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Symbiont Diversity of Zooxanthellae (*Symbiodinium* spp.) in *Porites astreoides* and *Montastraea cavernosa* from a Reciprocal Transplant in the Lower Florida Keys

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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 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. This variability may be key 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 withstand thermal stress.

Keywords: coral, inshore, ITS2, offshore

1. Introduction

Since the 1980's, coral of the Florida Keys Reef Tract have suffered severe mortalities (Glynn, Corte, Guzman, & Richmond, 1982; Glynn, 1991; Gardner, Cote, Gill, Grant, & Watkinson, 2003) due to bleaching, disease and poor water quality (Dustan, 1977; Precht & Miller, 2007). Coral bleaching is the result of the expulsion of symbiotic, single-celled dinoflagellate algae known as zooxanthellae (*Symbiodinium* spp.; Fitt, Brown, Warner, & Dunne, 2001). Many stressors may cause bleaching including, but not limited to, increasing sea surface temperatures and increased irradiance (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, Ridgway, Bongaerts, & Hoegh-Guldberg, 2008). Defining how specific reef systems and their resident symbionts respond to increases in bleach inducing stressors is important for delineating coral survivability and determining appropriate target populations for conservation efforts.

Symbiodinium spp. are classified into clades A-H and further divided into sub-clade types, with hermatypic coral known to form symbioses with members of clades A-D (reviewed by 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 increases in sea surface temperatures or irradiance.

Previous investigations demonstrated that stress tolerance varies between clades and even subclades (Rowan & Knowlton, 1995; Fitt et al., 2001; Berkelmans & vanOppen, 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, Goodbody-Gringley, Caillaud, Davies, Martels, & Matz, 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, Hauff, Strychar, & Cervino, in review). These studies, however, did not describe how zooxanthellae subclade type diversity between inshore and offshore coral responded to stress.

To expand our knowledge of how subclades vary among coral host conspecifics and how these assemblages respond to stress, we investigated patterns in the dominant populations of zooxanthellae from an inshore and offshore reef in the Lower Florida Keys. Additionally, we 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 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; Sampayo, Dove, & LaJeunesse, 2009; T. LaJeunesse pers. comm.).

2. Materials and Methods

2.1 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, species diversity, light).

2.2 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. Fragments were 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.

2.3 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).

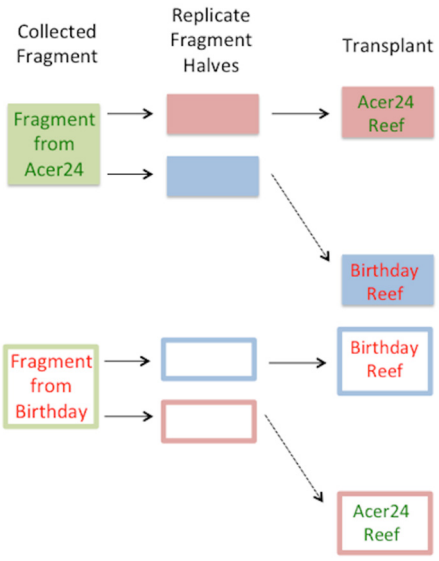

Figure 1. Experimental Design

Figure 1. Graphical representation of our 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 and both allowed to recover at Mote Marine Laboratory. 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 Reef. Dashed arrows represent transplanted fragments. For the experiment, the above was repeated ten times for each coral species resulting in n=80 fragments.

Transplant experiments 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. All cinder blocks received equal exposure to environmental conditions. Water temperature was monitored every 30 minutes via HOBO™ tags placed at each site.

2.4 Sample Collection for DNA Analysis

After the 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. Variation in storage technique resulted from the inability to extract viable DNA from *M. cavernosa* fragments stored in EtOH.

2.5 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 razor blade in 10 mL of zooxanthellae isolation buffer (ZIB, Rowan 1991). The homogenate was poured into centrifuge tubes and pelleted (500 x g for 10 minutes), and the supernatant decanted, washed with 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 manufacturer's 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 (GAATTGCAGAACTC CGTG) and ITS2-reverse (GGGATCCATATGCTTAAGTTCAGCGGGT)(2). PCR was performed 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 the Research Technology Support Facility (RTSF) at Michigan State University, respectively. Both sequencing reactions used the forward or reverse ITS2 primers listed above.

2.6 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 on 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.

