

Research Article

Coral Bleaching Susceptibility Is Decreased following Short-Term (1–3 Year) Prior Temperature Exposure and Evolutionary History

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Received 16 March 2011; Accepted 4 June 2011

Academic Editor: Baruch Rinkevich

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Coral exposed to short periods of temperature stress ($\geq 1.0^\circ\text{C}$ above mean monthly maximum) and/or increased frequencies of high temperatures may bolster resilience to global warming associated with climate change. We compared *Montastraea cavernosa* (Linnaeus, 1767; Cnidaria, Scleractinia, Faviidae) from the Florida Keys National Marine Sanctuary (FKNMS) and the Flower Garden Banks National Marine Sanctuary (FGBNMS). Thermal stress has been reported frequently within the FKNMS; however, corals in the FGBNMS experience nominal exposures to similar stressors. Corals were exposed to three temperatures (27°C , 31°C , and 35°C) for 72 h. Colonies from the FKNMS lost significantly fewer viable and necrotic zooxanthellae under conditions of acute stress (35°C) than the FGBNMS colonies. This indicates that the FKNMS corals are less temperature-sensitive than those in the FGBNMS. The observed differences point to greater prior temperature exposure and adaptation in the former versus the latter site when correlated to previous years of thermal exposure.

1. Introduction

The earliest reefs are believed to have existed more than 3.4 billion years ago, dating back to the Early Archean era [1]. Reefs at that time were likely dominated by cyanophyceans and other bacteria [2]. More “modern” reefs consisting of coral and algae evolved during the Oligocene and Miocene, ~5 million years ago [3]. Present day reefs, such as the Indo-Pacific Great Barrier Reef (GBR) have evolved over the last 500–600 K years [4]. During each of these major eras of reef development, major climate changes throughout the geological time are believed to have influenced the evolution of species and the subsequently, adaptation of surviving species to these changes [1]. Such changes are believed to include the evolution of symbiosis. To better understand how future reefs will adapt to present-day climate changes, numerous studies have been done (reviewed in [5–10]). The causes of present-day crises on coral reefs is believed to be most likely a synergism between natural climate-related stress exacerbated by human-imposed stress. In 1998, heat stress is believed to

have caused 48% of western Indian Ocean reefs and 16% of all reef areas globally to bleach [11, 12]. In 2002, extensive bleaching occurred on 60–95% of the world’s barrier reefs, causing the loss of 50–90% of the corals there [12]. On the Great Barrier Reef, Australia, 50–60% of the reefs bleached, affecting 75,000 to 210,000 km² [13, 14].

Disease has also been a major cause of loss of coral cover in the Caribbean Sea. Changes in climate in combination with an increase in the incidence of coral disease [15–20] have resulted in a major decrease in the live coral cover. Between 1984 and 1991, the live coral cover decreased by as much as 49% at certain sites in the Florida Keys [21]. In fact, damage inflicted on Caribbean corals by heat stress (i.e., increased seawater temperatures; SWTs) and disease is causing a shift from a coral-dominated community [22] to one dominated by algae [20, 23–25]. There have been numerous studies in the Caribbean on the causes and mechanisms of corals bleaching [6, 26], including the probability that some corals can “cope” with heat stress better than their symbionts [10], which may be related to their evolutionary age and/or

their prior experiences/exposures to specific stressors. In general, corals in the Florida Keys are relatively younger (5–7 K years old; [27]) than those in the Gulf of Mexico (i.e., 10 K years old; [28]) but have been exposed to a greater number of heat waves, pollution, and diseases. Here, we ask whether corals in the Florida Keys are better adapted for long-term survival of increasing seawater temperatures than those in the Gulf of Mexico because of prior exposure to such stresses (e.g., bleaching) through time in terms of frequency and level.

The coral reefs of the FGBNMS occur at ~180 kilometers offshore from Galveston, Tex, USA, in clear, pristine waters where visibility is ≥ 30 m ([29]; J. A. Haslun, K. B. Strychar, P. W. Sammarco, pers. obs.). Although past monitoring efforts indicate a healthy reef environment with little to no bleaching, a high degree of bleaching was recorded in the fall of 2005 when sustained temperatures above mean monthly maximums (MMM) were recorded at the FGBNMS. Bleaching in the fall of 2005 was observed in 42–46% of all coral on both the East and West banks; this percentage of corals affected by bleaching decreased to 4–10% by January (2006), with most recovering over that three to four month seasonal time period [30]. This paper is believed to be the first of any type of massive bleaching experienced by these reefs [31].

Bleaching is defined as the whitening in appearance of a coral when the endosymbiotic zooxanthellae die or are lost, causing the underlying white calcium carbonate skeleton to become visible through the transparent coral tissue [32–34], particularly after exposure to acute or chronic increases in SWTs. Small changes to a reef ecosystem, particularly shifts in SWTs by 0.5–1.0°C above the MMM, can result in bleaching [35, 36]. Corals have also exhibited resilience following a bleaching event, recovering weeks to months later following thermal stress [6, 37]. Jones et al. [38] discuss two mechanisms by which this can occur: (1) the colony is either able to recover fully from thermal stress, or suffer only partial mortality due to the independence of individual polyps increasing their feeding rate within a colony; or (2) following a bleaching event, the coral is unable to respond to the loss of the symbiont resulting in colony mortality. Both of these trends have been well documented. For example, on the Great Barrier Reef (GBR) of Australia, *Acropora spp.* corals were subjected to uncharacteristically high SWTs, causing up to 88% mortality [39]. Oxenford et al. [40], however, reported bleaching equivalent to $\geq 70\%$ of coral species, located near Barbados in the southeastern Caribbean in 2005. They categorized this as the most severe bleaching event recorded in Barbados; however, mortality was minimal as many coral recovered. Such corals are hypothesized to “reassociate” with better-adapted zooxanthellae. This concept has been termed the “Adaptive Bleaching Hypothesis” (ABH) [41–43], and its effectiveness is dependent upon both the intensity and duration of the stress [6].

The symptoms of bleaching have also been traditionally broken down into 2 cell phenomena: (1) apoptosis and (2) necrosis [44–46], both collectively part of cell death processes. Apoptosis is a preprogrammed or encoded event that is initiated following a stress stimulus and subsequent signaling cascade within the cell, including a reduction in cell

size over time concurrent with the externalization of the membrane-bound molecule phosphatidylserine resulting in its dismantling without inflammation or the stress signaling of other cells [47–50]. Other characteristics of apoptosis include symmetrical DNA cleavage patterns (multiple integers of ~180–200 bp) for apoptosis, versus random patterns associated with necrosis. Necrosis, on the other hand, is indicative of extreme trauma, with characteristic increases in cell size followed by cell rupture and inflammation at the site of insult, resulting in cell death [47, 51]. Strychar et al. [52] documented these processes to occur not only in the zooxanthellae *in situ* but also in the tissue of host corals. Using flow cytometry, Strychar et al. [52] determined that the concentration of live zooxanthellar cells in water samples obtained from corals subjected to hyperthermal stress (34°C) decreased with time while the concentration of apoptotic and necrotic cells increased. Lower experimental temperatures did not induce significant apoptosis or necrosis. This was an indication that such corals were already adapted (or exapted) to higher seawater temperatures [10].

