanta $m^{-2} s^{-1}$. The Wilcoxon Sign-Rank test showed that median light intensity values were significantly higher and different for lifted versus control blocks (z = 37,528, p-value < 0.000 [two-tailed]). Midday (11 am – 2 pm) values of light intensity were also compared for lifted versus control blocks. The distributions of light intensity for both lifted and control blocks were not normal, and the Wilcoxon Sign-Rank test was used to compare light intensity values for lifted versus control blocks. The median midday light intensity value for lifted blocks was 389 μmol quanta $m^{-2} s^{-1}$ with an interquartile range of 213 – 549 whereas control blocks had a median midday light intensity value of 264 with an interquartile range of 135 – 351μmol quanta $m^{-2} s^{-1}$. The Wilcoxon Sign-Rank test showed that midday median light intensity values were significantly higher and different for lifted versus control blocks (z = 2566, p-value < 0.000 [two-tailed]).

Maximum quantum yield

On the night prior to transplantation, maximum quantum yield was calculated from F_o and F_m measurements taken from all fragments on all blocks. The mean maximum quantum yields for lifted and control blocks were 0.642 and 0.636 with standard deviations of 0.381 and 0.376 respectively. A matched pairs t-test showed that there were no significant differences in maximum quantum yield for fragments to be lifted versus control fragments on the night prior to the start of experimentation (degrees of freedom = 24, t = 1.438, p-value = 0.163) (Figure 9A).

To determine whether there were significant differences in maximum quantum yield over time, by treatment, and by the interaction of time and treatment, a repeated measures ANOVA was conducted for the transplantation experiment. The distributions

by date were approximately normal for 8/21 and 8/27 whereas 8/23 had a single outlier. The means for maximum quantum yield for 8/21, 8/23, and 8/27 were 0.625, 0.623, and 0.642 (standard deviations were 0.405, 0.416, 0.371 respectively; raw values, not transformed). The mean maximum quantum yield values by treatment were 0.623 and 0.632 with standard deviations of 0.416 and 0.389 for up and down blocks respectively (raw data). Means and standard deviations for date x treatment are in Table 8. There were no significant differences in maximum quantum yield by date, treatment (up versus down), or by the interaction of date and treatment (Figure 9B and Table 9).

Table 8. The means and standard deviations (Std. Dev.) by date and treatment for the transplantation experiment.

	8/21 Up	8/21 Down	8/23 Up	8/23 Down	8/27 Up	8/27 Down
Mean	0.626	0.622	0.633	0.610	0.637	0.646
Std. Dev.	0.019	0.015	0.016	0.023	0.015	0.020

Variance component analysis showed that maximum quantum yield measurements from fragments on lifted blocks had 100% variability due to within colony variation and large standard deviations (Figure 10A and Table 10). In comparison, maximum quantum yield measurements from fragments in control blocks had a majority of variability due to within colony variation, but also some variability associated with between colony measurements of maximum quantum yield with much smaller standard deviations (Figure 10B and Table 10). No variances component estimates were computed for 8/27 because only a single fragment from each colony remained in each treatment (all variability was accounted for by between colony variation). Overall, between colony variability accounted for 10.2% of the variation in maximum quantum yield while within colony variability accounted for 89.8% of the variation in maximum

Table 9. Repeated measures ANOVA table from the transplantation experiment, where SS = sum of squares, MS Num = means square numerator, MS Den = means square denominator, DF Num (adj) = adjusted degrees of freedom for the numerator, and DF Den (adj) = adjusted degrees of freedom for the denominator, F-ratio = F statistic, p-value = p-value of the F-statistic.

Factor	SS	MS Num	MS Den	DF Num (adj)	DF Den (adj)	F ratio	p-value
Date	0.00263	0.00131	0.00065	1	25	2.033	0.1663
Treatment	0.00136	0.00136	0.00065	1	50	2.111	0.1525
Date*Treatment	0.00088	0.00044	0.00065	1	25	0.679	0.4177

quantum yield.

Genotyping colonies of M. capitata

Colonies A-M were genotyped using the ATPSß primer set, and seven variable sites were identified within the 217 bp intron, located at positions: 42, 99, 142, 154, 156, 177, 204 bp within the intron (Figure 11). All PCR products amplified from the ATPSß primer set were sequenced directly from PCR products and were not cloned to distinguish alleles. For homozygotes, sequence reads were clean, which means at each base position, there would only be a single peak in the sequence electrogram. Heterozygotes contained a mixed template or unclean sequence electrogram, which means that at each variable site two peaks would represent a single base position. Colonies A, L, and M were homozygotes (A and M had the same genotype, which means the sequence electrogram was identical). Colonies B, E, G, J, and K were heterozygotes, with B and E having the same genotype (identical sequence electrograms) and all others had distinct genotypes.

Table 10. Variance component estimates and percentages for between and within colony values of maximum quantum yield by date and treatment for August 21st and 23rd from the transplantation experiment.

	8/21 Up	8/21 Down	8/23 Up	8/23 Down
Colony	0.0000	0.0002	0.0000	0.0001
Within	0.0009	0.0004	0.0012	0.0003
Total	0.0009	0.0006	0.0012	0.0004
Percentage				
Colony	0.0	31.0	0.0	19.6
Within	100.0	69.0	100.0	80.4
Total	100.0	100.0	100.0	100.0

Chapter 4.0. Discussion

Gene identification

Sequences of hsp70 in the coral host *M. capitata* and *Symbiodinium* were successfully amplified and isolated using degenerate PCR. Although time consuming, this approach can be extremely useful and successful for isolating highly conserved genes present in the coral-algal symbiosis when genome information is not available (Finnerty 2001). For cost effective studies, degenerate PCR is a rapid and sensitive technique for the isolation of conserved genes such as the heat shock proteins and homeobox genes (Murtha *et al.* 1991).

Validating and normalizing qRT-PCR

Amplification of the SL DNA spike added to each sample was clearly quite robust among different samples and within replicates. All standard deviations with the exception of 3 samples were below 0.300, indicating high reproducibility within replicate samples. In addition, all samples amplified within the quantifiable 10 - 30 C_T range, although some samples amplified prior to the highest standard. To increase accurate quantification, dilutions of the standard curve should be made over the range of copy numbers that includes the expected mRNA quantities in unknown samples (Bustin 2000). Ideally in qRT-PCR, amplification of the housekeeping gene should have a similar primer set efficiency as well as be expressed, or in the case of an exogenous spike, be present with roughly the same amount of RNA (Bustin 2000). In this case, the SL DNA spike amplified several cycles before hsp70 in the symbiont from gDNA.

There are two ways to troubleshoot amplification so that threshold values overlap for the SL DNA spike and hsp70 in the symbiont. A simple test run altering the amount

of spike taken through the TRIzol®Reagent DNA extraction and then amplified in qRT-PCR can easily identify how much spike is needed to amplify within the same range of hsp70 in the symbiont. Or alternatively, reducing the dilution factor of the starting template will help to decrease C_T values of hsp70 in the symbiont so that they fall within the range of the SL DNA spike and the range of the standard curve. To ensure accurate quantification of hsp70 in the symbiont, different dilutions of starting template should be run through qRT-PCR to find the optimal concentration of gDNA.

For experimental treatments or from sample to sample, however, amplification of the SL DNA spike and hsp70 in the symbiont from gDNA will differ and may not have the same range of threshold values. Corals that bleach as a result of experimental treatment will contain fewer symbionts such that they will contain fewer copies of hsp70 in the symbiont. These samples will amplify with higher C_T values, and may not fall within the same range of the SL DNA spike. To avoid this problem, it may be necessary to add altered amounts of spike to each sample depending on the amount of starting biological material (Smith *et al.* 2003) as opposed to a fixed amount of spike to each sample. Calculating the amount of spike to add to each sample based on the amount of starting material is preferable when valid quantification of RNA and DNA is not possible (i.e. in single cells or microdissected tissues) (Gilsbach *et al.* 2006), which is the case with coral samples. Accurately quantifying biological material in corals can be complicated by the presence of the calcium carbonate skeleton, which is not easily separated from live tissue containing host and symbiont cells. This can lead to inaccurate calculations of starting material (Bak 1973). Despite these difficulties, the SL DNA spike can still be used as a reference gene if its amplification efficiency is similar to

hsp70 in the symbiont, because this suggests that both primer sets are equally efficient at amplifying their respective target amplicons despite variable amounts of the SL DNA spike or hsp70 in the symbiont from a particular sample.

Normalizing gene expression studies using SMP will more accurately quantify changes in gene expression because the ratio of host and symbiont tissue from the initial coral extraction will be accounted for as well as extraction efficiency between samples. The additional step of calculating SMP and the qRT-PCR reactions are easy to complete, fast (runs typically take only 2 hours even with the melting curve analysis), and efficient. The 96-well plates make it easy to run several samples, standards, and no template controls within a single day. Unfortunately, the cost of reaction components can be prohibitively expensive (Bustin 2002). Once primer sets have been properly tested (no primer dimers, high efficiency, single melting peaks), however, multiplex reactions using gene specific probes can allow amplification of several products within the same tube and detected in parallel (Wittwer *et al.* 2001). This will reduce the number of reactions, the price, and the run time (melting curves are not required).

The SL RNA spike and hsp70 in the host and symbiont did not amplify from cDNA such that quantification was possible. In order to use an exogenous RNA spike as an internal reference gene and to control for extraction efficiency, the spike needs to amplify in every sample, with tight replication, and with comparable efficiency to genes of interest (Johnson *et al.* 2005, Bower *et al.* 2007). Amplification of the RNA spike in unknown coral samples from both experiments amplified above 30 cycles. Although several samples were within the range of the standard curve, amplification above 30 cycles is within the range where the probability of the primer binding to the template is

significantly lower and not as reproducible (Bustin 2000). For hsp70 in the host and symbiont, most samples did not amplify, and those that did, were well above the 30 cycles. Samples that did amplify, had C_T values in the high 30's and had only one replicate amplify out of three (data not shown). The qRT-PCR data indicate the following problems: 1) poor efficiency of the cDNA synthesis step, 2) degradation of cDNA during storage or freeze-thaw, and/or 3) a PCR inhibitor. Clearly, steps need to be taken to improve the amplification of the SL RNA spike and hsp70 from cDNA.

The ability to accurately quantify copy number in qRT-PCR is limited by the efficiency of converting RNA to cDNA, which relies on the enzyme used in reverse transcription. The RT step is a major source of variability in qRT-PCR experiments, due in part to the sensitivity of reverse transcriptase enzymes to salts, alcohols, and phenol, which are commonly used in many RNA extractions (Freeman *et al.* 1999). RNA isolated samples that have been precipitated prior to use in cDNA synthesis, such as in this study, often contain an excess of salt that can greatly impact the downstream quantification of target genes in qRT-PCR. In some cases, low concentrations of added salt ($\leq$10 mM) enhance amplification, while in other cases high levels of salt impede amplification ($\geq$50 mM) (Freeman *et al.* 1999). Additionally, if the reverse transcriptase is compromised prior to use, there will be a concomitant reduction in cDNA synthesis that will affect downstream quantification of target genes. That the SL DNA spike amplified so that quantification was possible and the SL RNA spike did not even though the same concentration of both was added prior to extraction, suggests that the reverse transcriptase was not functioning properly. The results from this study reveal that qRT-

PCR quantification was greatly inhibited by the cDNA synthesis step due to excess salt and or/a compromised reverse transcriptase.

The cDNA was stored at -80°C until it was needed for use in qRT-PCR (1 month). Prior to amplification, cDNA was diluted 1/20 using DEPC treated water and stored at -80°C when not in use. It is possible that cDNA stored at this temperature degrades more rapidly than RNA stored at the same temperature. In a previous study, mRNA and first strand cDNA stability were compared at three different temperatures after three weeks of storage. The mRNA was more stable than cDNA at 4°C, while at -21°C and -70°C there were no significant differences between mRNA and cDNA (Wilkening and Bader 2004). The mRNA appeared to be more stable than cDNA at both -21°C and -70°C (slightly more stable at -70°C). After cDNA synthesis, hybrids of cDNA:RNA exist that may be more susceptibility to degradation from RNases, leaving single stranded cDNA. It is unclear how stable single stranded cDNA is and whether long-term storage results in degradation. cDNA can also be susceptible to degradation after many cycles of freeze-thaw during sample preparation for qRT-PCR. Although steps were taken to minimize freeze-thaw, this can greatly decrease the stability of cDNA used in qRT-PCR. To remove both of these elements of variability to qRT-PCR analysis, RNA should be stored at -80°C and cDNA synthesis reactions and dilutions should be completed just prior to use.

The presence of PCR inhibitors in the reaction could be due to oligos, excess buffer, or enzyme left over from the cDNA synthesis step or excess lipids from coral samples (Akane *et al.* 1994). Although an extra step was included in the RNA extraction step to remove excess polysaccharides and lipids, it is possible that inhibitors were still

present. Corals contain high levels of lipids (Benson and Muscatine 1974), which often prevent high quality DNA and RNA extractions. Inhibitors can be removed by dilution, but sometimes this does not fix the problem (Kubista 2006). Results from qRT-PCR using gDNA suggest, however, that PCR inhibitors present or left over from the original extractions were not a problem, because amplification of the SL DNA spike and hsp70 in the symbiont from gDNA was quantifiable in qRT-PCR. It is more likely that the reverse transcriptase was inefficient and not functioning properly.

Lastly, efficiency of the primer sets hsp70 in the host and symbiont were low, which suggests that the amplicon was not doubling at every cycle. The benefit of using a standard curve for quantification is that it is possible to determine the limits of amplification for a particular primer set (Bustin 2000). The lowest standard dilution often did not have as tight replication as the other four dilutions and had C_T values above 30. The primers were having difficulty sitting down on the right target for the lower end of standard dilutions used in qRT-PCR. This suggests the lower end of the standard curve was difficult to accurately quantify and contributed to the low efficiency of the primer set. As a general rule, eight dilutions of the standard curve should be run alongside several samples prior to final qRT-PCR to determine: 1) the limits of amplification at the lowest and highest end of the dilution series and 2) where the majority of samples fall within the standard curve so that future quantification is possible (Bustin 2000). If the standard curve still results in low efficiency, the primer set should be re-designed (Bustin 2000).

Although it was not possible to quantify relative gene expression for hsp70 in the host or symbiont, several steps were taken to properly normalize gene expression and to

understand the limitations of qRT-PCR. An exogenous reference gene can be used to normalize gene expression, but amplification of this spike needs to be thoroughly validated. The RNA spike must amplify in every sample, with tight replication, and with a single melting curve peak. To quantify genes of interest in qRT-PCR, it is essential that the RNA template is of high quality and quantity and that the cDNA synthesis reaction working properly with high efficiency. In addition, efficiency of all primer sets need to be within 90 -100%, so that doubling of the amplicon occurs at every cycle. Once the average C_T, efficiency, and SMP has been calculated, they can all be related using the following equation to calculate relative gene expression (Mayfield *et al.* accepted):

$$(7)\ E_{SL}^{(SL\ C_T)} / E_{target}^{(target\ C_T)} / SMP,$$

E_{SL} = the PCR efficiency of the tilSLα primer set

${}^{SL\ C}{}_{T}$ = threshold value of SL in the specific cDNA amplified sample

E_{target} = the PCR efficiency of the gene of interest primer set

${}^{target\ C}{}_{T}$ = the threshold value of the gene of interest from the cDNA amplified sample.

