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The Effects of Thermal Stress and Algal Competition on the Early Life-History Stages of Porites astreoides and the Development of Stress-Detecting Biomarkers for Use in Scleractinan Corals

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The effects of thermal stress and algal competition on the early life-history stages of *Porites astreoides* and the development of stress-detecting biomarkers for use in scleractinian corals

by

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CERTIFICATE OF APPROVAL

The effects of thermal stress and algal competition on the early life-history stages of *Porites astreoides* and the development of stress-detecting biomarkers for use in scleractinian corals by Kevin Calvin Olsen

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I wish to specially thank my mother Eileen Olsen for unwavering support and instilling in me the importance of hard work and higher education.

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Abstract

Scleractinian coral populations are declining worldwide in response to numerous stressors operating on both global and regional scales. Rising sea surface temperatures associated with global climate change and the increasing frequency of coral-macroalgae competitive interactions are two of the gravest ecological drivers facing coral reef ecosystems. However, little is known about how these stressors interact to impact corals, their health, and potential modes of population recovery. These threats also highlight the need to develop reliable techniques that detect stress in multiple life-history stages of hermatypic corals prior to the degradation of coral reef habitats. To address these concerns we evaluated the effects of elevated sea surface temperatures (+3.5°C), *Dictyota menstrualis* competition, and their combined impacts on three life-history stages of the reef-building coral *Porites astreoides*. Elevated temperature induced sub-lethal stress yet had varied responses that were contingent on the life-history stage being examined. Hyperthermal stress did not consistently effect the transcriptional expression of heat shock proteins (Hsp) 16 or 60, but was readily detected utilizing biomarkers of the oxidative stress pathway. The presence of *D. menstrualis* significantly reduced coral survival and recruitment beyond simple space occupation in every coral life-history stage examined. While macroalgal exposure and elevated temperature had distinct effects on coral survival and physiological condition, the combination of both stressors induced a synergistic impact on biomarkers of oxidative stress in coral larvae. The results highlight the potential of biomarkers of oxidative stress for detecting hyperthermal stress in scleractinian corals. They also support the accepted notion that benthic macroalgae compete with reef-building corals via direct contact for space on coral reefs and that elevated temperatures can reduce the health of the coral holobiont. In addition, the results indicate that larvae from *P. astreoides* are more susceptible to

the impacts of hyperthermal stress compared to established corals and that multiple perturbations can interact to exacerbate coral health.

1.

Introduction

1.1 Hermatypic Corals

Hermatypic corals are an ecological functional group composed of sedentary cnidarians characterized as being reef-building or, "having the capability to erect a wave resistant skeleton" and by possessing symbiotic zooxanthellae within their tissues (Wells 1933; Shumacher and Zibrowius 1985) (Fig.1.1). As ecological engineers, hermatypic corals are vital for building and maintaining the foundation of coral reef ecosystems. Coral reefs are key coastal habitats that promote marine biodiversity and provide a multitude of services that benefit human societies (Barbier et al. 2011).

Over the past 30 years however, coral reefs have faced significant global losses in live coral cover (Knowlton 2001; Hughes et al. 2007). These coral reef systems are becoming increasingly impacted by a myriad of interacting stressors, operating on both global and regional scales, resulting in mass degradation (Hoegh-Guldberg 1999). Reef degradation often results in community shifts from topographically complex coral systems to species-poor habitats that are dominated by macroalgae (Hughes 1994; McCook 1999; Hughes et al. 2007). These "phase shifts" have been most pronounced in the Caribbean where reefs have suffered a ~ 80% loss in live coral cover over the last three decades (Gardner et al. 2003).

While numerous studies have examined the effects of independent stressors on adult corals (Glynn and Croz 1990; Jokiel and Coles 1990; Desalvo et al. 2008; Rasher and Hay 2010), little is known about how compounding stressors impact multiple coral life-history stages and the recruitment process. Larval supply, settlement, and subsequent growth to sexual maturity are all key steps in the process of coral recruitment (Fig.1.2). Consequently, successful recruitment is

Figure 1.1 The Caribbean hermatypic coral *Porites astreoides* (photo: Kevin C. Olsen)

Figure 1.2 Larva from the coral *Porites astreoides* (DIC image: Kevin C. Olsen)

necessary for maintaining coral populations and for contributing to the resiliency of coral reefs (Ritson-Williams et al. 2009).

1.2 Climate Change and Associated Thermal Stress

Rising ocean temperatures associated with global climate change represents a major threat to tropical marine ecosystems (Koch et al. 2013). Since many reef-building corals are already near their thermal maxima, modest temperature increases of 1-2 °C can be particularly damaging to the health of corals (Hoegh-Guldberg 1999). Elevated temperature can promote the overproduction of reactive oxygen species (ROS) in the symbiotic zooxanthellae of corals (Lesser 1997), subsequently rendering corals prone to large-scale disease, bleaching, and mortality events (Jokiel and Coles 1990; Boyett et al. 2007; Bruno et al. 2007).

Due to increasing concentrations of anthropogenic CO_2 in the atmosphere, global ocean temperatures have increased by an average of 0.74 ^{o}C and are expected to increase 1-4 ^{o}C by the end of the next century (Hoegh-Guldberg et al. 2007; IPCC 2007). Based upon current greenhouse gas emission scenarios (IPCC 2007), sea surface temperatures are projected to increase rapidly over the next hundred years, from 1.8 ^{o}C above the current average (Low-emission B1 scenario) to 4.0 ^{o}C on average above current mean ocean temperatures (2.4 ^{o} to 6.4 ^{o}C) under the High-emission A1F1 scenario (IPCC 2007).

The negative effects of elevated seawater temperature on larval and adult mortality, as well as adult bleaching, have been relatively well studied (Edmunds et al. 2001; Lesser 1997; Weis 2008). Elevated seawater temperatures have been shown to affect the survival, settlement, and metamorphosis of corals (Bassim and Sammarco 2003; Nozawa and Harrison 2007; Randall

and Szmant 2009), yet only a few studies have described the physiological sublethal stress responses of corals exposed to thermal stress (Yakovleva et al. 2009; Kenkel et al. 2011).

Gene expression analysis has proven to be a useful tool for studying the physiological response of corals to a variety of stressors. Micro-array and/or quantitative-PCR (Q-PCR) analyses have been utilized to evaluate gene regulation in adult corals exposed to light and thermal stress (Desalvo et al. 2008; Desalvo et al. 2010; Csaszar et al. 2009; Kenkel et al. 2011), toxicants (Morgan et al. 2001) and elevated salinity (Edge et al. 2005). Limited studies however have described the effects of hyperthermal stress on gene regulation during the early life history stages of corals (Rodriquez-Lanetty et al. 2009; Voolstra et al. 2009). The expression and production of heat shock proteins (Hsps) in particular, have been used to characterize the response of many organisms to a variety of environmental stressors (Lindquist 1986). Hsps are molecular chaperones with a variety of functions, including protein folding, and the attainment of enhanced thermotolerance (Welch 1992; Parsell and Lindquist 1993). Thus, evaluating the transcript abundances of Hsp encoding genes in conjunction with established metrics of coral health (photophysiology, biomarkers of oxidative stress) can provide insight to the physiological condition of the coral.

1.3 "Phase-Shifts" and Coral-Algae Competition

Benthic macroalgae are important members of reef communities and provide vital ecological functions necessary for sustaining healthy coral reef ecosystems (reviewed in Fong and Paul 2011). Over the last few decades however, the distribution and abundance of fleshy macroalgae have increased significantly on coral reefs and have greatly replaced the historically dominant encrusting crustose coralline algae (Littler and Littler 1988; Lirman and Biber 2000;

Kuffner et al. 2008). The proliferation of fleshy macroalgae is often associated with the declining status of coral reefs globally and increases the frequency of coral-algae competitive interactions (Miller 1998; McCook 1999) (Fig 1.3).

Competition for space is an important factor in structuring the distribution of organisms in benthic communities (Jackson and Buss 1975; Thacker et al. 1998; Chadwick and Morrow 2011). In particular, coral-algae interactions are considered fundamental to the overall status of coral reefs, especially in the context of regional community dynamics and multiple shifting stable states (Knowlton 1992). Macroalgae can overgrow, chemically exclude, and replace reef-building corals, significantly reducing the functionality of coral reefs and their resiliency to disturbance (Hughes 1994; Ostrander et al. 2000; Nugues and Bak 2006; Rasher and Hay 2010; Paul et al. 2011) (Fig.1.3). In the Caribbean, depressed herbivory rates due to overfishing and the massive die-off of the sea urchin *Diadema antillarum* have increased macroalgal abundance and the frequency of coral-algae interactions (Hughes 1994; Mumby and Steneck 2008). Specifically, members of the brown macroalgal genus *Dictyota spp.* are conspicuous and have been shown to reduce coral survival (Rasher and Hay 2010) and larval settlement (Kuffner et al. 2006; Paul et al. 2011). *Dictyota menstrualis* is common on reefs in the Caribbean and possesses a suite of secondary metabolites with anti-herbivory properties (Pereira et al. 2000; Tiexeira et al. 2001). The chemical potency of this algae is pertinent, as allelopathy is believed to be one of the main mechanisms utilized by macroalgae in competing with reef-building corals (reviewed in McCook et al. 2001; Rasher and Hay 2010; Paul et al. 2011). Furthermore, the presence of *D. menstrualis* has been shown to negatively affect the process of recruitment, which is necessary for the recovery of coral populations (Kuffner et al. 2006; Box and Mumby 2007).

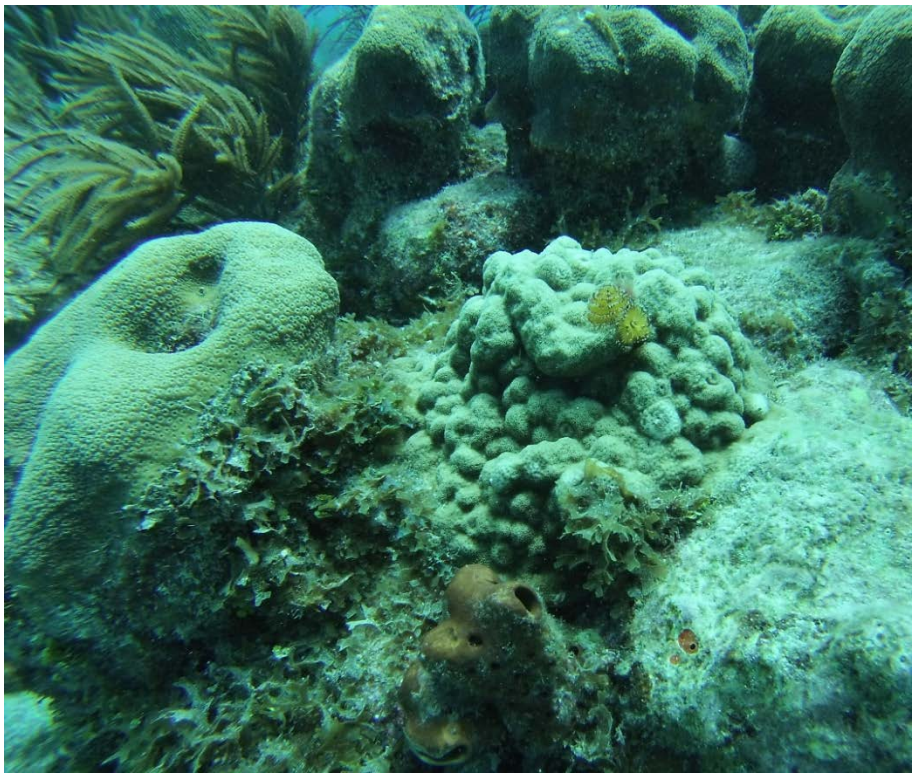

Figure 1.3 Coral-macroalgal competition between *Dictyota spp.* and several hermatypic corals (photo: Kevin C. Olsen)

The previous research involving hermatypic corals, thermal stress and macroalgal competition raise several questions in the context of coral recruitment in the face of interacting global and local stressors:

1. Can Hsp transcript levels be used as a biomarker of thermal stress in *P. astreoides*?
2. What roles do thermal stress and *Dictyota menstrualis* presence play in the context of coral recruitment?
3. When combined do the two stressors interact synergistically to further reduce coral recruitment?
4. Do corals become more resistant to these stressors as they age?

The goal of this thesis was to address these questions by conducting two major studies. The first evaluated the effects of elevated seawater temperature (+3.5°C) on *P. astreoides* larvae. This study utilized quantitative reverse transcription PCR (q-RT-PCR) to describe the transcriptional regulation of Hsp in corals exposed to thermal stress and provided a baseline on a technique rarely used in coral larvae. The second study evaluated the effects of elevated temperature (+3.5°C) and *D. menstrualis* presence on three life history stages of *P. astreoides* [larvae, juvenile (6 weeks-old), and adults (1-2 years-old)]. Both studies utilized integrative methodologies (ecology, physiology, and molecular biology) to evaluate the effects of these stressors on coral health, mortality, and recruitment.

2.

Detecting hyperthermal stress in larvae of the hermatypic coral *Porites astreoides*: The suitability of using biomarkers of oxidative stress versus heat shock protein transcriptional expression

Abstract

Scleractinian coral populations are declining worldwide in response to a variety of factors including increases in sea surface temperatures. To evaluate the effects of predicted elevated seawater temperatures on coral recruitment, larvae from the coral *Porites astreoides* were exposed to seawater at ambient (27.3°C) or elevated temperature (30.8°C) conditions for 4, 24 or 48 h. Following exposure, larvae were tested for survival and settlement, oxidative stress, respiratory demand, and mRNA expression of heat shock proteins (Hsps) 16 and 60. While elevated temperature had no effect on larval survival, settlement, or expression of Hsps, it did cause a significant increase in larval respiration, oxidative damage (lipid peroxidation) and antioxidant enzyme activity (catalase). The absence of a significant up-regulation of Hsp 16 or 60 expression in response to thermal stress suggests that the transcriptional expression of these genes is a less sensitive diagnostic tool compared to biomarkers of oxidative stress at the temperatures examined. The results of this study provide evidence that enhanced levels of oxidative stress are encountered in zooxanthellae-containing coral larvae in response to elevated temperatures, and that this occurrence should be strongly considered for use as a biomarker when monitoring sub-lethal cellular responses to rising sea surface temperatures.

2.1 Introduction

Reef-building corals are ecosystem engineers that build and sustain the foundation of systems that promote marine biodiversity and provide a multitude of services that benefit human societies (Barbier et al. 2011). Unfortunately, as a result of increasing environmental stress, coral cover is rapidly declining on a global scale (Gardner et al. 2003; Bruno and Selig 2007). While attention has been focused on how global scale stressors (e.g. elevated seawater temperatures and ocean acidification [OA]) will impact adult corals (Hoegh-Guldberg et al. 2007), local stressors such as overfishing and land-based sources of pollution are adding to the complexity of how stressors interact to reduce coral cover. Furthermore, these stressors can negatively impact coral survival during all life history stages and can inhibit the process of coral recruitment (Edmunds et al. 2001; Kuffner et al. 2006; Ritson-Williams et al. 2009), thus highlighting the need to develop reliable technologies to efficiently monitor coral health prior to the onset of death.

Climate change and the associated increases in ocean temperature represent a major threat for tropical marine ecosystems (Hoegh-Guldberg et al. 2007; Koch et al. 2013). Studies documenting the sensitivity of adult corals and their residential endosymbiotic zooxanthellae to thermal stress are extensive (Jokiel and Coles 1990; Lesser 1997; Hoegh-Guldberg 1999; Downs et al. 2002; Weis 2008). Conversely, there is much less work describing the impacts of elevated temperatures on coral larvae viability, settlement, and post-settlement survivorship (Edmunds et al. 2001; Bassim and Sammarco 2003; Nozawa and Harrison 2007; Randall and Szmant 2009; Ross et al. 2013). The short-term planktonic period is a critical life history phase of many marine organisms and has a major impact on dispersal, connectivity, and recruitment (reviewed in Pechenik 1999; Graham et al. 2008; O'Connor et al. 2007).

Over the last decade, several key studies have identified early molecular responses of coral larvae to hyperthermal stress (Rodriguez-Lanetty et al. 2009; Voolstra et al. 2009; Yakovleva et al. 2009; Polato et al. 2010). Two ubiquitous biological groups that consistently emerge as putative markers of temperature-induced cell stress responses are the oxidative stress pathway and the involvement of molecular chaperones such as heat-shock proteins (Hsps). Oxidative stress arises from the imbalance of the generation and removal of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) (Halliwell 2006; reviewed in Lesser 2006). The accumulation of these compounds in the coral holobiont can damage multiple intracellular targets, including proteins, lipids, and nucleic acids (Weis 2008), and can be quantified by measuring levels of protein carbonylation, lipid peroxidation, and cyclobutane pyrimidine dimer formation, respectively (Downs et al. 2000; Downs et al. 2002; Lesser and Farrell 2004; Ross et al. 2010; Ross et al. 2013). In addition, the up-regulation of antioxidant enzymatic machinery (e.g. catalase, superoxide dismutase) in response to elevated ROS levels can serve as a secondary means to detect the sub-lethal stress response (Downs et al. 2000; Downs et al. 2002; Griffin et al. 2006; Ross et al. 2010; Ross et al. 2013).

Heat-shock proteins are ubiquitous components of cells that serve as molecular chaperones to facilitate the folding of newly translated proteins, assist with protein translocation, and regulate apoptosis (reviewed in Feder and Hofman 1999). While some coral-based studies have demonstrated that selected Hsp levels increase in response to elevated temperature (Black et al. 1995; Hayes and King 1995; Sharp et al. 1997; Fang et al. 1997; Branton et al. 1999; Gates and Edmunds 1999; Downs et al. 2000; Downs et al. 2002; Kingsley et al. 2003; Robbart et al. 2004; Desalvo et al. 2008; Fitt et al. 2009; Desalvo et al. 2010), other studies reported no change

or even a decrease in Hsp content following thermal stress (Downs et al. 2000; Edge et al. 2005; Csaszar et al. 2009; Voolstra et al 2009; Mayfield et al. 2011). While differences occur in Hsp expression likely due to the natural variability within a population (Crawford and Oleksiac 2010) and the degree of inducibility of the particular Hsp of interest (Feder and Hofman 1999), the experimental conditions and detection techniques employed (i.e. immunoblot versus gene expression) undoubtedly contribute to the different activity of Hsps documented in the literature. The natural variability of transcriptomic expression both within and between species suggests that robust stress detection, based on gene expression of Hsps, may be problematic in scleractinian corals.

Most recently, Kenkel et al. (2011) developed a highly sensitive "*Porites* stress index" (PSI) based upon the magnitude of differential expression between the up-regulation of Hsp 16 and the down-regulation of actin in adult *P. astreoides*. In response to heat-light stress, fragments of *P. astreoides* displayed a ~800-fold up-regulation in the real-time expression of Hsp 16. These results prompted our efforts to determine if Hsp 16 expression could be used as a sensitive biomarker of sub-lethal thermal stress in larvae of *P. astreoides* in comparison to oxidative stress assays (Ross et al. 2010; Ross et al. 2013). This study tested whether Hsp expression (utilizing quantitative PCR to monitor Hsp 16 and 60 transcript levels) could serve as an accurate means of identifying sub-lethal stress associated with elevated temperature, in comparison to oxidative stress assays that specifically targeted lipid peroxidation and catalase activity.

