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ORIGINAL PAPER

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

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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.

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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 responses that consistently emerge as putative markers of temperature-induced cell stress 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^-), 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, 2002; Lesser and Farrell 2004; Ross et al. 2010, 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, 2002; Griffin et al. 2006; Ross et al. 2010, 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 Hofmann 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, 2002; Kingsley et al. 2003; Robbart et al. 2004; Desalvo et al. 2008, 2010; Fitt et al. 2009), other studies reported no change or even a decrease in Hsp content following thermal stress (Edge et al. 2005; Császár 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 2007) and the degree of inducibility of the particular Hsp of interest (Feder and Hofmann 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. Furthermore, the natural variability of transcriptomic expression both within and between species suggests that robust stress detection, based upon 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 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. In addition, Hsp 60 was found to be up-regulated ~fourfold. These results prompted our efforts to determine whether Hsp 16 or Hsp 60 expression could be used as sensitive biomarkers of sub-lethal thermal stress in larvae of *P. astreoides* in comparison with oxidative stress assays (Ross et al. 2010, 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 with oxidative stress assays that specifically targeted lipid peroxidation and catalase activity.

Materials and methods

Larval collection and treatments

Thirty adult colonies of *P. astreoides* were collected off of Wonderland reef at 6 m depth (GPS N24°33.62′; W81°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 aquaria 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/10 ambient aquaria). In turn, every aquarium housed three 400-ml tri-pour beakers which contained the coral larvae and corresponded to treatment duration (4,

24, or 48 h). Each of the 400-ml beakers was considered a replicate and contained 125 larvae in 200 ml 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 (qRT-PCR) analyses, respectively.

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.

Oxidative stress assays

Oxidative stress in larvae of *P. astreoides* was measured using the methods described by Ross et al. (2013). 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×*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). Up-regulation 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. Several replicates had to be excluded from the analysis due to their lack of sufficient protein content.

Quantitative reverse transcription PCR (qRT-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 to 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 1 μL forward primer, 1 μL 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 18 s). 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 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 ($n = 5$ for both Hsps and all treatments). 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.

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 × 4.5 cm × 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).

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 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).

Results

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. 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 ($F_{(18,\ 36)} = 0.778$, $p = 0.710$).

At each time point, larvae exposed to elevated temperature experienced a greater degree of lipid peroxidation compared to larvae maintained under ambient seawater conditions (Fig. 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

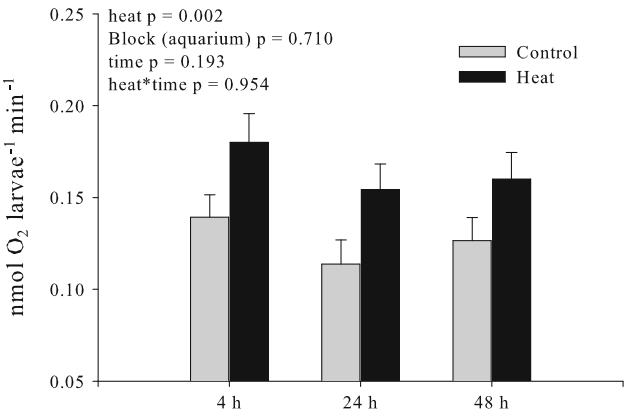

Fig. 1 Cellular respiration of *P. astreoides* larvae following 4, 24, or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control). $n = 10$ for all treatments. *Bars* represent mean +1 SE