Equation 7 can be further divided by total RNA or another aspect that takes into account differences in overall tissue amounts for each sample (Bustin 2000, Hugget *et al.* 2005, Mayfield *et al.* accepted).

Field experiments: Exploring fragmentation and transplantation

Fragmentation and recuperation experiment

Symbiodinium cell densities

In order for a symbiosis to achieve stability, the symbiont must grow at a similar rate to the host, especially, if the host growth rate is at its maximum (Muscatine and Pool

1979). Under conditions of illumination and external feeding that favor growth of both components, a steady-state ratio of symbionts per host cell exists. This steady-state condition will change when the symbiosis is exposed to changes in environmental conditions. In this study, coral branches broken off from parent colonies contained a lesion site, which was exposed to the external environment. Eventually live coral tissue will grow over the lesion site and subsequently symbiont cells will re-populate the new host tissue. If symbiont cell densities had been measured from these lesion sites over time, densities would likely have increased over time. Since sampling occurred 1 cm from the tip of the branch, far from the lesion site, symbiont cell densities did not change over time because fragmentation did not directly affect this area. It is apparent from the data set that *Symbiodinium* cell densities measured 1 cm from the tip of a coral fragment may not be the best indicator of stress following fragmentation.

The high within-colony variability and large variance components for *Symbiodinium* cell densities justifies systematic repeated measurements in surveys of cell densities over time. The standard deviations associated with biological replicates as well as repeated measures within the same coral colony were very large, which suggests a huge spread or dispersion of the data. This makes it difficult to interpret the results of the statistical tests because there is such high variability associated with measuring *Symbiodinium* densities. In a study such as this, where the number of colonies is limited, repeated measures within the same colony will reduce the total variance associated with within colony measurements and increase the power of the study (Stengel *et al.* 1999). Increasing the sample size and further testing of the qRT-PCR method will help to explain whether this variability is natural or due to the small sample size in this study.

Maximum quantum yield

Measurements of F_v/F_m from July 24th were significantly different and higher than August 2nd even given the conservative nature of the matched pairs t-test. The overall pattern of F_v/F_m reveals a slow decline through August 2nd with a subsequent rise in F_v/F_m through until the last date of sampling (August 16th). The results from this experiment are somewhat surprising, because if fragmentation was greatly affecting F_v/F_m, it is likely that the first week of sampling would reveal the lowest values of F_v/F_m. Instead, maximum quantum yield values are highest during the first week of sampling. There are several other possible explanations for the decline and rebound of F_v/F_m including: 1) chronic photoinhibition, 2) normal seasonal and daily fluctuations in F_v/F_m, and/or 3) abiotic factors.

The pattern of F_v/F_m in coral fragments suggests a chronic as opposed to dynamic photoinhibition. The former refers to a photoprotective regulatory process that is initiated in the photosynthetic apparatus when there is severe damage (Gorbunov *et al.* 2001). Normally this process takes place over an extended period of time and recovery of the PSII is slow (on the time-scale of weeks) whereas recovery form dynamic photoinhibition takes place within minutes to days. The data suggest a progressive decline over two weeks with a subsequent recovery in the last two weeks, which is commiserate with disruption of PSII and a slow recovery time typical of chronic photoinhibition.

Alternatively, photosynthetic efficiency of corals varies significantly over time-scales (i.e. time of day and/or season), which suggests that care must be taken in interpreting data from experiments (Fitt *et al.* 2001). For example, a decline in F_v/F_m

value may be statistically significant, such as in this experiment, but may actually be reflecting seasonal variation of normal photosynthetic efficiency of corals instead of chronic photoinhibition or algal dysfunction. It is difficult to differentiate from this study whether changes in F_v/F_m are the result of chronic photoinhibition or simply natural variability. In order to accurately assess changes in fluorescence data, other biochemical, molecular, or physiological data should be collected in tandem to ensure accurate interpretation of maximum quantum yield data. In addition, any prior knowledge of environmental parameters such as temperature, light intensity, tidal history, and characteristics that are specific to the site(s) is essential when analyzing photophysiological data in corals from the field (Fitt *et al.* 2001).

Previous studies have found that both temperature and light intensity can affect photosynthetic capacity, and so both factors were measured in this experiment (Iglesias-Prieto 1992). There is a slight increase in the temperature maxima from July 24th through August 12th as well as an increase in light intensity maxima from August 3rd through August 13th (Figure 12). The progressive decline and rebound of F_v/F_m, however, is not directly explained by these changes in temperature and/or light intensity. It is possible that other abiotic factors may be responsible for the observed changes in F_v/F_m, but were not directly measured. The water clarity during the course of the experiment did vary (personal observation), which suggests turbidity at the study site may influence fluorescence measurements. In order to more conclusively interpret F_v/F_m data in the future, several factors should be measured in corals to obtain a more comprehensive picture of how fragmentation can affect the host and symbiont from the molecular to the physiological level. This will allow for a distinct interpretation between natural

variability and true stress. This type of data will also detect which factors are most sensitive and at what biological level (subcellular, cellular, physiological) corals are affected by fragmentation, handling, and/or a specific stress.

Measurements of maximum quantum yield were highly variable within each colony but less so among different colonies. It is important to note, however, that between and within colony variance component estimates at each time point were small. This suggests that the within and between colony variances are negligible in determining F_v/F_m (Yandell 1997). Based on these results, the colony factor could be ignored in future experiments because its variability is negligible. Due to the small sample size for both within and between colony measurements of F_v/F_m in this study (n = 3 within, n = 5 biological replicates), repeat measurements and a larger sample size should be used to confirm this result.

Genotyping colonies of *M. capitata*

For the fragmentation and recuperation experiment, all hosts had different genotypes except colonies B and E, which were identical heterozygotes. The goal of genotyping coral host colonies was to ensure that colonies designated as biological replicates were in fact genetically distinct. *M. capitata* can reproduce asexually by cloning or fragmentation, which was a concern. That colonies A, C, and D contained distinct genotypes suggests that these colonies are not asexual propagations or fragments of each other. Even though colonies B and E had the same genotype at the ATPSβ locus, this does not necessarily mean they are genetically identical. Colonies B and E were separated by several meters which suggests that asexual propagation or fragmentation is unlikely. It is impossible to determine the probability that colonies B and E would be

different at other loci, because all loci are independent of each other. The probability that colonies B and E are identical at the ATPSβ locus assuming that each genotype sampled in this study has only 2 alleles is:

$$(1)\ P(A1) \times P(A2) + P(A2) \times P(A1),$$

where P(A1) = the probability of encountering allele 1 for genotype B (or E) from the 10 colonies sampled in this study

P(A2) = the probability of encountering allele 2 for genotype B (or E) from the 10 colonies sampled in this study. This would be equal to:

$$(2)\ 2/20 \times 2/20 + 2/20 \times 2/20 = 8 / 400 = 2\%.$$

Transplantation experiment

Temperature and light intensity differences: Lifted vs. control blocks

There were statistically significant differences between lifted and control blocks for both temperature and light intensity for the duration of the entire experiment and at midday (11 am – 2 pm). Only those differences during midday were statistically significant and greater than the resolution of the data loggers. This difference was still very small. In terms of biological significance, these changes in temperature and light intensity did not produce significant differences in F_v/F_m over time, by treatment, or the interaction of both terms. If this type of experiment was repeated or used in a gene expression study, it would be better to conduct it from mid May through to mid August over a deeper depth in Kaneohe Bay, Hawaii. There is a weak thermocline from May to August at about 8.5 meters, but this can vary 1 to 2 m either shallower or deeper during the summer (Jokiel, CRAMP publication). Data loggers could easily be placed at

different depths to confirm a difference in temperature and light intensity that was both statistically significant and biological relevant.

Maximum quantum yield

Measurements of F_v/F_m did not significantly differ by time, treatment, or the interaction of the two terms. This suggests that over the time frame that fragments were measured there were no changes in F_v/F_m even though significant differences were found in temperature and light intensity at midday between lifted versus control blocks. Clearly, the difference was not enough to elicit a differential response in F_v/F_m. Alternatively, the experiment took place over the course of a single week. Perhaps if fragments had remained in the field for a longer period of time, these subtle changes in temperature and light intensity would elicit a significant response over time and by treatment. This result seems unlikely, because the difference in treatments was so small.

Similarly to F_v/F_m values from the fragmentation and recuperation experiment, the majority of variability in F_v/F_m was due to within colony measurements. Interestingly, for fragments that were lifted, 100% of the variability in F_v/F_m was due to within colony measurements (Table 10). The variability within fragments from lifted blocks could be due to differences in fragments within the same colony or differences between blocks based on the experimental design. The variance component estimates were very small, which also suggests that variability associated with between and within colony measurements could be ignored in future studies. Due to the small number of within colony measurements (three for August 21st and two for August 23rd), the small sample size (n = 5), and the block design of this study, it is unclear whether colony variability is important in interpreting F_v/F_m results. In the future, repeated

measurements of F_v/F_m should be completed for each fragment and the sample size should also be increased to determine what influence colony has on F_v/F_m. These changes will allow for more predictive power in interpreting colony effects for future experiments.

Genotyping colonies of *M. capitata*

All coral colonies from the transplantation experiment had different genotypes based on the ATPSβ locus. This confirms that colonies are in fact genetically distinct at the ATPSβ locus and are not asexual or fragmented propagations of the same colonies.

Conclusions

Applying molecular tools, and specifically qRT-PCR, to the coral-algal symbiosis paves the way for exploring gene response during the onset of symbiosis (deBoer *et al.* 2007), bleaching (Mitchelmore *et al.* 2002, Smith-Kuene and Dove 2007), and stress events that occur at the molecular level (Downs *et al.* 2000, Brown *et al.* 2002). In comparison to other physiological measurements like growth rates, photophysiological data, reproductive output, or the presence of bleaching, changes in gene response due to the onset of stress or change in the symbiotic state are fast (van Oppen and Gates 2006). Stress genes, in particular, can provide information about how a specific stress affects the symbiosis and how long it takes to respond. Although previous studies have used qRT-PCR to explore gene expression (Mitchelmore *et al.* 2002, deBoer *et al.* 2007), none of these studies accounted for the ratio of coral host and algal symbiont tissue when normalizing gene expression data. In order to expand the current understanding of coral-algal symbiosis, it is essential that gene expression studies utilizing qRT-PCR are properly validated and normalized to a reference gene as well as ratios of host and

symbiont tissues between samples and treatments. The SMP method for normalizing for the presence of two study organisms in qRT-PCR is robust, and can easily be applied in future studies. The focus for further study with qRT-PCR should be optimizing the quality and efficiency of the RT step. This will provide a framework to expand accurate gene expression research within the complex cnidarian-dinoflagellate symbioses.

In order to produce high quality studies that can be used to measure gene regulation and transcription, it is important to ensure that study organisms are not experiencing stress prior to experimentation. In this study, branches were removed from parent colonies and recuperated in the field while indices of algal health were measured to determine the impact of fragmentation. Although significant changes in F_v/F_m were detected over time, it was difficult to identify whether this was the result of stress in the algal symbiont or simply due to natural variability in fluorescence measurements during the summer. In order to more thoroughly explore the sensitivity of corals to fragmentation, several factors (molecular to physiological) specific to the symbiont and host should be measured following fragmentation and during recovery. This type of study could provide a benchmark of sensitivity indices, identifying those factors with the highest and lowest sensitivity following fragmentation or coring in corals. The results would establish at what biological level and to what degree corals are sensitive to handling prior to experimentation and differentiate natural variability in F_v/F_m versus photoinhibition.

Lastly, many scientists prefer to work in the laboratory because it is easier to control factors and assess the results of manipulating a single variable. Fundamentally, corals live in the natural environment where they are exposed to a myriad of conditions

that vary on several scales (Lejeusne *et al.* 2006). As part of conducting an experiment in the field, it is imperative that treatments for the study are in fact different. While this may seem intuitive, it is difficult in practice to ensure given the variable nature outside the laboratory. In this study, despite the fact that blocks were lifted several meters in the water column compared to controls and that significant differences were found in temperature and light intensity during midday, no changes were found in F_v/F_m. The physical differences in temperature and light intensity were not enough to elicit a physiological response. The transplantation experiment highlights an important aspect of field research; it is essential to have a thorough understanding of the natural system in which the organism of study is found and specifically the areas in which field studies are conducted. This includes pilot studies measuring a variety of physical factors from the study site as well as understanding the physiological response (range) of corals to the stresses they will be exposed to in the natural environment. As long as experiments are conducted to ensure that: 1) stress to the organism prior to manipulation is minimized and 2) treatments are different and biologically relevant, experiments can be conducted *in situ* and be used to explore the initiation, regulation, and expression of genes from the cnidarian-dinoflagellate symbiosis.

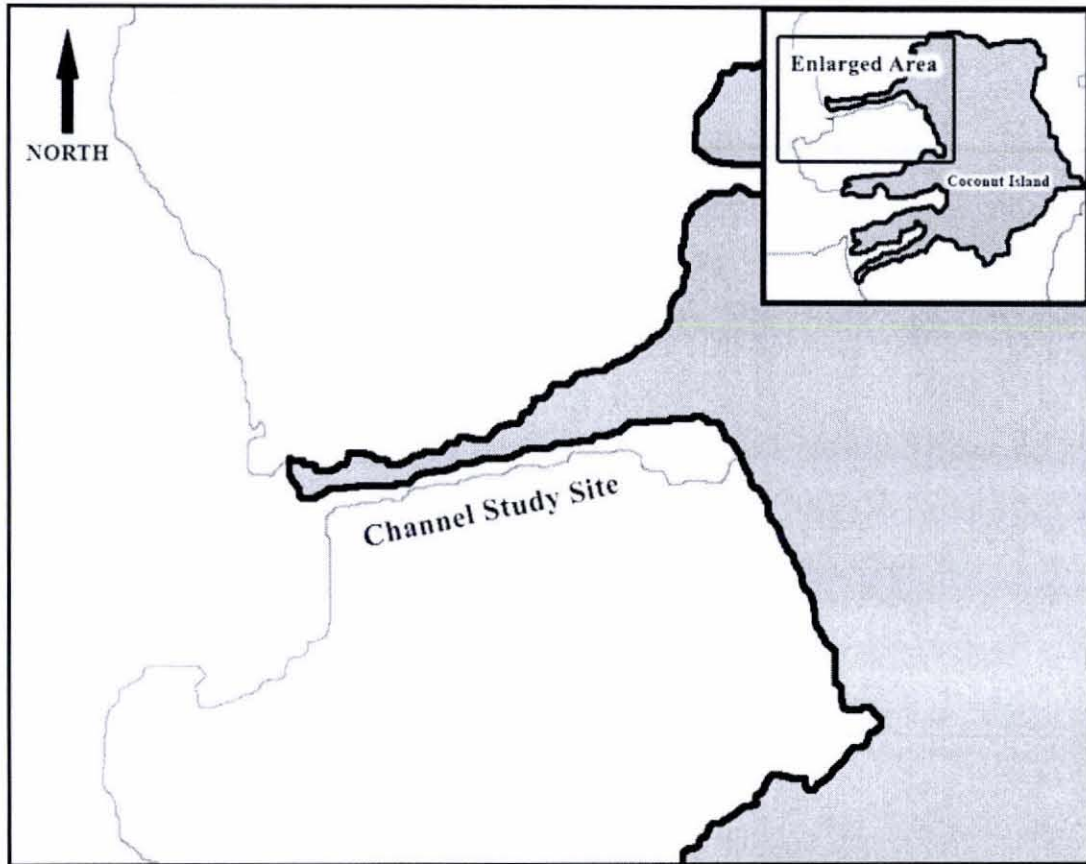

Figure 1. Study site for the fragmentation and recuperation and transplantation experiments. The inset shows Coconut Island and surrounding reefs flats (gray stroke). The channel where both studies were completed is labeled as channel study site. Satellite images were taken from Google™ Earth, © 2007 Navteq and © Europa Technologies, USGS and modified in Adobe® Photoshop® CS3 ed.