A third mode of cell death, autophagy [53, 54], has recently been observed to participate in cnidarian bleaching. Autophagy is a stress-dependent pathway in which identified targets including organelles, protein, and foreign intracellular pathogens are removed from the cell through the production of an autophagosome and digestion from hydrolytic enzymes therein ([55], reviewed by [56]). Dunn et al. [53] determined that autophagy occurs to some extent along with apoptosis during bleaching. Further, Downs et al. [54] reported on the autophagy of symbiotic dinoflagellates referring to this particular process as symbiophagy during the cnidarian bleaching response. Although these three modes of cell death appear to play a role in the bleaching response, their mechanisms still remain unclear.

The objectives of this study are to compare and contrast the physiology of zooxanthellae expelled from *Montastraea cavernosa* collected from two geographically separated sites—the FGBNMS and FKNMS. We will attempt to determine whether corals from the FGBNMS are more susceptible to thermal stress and exhibit a higher frequency of cell death responses (necrosis and apoptosis) than corals from the FKNMS, which are frequently reported to experience high seawater temperatures and thermal stress.

2. Methods

2.1. Target Species. We chose to use the host species *Montastraea cavernosa* as our experimental coral because it is well represented in both the FGBNMS (Texas) and FKNMS (Florida) and may be a good bioindicator species for thermal stress. The corals *M. cavernosa* (Linnaeus, 1767) and *Millepora alcicornis* (Linnaeus, 1758) are reported to be the most sensitive to thermal stress at the FGBNMS [30, 31]. Most other massive and encrusting species present, such as poritids and faviids, are much less sensitive [57–59]. *Montastraea cavernosa* is also abundant, easily recognizable, and its taxonomy has been well described.

2.2. Collection Sites. We sampled from one site within the Gulf of Mexico (GOM)—the West Flower Garden Bank (27° 52' 35.1'' N, 93° 48' 54.1'' W) National Marine Sanctuary (FGBNMS) located in the northwestern GOM, ~190 km SE of Galveston, Tex, USA [29]. The FGBNMS is comprised of two reefs, the East Bank (27° 54' 35.9'' N, 93° 35' 49.7'' W) and the West Bank, which are 19 km apart and 65.8 km² and 77.2 km² in area, respectively [60]. Seasonal water temperatures range from ~20°C in mid-February to 30°C in August [30]. The second site was the Florida Keys National Marine Sanctuary (FKNMS) which is ~4,506 km² and is located at the southern tip of Florida where the GOM loop current exits into the Caribbean [27].

2.3. Coral Collection and Maintenance. Twenty-four fragments of *Montastraea cavernosa* were collected from the West Bank of the FGBNMS with the assistance of SCUBA divers. Coral fragments ~2.5 cm² in size were excised from parent colonies at 23–25 m depth with a cold chisel and mallet (one fragment per colony) and placed in individual 50 mL plastic cylindrical bottles prefilled with water derived from the site of collection. Bottles were placed in netted collection bags and transported to the surface.

Aboard the vessel, individual coral fragments were removed from the bottles and submerged in a stress-relieving solution (Kent Marine Tech D parasite and bacterial dip) for 5 mins to prevent bacterial infection caused by excision from the parent colony. Samples were then placed and stored in a modified cooler fed with site-derived water pumped continuously from a depth of ~1 m using a submersible pump and a flow-through system to maintain a suitable environment for the coral during transport to Texas A & M University-Corpus Christi (TAMUCC). A portable aeration system provided oxygen during transport. At TAMUCC, corals were transferred to a 1,703 L quarantine tank consisting of a protein skimmer, refugium, UV light, water heater/chiller, and an automatic reverse osmosis deionized (RO DI) water-topping system for salinity maintenance. Fragments were acclimated to tank conditions for a minimum of two weeks prior to treatments and allowed to recover following collection. Coral color was monitored over this period using the Coral Color Reference Card technique developed by Siebeck et al. [61]. Specimens of *M. cavernosa* were used if they expressed the red color morph and were comparable to condition C6 of hue 8 on the reference card, indicating an abundance of symbionts equivalent to ~3.0 × 10⁶ zooxanthellae cm².

Coral representatives of the FKNMS were obtained with assistance from Mote Marine Laboratory Tropical Research Station, Key West, Florida. Twenty-four coral fragments ~2.5 cm² were collected in and around the Naval Air Station (NAS) ship channel from a depth of 11–12 m. The fragments were shipped to TAMUCC and placed in a quarantine tank similar to the FGBNMS fragments where they were monitored over the next two weeks using the Coral Color Reference Card [61].

Although the collection depths of each coral sample population were different, evidence from Lesser et al. [62] indicates that *M. cavernosa* populations lying between 11 and

30 m are not significantly different with respect to zooxanthellae population (cells cm⁻²), quantum yields, and chlorophyll concentration (total µg chl cm⁻³, total pg chl cm⁻³, and chl *a* : *c*₂ ratio) within the depth range of this study. Further, the cladal diversity of zooxanthellae within large geographic areas with respect to *M. cavernosa* has been shown to be consistently clade C although subtypes are present [63–68]. The areas of these studies span the Gulf of Mexico from the Bahamas to the Yucatan peninsula and to Bermuda. We also chose to only collect the adult red color morph of *M. cavernosa* in order to minimize the influence in host-derived pigments between fragments if present.

2.4. Experimental Design. We followed a Model I, repeated measures, three-way orthogonal experimental design in order to determine the thermal stress thresholds of *Montastraea cavernosa* from the FKNMS and FGBNMS. The three primary factors were water temperature, geographic site, and duration of exposure to water temperature. Each experimental trial consisted of twelve individual coral fragments collected as previously discussed. Four fragments were randomly assigned to each of three thermal stress temperatures (28, 31, and 35°C) for a total of *n* = 4 replicates. Fifty-milliliter water samples were collected from each replicate in 6 h intervals over 72 h in order to quantify and determine the physiological condition of expelled zooxanthellae. To increase the total number of replicates sampled, each trial was repeated twice (*n*_i = 8).

2.5. Flow-Through Seawater System. Thermal stress experiments were carried out in a “Flow-Through Seawater System” (FTSWS). The seawater used for this study was obtained from the Upper Laguna Madre, Corpus Christi, Tex, USA and delivered by Texas Parks and Wildlife Department (TPWD) to our FTSWS wet lab located at TAMUCC (Figure 1). All seawater was filtered through a 20 µm filter by TPWD prior to delivery. We then pumped the seawater through a 5 µm filter into five 189 L fiberglass cylindrical storage tanks (Figure 1). Practical salinity was maintained at 34 by the addition of deionized water when necessary. In this study, we are using practical salinity which is a dimensionless unit as recommended by the International Association for the Physical Sciences of the Oceans [59] and adopted by the International System of Units (SI) in 1985. A closed recirculation system consisting of a sand filter coupled with a UV filter (Figure 1) served as a tertiary filtration system for the experimental water, which was run for ≥2 days prior to any experimentation. Oxygen was delivered continuously via a Thomas 5030A electrical air pump, common to each of the five diffuser lines feeding the five cylindrical water tanks (Figure 1); oxygen levels were maintained at ~5–6 mg L⁻¹.

