THE RESPONSE OF SYMBIOTIC ZOOXANTHELLAE (*SYMBIODINIUM* SPP.) DIVERSITY AND GENE EXPRESSION TO STRESS IN GEOGRAPHICALLY DISTINCT REEFS

By

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ABSTRACT

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The persistence of coral reefs in the Florida Keys reef tract is of concern as coral bleaching, due to increased ocean temperatures, and human-linked disease outbreaks have led to a reduction in coral cover of 40% since the 1980's. Evidence suggests a variation in stress susceptibility of conspecific coral from inshore and offshore reefs in the Florida Keys. However, the mechanism behind the disparity in stress susceptibility is unknown. Variation in genetic composition of a coral's symbiotic algae (*Symbiodinium* spp.; referred to as zooxanthellae) has been proposed as a mechanism to withstand stress. As such, I investigated zooxanthellae composition of *Porites astreoides* and *Montastraea cavernosa* from an inshore and offshore reef in the Lower Florida Keys to determine links to stress susceptibility. Additionally, I investigated variation in the expression of metabolically related genes in zooxanthellae of *P. astreoides* reciprocally transplanted between reefs, as well as exposed to elevated temperatures and disease.

Chapter one examines changes in the dominant zooxanthellae subclade type in *P. astreoides* and *M. cavernosa* throughout a two-year reciprocal transplant study. The goal of this study was to determine if zooxanthellae subclade type could explain higher rates of bleaching in offshore reef coral, as well as assessing the possibility of acclimatization to different environments. Increased complexity and diversity was seen in the composition of zooxanthellae subclade types from coral collected at offshore reefs,

compared to inshore reefs. As a result, offshore reef zooxanthellae displayed less stability, possibly explaining higher bleaching susceptibility. Additionally, zooxanthellae composition patterns were retained throughout the reciprocal transplant, demonstrating a lack of acclimatization.

Chapter two examined site-specific variation in the expression of metabolically related genes in zooxanthellae from *P. astreoides* following the reciprocal transplant. Symbionts from offshore corals experienced significantly increased expression in *PCNA, SCP2, G3PDH, PCP* and *psaE* ($p<0.05$) compared to inshore symbionts, a pattern consistent with increased bleaching susceptibility. Significant differences in gene expression between zooxanthellae from inshore and offshore reef indicate functional variability and are likely a result of localized adaptation. Similar to results in chapter one, gene expression patterns from site of origin were retained throughout the reciprocal transplant, suggesting no acclimatization.

Chapter three investigated variation in the response of the same zooxanthellae genes when *P. astreoides* was exposed to extreme temperatures and disease. Here disease was mimicked by the application of lipopolysaccharide from *Serratia marcescens*, the causative agent of acroporid serratosis. Gene expression did not differ in zooxanthellae from inshore and offshore reefs, nor as a consequence of extreme temperature or disease. Several factors may explain the lack of variation including zooxanthellae response to acute versus moderate stress, host protection and targeting of symbionts by *S. marcescens*. These results suggest that symbionts of *P. astreoides* may be locally adapted to chronic moderate stress, but respond similarly to acute extreme conditions.

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My friend and personal editor Sam Kerath, who has taught me the art of prose since high school.

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A note on authorship:

I have had the pleasure of collaborating with a multitude of brilliant scientists to publish the work you are about to read. The first chapter of my dissertation will be published in The International Journal of Biology in January 2016, with coauthors: Joshua Haslun, Kevin Strychar, Peggy Ostrom and James Cervino. Chapters two and three will be submitted for publication with the same coauthors.

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CHAPTER 1

SYMBIONT DIVERSITY OF ZOOXANTHELLAE (*SYMBIODINIUM* SPP.) IN *PORITES ASTREOIDES* AND *MONTASTRAEA CAVERNOSA* FROM A RECIPROCAL TRANSPLANT IN THE LOWER FLORIDA KEYS

ABSTRACT

In recent years coral reefs worldwide have suffered high mortality rates due to coral bleaching, a phenomenon contributing to a 40% decrease in coral cover in the Florida Keys since the 1997/98 El Niño event. In the Florida Keys, coral from inshore reefs are known to be more thermotolerant than their conspecifics from offshore reefs, but the mechanism behind this difference is unclear. In this study we conducted a two-year, reciprocal transplant of *Porites astreoides* and *Montastraea cavernosa* from an inshore and offshore reef in the lower Florida Keys to determine if changes in the dominant symbiotic algae (*Symbiodinium* spp.) could explain variation in holobiont tolerance as well as to assess the possibility of acclimatization to a changing stress regime. Increased complexity and diversity was demonstrated in the composition of *Symbiodinium* spp. from both coral species collected at the offshore reef when compared to conspecifics collected inshore. As a result of this complexity, the offshore reef samples displayed higher numbers of transitions of zooxanthellae subclade types between seasons, while inshore fragments demonstrated more stability and this observation may explain previously measured thermotolerance. Additionally, the known thermotolerant subclade type D1 was associated with one *M. cavernosa* fragment from the inshore reef. When fragments were transplanted, compositional patterns of *Symbiodinium* spp. were retained from site of collection, indicating a lack of acclimatization to a new environment over the lengthy two-year experiment. These results demonstrate variability in the dominant *Symbiodinium* spp. of *P. astreoides* and *M. cavernosa* conspecifics from inshore and offshore reefs in the lower Florida Keys and point to

possible patterns in holobiont thermotolerance. Dominant symbiont variability may contribute to the continued persistence of these species in the face of climate change, but future studies are needed to determine the mechanisms and range in which these subclade types are able to withstand thermal stress.

INTRODUCTION

Since the 1980's, coral of the Florida Keys Reef Tract have suffered severe mortalities (Glynn *et al.*, 2001; Gardner *et al.*, 2003) due to bleaching, disease and poor water quality (Dustan, 1977; In *et al.*, 2007). Coral bleaching is the result of the expulsion of symbiotic, single-celled dinoflagellate algae known as zooxanthellae (*Symbiodinium* spp.) from the tissues of coral animals, and may be caused by many stressors including, but not limited to, elevated sea surface temperatures and increased irradiance (Fitt *et al.*, 2001; Lesser & Farrell, 2004). Bleaching can be fatal depending on the intensity and duration of a bleaching event (Brown, 1997), as many coral derive up to 95% of their daily metabolic needs from the byproducts of zooxanthellae (Muscatine, 1990). However, through genetic variability of their in-hospite zooxanthellae, coral-algal assemblages vary in their ability to tolerate bleaching conditions (Sampayo *et al.*, 2008). Defining how specific reef systems and their resident symbionts respond to increases in bleaching inducing stressors is important for delineating coral survivability and determining appropriate target populations for conservation efforts.

Symbiodinium spp. are classified into clades A-I and are further divided into sub-clade types, with hermatypic coral known to form symbioses with members of clades A-D (Baker, 2003). In response to stress, coral may shuffle or increase the abundance of a more tolerant type (Baker, 2001; Rowan, 2004). The ability to increase the relative frequency of stress tolerant

symbiont subclade types may enhance the survival of corals experiencing long or short-term deterioration of environmental quality such as sea surface temperatures above mean averages or irradiance.

Previous investigations demonstrated that stress tolerance varies between clades and even subclades (Rowan & Knowlton, 1995; Fitt *et al.*, 2001; Berkelmans & van Oppen, 2006; Hauff *et al.*, 2014), the latter demonstrated by Sampayo et al. (2008). They showed that while variation in susceptibility to bleaching was attributed to specific thermotolerant subclade types in *Stylophora pistillata*, zooxanthellae tolerance was also assemblage specific. Thus, to relate survival to the composition of symbionts, we need to learn more about how stress tolerance is related to subclade type and assemblage specificity.

Inshore and offshore reefs of the Florida Keys display distinct environmental parameters, which may result in locally adapted coral populations (Kenkel *et al.*, 2013). There is significant population genetic subdivision between host coral populations originating from offshore and inshore reefs, with inshore coral demonstrating higher thermotolerance (Kenkel *et al.*, 2013). Additionally, recent work documented changes in holobiont health and photosynthetic efficiency of zooxanthellae in response to reciprocal transplants between inshore and offshore reefs (Haslun *et al.* In Review). These studies, however, did not describe how zooxanthellae subclade type diversity between inshore and offshore coral responded to stress.

To expand my knowledge of how subclades vary among coral host conspecifics and how these assemblages respond to stress, I investigated patterns in the dominant populations of zooxanthellae from an inshore and offshore reef in the Lower Florida Keys. In addition, I investigated changes in the dominant populations of zooxanthellae from these coral in response to a two-year reciprocal transplant to assess the capacity of symbiont shuffling, and therefore

acclimatization, to an altered stress regime. *Porites astreoides* and *Montastraea cavernosa* were used as they commonly occur in both inshore and offshore patch reefs of the Florida Keys and are likely targets of conservation and repopulation efforts. These species also possess distinct life strategies and modes of symbiont acquisition and therefore, are likely to alter their zooxanthellae subclade types differently in response to stress. Fragments were sampled biannually and the predominant zooxanthellae were identified down to the subclade type by direct sequencing of the ITS2 region (LaJeunesse *et al.*, 2003; Thornhill *et al.*, 2009; T. LaJeunesse pers. comm.).

METHODS

Study sites

Two reefs off the coast of Summerland Key, Florida near Mote Marine Tropical Laboratory (MMTL) were used for collection of coral samples for genetic analyses and reciprocal transplant experiments. Birthday reef (inshore, 24.57917N, 81.49693W) and Acer24 reef (offshore, 24.55268N, 81.43741W) are patch reefs separated by Hawk Channel. These two reefs experience notably distinct temperature and turbidity regimes, with Birthday reef experiencing higher turbidity and higher annual average temperatures (~1°C) than Acer24 reef (Erich Bartels, pers.com.), but are otherwise similar (i.e. depth, coral and fish species diversity).

Coral fragment collection

In September 2011, fragments of *Montastraea cavernosa* and *Porites astreoides* were collected from Acer 24 and Birthday reef. At each site, ten 16x16 cm coral fragments of *P. astreoides*, and ten fragments of *M. cavernosa*, were obtained (Permit # FKNMS-2011-107, n=40) using a hammer and chisel and stored in coolers with site-derived seawater during

transport to MMTL where they were sectioned into two pieces (16x8 cm) using a table saw. The fragments were allowed to recover for 24 hours in a flow through water table shaded from direct sunlight. Following this recovery period, fragments were attached to labeled concrete pucks (1 part concrete: 3 parts sand) using a two-part epoxy (All Fix Epoxy®, AFP; Philadelphia, PA USA) and allowed to recover for an additional 72 hours under the same conditions described above. These fragments were then used for the reciprocal transplant experiment.

Reciprocal Transplant

A reciprocal transplant experiment was designed such that half of the original sample fragment was placed back at the site of origin and the other replicate fragment was placed at the transplant site (i.e. the other reef site, Figure 1).

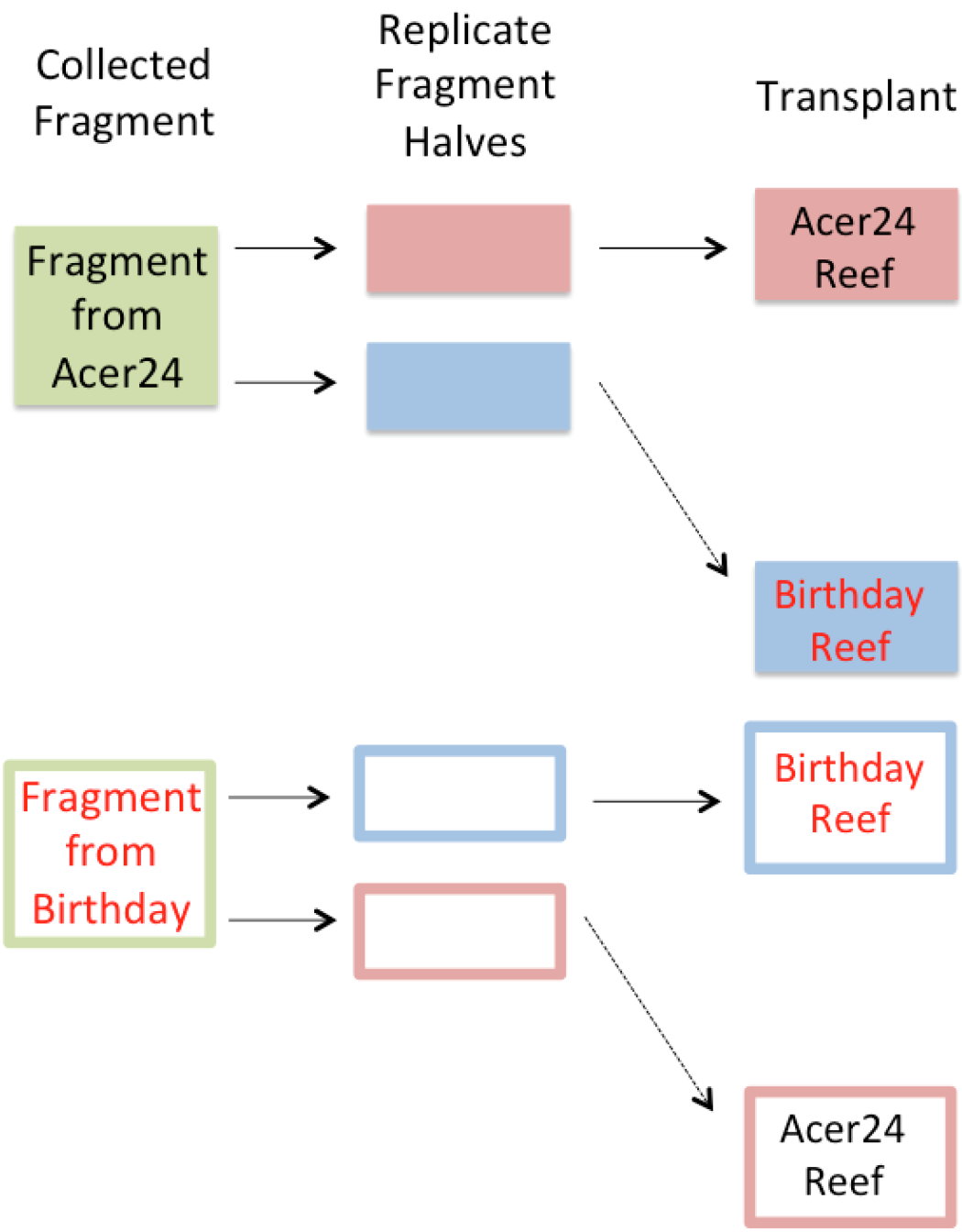

Figure 1. Experimental Design. Graphical representation of my reciprocal transplant design for a single coral species at one experimental level. A fragment of one species was collected at each of the two sites. Solid rectangles represent coral from Acer24 Reef and hollow rectangles represent coral from Birthday Reef. Those fragments were sectioned into two fragments. Each fragment was then placed back out on to a reef with one fragment half remaining at the site of origin while the other fragment half was transplanted to the companion site. The fragments colored in red were placed on to Acer24 Reef while the fragments colored in blue were placed at Birthday

Figure 1 (cont'd). Reef. Dashed arrows represent transplanted fragments. For the experiment, the above was repeated ten times for each coral species resulting in n=80 fragments.

Transplants were established at each reef using six concrete cinder blocks placed on a level benthic substrate in the shape of a hexagon. Each cinder block was attached to the substrate using AFP. A total of 40 coral were placed at each site, ten fragments of *P. astreoides* from Acer24 reef and ten fragments of *P. astreoides* from Birthday reef, as well as ten fragments of *M. cavernosa* from Acer24 reef and ten fragments of *M. cavernosa* from Birthday reef. This design yielded a total of 80 coral fragments.

Fragment halves were arranged on cinder blocks as follows. Due to unequal division of samples to cinder blocks, six or seven coral replicate fragment halves were attached to each cinder block using AFP. Individual cinder blocks contained one species of coral (i.e. either *P. astreoides* or *M. cavernosa*), with neighboring blocks containing the other species. Within each cinder block, replicate fragment halves were placed randomly. This experimental design was conducted to assure that all cinder blocks received equal exposure to homogenous environmental conditions. Temperature was monitored every 30 minutes via HOBO™ tags placed at each site.

Sample Collection for DNA Analysis

After transplant, coral fragments remained at their placement site for two years (i.e. August 2011 through August 2013). Sampling for DNA analysis was conducted biannually (February and August, 2012-2013, n=4) to reflect both conditions in which temperature stress is high (summer/August) and low (winter/February). These events will be referenced as sampling times throughout the manuscript. Sampling was achieved by chipping off a 1x1 cm piece of coral from fragments using a hammer and chisel. *Porites astreoides* pieces were stored in 70% ethanol (EtOH), while *Montastraea* cavernosa pieces were flash-frozen in liquid nitrogen and stored at -

80°C (n=80/field season) within 15 minutes of initial collection. The distinction in storage technique resulted from an inability to extract viable DNA from *M. cavernosa* fragments stored in EtOH.