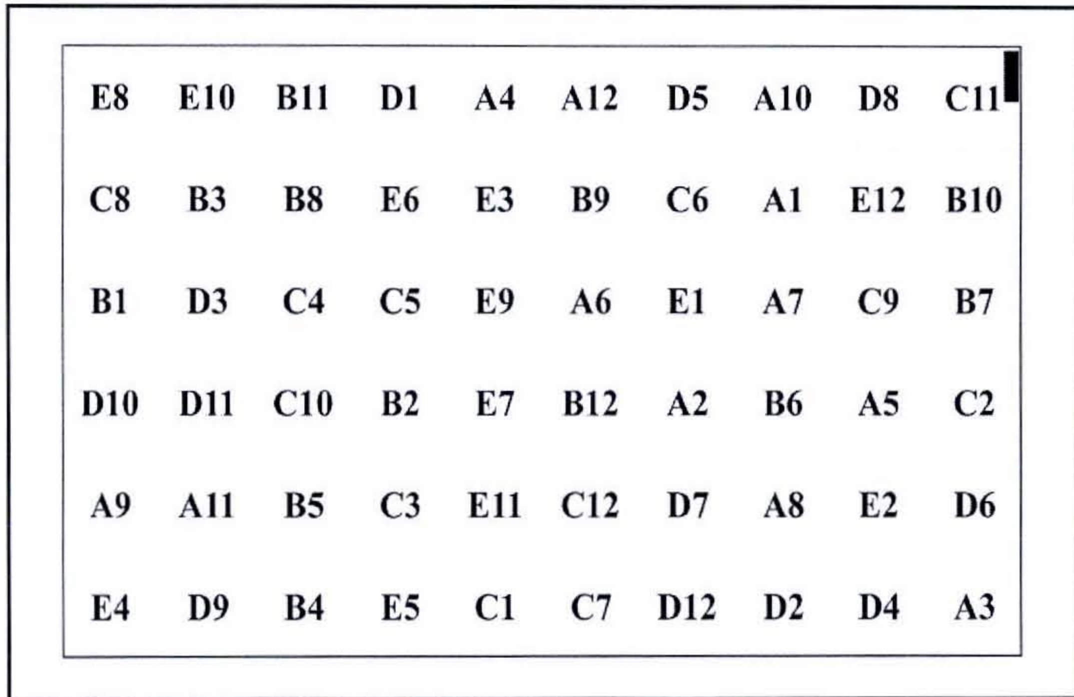

Figure 2. Experimental set-up for colony and fragment orientation used in the fragmentation and recuperation experiment. The black rectangle at the top right hand corner of the sheet represents the HOBO® Pendant data logger.

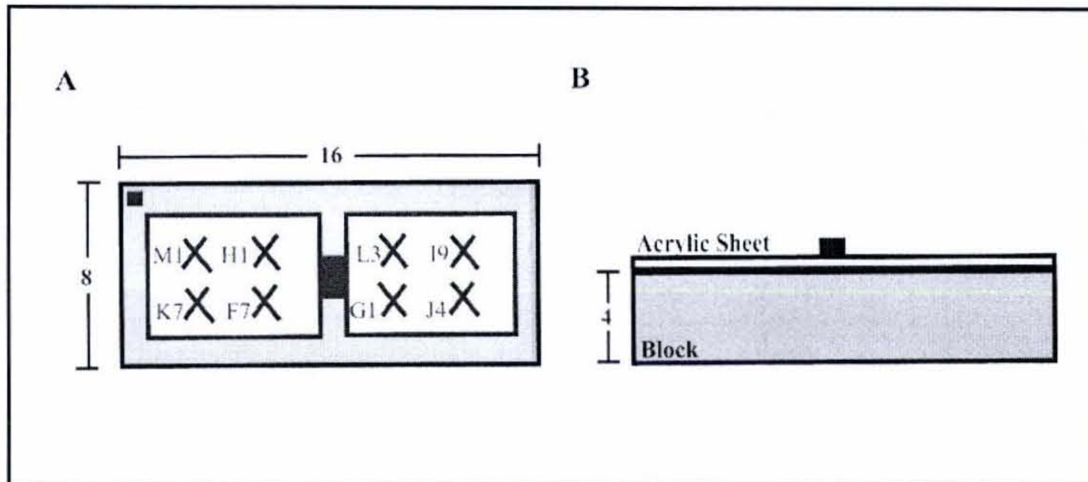

Figure 3. Blocks designed for the transplantation experiment, A) overhead view of the block and B) side view of the block. The black square at the top left corner of A) represents the pink cable tie attached to the top left corner of each block for orientation purposes. The letters and numbers in A) represent colony and fragment number as an example. A single plastic sheet was attached to each block and HOBO® Pendant data loggers were attached to the plastic sheet at the middle all blocks in A) and B).

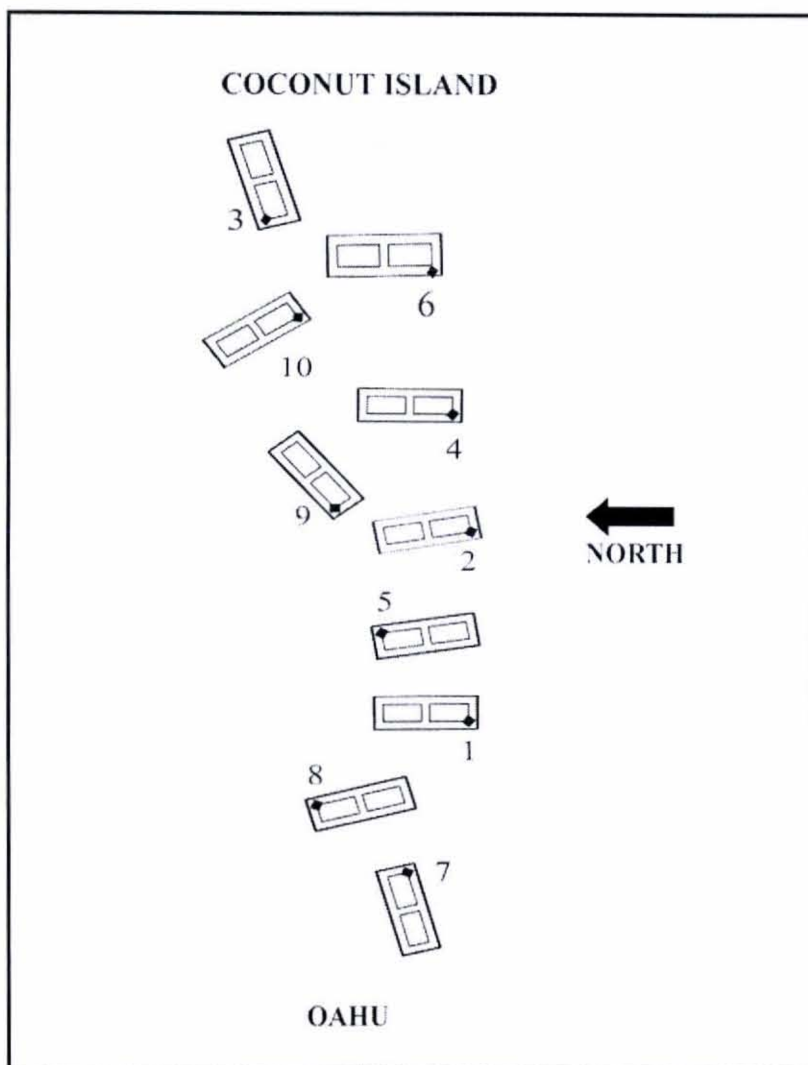

Figure 4. The location and orientation of the blocks for the transplantation experiment within the channel study site. Blocks labeled 1, 2, 3, 4, and 6 were designated as "up" while blocks 5, 7, 8, 9, and10 were designated as "down" blocks. Block 7 is located the farthest from Coconut Island and fragments were collected starting at this block. The black filled-in circle on each block represents the pink cable tie attached to each block.

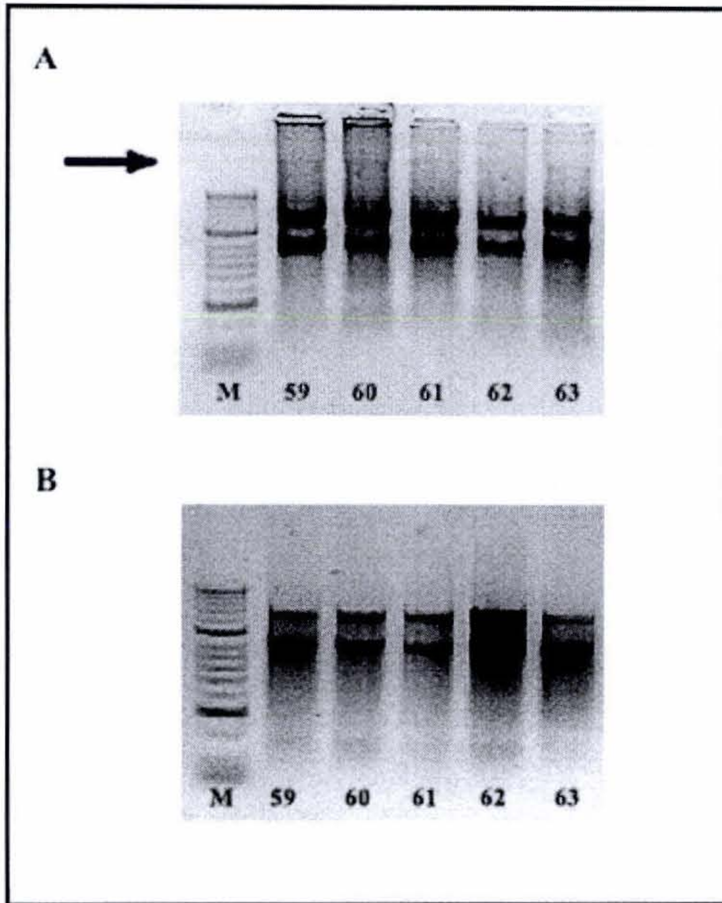

Figure 5. RNA extracted from 5 coral samples prior to DNase treatment (A) and the same 5 samples post-DNase treatment and precipitation (B). The arrow represents high molecular weight DNA that is removed after DNase treatment. Lane M represents the molecular marker (Hyperladder II from Bioline).

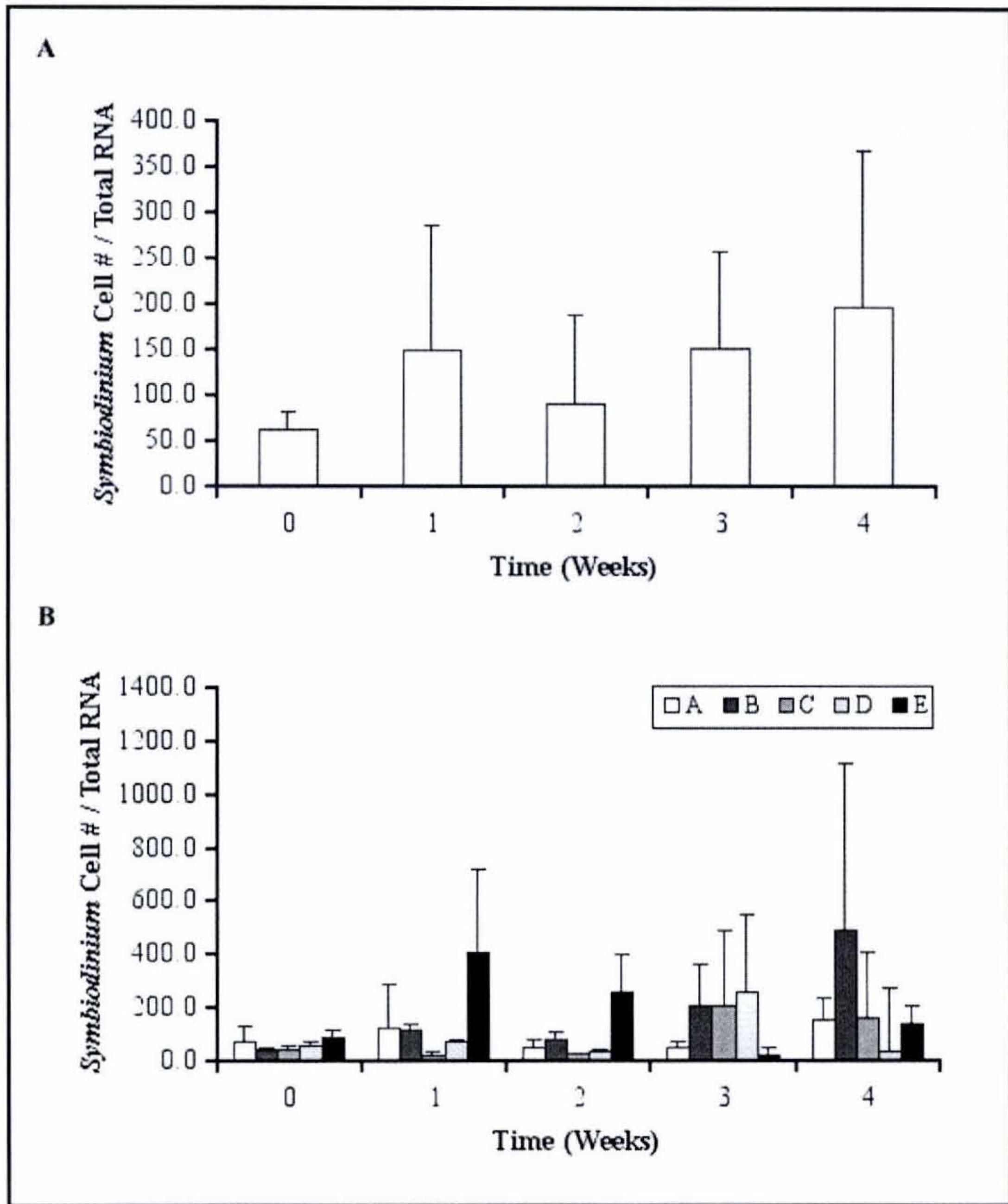

Figure 6. *Symbiodinium* cell density over time for the fragmentation and recuperation experiment. Values mean + 1 SD by week (n = 5) (A) and average *Symbiodinium* cell densities + 1SD for each colony by week (n = 3, except for those noted in the Appendices with a * were not included) (B).