Seawater was gravity-fed from the five storage tanks into two manifolds. Each manifold fed six 19 mm hose barbs with Nalgene tubing. Each of these was attached to one of two 6-channel peristaltic pumps (PP; Figure 1). The PP controlled the rate of seawater entering each of the twelve 1 L incubation chambers (IC), delivering a constant flow rate of 12.5 mL min⁻¹ ± 0.3 mL to each IC (Figure 2). To help

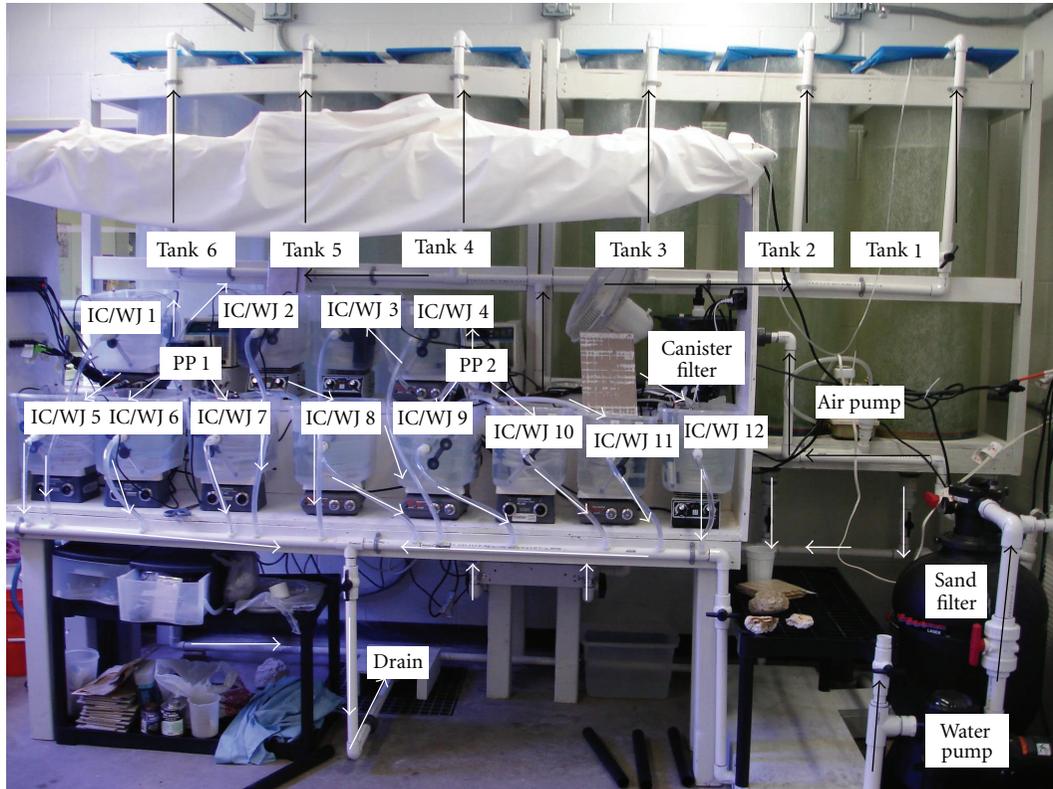


FIGURE 1: Flow-through seawater system consisting of sand and UV light filtration. Water was pumped into five of the six storage tanks through the filter system by a 1.5 hp centrifugal Jacuzzi pump (pump and filter system used only prior to experimentation to prevent pump cavitation). Incoming water enters the pump, passes through the sand filter and a 5 μm Canister filter coupled with UV-light sanitization, and is then passed to the storage tanks. During experimental trials, water is pumped to the twelve incubation chambers (IC), exiting *via* a drain.

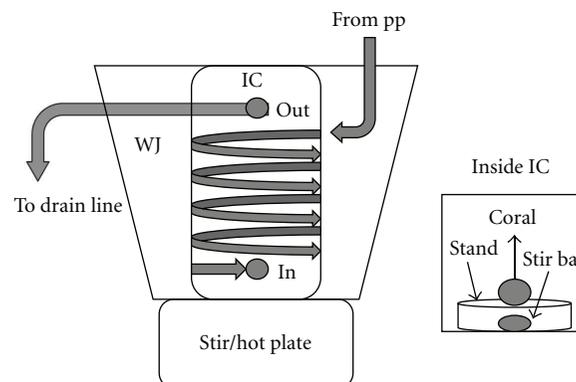


FIGURE 2: Schematic representation of a single incubation chamber (IC) and water jacket (WJ). Upper arrows represent the direction of water flow. Arrows encircling the IC represent tubing bathed within the WJ. Inset: magnified view of contents within an IC.

maintain IC seawater temperatures, each IC was housed in a 17.9 L water jacket (WJ), and each of these contained a 75 W Jager aquarium heater. In addition, 3 m of Nalgene tubing wrapped around the peripheral of the IC also helped maintain constant temperatures (Figure 2). From each PP, water was pumped through this tubing, subsequently entering each IC at one of three temperatures: 27°C (control), 31°C, or 35°C. Each IC contained a coral fragment, which

rested on a stand below which a single stir bar was placed (Figure 2). Each WJ was placed on top of a stir plate turned on at ~ 300 rpm in order to maintain a thermally well-mixed water column. Excurrent tubing from each IC was attached to a single drain line common to all chambers (Figure 1).

Environmental parameters were monitored every 12 h including flow rate (mL min^{-1}), practical salinity, temperature ($^{\circ}\text{C}$), and photosynthetically active radiation (PAR in

$\mu\text{E m}^{-2} \text{s}^{-1}$); photographs were also taken of coral fragments throughout each trial.

2.6. Sampling of Expelled Zooxanthellae. Sampling methods were adapted from Strychar et al. [44, 46]. Seawater samples (50 mL) were obtained every 6 h for 72 h using plastic syringes, one for each individual IC. Seawater samples were deposited into separate 50 mL Falcon conical centrifuge tubes and centrifuged at a relative centrifugal field of $700 \times g$ for 5 min in a Beckman Coulter Allegra 25R centrifuge. The supernatant was decanted leaving 5 mL remaining in the sample tube, and 2 mL of the suspended solution was equally separated into two 1.5 mL microcentrifuge tubes prior to centrifuging again at $700 \times g$ for 5 min in an Eppendorf Centrifuge 5415D. One group of aliquots (12 tubes) was used to determine the number of live, dead, and mitotic cells using light microscopy and a hemocytometer while a second group of twelve aliquots was used to assess cell viability using flow cytometry (FCM).