2.2 Materials and Methods

2.2.1 Larval Collection and Treatments

Thirty adult colonies of *P. astreoides* were collected off of Wonderland reef at 6 m depth (GPS N 24°33.62□; W 81°30.08□), transported to Mote Tropical Research Laboratory (Summerland Key, FL) in coolers, and maintained in running seawater. Brooded larvae were collected 1 day prior to the new moon (night of May 2, 2011; McGuire 1998). Larvae were collected and pooled as previously described by Ross et al. (2013) to ensure that the planula population was well mixed and to reduce any maternal effect. Adult colonies were later returned to the site of collection and reattached with Z-Spar Splash Zone Compound® underwater epoxy.

One day-old larvae (newly released) were exposed to either control (27.3°C) or elevated temperature (30.8°C) treatments for 4, 24, or 48 h using the following split-plot factorial design. Twenty, 7 L plastic aquaria were held in one of three different flow-through seawater tables. Each aquarium served as an independent water bath and was randomly assigned to be either heated +3.5°C using a 75-watt adjustable aquarium heater (Commodity Axis, Inc. Camarillo, CA, USA) or maintained at ambient temperature (10 heated aquaria/10 ambient aquaria). In turn, every aquarium housed three 400 ml tri-pour beakers which contained the larvae and corresponded to treatment duration (4, 24 or 48 h). Each of the 400 ml beaker replicates contained 125 larvae in 200ml of seawater.

The tri-pours were clipped onto the inside of the aquaria via clothespins and were continuously monitored to maintain their respective temperatures using a YSI model 85 multiprobe meter (YSI, Yellow Springs, OH, USA). All replicates were maintained at a constant salinity of 35‰ and were shaded allowing penetration of 10% photosynthetic active radiation (< 200 μmol $m^{-2}s^{-1}$). Photosynthetic photon flux fluence rates (PPFFR) were measured using a LI-

193 underwater spherical quantum sensor in conjunction with a LI-250A light meter (LI-COR, Lincoln, NE, USA).

Following 4, 24 or 48 h of exposure, 50 larvae from each replicate were removed for settlement and survival assays. Five larvae per replicate were used for oxygen respiration studies. Fifty and 20 larvae were separately flash frozen in liquid N_2, transported to UNF on dry ice and stored at -80°C for oxidative stress and quantitative reverse transcription PCR (q-RT-PCR) analyses, respectively.

2.2.2 Larval Oxygen Consumption

Larvae of *P. astreoides* were analyzed for their respiration post-treatment according to the methods of Ross et al. (2013) with minor modification. Respiration studies were conducted using an Oxygraph system outfitted with a DW3 liquid-phase electrode chamber (Clark type polarographic sensor; Hansatech Instruments, Norfolk, UK). Each replicate (n = 10 for all treatments) consisted of five larvae contained within a 2.5-ml reaction volume of O_2-saturated filtered seawater (0.45 µm). All samples were dark adapted for 1 h prior to any measurement. The system was calibrated by eliminating oxygen with the addition of sodium hydrosulfite. An O_2 maximum was created by bubbling air into filtered seawater. Oxygen uptake was measured in the dark over the course of 10 min for each replicate. Control blank runs, consisting of filtered seawater, were not subtracted from experimental runs as it was noted that filtered seawater alone did not undergo any change in oxygen consumption over the time course studied in this experiment. Respiration was calculated as nanomoles of oxygen consumed per minute per larva.

2.2.3 Oxidative Stress Assays

Oxidative stress in larvae of *P. astreoides* was measured using the methods described by Ross et al. (2013). The fifty frozen larvae were thawed to room temperature and each sample was extracted in 2.5 mL of buffer (50 mM potassium phosphate buffer [pH 7.0] containing 10% [w/v] polyvinylpolypyrrolidone [PVP]-40, 0.25% Triton X-100, and 1% [v/v] plant cell protease inhibitor cocktail [Sigma–Aldrich, St. Louis, MO, USA]). Larvae were homogenized with a Fast Prep 24 bead homogenizer (MP Biomedicals, Irvine, CA, USA) and centrifuged at 16,000 x g for 10 min. The resulting supernatants were used for oxidative stress assays and normalized for protein content using the Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Pittsburgh, PA, USA).

To assess the impacts of ROS on cellular integrity, lipid peroxidation was assayed. Unsaturated lipids of cell membranes are common targets of oxidative attack by ROS. The end result is lipid peroxidation, a destructive process that compromises normal cellular function. Lipid peroxidation was measured using a Lipid Hydroperoxide Assay kit (Cayman Chemical, Ann Arbor, MI, USA). Catalase (CAT) is a widely distributed enzyme that destroys H_2O_2 by dismutation to O_2 and H_2O (Halliwell 2006). Upregulation of CAT reflects an organism's response to counteract the presence of damaging ROS. Catalase was assayed using Amplex Red (Invitrogen Corporation, Eugene, OR, USA) as per the manufacturer's instructions. A few replicates were not included in the analysis due to no protein content (4 h control n = 6, 4 h heat n = 5, 24 h control and heat n = 9, 48 h control and heat n = 8) for both lipid peroxidation and CAT activity.

2.2.4 Quantitative Reverse transcription PCR (q-RT-PCR)

RNA Isolation

Total RNA was isolated from twenty larvae per replicate using the TRI reagent protocol (Molecular Research Center, Cincinnati, OH, USA). Total RNA concentrations and purities were calculated using absorbance values of 260 and 280 nm measured with a Bio-Tek® plate reader (Bio-Tek, Winooski, VT, USA).

Quantitative One-Step Reverse Transcription-PCR

Porites sp. gene specific primers were designed and validated by Kenkel et al. (2011). Gene specific primers were diluted to 5 µM and relative standard curves for each primer set were generated using serial dilutions of total RNA encompassing two orders of magnitude from 0.078-5.0 ng. Reverse Transcription-PCR was performed utilizing 1 ng total RNA per reaction. The appropriate volume from each RNA sample equivalent to 1 ng total RNA was added to 1µL forward primer, 1µL reverse primer, 1.2 µL random hexamer primer (Ambion Life Technologies, Grand Island, NY, USA), 12.5 µL SYBR Green Reaction Mix® (Bio-Rad, Hercules, CA, USA) and enough nuclease-free water to sum to 25 µL of volume for each gene primer set (Hsp 16, Hsp 60, and 18s). All runs were performed on a MiniOpticon Real-Time PCR system® (Bio-Rad) using the default protocol (50°C for 10 min, 95°C for 5 min, followed by 49 cycles of 95°C for 10 s, 55°C for 30 s, then 95°C for 1 min, 55°C for 1 min and finally increments of 0.5°C for 10 s from 55.0°C to 95.0°C). The expression of heat shock proteins 16 and 60 in the control and heat-treated samples were normalized using 18s ribosomal RNA (rRNA) as a house keeping gene. 18s rRNA was selected as a normalizing gene on the basis that rRNA is the major component of total RNA and has commonly been utilized as a reference gene for evaluating the transcriptional abundances of genes of interest (Ochrietor et al. 2003;

Schmittgen and Livak 2008; Meyer et al. 2009). Relative expression of each gene of interest was compared between controls and heat-treated samples.

2.2.5 Larval Settlement and Survival

Settlement and survival assays were conducted at ambient temperature (27°C) and followed the methods described in Ross et al. (2013). Post-treatment, 50 larvae were placed in 800 ml plastic tri-pour beakers with 180-µm nitex mesh bottoms to allow exchange of seawater with the outdoor flow-through water tables. Each beaker contained a single terracotta tile (4.5 cm x 4.5 cm x 1 cm; Sunshine Pavers®), which served as positive settlement substrata. All tiles were pre-conditioned at a depth of 6 m on an offshore reef in the Florida Keys for 5 weeks before use in the experiments. After 72 h, the number of larvae that remained as swimmers (still in planula phase) and those that settled and underwent metamorphosis were counted. The total (swimmers + settlers) and the number that had settled and metamorphosed were divided by 50 (the initial number of larvae) to calculate the proportion survival and settlement respectively (n = 10 for settlement and survival and all treatments).

2.2.6 Statistical Analysis

Data for all experiments were analyzed using a two factor split-plot ANOVA design (see larval collection and treatments). Since each aquarium received all three levels of the treatment duration factor (4, 24, and 48 h) but only one level of the temperature factor (either ambient or heated), an incomplete block design was utilized to determine the effects of temperature and time on larval dependent variables. Both temperature and exposure duration were treated as fixed factors, while block of the aquaria was used as a random factor. Larval lipid peroxide levels were

normally distributed and contained homogenous variances, thus meeting the assumptions of ANOVA. Data for respiration, percent survival, and percent settlement were arcsine square root transformed; catalase activity and normalized Hsp 16 expression were rank transformed; and normalized Hsp 60 expression was transformed using natural log to meet the assumptions of ANOVA. The normality of all variables was assessed using the Shapiro-Wilk test and the equality of error variances was analyzed using Levene's test. All statistical analyses were conducted using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA).

2.3 Results

2.3.1 Thermal Stress Elevates Larval Oxygen Demand

Over the 48 h time frame, the mean temperatures (±SE) were 30.83 ± 0.07°C and 27.29 ± 0.05°C for elevated and ambient temperatures, respectively. At each time point (4, 24 and 48 h), larval respiration was measured for both heated and ambient aquariums (Fig.2.1). Elevated temperature (heat) significantly increased the respiratory demand of dark-adapted larvae ($F_{(1, 18)} = 13.920$, $p = 0.002$), but duration of treatment (time) had no effect on larval oxygen consumption ($F_{(2, 36)} = 1.721$, $p = 0.193$) and there was no significant interaction (heat*time) between the two factors ($F_{(2, 36)} = 0.047$, $p = 0.954$). Furthermore, larval oxygen consumption did not vary significantly between experimental units (aquaria) within treatments ($F_{(18, 36)} = 0.778$, $p = 0.710$).

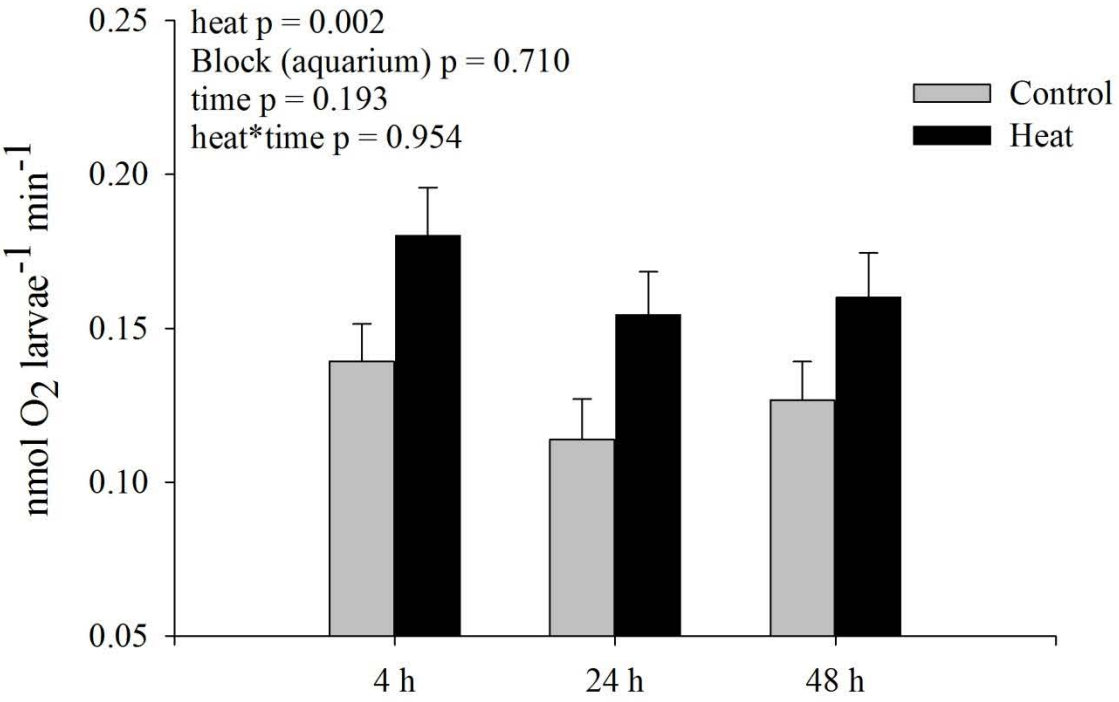

Figure 2.1 Cellular respiration of *P. astreoides* larvae following 4, 24 or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control). n = 10 for all treatments. Bars represent mean +1SE

2.3.2 Thermal Stress Elevates Lipid Peroxidation

At each time point larvae exposed to elevated temperature experienced greater degree of lipid peroxidation levels compared to larvae maintained under ambient seawater conditions (Fig.2.2). By the 48 h mark, heat treated larvae had a ~2.5-fold increase in lipid peroxidation compared to controls. Thus, the elevated temperature treatment significantly increased lipid peroxidation levels compared with controls ($F_{(1, 23.5)} = 24.400$, $p < 0.001$). Lipid peroxidation did not vary significantly as a function of time ($F_{(2, 21)} = 2.990$, $p = 0.072$), and the interaction between the two factors was not significant ($F_{(2, 21)} = 1.680$, $p = 0.210$). Individual aquaria within treatments did not differ significantly in their effect on lipid peroxidation ($F_{(18, 21)} = 0.804$, $p = 0.678$).

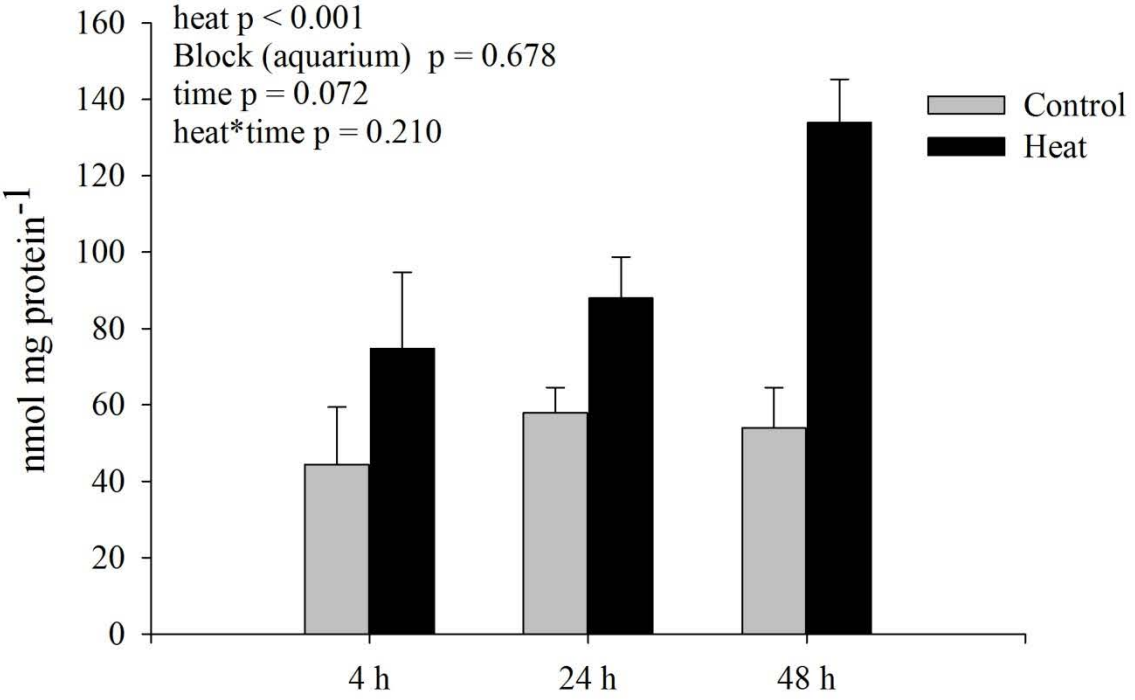

Figure 2.2 Lipid hydroperoxides, a biomarker of oxidative stress in *P. astreoides* larvae following 4, 24 or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control) (4 h control n = 6, 4 h heat n = 5, 24 h control and heat n = 9, 48 h control and heat n = 8). Asterisk indicates a significant difference between treatment and control (simple effects test). Bars represent mean +1SE

2.3.3 Thermal Stress Elevates Catalase Activity

Catalase activity varied significantly between treatments (Fig.2.3), as CAT activity was significantly greater in larvae exposed to 30.8°C compared to 27.3°C ($F_{(1, 20.5)} = 14.150$, $p = 0.001$). Exposure time did not significantly affect cellular CAT activity ($F_{(2, 21)} = 1.889$, $p = 0.176$), and the interaction between the two factors was not significant ($F_{(2, 21)} = 1.751$, $p = 0.198$). Moreover, CAT activity did not significantly vary between aquaria within the same treatment ($F_{(18, 21)} = 1.770$, $p = 0.105$).