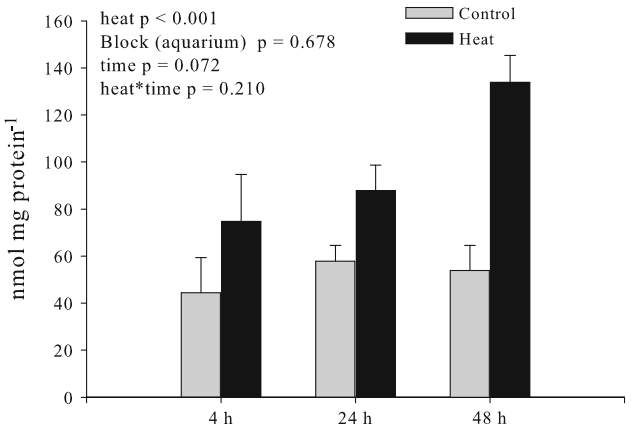

Fig. 2 Lipid hydroperoxides, a biomarker of oxidative stress in *P. astreoides* larvae following 4, 24, or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control) (4 h control $n = 6$, 4 h heat $n = 5$, 24 h control and heat $n = 9$, 48 h control and heat $n = 8$). *Bars* represent mean +1 SE

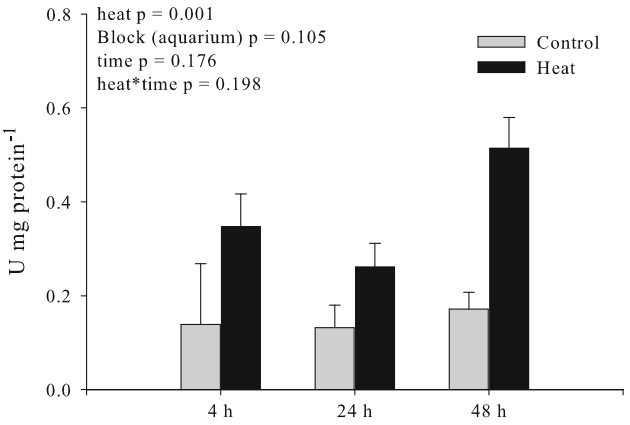

Fig. 3 Catalase, an enzyme that is a biomarker of oxidative stress in *P. astreoides* larvae following 4, 24, or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control) (4 h control $n = 6$, 4 h heat $n = 5$, 24 h control and heat $n = 9$, 48 h control and heat $n = 8$). *Bars* represent mean +1 SE

differ significantly in their effect on lipid peroxidation ($F_{(18,\ 21)} = 0.804$, $p = 0.678$).

Catalase activity varied significantly between treatments (Fig. 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 ($F_{(18,\ 21)} = 1.770$, $p = 0.105$).

As a measure of quality control, the differential expression of the housekeeping gene 18S ribosomal RNA was analyzed between treatments and time points (data not shown). 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 was not significantly different in larvae exposed to 30.8 or 27.3 °C (Fig. 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 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