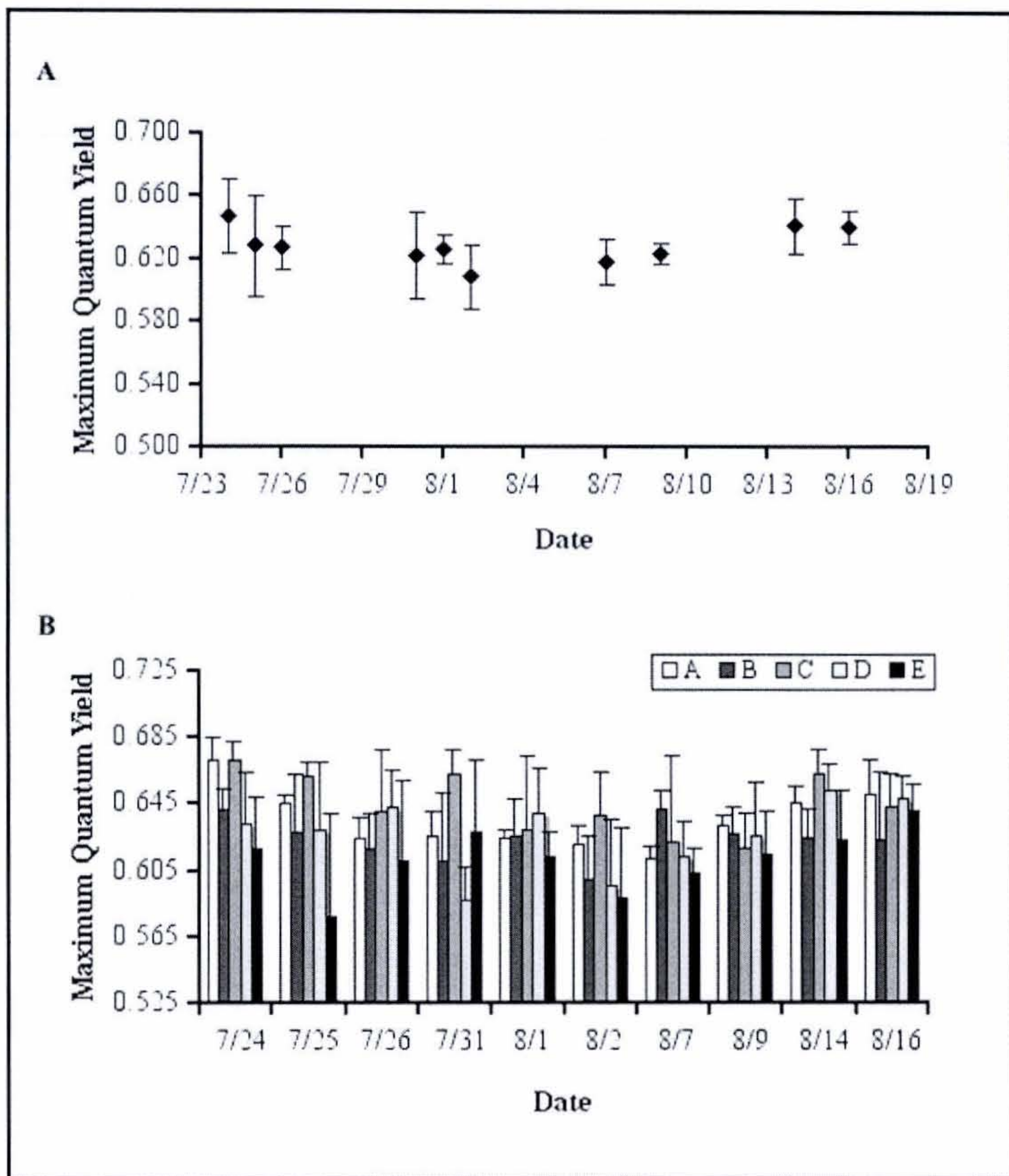

Figure 7. Average maximum quantum yield by date for the fragmentation and recuperation experiment. Values represent mean + 1 SD for each date (n = 5) (A) and average maximum quantum yield + 1 SD for each colony by date (n = 3).

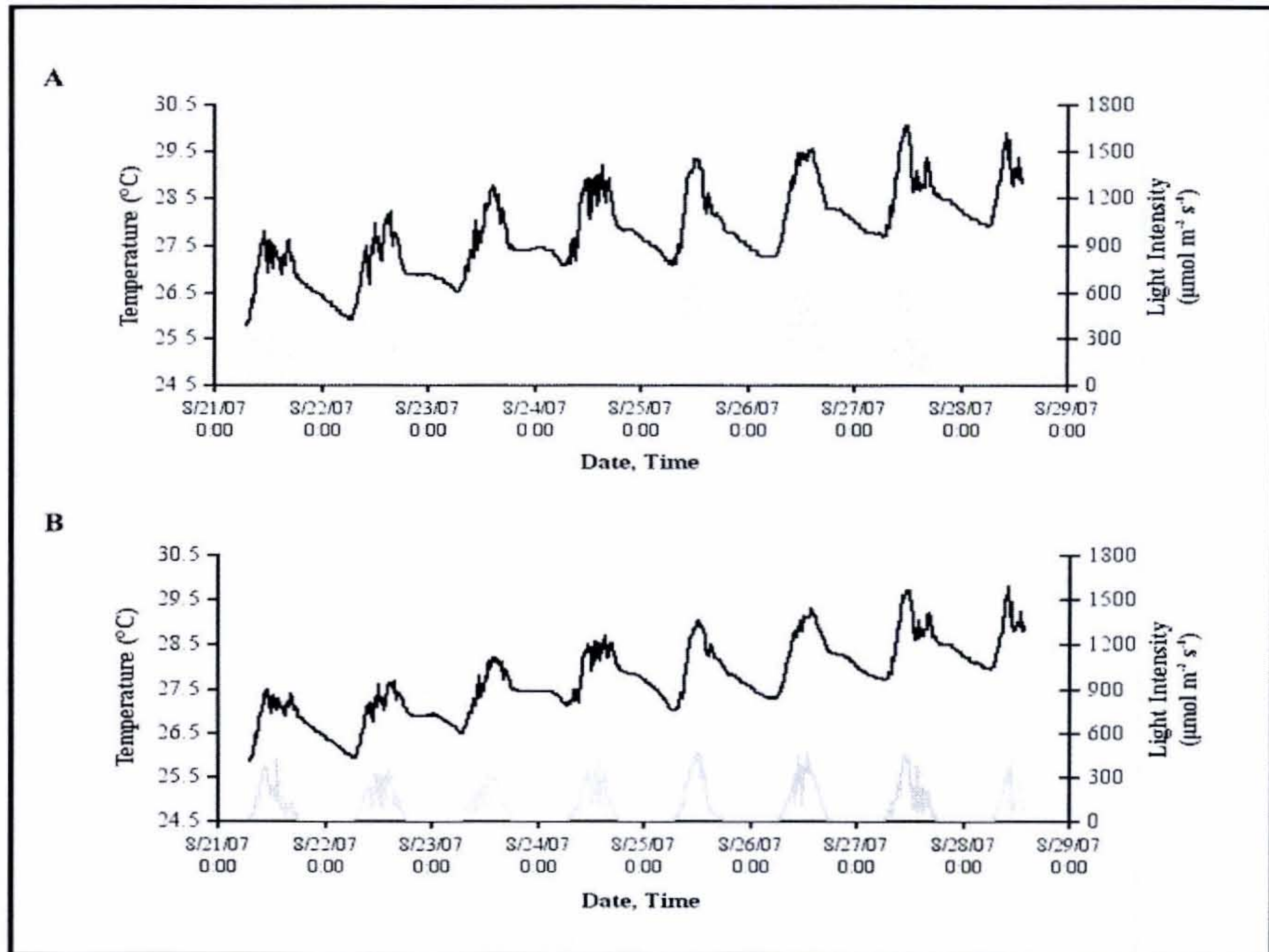

Figure 8. Average temperature (■) and light intensity ( ) by date and time for up blocks (A) and down blocks (B) over the course of the transplantation experiment (n = 5 measurements for each date and time).

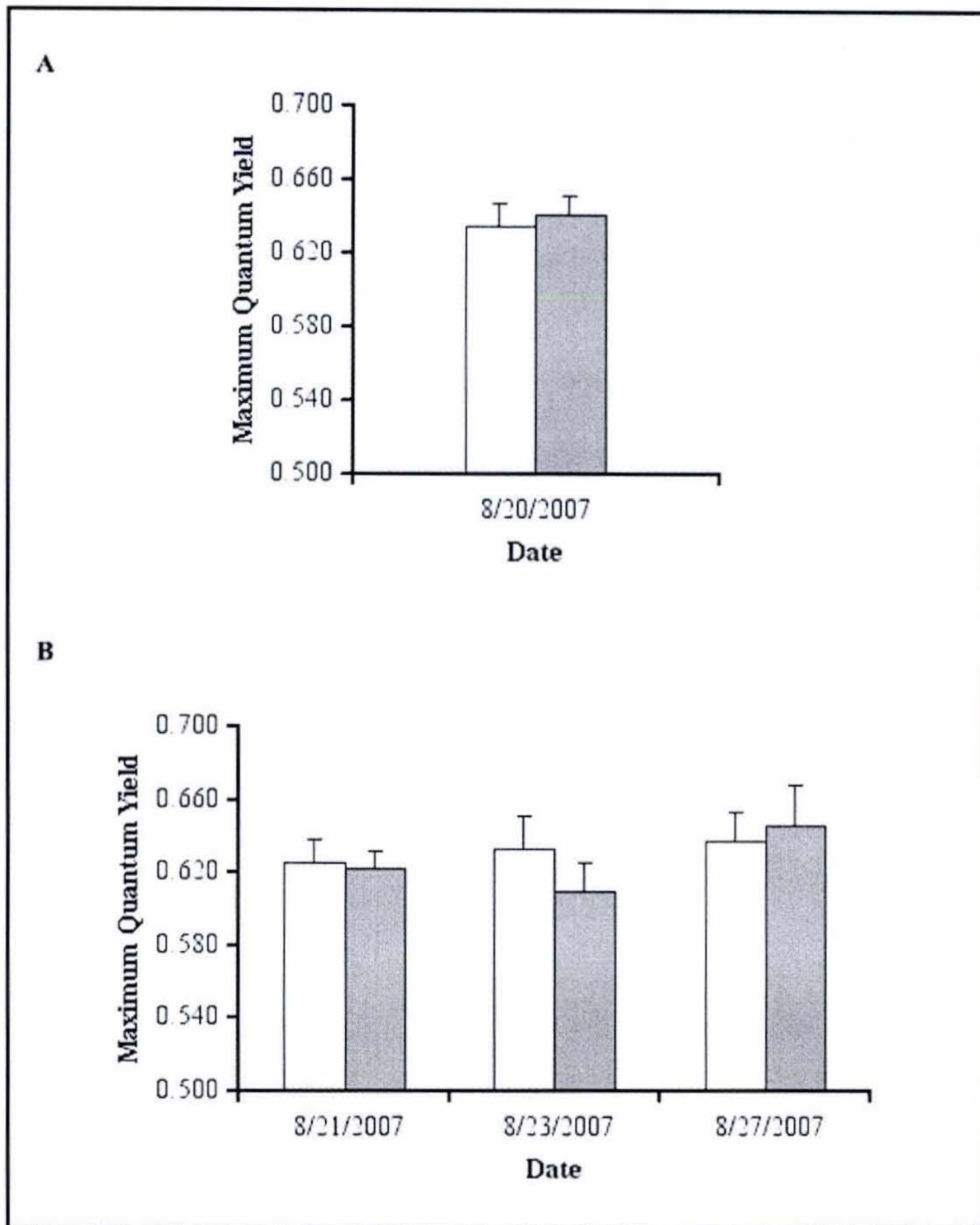

Figure 9. Average maximum quantum yield (mean + 1 SD) on August 20, 2007 for up (□) and down (■) blocks (n = 5) (A) and for subsequent dates during the transplantation experiment (B).

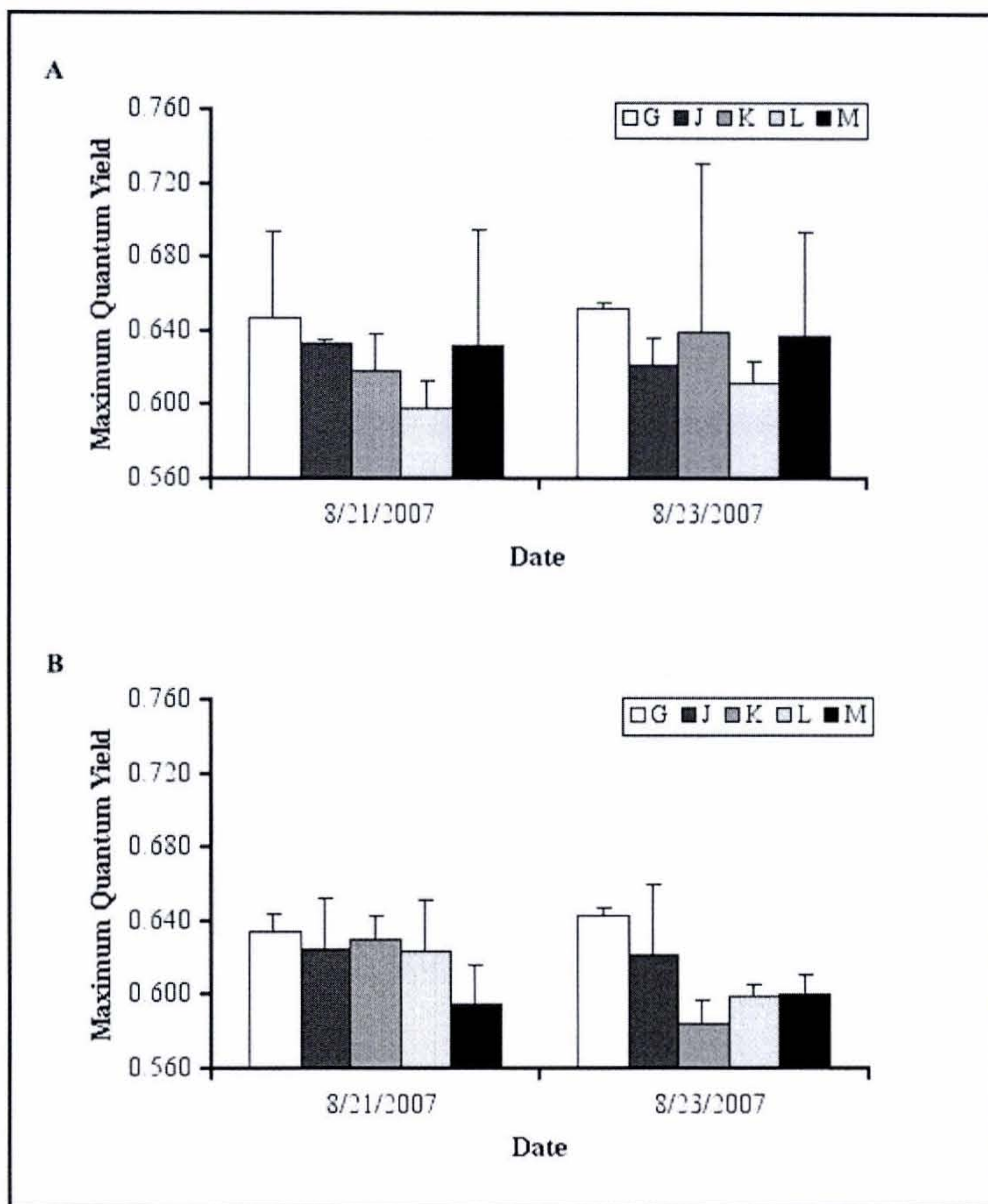

Figure 10. Average maximum quantum yield (mean + 1 SD) for colonies G, J – L, and M by date for up (A) and down blocks (B) (n = 3).