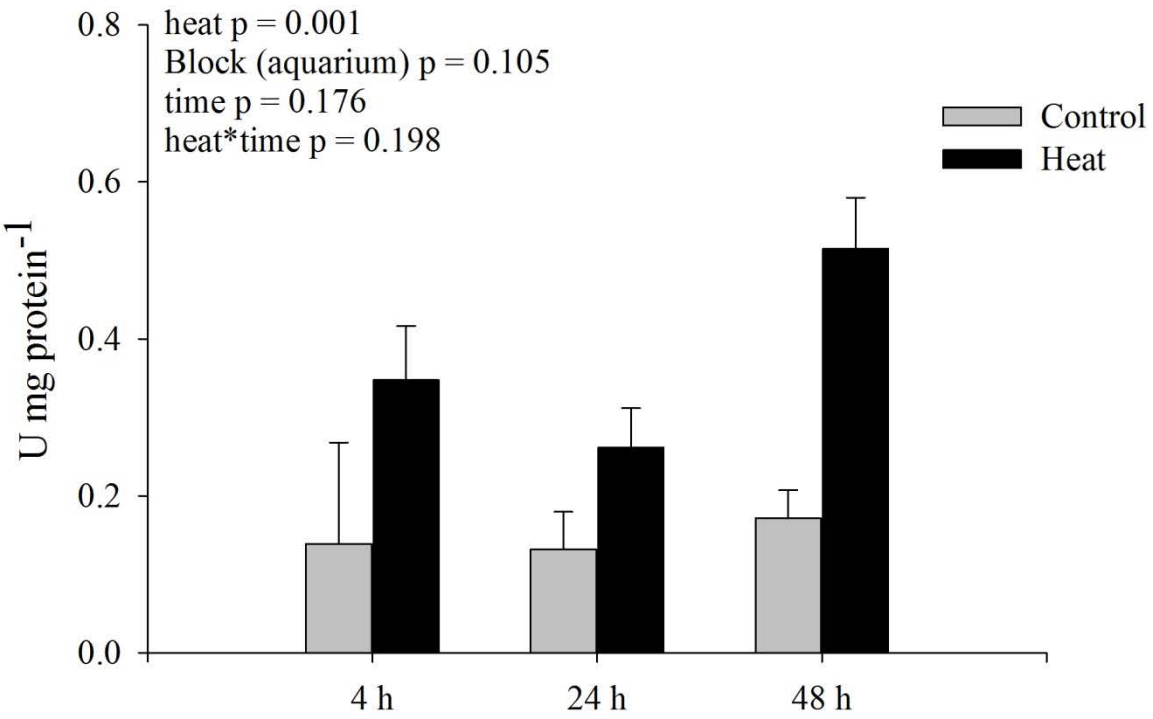

Figure 2.3 Catalase, an enzyme that is a biomarker of oxidative stress in *P. astreoides* larvae following 4, 24 or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control) (4 h control n = 6, 4 h heat n = 5, 24 h control and heat n = 9, 48 h control and heat n = 8). Bars represent mean +1SE

2.3.3 Elevated Temperature Does Not Impact mRNA Transcript Abundances of Hsp 16 or 60

As a measure of quality control, the differential expression of the housekeeping gene 18s ribosomal RNA was analyzed between treatments and time points. The transcript abundance of 18s rRNA did not vary significantly as a function of temperature ($F_{(1, 8)} = 0.244$, $p = 0.634$), time ($F_{(2, 16)} = 0.540$, $p = 0.593$), or within treatment aquarium ($F_{(8, 16)} = 0.822$, $p = 0.595$) and the interaction between the two factors was not significant ($F_{(2, 16)} = 0.478$, $p = 0.629$). The normalized abundance of Hsp 16 and 60 transcripts demonstrated substantial variability within treatments and were not significantly different in larvae exposed to 30.8°C or 27.3°C (Fig.2.4). Elevated temperature did not affect the transcript abundance of heat shock protein 16 ($F_{(1, 8)} = 0.042$, $p = 0.843$), and duration of treatment did not significantly impact Hsp 16 transcript levels ($F_{(2, 16)} = 0.035$, $p = 0.966$). The interaction between temperature and treatment duration was not significant ($F_{(2, 16)} = 0.105$, $p = 0.901$), and Hsp 16 transcript levels did not vary significantly between experimental units within treatments ($F_{(8, 16)} = 0.612$, $p = 0.756$). Hsp 60 transcript levels did not vary significantly as a function of treatment ($F_{(1, 8)} = 1.375$, $p = 0.275$) or duration ($F_{(2, 16)} = 0.569$, $p = 0.577$) and the interaction between the two factors was not significant ($F_{(2, 16)} = 0.603$, $p = 0.559$). Furthermore, Hsp 60 transcript levels did not significantly vary between experimental units within treatments ($F_{(8, 16)} = 0.300$, $p = 0.955$).

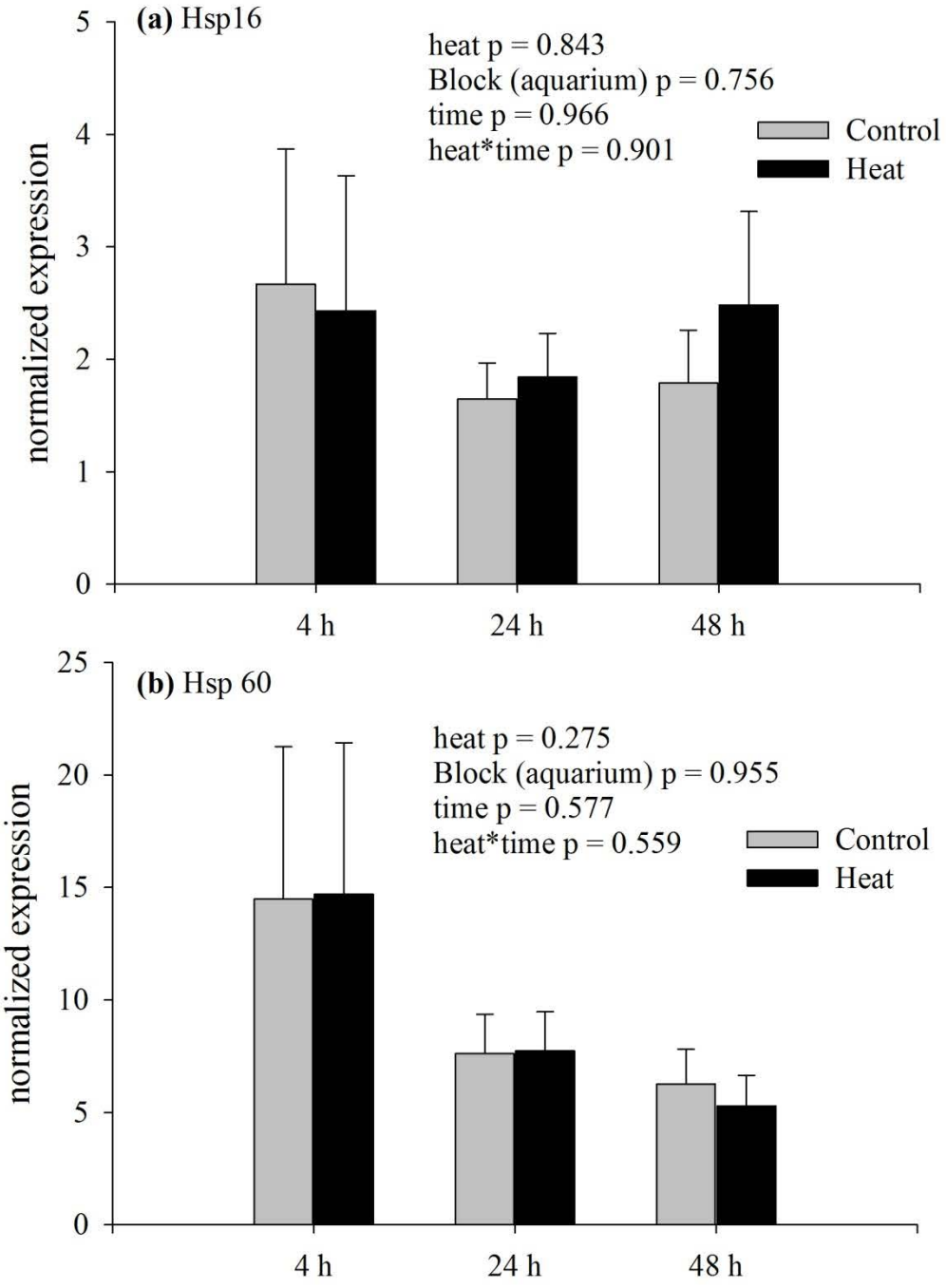

Figure 2.4 Normalized heat shock protein transcriptional expression in *P. astreoides* larvae following 4, 24 or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control): (a) Hsp 16, (b) Hsp 60. n = 5 for both heat shock proteins and all treatments. Bars represent mean +1SE

2.3.4 Elevated Temperature Does Not Impact Larval Settlement or Survival

Post-treatment, the larvae were allowed 72 h to settle and metamorphose under ambient seawater conditions (27°C) (Fig.2.5a). Larval survivorship did not vary significantly as a function of temperature treatment ($F_{(1, 18)} = 0.003$, $p = 0.954$), yet both control and heat treatments were affected by time ($F_{(2, 36)} = 4.137$, $p = 0.024$). There was no significant interaction between the two factors ($F_{(2, 36)} = 1.457$, $p = 0.246$) and survival did not vary significantly between aquaria within treatments ($F_{(18, 36)} = 0.693$, $p = 0.795$). Thermal stress did not significantly affect the process of settlement and metamorphosis ($F_{(1, 18)} = 0.127$, $p = 0.726$) (Fig.2.5b). Just as with survival, it was noted that duration of treatment significantly reduced larval settlement in both the control and heat treatments ($F_{(2, 36)} = 9.473$, $p < 0.001$). The interaction between temperature and time was not significant ($F_{(2, 36)} = 1.383$, $p = 0.264$) and settlement did not vary significantly between experimental units within treatments ($F_{(18, 36)} = 1.281$, $p = 0.256$).

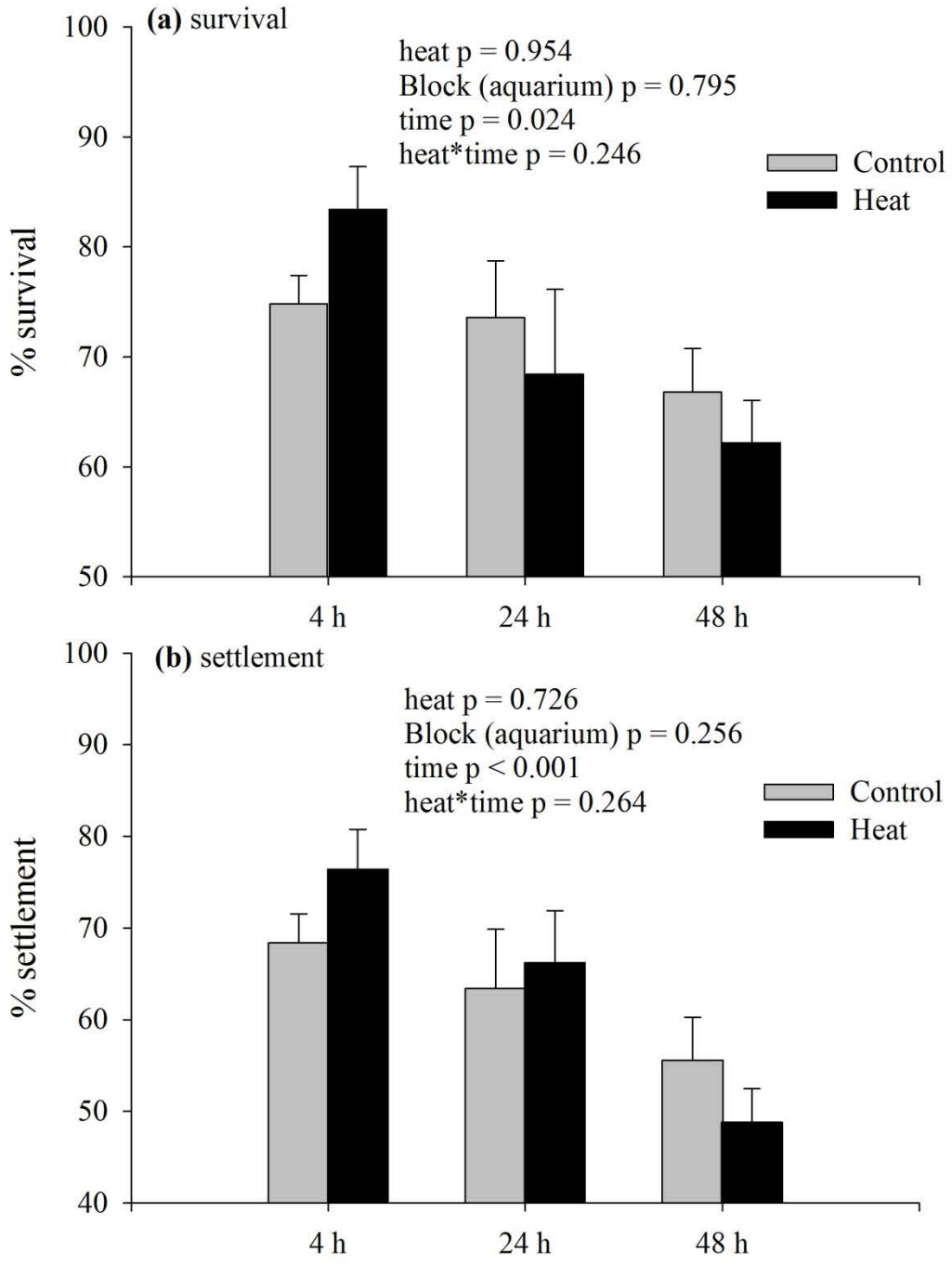

Figure 2.5(a) Percent survival and (b) settlement of *P. astreoides* larvae following 4, 24 or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control). Larvae were offered conditioned settlement tiles, and survival and settlement were measured after 72 h. n = 10 for survival and settlement and all treatments. Bars represent mean +1SE

2.4 Discussion

The elevated temperature treatment used in this study is an expected prediction for rising sea surface temperatures over the next century (IPCC 2007). Our results indicate that exposure to a modest elevation in temperature (+3.5°C) for 4, 24 or 48 h increased the respiratory demand and level of oxidative stress in *P. astreoides* larvae, but did not induce the transcript expression of Hsps 16 and 60. While the intensity and duration of elevated temperature examined in this study does not appear to directly affect the survival and settlement of *P. astreoides* larvae, it is apparent that cellular constituents are being impacted through oxidative damage. The statistically-insignificant up-regulation of Hsp 16 or 60 in response to thermal stress suggests that the change in transcript expression of these genes serves as a less sensitive diagnostic tool compared to biomarkers of oxidative stress in *P. astreoides* larvae.

2.4.1 Thermal Stress Elevates Larval Oxygen Demand

At 27.3°C, the rates of larval respiration were comparable to results from Edmunds et al. (2001) and Ross et al. (2010; 2013). Over the 48 h time course, the elevated temperature treatment caused increased respiratory demand compared to controls. Conversely, other studies utilizing *P. astreoides* larvae have found no significant change in respiration when temperatures were maintained at +3.5°C or even +5°C over the course of 24 h (Edmunds et al. 2001; Ross et al. 2013). Recent work examining the effects of temperature and ocean acidification on larval respiration has yielded mixed results as well. Putnam et al. (2012) found a 32% reduction in respiratory demand in *Pocillopora damicornis* larvae that were incubated at 29°C compared to controls maintained at 25°C. However, it was noted that pCO_2 had no effect. Nakamura et al. (2011) also found no significant difference in the respiratory rates of *Acropora digitifera* larvae

as a function of increasing pCO_2. Conversely, Albright and Langdon (2011) reported that *P. astreoides* larvae underwent a significant decrease in respiration in response to increasing pCO_2 levels. The inter-individual variation naturally found within populations and species, in conjunction with differences in sample size and experimental design, are likely to be contributing aspects to these discrepancies. Regarding our study, elevated temperatures likely caused an increase in respiratory demand due to several factors, including the effects of temperature on metabolism, elevated energetic costs associated with repairing damage caused by excessive temperature-induced oxygen intermediates, and the consumption of molecular oxygen during the formation of reduced oxygen (Lesser 1997).

2.4.2 Thermal Stress Elevates Biomarkers of Oxidative Stress

The onset of oxidative stress in response to elevated temperatures has been well documented in corals (Lesser 1996; 1997) and can provide a quantitative marker of the stress level experienced by the holobiont prior to coral bleaching (Downs et al. 2002). The presence of zooxanthellae in larvae likely enhances oxidative damage, as has been suggested by Yakovleva et al. (2009). Upon thermal stress, photosystem II generates excess superoxide anion, which can subsequently dismutate to hydrogen peroxide. In the presence of iron, extremely reactive hydroxyl radicals can form via the Fenton reaction, causing host tissue damage (Weis 2008; Pospisil 2012). Even small temperature increases (+3.5°C) over short time scales caused oxidative damage and increased antioxidant enzyme activity (lipid peroxidation and CAT activity, respectively). Recent work examining the effects of high temperatures on larval oxidative stress has chiefly focused on transcriptional responses (Rodriguez-Lanetty et al. 2009; Voolstra et al. 2009; Polato et al. 2010). These studies, based upon the description of the

transcriptome, may not be capturing post-transcriptional and post-translational regulatory processes that could impact enzyme activity. The up-regulation of catalase activity in response to thermal stress has been reported in both adult corals (Yakovleva et al. 2004; Higuchi et al. 2008) and larvae (Ross et al. 2013). Simultaneously, the degree of lipid peroxidation has been employed as a tool to identify thermal damage in adults (Downs et al. 2000; Downs et al. 2002) and larvae (Yakovleva et al. 2009) as well. Based upon the ubiquitous nature and sensitivity of these metrics, oxidative damage and antioxidant enzyme activity are useful markers for monitoring temperature-induced sub-lethal effects in corals.

2.4.3 Elevated Temperature Does Not Impact mRNA Transcript Abundances of Hsp 16 or 60

Modest increases in sea surface temperatures of 1-2°C can induce coral bleaching, yet several studies have failed to detect Hsp induction below 33°C (Black et al. 1995; Sharp et al. 1997; Voolstra et al. 2009). We were not able to detect a significant change in Hsp 16 or 60 mRNA expression over the course of 48 h when *P. astreoides* larvae were incubated at 30.8°C. Kenkel et al (2011) detected an ~800 fold and ~4 fold up-regulation in Hsp16 and 60 expression respectively, in heat-light stressed *P. astreoides* adult fragments obtained from the same habitat as the parent colonies used in the present study. However, this 48 h treatment was maintained at 35-36°C under full sunlight, deliberately exceeding natural environmental conditions. While the transcriptional expression of Hsps in response to thermal stress is universal, the specific environmental conditions, duration of treatment, organismal life history stage, and the functional role of the Hsp of interest all influence the degree of expression. For example, Polato et al. (2010) detected a down-regulation of Hsp 90α in *Montastrea faveolata* larvae that were maintained at 31.5°C for 48 h. Conversely, Voolstra et al. (2009) did not detect any change in the

expression of Hsp 90 in the same species of larvae that were exposed to 29°C or 31.5°C at 12 or 48 h. Rodriguez-Lanetty et al. (2009) provided evidence for the up-regulation of Hsp 70 and 90 in *Acropora millepora* larvae at 28 and 31°C for 3 h. However, these transcriptional responses were diminished by 10 h. The differential expression of Hsps has been reported in adult scleractinian corals as well, as Desalvo et al. (2008) showed an increased expression of Hsp 90 in adult *M. faveolata* that were incubated at 32°C for 10 days. On a shorter time scale, Desalvo et al. (2010) showed an increased expression of Hsp 90 and 70 in *Acropora palmata* maintained at 32°C for 24 h, yet no enhanced expression was detected at 48 h. Similarly, Mayfield et al. (2011) found no differential expression of Hsp 70 in *Seriaptopera hystrix* after 48 h of exposure at 30°C. Finally, work by Bellantuono et al. (2012) detected no change in the differential expression of any Hsp when specimens of *Acropora millepora* were challenged with a maximal temperature of 31°C for 8 days. The authors noted that the high variance obtained in their results was partially attributed to large biological variability. The concept of coral population-scale variability was probably best exemplified by Csazsar et al. (2009) who found a notable intra- and inter-colony variation in Hsp 70 expression in colonies of *A. millepora* that were exposed to temperatures of 32°C for up to 9 days. Intraspecific colonies of *A. millepora* exposed to the same level of stress for the same duration of time differentially up and down-regulated genes associated with the thermal stress response including Hsp 70. Furthermore, significant inter-colony variability has also been demonstrated in the transcriptional expression of Hsp 16 in heat stressed larvae of the same coral (Meyer et al. 2009).

