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REPORT

Nitric oxide and heat shock protein 90 co-regulate temperature-induced bleaching in the soft coral *Eunicea fusca*

Cliff Ross

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Abstract Coral bleaching represents a complex physiological process that is affected not only by environmental conditions but by the dynamic internal cellular biology of symbiotic dinoflagellates (*Symbiodinium* spp.) and their cnidarian hosts. Recently, nitric oxide (NO) has emerged as a key molecule involved with the expulsion of *Symbiodinium* from host cnidarian cells. However, the site of production remains under debate, and the corresponding signaling pathways within and between host and endosymbiont remain elusive. In this study, using freshly isolated *Symbiodinium* from the soft coral *Eunicea fusca*, I demonstrate that thermally induced stress causes an upregulation in *Symbiodinium* heat shock protein 90 (Hsp90). In turn, Hsp90 shows a concomitant ability to enhance the activity of a constitutively expressed isoform of NO synthase. The resulting production of NO constitutes a signaling molecule capable of inducing *Symbiodinium* expulsion. Using nitric oxide synthase (NOS) and Hsp90 polyclonal antibodies, thermal stress-induced Hsp90 was shown to co-immunoprecipitate with a constitutive isoform of NOS. The specific blocking of Hsp90 activity, with the Hsp90 inhibitor geldanamycin, was capable of inhibiting NO production implicating the involvement of a coordinated regulatory system. These results have strong evolutionary implications for Hsp90–NOS chaperone complexes among biological kingdoms and provide evidence for a new functional role in symbiotic associations.

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C. Ross (✉)
Department of Biological Sciences, University of North Florida, Jacksonville, FL 32224, USA
e-mail: cliff.ross@unf.edu

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Introduction

Scleractinian (reef-building) corals are in serious decline as they are exposed to persistent threats that operate at both regional (e.g., nutrient addition via land-based activities and macroalgal competition) and global (e.g., elevated sea surface temperatures and ocean acidification) scales (Gardner et al. 2003; Hoegh-Guldberg et al. 2007; Anthony et al. 2008; Rasher and Hay 2010). As adult coral cover throughout the Caribbean continues to decline at an alarming rate (Gardner et al. 2003), the need exists to better understand the physiological effects of environmental stressors on coral health and potential modes of population recovery. Based upon current greenhouse gas emission scenarios, sea surface temperatures are projected to increase rapidly from 1.8 to 4.0 °C, on average, above current mean ocean temperatures (Sheppard and Rioja-Nieto 2005; IPCC 2007). Since some coral species are living at their upper thermal limits, even modest increases in sea surface temperature can cause stress, affecting the dynamic internal cellular biology of symbiotic dinoflagellates (*Symbiodinium* spp.) and their cnidarian hosts. Coral bleaching, the loss of the endosymbionts and/or their photosynthetic pigments, is perhaps the most dramatic example of the induced disruption of this symbiosis (Hoegh-Guldberg 1999).

While the molecular and cellular signaling events behind coral bleaching are not entirely understood, significant advancements have shed light on the mechanisms driving the early stages of the collapse of endosymbiosis (Weis 2008; Davy et al. 2012). Recent

years have provided a substantial amount of work recognizing the importance of reactive oxygen species (ROS) (Franklin et al. 2004; Lesser 2006; Suggett et al. 2008; McGinty et al. 2012) and the emerging role of reactive nitrogen species (RNS) (Perez and Weis 2006; Bouchard and Yamasaki 2008; Hawkins and Davy 2013; Hawkins et al. 2013) on temperature-induced bleaching. Thermal stress can disrupt *Symbiodinium* photochemistry and carbon acquisition processes (e.g., D1 protein of photosystem II and ribulose bisphosphate carboxylase oxygenase activity, respectively), as well as thylakoid membrane integrity (Iglesias-Prieto and Trench 1997; Jones et al. 1998; Warner et al. 1999; Tchernov et al. 2004), resulting in the buildup of excess electrons. As a consequence, the reduction of diatomic oxygen (via the Mehler reaction of photosystem I) may ensue, leading to the production of highly reactive ROS superoxide anions, O_2^-. The accumulation, sequential reduction, or interconversion of these anions into other ROSs (hydrogen peroxide [H_2O_2], hydroxyl radical [$^{\cdot}OH$], or singlet oxygen [$^1O_2$]) may lead to the disfunction of multiple intracellular targets including proteins, lipids, and nucleic acids (Halliwell 2006).