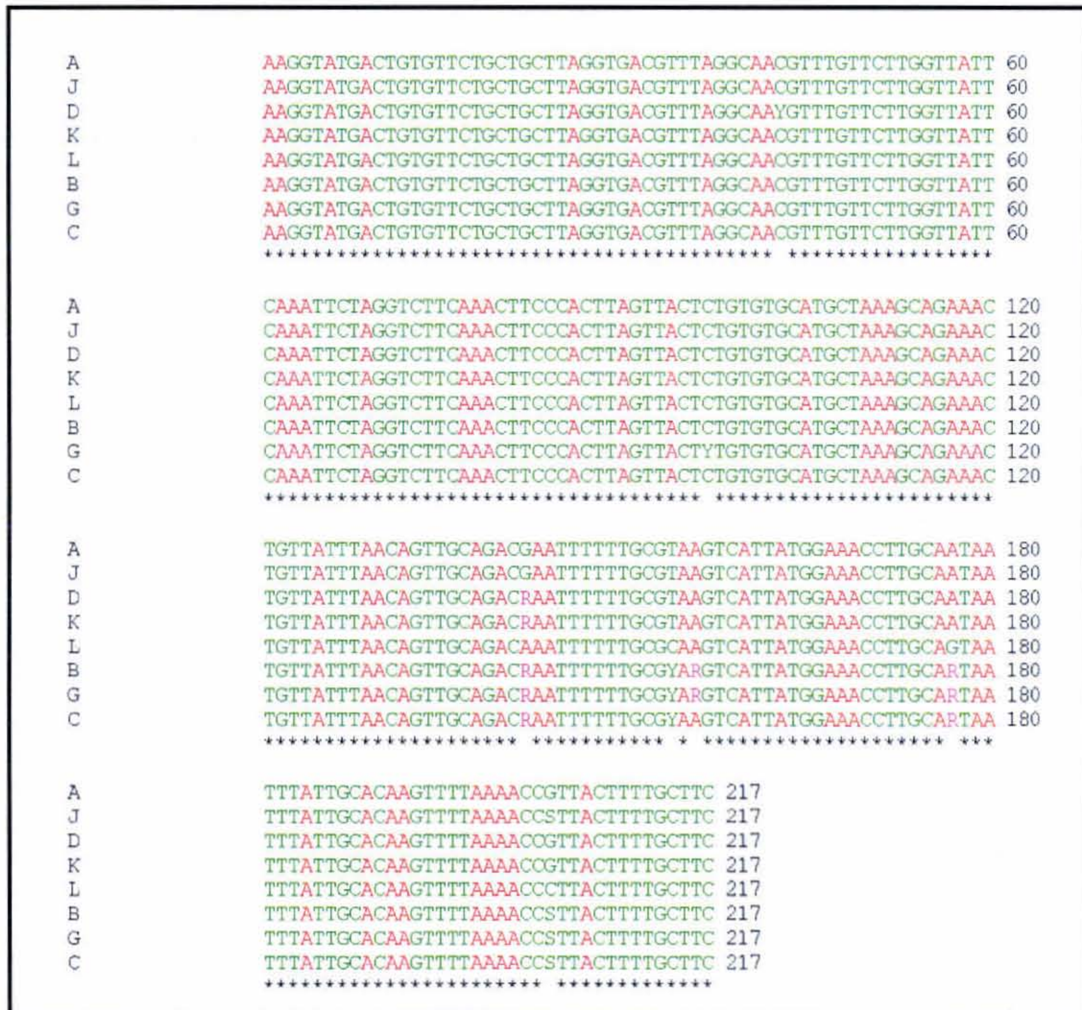

Figure 11. ClustalW (European Bioinformatics Institute) alignment of the 217 bp intron in the ATP Synthase ß subunit for colonies A - D, G, J - K. Colonies E and M are not shown because their sequence is identical to B and A respectively. The Codes of the International Union of Biochemistry correspond to the following bases: 1) Y = C, T, 2) R = A, G, 3) S = C, G. The stars below the sequence information show consensus bases between all colonies.

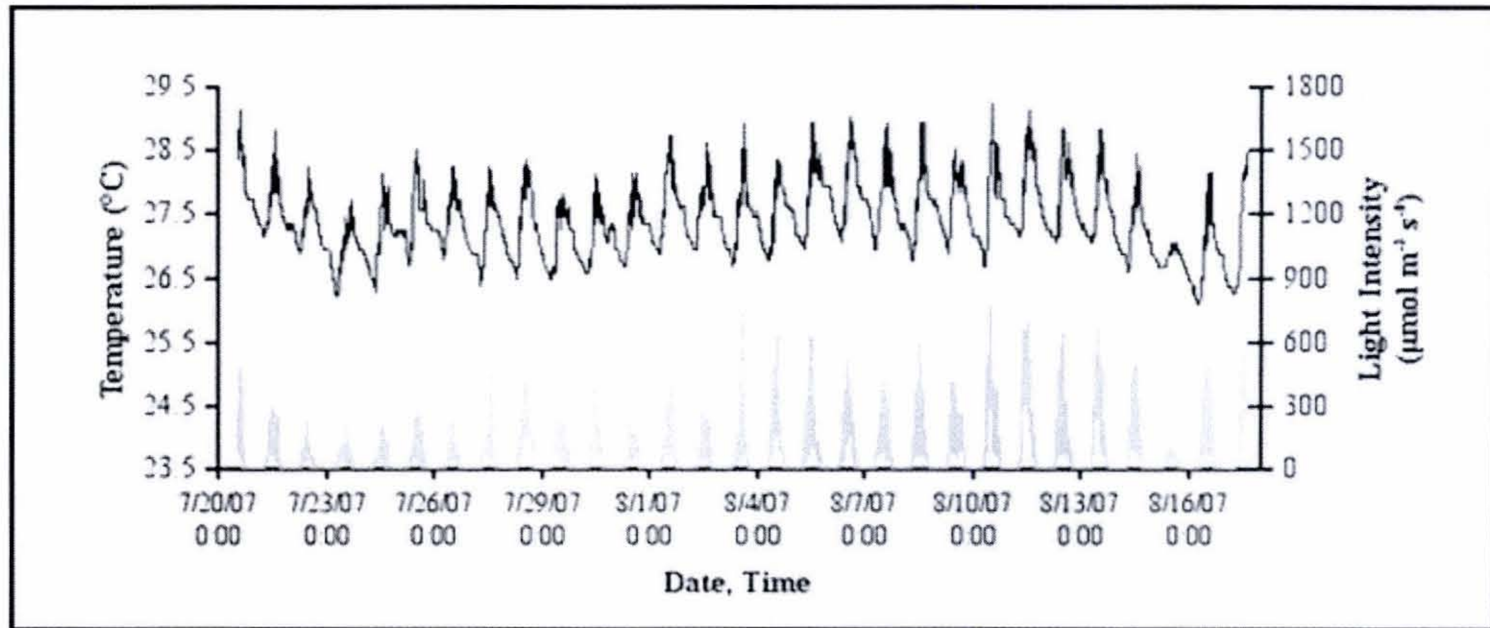

Figure 12. Temperature (■) and light intensity ( ) by date and time over the course of the fragmentation and recuperation experiment.

Appendix A. SL DNA spike from the fragmentation and recuperation experiment

qRT-PCR data for the SL DNA spike from the fragmentation and recuperation experiment, where Col = coral colony, Frag # = coral fragment number, Time (Wk) = time in weeks, C_T 1 (2, 3) = triplicate C_T values for each coral sample, Avg C_T = average of tripicate C_T values, Std Dev = standard deviation of triplicate C_T values, $E_{SL}{}^{(SLC_T)}$ = efficiency of the tilSα primer set to the power of the average C_T value for the SL DNA spike of that specific sample. Empty cells represent removed C_T values because of a replicate with different amplification kinetics.

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{SL}{}^{(SLC_T)}$
A	13	0	17.61	17.46	17.40	17.49	0.108	3.842
A	14	0	15.07	14.97	14.85	14.96	0.110	3.163
A	15	0	13.26	13.51	13.46	13.41	0.132	2.807
B	13	0	13.69		14.04	13.87	0.247	2.907
B	14	0	13.12	12.98	12.77	12.96	0.176	2.711
B	15	0	12.92	12.93	12.98	12.94	0.032	2.708
C	13	0	13.36	13.39	13.09	13.28	0.165	2.779
C	14	0	15.68	16.13	16.26	16.02	0.304	3.432
C	15	0	14.64	14.48	14.35	14.49	0.145	3.050
D	13	0	17.87	17.73		17.80	0.099	3.935
D	14	0	17.27		17.12	17.20	0.106	3.756
D	15	0	16.98	17.01	16.83	16.94	0.096	3.683
E	13	0	15.70	15.26	15.46	15.47	0.220	3.290
E	14	0	17.77	17.90	17.82	17.83	0.066	3.944
E	15	0	13.66	13.95	13.89	13.83	0.153	2.900
A	6	1	17.24	17.21		17.23	0.021	3.765
A	7	1	13.18	13.66	13.51	13.45	0.246	2.815
A	8	1	12.49		12.87	12.68	0.269	2.653
B	1	1	16.88	16.83	16.63	16.78	0.132	3.638
B	6	1	18.13	18.05	18.00	18.06	0.066	4.015
B	12	1	13.78	13.63	14.09	13.83	0.235	2.900
C	5	1	14.69	14.54	14.92	14.72	0.191	3.104
C	8	1	13.56	13.82	13.65	13.68	0.132	2.865
C	11	1	13.56		13.41	13.49	0.106	2.823
D	1	1		15.89	16.07	15.98	0.127	3.421
D	5	1	15.30	15.66		15.48	0.255	3.292
D	12	1	14.15	14.17	14.19	14.17	0.020	2.976
E	5	1	14.93	15.19	14.82	14.98	0.190	3.167
E	6	1		13.61	13.83	13.72	0.156	2.875
E	10	1	15.22	15.03	15.25	15.17	0.119	3.213
A	1	2	13.66	13.83	13.70	13.73	0.089	2.877

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{SL}^{(SLC_T)}$
A	5	2	12.61	12.57	12.76	12.65	0.100	2.647
B	2	2	13.04		13.40	13.22	0.255	2.766
B	10	2	13.47	13.59	13.98	13.68	0.267	2.866
B	11	2	14.38	14.39	14.35	14.37	0.021	3.023
C	1	2			18.10	18.10	0.000	4.027
C	4	2	14.07	14.08	14.45	14.20	0.217	2.983
C	10	2	13.36	13.46	13.82	13.55	0.242	2.836
D	3	2	14.69	14.25	14.64	14.53	0.241	3.059
D	6	2	14.12	14.27		14.20	0.106	2.982
D	7	2	14.03	13.76		13.90	0.191	2.914
E	1	2	15.00	15.31	15.32	15.21	0.182	3.224
E	8	2	15.89	15.99	16.15	16.01	0.131	3.429
E	9	2	15.59	15.55	16.01	15.72	0.255	3.352
A	3	3	14.45	14.39	14.15	14.33	0.159	3.013
A	9	3	13.18	13.23	13.34	13.25	0.082	2.772
A	12	3	14.38	14.15	14.34	14.29	0.123	3.003
B	3	3	16.00	16.10		16.05	0.071	3.439
B	4	3	14.73	14.57	14.73	14.68	0.092	3.094
B	8	3	13.95	13.53	13.74	13.74	0.210	2.879
C	3	3	13.46	13.61	13.81	13.63	0.176	2.854
C	6	3	17.47	16.61		17.04	0.608	3.711
C	7	3	14.70	14.66	14.86	14.74	0.106	3.109
D	2	3	13.85	13.93	13.91	13.90	0.042	2.914
D	4	3	14.60	14.90	14.61	14.70	0.170	3.101
D	10	3	16.36	16.20	16.49	16.35	0.145	3.519
E	3	3	14.81	14.34	14.49	14.55	0.240	3.063
E	4	3	14.13	14.01	14.05	14.06	0.061	2.952
E	7	3	14.45		14.80	14.63	0.247	3.082
A	4	4	16.28	15.88	16.08	16.08	0.200	3.447
A	10	4	13.61	13.33		13.47	0.198	2.820
A	11	4	15.23	15.35	15.72	15.43	0.255	3.280
B	5	4	16.42	16.41	16.53	16.45	0.067	3.548
B	7	4	14.64	14.50	14.83	14.66	0.166	3.089
B	9	4	13.02	13.31	13.44	13.26	0.215	2.774
C	2	4	13.74	13.69	13.73	13.72	0.026	2.875
C	9	4	15.11	15.38	15.52	15.34	0.208	3.255
C	12	4	15.09	15.08		15.09	0.007	3.193
D	8	4	13.90	13.54		13.72	0.255	2.875

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{SL}^{(SLC_T)}$
D	9	4	13.54	12.97	13.13	13.21	0.294	2.765
D	11	4	17.28	17.56	17.50	17.45	0.147	3.829
E	2	4	15.52	15.47	15.62	15.54	0.076	3.306
E	12	4	15.63	15.48	15.59	15.57	0.078	3.314

Appendix B. DNA hsp70 in the symbiont: Fragmentation and recuperation experiment

qRT-PCR data for hsp70 in the symbiont amplified from genomic DNA from the fragmentation and recuperation experiment, where Col = coral colony, Frag # = coral fragment number, Time (Wk) = time in weeks, C_T 1 (2, 3) = triplicate C_T values for each coral sample, Avg C_T = average of tripicate C_T values, Std Dev = standard deviation of triplicate C_T values, and $E_{hsp70z1}{}^{(hsp70z1C_T)}$ = efficiency of hsp70z1 primer set to the power of the average C_T value for hsp70 in the symbiont of that specific sample. Empty cells represent removed C_T values because of multiple melting peaks or a replicate with different amplification kinetics. Samples with a * were removed from any further analysis because the standard deviation of the triplicate C_T values was too high.