The lack of consistent up-regulation of genes associated with the heat stress response make the quantification of transcript abundance a metric that should be approached with caution when quantifying population-scale temperature induced stress in hermatypic corals. The

disparity between mRNA abundance and enzyme activity makes it difficult to predict enzyme activity based upon quantitative transcriptome data (Glanemann et al. 2003). To date there have been no studies identifying a correlation between mRNA transcript level and the enzyme activity of the corresponding translated protein in coral systems. One report by Wiens et al. (2000) demonstrated that when *Dendronephthya klunzingeri* was exposed to +4°C for 2 h, Hsp 90 transcriptional expression increased > 3-fold with a concomitant increase in Hsp 90 protein abundance as determined by immunoblotting. Enzyme activity, or better yet, enzyme activity coupled with gene expression, would offer a valuable assessment of the true physiological capability of the organism in response to stress.

2.4.4 Elevated Temperature Does Not Impact Larval Settlement or Survival

Larval settlement is a critical step in the process of recruitment and can significantly affect adult population dynamics (Ritson-Williams et al. 2009). The fact that survival and settlement were not immediately affected in our study suggests that exposure to +3.5 °C does not overly tax the short-term adaptive response of *P. astreoides* larvae. Similar results were obtained by Edmunds et al. (2001) and Ross et al. (2013) who found that *P. astreoides* larvae treated with +5°C or +3.5°C, respectively, failed to undergo a reduction in survival or settlement. Conversely, studies on other coral species reported negative impacts on both larval survival and settlement when exposed to +4.0°C in *Diploria strigosa* (Bassim and Sammarco 2003), +3.0°C in *Favia fragum* (Randall and Szmant 2009), and +6.0°C in *Acropora intermedia* (Yakovleva et al. 2009). While differences in treatment intensity and duration may explain the variation in survival across heterospecifics, it appears likely that *P. astreoides* larvae have a greater thermal tolerance

compared to other species of reef-building corals. However, this hypothesis warrants further investigation.

Both control and heat treatments experienced reduced survival and settlement following 48 h of treatment and subsequently 72 h in the settlement chambers. The larvae exposed to the 48 h treatments were 6 days old and may have been reaching their endpoint of window of competence for settlement (Carlon and Olson 1993). The duration and behavior of many marine larvae demonstrate considerable intraspecific variation even under controlled laboratory settings and may demonstrate reduced or prolonged planktonic existence (Thorson 1950; Raimondi and Keough 1990). Thus, longer incubation periods may have confounding effects on larval survival, and behavior and should be considered when designing larval experiments, particularly for brooding corals.

2.4.5 Conclusion

Developing reliable techniques to assess stress in multiple life history stages of corals is critical, as they are exposed to an increasing frequency of local and global scale stressors. Exposure to elevated seawater temperature (+3.5°C) for 4, 24, or 48 h had no effect on mRNA expression of Hsp 16 or 60, settlement, or survivorship of *P. astreoides* larvae, but elevated temperature induced oxidative stress and elevated respiratory demand. Oxidative stress has been reported to impair the heat stress response in human cell lines (Adachi et al. 2009), but the relationship between ROS production and Hsp regulation has not been investigated in corals. When *P. astreoides* larvae were exposed to elevated temperature (+3.5°C), there was a reduction in post-settlement survival, potentially due to energy dedicated to the thermal stress response (Ross et al. 2013). Thus, the deleterious effect of elevated temperature on larval condition,

indicated by elevated respiration, lipid peroxidation, and CAT abundance, may have indirect trade-off effects on coral recruitment following metamorphosis (reviewed in Monaghan et al.2009; Schnitzler et al. 2012). Our results have implications for the effects of climate change on coral reproduction and the potential techniques for evaluating heat-induced stress in hermatypic corals.

3.

Negative effects of macroalgal competition and elevated temperature on multiple life-history stages of the scleractinian coral *Porites astreoides*

Abstract

Coral reefs have suffered substantial global declines due to numerous stressors operating on both global and regional scales. However, little is known about how these stressors interact to impact corals and their potential modes of recovery. To address this void we evaluated the effects of contact with *Dictyota menstrualis*, elevated sea surface temperatures (+3.5°C), and their combined effects on three life-history stages of the reef-building coral *Porites astreoides*. *P. astreoides* larvae, juveniles (6 week-old), and adults (1-2 year-old) were exposed to a series of treatments and then subsequently evaluated for signs of sub-lethal stress and other associated metrics of recruitment. The presence of *D. menstrualis* reduced coral survival by ~ 40% and inhibited recruitment beyond simple space occupation. Elevated temperature induced sub-lethal stress yet had varied responses that were contingent on the life-history stage being examined. Thermal stress increased oxidative damage 2-fold and significantly reduced the F_v/F_m in coral larvae. While macroalgal exposure and elevated temperature had distinct effects on coral survival and physiological condition, the combination of both stressors induced a synergistic impact on the oxidative stress pathway in larvae following prolonged exposure. Our results support the growing evidence that benthic macroalgae compete with reef-building corals via direct contact for space on coral reefs and that elevated temperature can reduce the health of the coral holobiont. The results also suggest that larvae from *P. astreoides* are more susceptible to

the impacts of hyperthermal stress compared to established corals and that multiple perturbations can interact to exacerbate coral health.

3.1 Introduction

Hermatypic (reef-building) corals are critical for building and maintaining habitats that provide a multitude of ecosystem services that benefit human societies (Barbier et al. 2011). Unfortunately, coral reef degradation is occurring at an unprecedented rate (Knowlton 2001; Pandolfi et al. 2003), often resulting in community shifts from topographically complex coral systems to species-poor habitats that are dominated by macroalgae (Hughes 1994; McCook 1999; Hughes et al. 2007). These phase shifts have been most pronounced in the Caribbean (Gardner et al. 2003), where reefs are being increasingly exposed to environmental stressors that operate on both global (e.g. ocean acidification and warming sea surface temperatures) (Albright et al. 2010; Eakin et al. 2010) and local (e.g. competition with macroalgae) (Nugues and Bak 2006; Box and Mumby 2007) scales. While numerous studies have examined the effects of independent stressors on coral biology both under laboratory conditions and *in situ* (Glynn and Croz 1990; Jokiel and Coles 1999; Desalvo et al. 2008; Rasher and Hay 2010), there are fewer reports that have directly tested the impacts of multiple stressors simultaneously (Coles and Jokiel 1978; Porter et al. 1999; Darling et al. 2010; Dunne 2010). If multiple stressors interact to produce effects beyond that of each stressor alone, the result would be a major source of uncertainty for projections of coral reef stability (Paine et al. 1998; Dunne 2010). Thus, an understanding of how multiple stressors interact to affect established corals as well as their modes of population recovery is critical for managing coral reef habitats in the future.

Competition for space is an important factor in structuring the distribution of organisms in benthic communities (Jackson and Buss 1975; Thacker et al. 1998; Chadwick and Morrow 2011). In particular, coral-algae interactions are considered fundamental to the overall status of coral reefs, especially in the context of regional community dynamics and multiple shifting stable states (Knowlton 1992). Macroalgae can overgrow, chemically exclude, and replace reef-building corals, significantly reducing the functionality of coral reefs and their resiliency to disturbance (Hughes 1994; Ostrander et al. 2000; Nugues and Bak 2006; Rasher and Hay 2010; Paul et al. 2011). In the Caribbean, depressed herbivory rates due to overfishing and the massive die-off of the sea urchin *Diadema antillarum* have increased macroalgal abundance and the frequency of coral-algae interactions (Hughes 1994; Rogers and Miller 2006; Mumby and Steneck 2008). Specifically, members of the brown macroalgal genus *Dictyota spp.* are conspicuous and have been shown to reduce coral survival (Rasher and Hay 2010) and larval settlement (Kuffner et al. 2006; Paul et al. 2011). *Dictyota menstrualis* is common on reefs in the Caribbean and possesses a suite of secondary metabolites with anti-herbivory properties (Pereira et al. 2000; Tiexeira et al. 2001). The chemical potency of this algae is pertinent, as allelopathy is believed to be one of the main mechanisms utilized by macroalgae in competing with reef-building corals (reviewed in McCook et al. 2001; Rasher and Hay 2010; Paul et al. 2011). Furthermore, the presence of *D. menstrualis* has been shown to negatively affect the process of recruitment, which is necessary for the recovery of coral populations (Kuffner et al. 2006; Box and Mumby 2007).

Increasing ocean temperatures associated with global climate change represents a second major threat encountered in tropical marine ecosystems (Hoegh-Guldberg et al. 2007; Koch et al. 2013). Since many reef-building corals are already near their thermal maxima, modest

temperature increases of 1-2 °C can be particularly damaging to the health of coral reef ecosystems (Hoegh-Guldberg 1999). Elevated temperature can promote the overproduction of reactive oxygen species (ROS) in the symbiotic zooxanthellae of corals (Lesser 1997), subsequently causing coral bleaching and mortality of the holobiont (Weis 2008). This stress, in addition to the increasing abundance of macroalgae is believed to be one of the gravest ecological drivers facing coral ecosystems (Hoegh-Guldberg 1999; McCook 1999; McCook et al. 2001). Unfortunately, little is known about how these two stressors interact to affect reef-building corals and their potential recovery. In the context of compounding global and local stressors, a significant source of uncertainty is whether coral decline is due primarily to large-scale forces, regional perturbations, or multiple stressors interacting synergistically (Downs et al. 2005). To address this concern we evaluated the independent and combined impacts of elevated temperature and *D. menstrualis* presence on the survival, health, and recruitment of the common hermatypic coral *Porites astreoides*.

3.2 Materials and Methods

3.2.1 General Experimental Design

This study was composed of two experiments that evaluated the effects of elevated seawater temperatures, *D. menstrualis* presence, and the combination of these two stressors on three distinct life-history stages of the scleractinian coral *P. astreoides*. The first experiment was conducted on larvae obtained from *P. astreoides* at Mote Marine Tropical Research Laboratory, Summerland Key, FL, USA from 18 to 23 May 2012. The second was conducted on juvenile (~6 weeks of age) and adult corals (>1 year of age) simultaneously at the Smithsonian Marine Station, Fort Pierce, FL, USA from 30 June to 7 July 2012. *P. astreoides* was chosen for

experimentation due to its abundance on the reefs in Florida, the availability of its brooded larvae, and its previous use as an experimental hermatypic coral (Kuffner et al. 2006; Paul et al. 2011; Ross et al. 2010; 2013; Olsen et al. 2013). The macroalgae *D. menstrualis* was selected because it is common on reefs throughout the Caribbean, has previously been shown to reduce the survival of *P. astreoides* recruits (Kuffner et al. 2006), and this genus is known to have an allelopathic impact (via non-polar terpenoid compounds) on corals (Teixeira et al. 2001; Rasher and Hay 2010; Paul et al. 2011).

A single experimental design was used on all three life history stages with each stressor being tested independently and in combination. Treatments included; 1) ambient temperature and a plastic algae mimic (Control), 2) elevated temperature (+3.5°C) and a plastic algae mimic (Heat), 3) ambient temperature with *D. menstrualis* (Algae), and 4) elevated temperature (+3.5°C) with *D. menstrualis* (Heat+Algae). Larval, juvenile, and adult corals were maintained in ambient salinities and under shade cloth allowing penetration of 10 % photosynthetic active radiation (<200 µmol $m^{-2}s^{-1}$). The amount of *D. menstrualis* used was consistent across treatments and life history stages and approximated 3 cm^3 volume (Kuffner et al. 2006). The dependent variables evaluated were coral survival, photosynthetic efficiency of *in hospite* zooxanthellae, two biomarkers of oxidative stress (lipid peroxidation and catalase activity), and mRNA transcript abundances of Hsps 16 and 60.

3.3.2 Larval Collection

On May 18 2012, thirty colonies of *P. astreoides* were collected at 6 m depth from Wonderland reef (GPS N 24°33.62□; W 81°30.08□), transported to Mote Marine Tropical Research Laboratory in coolers, and maintained in raceway tables with running seawater. Larvae

were collected during the night of 18 May 2012 prior to the new moon (21 May) when adult colonies release brooded larvae (McGuire 1998). Adult colonies were later returned to the site of collection and reattached with Z-Spar Splash Zone Compound® underwater epoxy following experimentation.

To obtain larvae, each colony was placed in an individual 3 L Rubbermaid Grip's Mix bowls® supplied with continuously running seawater. The bowls were tilted so that the larvae spilled over the handles of the bowls into plastic tri-pour beakers fitted with a mesh bottom (mesh diameter:180 µm) supported 3 cm off the sea table bottom by 3 PVC stopper feet. The water level inside the tanks was maintained at 15 cm so the larvae remained in the tri-pour beakers until the next morning when larvae were pooled and assigned to treatments.

3.2.3 Larval Treatments

To evaluate the effects of elevated temperature and *D. menstrualis* presence on coral larvae and to assess for a possible interaction, each stressor was tested independently and in combination (see general experimental design). Forty plastic aquaria were held in one of three raceway tables and served as independent water baths. Each replicate water bath was randomly assigned to one of the four previously described treatments (n = 10/treatment). Water baths were either maintained at ambient temperature or heated +3.5°C using a 75-watt adjustable aquarium heater (Commodity Axis, Inc. Camarillo, CA, USA). Each replicate aquarium housed two acrylic treatment chambers (10 × 5.5 cm) with 180 µm mesh bottoms. Every aquarium contained 250 *P. astreoides* larvae that were split between the two chambers; one hundred fifty larvae were aliquoted into one acrylic treatment chamber which contained either a plastic algae mimic or live *D. menstrualis*. The remaining 100 larvae were placed in the other acrylic settlement chamber

that contained a single conditioned (i.e., 4 week accumulated biofilm) terracotta tile (4.5 x 4.5 x 1 cm; Sunshine Pavers ®), which served as a settlement substrate with either an affixed plastic algae mimic or *D. menstrualis*. Plastic algae mimics and live *D. menstrualis* were attached to settlement tiles via cable ties.

Of the 150 larvae that were placed into one treatment chamber; 50 were removed following 16 h of treatment for quantitative reverse transcription PCR (q-RT-PCR) analysis. At the 48 h time point, 90 and 10 larvae were removed for oxidative stress and photochemical assessments, respectively. At the 68 h time point, those larvae remaining in the swimming planula stage (following settlement assays in the other chamber) were removed to evaluate the impact of extended treatment exposure on oxidative stress. Larvae removed for q-RT-PCR and oxidative stress assays were flash frozen in liquid N_2 and stored at -80°C until analysis.

3.2.4 Larval Survivorship, Settlement, and Post-Settlement Survivorship

Larvae were allowed to settle for 68 h, after which the number of larvae that remained in the settlement chamber as swimmers (still in planula phase) and those that settled and underwent metamorphosis were counted. The total (swimmers + settlers) was divided by 100 (the initial number of larvae) to determine the percentage survival. The number of larvae that had settled and metamorphosed was divided by 100 to determine the percentage settled.

To assess the effects of acute treatment exposure on subsequent recruitment processes, the survival of individual spat was evaluated post-settlement. Following survival and settlement assessments, the tiles containing newly settled recruits were randomly arranged on fiberglass rods (one individual tile from each treatment on each rod) and then cable-tied to a patch reef east of Looe Key research area (N 24°34.130; W 81°22.868; ~7 m depth) for 25 days similar to

methods by Ross et al. (2013). Post-settlement survival was calculated by dividing the number of live coral recruits after 25 days by the initial number of spat that were previously recorded on each tile before deployment to the patch reef.

3.2.5 Larval Photophysiology

Ten larvae were removed from the treatment chambers of each replicate following 48 h of exposure for photochemical assessments. The photochemical efficiency of symbiotic zooxanthellae was examined using pulse amplitude modulated (PAM) fluorometry (Diving-PAM; Walz, GmbH, Germany). Changes in dark-adapted maximal quantum yield [$F_v/F_m = (F_m - F_o)/F_{m'}$] of PS II were assessed using the methodology of Ross et al. (2013). For analysis, 10 larvae in 25 µl of seawater were pipetted onto the tip of the fiber-optic cable. All larval samples were dark adapted for 1 hour prior to any photochemical measurement.

3.2.6 Juvenile and Adult Rearing

On 20 May 2012 at Mote Marine Tropical Research Laboratory, 50 preconditioned terra cotta tiles (4.5 x 4.5 x 1 cm) were individually placed in plastic tripour containers containing a haphazardly selected number of larvae originating from ~25 colonies. The larvae were allowed to settle for 4 days at ambient temperature (~27.5°C), after which the new recruits were transported in coolers to raceways at the Smithsonian Marine Station. The juvenile spat were reared in running seawater from 24 May to 29 June 2012 (5 weeks). Following 6 weeks total incubation from larval release, the 40 tiles with the greatest number of individuals on the top of the tile were selected for experimentation (n = 10/treatment).

Twenty eight adult colonies were raised from newly settled larvae on preconditioned terra cotta tiles (4.5 x 4.5 x 1 cm) in raceway tables containing running seawater at the Smithsonian Marine Station. Individual colonies were grown over the course of one to two years and varied in surface area from 0.8 cm^2 to 45 cm^2. At the start of the experiment, each terra cotta tile contained a single adult colony and tiles were randomly assigned to treatments (n = 7/treatment) to control for varying colony size and age.

3.2.7 Juvenile and Adult Treatments

The effects of thermal stress and *D. menstrualis* presence were tested independently and in combination (see general experimental design) on juvenile and adult corals simultaneously. Forty plastic aquaria, serving as independent water baths, were held in one of two raceway tables. In turn, each aquarium housed a tile containing juvenile recruits and a second tile containing an adult coral. As in the larval experiments, each water bath was randomly assigned to one of the four previously described treatments. Water baths were either maintained at ambient temperature or heated +3.5°C using a 75-watt adjustable aquarium heater (Commodity Axis, Inc.). A single replicate from the Heat treatment of both life-history stages was lost due to a faulty aquarium heater and was not used in any analysis (juveniles Control, Algae, Heat+Algae treatments n = 10, Heat treatment n = 9) (adults Control, Algae, Heat+Algae treatments n = 7, Heat treatment n = 6). Tiles containing 6-week-old recruits or adult corals had either a plastic algal mimic or live *D. menstrualis* affixed to one half of the available substrate. Plastic algal mimics and live *D. menstrualis* were placed in direct but loose contact with juvenile and adult corals (Kuffner et al. 2006). Treatment duration was based on the availability of live *D.*

menstrualis and in total lasted 7 days. Following 96 hours, all tiles were removed from treatments to replace potentially decaying algae.

At the conclusion of the experiment (7 d), juvenile and adult corals were biopsied (~ $2cm^3$ of tissue) from directly underneath either the plastic algal mimic or live algae and this sample was flash frozen in liquid N_2 and stored at -80°C for subsequent q-RT-PCR and oxidative stress assays.

3.2.8 Juvenile and Adult Survival

Tiles containing juvenile recruits were scored prior to the experiment to determine the original number and location of spat on each tile. Following 7 d of treatment the tiles were evaluated again and the number of living and dead colonies was recorded. Colonies were scored as dead if no visible live tissue remained on the skeleton. The counts of dead colonies were used to insure that each tile was being assessed consistently by comparison to the original evaluation.

Survival in the adult life history stage was assessed by comparing the surface area of live tissue to the surface area of dead and bare skeleton following treatment. Surface areas were estimated in duplicate following 7 d of treatment using the tracing function in ImageJ® (nih.gov).