DNA Extraction and Sequencing

Tissues of *P. astreoides* were collected from coral fragments stored in 70% EtOH. Tissue was removed from the skeleton using a razorblade in 10 mL of zooxanthellae isolation buffer (ZIB, Rowan & Powers, 1991). The homogenate was poured into centrifuge tubes and pelleted (500 x g for 10 minutes), and the supernatant decanted, washed in 5 mL of ZIB, and pelleted (500 x g for 10 minutes) a second time. Upon collection of the tissue pellet, DNA was extracted using a plant DNA extraction kit (MoBio Laboratories) according to manufacturers instructions.

Samples of *M. cavernosa*, stored at -80°C were macerated into a fine powder using a mortar and pestle pre-chilled in liquid nitrogen. DNA was extracted (Extract-n-amp, Sigma Aldrich) from 0.05 g of powder following the manufacturer's instructions.

For polymerase chain reactions (PCR) of the internal transcriber 2 region (ITS2), 1 µL of DNA was added to 10uL of GoTaq (Promega), 7 µL of nuclease free H_2O and 1 µL each of primers ITSintfor2 (GAATTGCAGAACTCCGTG) and ITS2-reverse (GGGATCCATATGCTTAAGTTCAGCGGGT)(Lajeunesse & Trench, 2000) using a touchdown PCR method outlined in LaJeunesse et al. (2003). Amplification was verified using 5 µL of PCR product examined on a 2% agarose gel (60 minutes and 110 volts). PCR products were purified (GeneElute PCR purification kit, Sigma Aldrich) and quantified. *Porites astreoides* and *Montastraea cavernosa* samples were subsequently sequenced using a capillary ABI 3130xl platform at the DNA sequencing facilities located at the Annis Water Resources Institute, Grand

Valley State University and at the Research Technology Support Facility (RTSF) at Michigan State University, respectively. Both sequencing reactions used the forward or reverse ITS2 primers listed above.

Data Analysis

Chromatograms were visually inspected and aligned using BioEdit (v7.2.5; Hall, 1999). All statistical analyses were conducted in R (Version 3.1.3; R Core Team, 2015). Sequences from each coral species were divided into four groups based upon fragment origin and transplantation (see Figure 2). For example, sequences of samples taken from *Porites astreoides* fragments were categorized into two main categories, from Acer 24 reef or Birthday reef, with their site of relocation determining their further classification (i.e. four groups). Sequences grouped as "PAA" are those of *P. astreoides* fragments originating from Acer 24 reef that were placed back on Acer 24 reef. Sequences grouped as "PAB" are those from *P. astreoides* fragments originating from Acer 24 reef that were placed back on Birthday reef. An analogous sample identification scheme was used for sequences obtained from *Montastraea cavernosa* samples.

A multinomial model was used to analyze differences in population frequencies of clade subtypes within and between sampling periods. Overall, four models were run, one for each coral species and one for each collection site. For example, one model was run comparing *P. astreoides* native to Acer24 reef with coral native to Acer24 but transplanted to Birthday reef.

Within each model, frequency of zooxanthellae subclade types were used as a response variable, with sampling season and treatment (i.e. transplant *vs*. non-transplant) as predictors. Model outputs, termed coefficients, represent probabilities of individual response variables.

Residual deviances of models with and without the interaction term (sampling time:treatment) were compared to determine any significance of interaction, while AIC values of full models and individual response variables were compared to chose best fit models for further analysis. In order to perform t-tests on individual subclade type abundance comparisons, fitted values and standard errors were calculated from coefficients.

RESULTS

A total of 139 zooxanthellae sequences were obtained from samples of *Porites astreoides* fragments and 111 zooxanthellae sequences obtained from samples of *Montastraea cavernosa* fragments. To identify these samples, *Symbiodinium* spp. sequences or subclade types were categorized into codes describing the (1) species (P or M for *P. astreoides* or *M. cavernosa*, respectively), (2) collection site (A or B for Acer24 reef or Birthday reef, respectively) and (3) transplant site (A or B for Acer24 reef or Birthday reef, respectively). For example, sequences or subclade types obtained from fragments collected and replaced at Birthday reef will be referred to as "PBB". Those collected at Birthday Reef and transplanted to Acer24 reef will be referred to as "PBA".

Porites astreoides collected from Birthday Reef (PBB and PBA)

A total of three subclade types were recovered from transplant and non-transplant *Porites astreoides* originating from Birthday reef. These types included members of subclades A4.1, A4.2 and A4.3. Subclade type A4.1 was the predominant type between PBB and PBA samples (Figure 2A). Subclade type A4.2 was absent in PBB samples in Summer 2012 as well as PBA samples in Winter 2012 and Winter 2013. Subtype A4.3 was absent only in PBA samples from

Summer 2012, but reemerged in the follow two field seasons.

In a multinomial model, the interaction term between experimental treatment and field season was not significant ($p>0.25$). Based upon AIC values of the full model (zooxanthellae subclade types explained by both experimental treatment and sampling time) and those of the individual independent variables, the model containing experimental treatment only (transplant *vs*. non-transplant) fit the data best and was used for further analysis. T-tests of individual subclade types between experimental treatments (i.e. subclade type A4.1 in PBB *vs*. PBA samples) were insignificant ($p>0.05$). In PBB samples, the probability of subclade type A4.1 was significantly higher than A4.2 ($p=0.000001$) and A4.3 ($p=0.0005$). Additionally, the probability of subclade type A4.3 was significantly higher than A4.2 in PBB samples ($p=0.01$). In PBA samples, the probability of subclade type A4.1 was significantly higher than A4.2 ($p=0.001$) and A4.3 ($p=0.008$) (Figure 2A).

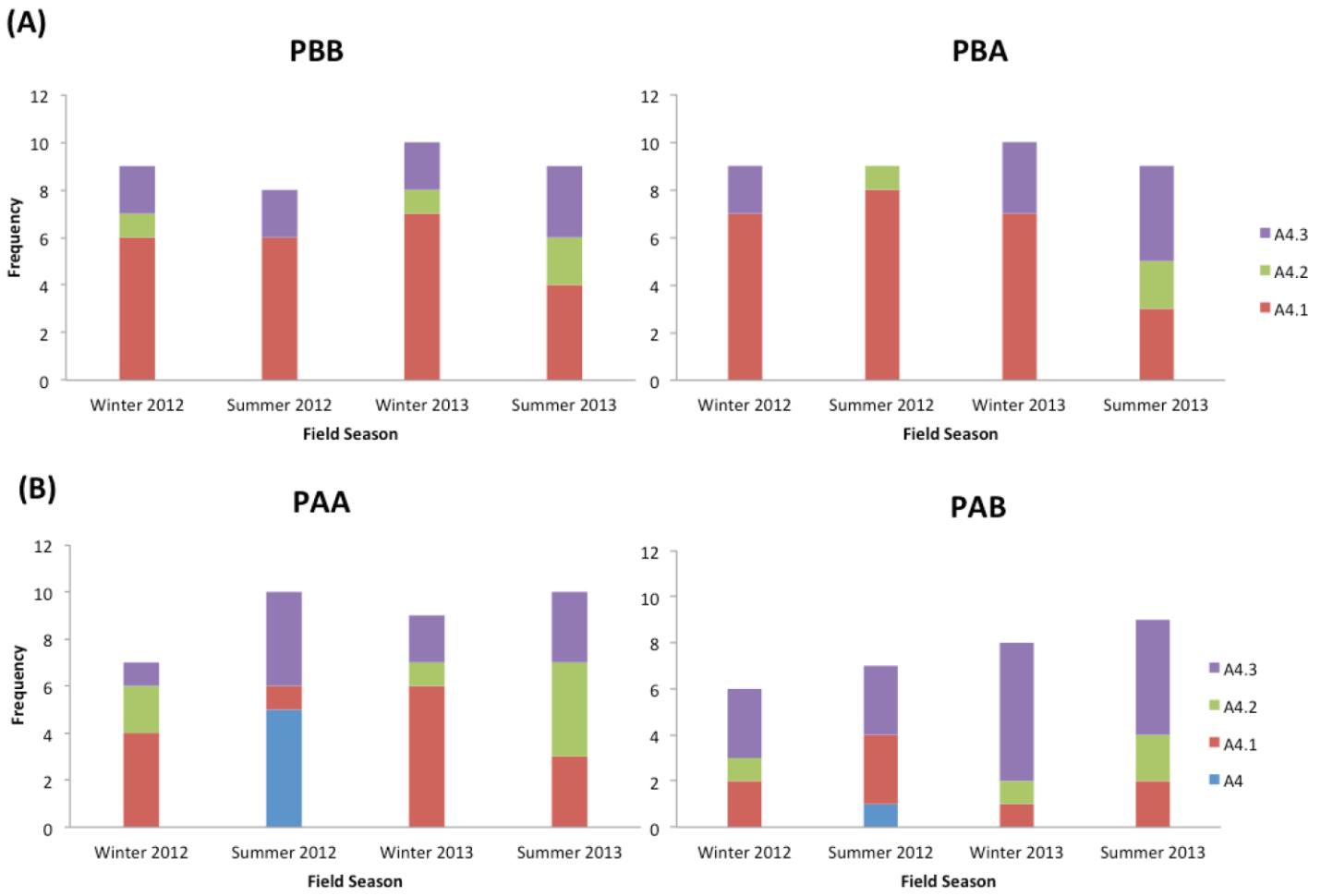

Figure 2. *Symbiodinium* subclade type frequencies for *Porites astreoides* samples. Graphs are labeled with acronyms describing the

Figure 2 (cont'd). (1) coral species, (2) field site of original collection (i.e. Acer24 or Birthday Reefs) and (3) site of transplant (i.e. Acer24 or Birthday Reefs). For example, PBA are samples from *Porites astreoides* fragments collected at Birthday Reef and transplanted to Acer24 Reef. Graphs in the left-hand column represent non-transplanted coral while graphs on the right represent transplanted coral. Although each condition began at n=10, note variation in group sizes due to sequencing error.

Figure 3 outlines the specific changes in zooxanthellae subclade type observed within individual sample fragments between sampling times (i.e. subclade type change from Summer 2012 to Winter 2012), here referred to as transitions. Greater than 50% of subclade types present in PBB and PBA samples did not change throughout the field seasons (Figure 3A). Additionally, with the exception of PBA in Winter 2013 to Summer 2013, more than 50% of the samples were of subclade type A4.1. Not all A4.1 samples transitioned, however. Many A4.1 samples from PBA transitioned to A4.3, specifically in the Summer 2012 to Winter 2013 transition. There were also high transitions to A4.2 in the 2013 Winter to Summer field season.

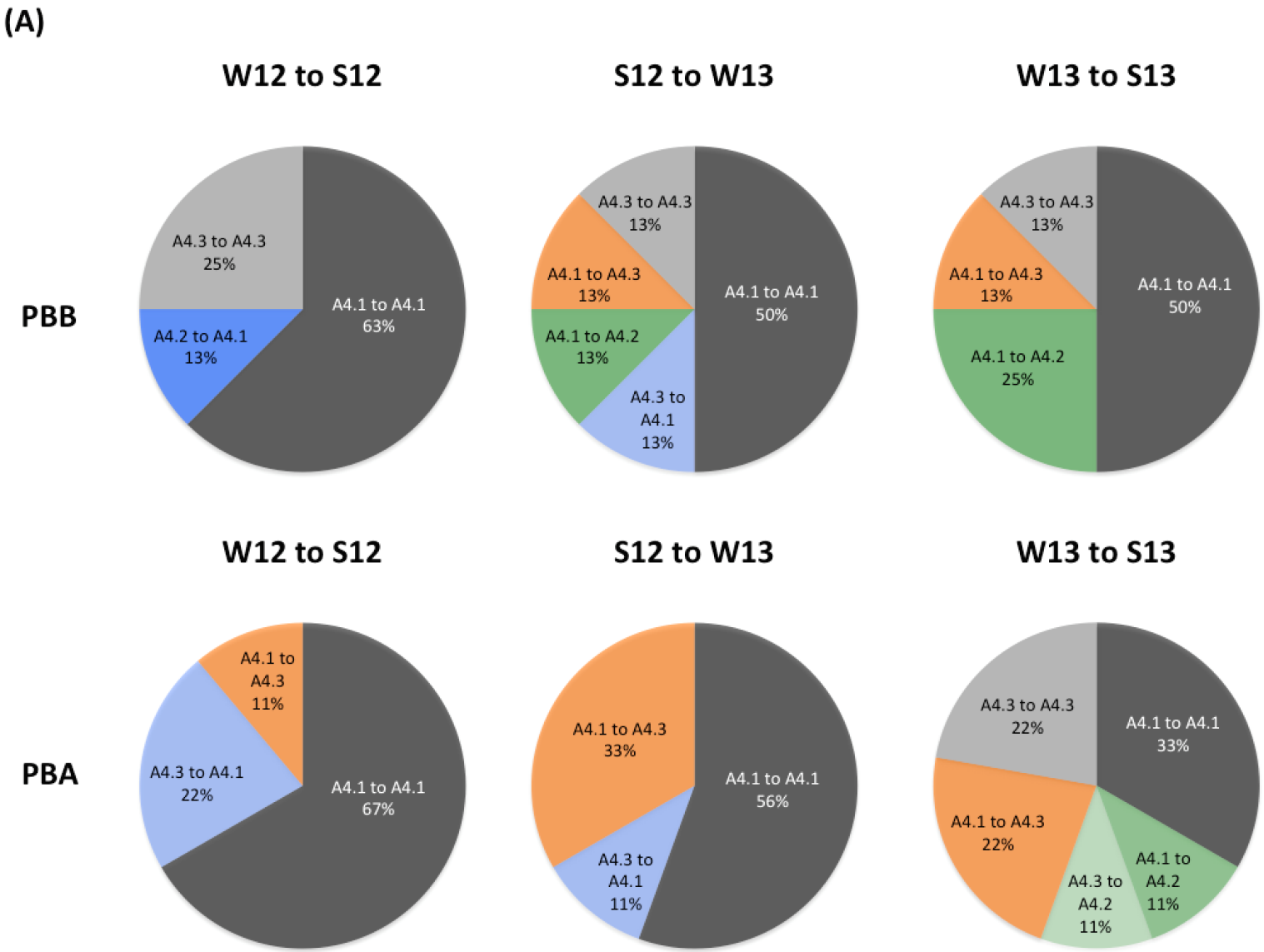

Figure 3. *Porites astreoides* zooxanthellae transitions. Transitions in zooxanthellae subclade type occurring within the same coral

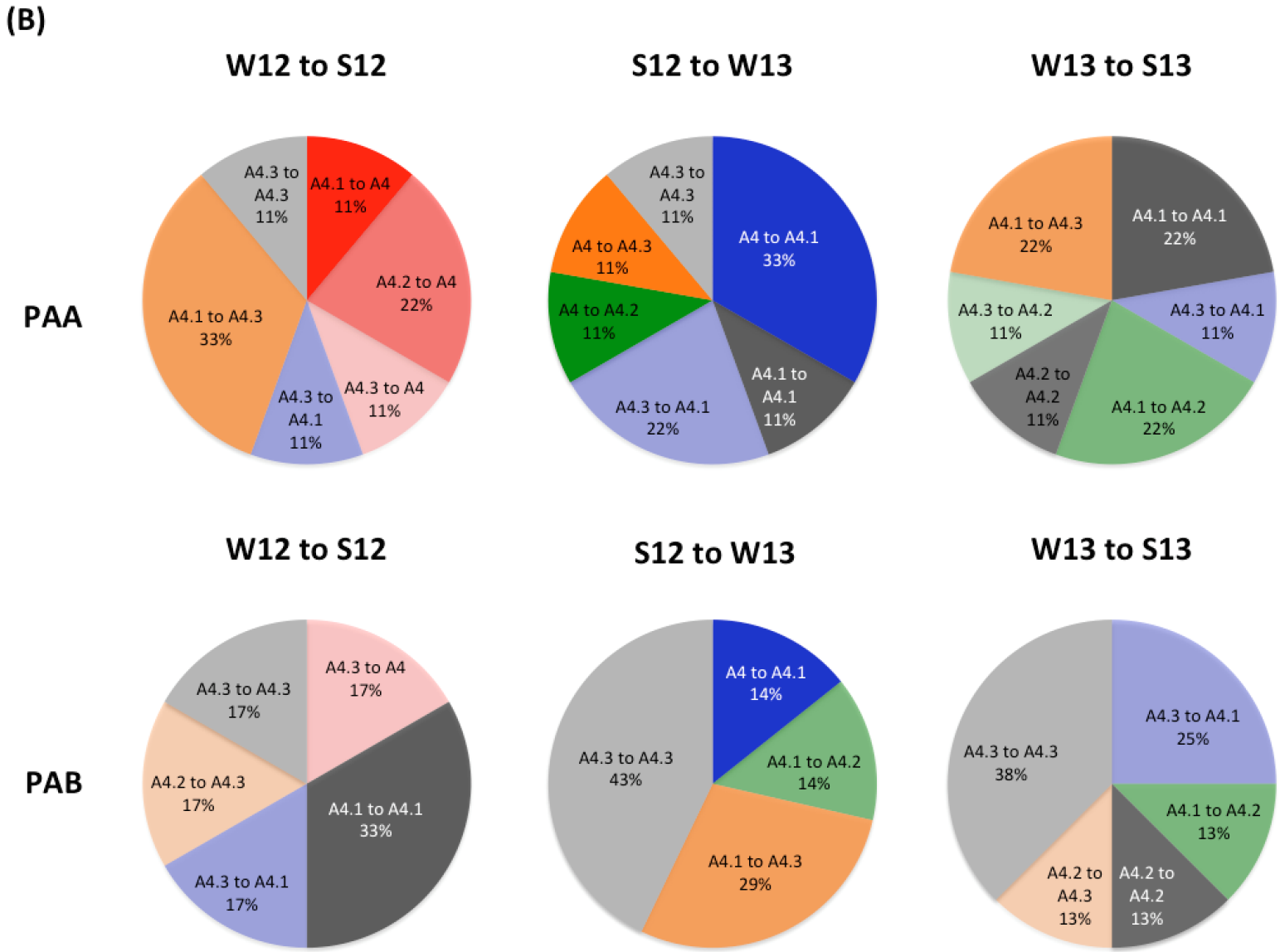

Figure 3 (cont'd). fragment between sampling times for all fragments of *Porites astreoides* zooxanthellae. Pie charts represent the

Figure 3 (cont'd). relative frequencies of individual transitions of clade subtypes through each field season. Graphs are labeled with acronyms describing the (1) coral species, (2) field site of original collection (i.e. Acer24 or Birthday Reefs) and (3) site of transplant (i.e. Acer24 or Birthday Reefs). For example, PBA represents samples from *P. astreoides* fragments collected at Birthday Reef and transplanted to Acer24 Reef. Color scales are determined by the outcome of the transition. For example, slices represented in shades of red describe transitions that resulted in A4 zooxanthellae, transitions to A4.1 in blue, A4.2 in green and A4.3 in orange. Transitions that stayed the same are represented by a gray scale and outlined in black. Sampling times are abbreviated above each graph with "W" for Winter and "S" for Summer. Years correspond to 2012 or 2013.