Nitric oxide (NO) is a ubiquitous membrane-permeable molecule implicated in the regulation of numerous metabolic activities in organisms, including endosymbiosis (Palumbo 2005; Mur et al. 2013). It is capable of reacting with O_2^- to form peroxynitrite ($ONOO^-$), which is highly diffusible and capable of serving as a potent redox regulator and mediator of cellular damage (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011). NO has been hypothesized to be involved in the cnidarian innate immune-like pathway resulting in bleaching and apoptotic processes (Weis 2008), yet its site of origin remains unclear. Perez and Weis (2006) demonstrated that the addition of NO to the symbiotic anemone *Aiptasia pallida* induced bleaching and that upon thermal stress nitric oxide synthase (NOS; EC 1.14.13.165) activity could be localized to host tissue. Supporting evidence for the localization of NOS activity specifically to cnidarian host tissue was provided by Safavi-Hemami et al. (2010). Conversely, work by Bouchard and Yamasaki (2008) and Trapido-Rosenthal et al. (2001) provided evidence for the production of NO in cultured and freshly isolated symbionts, respectively, in response to elevated temperature. Furthermore, Hawkins and Davy (2012) demonstrated that NO was produced by *Symbiodinium* upon thermal stress, and this production could be differentially regulated based upon the thermotolerance of the clade. Collectively, these data suggest that while NO has emerged as a key molecule associated with the expulsion of *Symbiodinium* from host cnidarian cells, the corresponding signaling pathways remain elusive.

Several sources of NO have been described in algae, plants, and animals. In photoautotrophs, evidence suggests that NO may arise from NOS, nitrate reductase (EC 1.7.1.2), or nonenzymatic sources (del Rio et al. 2004; Ross et al. 2006; Bouchard and Yamasaki 2008; Frohlich and Durner 2011). In animals, NOS catalyzes the NADPH and O_2-dependent oxidation of L-arginine to NO and L-citrulline and has been reported to require the interaction with heat shock protein 90 (Hsp90) for enhanced activation (Garcia-Cardena et al. 1998). Hsp90 has important roles in the conformational regulation of key signaling molecules and has been shown to facilitate the activation of inducible (iNOS), neuronal (nNOS), and endothelial NOS (eNOS) isoforms in mammalian systems (Garcia-Cardena et al. 1998; Bender et al. 1999; Ilangovan et al. 2004). Furthermore, the interaction between Hsp90 and NOS has been implicated in the regulation of metamorphosis in several taxa of marine invertebrates including urochordates and echinoderms (Bishop and Brandhorst 2001; Bishop et al. 2001; Ueda and Degnan 2013). This suggests that the requirements of Hsp90 function for NOS activity represent a rather ubiquitous molecular interaction not just restricted to mammalian taxa.

To better understand the mechanistic framework driving temperature-induced bleaching, I conducted experiments using the symbiotic soft coral *Eunicea fusca*. *E. fusca* is commonly found throughout the Caribbean basin and has been previously studied for its secondary metabolite pharmacology (Shin and Fenical 1991; Jacobson and Jacobs 1992), inducible release of ROS (Mydlarz and Jacobs 2006), and microbial community composition (Duque-Alarcon et al. 2012). By coupling pharmacological treatments with analysis of protein expression, I provide evidence that *Symbiodinium* expulsion involves a coordinated regulatory system involving Hsp90 and NO.

Materials and methods

Coral collection and experimental design

Colonies of *E. fusca* (Duchassaing & Michelotti) were collected off of Hillsboro Ledge (N26°18.04′; W80°04.09′), Deerfield Beach, Florida via SCUBA at a depth of approximately 10 m. Specimens were immediately transported to the Smithsonian Marine Station at Ft. Pierce (Florida) in coolers and acclimated in aerated seawater aquaria for 24 h.

To evaluate the effects of thermal stress on the regulation of NO production and bleaching, individual colonies were exposed to selected treatments for 10 h under constant ambient light levels of 100 μmol m^{-2} s^{-1}. Each colony (8 × 5 cm; ~6.0 g fresh weight) was placed into an individual plant tissue culture box (9.5 × 9.5 × 3.5 cm,

ICN Biomedicals, Inc., Aurora, OH, USA) containing 250 ml of 0.22 μm filtered seawater. Culture boxes were maintained at ambient temperature (25 °C) or heated to 31 °C using 25-W adjustable aquarium heaters (Commodity Axis, Inc., Camarillo, CA, USA). Coral colonies were randomly assigned to the following treatments ($n = 5$/treatment): (1) control temperature (25 °C), (2) control temp + 1 mM of the NO donor sodium nitroprusside (SNP; Sigma, St. Louis, MO, USA), (3) control temp + 1 mM SNP + 20 mM of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Molecular Probes, Eugene, OR, USA), (4) elevated temperature (31 °C), (5) elevated temperature + 5 mM of the NOS inhibitor NG-methyl-L-arginine (L-NMMA; Sigma), (6) elevated temperature + 20 mM cPTIO, or (7) elevated temperature + 0.5 mM of the Hsp90 inhibitor geldanamycin (GDA, Sigma). SNP, cPTIO, and L-NMMA were dissolved in H_2O and stored at −80 °C as 100-mM stock solutions. GDA was solubilized in DMSO and stored as a 1-M stock solution. All compounds were pre-incubated with their respective coral treatments for 30 min prior to the start of any experiment.