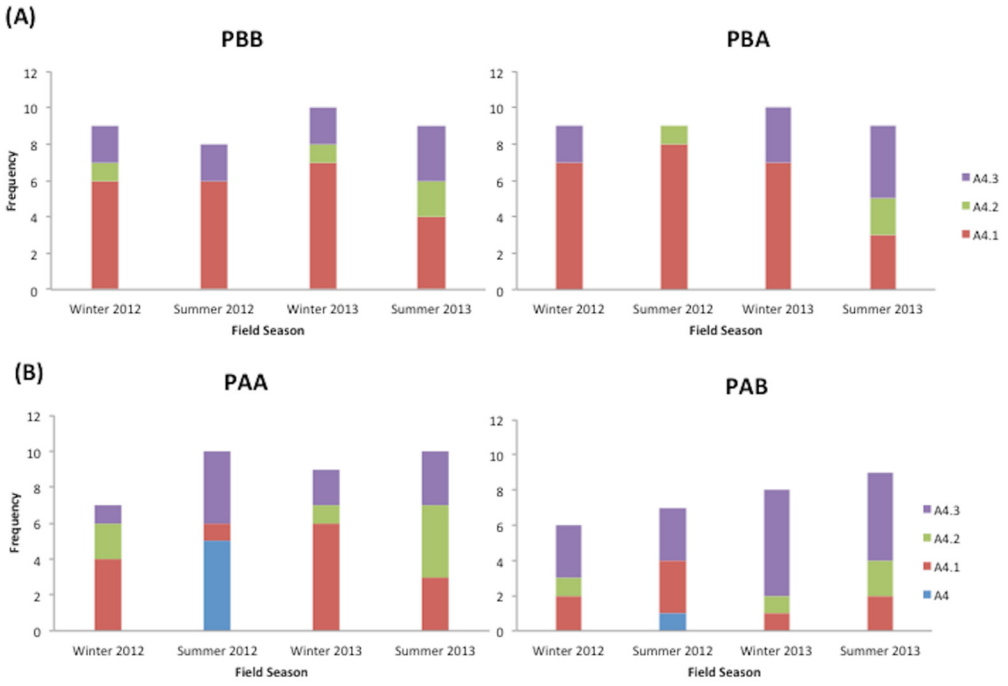

Figure 2. *Symbiodinium* subclade type frequencies for *Porites astreoides* samples

Figure 2. 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 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.

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 choose best fit models for further analysis. To perform t-tests on individual subclade type abundance comparisons, fitted values and standard errors were calculated from coefficients.

3. 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* sp. 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".

3.1 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 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 seasons.

Figure 3. Transitions in zooxanthellae subclade type occurring within the same coral fragment between sampling times for all fragments of *Porites astreoides* zooxanthellae. Pie charts 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, 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.