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{hsp70z1}{}^{(hsp70z1C_T)}$
A	13	0	32.75	32.98	32.51	32.75	0.235	0.186
A	14	0	31.33	31.15	31.35	31.28	0.110	0.201
A	15	0		29.23	29.51	29.37	0.198	0.222
B	13	0	33.02		32.73	32.88	0.205	0.185
B	14	0		30.10	30.44	30.27	0.240	0.212
B *	15	0	28.58	29.35	28.07	28.67	0.644	0.230
C	13	0	22.32	22.38		22.35	0.042	0.318
C	14	0	23.02		22.89	22.96	0.092	0.308
C	15	0	23.47	23.77	23.26	23.50	0.256	0.300
D	13	0	23.41	23.78	23.65	23.61	0.188	0.298
D	14	0	23.03	23.02	23.07	23.04	0.026	0.307
D	15	0	23.35	22.84	23.07	23.09	0.255	0.306
E	13	0		28.90	28.74	28.82	0.113	0.228
E	14	0		31.91	31.96	31.94	0.035	0.194
E	15	0	31.87		31.49	31.68	0.269	0.197
A	6	1		30.62	30.99	30.81	0.262	0.206
A	7	1	29.60	29.38	29.11	29.36	0.245	0.222
A	8	1	27.30	27.31	27.71	27.44	0.234	0.245
B	1	1	32.32	32.14	31.91	32.12	0.206	0.192
B *	6	1	32.33	31.15		31.74	0.834	0.196
B	12	1		30.92	30.74	30.83	0.127	0.206
C	5	1	21.82	22.09	21.60	21.84	0.245	0.326
C	8	1		22.80	22.72	22.76	0.057	0.311
C	11	1	22.04	22.26	21.72	22.01	0.272	0.323
D *	1	1			23.40	23.40	0.000	0.301
D	5	1	23.45		23.65	23.55	0.141	0.299
D	12	1	24.15	24.12		24.14	0.021	0.290
E	5	1	28.01	27.87	28.00	27.96	0.078	0.238
E	6	1	24.45	24.75	24.28	24.49	0.238	0.285

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{hsp70z1}^{(hsp70z1C_T)}$
E	10	1		26.68	26.63	26.66	0.035	0.255
A	1	2	30.13	30.29	29.87	30.10	0.212	0.214
A	2	2	29.12	29.03	29.12	29.09	0.052	0.225
A	5	2	29.42	29.35	29.39	29.39	0.035	0.221
B	2	2	29.63		29.83	29.73	0.141	0.218
B	10	2	29.60		29.41	29.51	0.134	0.220
B	11	2	31.24		31.28	31.26	0.028	0.201
C	1	2	21.31	21.54	21.71	21.52	0.201	0.332
C	4	2	23.17	23.04	23.21	23.14	0.089	0.305
C	10	2	21.92		21.57	21.75	0.247	0.328
D *	3	2	21.00	22.29	21.62	21.64	0.645	0.330
D	6	2	22.41	22.58	22.99	22.66	0.298	0.313
D	7	2	22.65	22.65	23.12	22.81	0.271	0.310
E	1	2		29.51	29.31	29.41	0.141	0.221
E	8	2	26.21		26.28	26.25	0.049	0.260
E	9	2	26.98	27.00	27.00	26.99	0.012	0.250
A	3	3	31.65	31.35	31.46	31.49	0.152	0.199
A	9	3	29.93		29.63	29.78	0.212	0.217
A	12	3	32.19	31.91	32.14	32.08	0.149	0.193
B	3	3	30.50	30.41	30.50	30.47	0.052	0.210
B	4	3	30.92	30.59	30.57	30.69	0.197	0.207
B	8	3	32.89	32.76	32.73	32.79	0.085	0.186
C	3	3	21.26	21.41	21.12	21.26	0.145	0.336
C	6	3	23.21	23.37	23.21	23.26	0.092	0.303
C	7	3	23.43	23.94	23.69	23.69	0.255	0.297
D *	2	3	24.75		24.29	24.52	0.325	0.284
D	4	3	24.04	24.47	24.30	24.27	0.217	0.288
D	10	3	24.70	24.39	24.77	24.62	0.202	0.283
E	3	3	29.05	29.28		29.17	0.163	0.224
E *	4	3	28.09	28.68	27.45	28.07	0.615	0.237
E	7	3		25.65	25.64	25.65	0.007	0.268
A	4	4	30.88	31.06	30.72	30.89	0.170	0.205
A	10	4	29.94	29.99	30.49	30.14	0.304	0.213
A	11	4	29.73	29.58	29.88	29.73	0.150	0.218

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{hsp70z1}^{(hsp70z1C_T)}$
B *	5	4		32.40	31.33	31.87	0.757	0.195
B	7	4	27.66	28.05	28.16	27.96	0.263	0.238
B	9	4	28.51		28.81	28.66	0.212	0.230
C	12	4	23.76	23.98	23.97	23.90	0.124	0.293
D	8	4		22.58	22.43	22.51	0.106	0.315
D	9	4	23.14	23.54	23.29	23.32	0.202	0.302
D	11	4	23.38	23.55	23.57	23.50	0.104	0.300
E	2	4	29.84	29.99		29.92	0.106	0.216
E	11	4	26.72	26.88		26.80	0.113	0.253
E	12	4	27.12	27.64	27.11	27.29	0.303	0.247

Appendix C. Symbiodinium cell number / Total RNA (ng): Fragmentation and recuperation experiment

Symbiodinium cell densities for the fragmentation and recuperation experiment, where RNA Total (ng) = the total RNA extracted from each sample after DNase treatment and precipitation, SMP = symbiont molecular proxy, and Symbio Cell # = *Symbiodinium* cell number. Samples with a * were removed from statistical analysis of *Symbiodinium* cell densities.

Col	Frag #	Time (Wk)	RNA Total (ng)	SMP	Symbio Cell #	Symbio Cell # / Total RNA (ng)
A	13	0	8136	20.656	1193983	147
A	14	0	22853	15.736	909614	40
A	15	0	16304	12.644	730875	45
B	13	0	17344	15.714	908296	52
B	14	0	18617	12.788	739175	40
B *	15	0	40355	11.774	680573	17
C	13	0	15184	8.739	505144	33
C	14	0	10684	11.143	644096	60
C	15	0	12719	10.167	587669	46
D	13	0	12037	13.205	763277	63
D	14	0	25951	12.235	707198	27
D	15	0	8488	12.036	695720	82
E	13	0	10164	14.430	834094	82
E	14	0	9829	20.330	1175139	120
E	15	0	14028	14.721	850914	61
A	6	1	24852	18.277	1056457	43
A	7	1	2304	12.680	732958	318
A	8	1	32994	10.829	625929	19
B	1	1	10873	18.948	1095255	101
B *	6	1	9501	20.485	1184086	125
B	12	1	5988	14.078	813738	136
C	5	1	14754	9.521	550374	37
C	8	1	52018	9.212	532498	10
C	11	1	17541	8.740	505199	29
D *	1	1	19710	11.365	656962	33
D	5	1	7936	11.010	636418	80
D	12	1	9465	10.262	593183	63
E	5	1	2003	13.307	769175	384
E	6	1	790	10.088	583105	738
E	10	1	6475	12.600	728324	112
A	1	2	9555	13.444	777106	81
A	2	2	26681	12.609	728838	27

Col	Frag #	Time (Wk)	RNA Total (ng)	SMP	Symbio Cell #	Symbio Cell # / Total RNA (ng)
A	5	2	15480	11.977	692334	45
B	2	2	16600	12.688	733415	44
B	10	2	7525	13.027	753022	100
B	11	2	9331	15.040	869353	93
C *	1	2	28100	12.130	701128	25
C	4	2	18007	9.780	565337	31
C	10	2	18464	8.646	499789	27
D *	3	2	3441	9.270	535821	156
D	6	2	22197	9.527	550703	25
D	7	2	10399	9.400	543353	52
E	1	2	4634	14.588	843251	182
E	8	2	1795	13.188	762339	425
E	9	2	4301	13.408	775029	180
A	3	3	12109	15.141	875185	72
A	9	3	23640	12.774	738393	31
A	12	3	14332	15.560	899398	63
B	3	3	2458	16.376	946601	385
B	4	3	5587	14.947	863980	155
B	8	3	9295	15.478	894711	96
C	3	3	85121	8.494	490985	6
C *	6	3	9254	12.248	707949	76
C	7	3	1481	10.468	605087	409
D *	2	3	7947	10.261	593096	75
D	4	3	12208	10.767	622391	51
D	10	3	1509	12.435	718765	476
E	3	3	18975	13.674	790411	42
E *	4	3	15516	12.456	719982	46
E	7	3	95600	11.500	664740	7
A	4	4	5255	16.815	971944	185
A	10	4	13441	13.239	765285	57
A	11	4	3982	15.046	869704	218
B *	5	4	7297	18.195	1051727	144
B	7	4	804	12.979	750231	934
B	9	4	14323	12.061	697160	49
C	2	4	23374	9.215	532644	23
C	9	4	1436	11.034	637798	444
C	12	4	18461	10.898	629920	34

Col	Frag #	Time (Wk)	RNA Total (ng)	SMP	Symbio Cell #	Symbio Cell # / Total RNA (ng)
D	8	4	13169	9.127	527571	40
D	9	4	17066	9.156	529227	31
D	11	4	16932	12.763	737765	44
E	2	4	8620	15.306	884714	103
E	11	4	7014	12.514	723343	103
E	12	4	3508	13.417	775549	221

Appendix D. SL RNA spike: Fragmentation and recuperation experiment

qRT-PCR data for the SL RNA spike from the fragmentation and recuperation experiment, where Col = coral colony, Frag # = coral fragment number, Time (Wk) = time in weeks, C_T 1 (2, 3) = triplicate C_T values for each coral sample, Avg C_T = average of tripicate C_T values, Std Dev = standard deviation of triplicate C_T values, and $E_{SL}^{(SLC_T)}$ = efficiency of the tilSα primer set to the power of the average C_T value for the SL RNA spike of that specific sample. Empty cells represent removed C_T values because of multiple melting peaks or a replicate with different amplification kinetics.

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{SL}^{(SLC_T)}$
A	13	0	28.37	28.31	28.25	28.31	0.060	6.790
A	14	0	29.15	29.03	29.17	29.12	0.076	7.171
A	15	0	31.74		31.33	31.54	0.290	8.445
B	13	0	33.76	33.41	33.93	33.70	0.265	9.778
B	14	0		32.28	32.26	32.27	0.014	8.876
B	15	0		31.48	31.53	31.51	0.035	8.428
C	13	0			34.19	34.19	0.000	10.107
C	14	0	32.51	32.87	32.41	32.60	0.242	9.074
C	15	0		32.74	32.47	32.61	0.191	9.079
D	13	0	30.79	30.46	30.78	30.68	0.188	7.969
D	14	0			33.47	33.47	0.000	9.627
D	15	0	24.94	24.68	24.89	24.84	0.138	5.368
E	13	0	31.47		31.3	31.39	0.120	8.360
E	14	0		32.33	32.74	32.54	0.290	9.037
E	15	0	33.18	32.93	32.82	32.98	0.184	9.311
A	6	1	26.32	26.58	26.27	26.39	0.166	5.963
A	7	1	25.94	26.08	26.14	26.05	0.103	5.828
B	1	1	25.48	25.58	25.02	25.36	0.299	5.561
B	6	1	23.74	23.72	23.51	23.66	0.127	4.956
B	12	1		31.64	32.14	31.89	0.354	8.651
C	5	1	29.98		29.84	29.91	0.099	7.566
C	8	1	30.73	30.44		30.59	0.205	7.920
C	11	1	30.69	30.5	30.94	30.71	0.221	7.987
D	1	1	29.83	29.78	29.6	29.74	0.121	7.478
D	5	1	26.34	26.47	26.49	26.43	0.081	5.980
D	12	1	31.12	31.24	31.7	31.35	0.306	8.342
E	5	1	25.59	25.48	25.52	25.53	0.056	5.626
E	6	1	27.48	26.98	26.99	27.15	0.286	6.277
E	10	1	31.41	30.92	31.31	31.21	0.259	8.264
A	1	2		32.56	32.2	32.38	0.255	8.942
A	2	2	30.95	30.84	30.89	30.89	0.055	8.087
A	5	2			29.72	29.72	0.000	7.469

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	E_{SL} $^{(SLC_T)}$
B	2	2		33.03	32.78	32.91	0.177	9.266
B	10	2	32.73	33.08		32.91	0.247	9.266
B	11	2		30.67	30.88	30.78	0.148	8.022
C	1	2	23.69	23.71	23.84	23.75	0.081	4.986
C	4	2		29.45	29.75	29.60	0.212	7.409
C	10	2	32.16		32.33	32.25	0.120	8.861
D	3	2	25.15	25.13	24.97	25.08	0.099	5.458
D	6	2	32.79	32.37		32.58	0.297	9.064
D	7	2	35.89	35.59	35.26	35.58	0.315	11.104
E	1	2	31.44	31.12	31.01	31.19	0.223	8.250
E	8	2	25.78	25.77	25.73	25.76	0.026	5.714
E	9	2	24.83	24.91	24.94	24.89	0.057	5.388
A	3	3	31.83	31.69		31.76	0.099	8.575
A	9	3	31.79		31.7	31.75	0.064	8.566
A	12	3	33.64	33.49	33.52	33.55	0.079	9.679
B	3	3	24.62	24.61	24.62	24.62	0.006	5.288
B	4	3	28.45	28.73	28.53	28.57	0.144	6.910
B	8	3	33.98	33.42	33.93	33.78	0.310	9.828
C	3	3	33.84			33.84	0.000	9.871
C	6	3	33.26	33.1		33.18	0.113	9.440
C	7	3	25.92	25.94	25.96	25.94	0.020	5.784
D	2	3		31.93	31.93	31.93	0.000	8.674
D	4	3	29.7		29.4	29.55	0.212	7.384
D	10	3	28.58	28.59	28.68	28.62	0.055	6.932
E	3	3	30.99		30.55	30.77	0.311	8.019
E	4	3		23.93	24.21	24.07	0.198	5.096
A	4	4	32.51	32.21	32.22	32.31	0.170	8.902
A	4	4	32.51	32.21	32.22	32.31	0.170	8.902
A	10	4	31.79	31.58		31.69	0.148	8.531
A	11	4	24.87	24.71	24.8	24.79	0.080	5.352
B	5	4	26.19	26.27	26.46	26.31	0.139	5.929
B	7	4	25.73	25.62	25.63	25.66	0.061	5.675
B	9	4	29.68		29.93	29.81	0.177	7.512
C	2	4	32.84	32.85		32.85	0.007	9.228
C	9	4	25.39	25.32	25.33	25.35	0.038	5.556
C	12	4	30.28	30.35		30.32	0.049	7.776
D	8	4		32.39	32.34	32.37	0.035	8.933
D	9	4	31.41		31.74	31.58	0.233	8.468

Col	Frag #	Time (Wk)	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	E_{SL} (SLC_T)
D	11	4	32.79	32.82	32.3	32.64	0.292	9.099
E	2	4	32.29			32.29	0.000	8.888
E	11	4	29.17	29.27	29.35	29.26	0.090	7.242
E	12	4	26.31	26.28	26.29	26.29	0.015	5.924

Appendix E. SL DNA spike: Transplantation experiment

qRT-PCR data for the SL DNA spike from the transplantation experiment, where Col = coral colony, Frag # = coral fragment number, Time (hr) = time in hours, Bl = block #, Up or Down = treatment, C_T 1 (2, 3) = triplicate C_T values for each coral sample, Avg C_T = average of tripicate C_T values, Std Dev = standard deviation of triplicate C_T values, $E_{SL}^{(SLC_T)}$ = efficiency of the tilSα primer set to the power of the average C_T value for the SL DNA spike of that specific sample. Empty cells represent removed C_T values because of multiple melting peaks or a replicate with different amplification kinetics.