Porites astreoides collected from Acer24 Reef (PAA and PAB)

A total of four subclade types were observed from sequences originating from transplant and non-transplant *Porites astreoides* collected from Acer24 reef, including members of subclade types A4, A4.1, A4.2 and A4.3. Subclade type A4 was present only during the Summer 2012 field season in PAA and PAB samples (Figure 2B). Additionally, subclade type A4.2 was present during each sampling time except Summer 2012 for PAA and PAB samples. The other three subclade types were present at all other sampling times in both PAA and PAB samples.

Experimental treatment (transplant *vs.* non- transplant) did not have a significant effect on sequence frequencies ($p>0.05$). Additionally, the interaction term between experimental treatment and sampling time was not significant ($p>0.25$). According to AIC values of individual models, the model containing only sampling time fit the data best and was used for further analysis.

The probability of having subclade type A4 was significantly higher in Summer 2012 than the other sampling times ($p<0.03$). Although the probability of subclade type A4.1 was higher in both Winter sampling times compared to Summer sampling times, these increases were not significant ($p>0.3$) (Figure 2B).

A higher number of transitions to different subclade types were seen in PAA samples than in PAB samples (Figure 3B). Overall, ~78% of the zooxanthellae subclade types from PAA

changed between field seasons, where as 52% changed in PAB samples (i.e. the difference between the total number of transitions and those in grey). Notable transitions are those of PAA samples from Winter 2012 to Summer 2012 (Figure 3B). Approximately 50% of the transitions resulted in the presence of the A4 subtype, a subtype that was only observed again in the same transition time point in PAB samples, but in fewer numbers than in PAA samples. Overall, PAB samples were dominated by transitions to A4.3, particularly from summer to winter, where as PAA had a high number of transitions to A4.1.

Montastraea cavernosa collected from Birthday Reef (MBB and MBA)

A total of 59 sequences were recovered from transplant and non-transplant *Montastraea cavernosa* originating from Birthday reef. These sequences were representative of seven different subclade types, including A4.1, A4.2, A4.3, C1, C3 and D1. A large range of sample sizes between field sites in *M. cavernosa* samples resulted. Although the exact subclade types are detailed here, for analytical purposes and in corresponding figures, subclade types were binned by clade type (clade type A or clade type C) due to low replicate numbers (Figure 4A). Clade C subclade types were the dominant clade represented in MBB and MBA samples. Some subclade types were only represented in one sampling time, such as A4.3 in Summer 2012 (MBB), A4.2 in Winter 2012 (MBA) and D1 in Summer 2013 (MBA). The presence of subclade type D1 in MBA samples in the Summer of 2013 marks the only appearance of a D subclade type in all samples tested.

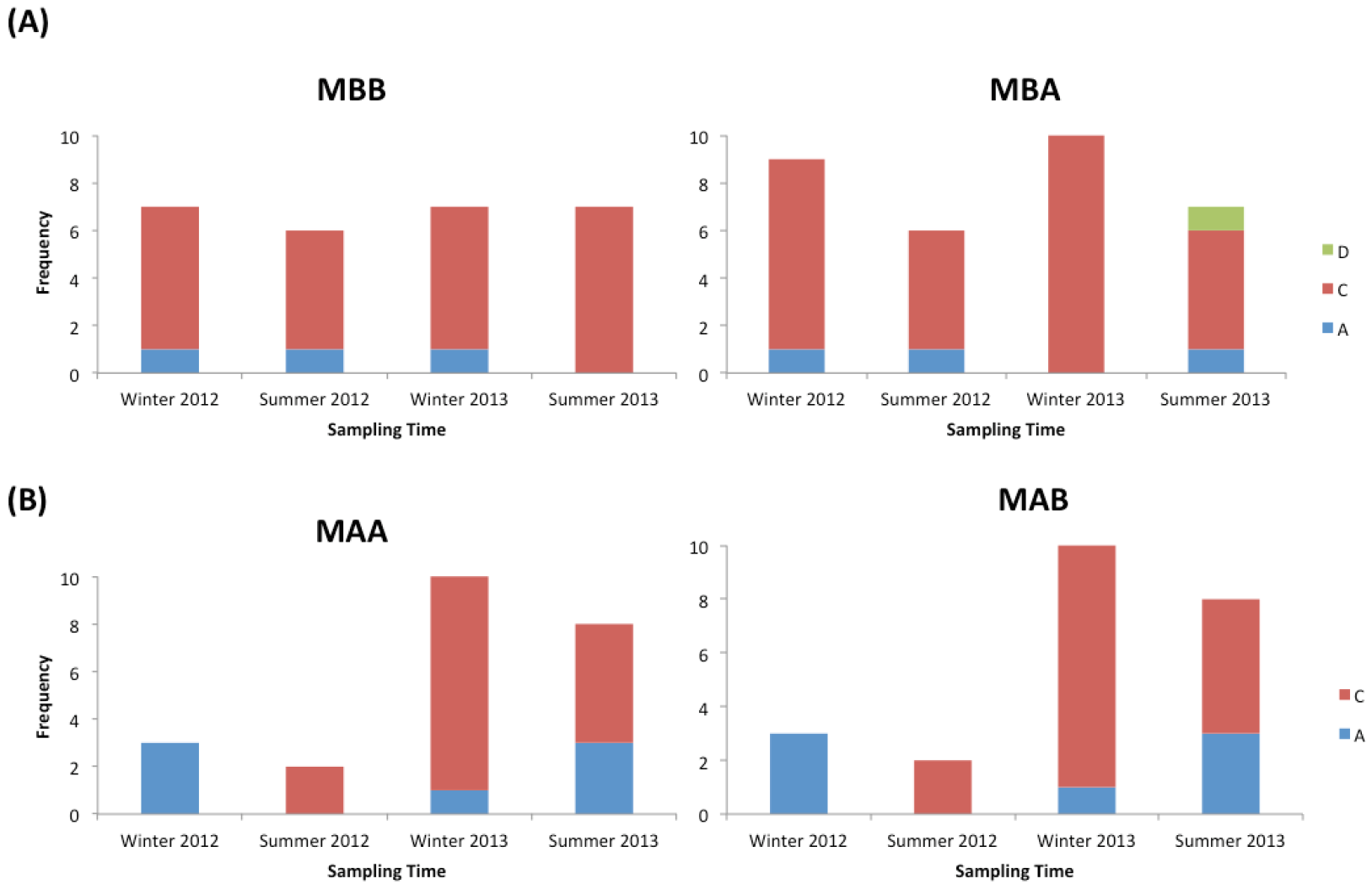

Figure 4. *Symbiodinium* subclade frequencies for *Montastraea cavernosa* samples. Graphs are labeled with acronyms describing the

Figure 4 (cont'd). (1) coral species, (2) field site of original collection (i.e. Acer24 or Birthday Reefs) and (3) site of transplant (i.e. Acer24 or Birthday Reefs). For example, MBA represents samples from *Montastraea cavernosa* fragments collected at Birthday Reef and transplanted to Acer24 Reef. Graphs in the left-hand column represent non-transplanted coral while graphs on the right represent transplanted coral. Although each condition began at n=10, note variation in group sizes due to sequencing error.

A comparison of MBB and MBA samples described an insignificant interaction between experimental treatment and field season ($p>0.25$). Additionally, when comparing AIC values between the full model and those of the individual independent variables, the model containing only the experimental treatment provided the best fit to the data and was used for further analysis.

A comparison of fitted values of the probability of clades A, C and D in MBB and MBA samples displayed no significant difference in the probability of any clade type between experimental treatments. For example, there was no significant difference in the presence of clade A in MBB *vs.* MBA samples. However, there was a significant difference in the probability of clade A and C within MBB samples ($p=0.0000003$), and within MBA samples ($p=0.0000002$), with probabilities of clade C being higher than clade A in both cases (Figure 4A).

The majority of transitions seen in MBB samples resulted in clade C types (Figure 5A). Interestingly, a few transitions resulted in changes from clade A to C or vice versa. The single appearance of clade D results from a transition from clade C in MBA samples during the Winter 2013 to Summer 2013 seasons.

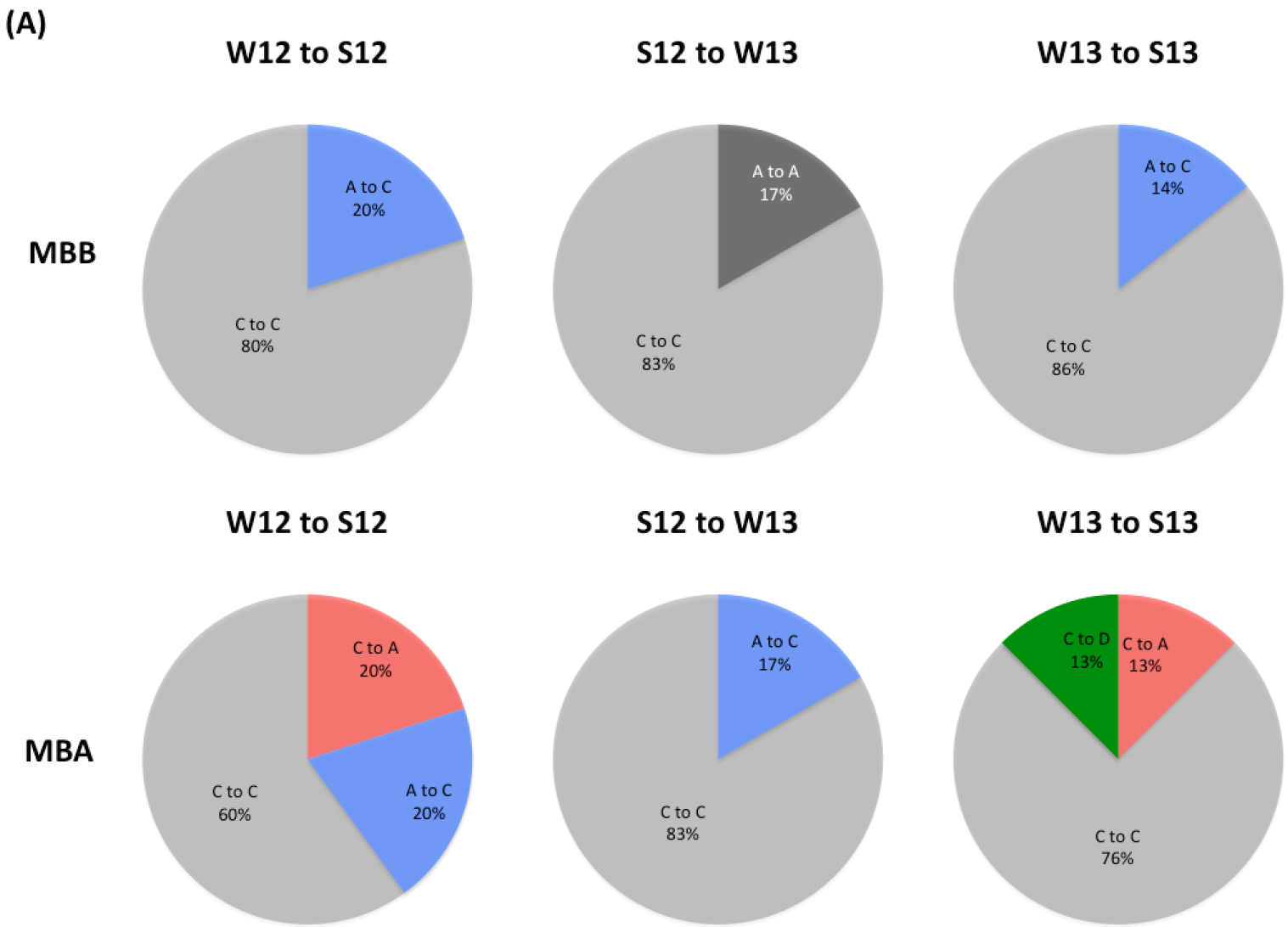

Figure 5. *Montastraea cavernosa* zooxanthellae Transitions. Transitions in zooxanthellae subclade type occurring within the same

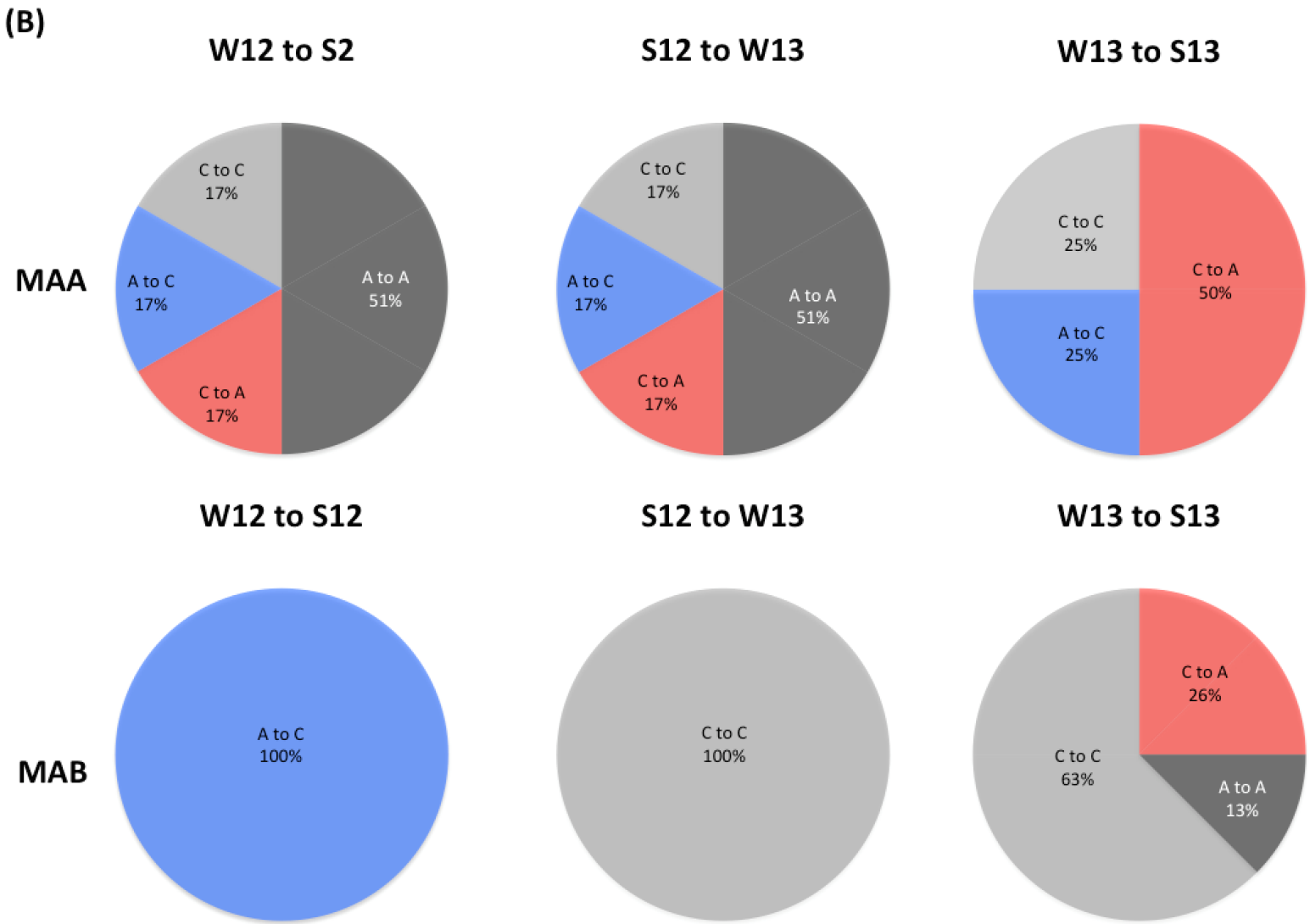

Figure 5 (cont'd). coral fragment between sampling times for all fragments of *Montastraea cavernosa* zooxanthellae. Pie charts

Figure 5 (cont'd). represent the relative frequencies of individual transitions of clade subtypes through each field season. Graphs are labeled with acronyms describing the (1) coral species, (2) field site of original collection (i.e. Acer24 or Birthday reefs) and (3) site of transplant (i.e. Acer24 or Birthday reefs). For example, MBA represents samples from *M. cavernosa* fragments collected at Birthday reef and transplanted to Acer24 reef. Color scales are determined by the outcome of the transition**.** For example, slices represented in shades of red describe transitions that resulted in subtypes belonging to clade A zooxanthellae, transitions to C in blue, D in green. Transitions that stayed the same are represented by gray. All transitions resulting in the same subtype are represented by the same color, regardless of the original zooxanthellae subtype. Field seasons are abbreviated above each graph with "W" for Winter and "S" for Summer. Years correspond to 2012 or 2013.