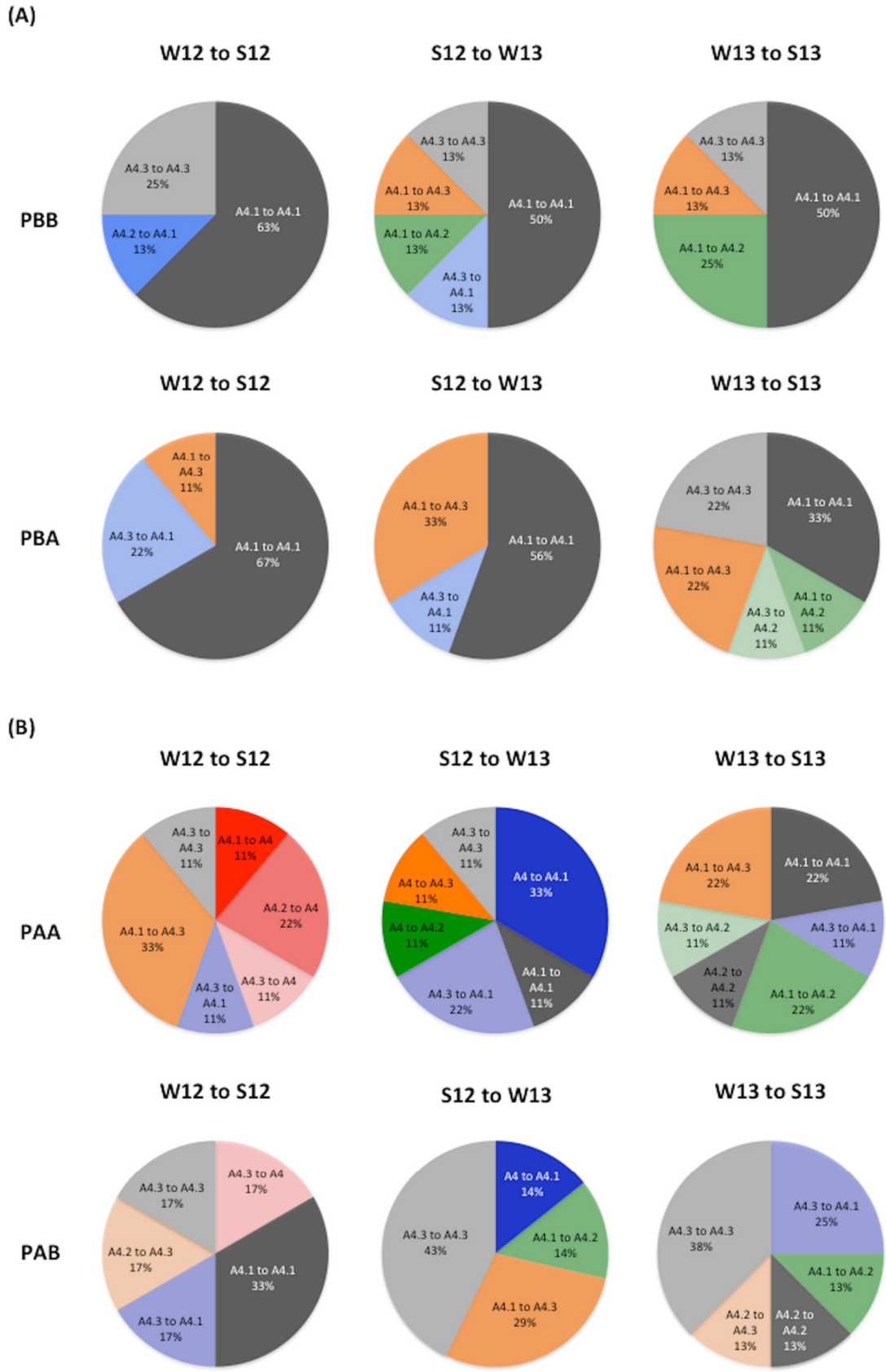

Figure 3 *Porites astreoides* zooxanthellae transitions.

3.2 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, whereas 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, whereas PAA had a high number of transitions to A4.1.