3.2.9 Juvenile and Adult Photophysiology

The photochemical efficiency of zooxanthellae within juvenile and adult corals were assessed using pulse amplitude modulated (PAM) fluorometry (Diving-PAM). Treatment effects on effective quantum yield (EQY) were assessed following 7 days. For analysis, three individual

readings were taken using the fiber-optic cable directly underneath either the plastic or live algae. The average of the three measurements was used as a single value for each replicate.

3.2.10 Oxidative Stress Assays

Oxidative stress in *P. astreoides* larvae, juveniles, and adults were measured using the methods described by Ross et al. (2013). Larval, juvenile, and adult samples were thawed to room temperature and each sample was extracted in 2.5 mL of buffer (50 mM potassium phosphate buffer [pH 7.0] containing 10% [w/v] polyvinylpolypyrrolidone [PVP]-40, 0.25% Triton X-100, and 1% [v/v] plant cell protease inhibitor cocktail [Sigma–Aldrich, St. Louis, MO, USA]). Corals were homogenized with a Fast Prep 24 bead homogenizer (MP Biomedicals, Irvine, CA, USA) and centrifuged at 16,000 x g for 10 min. The resulting supernatants were used for oxidative stress assays and normalized for protein content using the Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Pittsburgh, PA, USA).

To assess the impacts of ROS on cellular integrity, lipid peroxidation was assayed. Unsaturated lipids of cell membranes are common targets of oxidative attack by ROS. The end result is lipid peroxidation, a destructive process that compromises normal cellular function. Lipid peroxidation was measured using a Lipid Hydroperoxide Assay kit (Cayman Chemical, Ann Arbor, MI, USA). Catalase (CAT) is a widely distributed enzyme that destroys H_2O_2 by dismutation to O_2 and H_2O (Halliwell 2006). Upregulation of CAT reflects an organism's response to counteract the presence of damaging ROS. Catalase was assayed using Amplex Red (Invitrogen Corporation, Eugene, OR, USA) as per the manufacturer's instructions.

3.2.11 Quantitative Reverse transcription PCR (q-RT-PCR)

RNA isolation

Total RNA was isolated from larvae, juvenile, and adult corals using the TRI reagent protocol (Molecular Research Center, Cincinnati, OH, USA). Total RNA concentrations and purities were calculated using absorbance values of 260 and 280 nm measured with a Bio-Tek® plate reader (Bio-Tek, Winooski, VT, USA).

Quantitative One-Step Reverse Transcription-PCR

Porites sp. gene-specific primers were designed and validated by Kenkel et al. (2011). Gene specific primers were diluted to 5 µM and relative standard curves for each primer set were generated using serial dilutions of total RNA encompassing two orders of magnitude from 0.078-5.0 ng. Reverse Transcription-PCR was performed utilizing 1 ng total RNA per reaction. The appropriate volume from each RNA sample equivalent to 1 ng total RNA was added to 1µL forward primer, 1µL reverse primer, 1.2 µL random hexamer primer (Ambion Life Technologies, Grand Island, NY, USA), 12.5 µL SYBR Green Reaction Mix® (Bio-Rad, Hercules, CA, USA) and enough nuclease-free water to sum to 25 µL of volume for each gene primer set (Hsp 16, Hsp 60, and 18s). All runs were performed on a MiniOpticon Real-Time PCR system® (Bio-Rad) using the default protocol. The expression of heat shock proteins 16 and 60 in all treatments were normalized using 18s ribosomal RNA (rRNA) as a house keeping gene. 18s rRNA was selected as a normalizing gene on the basis that rRNA is the major component of total RNA and has commonly been utilized as a reference gene for evaluating the transcriptional abundances of genes of interest (Ochrietor et al. 2003; Meyer et al. 2009; Olsen et al. 2013). Relative expression of each gene of interest was compared between treatments.

3.2.12 Statistical Analysis

Dependent variables were analyzed within each coral life history stage. Data from larval and adult corals were analyzed using a two-way analysis of variance (ANOVA), with temperature and algal presence treated as fixed factors. Within the juvenile experiment, since the number of colonies on each tile was not consistent, the effect of juvenile density on survival and photosynthetic efficiency was evaluated as a covariate in analyses of co-variance (ANCOVA). Temperature and algal presence were again treated as fixed factors while juvenile density was evaluated as a random factor. This design was used to avoid reducing the number of coral colonies in the majority of replicates. Data for percent survival, percent settlement, and post-settlement survival were arcsine square root transformed prior to analysis since they were proportions. For dependent variables with a significant interaction term, a Tukey's post hoc test was subsequently performed to determine significant groupings. The normality of all variables was assessed using the Shapiro-Wilk test and the equality of error variances was analyzed using Levene's test. Those variables with data that could not be transformed to meet the assumptions of ANOVA were rank transformed prior to analysis. All statistical analyses were conducted using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA).

3.3 Results

3.3.1 Larval Recruitment

Throughout the course of the larval experiment, the mean temperatures of each treatment (±SE) were 27.485±0.10 °C (Control), 31.025±0.12 °C (Heat), 27.523±0.10 °C (Algae), and 31.213±0.12 °C (Heat+Algae). Treatments exhibited consistent impacts on several parameters evaluating larval supply and recruitment (Fig.3.1). Exposure to seawater elevated ~ +3.5°C (heat) for 68 h did not significantly affect larval survival (Fig.3.1a), settlement (P=0.414,

Fig.3.1b), or post-settlement survival as juvenile spat (P=0.709, Fig.3.1c). However, the presence of live *D. menstrualis* (algae), significantly reduced the survival of larvae (Fig.3.1a), deterred their settlement and metamorphosis (P=0.002, Fig.3.1b), and reduced their survival following settlement as new recruits (P=0.014, Fig.3.1c).

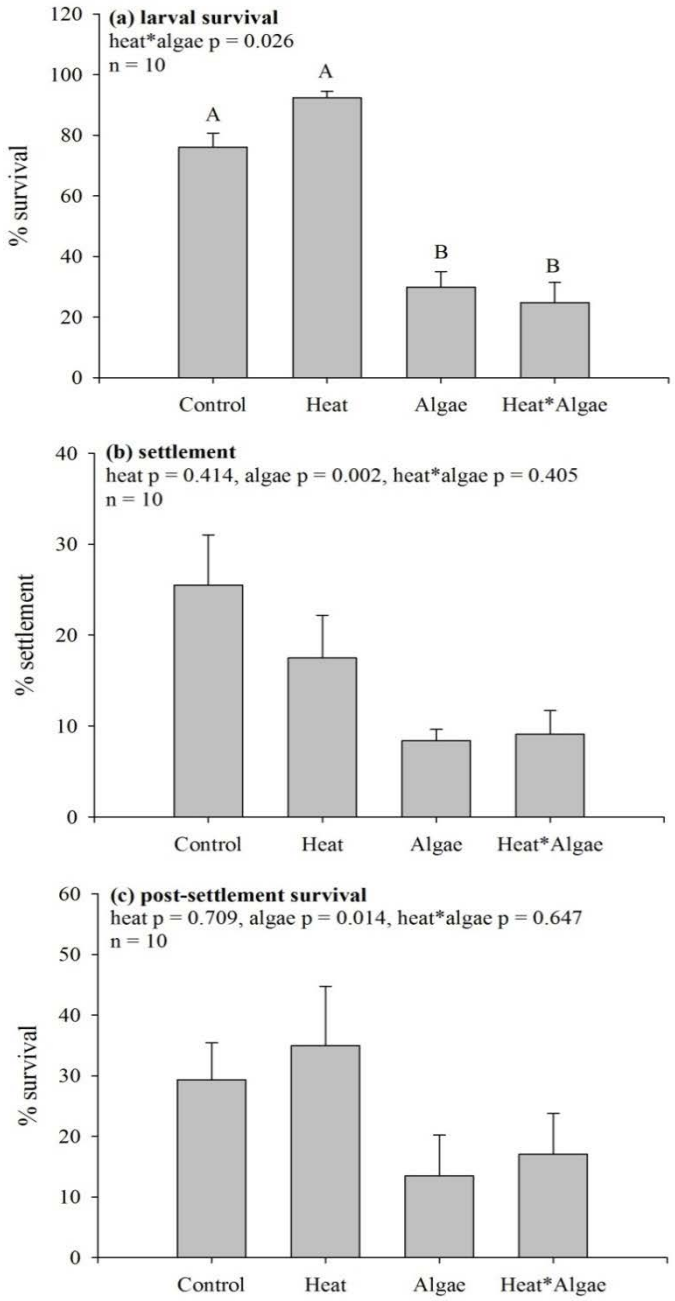

Figure 3.1 (a) Percent survival, (b) settlement, and (c) post-settlement survival of larvae from *P. astreoides* following 68 h of exposure to treatment (survival and settlement) and subsequently 25 d on a patch reef (post-settlement survival). Bars represent mean +1SE. The same letters above the bars indicate means that are not significantly different as determined by a Tukey's post hoc test

3.3.2 Larval Photophysiology

The photochemical efficiency (maximal quantum yield) of *in hospite* zooxanthellae was evaluated using in situ PAM fluorometry, a method that quantifies sub-lethal stress in the coral holobiont (Putnam et al. 2012) (Fig.3.2). Symbiont photochemical efficiency was significantly reduced by thermal stress ($P<0.001$), but was unaffected by the presence of macroalgae ($P=0.267$).

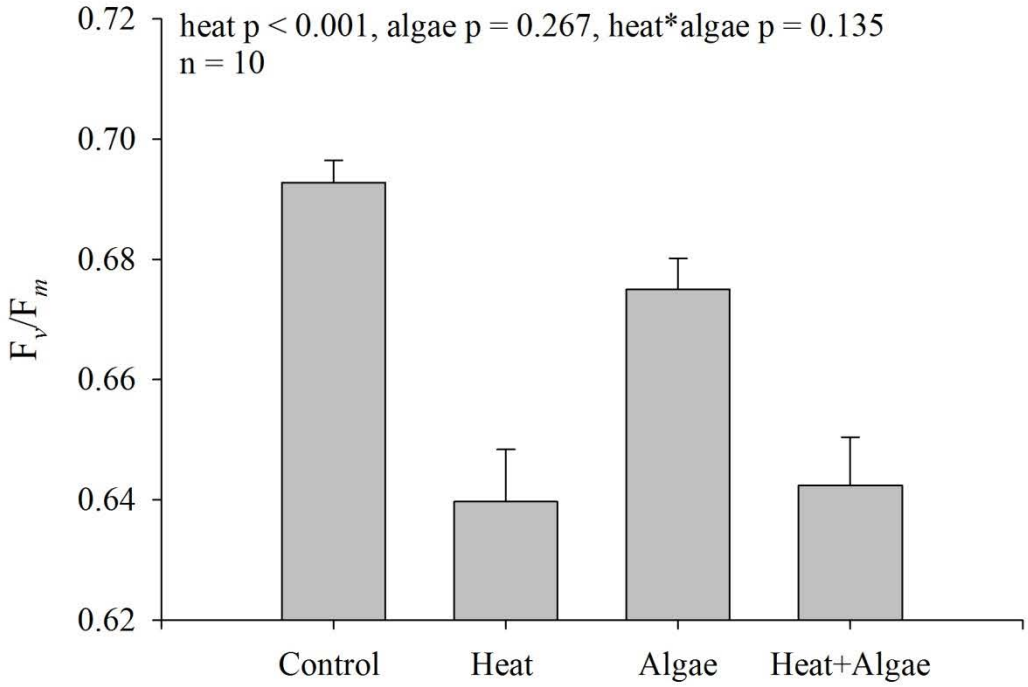

Figure 3.2 Treatment effects on photochemical efficiency (MQY) of zooxanthellae-containing larvae from *P. astreoides*. Bars represent mean +1SE

3.3.3 Larval Oxidative Stress

Sub-lethal stress in coral larvae was also quantified using two biomarkers of oxidative stress (CAT activity and lipid peroxidation) following two time points of treatment exposure (48 h and 68 h) (Fig.3.3). Exposure to elevated temperature or live *D. menstrualis* for 48 h did not significantly impact larval CAT activity (Fig.3.3a), but those larvae exposed to the combined treatment (Heat+Algae) for 68 h underwent a ~2.5 fold increase in CAT activity compared to controls or larvae treated with heat alone (Fig.3.3b).

Elevated temperature significantly increased the degree of oxidative damage in the form of lipid peroxidation in coral larvae following 48h (Fig.3.3c). Just as with CAT activity, those larvae exposed to the combined treatment for 68 h demonstrated a significant increase in lipid peroxidation compared to controls or larvae treated with heat or live algae alone (Fig.3.3d).

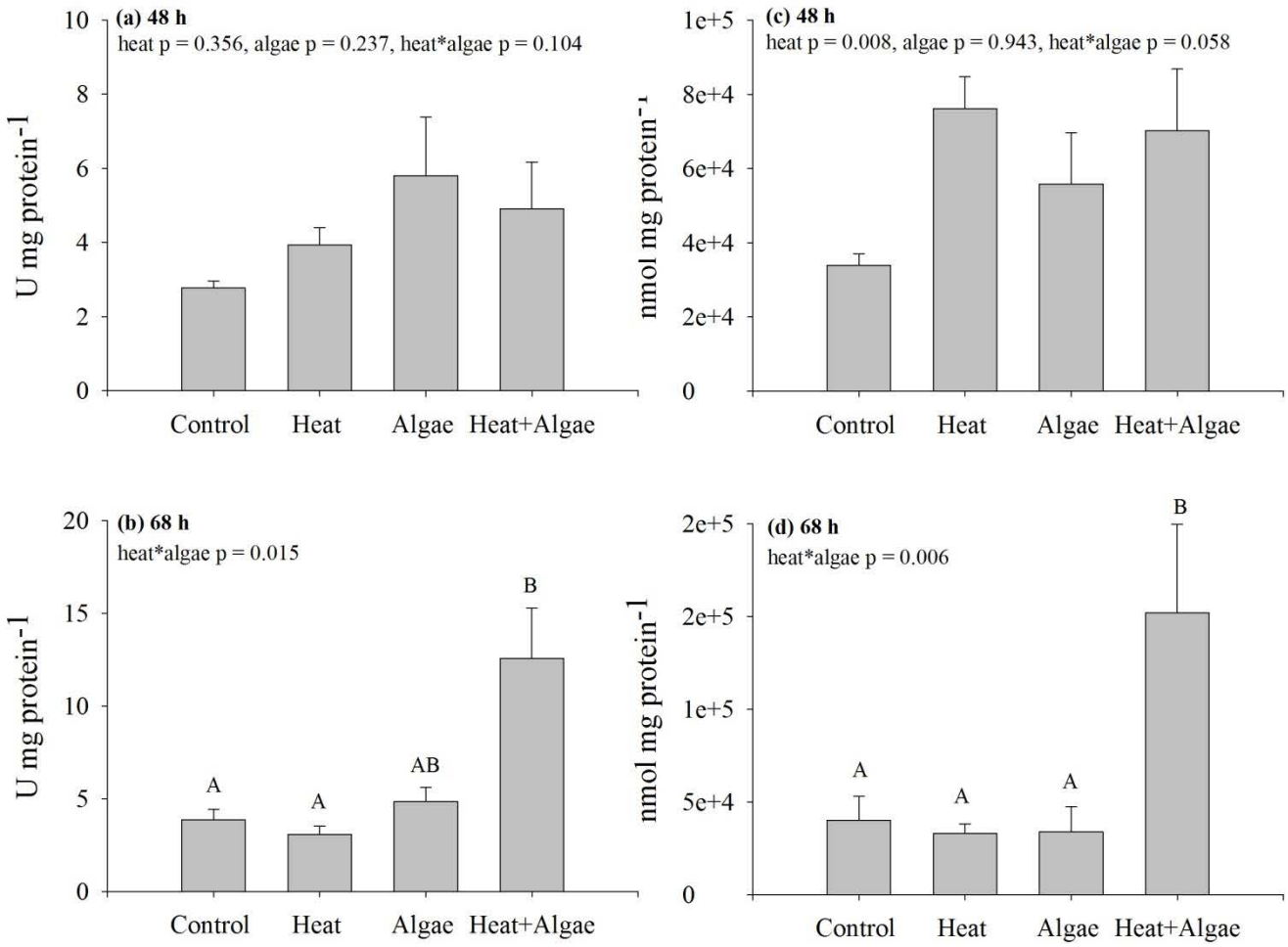

Figure 3.3 Oxidative stress biomarkers in larvae of *P. astreoides*: (a) activity of the enzyme catalase (CAT) following 48 h of exposure to treatments (n = 10 for Control and Heat+Algae treatments, n = 9 for Heat and Algae treatments), (b) CAT activity following 68 h of exposure (n = 8 for Control, Algae, and Heat+Algae treatments, n = 10 for Heat treatment) , (c) lipid hydroperoxides following 48 h of exposure (n = 6 for Control treatment, n = 4 Heat and Heat+Algae treatments, and n = 5 for Algae treatment), and (d) lipid hydroperoxides following 68 h of treatment (n = 7 for Control and Algae treatments, n = 6 for Heat and Heat+Algae treatments). Bars represent mean +1SE. The same letters above the bars indicate means that are not significantly different as determined by a Tukey's post hoc test

3.3.4 Larval Hsp 16 and 60 Transcript Abundances

The normalized abundance of Hsp 16 was elevated in larvae exposed to heated conditions but results bordered statistical significance (P=0.048, Fig.3.4a), and the presence of macroalgae had no impact on the normalized abundance of Hsp16 (P=0.092). Neither thermal stress nor algal exposure significantly impacted the normalized expression of Hsp 60 (Fig.3.4b). As a measure of quality control, the differential expression of the housekeeping gene 18s ribosomal RNA was analyzed among treatments. The transcript abundance of 18s rRNA did not vary significantly as a function of temperature (P =0.675), or algal exposure (P=0.894), verifying its use as a normalizing gene (data not shown).