Montastraea cavernosa collected from Acer24 Reef (MAA and MAB)

A total of 52 sequences were recovered from transplant and non-transplant *Montastraea cavernosa* originating from Acer24 reef. These sequences were representative of seven different subclade types, including A3, A4, A4.1, A4.2, A4.3, C1 and C3. Again, although detailed here, subclade types were binned by overall clade for analysis and outlined by clade in corresponding figures (Figure 4B).

According to the mutinomial model frequencies of clade types in MAA and MAB samples, experimental treatment (transplant *vs*. non- transplant) did not have a significant effect on clade frequencies ($p>0.05$). In addition, the interaction term between experimental treatment and sampling time was not significant ($p>0.25$). When comparing AIC values between the full model and those of the individual independent variables, the model containing only sampling time provided the best fit to the data and was used for further analysis. Across all samples, the Winter 2013 sampling time was associated with a significant increase in the probability of clade type C ($p=0.003$) (Figure 4B).

All but one MAA sample transitioned to a different subclade type over the sampling times (Figure 5B). All but two MAB samples transitioned to a different subclade type over the sampling times. Interestingly, there were multiple occurrences of A to C, or C to A subclade type

shifts in both MAA and MAB samples.

DISCUSSION

Since the 1997/98 El Niño event coral cover on offshore reefs in the Florida Keys Reef Tract have remained at low levels ≤5% (Ruzicka *et al.*, 2013). Prior studies have demonstrated differences in the ability of certain coral assemblages to endure stress. Kenkel et al. (2013) found that coral assemblages from inshore reefs exhibited higher thermotolerance than their offshore conspecifics. Additionally, Haslun *et al.* (In Review) found lower incidences of chronic bleaching inshore relative to assemblages from offshore. Zooxanthellae subclade type may be an important driver of holobiont stress tolerance (Sampayo *et al.*, 2008). In this study, I investigated whether observable patterns of change in the abundances of dominant zooxanthellae subclade types from fragments of *Porites astreoides* and *Montastraea cavernosa* provide information regarding inshore and offshore reef coral stress tolerance.

Porites astreoides

Subclade type A4.1 was the predominant subclade type among fragments collected at Birthday Reef (Figure 2A). Haslun *et al.* (In Review) proposed that due to moderate temperatures and low irradiance, coral inhabiting the inshore (Birthday) reef experience less stress than their conspecifics at my offshore (Acer24) reef. Temperatures at Birthday reef during summer months were within the mean summer maximum for this reef. These similar temperatures associated with presumed low-level irradiance are likely responsible for the relatively stable associations at Birthday reef compared to assemblages from Acer24 reef that demonstrated fewer transitions. Additionally, associations at Birthday reef consisted mostly of

subclade type A4.1 and A4.3. Further studies will be needed to determine if the prominence of subclade type A4.1 found at the inshore site compared to the offshore site is related to a lower stress environment.

According to Haslun *et al.* (In Review), coral offshore experience higher bleaching than coral at the inshore site presumably due to higher irradiance. This implies that coral fragments transplanted from Birthday Reef to the offshore Acer24 reef (PBA) may experience more stress than coral fragments that remained inshore (PBB). As a consequence, I expected a transition in subclade types in PBA fragments to more stress tolerant subclades. Yet there is no significant difference in the probabilities of occurrence for individual subclade types between transplanted and non-transplanted *P. astreoides* fragments from Birthday reef ($p>0.05$). In fact, composition of zooxanthellae from PBB and PBA samples are very similar, indicating a lack of acclimatization to a presumed higher stress environment. The lack of a transition in subclade abundance may indicate that *Porites astreoides* fragments originating from the inshore site do not possess a *Symbiodinium* spp. subclade type adapted to higher irradiance.

P. astreoides fragments collected at the offshore site, I observed zooxanthellae subclade type A4 in fragments of *P. astreoides* solely during the Summer of 2012 sampling time (Figure 2B). Further, subclade type A4 was never observed in *P.* astreoides collected at the inshore site, indicating that this subclade type may originate from and confer a specific advantage to environmental parameters experienced offshore. Summer 2012 was the warmer of the two summer sampling times (Figure 6). The combination of higher irradiance levels characteristic of summer and higher temperatures may have favored the higher relative abundance of subclade type A4 during the summer of 2012 relative to the summer of 2013 offshore.

Porites astreoides is known to contain multiple subclade types in addition to members of A4 (LaJeunesse, 2002). However, little variability in subclade type abundance was found in this study as conspecifics of *P. astreoides* displayed subclade types A4, A4.1, A4.2 and A4.3. This lack of variability in zooxanthellae subclade type is common in corals that reproduce *via* brooding as their zooxanthellae are acquired *via* vertical transmission (Van Oppen, 2004). The long-term coevolution of the mutualism between the coral host and zooxanthellae likely leads to a tight symbiosis, with little flexibility, and may explain the lack of marked changes in subclade type abundances observed in this study (Thornhill *et al.*, 2006).

The altering of zooxanthellae subclade types, here demonstrated *via* transitions, is one mechanism that can alter the relative abundance of subclade types (Kinzie *et al.*, 2001; Rowan, 2004). Less than 50% of zooxanthellae subclade types in fragments originating from the inshore reef displayed transitions (Figure 3A), demonstrating a relatively stable association even at the higher stress reef. In contrast, transitions between subclade types were much more frequent for coral fragments originating from the offshore reef (Figure 3B). Retention of zooxanthellae composition and diversity even though transferred to new sites indicates a lack of acclimation to changing stress regimes, particularly notable after a lengthy transplant period of two years.

Montastraea cavernosa

Fragments of *M. cavernosa* collected from the inshore reef contained zooxanthellae subclade types representative of clades A, C and D (Figure 4A). To my knowledge this is the first time that subclade types of clade A have been found in association with *M. cavernosa* in this region. However, members of clade A are frequnetly isolated from species within the genus

Montastraea (Garren *et al.*, 2006). Clade A is also a common zooxanthellae type in this region, and is common in *P. astreoides* for example.

Subclade type C3 is commonly associated with *M. cavernosa* (LaJeunesse, 2002; Banaszak, 2007; Serrano *et al.*, 2014) and has been described as a pandemic host generalist commonly associated with *M. cavernosa* in times of low stress (Lajeunesse, 2005). The probability of finding clade type C at the inshore reef was significantly higher than offshore and likely reflects the lower stress environmental conditions inshore and the generalist nature of clade type C. A notable exception in the subclade type abundances found in MBA samples (Figure 4A) was the presence of a D1 subclade type in the Summer of 2013. Although this subclade type was only present in one fragment and at one sampling time, clade D has many implications for holobiont thermotolerance. For instance, the dominance of clade D subclade types relative to other subclade types during stress events has been demonstrated repeatedly in the literature (Baker, 2001; Thornhill *et al.*, 2006; Silverstein *et al.*, 2015), leading to the belief that clade D subclade types confer a specific advantage to holobionts during times of stress. Ulstrup and Van Oppen (2003) found that subclade types of clade D may be present in and acquired from the surrounding environment, but may also exist at low concentrations within the host. Subclade types of clade D become more prevalent when environmental stress increases due to elevated temperatures or changes in turbidity. This confers resistance to stressful conditions (Berkelmans & van Oppen, 2006) and may explain why inshore coral have been found to be more thermotolerant than offshore conspecifics.

In contrast to the brooder *Porites astreoides*, *Montastraea cavernosa* is a broadcast spawner, whose gametes lack zooxanthellae. Thus, they acquire symbionts *via* horizontal transmission, from the surrounding environment (Van Oppen, 2004), as their eggs are void of

symbiont cells (Szmant, 1991). This ability to acquire zooxanthellae from the surrounding environment may also explain the presence of clade type A in *M. cavernosa* samples. Compared to brooders, broadcast spawners are thought to rely more heavily on zooxanthellae as a mechanism for surviving temperature stress as they possess the ability to switch and shuffle their zooxanthellae (Silverstein *et al.*, 2015). While I did not attempt to identify the origin of zooxanthellae clade types, the presence of D1 in MBA samples from Summer 2013 suggest stressful conditions during this sampling time, likely due to increases in irradiance demonstrated by Haslun *et al.* (In Review) and as a means to counteract this stress by the holobiont.

Fragments of *Montastraea cavernosa* collected from the offshore site contained zooxanthellae subclade types representative of clades A and C. Similarly to fragments of *Porites astreoides*, subclade type A4 was isolated only from *M. cavernosa* fragments from the offshore site, supporting the notion that subclade type A4 may be endemic to offshore sites. Symbionts from clade C, however, were the predominant clade type observed in *M.* cavernosa fragments. The winter of 2013 was warmer, and therefore milder, than winter of 2012 (Figure 6). This mild winter, combined with the generalist nature of subtype C3 mentioned earlier, may explain the significant increase in clade C displayed by *M. cavernosa* fragments collected from the offshore reef at that time.

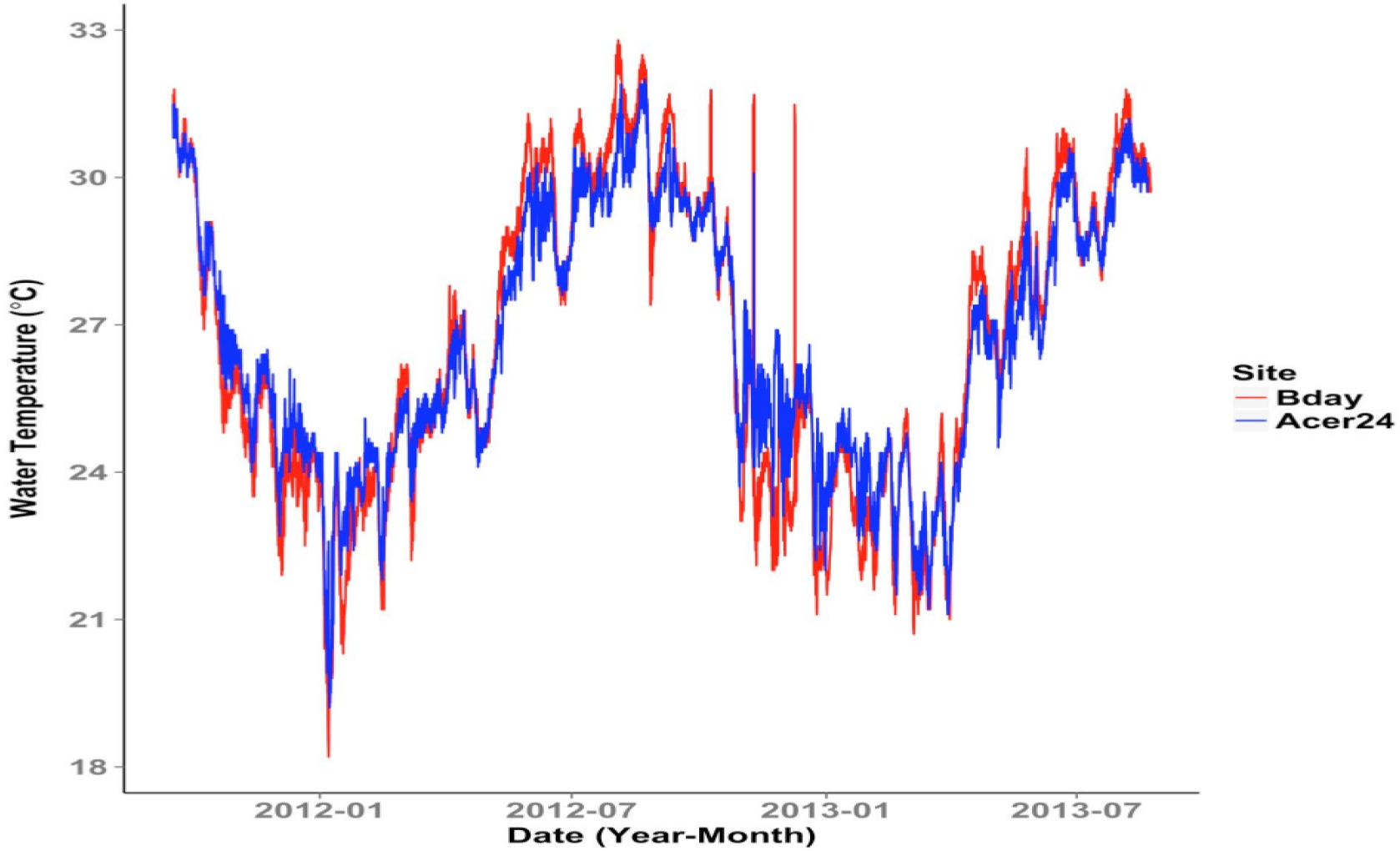

Figure 6. Temperatures at Birthday and Acer24 reefs. Temperature data for inshore (Birthday) and offshore (Acer24) reefs. Temperatures were taken every 10 minutes with HOBO™ tags placed on each reef site

Transitions between zooxanthellae clade types, rather than subclade types, are significant as individual clades can differ drastically physiologically. We observed multiple instances of transitions between subclade types A and C in *M. cavernosa* fragments collected at the inshore and offshore reef (Figure 5). Transitions between clade types are commonly reported in *M. cavernosa* (Ulstrup & Van Oppen, 2003; Thornhill *et al.*, 2006; Silverstein *et al.*, 2015) and are likely due to their reproductive strategy and ability to acquire symbionts from the surrounding environment. Similar to trends observed in *P. astreoides*, fragments of *M. cavernosa* collected inshore displayed fewer transitions, and therefore more stability, compared to fragments of *M. cavernosa* collected offshore. Additionally, similar transition frequencies, as well as zooxanthellae composition, within samples from the same collection site suggest a lack of acclimatization to the new environmental conditions present at the transplant site.

In response to global climate change, many researchers are focused on finding particular coral assemblages that are better adept at surviving a myriad of environmental stresses that climate warming will impose. My study found significant differences in subclade type frequencies between inshore and offshore *P. astreoides* and *M. cavernosa*, suggesting a mechanistic role of zooxanthellae subclade type in their thermotolerance variation. It also showed a site-dependent effect of seasonal zooxanthellae compositional changes suggesting a lack of acclimatization by zooxanthellae to the new environment. This observation has important implications for the choice of which coral populations to target for conservation or repopulation efforts. While these findings are the first step in identifying specific holobiont populations best equipped to survive stressors associated with global climate change, future studies will be required to determine differences in these subclade types at the functional genetic

level, as well as the response of these subclade types to bleaching temperatures and other local synergistic stress.

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LITERATURE CITED

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CHAPTER 2

SITE-SPECIFIC VARIATION IN GENE EXPRESSION FROM *SYMBIODINIUM* SPP. ASSOCIATED WITH OFFSHORE AND INSHORE *PORITES ASTREOIDES* IN THE LOWER FLORIDA KEYS

ABSTRACT

Scleractinian coral are experiencing unprecedented rates of mortality due to increases in sea surface temperatures in response to global climate change. Some coral species however, survive high temperature events due to a reduced susceptibility to bleaching. I investigated the relationship between bleaching susceptibility and expression of five metabolically related genes of *Symbiodinium* spp. from the coral *Porites astreoides* originating from an inshore and offshore reef in the Florida Keys. The acclimatization potential of *Symbiodinium* spp. to changing temperature regimes was also measured *via* a two-year reciprocal transplant between the sites. Offshore coral fragments displayed significantly higher expression in *Symbiodinium* spp. genes *PCNA, SCP2, G3PDH, PCP* and *psaE* than their inshore counterparts ($p<0.05$), a pattern consistent with increased bleaching susceptibility in offshore corals. Additionally, gene expression patterns in *Symbiodinium* spp. from site of origin were conserved throughout the two-year reciprocal transplant, indicating acclimatization did not occur. Gene expression reported here indicates functional variation in populations of *Symbiodinium* spp. associated with *P. astreoides* in the Florida Keys, and is likely a result of localized adaptation.