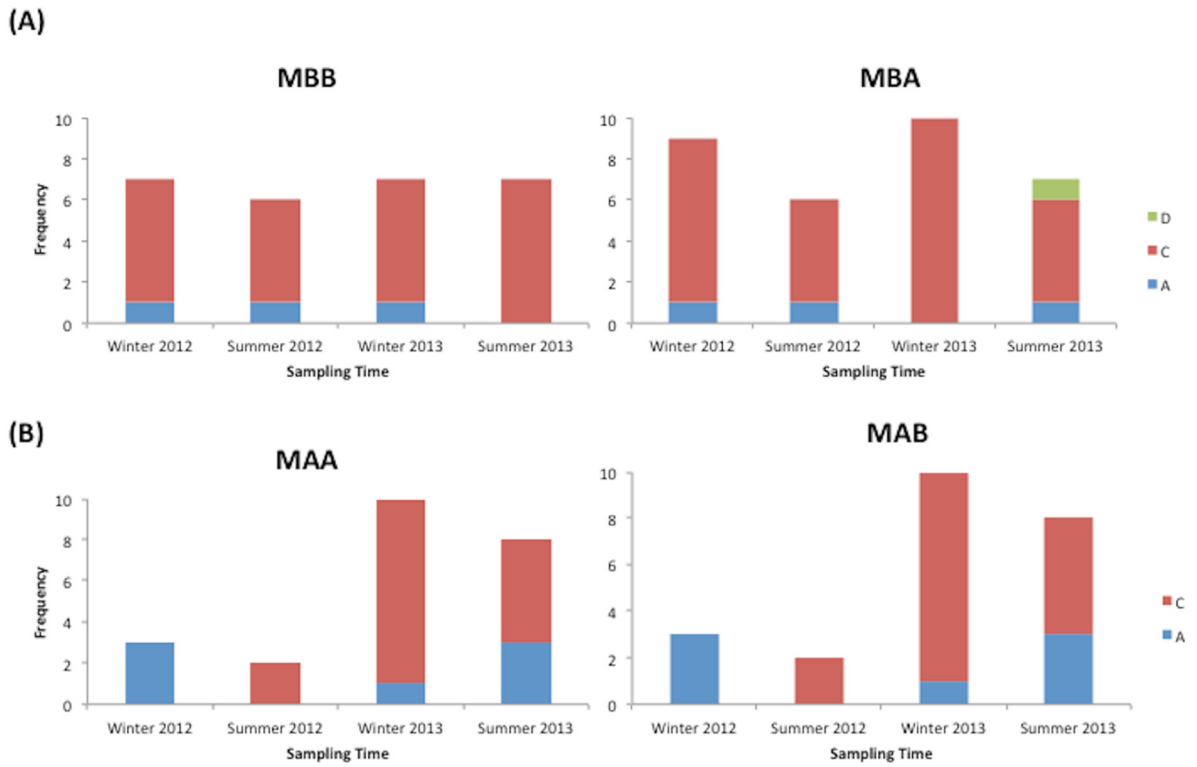

Figure 4: *Symbiodinium* subclade frequencies for *Montastraea cavernosa* samples.

Figure 4. 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 *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.