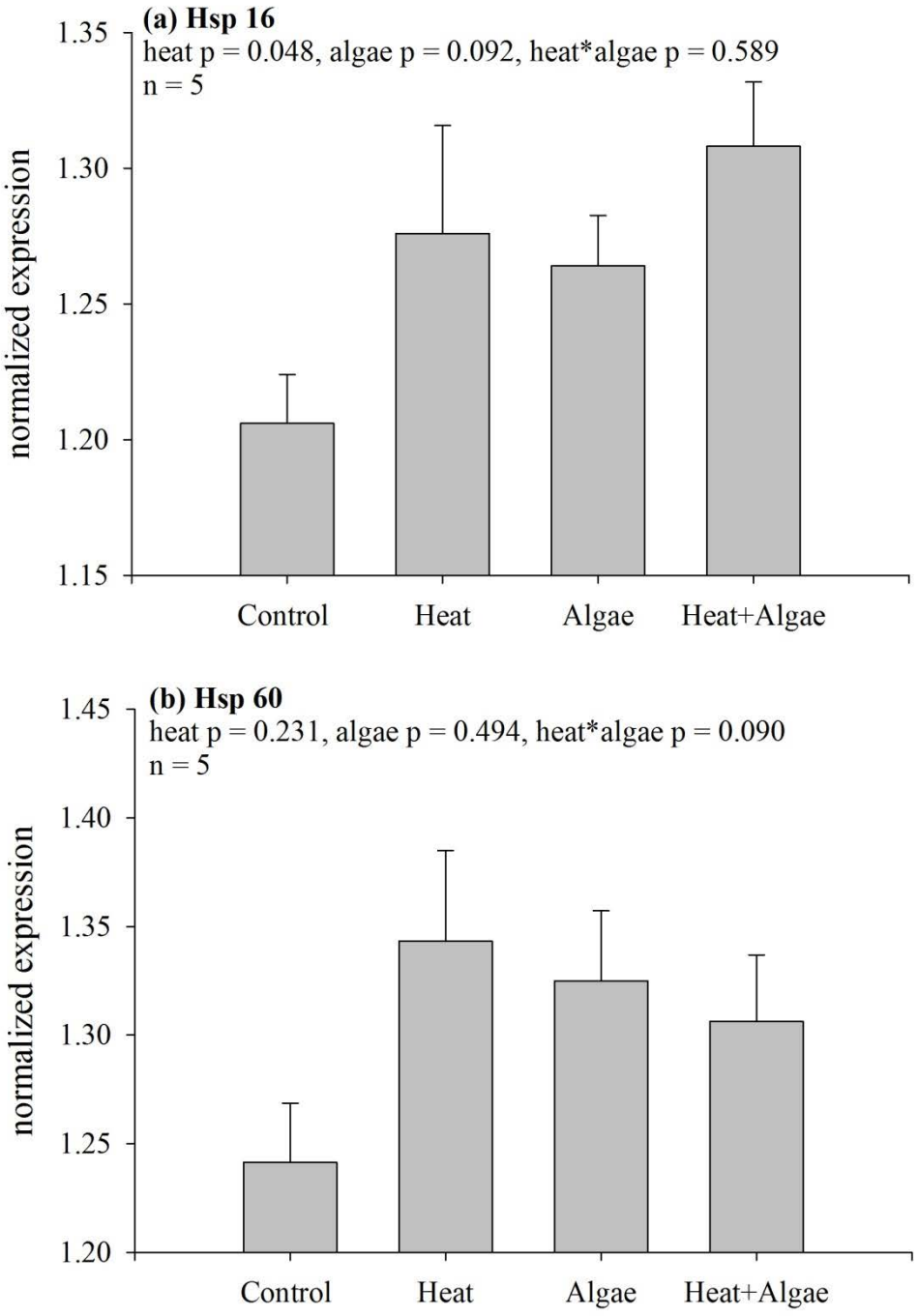

Figure 3.4 Normalized heat shock protein transcriptional expression in larvae from *P. astreoides* following 16 h of exposure to treatments: (a) Hsp 16, (b) Hsp 60. Bars represent mean +1SE

3.3.5 Juvenile Survival

The mean temperatures (±SE) of each treatment through the duration of the experiment on juvenile and adult corals were 29.47±0.07 °C (Control), 32.49±0.10 °C (Heat), 29.49±0.07 °C (Algae), and 32.75±0.09 °C (Heat+Algae).The survival and photochemical efficiency of 6-week-old corals was evaluated using recruit density as a covariate to control for varying numbers of colonies between replicates (see statistical analysis). The differing number of juveniles among replicates [covariate (density)], did not significantly affect recruit survival (P=0.931) or photochemical efficiency (P=0.205). As in the larval experiment, the survival of juvenile corals was not affected by elevated temperature (P=0.182, Fig.3.5), but was significantly reduced in the presence of live *D. menstrualis* ($P < 0.001$, Fig.3.5).

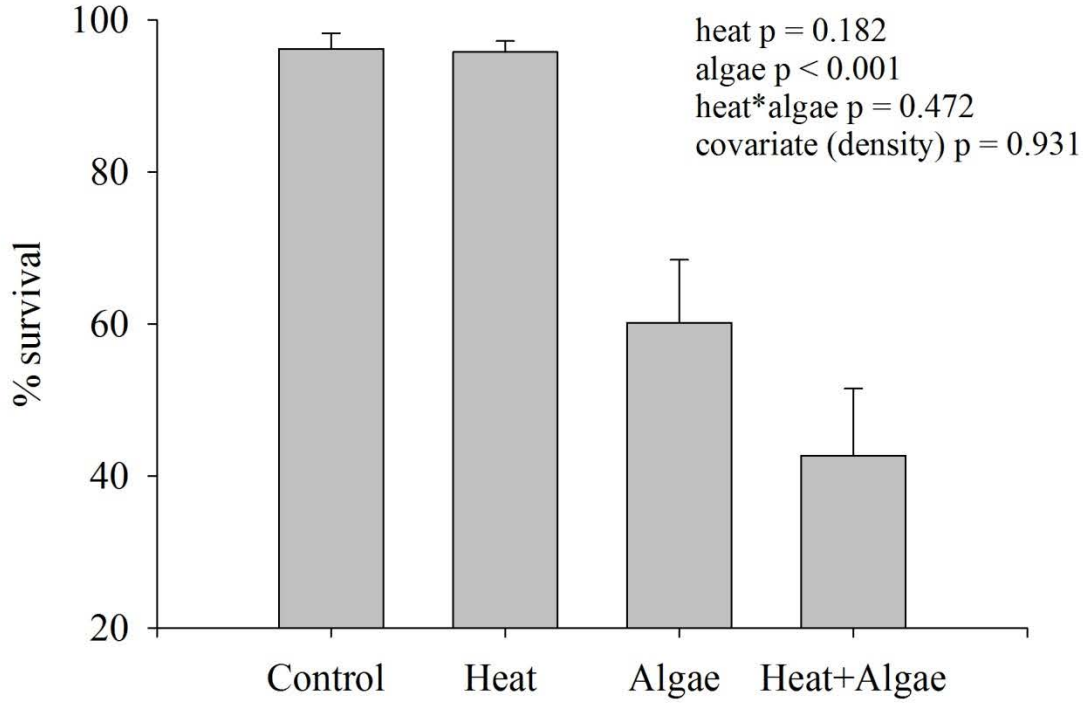

Figure 3.5 Percent survival of 6-week-old *P. astreoides* following 7 d of exposure to treatments (n = 10 for Control, Algae, and Heat+Algae treatments, n = 9 for Heat treatment). Bars represent mean +1 SE

3.3.6 Juvenile Photophysiology

The photochemical efficiency (effective quantum yield) of zooxanthellate 6-week-old corals was evaluated following 7 d of treatment (Fig.3.6). The elevated temperature treatment did not affect the photochemical efficiency of juvenile corals ($P=0.135$), but recruits in direct contact with *D. menstrualis* had a significant reduction in effective quantum yield ($P< 0.001$).

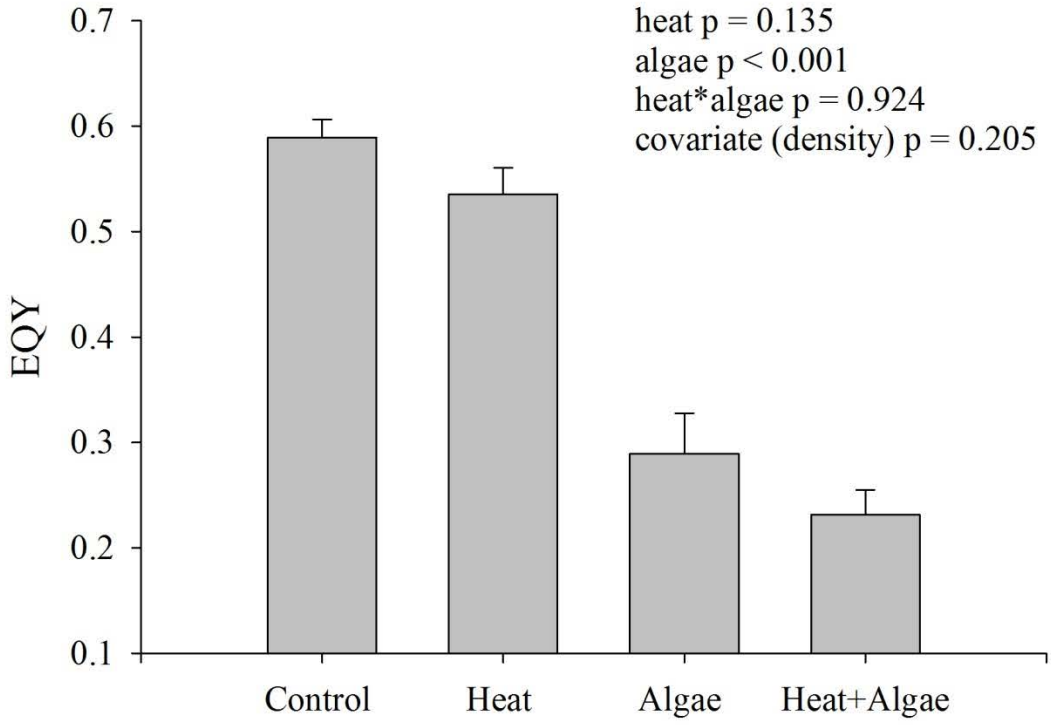

Figure 3.6 Treatment effects on the photochemical efficiency (EQY) of *in hospite* zooxanthellae within 6-week-old *P. astreoides* following 7 d of exposure (n = 10 for Control, Algae, and Heat+Algae treatments, n = 9 for Heat treatment). Bars represent mean +1SE

3.3.7 Juvenile Oxidative Stress

CAT activity and lipid peroxidation were examined as a measure of oxidative stress in juvenile corals following 7 d of treatment (Fig.3.7). Neither thermal stress nor algal exposure affected CAT activity in juvenile corals (Fig.3.7a). Unlike recruit survival and photochemical efficiency, heated seawater had a significant treatment effect on lipid peroxidation in juvenile corals ($P=0.023$, Fig.3.7b). However, direct contact with live *D. menstrualis* did not significantly affect lipid peroxidation in 6-week-old corals ($P= 0.554$).

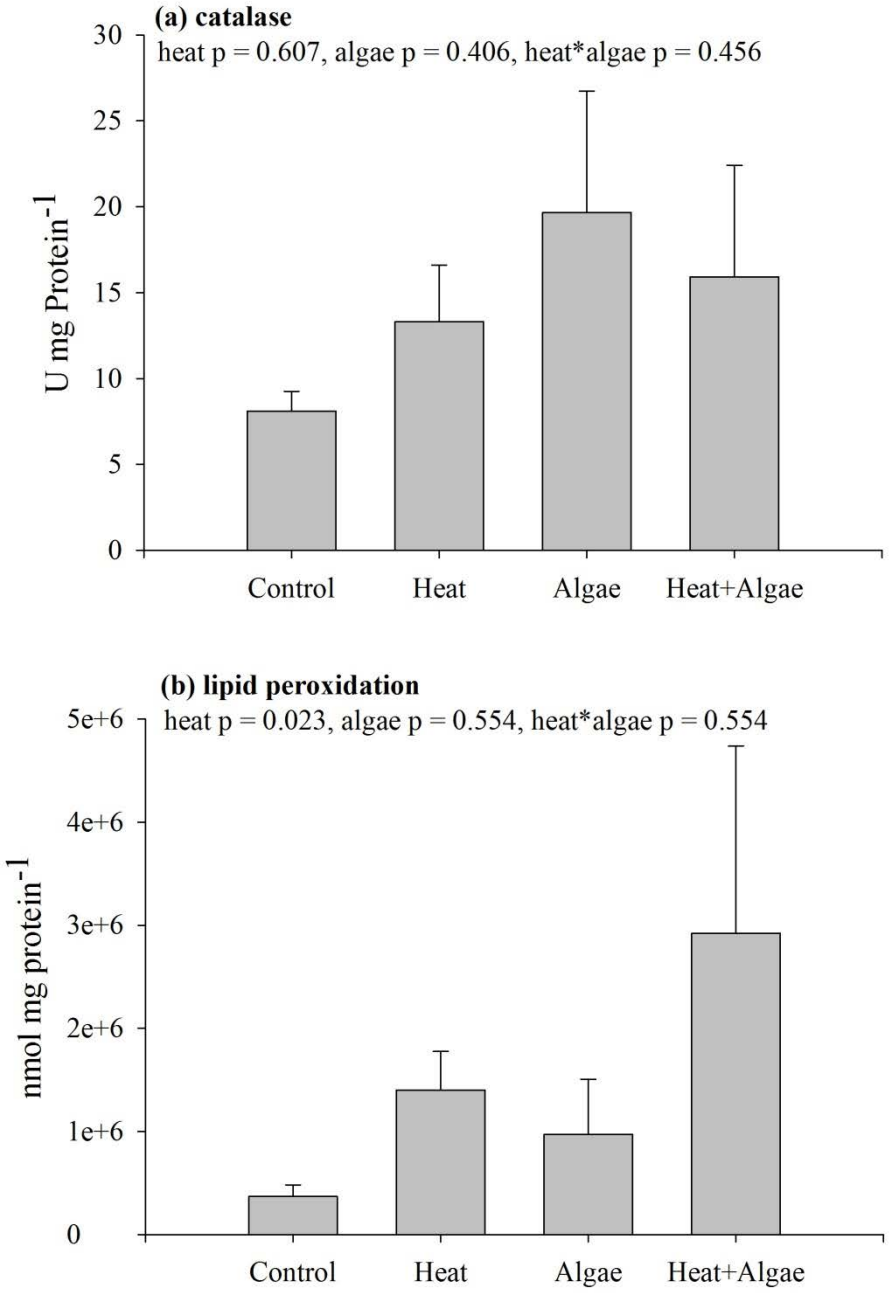

Figure 3.7 Oxidative stress biomarkers in 6-week-old *P. astreoides* following 7 d of exposure: **(a)** activity of the enzyme catalase (CAT) (n = 6 for Control and Algae treatments, n = 4 for Heat and Heat+Algae treatments), and **(b)** lipid hydroperoxides (n = 6 for Control treatment, n = 4 for Heat and Heat+Algae treatments, and n = 5 for Algae treatment). Bars represent mean +1SE

3.3.8 Juvenile Hsp 16 and 60 Transcript Abundances

As in the larval experiment, the transcript abundance of 18s rRNA did not vary significantly as a function of temperature ($P = 0.942$), or algal exposure ($P = 0.288$) (data not shown). Similarly, neither exposure to elevated temperature, algal presence, or their combined treatment significantly affected the normalized transcript abundances of Hsp 16 (Fig.3.8a) or 60 (Fig.3.8b) in juvenile corals.

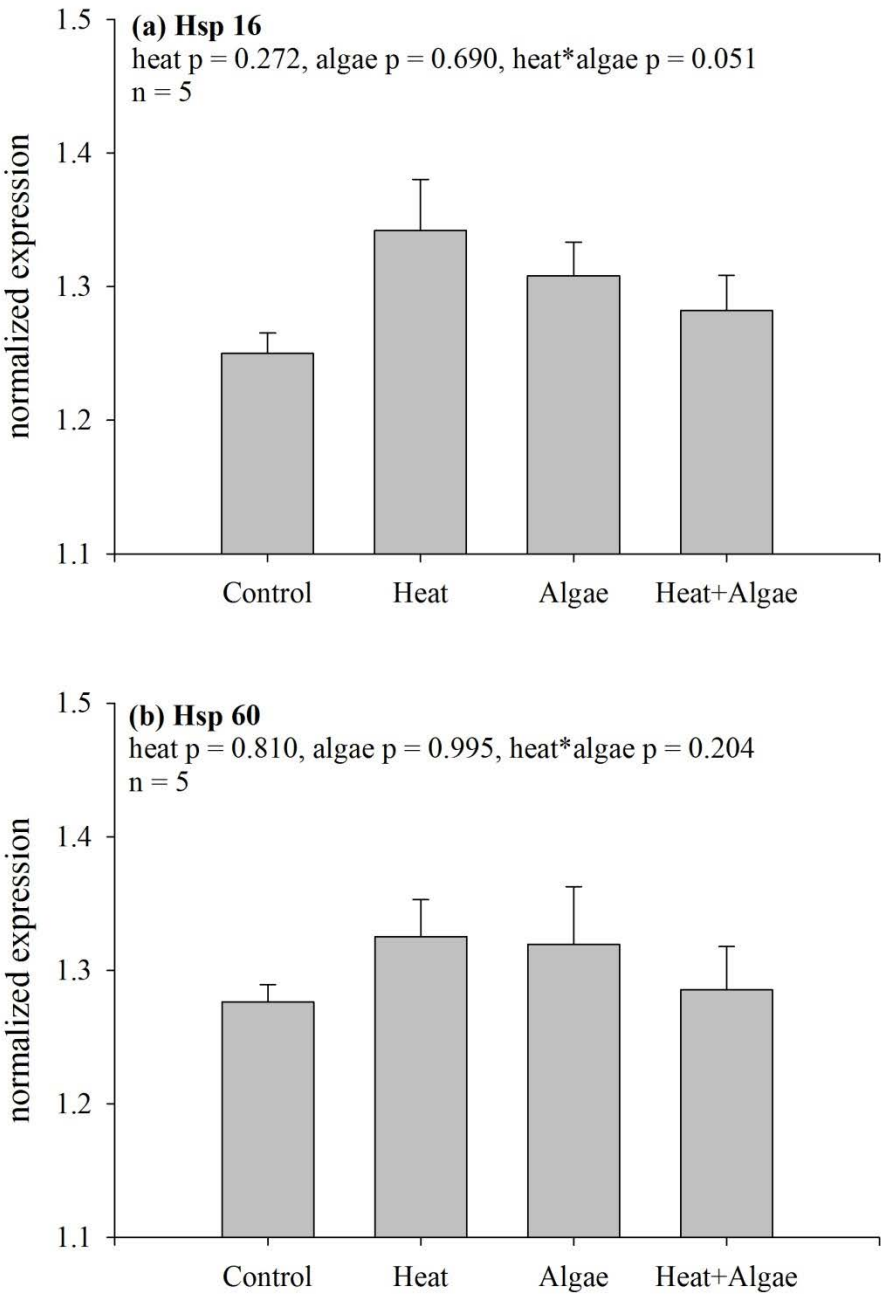

Figure 3.8 Normalized heat shock protein transcriptional expression in 6-week-old *P. astreoides* following 7 d of exposure to treatments: **(a)** Hsp 16, **(b)** Hsp 60. Bars represent mean +1SE

3.3.9 Adult Survival

Treatment effects on the survival of adult corals following 7 d of exposure were analogous to the larval and juvenile experiments (Fig.3.9). Seawater elevated +3.5°C did not significantly affect the survival of adult corals ($P=0.130$), whereas corals in direct contact with live *D. menstrualis* exhibited significant reductions in the ratio of live tissue to dead and bare skeleton ($P<0.001$).