2.7. Light Microscopy and Hemocytometry. The supernatant from each tube of the first set of twelve aliquots was discarded, leaving 200 μL in each tube without disturbing the pelleted cells. The cells were stained with trypan blue (0.2%, BDH Chemicals) in phosphate-buffered saline (PBS; 1 : 1) and gently vortexed for 5 sec before incubating at room temperature for 5 min. Two 10 μL subsamples were then loaded into each side of a Neubauer-improved hemocytometer (Marienfeld), which were then visualized using a XDY-1 inverted microscope at $100\times$ magnification. Light microscopy helped to determine zooxanthellar density and cell viability. Cells were considered viable if their membranes were intact and the DNA-binding Trypan blue was excluded from the cell. Cells were considered mitotic if “doublets” were observed. Necrotic cells appeared blue and exhibited either the basic shape of live cells or the expulsion of their contents. Concentrations of cell types were determined using the following equation (traditional hemocytometer calculations, Hausser Scientific Co.):

$$\frac{\text{Zooxanthellae}}{\text{mL}} = \frac{((\text{total}/2 \text{ replicates})/5.0 \text{ mm}^2) \times 1000}{50 \text{ mL}} \times \frac{15 \text{ samples}}{\text{IC}}. \quad (1)$$

2.8. Flow Cytometry (FCM). The second set of aliquots consisting of twelve microcentrifuge tubes used for FCM were prepared using 100 μL of $1 \times$ Annexin-binding buffer (ABB) added to each microcentrifuge tube following the recommendations of the manufacturer. This step was followed by the addition of 1 μL propidium iodide (PI; 100 $\mu\text{g mL}^{-1}$; Invitrogen) and 3 μL of Annexin V-fluor 488 nm conjugate (Invitrogen). The 12 microcentrifuge tubes were then incubated at room temperature in the dark for 30 min. Following incubation, an additional 400 μL of ABB was added to each microcentrifuge tube and the contents gently vortexed for 1 min. After vortexing, the solution was transferred by

pipette to a 5 mL BD Falcon round-bottom tube (one tube per microcentrifuge tube) for analysis *via* FCM.

A FACSVantage SE flow cytometer (Becton Dickinson) equipped with a 488 nm laser was used to identify and quantify viable, necrotic, apoptotic, and postapoptotic zooxanthellar cells in each sample. A fluorescein isothiocyanate (FITC) 530/30 band-pass FL-1 filter detected green fluorescence whilst a LSRI Red 630/22 band-pass FL-3 filter was used to detect red fluorescence, in order to differentiate between each of the four cell death categories. On a scatter-plot of FL-1 versus FL-3 cells displaying increased phosphatidylserine exposure and thus apoptosis, such cells would be stained with the fluorescent green Annexin V-fluor and thus, display an increase in the FL-1 parameter. Cells displaying necrotic characteristics would be stained red by the membrane-impermeable dye PI and green as all phosphatidylserine will be stained by Annexin V-fluor. This results in live cells predictably in the lower left quadrant (no stains), apoptotic cells in the lower right quadrant (green fluorescence only), and dead cells in the upper right quadrant (red and green fluorescence). All data were visualized and plotted using the manufacturers CwllQuest-Pro software.

2.9. Statistical Analyses. Refer to section 2.4 for statistical model parameters. Coral fragments were chosen and assigned to treatments randomly. Data were analyzed using SPSS V17 and the general linear model (GLM) repeated-measures, analysis of variance (ANOVA). Hemocytometry and FC data were not compared directly due to differences in data format (percent versus count).

All data were tested for significant variation due to sphericity using the Greenhouse-Guysler correction (Sphericity < 0.75) or the Huynh-Feldt correction (Sphericity > 0.75; Field, 2009). Since the data lacked sphericity, Tukey's and Games-Hollow *post hoc* tests were applied after analysis *via* GLM repeated-measure ANOVAs to examine specific significant differences between various means. Data displaying significant variations over time (h) were Bonferroni corrected. Higher-order interactions will only be mentioned and discussed if significant.

3. Results

The environmental variables that were monitored, including temperature and PAR, were stable over the 72 h time period.

3.1. Hemocytometry: Flower Garden Banks National Marine Sanctuary (FGBNMS). The number of viable zooxanthellae expelled from the coral per unit time was initially very high and then decreased through time. When stressed at 35°C (maximum temperature treatment), a maximum of 2,750 cells mL^{-1} were expelled at 36 h; this dropped to 1,250 cells mL^{-1} at 66 h. At the lower temperatures, loss of cells from the holobionts was <1,000 cells mL^{-1} during any given time period (Figure 3(a1)). Necrotic zooxanthellar cells expelled from corals exposed to 35°C followed an increasing then decreasing trend, starting at 400 cells mL^{-1} at 6 h, increasing to 1,050 cells mL^{-1} at 42 h, and then falling to