Quantification of expelled *Symbiodinium*

Following the 10-h treatment, corals were removed from their respective plant tissue culture boxes and split into two equal pieces. One piece was used to determine the density of *Symbiodinium* that remained *in hospite* following treatment. The other piece was used to obtain a clean *Symbiodinium* preparation, which was used for all other assays (described below).

To quantify *Symbiodinium* retained within the host tissue, the first piece of coral from each pair was cut up into 1-inch pieces with scissors and homogenized in a blender (~1.5 g coral: 100 ml filtered seawater). The resulting homogenate was filtered through cheesecloth and collected in 50-ml falcon tubes. Samples were vortexed for 20 s, and a 25 μl subsample was taken for hemocytometer-based *Symbiodinium* cell counts. Subsampling and cell counts were performed in triplicate to obtain mean values. The total number of *Symbiodinium* in the 100 ml homogenate was calculated and multiplied by two (since only one half of the colony was used) to determine the approximate number of *Symbiodinium* still residing within the entire coral sample.

The seawater (containing the expelled symbionts) was poured from the plant tissue containers into 50-ml falcon tubes. Tubes were centrifuged for 10 min, and the *Symbiodinium* pellets were retained, pooled, and re-suspended in 1 ml of seawater. *Symbiodinium* cell numbers were determined via hemocytometer. *Symbiodinium* expulsion was expressed as % cell loss according the methods of Perez et al. (2001). In this case, % cell loss = expelled algae/(expelled algae + *in hospite* algae).

Clean *Symbiodinium* preparation from host tissue

Following treatment, the second piece of coral from each pair was cut up into 1-inch pieces with scissors and homogenized in a blender (~3 g coral: 25 ml filtered seawater). The resulting homogenate was filtered through cheesecloth and collected in 50-ml falcon tubes. Filtered material was centrifuged at 400×*g* for 3 min. Pellets were collected and suspended in 40 ml of seawater. Samples were vortexed for 20 s and re-centrifuged. The pellets were retained, and this process was repeated until the supernatants were void of pigment. The end product yielded clean *Symbiodinium* preparations that were used in subsequent confocal microscopy, fluorometric, and Western blot assays.

Laser scanning confocal microscopy

DAF-FM diacetate (4-amino-5-methylamino-2′7′-difluorofluorescein diacetate, Molecular Probes, Eugene, OR, USA) is a cell-permeable probe that passively diffuses across cellular membranes. Once inside the cell, it is deacetylated by intracellular esterases to become DAF-FM. Oxidation of DAF-FM by NO yields a fluorescent benzotriazole derivative. DAF-FM diacetate was dissolved in DMSO in 5-mM aliquot stocks (stored at −80 °C).

Posttreatment, *Symbiodinium* were isolated as described above. A small aliquot of cells were transferred into an incubation mixture of 2 ml filtered seawater and 20 μl of DAF-FM diacetate (final concentration of 20 μM DAF-FM diacetate). The algae were incubated in the dark for 30 min. The specimens were then washed in 5 ml of fresh seawater and subsequently imaged. Confocal laser scanning microscopy (CLSM) was performed using a Nikon Eclipse E800 compound microscope (Nikon Instruments, Kanagawa, Japan) equipped with a Bio-Rad Radiance 2000 laser system (Bio-Rad, Hercules, CA, USA). Laser power was set at 20 % with an excitation of 488 nm and an emission of 580 nm (channel 1—red) or 525 nm (channel 2—green). Series of 0.2-μm optical sections with maximum intensity projection along the *z* axis were made into one 2D image with greater focal depth. Bio-Rad images were imported into Confocal Assistant 4.02 and converted into TIF files.