INTRODUCTION

Over the last 30-40 years, a decline in global coral populations as high as 50% has been recorded and linked to coral bleaching (Bruno *et al.*, 2007; De'ath *et al.*, 2012; Jackson *et al.*, 2014). Bleaching is a response to elevated seawater temperatures and high irradiance, and results

when the density of symbiotic algae (*Symbiodinium* spp. commonly referred to as zooxanthellae) declines severely in the host coral allowing the white skeleton below to show (Gates *et al.*, 1992). Considering that coral hosts derive up to 95% of their daily metabolic needs from zooxanthellae (Muscatine *et al.*, 1989), the loss of symbionts compromises the host during a bleaching event. Depending on the extent and duration of a bleaching event, loss of zooxanthellae may lead to coral host mortality (Brown, 1997). Given the importance of the host-zooxanthellae symbiosis, plasticity in algal response to stress is an important area of coral research (Baker, 2003).

Coral susceptibility to bleaching is influenced by the *Symbiodinium* spp. clade type harbored by the host (Rowan, 2004). For example, Sampayo *et al.* (2008) demonstrated that bleaching susceptibility of *Stylophora pistillata* colonies was determined by compositional differences at the subclade level within *Symbiodinium* spp. of clade C. In the Florida Keys, several studies found variation in bleaching susceptibility between inshore and offshore communities of *P. astreoides* (Kenkel *et al.*, 2013, 2015; Haslun *et al.*, In Review). The observation of variation in subclades between proximate inshore and offshore reefs prompts questions of the role of genetically associated functional variation in zooxanthellae to bleaching susceptibility (Hauff *et al.* In Press). However, the role of zooxanthellae in bleaching susceptibility variation in these coral is unknown (Kenkel *et al.*, 2015).

Changes in metabolic processes as markers of bleaching susceptibility have a long-standing history in the study of zooxanthellae response to stress (Szmant & Gassman, 1990; Jones & Hoegh-Guldberg, 2001; Rodrigues & Grottoli, 2007). Changes in metabolism are commonly assessed *via* gene expression analysis, which targets genes related to the production of metabolically important proteins. Common examples include, proliferating cell nuclear

antigen (*PCNA*), a protein involved in DNA synthesis and replication (Prelich *et al.*, 1987), non-specific lipid-transfer protein (*SCP2*), which mediates the transfer of all common lipids (Thoma & Kaneko, 1991), Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), an enzyme involved in glycolysis (Sirover, 1997), peridinin chlorophyll alpha binding protein I chloroplastic (*PCP*) and photosystem I reaction center subunit IV (*psaE*). *PCP* is involved in the capture of solar energy (Norris & Miller, 1994) and *psaE* stabilizes interactions within the photosystem 1 complex (Bengis & Nelson, 1977). Variation in the expression of these metabolic genes affords functional variability to zooxanthellae and be involved in acclimatization to bleaching conditions (Rosic *et al.*, 2010, 2011a; Leggat *et al.*, 2011).

We studied the role of zooxanthellae in bleaching susceptibility of *P.* astreoides by evaluating symbiont gene expression in reciprocal transplants of *P.* astreoides in the Florida Keys. The expression of zooxanthellae genes *PCNA, SCP2, G3PDH, PCP* and *psaE* was measured in coral sub-samples from inshore and offshore reefs differing in thermal and irradiance regimes. The offshore Acer24 reef experiences lower temperatures and higher irradiance than the inshore counterpart, Birthday reef. Differences in patterns of gene expression in the reciprocal transplant allowed us to evaluate the role of these genes in acclimatization to environmental stress.

METHODS

In this study, *Symbiodinium* spp. from *Porites astreoides* reciprocally transplanted from an inshore and offshore site in the lower Florida Keys were analyzed for changes in gene expression over two years. Study sites, coral collection, reciprocal transplant design and sample collection for analyses were completed as outlined in Hauff *et al.* (In Press). Briefly, in

September 2011, ten 16 cm^2 fragments of *Porites astreoides* were collected from Birthday reef (inshore: 24.57917N -81.49693W) and an additional ten 16 cm^2 fragments were obtained from Acer24 reef (offshore: 24.55268N -81.43741W). Birthday reef and Acer24 reef are patch reefs separated by Hawk Channel and have notably distinct temperature and turbidity regimes, but are otherwise similar (i.e. depth, species diversity). Relative to Acer24 reef, Birthday reef has higher turbidity, lower light and higher annual average temperatures (~1°C) (Haslun *et al.*, In Review, Erich Bartels, pers.com.).

The 20 *Porites astreoides* fragments taken from Birthday reef and Acer24 reef were sectioned into nearly equal halves. These small fragments were approximately 8 cm^2 each. One small fragment was placed at the site of origin and the other transplanted to the alternate site (i.e. fragments from offshore transplanted to inshore site) (n=40). Small coral fragments remained at each site for a total of two years, but were sub-sampled biannually (i.e. February and August of 2012 and 2013) to capture gene expression during winter and summer conditions. At each sub-sampling time, 1 cm^2 sub-samples were taken with hammer and chisel and flash frozen in liquid nitrogen within 15 minutes of initial collection and stored at -80°C.

Sub-samples from six of the small coral fragments from each treatment within the reciprocal transplant experiment were randomly chosen for qRT-PCR analysis. For example, six small fragments of *P. astreoides* collected and replaced at Birthday Reef and sub-sampled over the four sampling times were analyzed and six small fragments of *P. astreoides* collected at Birthday Reef, transplanted to Acer24 reef, and sampled over the four sampling times, were analyzed. Sample analysis for sub-samples originating from Acer24 reef was the same. This sampling design yielded a total of n=96 sub-samples.

RNA extraction and cDNA preparation

Sub-samples of *P. astreoides* were stored at -80°C and crushed to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. Between samples, mortar and pestles were cleaned with liquid detergent and RNase AWAY® (Sigma-Aldrich, St. Louis MO, USA) and rinsed in ultrapure water.

TRI Reagent® (Sigma-Aldrich, St. Louis MO, USA) was used to extract RNA from 0.1 g coral powder following a modified manufacturer's protocol. Extracted RNA was quantified and two-50 µL samples were stored at -80°C. Prior to reverse transcription, samples of RNA were diluted to 5 ng/µL and examined on a bioanalyzer to check for RNA integrity and re-extracted if necessary. Once proper integrity was obtained, 2.5 ng/µL of RNA was treated with DNase I (Life Technologies, San Antonio TX, USA) and reverse transcribed into cDNA following manufacturer instructions using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies, San Antonio TX, USA).

qRT-PCR

Fully transcribed cDNA was diluted 1:16 for qRT-PCR. Reaction volumes for qRT-PCR were 10 µL and as follows: 5 µL Power SYBR® Green PCR Master Mix, 3.5 µL H_2O, 1µL cDNA, 0.5µL primer. Cycling conditions for all reactions were: 50°C-2 minutes (1x), 95°C-10 minutes (1x), 95°C-15 seconds and 60°C-1 minute (40x). Primer information for all genes can be found in Table 1.

Gene	Abbreviation	Function
Cytochrome Oxidase Subunit 1	Cox	Housekeeping Gene
Proliferating Cell Nuclear Antigen	PCNA	DNA Synthesis
Non-Specific Lipid-Transfer Protein	SCP2	Lipid Transfer
Glyceraldehyde-3-Phosphate Dehydrogenase	G3PDH	Metabolism
Peridinin Chlorophyll α-Binding Protein 1	PCP	Photosynthesis
Photosystem I Reaction Center Subunit IV	psaE	Photosynthesis

	Forward Primer		Reverse Primer		
Gene	Sequence (5'-3')	Melting Temp (°C)	Sequence (5'-3')	Melting Temp (°C)	Amplicon Length (bp)
Cox	GGTCATTTCCATAATAATTTCTGGTGTT	60	CAAGACCTCCAAGAAGAGAAATAGATG	59	101
PCNA	GGACGTGGTGAACTTCCAGT	60	CCACCTTGTAGTGCTGCTCA	60	116
SCP2	CCAGAAGCTCCAGTCCATTC	59.8	GAGTTGCCCGGATTACACAT	59.8	110
G3PD	ACGACAAGGCCAACCATAAC	59.9	AGGAGTGGATGGTGGTCATC	59.9	129
PCP	GACTGGACCTCCGACGTTTA	60.1	GTCCATTGAAGCACCCATCT	59.9	99
psaE	ACCTGGTGAGGTTTGAATGG	59.8	CGAAGCCAAGTACAGGAAGG	59.9	122

Table 1. Genes used in this study, abbreviations, function, primer sequences, melting temperatures and amplicon length for primers used for qRT-PCR.

In this study, six zooxanthellae-specific genes were analyzed across all sub-samples, and include one housekeeping gene and five experimental genes (Table 1). The housekeeping gene, cytochrome oxidase subunit 1 (*Cox*) has previously been identified as a viable housekeeping gene in *Symbiodinium* spp. (Rosic *et al.*, 2011b). Technical replicates were run in duplicate and negative controls were run to confirm no genomic DNA contamination for a total of n=1,152 reactions. All analyses were completed at the Research Technology Support Facility at Michigan State University on an Applied Biosystems® Prism 7900HT (Thermo Scientific, New York, NY USA).

Data Analysis

All analyses were performed in R (v3.1.3) using the specialized package *MCMC.qpcr* (Matz *et al.*, 2013; R Core Team 2014). In this analysis, raw qRT-PCR data (i.e. Ct values) is represented as molecule counts and described under a Poisson-lognormal error using generalized linear mixed models. There are numerous benefits to this approach. It is fully flexible for all levels of random and fixed effects, enables evaluation of unlimited interactions, increases power *via* simultaneous analysis of all genes in one model, accounts for low amplification targets by using molecule counts, and eliminates the need for control genes (Matz *et al.*, 2013). For this study, however, a control gene was used to balance conservatism and the risk of bias, as suggested by Matz *et al.* (2013).

For analysis, a two-way model was fit using "treatment" (i.e. transplant versus non-transplant) and "time" (i.e. winter and summer) as fixed factors, as well as their interaction, "treatment:time". This resulted in a model describing the individual effect of each factor, as well as the effect of their interaction (i.e. the treatment dependent effect of sampling time). Two models were run in order to report all relevant comparisons. The first model used fragments

originating from, and replaced at, Acer24 reef as a baseline comparison, with the second using fragments originating and replaced at Birthday reef for baseline comparison. Additionally, the model was "informed" using qRT-PCR data generated here from one previously established control gene (*Cox*, Table 1, Rosic *et al.*, 2011b). Diagnostic plots using the function diagnostic.mcmc() were analyzed to confirm linear modeling as an appropriate application for this data set.

RESULTS

There were no significant effects in gene expression between winter and summer observed, however, significant differences in zooxanthellae gene expression were found between sub-samples from Acer24 and Birthday reefs ($p>0.05$). Overall, gene expression from zooxanthellae was higher in algae from Acer24 reef than those present at Birthday reef. Gene expression patterns observed in zooxanthellae from the original site were retained in the symbiont throughout the transplant period for both collection reefs. For example, *Symbiodinium* spp. from Acer24 reef that were transplanted to Birthday reef displayed gene expression patterns similar to zooxanthellae that were from, and held, at Acer24 reef.

Several genes from zooxanthellae harbored in *P. astreoides* sub-samples originating from Birthday reef exhibited a significant down regulation relative to symbionts originating from Acer24 reef. These included *PCP* ($p=0.002$), *PCNA* ($p=<0.001$) and *psaE* ($p=0.044$) and *G3PDH* ($p=0.052$) (Figure 7, Table 2). Relative to zooxanthellae from sub-samples originating at Acer24 reef, *Symbiodinium* spp. originating from Birthday reef and transplanted to Acer24 reef exhibited significant down-regulation of the zooxanthellar genes *PCP* ($p<0.001$), *PCNA* ($p<0.001$), *psaE* ($p=0.018$), and *G3PDH* ($p=0.024$)(Figure 7, Table 2). Relative to zooxanthellae originating at

Birthday reef, *Symbiodinium* spp. originating at Acer24 reef and transplanted to Birthday reef exhibited significant up-regulation of zooxanthellar genes *PCP* ($p<0.001$), *PCNA* ($p<0.001$), *psaE* ($p=0.002$), *G3PDH* ($p<0.001$) and *SCP2* ($p=0.028$)(Figure 7, Table 3).

Sample	Gene	Post.mean	l-95% CI	u-95% CI	Eff.samp	pMCMC	
AcerBirthday	G3PD	0.75786	-0.32954	1.81690	1100.1	0.182	
AcerBirthday	PCP	0.57277	-0.65011	1.58568	1000.0	0.334	
AcerBirthday	PCNA	0.45110	-0.85448	1.74646	1000.0	0.472	
AcerBirthday	PSAE	0.82805	-0.24776	1.92126	1095.1	0.146	
AcerBirthday	SCP2	0.51517	-0.62875	1.57658	1140.2	0.368	
AcerBirthday	Cox	0.29714	-0.81822	1.34287	1000.0	0.598	
BirthdayAcer	G3PD	-1.27444	-2.32264	-0.17412	1000.0	0.024	*
BirthdayAcer	PCP	-1.99936	-3.00025	-0.71156	1000.0	<0.001	*
BirthdayAcer	PCNA	-2.41345	-3.72389	-1.14470	1000.0	<0.001	*
BirthdayAcer	PSAE	-1.33718	-2.43305	-0.23223	1000.0	0.018	*
BirthdayAcer	SCP2	-1.04736	-2.08630	0.05666	1000.0	0.072	
BirthdayAcer	Cox	-1.15290	-2.32132	-0.11005	1000.0	0.056	
BirthdayBirthday	G3PD	-1.03375	-2.11422	-0.03793	1024.5	0.052	
BirthdayBirthday	PCP	-1.79766	-2.94654	-0.74262	1122.8	0.002	*
BirthdayBirthday	PCNA	-2.49322	-3.63201	-1.13299	1000.0	<0.001	*
BirthdayBirthday	PSAE	-1.05099	-2.07456	-0.01014	1063.0	0.044	*
BirthdayBirthday	SCP2	-0.80628	-1.87348	0.26407	1046.7	0.156	
BirthdayBirthday	Cox	-0.86286	-1.96599	0.21116	1034.0	0.140	
Summer	G3PD	-0.42471	-1.44246	0.57653	1000.0	0.400	
Summer	PCP	-0.96875	-2.08771	0.07127	1000.0	0.076	
Summer	PCNA	-0.90811	-2.04225	0.42268	1000.0	0.136	
Summer	PSAE	-0.53284	-1.49807	0.53535	1000.0	0.298	
Summer	SCP2	-0.50555	-1.67745	0.41615	1000.0	0.336	
Summer	Cox	-0.66882	-1.56163	0.42765	1000.0	0.176	
AcerBirthday:Summer	G3PD	-1.10678	-2.53604	0.23038	1000.0	0.146	
AcerBirthday:Summer	PCP	-0.71755	-2.36609	0.71800	1000.0	0.366	
AcerBirthday:Summer	PCNA	-0.42148	-2.29178	1.34468	1102.2	0.660	
AcerBirthday:Summer	PSAE	-0.88605	-2.41830	0.49638	1000.0	0.258	
AcerBirthday:Summer	SCP2	-0.73566	-2.13112	0.78400	1000.0	0.346	
AcerBirthday:Summer	Cox	-0.15287	-1.43671	1.36536	1000.0	0.860	
BirthdayAcer:Summer	G3PD	0.48370	-1.05469	1.93171	1000.0	0.528	
BirthdayAcer:Summer	PCP	1.17241	-0.36614	2.84003	1000.0	0.162	
BirthdayAcer:Summer	PCNA	-0.44314	-2.25117	1.56216	1000.0	0.648	
BirthdayAcer:Summer	PSAE	0.51274	-1.06876	2.00907	1043.4	0.506	
BirthdayAcer:Summer	SCP2	0.24851	-1.14452	1.85562	1000.0	0.748	
BirthdayAcer:Summer	Cox	0.35344	-1.07449	1.79065	1121.9	0.672	
BirthdayBirthday:Summer	G3PD	-0.11873	-1.52551	1.26020	1000.0	0.860	
BirthdayBirthday:Summer	PCP	0.28191	-1.21268	1.80808	1000.0	0.704	
BirthdayBirthday:Summer	PCNA	0.17796	-1.53111	2.15363	1000.0	0.822	
BirthdayBirthday:Summer	PSAE	-0.06285	-1.50997	1.28971	1000.0	0.940	
BirthdayBirthday:Summer	SCP2	-0.08662	-1.60147	1.34879	1000.0	0.900	
BirthdayBirthday:Summer	Cox	-0.16014	-1.51131	1.16904	1000.0	0.818	

Table 2. Output for two-way model using "time", "transplant" and their interaction as fixed factors under the MCMC.qpr package for zooxanthellae gene expression from subsamples collected at Birthday reef and Acer24 reef. Post means are reported as well as lower and upper credible intervals, effective sample size and p-values. "AcerAcer" represents non-transplanted

Table 2 (cont'd). sub-samples that originated at Acer24 reef, whereas "AcerBirthday" represents transplanted sub-samples that were collected at Acer24 reef and transplanted to Birthday reef. "BirthdayBirthday" represents non-transplanted sub-samples that originated at Birthday reef, whereas "BirthdayAcer" represents transplanted sub-samples that were collected at Birthday reef and transplanted to Acer24 reef. Baseline comparison, or reference factor, included non-transplanted sub-samples from Acer24 reef (i.e. AcerAcer) at the winter sampling time. Asterisk denotes a p-value <0.05.