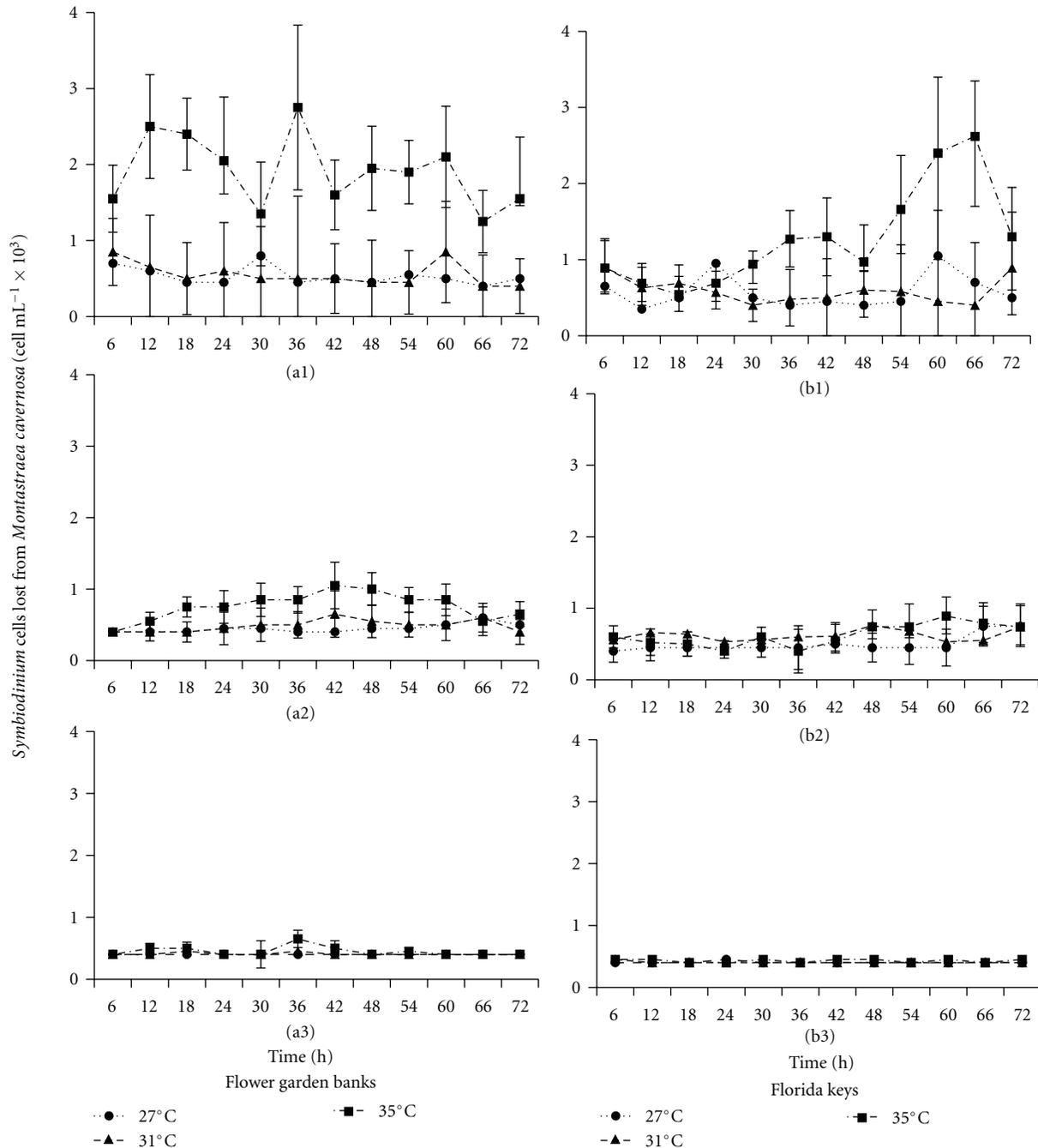


FIGURE 3: Hemocytometry data showing the mean temporal loss of viable (a1, b1), necrotic (a2, b2), and mitotic (a3, b3) zooxanthellae cells ($\times 10^3 \text{ mL}^{-1}$) expelled from *Montastraea cavernosa* collected from the Flower Garden Banks National Marine Sanctuary (a1–3)) and the Florida Keys National Marine Sanctuary (b1–3)) sampled every 6 h over a 72 h time period at 27°C (●), 31°C (▲), and 35°C (■). Error bars represent 95% confidence intervals ($n_i = 8$); some error bars are too small to be seen.

550 cells mL^{-1} at 66 h (Figure 3(a2)). There were no significant differences in the concentrations of mitotic symbiont cells expelled between any of the temperature treatments (Figure 3(a3)).

In terms of cumulative loss, both viable (Figure 4(a1)) and necrotic (Figure 4(a2)) expelled zooxanthellae were significantly greater in the 35°C treatment than in the 27°C

control and 31°C treatments (ANOVA; $P < 0.005$). The concentration of mitotic cells lost, however, did not vary significantly (Figure 4(a3)). Concentrations of live zooxanthellae were consistent across temperatures and time, with the exception of $t = 30, 36,$ and 66 h (Figure 4(a1): $P < 0.05$, Games-Hollow *post hoc* test). Significant differences in necrotic zooxanthellae concentrations were observed

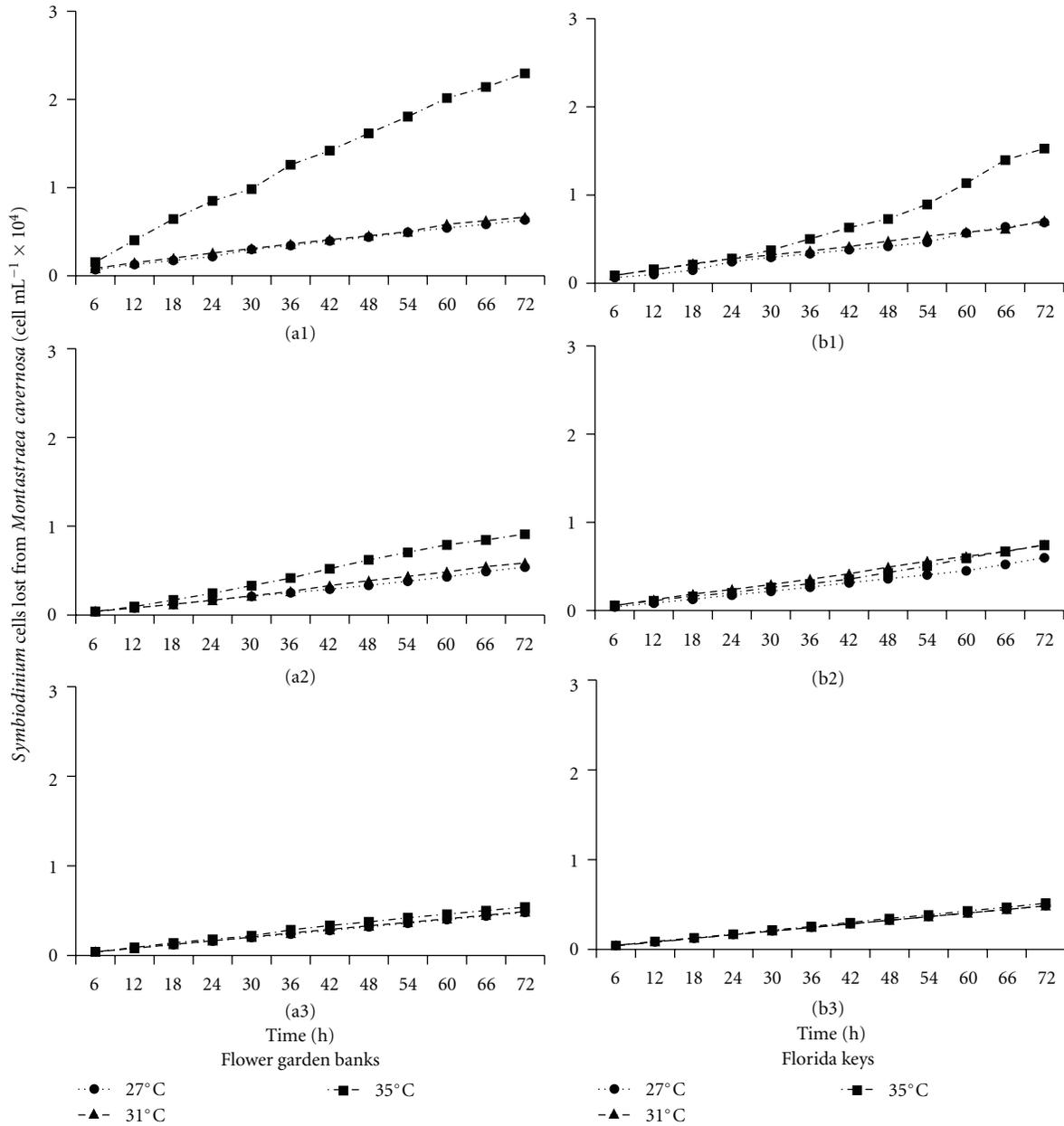


FIGURE 4: Hemocytometry data showing the mean cumulative loss of viable (a1, b1), necrotic (a2, b2), and mitotic (a3, b3) zooxanthellae cells ($\times 10^3 \text{ mL}^{-1}$) expelled from *Montastraea cavernosa* collected from the Flower Garden Banks National Marine Sanctuary (a1–3)) and the Florida Keys National Marine Sanctuary (b1–3)) sampled every 6 h over a 72 h time period at 27°C (●), 31°C (▲), and 35°C (■). Error bars represent 95% confidence intervals ($n_i = 8$); some error bars are too small to be seen. Note how cumulative graphs illustrate the effect of temperature on overall depletion of a coral’s *Symbiodinium* complement over 72 h.

between the control and maximum temperature treatment at $t = 18, 36, 42, 48,$ and 54 h ($P < 0.05$; Figures 3(a2) and 4(a2)). Total cumulative concentrations of viable, dead, and mitotic zooxanthellae at 27°C (control) and 31°C (Figure 4(a1-2)) did not vary significantly through time.