Fluorometric quantification of nitric oxide production

The presence of NO from *in hospite Symbiodinium* was quantified by measuring the oxidation of DAF-FM diacetate. Posttreatment, *Symbiodinium* cell density was normalized, via hemocytometer, to a concentration of 5×10^7

cells in a 50 ml volume of seawater. 20 μM DAF-FM (final concentration) was added to each 50 ml reaction and incubated at room temperature in the dark for 30 min. Two thousand microliters of the reaction mixture was placed into a cuvette, and NO was detected using a VersaFluor fluorometer (Bio-Rad). Results were expressed in relative fluorescent units (RFUs).

SDS-PAGE and western blotting

Isolated *Symbiodinium* were ground in liquid nitrogen and solubilized in 50 mM potassium phosphate buffer (pH 7.0) containing 10 % (w/v) polyvinylpolypyrrolidone and 0.25 % Triton X-100. The extract was centrifuged at 10,000×*g* for 10 min at 4 °C, and each sample was normalized by total soluble protein content using a Quick Start™ Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions. Laemmli sample buffer (Bio-Rad) was added to each sample (1:1) and subsequently heated at 100 °C for 5 min. Samples were run under non-denaturing SDS-PAGE conditions (10 % Tris–HCl Ready Gels™, Bio-Rad) and transferred to PVDF (Polyscreen®, Perkin Elmer, Wellesley, MA, USA) using a Mini Trans-Blot Elecrophoretic Transfer Cell (Bio-Rad). The blot was blocked in phosphate-buffered saline containing 3 % nonfat dry milk (PBS-MLK) for 60 min at 25 °C with constant agitation. The blot was subsequently incubated with a 1:1,000 solution of rabbit polyclonal uNOS (#PA1039; Affinity Bioreagents, Golden, CO, USA) or 1:2,000 solution of rabbit polyclonal anti-Hsp 90 (#SPA-846; Stressgen, San Diego, CA, USA) in freshly prepared PBS-MLK for 2.5 h with agitation at room temperature. A secondary horseradish peroxidase conjugated IgG goat anti-rabbit antibody was added (1:3,000) in PBS-MLK and incubated for 1 h. Band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation

One hundred microliters of a 1:100 dilution of rabbit polyclonal anti-uNOS or rabbit polyclonal anti-Hsp 90 was added to a 1 ml bed volume of hydrated protein A-Sepharose conjugate (Sigma, #P3391). The mixture was incubated for 1 h at room temperature on a rotary shaker. Following incubation, the suspension was centrifuged for 3,000×*g* for 2 min. The supernatant was discarded, and the pellet was resuspended with 1 ml of 10 mM Tris HCl (pH 7.5). Following centrifugation (3,000×*g* for 2 min), the supernatant was discarded and 1 mg ml^{-1} solution of *Symbiodinium* extract was added. The mixture was incubated overnight on a rotary shaker. The immunoprecipitated complexes were collected by centrifugation at 3,000×*g* for 2 min. The pellet was collected and washed in 1 ml 10 mM Tris–HCl (pH 7.5) several times. The resulting pellet was suspended in Laemmli sample buffer (Bio-Rad) and heated at 100 °C for 5 min.

Statistical analysis

Data for Hsp90 abundance and immunoprecipitation experiments were analyzed using a student's *t* test. Data for NOS abundance and NO quantification were analyzed using a one-way ANOVA. Data for percent *Symbiodinium* cell loss were arcsine square-root transformed since it was a proportion and subsequently analyzed with a one-way ANOVA. Following one-way ANOVAs, a Tukey's post hoc test was performed to determine significant groupings. A Pearson product-moment correlation was used to correlate *Symbiodinium* cell loss and NO production. The data were normally distributed as was determined by the Shapiro–Wilk test. All statistical analyses were conducted using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA).

Results

Fusca Symbiodinium cell loss in response to elevated temperature, NO presence, and Hsp90 inhibition

Throughout the course of the experiment, the mean temperatures (±SE) were 25.32 ± 0.16 and 31.08 ± 0.09 °C for ambient and elevated temperatures, respectively. Following the 10-h treatment period, corals were assessed to determine whether elevated temperature or NO exposure induced *Symbiodinium* release. A significant change in *Symbiodinium* cell loss occurred as a function of treatment (Fig. 1; one-way ANOVA, $F_{(6,34)} = 0.12$, $p = 0.022$) with thermal stress having the strongest effect. The addition of SNP-derived NO caused a fivefold increase in *Symbiodinium* loss (equating to ~5 % *Symbiodinium* loss) compared to controls (Fig. 1). This was associated with a pronounced discoloration of host tissue (Fig. 2). The co-incubation of the NO scavenger cPTIO with SNP reduced *Symbiodinium* loss by 43 % compared to treatment with SNP alone. Exposure to elevated temperature (31 °C) caused a 1,680 % increase in *Symbiodinium* cell loss compared to control corals (25 °C). Furthermore, the presence of L-NMMA, cPTIO, and GDA reduced the effects of temperature-induced *Symbiodinium* loss by 36, 51, and 26 %, respectively (Fig. 1).