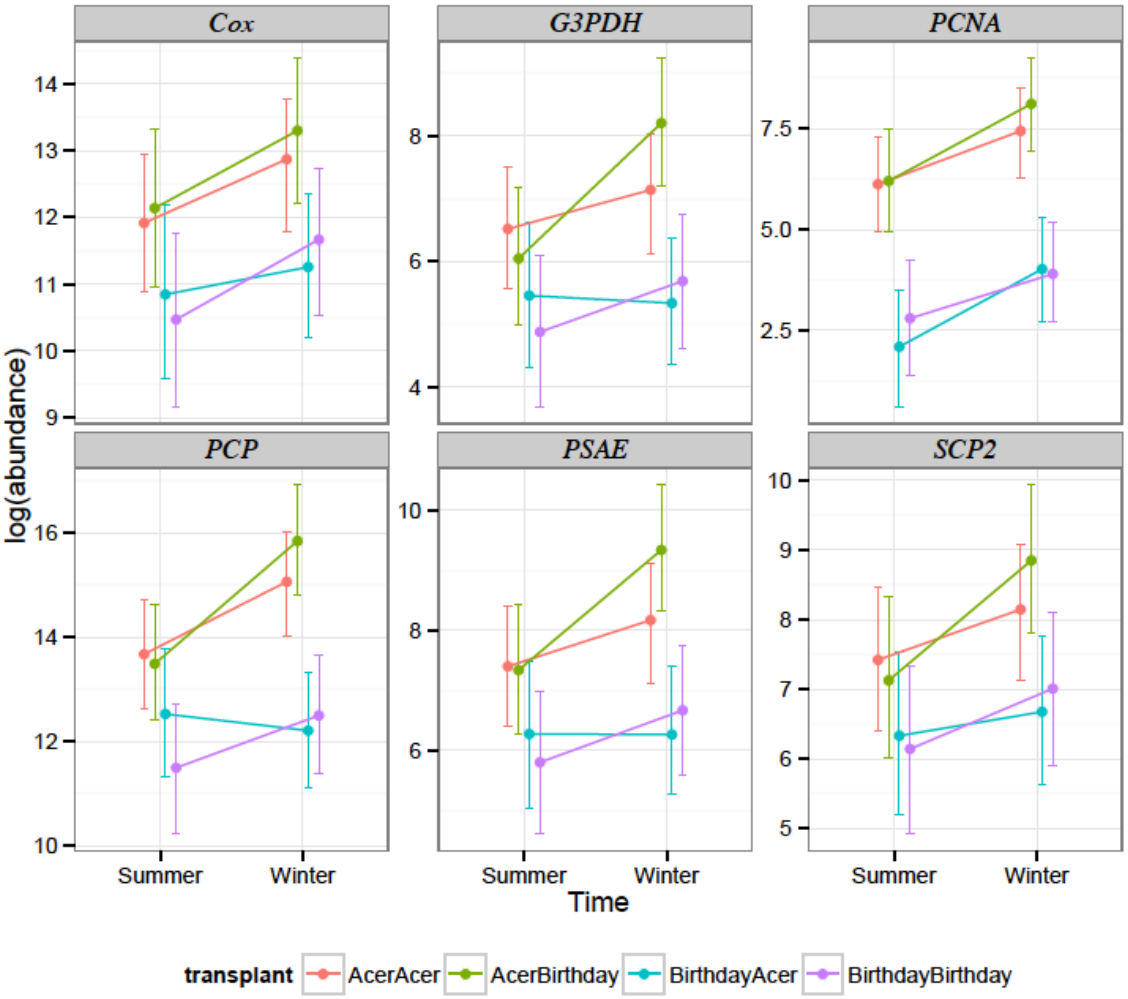

Figure 7. By-gene plot of transcript abundance for zooxanthellae genes obtained from sub-samples of *Porites* astreoides taken from Birthday reef and Acer24 reef during winter and summer sampling efforts. "AcerAcer" represents non-transplanted sub-samples that originated at Acer24 reef, whereas "AcerBirthday" represents transplanted sub-samples that were collected at Acer24 reef and transplanted to Birthday reef. "BirthdayBirthday" represents non-transplanted sub-samples that originated at Birthday reef, whereas "BirthdayAcer" represents transplanted sub-samples that were collected at Birthday reef and transplanted to Acer24 reef. Sub-samples collected in summer months are represented in orange, while sub-samples collected in winter months are represented in blue. Whiskers denote 95% credible intervals.

In contrast to the cases above, no significant effect of site was found when comparing zooxanthellae gene expression from sub-samples originating at Acer24 reef and zooxanthellae originating from Acer24 reef sub-samples transplanted to Birthday reef ($p>0.05$, Table 2). Furthermore, gene expression did not vary significantly between zooxanthellae originating from Birthday reef sub-samples and zooxanthellae originating from Birthday reef sub-samples that were transplanted to Acer24 reef (Table 3).

Sample	Gene	Post.mean	l-95% CI	u-95% CI	Eff.samp	pMCMC	
AcerAcer	G3PD	1.21755	0.17023	2.20626	1000.0	0.022	*
AcerAcer	PCP	1.99231	0.96816	3.19196	1000.0	<0.001	*
AcerAcer	PCNA	2.72957	1.49594	3.84646	1000.0	<0.001	*
AcerAcer	PSAE	1.25442	0.20186	2.25162	1000.0	0.022	*
AcerAcer	SCP2	1.00775	-0.02516	2.11803	1000.0	0.080	
AcerAcer	Cox	1.09524	0.05914	2.19882	1000.0	0.056	
AcerBirthday	G3PD	1.64635	0.54220	2.66718	1000.0	<0.001	*
AcerBirthday	PCP	2.22900	1.03429	3.28904	1000.0	<0.001	*
AcerBirthday	PCNA	2.83415	1.54539	4.01819	1000.0	<0.001	*
AcerBirthday	PSAE	1.74730	0.65014	2.81450	1000.0	0.002	*
AcerBirthday	SCP2	1.18808	0.16333	2.30082	1000.0	0.028	*
AcerBirthday	Cox	0.98921	-0.13981	2.08998	909.6	0.094	
BirthdayAcer	G3PD	-0.31557	-1.38858	0.74282	1092.6	0.580	
BirthdayAcer	PCP	-0.27211	-1.36499	0.85086	1000.0	0.650	
BirthdayAcer	PCNA	0.04449	-1.31269	1.16807	1136.6	0.934	
BirthdayAcer	PSAE	-0.35742	-1.51305	0.65777	943.5	0.518	
BirthdayAcer	SCP2	-0.30484	-1.42683	0.71932	1113.7	0.600	
BirthdayAcer	Cox	-0.41275	-1.52753	0.68653	1098.6	0.444	
Summer	G3PD	-0.42119	-1.38320	0.59371	1000.0	0.410	
Summer	PCP	-0.56929	-1.60197	0.51766	1000.0	0.300	
Summer	PCNA	-0.50935	-1.64950	0.90378	1110.9	0.414	
Summer	PSAE	-0.47024	-1.42073	0.61740	1107.8	0.370	
Summer	SCP2	-0.46287	-1.44652	0.61586	1000.0	0.398	
Summer	Cox	-0.69225	-1.64933	0.33131	1000.0	0.166	
AcerAcer:Summer	G3PD	-0.27623	-1.60932	1.10719	933.0	0.716	
AcerAcer:Summer	PCP	-0.67608	-2.14837	0.73583	888.2	0.388	
AcerAcer:Summer	PCNA	-0.68121	-2.33178	0.95846	1000.0	0.446	
AcerAcer:Summer	PSAE	-0.33763	-1.68846	1.13446	921.6	0.654	
AcerAcer:Summer	SCP2	-0.32461	-1.65484	1.17265	887.0	0.652	
AcerAcer:Summer	Cox	-0.31844	-1.64613	1.04231	959.7	0.658	
AcerBirthday:Summer	G3PD	-0.98030	-2.38653	0.45556	1000.0	0.182	
AcerBirthday:Summer	PCP	-0.97558	-2.51798	0.46522	1000.0	0.208	
AcerBirthday:Summer	PCNA	-0.60709	-2.36205	1.04494	1000.0	0.502	
AcerBirthday:Summer	PSAE	-0.81838	-2.28514	0.60443	1000.0	0.272	
AcerBirthday:Summer	SCP2	-0.65107	-2.10597	0.78232	1000.0	0.390	
AcerBirthday:Summer	Cox	0.01370	-1.42340	1.33326	1000.0	0.986	
BirthdayAcer:Summer	G3PD	0.52894	-0.79296	1.88105	1150.7	0.456	
BirthdayAcer:Summer	PCP	0.82061	-0.72111	2.21444	1125.8	0.278	
BirthdayAcer:Summer	PCNA	-0.75939	-2.51604	0.91120	1146.2	0.406	
BirthdayAcer:Summer	PSAE	0.51246	-0.88945	1.90230	1126.0	0.480	
BirthdayAcer:Summer	SCP2	0.25853	-1.00056	1.84046	1000.0	0.738	
BirthdayAcer:Summer	Cox	0.46346	-0.91224	1.83777	1125.6	0.506	

Table 3. Output for two-way model using "time" and "transplant" as fixed factors under the MCMC.qpr package for zooxanthellae gene expression from sub-samples collected at Birthday reef and Acer24 reef. Post means are reported as well as lower and upper credible intervals, effective sample size and p-values. "AcerAcer" represents non-transplanted sub-samples that originated at Acer24 reef, whereas "AcerBirthday" represents transplanted sub-samples that were collected at Acer24 reef and transplanted to Birthday reef. "BirthdayBirthday" represents non-transplanted sub-samples that originated at Birthday reef, whereas "BirthdayAcer" represents transplanted sub-samples that were collected at Birthday reef and transplanted to Acer24 reef.

Table 3 (cont'd). Baseline comparison, or reference factor, included non-transplanted sub-samples from Birthday reef (i.e. BirthdayBirthday) at the winter sampling time. Asterisk denotes a p-value <0.05.

For all of the zooxanthellae genes tested throughout all experimental conditions, expression was higher in winter, compared to summer, but the effect of season was not significant (p>0.07, Figure 7, Tables 2 & 3). In zooxanthellae from Birthday reef sub-samples transplanted to Acer24 reef, *G3PDH* and *PCP* experienced slight decreased expression in winter compared to summer, while *psaE* experienced no change in expression. Finally, the interaction between time and transplant was not significant (p>0.1, Table 2 & 3).

DISCUSSION

Gene expression analysis is a powerful tool increasingly utilized to determine responses of closely related coral and zooxanthellae populations to stress (Rosic *et al.*, 2010, 2011a; McGinley *et al.*, 2012a; Kenkel *et al.*, 2014). Traditional techniques used in the study of stress responses rely on phenotypic variation and disregard differences at the genetic level (Berkelmans & van Oppen, 2006; Middlebrook *et al.*, 2008; Downs *et al.*, 2012). Molecular stress markers, such as differences in gene expression, provide unique advantages over visual assessments. For example, molecular markers measure changes in stress response prior to the advent of phenotypic change (DeSalvo *et al.*, 2008; Rosic *et al.*, 2011a; Kenkel *et al.*, 2013). Coral host populations of *Porites astreoides* exhibit differential gene expression that has been linked to thermal tolerance (Kenkel *et al.*, 2013). To understand the role of zooxanthellae in bleaching susceptibility I studied gene expression in *Symbiodinium* spp. from sub-samples of *P. astreoides* from an inshore and offshore reef, as well as a reciprocal transplant conducted at the same reefs. Results demonstrating increased gene expression in zooxanthellae from the offshore reef

experiencing more bleaching and no change in gene expression patterns in reciprocal transplants, allowed me to make inferences regarding the roles of zooxanthellae and acclimatization in bleaching susceptibility.

Differences in zooxanthellae gene expression between *P. astreoides* sub-samples from inshore and offshore sites are indicative of functional variability within zooxanthellae. Elevated gene responses and higher rates of bleaching at Acer24 reef relative to inshore may be a consequence of higher irradiance (Haslun *et al.*, In Review). Owing to the elevated coral-host stress response at Acer24 reef, I interpreted patterns of zooxanthellae gene expression exhibited in Acer24 reef sub-samples to reflect bleaching susceptibility at their cellular level. With the exception of *SCP2* in zooxanthellae from sub-samples originating and retained at Acer24 reef, four symbiont genes (*PCNA, G3PDH*, *PCP* and *psaE*) displayed significantly higher expression in all offshore Acer24 reef sub-samples compared to inshore Birthday reef sub-samples (Tables 2 & 3). These zooxanthellae genes are associated with metabolic processes that increase with stress, such as DNA synthesis, lipid transfer, metabolism and photosynthesis (Karako-Lampert *et al.*, 2005; Leggat *et al.*, 2011; McGinley *et al.*, 2012b). Consequently, increased zooxanthellae gene expression observed at Acer24 reef is likely a function of differential bleaching susceptibility experienced by the symbiont.

Although normally involved in glycolysis, *G3PDH* expression has been associated with apoptosis activation (Tarze *et al.*, 2007). Apoptosis, also called programmed cell death, occurs during the beginning stages of host coral bleaching (Strychar *et al.*, 2004a, 2004b). The possibility of increased zooxanthellae *G3PDH* expression signaling symbiont apoptosis and subsequent bleaching in coral originating at Acer24 reef is consistent with significantly higher levels of bleaching among host coral fragments originating from Acer24 reef relative to those

originating from Birthday reef (Haslun *et al.*, In review). As such, this is the first report to use zooxanthellae gene expression (i.e *G3PDH*) as a precursor of apoptosis to document bleaching; *G3PDH* may also be a better marker of symbiont stress then apoptosis. Documenting zooxanthellae gene expression is an important mechanism to better understand symbiosis and climate stress in coral. The ease of sampling and analysis broadens the scope of potential analyses, as well as our understanding of the role of zooxanthellae apoptosis in host bleaching. It is important to note that as zooxanthellar *G3PDH* expression increases, downstream activation of apoptotic processes are stimulated and expressed on the surface of the symbiont cells (Strychar *et al.*, 2004b). It is likely that the host then "evicts" the dead and dying symbionts, as these cells are recognized as problematic by the host's innate immune system (Strychar *et al.*, 2004a). If zooxanthellae are being actively expelled from the host *via* phagocytic cells, or other removal mechanisms, as indicated by increases in *G3PDH* expression, an increase in symbiont replication rates to counteract symbiont loss may explain increases in *PCNA* and *SCP2*.

Increased expression of *PCNA* and *SCP2* in zooxanthellae from Acer 24 reef suggests higher rates of DNA synthesis and lipid transfer/synthesis, processes that occur during cell replication and division. My observation of increased expression of *PCNA* and *SCP2* suggests that rates of zooxanthellae replication and division increases with elevated stress (Suharsono & Brown, 1992). During a bleaching event in which stress results in host bleaching, it is plausible that the demands placed upon the symbiont *via* the host results in increased zooxanthellar lipid metabolism. As a consequence, the symbiont increases lipid use not only as a mechanism to increase its own metabolism, but also to increase lipid transfer to the host (Luo *et al.*, 2009). The drawback of increased expression of *SCP2*, however, is that the symbionts may become deficient in peroxisomes, thereby creating a negative feedback loop. Peroxisomes, found in most, if not

all, eukaryotic cells, play a major role in catabolism of long chain fatty acids (Gabaldon, 2010). As increased zooxanthellae *SCP2* expression is maintained, the symbiont will continue to utilize lipid stores for its own metabolism, as well as increased translocation that benefits the survivorship of the host.