3.2. Hemocytometry: Florida Keys National Marine Sanctuary (FKNMS). Concentrations of expelled viable zooxanthellar cells varied significantly between all temperature treatments

(ANOVA, $P < 0.01$; Figure 3(b1)). Corals incubated at 35°C experienced a greater loss of symbiont cells than at 27°C and 31°C (ANOVA; $P < 0.05$; Figure 3(b1)). Concentrations of viable zooxanthellae did not vary with temperature when exposed for $\leq 30 \text{ h}$ ($P > 0.05$, GLM ANOVA). After 30 h at 35°C, concentrations of viable cells lost from the coral at first decreased to $\sim 600 \text{ cells mL}^{-1}$ at 18 h, increased to $2,750 \text{ cells mL}^{-1}$ at 66 h, and then decreased again to $\sim 1,300 \text{ cells mL}^{-1}$ at 72 h (Figure 3(b1)). Concentrations of

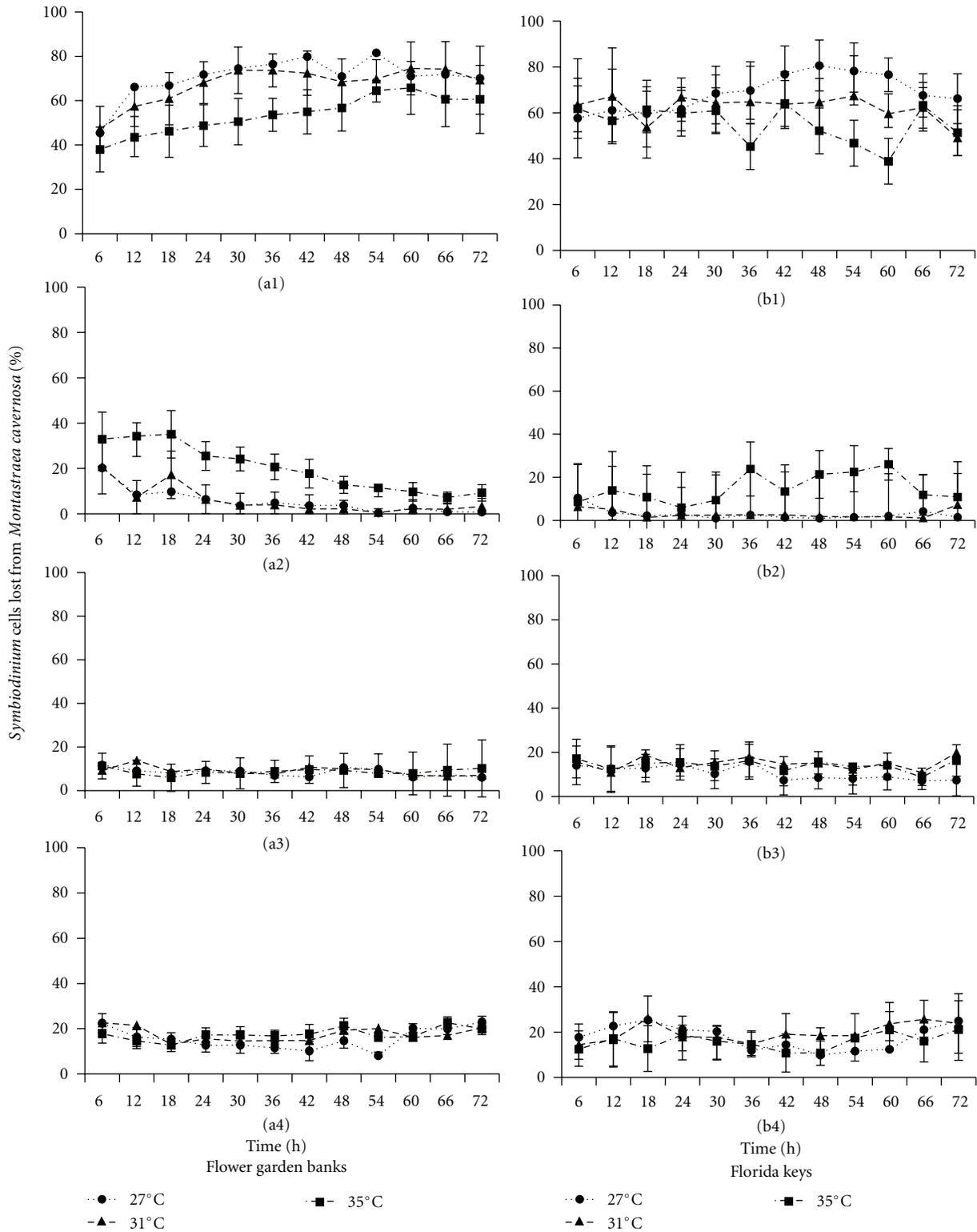


FIGURE 5: Flow cytometry data showing the mean temporal loss expressed as a percent (%) of viable (a1, b1), necrotic (a2, b2), apoptotic (a3, b3), and postapoptotic (a4, b4) *Symbiodinium* cells expelled from *Montastraea cavernosa* collected from the Flower Garden Banks National Marine Sanctuary (a1–3)) and the Florida Keys National Marine Sanctuary (b1–3)) sampled every 6 h over a 72 h time period at 27°C (●), 31°C (▲), and 35°C (■). Error bars represent 95% confidence intervals ($n_i = 8$); some error bars are too small to be seen.

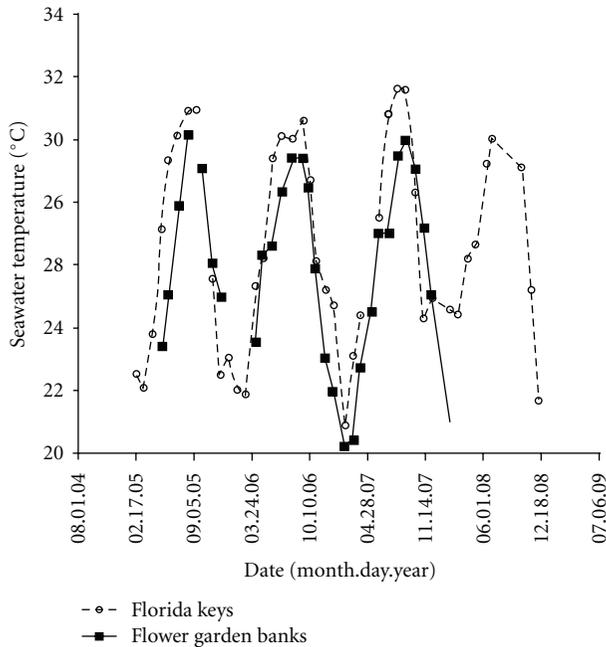


FIGURE 6: Temperature data spanning 2005–2008 from the West bank of the Flower Garden Banks National Marine Sanctuary (FGBNMS) and the Florida Keys National Marine Sanctuary (FKNMS). All measurements were taken with submerged YSI devices. The FGBNMS and FKNMS data set gaps were due to YSI maintenance and hurricane damage.

necrotic zooxanthellae released varied significantly between temperatures ($P < 0.01$) as well as time ($P < 0.05$, ANOVA). Concentrations of mitotic cells lost did not vary significantly between temperatures or through time (Figure 3(b3)).