Col	Frag #	Time (hr)	Bl	Up or Down	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{SL}^{(SLC_T)}$
G	2	0	1	Up	17.71	17.39	17.16	17.42	0.276	3.822
G	5	0	9	Down	15.94	15.70		15.82	0.170	3.379
J	1	0	8	Down		13.50	13.12	13.31	0.269	2.785
J	3	0	4	Up	16.91	16.83	16.98	16.91	0.075	3.674
K	4	0	3	Up		15.67	15.85	15.76	0.127	3.363
K	9	0	5	Down	13.46	13.41		13.44	0.035	2.812
L	3	0	10	Down	16.71	17.18	16.67	16.85	0.284	3.658
L	4	0	6	Up	17.69	18.11	17.78	17.86	0.221	3.953
M	4	0	7	Down	17.30	17.56	17.50	17.45	0.136	3.831
M	9	0	2	Up	18.36	18.61	18.17	18.38	0.221	4.115
G	1	6	10	Down	18.11	18.26	18.26	18.21	0.087	4.061
G	6	6	6	Up	16.35		16.32	16.34	0.021	3.515
J	8	6	7	Down	15.35	15.14	15.07	15.19	0.146	3.218
J	10	6	2	Up	15.86	15.54	15.56	15.65	0.179	3.336
K	6	6	4	Up		14.21	14.57	14.39	0.255	3.027
K	10	6	9	Down	15.99	16.22	15.82	16.01	0.201	3.429
L	1	6	5	Down	17.69	17.63	17.65	17.66	0.031	3.892
L	5	6	3	Up	17.95	17.81	17.59	17.78	0.181	3.930
M	6	6	1	Up	16.55	16.17	16.43	16.38	0.194	3.529
M	10	6	8	Down	15.34	15.76	15.34	15.48	0.242	3.292
G	8	30	5	Down	14.46	14.27	14.10	14.28	0.180	3.000
J	2	30	9	Down	14.40	14.27	14.23	14.30	0.089	3.006
J	5	30	1	Up	13.57	13.84	13.64	13.68	0.140	2.866
K	1	30	6	Up	15.19	15.42	15.36	15.32	0.119	3.252
K	2	30	8	Down	14.07	14.00	13.92	14.00	0.075	2.936
L	8	30	7	Down	14.77	14.88	14.85	14.83	0.057	3.132
L	10	30	2	Up	12.87	12.99	13.05	12.97	0.092	2.713
M	1	30	10	Down	14.26	14.54	14.51	14.44	0.154	3.038
L	8	30	7	Down	14.77	14.88	14.85	14.83	0.057	3.132
L	10	30	2	Up	12.87	12.99	13.05	12.97	0.092	2.713
M	1	30	10	Down	14.26	14.54	14.51	14.44	0.154	3.038

Col	Frag #	Time (hr)	Bl	Up or Down	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	E_{SL} (SLC_T)
M	7	30	3	Up	15.93	15.94		15.94	0.007	3.409
G	4	78	8	Down	16.84	16.66	16.79	16.76	0.093	3.633
G	7	78	2	Up	19.95	19.97	19.97	19.96	0.012	4.648
J	4	78	10	Down	14.12	14.30		14.21	0.127	2.985
J	9	78	3	Up	15.58	15.86	15.43	15.62	0.218	3.328
K	3	78	1	Up	20.46	20.52	20.56	20.51	0.050	4.849
K	5	78	7	Down	20.37	20.74	20.72	20.61	0.208	4.885
L	2	78	9	Down	15.12	15.17	15.02	15.10	0.076	3.197
L	9	78	4	Up	16.10	16.39	15.82	16.10	0.285	3.453
M	2	78	6	Up	18.92	19.27		19.10	0.247	4.347
M	8	78	5	Down	14.63	15.05	15.19	14.96	0.291	3.162
G	3	174	7	Down	15.17		15.25	15.21	0.057	3.224
G	10	174	3	Up	16.48	16.98	16.45	16.64	0.298	3.598
J	6	174	6	Up	14.45	14.34	14.46	14.42	0.067	3.033
J	7	174	5	Down	13.31	12.92	13.41	13.21	0.259	2.765
K	7	174	10	Down	15.19	15.68	15.27	15.38	0.263	3.266
K	8	174	2	Up	14.27	14.53	14.31	14.37	0.140	3.022
L	6	174	8	Down		14.00	13.76	13.88	0.170	2.910
L	7	174	1	Up	15.39	15.77	15.92	15.69	0.273	3.346
M *	3	174	9	Down	18.87	19.35		19.11	0.339	4.352
M	5	174	4	Up	15.29	15.16		15.23	0.092	3.228

Appendix F. DNA hsp70 in the symbiont: Transplantation experiment

qRT-PCR data for hsp70 in the symbiont amplified from genomic DNA from the transplantation experiment, where Col = coral colony, Frag # = coral fragment number, Time (hr) = time in hours, Bl = block #, Up or Down = Treatment, C_T 1 (2, 3) = triplicate C_T values for each coral sample, Avg C_T = average of tripicate C_T values, Std Dev = standard deviation of triplicate C_T values, and $E_{hsp70z1}{}^{(hsp70z1 C_T)}$ = efficiency of hsp70z1 primer set to the power of the average C_T value for hsp70 in the symbiont of that specific sample. Empty cells represent removed C_T values because of multiple melting peaks or a replicate with different amplification kinetics. Samples with a * would have been removed from any further analysis because the standard deviation of the triplicate C_T values was too high, but no *Symbiodinium* cell density data was analyzed because too many samples were removed.

Col	Frag #	Time (hr)	Bl	Up or Down	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{hsp70z1}{}^{(hsp70z1 C_T)}$
G	2	0	1	Up		22.54	22.16	22.35	0.269	0.318
G	5	0	9	Down	21.49	22.05	21.77	21.77	0.280	0.327
J	1	0	8	Down	20.09	19.74		19.92	0.247	0.360
J	3	0	4	Up		27.05	27.11	27.08	0.042	0.249
K	4	0	3	Up		23.18	22.85	23.02	0.233	0.307
K	9	0	5	Down	25.59	25.56	25.95	25.70	0.217	0.268
L	3	0	10	Down	31.49		31.07	31.28	0.297	0.201
L	4	0	6	Up	31.72	31.94		31.83	0.156	0.195
M *	4	0	7	Down	33.50			33.50		0.179
M	9	0	2	Up	32.17	32.35		32.26	0.127	0.191
G *	1	6	10	Down		22.97	23.43	23.20	0.325	0.304
G	6	6	6	Up	22.74	22.73		22.74	0.007	0.312
J	8	6	7	Down	29.46	29.45	29.18	29.36	0.159	0.222
J	10	6	2	Up	28.63	28.65	28.77	28.68	0.076	0.230
K	6	6	4	Up	26.01	25.47	25.69	25.72	0.272	0.267
K *	10	6	9	Down		24.70	25.20	24.95	0.354	0.278
L	1	6	5	Down	32.64	32.65	32.46	32.58	0.107	0.188
L *	5	6	3	Up		31.20		31.20		0.202
M	6	6	1	Up	35.21		35.10	35.16	0.078	0.165
M *	10	6	8	Down	34.26		33.68	33.97	0.410	0.175
G	8	30	5	Down	26.63	26.52		26.58	0.078	0.256
J	2	30	9	Down	24.03	23.89	23.81	23.91	0.111	0.293
J	5	30	1	Up	24.32	24.57	24.68	24.52	0.184	0.284
K	1	30	6	Up	32.24	32.03	31.71	31.99	0.267	0.194
K	2	30	8	Down	28.21	27.89	28.09	28.06	0.162	0.237
L *	8	30	7	Down	29.65	30.68	30.17	30.17	0.515	0.213
L	10	30	2	Up		24.04	24.19	24.12	0.106	0.290

Col	Frag #	Time (hr)	Bl	Up or Down	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{hsp70zl}^{(hsp70zl\ C_T)}$
M	1	30	10	Down	30.97	30.97	30.89	30.94	0.046	0.205
M	7	30	3	Up	34.44	34.13		34.29	0.219	0.172
G *	4	78	8	Down	25.20		25.65	25.43	0.318	0.271
G	7	78	2	Up	33.05		33.29	33.17	0.170	0.182
J	4	78	10	Down	26.23	26.10	25.96	26.10	0.135	0.262
J	9	78	3	Up	27.79	27.37	27.54	27.57	0.211	0.243
K	3	78	1	Up	31.92	31.68		31.80	0.170	0.196
K *	5	78	7	Down	35.94			35.94		0.158
L	2	78	9	Down	31.43		31.76	31.60	0.233	0.198
L	9	78	4	Up		31.33	31.26	31.30	0.049	0.201
M *	2	78	6	Up		33.30	33.89	33.60	0.417	0.178
M	8	78	5	Down	27.85	28.01	27.74	27.87	0.136	0.239
G	3	174	7	Down	28.33	28.39	28.75	28.49	0.227	0.232
G *	10	174	3	Up	31.91	31.24		31.58	0.474	0.198
J	6	174	6	Up	25.94	25.96	26.00	25.97	0.031	0.264
J	7	174	5	Down	29.12	29.48	29.27	29.29	0.181	0.223
K	7	174	10	Down	31.17		31.53	31.35	0.255	0.200
K	8	174	2	Up	28.22	28.27		28.25	0.035	0.235
L *	6	174	8	Down		32.52		32.52		0.189
L	7	174	1	Up	28.48	28.65	28.12	28.42	0.271	0.233
M	3	174	9	Down		34.45	34.85	34.65	0.283	0.169
M *	5	174	4	Up		34.19	33.54	33.87	0.460	0.176

Appendix G. SL RNA spike: Transplantation experiment

qRT-PCR data for the SL RNA spike from the transplantation experiment, where Col = coral colony, Frag # = coral fragment number, Time (hr) = time in weeks, Bl = block #, Up or Down = treatment, C_T 1 (2, 3) = triplicate C_T values for each coral sample, Avg C_T = average of tripicate C_T values, Std Dev = standard deviation of triplicate C_T values, and $E_{SL}^{(SLC_T)}$ = efficiency of the tilSα primer set to the power of the average C_T value for the SL RNA spike of that specific sample. Empty cells represent removed C_T values because of multiple melting peaks or a replicate with different amplification kinetics.

Col	Frag #	Time (hr)	Bl	Up or Down	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	$E_{SL}^{(SLC_T)}$
G	2	0	1	Up		32.52	32.44	32.48	0.057	9.003
G	5	0	9	Down	31.27	31.58	31.7	31.52	0.222	8.435
J	1	0	8	Down		31.38		31.38	0.000	8.357
J	3	0	4	Up	25.24	25.23	25.2	25.22	0.021	5.510
K	4	0	3	Up						
K	9	0	5	Down	32.94		32.45	32.70	0.346	9.135
L	3	0	10	Down	27.28	27.08	27.13	27.16	0.104	6.283
L	4	0	6	Up	26.75	26.32	26.79	26.62	0.261	6.056
M	4	0	7	Down		32.57	32.55	32.56	0.014	9.052
M	9	0	2	Up	26.79			26.79	0.000	6.126
G	1	6	10	Down						
G	6	6	6	Up		31.96	31.82	31.89	0.099	8.651
J	8	6	7	Down	24.84	24.88	24.73	24.82	0.078	5.361
J	10	6	2	Up	26.16	25.82	25.87	25.95	0.184	5.788
K	6	6	4	Up	32.23			32.23	0.000	8.852
K	10	6	9	Down			33.21	33.21	0.000	9.459
L	1	6	5	Down	24.23	24.23	24.23	24.23	0.000	5.152
L	5	6	3	Up		32.55	32.54	32.55	0.007	9.043
M	6	6	1	Up	23.46	23.58	23.58	23.54	0.069	4.917
M	10	6	8	Down	24.45	24.69	24.59	24.58	0.121	5.274
G	8	30	5	Down	31.19	31.48	31.52	31.40	0.180	8.367
J	2	30	9	Down						
J	5	30	1	Up	32.4		32.49	32.45	0.064	8.982
K	1	30	6	Up	25.85	25.83	25.78	25.82	0.036	5.737
K	2	30	8	Down	26.47	26.78	26.88	26.71	0.214	6.093
L	8	30	7	Down	25.04	25.13	24.71	24.96	0.221	5.413
L	10	30	2	Up	31.96	31.63	31.64	31.74	0.188	8.565
M	1	30	10	Down	31.82		31.81	31.82	0.007	8.607
M	7	30	3	Up	30.82		30.49	30.66	0.233	7.957
G	4	78	8	Down	31.89	31.91	31.76	31.85	0.081	8.629
G	7	78	2	Up	30.61	30.44	30.71	30.59	0.137	7.920