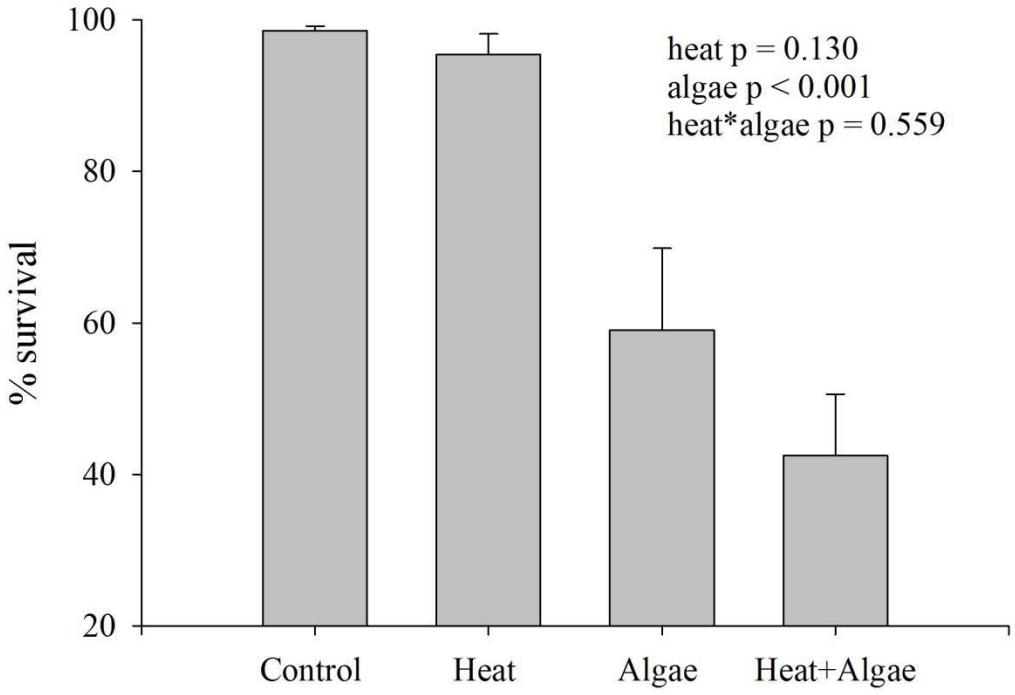

Figure 3.9 Percent survival of adult *P. astreoides* following 7 d of exposure to treatments (n = 7 for Control, Algae, and Heat+Algae treatments, n = 6 for Heat treatment). Bars represent mean +1 SE

3.3.10 Adult Photophysiology

PAM fluorometry provided a more rigorous quantification of survival and health in adult corals compared to visual assessments alone. The photochemical efficiency (effective quantum yield) of adult corals was evaluated following 7 d of exposure (Fig.3.10). Following treatment, both thermal stress ($P=0.016$) and algal presence ($P<0.001$) significantly reduced the effective quantum yield of zooxanthellate adult corals.

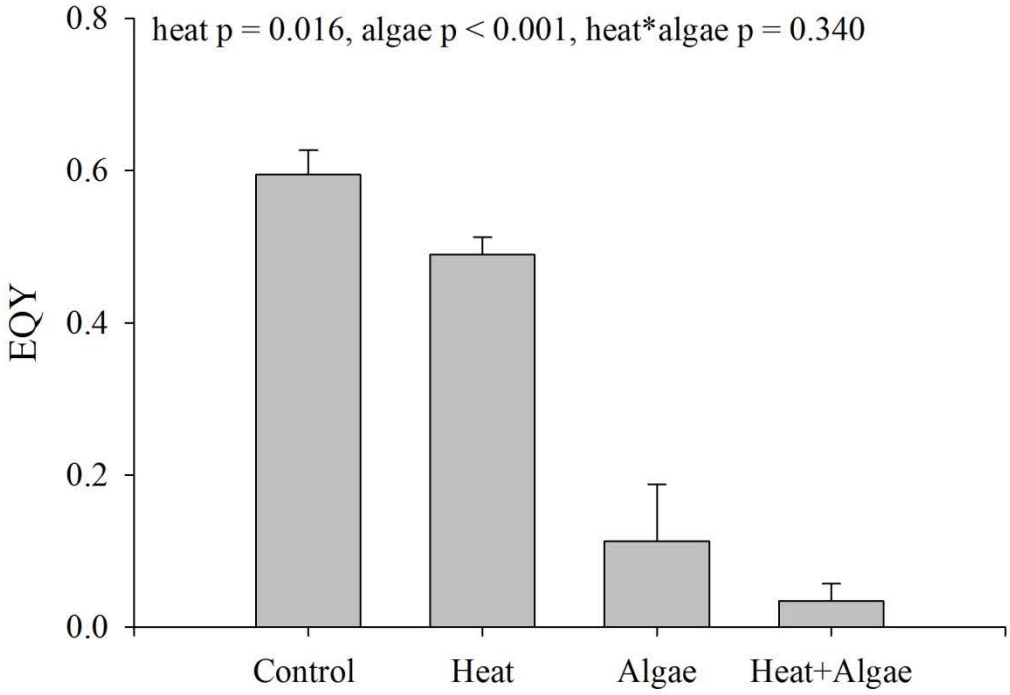

Figure 3.10 Treatment effects on the photochemical efficiency (EQY) of *in hospite* zooxanthellae within adult *P. astreoides* following 7 d of exposure (n = 7 for Control, Algae, and Heat+Algae treatments, n = 6 for Heat treatment). Bars represent mean +1SE

3.3.11 Adult Oxidative Stress

The health of adult corals was further evaluated using CAT activity and degree of lipid peroxidation as indicators of oxidative stress (Fig.3.11). In contrast to larval and juvenile experiments, neither elevated temperature, algal exposure, nor their combined treatment significantly affected CAT activity (Fig.3.11a) or lipid peroxidation (Fig.3.11b) in adult corals.

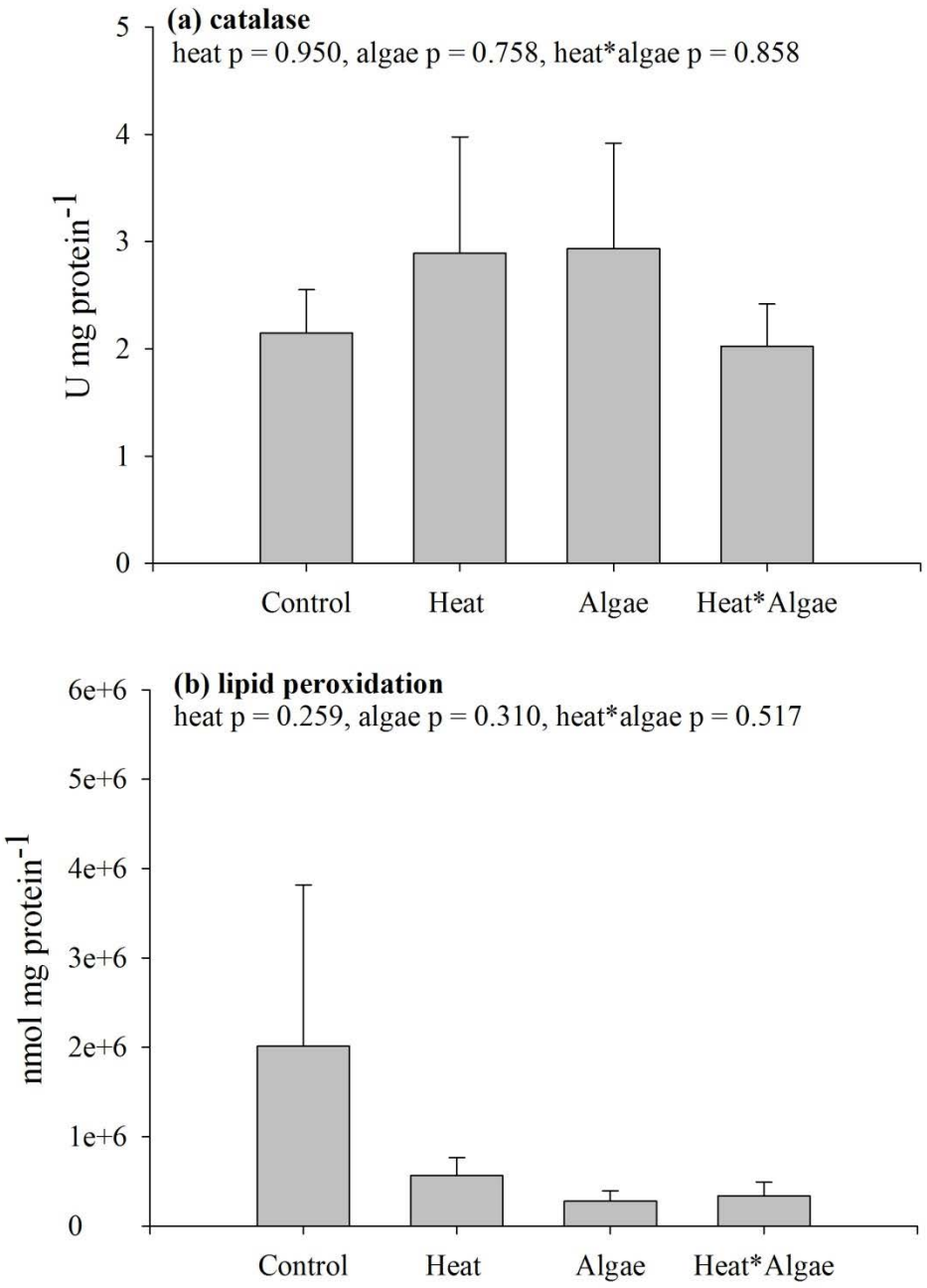

Figure 3.11 Oxidative stress biomarkers in adult *P. astreoides* following 7 d of exposure: (a) activity of the enzyme catalase (CAT) (n = 6 for Control and Algae treatments, n = 4 for Heat and Heat+Algae treatments), and (b) lipid hydroperoxides (n = 6 for Control treatment, n = 4 for Heat and Heat+Algae treatments, and n = 5 for Algae treatment). Bars represent mean +1SE

3.3.12 Adult Hsp 16 and 60 Transcript Abundances

Again the transcript abundance of 18s rRNA did not vary significantly as a function of temperature (P=0.773), or algal exposure (P=0.220), permitting its use as a normalizing agent (data not shown). Treatments did not significantly affect the normalized expression of Hsps 16 (Fig.3.12a) or 60 (Fig.3.12b) in adult corals following 7 d of exposure.

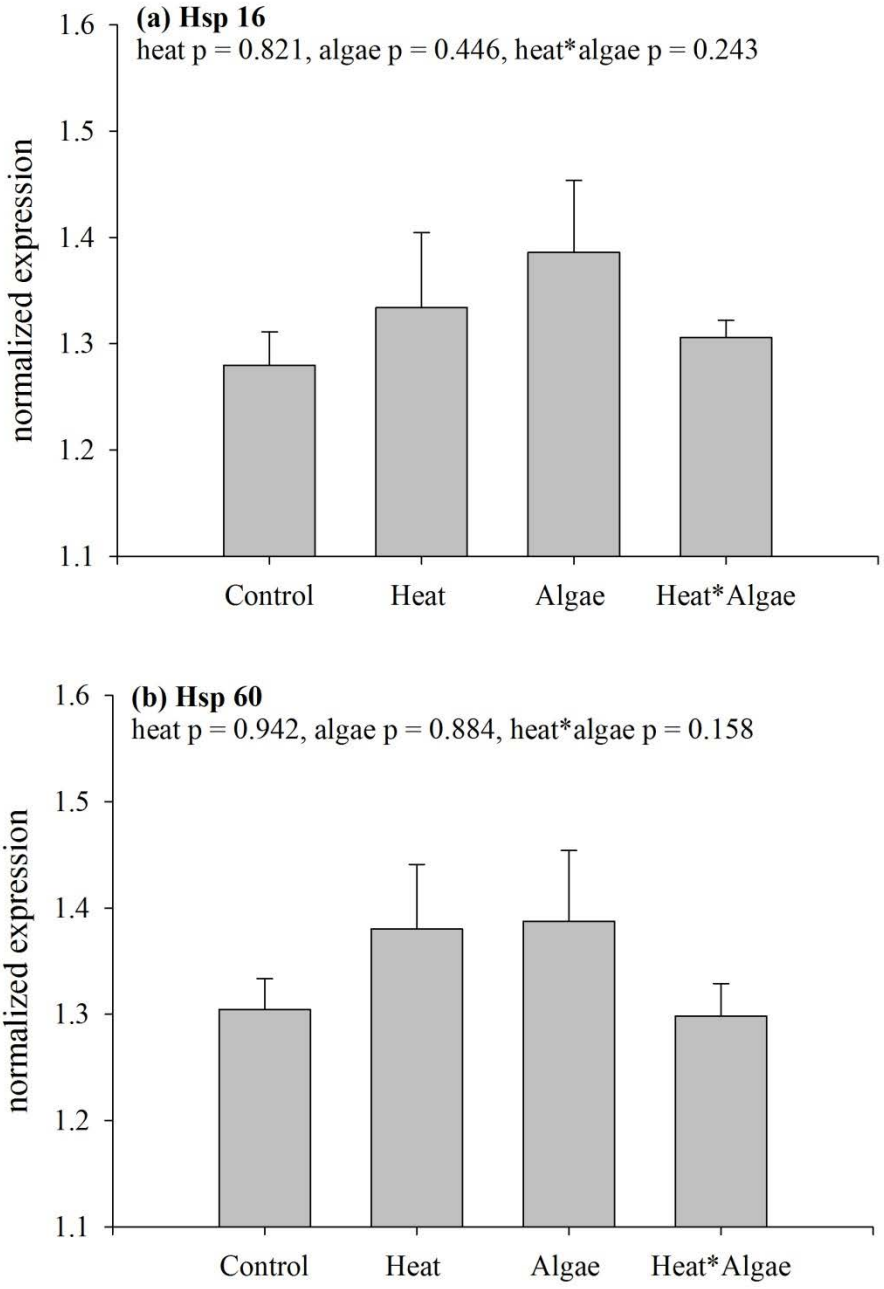

Figure 3.12 Normalized heat shock protein transcriptional expression in adult *P. astreoides* following 7 d of exposure to treatments: (a) Hsp 16, (b) Hsp 60. Bars represent mean +1SE

3.3.13 Statistics Summary

A summary of the statistical results from all experiments in this study is located in Table 3.1.

Life History Stage	Dependent Variable	heat	algae	heat*algae	covariate(density)
Larval	% survival	$F_{(1,36)} = 1.089$, $p = 0.304$	$F_{(1,36)} = 110.009$, $p < 0.001$	$F_{(1,36)} = 5.419$, $p = 0.026$	n/a
	% settlement	$F_{(1,35)} = 0.684$, $p = 0.414$	$F_{(1,35)} = 10.646$, $p = 0.002$	$F_{(1,35)} = 0.709$, $p = 0.405$	n/a
	% post-settlement survival	$F_{(1,36)} = 0.141$, $p = 0.709$	$F_{(1,36)} = 6.615$, $p = 0.014$	$F_{(1,36)} = 0.214$, $p = 0.647$	n/a
	F_v/F_m	$F_{(1,35)} = 40.914$, $p < 0.001$	$F_{(1,35)} = 1.270$, $p = 0.267$	$F_{(1,35)} = 2.341$, $p = 0.135$	n/a
	CAT activity (48 h)	$F_{(1,35)} = 0.877$, $p = 0.356$	$F_{(1,35)} = 1.445$, $p = 0.237$	$F_{(1,35)} = 2.782$, $p = 0.104$	n/a
	CAT activity (68 h)	$F_{(1,30)} = 0.277$, $p = 0.603$	$F_{(1,30)} = 14.241$, $p = 0.001$	$F_{(1,30)} = 6.679$, $p = 0.015$	n/a
	lipid peroxidation (48 h)	$F_{(1,34)} = 7.951$, $p = 0.008$	$F_{(1,34)} = 0.005$, $p = 0.943$	$F_{(1,34)} = 3.861$, $p = 0.058$	n/a
	lipid peroxidation (68 h)	$F_{(1,28)} = 7.695$, $p = 0.010$	$F_{(1,28)} = 1.3566$, $p = 0.069$	$F_{(1,28)} = 8.740$, $p = 0.006$	n/a
	18s ribosomal RNA	$F_{(1,16)} = 0.182$, $p = 0.675$	$F_{(1,16)} = 0.018$, $p = 0.894$	$F_{(1,16)} = 0.046$, $p = 0.832$	n/a
	normalized Hsp 16	$F_{(1,16)} = 4.573$, $p = 0.048$	$F_{(1,16)} = 3.222$, $p = 0.092$	$F_{(1,16)} = 0.305$, $p = 0.589$	n/a
	normalized Hsp 60	$F_{(1,16)} = 1.550$, $p = 0.231$	$F_{(1,16)} = 0.489$, $p = 0.494$	$F_{(1,16)} = 3.252$, $p = 0.090$	n/a
Juvenile	% survival	$F_{(1,34)} = 1.857$, $p = 0.182$	$F_{(1,34)} = 66.140$, $p < 0.001$	$F_{(1,34)} = 0.529$, $p = 0.472$	$F_{(1,34)} = 0.008$, $p = 0.931$
	effective quantum yield (EQY)	$F_{(1,34)} = 2.341$, $p = 0.135$	$F_{(1,34)} = 123.962$, $p < 0.001$	$F_{(1,34)} = 0.009$, $p = 0.924$	$F_{(1,34)} = 1.666$, $p = 0.205$
	CAT activity	$F_{(1,16)} = 0.275$, $p = 0.607$	$F_{(1,16)} = 0.729$, $p = 0.406$	$F_{(1,16)} = 0.584$, $p = 0.456$	n/a
	lipid peroxidation	$F_{(1,15)} = 6.397$, $p = 0.023$	$F_{(1,15)} = 0.367$, $p = 0.554$	$F_{(1,15)} = 0.367$, $p = 0.554$	n/a
	18s ribosomal RNA	$F_{(1,16)} = 0.005$, $p = 0.942$	$F_{(1,16)} = 1.209$, $p = 0.288$	$F_{(1,16)} = 0.650$, $p = 0.432$	n/a
	normalized Hsp 16	$F_{(1,16)} = 1.292$, $p = 0.272$	$F_{(1,16)} = 0.165$, $p = 0.690$	$F_{(1,16)} = 4.455$, $p = 0.051$	n/a
	normalized Hsp 60	$F_{(1,16)} = 0.060$, $p = 0.810$	$F_{(1,16)} < 0.001$, $p = 0.995$	$F_{(1,16)} = 1.751$, $p = 0.204$	n/a
Adult	% survival	$F_{(1,23)} = 2.472$, $p = 0.130$	$F_{(1,23)} = 37.203$, $p < 0.001$	$F_{(1,23)} = 0.352$, $p = 0.559$	n/a
	effective quantum yield (EQY)	$F_{(1,23)} = 6.814$, $p = 0.016$	$F_{(1,23)} = 55.628$, $p < 0.001$	$F_{(1,23)} = 0.951$, $p = 0.340$	n/a
	CAT activity	$F_{(1,20)} = 0.004$, $p = 0.950$	$F_{(1,20)} = 0.097$, $p = 0.758$	$F_{(1,20)} = 0.033$, $p = 0.858$	n/a
	lipid peroxidation	$F_{(1,21)} = 1.344$, $p = 0.259$	$F_{(1,21)} = 1.082$, $p = 0.310$	$F_{(1,21)} = 0.435$, $p = 0.517$	n/a
	18s ribosomal RNA	$F_{(1,16)} = 0.086$, $p = 0.773$	$F_{(1,16)} = 1.628$, $p = 0.220$	$F_{(1,16)} = 0.001$, $p = 0.976$	n/a
	normalized Hsp 16	$F_{(1,16)} = 0.053$, $p = 0.821$	$F_{(1,16)} = 0.611$, $p = 0.446$	$F_{(1,16)} = 1.468$, $p = 0.243$	n/a
	normalized Hsp 60	$F_{(1,16)} = 0.005$, $p = 0.942$	$F_{(1,16)} = 0.022$, $p = 0.884$	$F_{(1,16)} = 2.192$, $p = 0.158$	n/a

Table 3.1Summary of between-subjects effects for experiments on the larval, juvenile, and adult life-history stages of *P. astreoides*

3.4 Discussion

While it is believed that global and regional-scale stressors may be interacting synergistically to reduce the status of coral reefs in the Caribbean, few empirical studies have evaluated the effects of multiple stressors on coral health and recruitment. Our results indicate that *D. menstrualis* exposure and elevated temperature did not consistently interact to directly affect coral mortality. Instead, macroalgal presence and thermal stress had unique impacts on either coral survival or physiological condition. The presence of *D. menstrualis* significantly reduced the survival and recruitment of *P. astreoides* during every coral life-history stage examined. *D. menstrualis* presence significantly reduced coral larval survival, reduced settlement, and those larvae exposed to macroalgae for 68 h had significantly reduced survival following settlement as juvenile recruits. Furthermore, direct contact with *D. menstrualis* significantly reduced the survival of both 6 week-old and 1-2 year-old corals. Elevated temperature impacted coral condition yet had disparate effects across coral life-history stages. Within larvae, thermal stress reduced the photochemical efficiency of *in hospite* zooxanthellae, enhanced oxidative stress following 48 h, and elevated the transcriptional abundance of a heat shock protein associated with the hyperthermal stress response. In comparison, the effects of elevated seawater temperatures on more established juvenile (6 week-old) and adult (1-2 year-old) corals were seemingly diminished. While macroalgal exposure and elevated temperature typically had distinct effects on either coral survival or health, both stressors combined induced interactive impacts on CAT activity and a synergistic elevation in lipid peroxidation in larvae following 68 h. Our findings suggest that exposure to the conspicuous macroalgae *D. menstrualis* caused significant reductions in coral recruitment and that seawater elevated 3.5°C had deleterious impacts on coral health. In addition, coral larvae were more susceptible to the

effects of hyperthermal stress compared to established corals and both stressors combined induced interactive or synergistic responses with respect to biomarkers of oxidative stress in the larvae of *P. astreoides*.