In terms of cumulative loss, only viable (Figure 4(b1)) expelled symbiont cells show a significant overall loss through time ($P < 0.01$, ANOVA). This pattern was consistent throughout the experiment ($P < 0.05$, Games-Hollow *post hoc* test).

3.3. Flow Cytometry: Flower Garden Banks National Marine Sanctuary (FGBNMS). Flow cytometry revealed that zooxanthellar loss from FGB corals was significant through time (ANOVA, $P < 0.01$) and varied significantly across experimental temperatures (ANOVA, $P < 0.01$). The percent of viable zooxanthellae lost from the holobionts increased through time in all temperature treatments (Figure 5(a1)). Specifically, the mean percentage of viable expelled cells observed at 35°C was significantly lower than those at 27°C and 31°C ($P < 0.05$, Games-Hollow *post hoc* tests; Figure 5(a1)), with no significant differences between the latter two treatment effects ($P > 0.05$). The percentage of necrotic cells detected varied significantly between experimental temperatures ($P < 0.01$, ANOVA) as well as through time ($P < 0.01$). The mean percentage of necrotic cells expelled was the greatest at 35°C ($P < 0.01$, Games-Hollow *post hoc* test; Figure 5(a2)) with the greatest loss occurring within 24 h. There was a general decline in necrotic cells

over time ($p < 0.01$, ANOVA; Figure 5(a2)), although no significant differences occurred between the control and 31°C treatments. There was no significant variation between temperatures or through time in apoptotic ($P > 0.05$, Figure 5(a3)) and post apoptotic ($P > 0.05$, Figure 5(a4)) cells.

3.4. Flow Cytometry: Key West National Marine Sanctuary (FKNMS). Percent viable zooxanthellae did not vary significantly between temperature treatments ($P > 0.05$, ANOVA) or through time ($P > 0.05$, Figure 5(b1)). Necrotic cells were lost at significantly different rates between temperatures ($P < 0.01$, ANOVA) and times ($P < 0.01$, Figure 5(b2)). A higher number of necrotic zooxanthellae were expelled from coral at 35°C than at 27°C or 31°C, at 30, 60, and 66 h time intervals ($P < 0.01$). The percentage of apoptotic cells expelled did not vary significantly between temperatures; variation was detected, however, with time ($P < 0.01$, Figure 5(b3)). A difference in the incidence of postapoptotic cells was also evident through time ($P < 0.01$, Figure 5(b4)) but not between temperatures ($P > 0.05$). In general, the data followed a bimodal distribution through time for each temperature with two local maxima at ~25%, between 12 h and 18 h and another between 54 h and 72 h (Figure 5(b4)). Minima of ~10, 15, and 11% were recorded between 36 h and 48 h time intervals at 27°C, 31°C, and 35°C, respectively, (Figure 5(b4)).

4. Discussion

The rate at which corals can adapt to increases in seawater temperature is of great concern worldwide. Mean global SWTs have increased by ~0.5°C over the past century and are expected to continue to rise over the next several decades [69]. The deteriorating physiological state of expelled zooxanthellae is indicative of specific bleaching responses in a wide array of corals including both alcyonacean and scleractinian corals [45, 47]. We speculate that the observed differences we found in *Montastraea cavernosa* between the Flower garden banks and the Florida keys populations may be attributed to adaptation/exaptation [10] or symbiont subtype shuffling. That is, the adaptation may be associated with intermittent ~1 month-long increases in SWTs over the last few decades in corals in the Florida keys compared to that of the Flower garden banks (Figure 6).

The fact that increased concentrations of viable zooxanthellae were expelled by *M. cavernosa* fragments from the FGBNMS as opposed to those from the FKNMS at higher experimental temperatures implies that (1) symbionts in corals inhabiting the FGBNMS may be more heat-sensitive than those inhabiting the FKNMS; (2) symbionts in corals from FKNMS may have become better adapted or acclimated to increased seawater temperatures; (3) clade C symbionts with large temperature sensitivities are shuffled in *M. cavernosa*, or (4) the host itself has become better adapted to increased prevalence of heat stress in the FKNMS. Generally, bleaching occurs in corals exposed to temperatures $\geq 1^\circ\text{C}$ above the MMM in a given marine environment for a period

of time on the order of days or weeks, defined as “Degree Heating Weeks” [35, 36]. Goreau and Hayes [35] coined the term “Degree Heating Weeks” by developing a modeling program referred to as “Hotspot” in combination with a satellite thermal detection system to detect bleaching events. A “hotspot” was deemed a region where the sea surface temperature (SST: defined as the upper 1 m of ocean) exceeded the annual MMM climatological value by 1.0°C for an observed number of weeks. The fact that zooxanthellate coral from the FKNMS tolerated ~2 months of increased temperature (E. Bartels, pers. com.), reaching or surpassing the bleaching criteria suggested by Goreau and Hayes [35] in 2005 and 2007, suggests that FKNMS zooxanthellae corals appear better adapted or acclimatized to heat stress. Conversely, very few subsurface temperatures >30°C were recorded at the FGBNMS over a 3-year period during the three warmest months (August–October; J. DeBose, pers. comm.). Temperature rarely exceeds MMM temperatures although isolated mass bleaching events have been observed [30].

The increased frequency of longer-term (~2 months) low-intensity heat stress may have allowed the Florida keys *M. cavernosa* zooxanthellar population to become better adapted to these environmental changes. Middlebrook et al. [70] discussed the role of specific patterns of temperature change over time in a given region and its influence on temperature adaptation in populations. They noted that the zooxanthellate *Acropora* sp. (harboring solely clade C3) pre-stressed at 31°C for ≤2 weeks prior to a hyperthermic treatment could resist hyperthermic stress for at least 5 days (34°C); coral not receiving such pretreatment exposure experienced a large decrease in symbiont density. This significant change was attributed to adaptations within the host alone and not the zooxanthellae population, specifically photoprotective mechanisms. Our data support the conclusions of Middlebrook et al. [70]. Seawater temperatures that frequently exceed the MMM slightly over a period of ≤2 y in the Florida keys (E. Bartels, pers. com.) may have driven the coral host to become better adapted to heat stress. Although we cannot conclusively determine that clade C subtypes between sites had no effect on bleaching susceptibility and the expulsion of live, dead, or apoptotic zooxanthellae, authors from the published literature as previously discussed in section 2.3 suggest that the depths at which fragments were collected for this study, species utilized, and the condition of fragments at the point of collection [68, 71] allow for some interpretation with respect to the genetic diversity of clades within fragments between sites. Future studies should consider incorporating genetic studies to help determine if symbiont clade subtypes exist and whether those subtypes contribute to a host’s ability to withstand episodes of bleaching. Hence, it is highly possible that symbiont clade subtype shuffling may affect bleaching susceptibility. Lesser et al. [62] noted that unique subtypes in *M. cavernosa* were described at depth (>30 m), and these, therefore, may play an important role in the holobiont survivorship. Further, many coral species have been found to be host-specific subtypes especially in high temperature stressed areas. Oliver and Palumbi [72] report that the corals *Pocillopora damicornis*