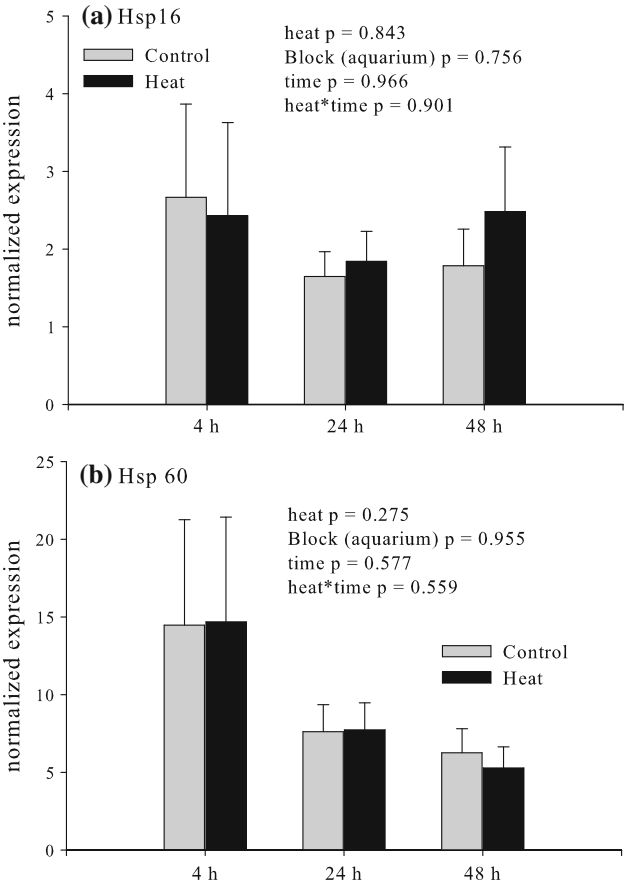

Fig. 4 Normalized heat-shock protein transcriptional expression in *P. astreoides* larvae following 4, 24, or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control): **(a)** Hsp 16, **(b)** Hsp 60. $n = 5$ for all heat-shock protein treatments. *Bars* represent mean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

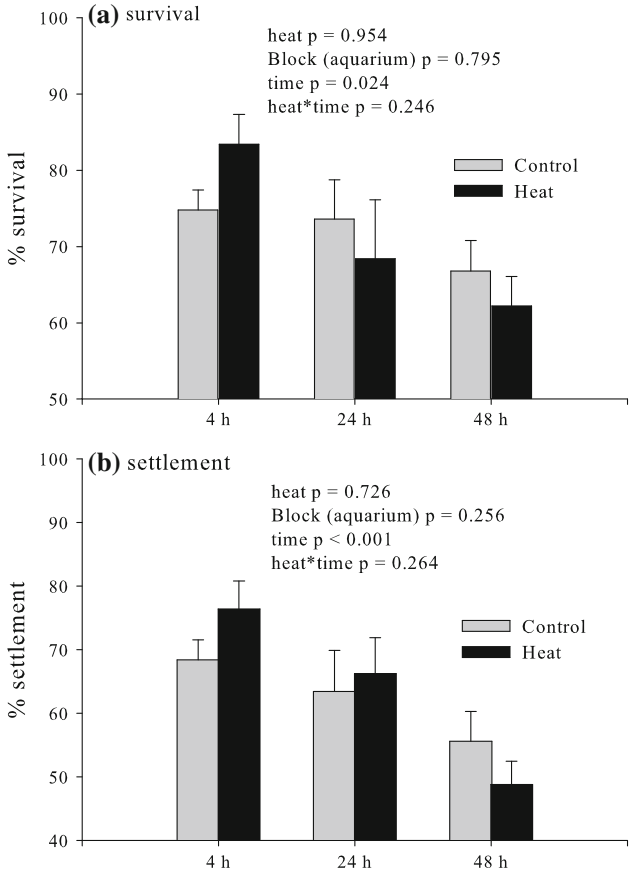

Fig. 5 **a** Percent survival and **b** settlement of *P. astreoides* larvae following 4, 24, or 48 h of exposure to elevated temperature (Heat) or ambient temperature conditions (Control). Larvae were offered conditioned settlement tiles, and survival and settlement were measured after 72 h. $n = 10$ for all survival and settlement treatments. *Bars* represent mean +1 SE

($F_{(8,\ 16)} = 0.612$, $p = 0.756$). The results obtained for Hsp 60 was similar in that 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 ($F_{(8,\ 16)} = 0.300$, $p = 0.955$).

Post-treatment, the larvae were allowed 72 h to settle and metamorphose under ambient seawater conditions (27 °C) (Fig. 5a). Larval survivorship did not vary 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 between aquaria within treatments ($F_{(18,\ 36)} = 0.693$, $p = 0.795$). Thermal stress did not affect the process of settlement and metamorphosis ($F_{(1,\ 18)} = 0.127$, $p = 0.726$) (Fig. 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 between experimental units within treatments ($F_{(18,\ 36)} = 1.281$, $p = 0.256$).

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.

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).

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.

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 ~fourfold 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 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 *A. 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 Császár 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 *A. millepora* (Meyer et al. 2009).

The lack of consistent up-regulation of Hsps 16 and 60 in response to thermal stress makes the quantification of their transcript abundance a metric that should be approached with caution when quantifying 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 >threefold 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.

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 acute 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 treatment 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 (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.

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.

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