Parallel to increases in *SCP2*, *PCNA* expression increased in zooxanthellae at Acer24 reef that is indicative of a stress response. Increased expression of *PCNA* during stress, when DNA repair and chromatin remodeling would be most important for symbiont survivorship, could be a driver of the development of new zooxanthellae subclade types. *PCNA* is involved in two processes of post replication repair (Lehman & Fuchs, 2006). The first, referred to as the "translesion pathway", occurs *via* DNA polymerases. The translesion pathway acts as a mechanism to incorporate damaged DNA bases into their active sites. In this pathway, polymerases bypass damage caused by a particular stress, where as "normal" replicative polymerases would stall during this process (Lehman & Fuchs, 2006). Incorporating damaged DNA bases may result in different gene sequences, which may contribute to an increase in the number of *Symbiodinium* spp. subclade type reported (LaJeunesse *et al.*, 2004; Sampayo *et al.*, 2008; Hauff *et al*. In Press). The second mechanism, called the "template switch", similarly attempts to bypass DNA damage, but this process involves homologous machinery (Lehmann and Fuchs, 2006). Regardless of which pathway is chosen, PCNA is a vital mechanism of pathway activation used by a cell.

Rates of photosynthesis increase in response to moderate stress in cultured *Symbiodinium* spp. (Iglesias-Prieto *et al.*, 1992). The increased expression of *PCP* in Acer24 reef samples is consistent with this observation as *PCP* is related to light harvesting. The scarcity of carbon dioxide (CO_2) in an aquatic environment results from slow diffusion rates coupled with CO_2

availability existing mostly as bicarbonate, as well as the non-specificity of RuBisCO for CO_2 over O_2 (Raven, 1970; Eckardt, 2012). To augment RuBisCO efficiency, symbionts increase carbon-concentrating mechanisms (CCM) when experiencing survivorship problems in a host coral (CCMs; Price *et al.*, 2013, Oakley *et al.* 2014). Found in nearly all terrestrial plants (Jungnick *et al*. 2014) and a variety of algae (Xu *et al*. 2012), there are two general types of CCMs, C4 and Crassulacean Acid Metabolism (CAM) pathways (Jungnick *et al.*, 2014). Fundamentally, both processes attempt to increase uptake and fixation of CO_2, concentrating carbon around RuBisCO (Price *et al.*, 2013). Increased *PCP* expression yields increases in light energy transfer and therefore, increased carbon availability, boosting potential survivorship of the symbiont (Reynolds *et al.*, 2008). Hence, it is plausible that my observation of increased *PCP* expression under stress is a mechanism used by zooxanthellae to increase CCM.

In addition to increased *PCP*, *PsaE*, a protein involved in the stabilization of interactions within the photosystem 1 complex (PSI), elevated expression in zooxanthellae from Acer24 sub-samples relative to those at Birthday reef. *PsaE* expression increases in the cyanobacterium *Synechocystis* spp. during light stress (Jeanjean *et al.*, 2008). The increases in *psaE* expression counteracts the effects of reactive oxygen species (ROS), whose presence breaks down photosystem II machinery (Mayfield *et al.*, 2011). ROS production is a common response of *Symbiodinium* spp. to bleaching conditions (Lesser, 2006). Increases in the expression of stabilizing protein *psaE* in Acer24 reef samples may represent an initial attempt to counteract degradation of photosynthetic machinery repair as reported in McGinley *et al.* (2012a) for *Symbiodinium* spp. A13. Increases in the expression of *psaE* also demonstrate the higher stressful environment offshore.

The lack of an effect of transplantation and the retention of zooxanthellae gene expression patterns from reef of origin, demonstrates that environmental change does not influence the expression patterns of zooxanthellae genes investigated in this study. If acclimatization were an important means of counteracting bleaching susceptibility, zooxanthellae gene expression patterns would have likely changed in the transplant experiment. Instead, gene expression may be genetically determined by adaptation, as was also proposed for the coral host (Kenkel *et al.*, 2015). The lack of acclimatization demonstrated in gene expression parallels previous work demonstrating a lack of change in zooxanthellae subclade type populations associated with *P. astreoides* from Acer24 and Birthday reefs in response to reciprocal transplant (Hauff *et al.,* In Review). Differences in zooxanthellae subclade populations seen in Hauff *et al.* (In Review) reflect variation in genetic material for natural selection to act upon, and may have resulted in locally adapted populations of zooxanthellae at Acer24 and Birthday reefs.

To predict how coral reefs will change in response to bleaching induced stress, it is important to understand mechanisms involved in bleaching susceptibility of zooxanthellae associated with scleractinian corals. Although demonstrating a lack of acclimatization to environmental change, my gene expression results suggest that zooxanthellae associated with *Porites astreoides* in the Florida Keys are functionally variable and the lower expression of stress response genes relative to the offshore likely reflects better adaptation to bleaching susceptibility inshore. If zooxanthellae are given time to adapt, *P. astreoides* may respond positively in a climate change scenario due to reduced bleaching susceptibility. Additionally, I demonstrated a link between *G3PDH* gene expression and bleaching, which may be a good bioindicator gene of zooxanthellae apoptotic activation. Future studies regarding responses of *P. astreoides* to heat

stress should focus on evaluating the response of *Symbiodinium* spp. gene expression under bleaching temperatures and other synergistic stressors such as disease.

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LITERATURE CITED

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CHAPTER 3

FUNCTIONALLY VARIABLE *SYMBIODINIUM* SPP. DISPLAY SIMILAR REPONSES TO BLEACHING AND DISEASE STRESS IN THE LOWER FLORIDA KEYS

ABSTRACT

Since the 1970's, corals of the Florida Keys have suffered severe declines in cover due to bleaching and disease. Recent variation in susceptibility to moderate bleaching stress has been reported for symbiotic algae (*Symbiodinium* spp.) of the coral *Porties astreoides* between inshore and offshore reefs in the Florida Keys. Continued survival of *P. astreoides* depends on the ability of the coral to survive extreme bleaching events and synergistic disease stress. I conducted a laboratory experiment to investigate the influence of acute high temperature (32°C for eight hours) and disease (lipopolysaccharide of *Serratia marcescens*) on five metabolically related genes of *Symbiodinium* spp. from inshore and offshore *P. astreoides*. Gene expression did not differ between reefs, or as a consequence of acute exposure to heat or heat and disease. This contrasts to results from a separate field study that showed differences in expression of several of the same genes between the same inshore and offshore reefs. Several factors may account for the lack of variation in gene expression such as zooxanthellae response to moderate chronic versus acute stress, host protection and/or the targeting of zooxanthellae by *S. marcescens*. Patterns reported here imply that functional variation in zooxanthellae observed under conditions of chronic moderate stress is lost under the acute extreme conditions studied here.

INTRODUCTION

Scleractinian coral have recently experienced up to 80% mortality in the Florida Keys (Ruzicka *et al.*, 2013). Coral bleaching and disease are the leading stressors threatening the mortality of coral reefs (Harvell *et al.*, 2002). Recent studies show that coral symbiont clade

types (*Symbiodinium* spp.), also known as zooxanthellae, vary in susceptibility to bleaching and disease stress (Sampayo *et al.*, 2008; Hauff *et al.*, 2014; Hauff *et al.*, In Review). However, these studies assessed stress responses of zooxanthellae in culture, or under conditions of chronic moderate stress. Chronic moderate stress refers to elevated levels of a stressor experienced over a lengthy period of time (i.e. weeks or months). Chronic moderate stress will produce a response such as bleaching, but may not result in death (Dikou & van Woesik, 2006). Acute, extreme stress is often associated with short periods of drastically elevated temperatures, but may also be related to extremes in irradiance, disease, or the synergistic effect of two or more environmental variables (Lirman *et al.*, 2011). Herein, I differentiate chronic and acute stress on the basis of time. Importantly, the continued success of coral reefs is likely dependent on the ability of zooxanthellae to survive acute extreme stress and the synergistic effects of bleaching and disease (Hauff *et al.*, 2014; Palumbi *et al.*, 2014).

Coral bleaching has contributed to considerable declines in coral cover in the Florida Keys (Ruzicka *et al.*, 2013). Bleaching is modestly defined as the expulsion of zooxanthellae from the host gastrodermal cavity resulting in the exposure of the white coral skeleton (Gates *et al.*, 1992). It can result in mortality and is tightly linked to elevated seawater temperatures (Goreau & Hayes, 1994), but can also be promoted by other stressors such as irradiance and sedimentation (Brown, 1997). In addition to bleaching, disease plays a large role in the mortality of coral in the Florida Keys. Increased disease related mortality is a function of both the number of coral species with diseases and the frequency of diseased reefs (Porter *et al.*, 2001). Acroporid serratosis refers to White Pox Disease specifically caused by *Serratia marcescens* (Patterson *et al.*, 2002). *S. marcescens* is derived from the gut of animals, including humans, and was

identified in wastewater of the Florida Keys (Sutherland *et al.*, 2011). Acroporid serratosis has caused a 70% loss of *Acropora palmata* species in the Florida Keys (Patterson *et al.*, 2002).

In the Florida Keys, higher rates of bleaching have been observed in offshore *Porites astreoides* relative to their inshore counterparts (Haslun *et al.*, In Review). The expression of metabolically related zooxanthellae genes was also higher in the offshore reef (Hauff *et al.*, Chapter 2). Both of these results likely reflect a response to chronic moderate stress. To my knowledge, the effects of disease on the expression of zooxanthellae genes have not been described previously.

In order to determine the synergistic effects of heat and disease on functionally variant populations of zooxanthellae, I measured the expression of five metabolically related *Symbiodinium* spp. genes, *Cox*, *G3PDH*, *SCP2*, *PCP* and *psaE* to bleaching temperatures and the combination of bleaching temperatures and lipopolysaccharide (LPS) from *S. marcescens*. Lipopolysaccharide are large molecules found on the outer membrane of gram-negative bacteria (Raetz & Whitfield, 2002). When present, LPS elicits a strong immune response in infected organisms (Maverakis *et al.*, 2015). Conducting a laboratory study to determine the effects of acute (8-hour) heat and disease stress allowed me to assess the response of zooxanthellae to acute bleaching and disease. Variation in the response of zooxanthellae to acute stress in the laboratory compared to the chronic moderate stress experienced in the field allowed us to make inferences of the role of the coral host in response to acute stress.

METHODS

Coral Collection and Preparation

My laboratory experiment involved fragments of *Porites astreoides* associated with a two-year field experiment conducted at offshore Acer24 reef (24.55268 N -81.43741 W) and inshore Birthday reef (24.57917 N -81.49693 W) (Permit # FKNMS-2011-107) (Haslun *et al.*, In Review; Hauff *et al.*, In Review). The two reefs experience distinct temperature and turbidity regimes. Birthday reef experiences higher mean average annual temperatures (~1°C) and turbidity than Acer24 reef. However, relative to Birthday reef, there is more bleaching at Acer24 reef, likely due to greater levels of irradiance (Haslun *et al.*, In Review). For field experiments, 16x16 cm^2 fragments of *P. astreoides* were sampled at each reef and sectioned into equal halves. Each half was either replaced at the reef of origin or transplanted to the counterpart reef. At the end of the field experiment, *P. astreoides* fragments originating and replaced at Birthday (n=6) and Acer24 (n=6) reef were brought back to Mote Marine Tropical Laboratory (MMTL). Upon arrival, all 12 fragments were sectioned again and allowed to recover for 72 hours in a shaded flow through water table prior to use in the laboratory experiment discussed herein.

For the current experiment, *P. astreoides* fragments were sectioned into nine 2.5 cm^2 fragments for a total of 108 samples (54 per site). The nine pieces resulting from each of the 12 fragments were used to accommodate a laboratory design consisting of three fragments for controls and three fragments for each of two treatment levels described below.

Temperature and Bacteria Experiments

Two treatment levels and one control were used in experiments: a temperature level known to cause bleaching (32°C, "Heat") and a treatment in which both the bleaching temperature and *S. marsescens* lipopolysaccharide were applied (LPS: 32°C, "Heat+LPS") (Sigma Aldrich, St. Louis MO, USA); the latter application was induced to mimic the onset of

disease. Purified LPS is a common reagent used to elicit immune responses in human and animal studies (Maverakis *et al.*, 2015). I chose to use purified LPS instead of *S. marsescens* colonies to minimize potential cross contamination of *S. marsescens* into the environment, as MMTL is located directly adjacent to a public waterway. Additionally, LPS was not added to flow-through sections of the experimental design, see below. Control fragments were maintained in experimental tanks at ambient temperature (28°C) without exposure to LPS.

Experiments were conducted by placing coral fragments in custom made acrylic boxes (2.5 x 7.5 x 7.5 cm) set within 38-liter fish tanks. The acrylic boxes included three distinct subdivisions each of which housed a single coral fragment. Because each subdivision was isolated from one another, individual coral fragments were entirely independent. Six 38-liter tanks containing five acrylic boxes each were used in the experiment. Because each acrylic box contained three subdivisions, this resulted in 90 experimental units. The experimental designs for the six tanks included two controls with a total of 30 experimental units and two tanks for each of the two treatments, with 30 experimental units for each. The six 38-liter tanks were filled with artificial sterile seawater (ASSW, Instant Ocean™, Blacksburg VA, USA) and placed in flow through water tables. Water tables were 680 liter, fiberglass troughs with seawater pumped through *via* the adjacent waterway to aid in temperature maintenance.

Coral fragments were rinsed in ASSW prior to placement in acrylic box subsections. ASSW held within the tanks was circulated *via* pumps inside the tanks but external to acrylic boxes. Heaters placed external to the acrylic box maintained temperatures characteristic of bleaching (32°C±0.5) and were monitored with HOBO™ tags (Onset Computer Corporation, Bourne MA, USA). For the disease response treatment, LPS from *S. marsescens* (1 mL, 5 μg/mL) was added using disposable plastic pipettes. Additionally, 1 mL of ASSW was added to

the heat treatment and control tanks for consistency.

Coral fragments were exposed to treatments for eight hours. Within that period of time, individual subdivisions were aerated each hour *via* air infusion using disposable pipettes. Water was aerated (C. Page MMTL, per. obs.) taking care not to disturb the fragments. At the end of each experiment, coral fragments were removed from their individual tanks using sterile tongs, wrapped in combusted foil and placed in freezer bags. Coral fragments were then immediately flash frozen in liquid nitrogen and stored at -80°C until processing.

RNA Extraction, qRT-PCR and Data Analysis

RNA extractions, cDNA preparations and qRT-PCR were conducted as outlined in Hauff *et al.*, (In Review). Gene expression of zooxanthellae genes *Cox, PCNA, SCP2, G3PDH, PCP* and *psaE* were evaluated in each control and treatment coral fragment. These were the same genes used in zooxanthellae gene expression studies by Hauff *et al.,* (In Review). Gene and primer information may be found in Hauff *et al.*, (In Review) (Table 1). Technical replicates were analyzed in duplicate and negative controls confirmed the absence of genomic DNA contamination. This yielded a total of n=1,296 analyses.

Statistical analyses were performed in R (v3.1.3) using the specialized package *MCMC.qpcr* (Matz *et al.*, 2013; R Core Team 2014). Within this package, raw qRT-PCR data is converted to molecule counts. Counts are then described under a Poisson-lognormal error using generalized linear mixed models. This approach was used as it allows any number of random and fixed effects, accounts for low amplification, increases model power by analyzing all genes simultaneously in one model, and allows for analysis without the use of housekeeping genes

(Matz *et al.*, 2013). The addition of a housekeeping gene *PCNA* however, decreases the risk of bias, and therefore, was used here.

A two-way model was fit using "reef" (i.e. Acer24 or Birthday reef) and "treatment" (i.e. control, heat or heat+LPS) as fixed factors, as well as their interaction term, "reef:treatment". This resulted in a model describing the individual effects of each factor, in addition to the interaction effect (i.e. the site dependent effect of treatment). I ran three Markov Chain Monte Carlo chains for 25,000 iterations with the first 4,000 discarded as burn-in. The model was "informed" using qRT-PCR data generated here from one previously established housekeeping gene (*PCNA*, McGinley *et al.*, 2012). Diagnostic plots using the function diagnostic.mcmc() were analyzed to confirm linear modeling as an appropriate application for this data set.

RESULTS

No significant effect of site or treatment was found on the expression of *Cox*, *G3PDH*, *SCP2*, *PCP* or *psaE* between Acer24 and Birthday reef zooxanthellae associated with the coral fragments ($p>0.05$, Table 4). For example, gene expression of *Cox* was not different between Acer24 and Birthday reef fragments or between control and experimental treatments from the same site. The interaction of site and treatment also did not have a significant effect on zooxanthellae expression ($p>0.05$, Table1). For instance, the gene expression of *Cox* in heat-stressed *Symbiodinium* spp. from Acer24 reef was not different from the expression of *Cox* heat-stressed zooxanthellae from Birthday reef. Pair-wise comparisons showed no significant differences in gene expression among treatments and controls. The expression of *Cox* in zooxanthellae harbored in control fragments from Birthday reef was not significantly different

from the expression of *Cox* in zooxanthellae harbored in fragments from Birthday reef exposed to heat or heat+LPS treatments ($p>0.05$).