Col	Frag #	Time (hr)	Bl	Up or Down	C_T 1	C_T 2	C_T 3	Avg C_T	Std Dev	E_{SL} (SLC_T)
J	4	78	10	Down	32.14	32.02		32.08	0.085	8.763
J	9	78	3	Up	23.52	23.36	23.26	23.38	0.131	4.864
K	3	78	1	Up	32.72	32.3		32.51	0.297	9.021
K	5	78	7	Down	31.84	31.82	32.21	31.96	0.220	8.690
L	2	78	9	Down	23.98	23.98	23.89	23.95	0.052	5.055
L	9	78	4	Up	32.63	32.43	32.56	32.54	0.101	9.040
M	2	78	6	Up	29.82	29.81	30.09	29.91	0.159	7.564
M	8	78	5	Down	30.33		30.2	30.27	0.092	7.750
G	3	174	7	Down	31.4	31.44	31.85	31.56	0.249	8.462
G	10	174	3	Up	23.82	23.77	23.82	23.80	0.029	5.005
J	6	174	6	Up	29.35	29.42	29.1	29.29	0.168	7.255
J	7	174	5	Down	32.9	32.67		32.79	0.163	9.191
K	7	174	10	Down	31.71	31.46		31.59	0.177	8.474
K	8	174	2	Up	32.33	32.28		32.31	0.035	8.897
L	6	174	8	Down	32.97	32.5	32.39	32.62	0.308	9.089
L	7	174	1	Up	25.88	25.76	25.7	25.78	0.092	5.722
M	3	174	9	Down	26.38	25.85	25.92	26.05	0.288	5.827
M	5	174	4	Up	31.48	31.69		31.59	0.148	8.474

References

Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K (1994) Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from blood stains, a major inhibitor of polymerase chain reaction (PCR) amplification. Journal of Forensic Science 39" 362-372

Anathan J, Goldberg AL, Voellmy R (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science 232 (4749): 522-524

Bak RPM (1973) Coral weight increment *in situ.* A new method fot determine coral growth. Marine Biology 20(1): 45-49

Bak RPM (1978) Lethal and sublethal effects of dredging on reef corals. Marine Pollution Bulletin 9: 14-16

Benson AA and Muscatine L (1974) Wax in coral mucus: Energy transfer from corals to reef fishes. Limnology and Oceanography 19(5): 810-814

Bierkens JGEA (2000) Applications and pitfalls of stress-proteins in biomonitoring. Toxicology 153: 61-72

Bower, NI, Moser RJ, Hill JR, Lehnert SA (2007) Universal reference method for real-time PCR gene expression analysis of preimplantation embryos. BioTechniques 42(2): 199-206

Brower DL, Brower SM, Hayward DC, Ball EE (1997) Molecular evolution of integrins: Genes encoding integrin ß subunits from a coral and a sponge. PNAS 94(17): 9182-9187

Brown BE (1997) Coral bleaching: causes and consequences. Coral Reefs 16 (S1): S129-S138

Brown BE, Downs CA, Dunne RP, Gibb SW (2002) Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. Marine Ecology Progress Series 242: 119-129

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25: 169-193

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. Journal of Molecular Endocrinology 29: 23-39

Bustin SA (2004) A – Z of quantitative PCR. International University Line, London, UK, pp 912

Connell DW, Lam PKS, Richardson BJ, Wu RRS (1999) Introduction to Ecotoxicology. Blackwell Science, Cornwall, pp 50-144

Davis, Jr. W, De Sousa PA, Shultz RM (1996) Transient expression of translation initiation factor eIF-4C during the 2-cell stage of the preimplantation mouse embryo: Identification by mRNA differential display and the role of DNA replication in zygotic gene activation. Developmental Biology 174: 190-201

deBoer ML, Krupp DA, Weis VM (2007) Proteomic and transcriptional analyses of coral larvae newly engaged in symbiosis with dinoflagellates. Comparative Biochemistry and Physiology, Part D 2: 63-73

Dempster E, Pryor K, Francis D, Young J, and Rogers H (1999) Rapid DNA extraction from ferms for PCR-based analyses. Biotechniques 27: 62-64

Depledge MH (1999) Recovery of ecosystems and their components following exposure to pollution. Journal of Aquatic Ecological Stress Recovery 6: 199-206

De Sousa PA, Westhusin ME, Watson AJ (1998) Analysis of variation in relative mRNA abundance for specific gene transcripts in single bovine oocytes and early embryos. Molecular Reproduction and Development 49: 119-130

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004) Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques 37: 112-119

Downs CA, Mueller E, Philips S, Fauth, JE, Woodley CM (2000) A molecular biomarker system for assessing the health of coral (*Montastrea faveolata*) during heat stress. Marine Biotechnology 2: 533-544

Farmer MA, Fitt WK, Trench RK (2001) Morphology of the symbiosis between *Corculum cardissa* (Mollusca: Bivalvia) and *Symbiodinium corculorum* (Dinophyceae). The Biological Bulletin 200: 336-343

Finnerty JR (2001) Cnidarians reveal intermediate stages in the evolution of hox clusters and axial complexity. American Zoologist 41(3): 608-620

Fitt WK, Brown BE, Warner ME, Dunne RP (2001) Coral bleaching: Interpretation of thermal tolerance limits and thermal thresholds in tropical corals. Coral Reefs 20: 51-65

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: Pitfalls and potential. BioTechniques 26: 112-115

Gilsbach R, Kouta M, Bönisch H, Brüss M (2006) Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. BioTechniques 40(2): 173-177

Glynn PW and D'Croz L (1990) Experimental evidence for high temperature stress as the cause of El Nino-coincident coral mortality. Coral Reefs 8:181-192

Glynn PW (1991) Coral bleaching in the 1980's and possible connections with global warming. Trends in Ecology and Evolution 6; 175-179

Gorbunov M, Kolber ZS, Lesser MP, Falkowski PG (2001) Photosynthesis and photoprotection in symbiotic corals. Limnology and Oceanography 46: 75-85

Guzmán HM, Jiménez CE (1992) Contamination of coral reefs by heavy metals along the Caribbean coast of Central America. Marine Pollution Bulletin 24:554-561

Heinz Center (2002) The State of the Nation's Ecosystems: Measuring the Lands, Waters, and Living Resources of the United States. Cambridge University Press, New York

Ho, Chai-Ling, Phang S, Sinnappah ND, Pang T (1996) Molecular approaches in the taxonomy of the red and brown seaweeds. In: Cytology, genetics and molecular biology of algae (eds. Chaudhary BR and Agrawal SB), SPB Academic Publishing, Amsterdam, pp 351-362

Hoegh-Guldberg O, Smith GJ (1989) The effect of sudden changes in temperature, light and salinity on the population density and export of zooxanthellae from the reef corals *Stylophora pistillata* Esper and *Seriatopora hystrix* Dana. Journal of Experimental Marine Biology and Ecology 129: 279-304

Hoegh-Guldberg O (1999) Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research 50: 839-866

Hugget J, Dheda K, Bustin SA, Zumla A (2005) Real-time RT-PCR normalization; strategies and considerations. Genes and Immunity 6: 279-284

Iglesias-Prieto R, Matt JL, Robins WA, Trench RK (1992) Photosynthetic response to elevated temperature in the symbiotic dinoflagellate *Symbiodinium microadriaticum* in culture. Proceedings of the National Academy of Sciences of the United States of America 89(21): 10302-10305

Johnson DR, Lee, PKH, Holmes VF, Avarez-Cohen (2005) An internal reference technique for accurately quantifying mRNAs by Real-Time PCR with application to the *tceA* reductive dehalogenase gene. Applied and Environmental Microbiology 71(7): 3866-3871

Jokiel PL Jokiel's illustrated scientific guide to Kane'ohe Bay, O'ahu. CRAMP publication Typescript pp 65.

Jones LJ, Yue St, Cheung CY and Singer VL (1998) RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterization. Analytical Biochemistry 265: 368-374

Karako S, Stambler N, Dubinsky N (2002) The taxonomy and evolution of zooxanthellae-coral symbiosis. In: Symbiosis: Mechanisms and Model Systems (eds. Seckback J), Kluwer Academic Publishers, Dordrecht, pp 541-557

Kortschak R, Samuel G, Saint R, Miller D (2003) EST analysis of the cnidarian *Acropora millepora* reveals extensive gene loss and rapid sequence divergence in the model invertebrates. Current Biology 13(24): 2190-2195

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) The real-time polymerase chain reaction. Molecular Aspects of Medicine 27: 95-125

Leggat W, Hoegh-Guldberg O, Dove S, Yellowlees D (2007) Analysis of an EST library from the dinoflagellate (*Symbiodinium sp.*) symbiont of reef building corals. Journal of Phycology 43(5): 1010-1021

Lejeusne C, Pérez T, Sarrazin V, Chevaldonné P (2006) Baseline expression of heat-shock proteins (HSPs) of a "thermotolerant" Mediterranean marine species largely influenced by natural temperature fluctuations. Canadian Journal of Fisheries Aquaculture Sciences 63: 2028-2037

Lesser MP (1989) Photobiology of natural populations of zooxanthellae from the sea anemone *Aiptasia pallida*: assessment of the host's role in protection against ultraviolet radiation. Cytometry 10: 653

Lesser MP, Shick JM (1989) Effects of irradiance and ultraviolet radiation on photoadaptation in the zooxanthellae of *Aiptasia pallida*: primary production, photoinhibition, and enzymatic defenses against oxygen toxicity. Marine Biology 102:243-255

Lesser MP, Stochaj WR, Tapley DW, Shick JM (1990) Bleaching in coral reef anthozoans: Effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. Coral Reefs 8: 225-232

Lewin RA and Cheng L (1989) Prochloron: A Microbial Enigma. Chapman & Hall, New York.

Mayfield AB, Hirst MB, Gates RD (accepted) Gene expression normalization in a dual-compartment system: A real-time quantitative PCR protocol for symbiotic anthozoans. Molecular Ecology Resources.

McCarthy JF, Shugart LR (1990) Biological markers of environmental contamination. In: Biomarkers of Environmental Contamination (eds. McCarthy JF, Shugart LR), Lewis Publishers, Boca Raton, Florida, pp 3-14

Mitchelmore CL, Schwarz JA, Weis VM (2002) Development of symbiosis-specific genes as biomarkers for early detection of cnidarian-algal symbiosis breakdown

Murtha MT, Leckman JF, Ruddle FH (1991) Detectionof homeobox genes in development and evolution. PNAS 88: 10711-10715

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

Muscatine L and Porter JW (1977) Reef corals: Mutualistic symbioses adapted to nutrient-poor environments. BioScience 27(7): 454-460

Muscatine L and Pool RR (1979) Regulation of numbers of intracellular algae. Proceedings of the Royal Society of London. Series B, Biological Sciences 204(1155): 131-139

Muscatine L, Falkowski PG, Porter JW, Dubinsky Z (1984) Fate of photosynthetic fixed carbon in light-and-shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. Proceedings of the Royal Society London B 222: 181-202

Nomura M, Gourse R, Baughman G (1984) Regulation of the synthesis of ribosomes and ribosomal components. Annual Review of Biochemistry 53: 75-117

Norton JH, Sheperd MA, Long HM, Fitt WK (1992) The zooxanthelallar tubular system in the giant clam. The Biological Bulletin 183: 503-506

Pearse VB and Muscatine L (1971) Role of symbiotic algae (zooxanthellae) in coral calcification. The Biological Bulletin 141: 350-363

Reynolds WS, Schwarz JA, and Weis VM (2000) Symbiosis-enhanced gene expression in cnidarian-algal association: Cloning and characterization of a cDNA, *sym32* encoding a possible cell adhesion protein. Computational Biochemistry and Physiology A 126: 33-44

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43(1): 223 - 225

Richier S, Furla P, Plantivaux A, Merle P, Allemand D (2005) Symbiosis-induced adaptation to oxidative stress. Journal of Experimental Biology 208: 277-285

Rodriguez-Lanetty M, Phillips WS, Weis VM (2006) Transcriptome analysis of a cnidarian – dinoflagellate mutualism reveals complex modulation of host gene expression. BMC Genomics 7: 23-33

Santiago-Vásquez LZ, Ranzer LK, Kerr, RG (2006) Comparison of two total RNA extraction protocols using the marine gorgonian coral *Pseudopterogorgia elisabethae* and its symbiont *Symbiodinium* sp. Electronic Journal of Biotechnology 9(5): 598-603

Schwarz J, Brokstein PB, Voolstra C, Terry AY, Miller DJ, Szmant AM, Coffroth MA, Medina M (2008) Coral life history and symbiosis: Functional genomic resources for reef buildig corals, *Acropora palmate* and *Montastraea favelata*. BMC Genomics 9: 97

Sharp VA, Brown BE, Miller D (1997) Heat shock protein (Hsp70) expression in the tropical reef coral *Goniopora djiboutiensis*. The Journal of Thermal Biology 22(1): 11-19

Smith-Kuene C and Dove S (2007) Gene expression of a green fluorescent protein homology as a host-specific biomarker of heat stress within a reef-building coral. Marine Biotechnology 0: 1-15

Smith RD, Brown B, Ikonomi P, Schechter AN (2003) Exogenous reference RNA for normalization of real-time quantitative PCR. BioTechniques 34: 88-91

Ståhlberg, A, Håkansson, J, Xian, X, Semb, H, Kubista, M (2004) Properties of the reverse transcription reaction in mRNA quantification. Clinical Chemistry 50(3): 509-515

Stat M, Carter D, Hoegh-Guldberg O (2006) The evolutionary history of *Symbiodinium* and scleractinian hosts— Symbiosis, diversity, and the effect of climate change. Perspectives in Plant Ecology, Evolution, and Systematics 8: 23-43

Stengel B, Watier L, Chouquet C, Cénée S, Philippon C, Hémon D (1999) Influence of renal biomarker variability on the design and interpretation of occupational or environmental studies. Toxicology Letters 106(1): 69-77

Sullivan JC, Ryan JF, Watson JA, Webb J, Mullikin JC, Rokhsar D, Finnerty JR (2006) *StellaBase*: The *Nematostella vectensis* genomics database. Nucleic Acids Research 34: 495-499

Temeles GL, Ram PT, Rothstein JL, Shultz RM (1994) Expression patterns of novel genes during mouse preimplantation embryogenesis. Molecular Reproduction and Development (37): 121-129

Trench RK (1979) The cell biology of plant-animal symbiosis. Annual Review of Plant Physiology 30: 485-531

van Oppen MJH and Gates RD (2006) Conservation genetics and the resilience of reef-building corals. Molecular Ecology 15(13): 3863-3883

Weis VM and Reynolds WS (1999) Carbonic anhydrase expression and synthesis in the sea anemone *Anthopleura elegantissima* are enhanced by the presence of dinoflagellate symbionts. Physiological Zoology 72(3): 307-31

Wilkening S and Bader A (2004) Quantitative Real-time PCR Reaction: Methodical analysis and mathematical model. Journal of Biomolecular Techniques 15: 107 – 111

Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson, KS (2001) Real-time multiplex PCR assays. Methods 25 (4): 430–442.

Yandell (1997). Practical Data Analysis. "Models with random effects." Chapman & Hall/CRC, Boca Raton FL, pp 297 - 312

Zhu G, Chang Y, Zuo J, Dong X, Zhang M, Hu G, Fang F (2001) Fudenine, a C-terminal truncated rat homologue of mouse prominin, is blood glucose-regulated and can up-regulate the expression of GAPDH. Biochemical and Biophysical Research Communications 281: 951-956