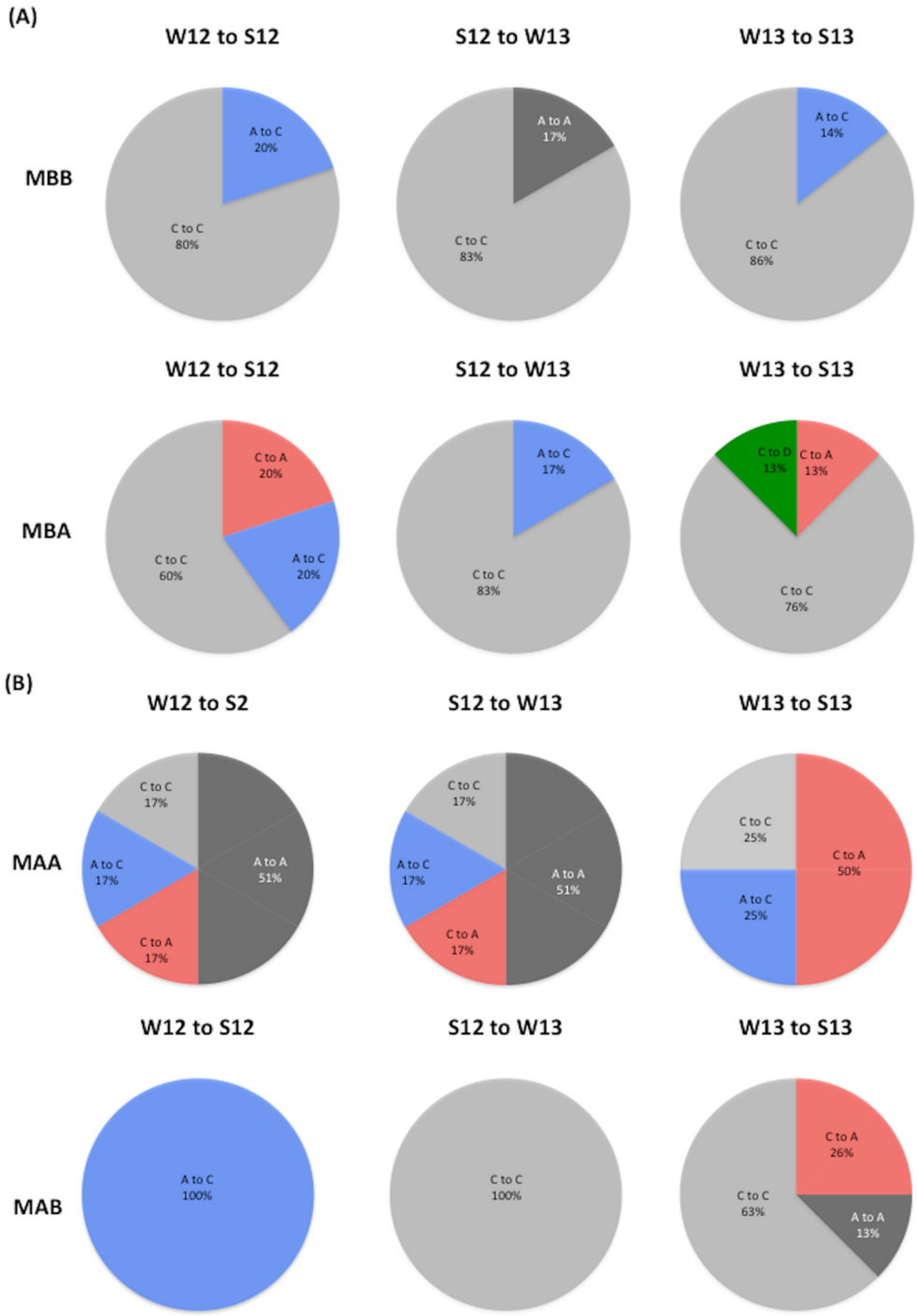

Figure 5. *Montastraea cavernosa* zooxanthellae Transitions

Figure 5. Transitions in zooxanthellae subclade type occurring within the same coral fragment between sampling times for all fragments of *Montastraea cavernosa* zooxanthellae. Pie charts 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 on a 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.

3.3 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.

Comparing MBB and MBA samples, the interaction term between experimental treatment and field season was not significant ($p>0.25$). 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.

When comparing fitted values of the probability of clades A, C and D in MBB and MBA samples, no significant difference was found 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.

3.4 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 comparing 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.

4. Discussion

Since the 1997/98 El Niño event coral cover on offshore reefs in the Florida Keys Reef Tract has been ≤5% (Ruzicka et al., 2013). Previously, studies have demonstrated differences in the ability of certain coral assemblages to withstand stress. Kenkel et al. (2013) found that coral assemblages from inshore reefs demonstrate higher thermotolerance than their offshore conspecifics. Additionally, Haslun et al. (in review) found inshore assemblages to demonstrate lower incidences of chronic bleaching compared to assemblages from offshore. Zooxanthellae subclade type may be an important driver of holobiont stress tolerance (Sampayo et al., 2008). We investigated whether observable patterns of changes in the dominant zooxanthellae subclade type from fragments of *Porites astreoides* and *Montastraea cavernosa* provide information regarding inshore and offshore reef coral stress tolerance and how these changes are reflected in responses to changing stress regimes.

4.1 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 our offshore (Acer24) reef. Temperatures at Birthday reef during summer months were within the mean summer maximum for this area. These similar

temperatures associated with low-level irradiance are likely responsible for the relatively stable associations at Birthday Reef compared to assemblages from Acer24 Reef, as demonstrated by fewer transitions. Additionally, associations 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 the lower stress environment.

According to Haslun et al. (in review), coral at the offshore site experienced higher bleaching than coral at the inshore site due to higher irradiance. Higher bleaching 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). Consequently, one might expect a transition in subclade type to more stress tolerant subclades. Yet there is no significant difference in the probabilities of individual subclade types between transplanted and non-transplanted *P. astreoides* fragments from Birthday Reef ($p>0.05$). In fact, the composition of zooxanthellae from PBB and PBA samples are very similar, indicating a lack of acclimatization to a presumed higher stress environment. Transitions may be absent if *Porites astreoides* fragments originating from the inshore site do not possess a zooxanthellae subclade type adapted to higher irradiance.

When examining *P. astreoides* fragments collected at the offshore site, we observed zooxanthellae subclade type A4 in fragments of *P. astreoides* solely during the Summer 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. Examining the temperature data from the offshore site (Figure 6), Summer 2012 was the warmer of the two summer sampling times. In addition to high irradiance levels characteristic of summer season, higher temperatures may have contributed to the higher frequency of subclade type A4 during Summer 2012 relative to Summer 2013.

Porites astreoides is known to contain multiple subclade types besides members of A4 (LaJeunesse, 2002). However, little variability was found in this study as conspecifics of *P. astreoides* from an inshore and offshore reef in the middle Florida Keys 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 (vanOppen, 2004). The long-term coevolution of this mutualism likely leads to a tight symbiosis, with little flexibility, and may explain the small changes observed in this study (Thornhill, Fitt & Schmidt, 2006).

Shuffling, here demonstrated *via* transitions, is one mechanism that can alter the relative abundance of subclade types (Kinzie, Takayama, Santos, & Coffroth, 2001; Rowan, 2004). Zooxanthellae subclade types from coral fragments originating from the inshore reef showed less than 50% of fragments displaying 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 site (Figure 3B). Retention of zooxanthellae composition and diversity based on collection site suggests a lack of acclimatization to changing stress regimes, even after a lengthy two-year transplant.