Treatment	Gene	Post.mean	l-95% CI	u-95% CI	Eff.samp	pMCMC
Reef	Cox	2.187638	-0.139690	4.479140	1000.0	0.068
Reef	G3PDH	0.504446	-0.644153	1.417098	1000.0	0.334
Reef	PCP	0.245573	-0.812485	1.259310	1000.0	0.636
Reef	PSAE	0.038782	-0.910168	1.093089	1000.0	0.914
Reef	SCP2	1.174183	-0.081910	2.613642	1000.0	0.102
Reef	PCNA	0.353228	-0.635999	1.392324	1000.0	0.538
Heat	Cox	0.631882	-1.126961	2.276879	1000.0	0.452
Heat	G3PDH	0.370865	-0.632919	1.328137	1000.0	0.466
Heat	PCP	0.410617	-0.656092	1.311776	1000.0	0.430
Heat	PSAE	0.418968	-0.651622	1.390965	1000.0	0.414
Heat	SCP2	0.603772	-0.547622	1.572087	1000.0	0.300
Heat	PCNA	0.024704	-0.753097	0.877509	1000.0	0.980
LPS	Cox	0.007293	-1.872295	1.749692	898.7	0.986
LPS	G3PDH	0.345562	-0.654212	1.385999	1000.0	0.520
LPS	PCP	0.613552	-0.434824	1.643339	798.5	0.262
LPS	PSAE	0.556251	-0.492400	1.607295	799.0	0.314
LPS	SCP2	0.223214	-0.903428	1.304645	1000.0	0.686
LPS	PCNA	-0.001164	-0.869460	0.914153	1000.0	0.982
Reef+Heat	Cox	-1.832442	-4.065119	0.415856	872.3	0.094
Reef+Heat	G3PDH	-0.088287	-1.412416	1.204037	1000.0	0.904
Reef+Heat	PCP	-0.061582	-1.457569	1.138234	709.4	0.916
Reef+Heat	PSAE	0.009594	-1.246905	1.411503	732.4	0.994
Reef+Heat	SCP2	-0.163576	-1.403211	1.327639	1000.0	0.816
Reef+Heat	PCNA	-0.145029	-1.095284	0.821031	867.0	0.768
Reef+LPS	Cox	-0.438337	-2.817670	1.779693	1000.0	0.712
Reef+LPS	G3PDH	-0.110682	-1.423377	1.182371	1000.0	0.848
Reef+LPS	PCP	-0.414284	-1.808073	0.857795	1000.0	0.526
Reef+LPS	PSAE	-0.086955	-1.474356	1.181806	1000.0	0.886
Reef+LPS	SCP2	0.069792	-1.298356	1.489236	1000.0	0.918
Reef+LPS	PCNA	-0.126965	-0.963841	0.885971	1121.8	0.770

Table 4. Two-way model for an experiment testing the effects of reef, heat and heat+LPS on the gene expression of *Cox*, *G3PDH*, *PCP*, *psaE*, *SCP2* and *PCNA* under the MCMC.qpr package. Post means are reported as well as lower and upper credible intervals, effective sample size and p-values. The model was "informed" using *PCNA* as a housekeeping gene and run with 25,000 iterations, with the first 4,000 discarded as burn-in. Coral colony individual was used as a random factor. Reef fragments were from Acer24 or Birthday reef and exposed to 28°C. Heat treatments were fragments from both reefs exposed to 32°C, and heat+LPS treatments were fragments from both reefs exposed to 32°C+LPS from *Serratia marcescens*. Asterisk denotes a p-value <0.05.

Although not significant, the cumulative effect of heat stress on the expression of each of these genes generally resulted in an increase in gene expression through time. For some genes (e.g. *Cox*), an increase in temperature resulted in a decrease in expression (Figure 8). The only other time a decrease in gene expression was observed was when *PCP* and *PSAE* were exposed to the heat+LPS treatment. In addition, depending upon treatment, the stress resulted in a 2-fold increase in expression relative to the control (e.g. *Cox*, *SCP2*; Figure 8).

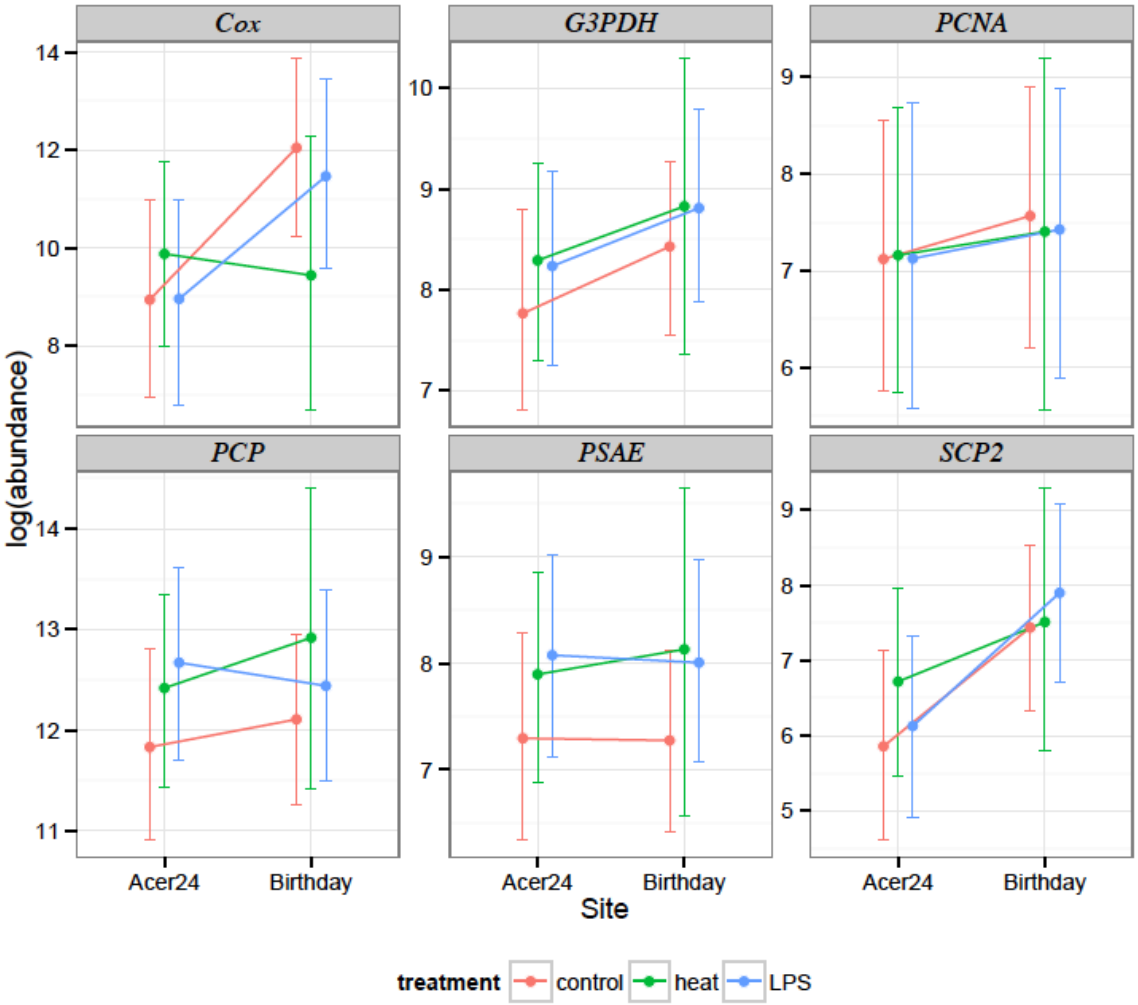

Figure 8: By-gene plot of normalized log_2-transformed expression values (±SEM) of experimental genes from all samples. Orange lines represent fragments exposed to control treatments (28°C), green lines represent fragments exposed to heat treatments (32°C) and blue lines represent fragments exposed to heat+LPS treatments (32°C+LPS from *Serratia marcescens*). Whiskers denote 95% credible intervals.

DISCUSSION

The continued success of coral reefs will likely be determined by the ability of coral-zooxanthellae association to survive exposure to chronic moderate stress and acute extreme bleaching events (Sampayo *et al.*, 2008; Palumbi *et al.*, 2014). While chronic moderate stress is commonly experienced by zooxanthellae on reefs in the Florida Keys (Ruzicka *et al.*, 2013; Haslun *et al.*, In Review) acute stress is a more transient phenomenon poorly described in the literature. Most studies report a response of the coral host and imply that such effects also impact the metabolism of the symbionts (Barshis *et al.*, 2013; Kenkel *et al.*, 2015). In a complimentary field study, I showed bleaching and elevated expression of zooxanthellar metabolic genes in response to chronic moderate stress at offshore Acer24 reef (Hauff *et al.*, In Review). To evaluate the influence of acute stress on zooxanthellae gene response I exposed coral from Acer24 and Birthday reef to a high temperature (32°C) stress, and high temperature combined with LPS from *S. marsescens*; a treatment that represents a primary stress coupled with a secondary stress (i.e. disease).

Although my data was not statistically significant, cumulative trends demonstrate increased expression of five of the six zooxanthellar genes relative to the experimental control in response to heat stress. During acute temperature exposure, symbiont cells show a "knee-jerk" reaction in which cumulative gene expression increases one or two-fold, increasing photosynthetic capacity (i.e. *PCP* and *psaE* increases, Figure 8). Up regulation of photosynthetic genes is likely a mechanism to amplify zooxanthellae metabolism (i.e. increased *G3PDH*, Figure 8). At a cellular level, the consequence of increased metabolism is likely an increase in symbiont DNA synthesis, as well as DNA repair due to mutations (Ruiz-Jones & Palumbi, 2015). Increased DNA synthesis was observed in this study *via* an overall trend of increased *PCNA*

concentration (Figure 8). As metabolic activity increases, *Symbiodinium* spp. also augment their ability to transfer lipids (i.e. the increase in lipid-transfer protein *SCP2,* Figure 8). My expectation is that as photosynthetic machinery ramps-up its production of photosynthate (i.e. lipids), lipid products need to be used or transferred to the host. *SCP2* is likely a mechanism to increase lipid transport to the host coral.

Although I observed a general cumulative increase in metabolic activity in zooxanthellae, it is possible that the algae are less stressed (i.e. non-significant gene expression) than the host in laboratory conditions experienced here. This result is perplexing as significant changes in algal gene expression were measured in the companion field study, even though summer temperatures and irradiance in the field study were not outside the norm for these reefs (Hauff *et al.,* In Review, Haslun *et al.,* In Review). As elevated temperatures and irradiance persisted for several weeks in the field, perhaps these environmental conditions manifested themselves as a chronic stress. I recommend that future studies pay close attention to subtle changes in temperature, even over a short duration. I suspect that zooxanthellae genes respond differently under acute and chronic stress, with this difference contributing to dissimilarities reported in the current and field experiment. As such, the implications to the dynamics of symbiosis may be considerable.

Contrary to chronic stress and variable gene expression observed from samples collected in the field in Hauff *et al.* (In Review), there were no significant differences in zooxanthellae gene expression between Acer24 and Birthday reef fragments exposed to high temperature as the primary stressor, and disease as the secondary stressor. However, I did observe a cumulative increase in expression of zooxanthellae genes. For instance, the only symbiont genes showing decreased expression with the addition of disease were *PCP* and *PSAE* - which are both related to photosynthesis. I viewed this decrease as peculiar as it would seem natural to increase

metabolism as a mechanism to counteract disease. Instead, however, the opposite occurred. I suspect that the algae's response to disease stress is evolutionary in nature.

The evolution of plant and pathogen is a dynamic interaction whereby pathogens evolve to maximize nutrient intake, ensuring their future success (Berger *et al.*, 2007). In this context, the synthesis of an energy source – photosynthate – and its availability for use, is a competition between organisms (Selvaraj & Fofana, 2012), in which a pathogen will often reprogram a plants metabolism to suit its own needs (Biemelt & Sonnewald, 2006). In this case, the application of LPS mimicked a pathogen attack, I believe that the zooxanthellae responded by undergoing a "source to sink transition" where the plant (i.e. *Symbiodinium* spp.) down-regulated photosynthesis as a mechanism to starve the pathogen (Selvaraj & Fofana, 2012). This is a common occurrence in terrestrial plants (Kocal *et al.*, 2008), but poorly described in aquatic habitats. Although not shown in this study Cervino *et al.* (2012) demonstrated decreased photosystem II quantum yields under disease stress, supporting my speculations.

Garavaglia *et al.* (2010) describes reprogramming of a hosts metabolism as a first step in a pathogens attempt to control its host. As the host attempts to starve the pathogen, the microbe works to reprogram a host's metabolism producing "host-selective toxins" that interact with host sensitivity genes, resulting in localized programmed cell death (PCD) and/or hypersensitive responses restricting pathogen growth but preventing its elimination. In terrestrial plants the phenotypic response of this stress yields irregular spots that are visualized as small dark brown spots with a tan/yellow border (Kim *et al.*, 2010). In coral reef ecology, the aforementioned phenotype displays resemblance to coral diseases such as Yellow Band Disease (Cervino *et al.*, 2001) and dark spot disease (Gil-Agudelo & Garzón-Ferreira, 2001).

I speculate that the differences observed here over a short time period (8 hours) may have been insufficient when compared to the chronic expression observed in an earlier study (Hauff *et al*. In Review). It is also plausible that this coral species is well adapted to short acute responses of temperature and/or temperature and disease as observed by the increased concentration of particular genes (i.e. G3PDH = metabolism) despite the lack of statistical significance. This is plausible as *P. astreoides* is considered a dominant coral on reefs of the Florida Keys in a region known to experience elevated temperatures and disease (Green *et al.,* 2008).

In addition to insufficient exposure time as a cause of the lack of a gene response, the coral host may influence zooxanthellae gene expression by providing protection to zooxanthellae. This possibility is suggested by the observation that *in hospite* zooxanthellae experience less photosynthetic degradation than cultured zooxanthellae when exposed to short-term bleaching conditions (Bhagooli *et al.*, 2010). Because the viability of zooxanthellae metabolism is not independent of the coral host, understanding the response of the coral host to heat and disease conditions is relevant to the current study. Using the same samples as this study, Haslun *et al*. (In Review B) evaluated the genetic response of the coral host. Coral host genes associated with bacterial recognition increased under acute heat and disease stress. This suggests that coral, rather than zooxanthellae, are the first responders to acute stress. Cultured zooxanthellae provide a unique way to further understand the role of host protection, as they are completely void of host tissues. In order to determine whether the host is indeed providing protection, gene expression of cultured zooxanthellae exposed to the same levels of heat and disease stress should be compared to responses demonstrated here. Deciphering which symbiotic partner experiences the lions-share of acute stress (Sammarco & Strychar, 2009) will be key in determining the range at which coral holobionts can resist acute extreme episodes of stress.

The response of zooxanthellae to heat and disease appears to vary among clades. Hauff *et al.* (2014) reported higher rates of apoptosis in *Symbiodinium* spp. clade C1 synergistically affected by heat and Yellow Band Disease (YBD) pathogens. The same synergistic effect was not demonstrated in *Symbiodinium* spp. clade B2. While YBD specifically targets zooxanthellae (Cervino *et al.*, 2004), *S. marcescens* does not. If *S. marcescens* targeted host coral cells as shown in Haslun *et al.*, (In ReviewB), rather than zooxanthellae, this could explain the absence of zooxanthellae gene expression in the current experiment. Owing to differences in variability among clades discussed above, and paucity of studies at different temperatures and disease regimes, the synergistic effect of elevated temperature and disease on zooxanthellae remains unresolved. Investigations into the impacts of *S. marcescens* specifically are a particularly important line of investigation. Increased virulence of *S. marcescens* is linked to elevated water temperatures and bleaching, yielding host tissue loss rates of up to 2.5 cm^2 per day (Patterson *et al.*, 2002).

While *S. marcescens* virulence is enhanced with elevated temperature (Patterson *et al.*, 2002), some pathogens are not able to establish themselves under extreme temperatures. Cervino *et al.* (2004) demonstrated increased spreading rates of YBD lesions with elevated temperatures, but only after infectivity of YBD at ambient temperatures. If exposure to ambient temperatures is similarly required for the *S. marcescens* pathogens, this could be another factor accounting for the absence of differential gene expression observed herein.

Short-term perturbations such as acute stress are difficult to capture in the field. As a counterpart to field studies, laboratory studies are important as they allow for manipulation and the capture of acute stress perturbations. My study demonstrated an absence of statistically significant functional variability to acute stress, even though functional variation in

zooxanthellae populations from *P. astreoides* as a consequence of chronic stress was established under similar or less stressful conditions in the field. The role of the host as a protective mechanism may explain the apparent lack of variability reported. Alternatively, the short time period may have also skewed the results, indicating longer sampling intervals are required to induce a significant stress response in zooxanthellae. The ecological implications of the field and lab findings suggest that the response of the coral holobiont to stress is dynamic. As such, the future persistence of *P. astreoides* is dependent on a myriad of environmental and biological parameters, as well as a complex interplay between the host and symbiont. To the authors' knowledge, this study marks the first study of zooxanthellae gene expression in response to synergistic temperature as a primary stress coupled to disease as a secondary stress.

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