3.4.1 Effects of Macroalgae on the Recruitment of Coral Larvae

Benthic macroalgae are important members of reef communities and provide vital ecological functions necessary for sustaining healthy coral reef ecosystems (reviewed in Fong and Paul 2011). Over the last few decades however, the distribution and abundance of fleshy macroalgae have increased significantly on coral reefs and have greatly replaced the historically dominant encrusting crustose coralline algae (Littler and Littler 1988; Lirman and Biber 2000; Kuffner et al. 2008). The proliferation of fleshy macroalgae is often associated with the declining status of coral reefs globally and increases the frequency of coral-algae competitive interactions (Miller 1998; McCook 1999). Coral susceptibility to macroalgal competition is species-specific and is assumed to be influenced by coral life-history stage (Tanner 1995; Lirman 2001). Our results indicated that the presence of *D. menstrualis* within larval settlement chambers significantly inhibited the successful recruitment of *P. astreoides*. Macroalgal presence elevated the mortality of coral larvae, reduced their settlement, and acute exposure to *D. menstrualis* during the planktonic stage had a latent effect on recruit survival post-settlement.

Larval supply, settlement, and subsequent growth to sexual maturity are key steps in the process of coral recruitment. Consequently, successful recruitment is necessary for maintaining coral populations and for contributing to the resiliency of coral reefs (Ritson-Williams et al. 2009). Stressors such as coral-algae competition that impact multiple life-history stages of corals have the most potential for reducing coral recruitment and are the most significant threat to the

recovery of coral populations (Hughes 1994; Ritson-Williams et al. 2009). *Dictyota spp*. have been shown to reduce the supply, survival, and settlement of coral larvae (Kuffner et al. 2006; Foster et al. 2008; Paul et al. 2011), as well as the survival of juvenile spat (Box and Mumby 2007). However, the effects of acute macroalgal exposure and the associated stress on subsequent recruitment processes are poorly understood. Exposure to stressful conditions during the planktonic larval stage can result in elevated energetic demands that reduce coral survival following settlement and metamorphosis (Monaghan et al. 2009; Ross et al. 2013). This may be particularly pertinent in the context of coral-algae competition due to the ephemeral nature of many reef-inhabiting fleshy macroalgae (Lirman and Biber 2000).

3.4.2 Effects of Macroalgae on Juvenile and Adult Corals

Dictyota spp. and non-polar extracts isolated from these macroalgae have also been shown to reduce the survival and growth of established corals (Tanner 1995; Rasher and Hay 2010; Paul et al. 2011). Similarly, in this study both 6 week-old and 1-2 year-old coral colonies had reduced survival when in direct contact with *D. menstrualis*. Plastic algal mimics held in direct contact with coral tissue had little to no effect on colony survival, suggesting that coral mortality was due to more than algal abrasion, shading, or physical effects alone. PAM fluorometry was utilized to supplement visual assessments of colony survival and focused on areas of coral tissue that were in direct contact with macroalgae. The effective quantum yield (EQY) of 6 week-old and 1-2 year-old coral colonies exposed to *D. menstrualis* paralleled visual assessments of coral survival and reflected values indicative of colony mortality (Smith et al. 2006; Rasher and Hay 2010). The consistent impact of *D. menstrualis* on the recruitment

parameters of all three coral life-history stages suggests that macroalgae effectively compete with reef-building corals during every critical step of the recruitment process.

While exposure to macroalgae caused coral mortality, *D. menstrualis* presence did not significantly impact the biomarkers of oxidative stress or the transcript abundances of the heat shock proteins evaluated in this study. Assessments of the effects of macroalgae exposure on coral oxidative stress and heat shock protein regulation are severely limited. In a study evaluating the effects of allelopathic extracts isolated from several species of macroalgae on the *Acropora millepora* transcriptome, Shearer et al. (2012) reported the up-regulation of several molecular chaperones and oxidative stress genes following 24 h and 20 d of exposure. However, the authors also concluded that the coral transcriptomic response was species-specific and varied considerably depending on the composition and concentration of the extract being tested. In addition, the study did not evaluate non transcriptional biomarkers of oxidative stress. Further investigation is certainly warranted to better characterize the effects of macroalgal exposure on coral oxidative stress and transcriptional regulation of Hsps.

3.4.3 Effects of Elevated Temperature on Coral Health

The elevated temperature treatment used in this study is an expected prediction for rising sea surface temperatures over the next century (IPCC 2007). Hyperthermal stress was quantified with several model biomarkers of coral health and included photosynthetic efficiency of *in hospite* zooxanthellae, the reduced oxygen species (ROS) sequestering activity of the enzyme catalase (CAT), oxidative damage in the form of lipid peroxidation, and the transcriptional abundance of heat shock proteins (Hsp) 16 and 60 (reviewed in Lesser 2006; Ross et al. 2010; Kenkel et al. 2011; Ross et al. 2013; Olsen et al. 2013). Elevated temperature consistently

impacted coral health but the magnitude of its effects was not detected equally across coral life-history stages. Thermal stress reduced the photosynthetic efficiency of *in hospite* zooxanthellae, elevated lipid peroxidation, and increased the transcriptional abundance of Hsp 16 in larvae from *P. astreoides*. Comparatively, seawater elevated +3.5°C caused oxidative damage in 6 week-old corals and reduced the effective quantum yield of zooxanthellae within 1-2 year-old corals.

Elevated temperatures are known to disrupt the symbiosis between coral hosts and symbiotic dinoflagellates (Weis 2008; Baird et al. 2009). Hyperthermal stress can cause reductions in the photosynthetic efficiency of *in hospite* zooxanthellae (Warner et al. 1999), and can elevate oxidative damage leading to the expulsion of the algal symbiont (Lesser 1997; Weis 2008; Baird et al. 2009). Correspondingly, biomarkers of oxidative stress (Lesser 1997; Downs et al. 2000; Yakovleva et al. 2009; Ross et al. 2013; Olsen et al. 2013) and the regulation of molecular chaperones including Hsps (Rodriguez-Lanetty et al. 2009; Kenkel et al. 2011) have been well characterized in corals in response to temperature perturbations. The results of the current study support the established notion that thermal stress reduces the health of the coral holobiont, rendering corals prone to large-scale disease, bleaching, and mortality events (Jokiel and Coles 1990; Boyett et al. 2007; Bruno et al. 2007). This study also highlights the differing response of corals from multiple life-history stages to elevated temperature.

3.4.4 Susceptibility of Coral Larvae to Hyperthermal Stress

The disparate impacts of elevated temperature on coral health between distinct life-history stages suggests that coral larvae are more susceptible to the damaging effects of hyperthermal stress compared to established 6 week-old and 1-2 year-old corals. Larvae of marine benthic invertebrates are often assumed to be particularly vulnerable to environmental

stress compared to post-metamorphic counterparts (Hunt and Scheibling 1997; Pechenik 1999), but this may be misleading as the opposite has also been shown to be true (Hamdoun and Epel 2007) . Furthermore few studies have directly compared the effects of environmental stressors on multiple life-history stages of hermatypic corals (Albright 2011). Putnam et al. (2010) found that the photochemical efficiency of *Pocillopora damicornis* and *Seriatopora hystrix* larvae were greatly reduced in comparison to adult corals exposed to identical elevated temperature treatments. The authors concluded that the larvae of these corals were more sensitive to the effects of elevated temperature than their adult counterparts. Our results support this conclusion and expand upon it by including juvenile corals, biomarkers of the oxidative stress response, and the transcriptional regulation of Hsps 16 and 60. Additionally, the results of the current study likely underestimate the discrepancy between life-history stages as exposure duration was significantly less in larval corals (68 h versus 7 days in juvenile and adult corals).

3.4.5 Effects of Compounding Stressors

There is growing evidence that multiple ecological perturbations can interact synergistically to accelerate biodiversity loss (Sala and Knowlton 2006; Darling and Cote 2008). An ecological synergy is said to occur when two or more physical factors have an interactive compounding affect on a biological parameter which is greater than the simple addition of the impacts of each factor alone (Paine et al. 1998; Dunne 2010). In the context of management, the unpredictable nature of synergistic relationships makes them particularly threatening to predictions of coral reef stability and resiliency (Hughes and Connell 1999; Darling and Cote 2008). Exposure to *D. menstrualis* in combination with elevated temperature induced interactive effects on CAT activity and synergistic elevations in oxidative damage in *P. astreoides* larvae

following 68 h. The synergistic impacts on lipid peroxidation in larval corals indicate that compounding stressors may have ecological consequences due to elevated energetic taxing associated with repairing oxidative damage (Lesser and Farrell 2004;Monaghan et al. 2009; Ross et al. 2013). Reductions in growth, immune function, and fecundity are all potential repercussions associated with increasing oxidative stress and have serious implications for the declining status of coral reefs (Hughes et al. 2007; Monaghan et al. 2009). Thus, the previously predicted effects of macroalgal proliferation and rising sea surface temperatures on coral reef resiliency may be grave overestimations.

3.4.6 Conclusion

In conclusion, the results indicate that fleshy macroalgae effectively compete with reef-building corals for space and resources on coral reefs and that rising sea surface temperatures associated with global climate change are deleterious to the health of the coral holobiont. Hyperthermal stress was also detected more readily in *P. astreoides* larvae compared to post-metamorphic corals and may indicate that the larvae of this species are more vulnerable to the damaging effects of thermal stress. Finally, the results suggest that compounding stressors can synergistically reduce coral health which may exacerbate the status of coral reefs in response to multiple perturbations.

4.

General Conclusions

In summary, the significant decline of live coral cover in the Caribbean highlights the need to develop reliable techniques that detect stress in multiple life-history stages of hermatypic corals prior to the degradation of coral reef habitats. Exposure to elevated sweater temperature did not consistently impact the mRNA expression of Hsp 16 or 60, settlement, or survivorship of *P. astreoides* larvae, but elevated temperature induced oxidative stress and elevated respiratory demand. These results have implications for the effects of climate change on coral reproduction and the potential techniques for evaluating heat-induced stress in hermatypic corals.

Furthermore, the results support the accepted notion that fleshy macroalgae compete with reef-building corals for space and resources on coral reefs and that rising sea surface temperatures associated with global climate change are deleterious to the health of the coral holobiont. The results also suggest that coral larvae are more vulnerable to the effects of hyperthermal stress compared to post-metamorphic corals and that compounding stressors can synergistically reduce coral health which may result in unpredicted ecological consequences.

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Vita

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Education

M.S., Biology, University of North Florida, Expected completion 2013
B.S., Biology, University of North Florida, 2011

Publications

Ross, C., Ritson-Williams, R., **Olsen, K**., Paul, V. J. (2012) Short term and latent post-settlement effects associated with elevated temperature and oxidative stress on larvae from the coral *Porites astreoides*. Coral Reefs 32: 71-79. DOI 10.1007/s00338-012-0956-2

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Presentations

Ross, C., **Olsen, K.**, Ritson-Williams, R., and Paul, V.J. Interactions among ocean acidification, elevated temperature and macroalgal presence on coral larvae stress and survivorship MOTE P.O.R. Project Review. Florida Keys Eco-Discovery Center, Key West, FL. April 23rd 2013.

Olsen, K., Paul V.J., Ritson-Williams, R., and Ross, C. The effects of elevated temperature (global-scale stressor), algal competition (local-scale stressor) and their combined impacts on the early life history stages of the Caribbean hermatypic coral *Porites astreoides.* 42nd Annual Benthic Ecology Meeting. Savannah, GA. March 20-24, 2013.

Olsen, K. Reef-building corals and the systems they engineer. Invited Speaker. University of North Florida Marine Biology Club Jacksonville, FL. February 28, 2013.

Eash-Loucks, W., and **Olsen, K.** Recruitment and distribution of biofouling invertebrates within the GTM estuary. State of the Reserve 2013 GTMNERR, FL. February 1, 2013.

Olsen, K., Paul V.J., Ritson-Williams, R., Ochrietor, J., and Ross, C. The effects of global and local-scale stressors on the early life history stages of the reef-building coral *Porites astreoides*. University of Florida Marine Biology Symposium 2013 St. Augustine, FL. January 17-18, 2013.

Olsen, K., Ochrietor, J., Ritson-Williams, R., Paul, V.J., Ross, C. Utilization of oxidative stress and heat shock protein biomarkers to detect heat-induced sublethal stress in larvae of the reef-building coral *Porites astreoides*. American Society for Cell Biology 2012 Annual Meeting San Francisco, CA. December 15-19, 2012.

Olsen, K., Ritson-William, R., Paul, V.J., Ross, C. Reef-building corals in a stressful world. Invited Speaker. Smithsonian Marine Station Fort Pierce, FL. July 27, 2012.

Olsen, K., Ritson-Williams, R., Paul, V.J., Ross, C. Effects of elevated temperature and oxidative stress on the physiology and post-settlement survival of larvae of the coral *Porites astreoides*. University of Florida Marine Biology Symposium St. Augustine, FL. January 19-20, 2012. – Runner up for best poster in session.

Ross, C., Ritson-Williams, R., **Olsen, K.,** Paul, V.J. Effects of oxidative stress and elevated temperature on coral larvae health and post-settlement survival. Coastal and Research Estuarine Foundation 2011 Daytona, FL. Nov. 6-10, 2011.

Ross, C., Ritson-Williams, R., **Olsen, K.,** Paul, V.J. Effects of oxidative stress and elevated temperature on coral larvae health and post-settlement survival. 40th Annual Benthic Ecology Meeting. Mobile Alabama. March 16-20, 2011.

Olsen, K., Ritson-Williams, R., Paul, V.J., Ross, C. Effects of thermal and oxidative stress on coral larvae health and post-settlement survivorship. Florida Statewide Student Research Symposium, UNF March 2011.

Olsen, K., Ritson-Williams, R., Paul, V.J., Ross, C. Effects of thermal and oxidative stress on coral larvae health and post-settlement survivorship. STARS Student Research Symposium, UNF March 2011.

Olsen, K., Ritson-Williams, R., Paul, V.J., Ross, C. Effects of thermal and oxidative stress on coral larvae health and post-settlement survivorship. University of North Florida Undergraduate Research Symposium. UNF, October 2010.

Grants and Fellowships

- UNF Graduate Summer Research Award, University of North Florida Biology Department April 2013.
- UNF Graduate School Travel Grant, University of North Florida Graduate School September 2012.
- UNF Coastal Biology Travel Grant, University of North Florida Biology Department October 2012.
- Link Foundation Fellowship, Smithsonian Marine Station Fort Pierce, FL. May-August 2012.
- UNF Coastal Biology Travel Grant, University of North Florida Biology Department October 2011.
- UNF Undergraduate Summer Research Award, University of North Florida Biology Department April 2010.
- Eddie M. Skofield Scholarship, University of North Florida Biology Department November 2010.

Research Experience

2012 Smithsonian Marine Station, Fort Pierce, FL. Fellowship research. Synergistic effects of elevated temperature and *Dictyota spp*. presence on the early life history stages of the scleractinian coral *Porites astreoides.*

2012 Mote Marine Laboratory, Summerland Key, FL. Thesis research. Synergistic effects of elevated temperature and *Dictyota sp*. presence on the health and post-settlement survival of larvae of the coral *Porites astreoides*.

2011 Smithsonian Marine Station, Carrie Bow Cay, Belize. Research Assistant, Effects of salinity stress on larvae of the coral *Favia fragum.*

2011 Mote Marine Laboratory, Summerland Key, FL. Thesis research. Effects of elevated temperature and oxidative stress on the health and post-settlement survival of larvae of the coral *Porites astreoides*.

2010 Mote Marine Laboratory, Summerland Key, FL. Research Assistant, Embryonic development in the marine Tiger Flatworm *Maritigrella crozieri*.

2010 St. Johns River Water Management District, Jacksonville, FL. Assisted with salt water intrusion research. Effects of salinity stress on the freshwater grass *Vallisneria americana*.

2010 Florida Institute of Oceanography Keys Marine Laboratory, Long Key, FL. Research Assistant, Embryonic development in the marine Tiger Flatworm *Maritigrella crozieri*.

2010 Mote Marine Laboratory, Summerland Key, FL. Undergraduate Research, Effects of thermal and oxidative stress on coral larvae health and post-settlement survivorship.

Teaching Experience

2013 University of North Florida, Jacksonville, Florida. Graduate Teaching Assistantship. General Biology 2. Spring 2013.
2012 University of North Florida, Jacksonville, Florida. Graduate Teaching Assistantship. General Biology 1. Fall 2012.
2011 University of North Florida, Jacksonville, Florida. COSEE Florida workshop instructor. Harmful Algae Blooms (HABs) and their effects on coastal ecosystems.

Skills and Qualifications

- PADI Open Water SCUBA Certification, August 2010.
- American Academy of Underwater Sciences (AAUS) Scientific Diver April 2011.