4.2 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 the author's 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 routinely isolated from species within the genus *Montastraea* (Garren, Wash, Caccone & Knowlton, 2006) as well as clade A being a common zooxanthellae type in this region, as demonstrated for *P. astreoides* species.

Subclade type C3 is commonly associated with *M. cavernosa* (LaJeunesse, 2002; Banaszak, Santos, LaJeunesse & Lesser, 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 clade type C was found to be significantly higher in fragments collected from the inshore reef. Increased in subclade type C is likely due to low stress environmental conditions and the generalist nature of clade type C. A notable exception in the subclade types found in MBA samples (Figure 4A) is the presence of a D1 subclade type in the Summer 2013 sampling time. 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 during stress events has repeatedly been demonstrated in the literature (Baker, 2001; Thornhill, LaJeunesse, Kemp, Fitt & Schmidt, 2006; Silverstein, Cunning & Baker, 2015). The dominane of clade D subclade types during stress leads to the belief that clade D subclade types confer a specific advantage to holobionts during times of stress. Ultrup and vanOppen (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, versus other

subclade types, become more prevalent when environmental stress increases due to elevated temperatures or changes in turbidity. Increases in clade D subclade types confer resistance to stressful conditions (Berkelmans & vanOppen, 2006) and may demonstrate 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. Their gametes are void of zooxanthellae. Thus, they acquire symbionts *via* horizontal transmission, from the surrounding environment (vanOppen, 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 these samples. Compared to brooders, broadcast spawners are thought to rely more heavily on zooxanthellae subclade type for holobiont thermotolerance, as they possess the ability to switch and shuffle their zooxanthellae subclade types (Silverstein et al., 2015). While the present study did not attempt to identify the source 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) as well as an attempt to counteract this *via* 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 seen in *M.* cavernosa fragments. Upon examining our temperature data the Winter 2013 sampling time was warmer, and therefore milder, than Winter 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.

Transitions between zooxanthellae clade types, rather than subclade types, are significant as individual clades can differ drastically at the genetic level. 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 & vanOppen 2003; Thornhill et al., 2006; Silverman 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 new stress regimes.

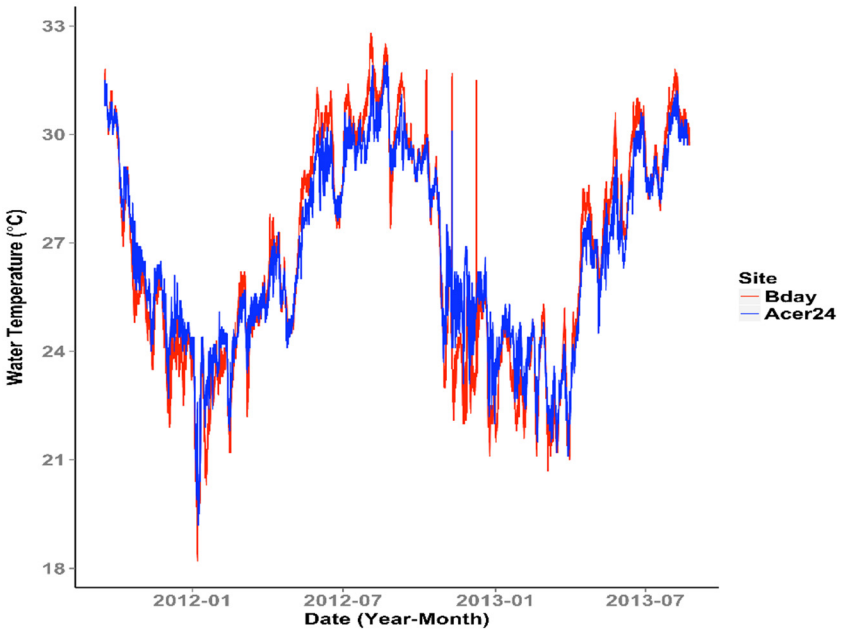

Figure 6. Temperature Birthday and Acer24 reefs

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

5. Conclusion

In the face of global climate change, many studies are focused on finding particular coral assemblages that are better adept at dealing with a myriad of environmental stresses. Our 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-derived effect of seasonal zooxanthellae compositional changes suggesting a lack of acclimatization to a new environment, an important concept when targeting coral populations 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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