and *Acropora pulchra* display distinct clade shuffling with respect to the frequency of high-temperature events. Clade C subtype C1 occurred in cooler pool inhabiting corals while Clade D was found in those coral inhabiting hotter pools. Although this dramatic difference in temperature was not the case with our particular sites, continued low degree temperature stress could impart a similar result. We, therefore, hypothesize that adaptation may be occurring in *M. cavernosa* at the FKNMS.

Low concentrations of apoptotic zooxanthellae cells were also detected in all temperature treatments from both experimental sites. The frequency of apoptosis in symbiont cells lost at all temperatures suggest that either the incubation at 31°C was not a significant stress, the length of time at each temperature treatment was too short, or the incubation at 35°C was too intense to allow the development of an apoptotic response, resulting in a larger concentration of necrotic zooxanthellar cells. By comparison, Strychar et al. [44] observed increased apoptotic zooxanthellae from 0 to ~30% in the Indo-Pacific coral *Acropora hyacinthus* when temperatures at 28°C and at 32°C were compared. They showed that zooxanthellate corals that were less susceptible to heat stress expelled higher concentrations of apoptotic zooxanthellae ranging from 0–10% under intense heat stress (34°C), with necrosis being more prominent (≤20%). Strychar and Sammarco [73, 74] and Sammarco and Strychar [10] have also shown that some host corals in the Indo-Pacific, specifically *Acropora hyacinthus*, *Porites solida*, and *Favites complanata* have a much broader temperature tolerance (i.e., 34–36°C) than their endosymbiotic zooxanthellae (30–32°C), exhibiting adaptation (or exaptation) towards increased SWTs. Fitt et al. [75] observed similar results in the Indo-Pacific corals *Porites cylindrica* and *Stylophora pistillata*, and reviews by Baird et al. [76], who comparatively assessed the role of coral hosts versus zooxanthellae in a wide variety of corals worldwide, support the concepts of Strychar and Sammarco [73, 74] and Sammarco and Strychar [10]—such that exaptation to heat stress by coral hosts may result from numerous prior exposures to varying climates.

The growth of *Symbiodinium* by mitosis *in situ* under bleaching conditions provides information regarding whether a host coral will survive, maintain its standing stock of zooxanthellae, or become reinfected by an alternate zooxanthellar clade. The standing stock density of zooxanthellae is regulated by symbiont reproduction *in hospite* as well as by the degradation and removal of zooxanthellae through the gastrodermal cavity and tentacles of the host [77–79]. Here, the expulsion of mitotic zooxanthellae was relatively constant in experimental corals from the FGBNMS and FKNMS. Strychar et al. [45], however, noted that expelled zooxanthellae exhibited higher growth rates at 34°C versus lower experimental temperatures. The low abundance of mitotic zooxanthellae we observed in *M. cavernosa* may indicate a preference toward host autotrophy (i.e., ingesting the zooxanthellae) versus increased host heterotrophy *via* polyps increasing their feeding rate. Increased heterotrophy is known to occur in some cnidarians when under stress (84, 85, Goreau, pers. com.).

Interestingly, we note that the homeostatic control of zooxanthellae, although not monitored extensively (zooxanthellae were not continuously collected) in this study, was observed. It should, therefore, be concluded that simple alterations to the methods used for such studies including the continual collection of zooxanthellae over a given period of time be employed in order to understand the control of endosymbiont concentration within a host coral. Information regarding the homeostatic control of endosymbionts with respect to sensitive and hearty coral species may provide further insight into the importance of host endosymbiont interactions as well as a means to determine the role of specific proteins on host control of zooxanthellae density.

5. Conclusion: Adaptation in *Montastraea cavernosa* to Heat Stress

Plant/animal symbioses require cooperative communication between the host and symbiont (i.e., cell signaling) in order to remain effective [80]. It is important to again note that the diversity and concentration of zooxanthellae within colonies of *M. cavernosa* has been shown to be consistently clade C and between $2.3\text{--}4.2 \times 10^6$ cells cm^{-2} [62–68]. Further this study utilized only the red color morph of *M. cavernosa*, and, therefore, the pigments associated with the host were similar for all fragments. Isolating the degree and duration of heat stress, we hypothesize that during heat stress, the sensitive connection that evolved over many millennia between the coral zooxanthellar symbioses is potentially caused by the disruption of the Calvin Cycle [42, 81]. Due to the large percentage of carbon supplied to the host by algal photosynthesis *in hospite*, the symbiosis has evolved mechanisms to reduce the impacts of harmful byproducts (e.g., radical oxygen species—ROS), most likely through fluorescent proteins [26]. In organisms not repeatedly exposed to such stressors, mechanisms to cope with increased stimulation of apoptosis and any associated byproducts may not be well developed and subsequently may cause particular species to show increased symptoms of bleaching. Those species that show increased sensitivity to bleaching, for example, FGBNMS coral as opposed to FKNMS coral, may require increased frequency of exposure to mild heat stress (i.e., acclimation) and/or an increased period of time over which seawater temperature increase (i.e., adaptation) to become less sensitive.

To our knowledge, this is the first study that has experimentally examined the effects of heat stress on *Montastraea cavernosa* in the Caribbean (i.e., FKNMS) versus the Gulf of Mexico (i.e., FGBNMS). Further, we believe these sites to be a unique opportunity to determine the response of coral to bleaching particularly with respect to the degree that host adaptation/exaptation as well as symbiont shuffling may interact to resist thermal stress. *In situ* bleaching observations by Precht et al. [30] indicate that 4–10% of all corals are suffering effects of increased thermal stress at the FGBNMS. Our data indicate a potential source for the variation in this susceptibility to heat stress and bleaching, which we propose as regional adaptation most likely based on range and variation of temperatures not normally experienced.

Acknowledgments

The authors thank the Texas Parks and Wildlife for seawater transportation services, Texas Flower Garden Banks National Marine Sanctuary (Permit No. FGBNMS-2007-010) and the Florida Keys National Marine Sanctuary (Permit No. FKNMS 0648-0141) for permits given K. B. Strychar. They also thank Texas Research Development Fund for grants to K. B. Strychar. Special thanks to G. P. Schmahl and E. Hickerson at NOAA (FGBNMS) for ship time and J. Delaney and E. Bartels at Mote Marine Lab for collecting coral.

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