To qualitatively determine whether NO was produced in response to thermal stress in *Symbiodinium* cells, laser scanning confocal microscopy and the NO-sensitive probe DAF-FM were employed (Fig. 3). Exposure to elevated temperature caused a notable increase in NO production

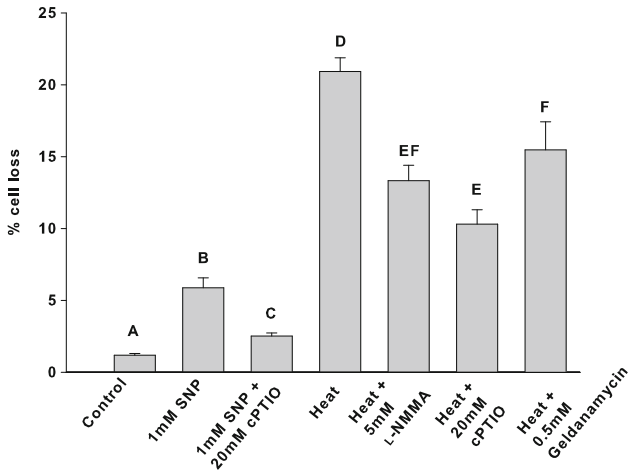

Fig. 1 Effects of temperature treatment on *Symbiodinium* expulsion following a 10-h exposure period. (*Bars* represent mean +1 SE; $n = 5$; one-way ANOVA, $p = 0.022$). The *same letters* above the *bars* indicate means that are not significantly different as determined by a Tukey's post hoc test

compared to controls. Fluorometric quantification of NO yielded similar results as 5 times the amount of NO was produced in heat-treated samples when compared to control specimens (Fig. 4; one-way ANOVA, $F_{(6,34)} = 10.75$, $p = 0.006$). The pre-incubation of samples with the NOS inhibitor L-NMMA or general scavenger cPTIO resulted in a significant reduction in NO content in heat-treated samples (Figs. 3c–f, 4). Furthermore, GDA depressed NO production by 32 % when compared to the heat treatment (Fig. 4). There was a very strong significant positive correlation between *Symbiodinium* cell loss and NO production ($p < 0.001$, $r = 0.88$).

Establishment of a temperature-induced Hsp90–NOS heterocomplex in *Symbiodinium*

Protein expression of *Symbiodinium* Hsp90 was assayed via Western blot following exposure to elevated temperature and was shown to increase significantly ~sixfold compared to controls (Fig. 5; *t* test, $t_{(1,5)} = 14.15$, $p < 0.050$). Conversely, the protein expression of NOS was not affected by thermal stress or the presence of L-NMMA (Fig. 6; one-way ANOVA, $F_{(3,9)} = 0.28$, $p = 0.386$).

The ability for Hsp90 and NOS to form a temperature-induced heterocomplex was tested through immunoprecipitation using polyclonal antibodies. Hsp90 was detected in the immunoprecipitate of NOS following heat stress. Quantitative analysis of band densities, using the mean pixel intensity of two experimentally independent bands (data not shown), indicated that this response was almost nonexistent under control conditions (Fig. 7a; control versus heat: *t* test, $t_{(1,3)} = 13.84$, $p = 0.001$) or when GDA was pre-incubated with the heat-treated corals (GDA + heat versus heat: *t* test, $t_{(1,3)} = 12.56$, $p = 0.001$). Similarly, NOS was detected in the immunoprecipitate of Hsp90 following exposure to 31 °C but not at 25 °C (Fig. 7b; *t* test, $t_{(1,3)} = 9.65$, $p = 0.002$). The presence of GDA appeared to only cause a slight reduction in the levels of heterocomplex formation, but the band intensity was not significantly different compared to controls (Fig. 7b; *t* test, $t_{(1,3)} = 0.84$, $p = 0.326$).

Discussion

Despite the wealth of knowledge on NO as a key component behind numerous biochemical processes in animals and plants, the mechanisms underlying the regulatory roles of this compound in symbiotic associations remain less well studied (Hentschel et al. 2000; Davidson et al. 2004; Meilhoc et al. 2011; Wang and Ruby 2011). The current study demonstrates that the temperature-dependent expulsion of *Symbiodinium* from *E. fusca* involves a coordinated regulatory system involving Hsp90 and nitric oxide. There are three general lines of evidence that support this finding.

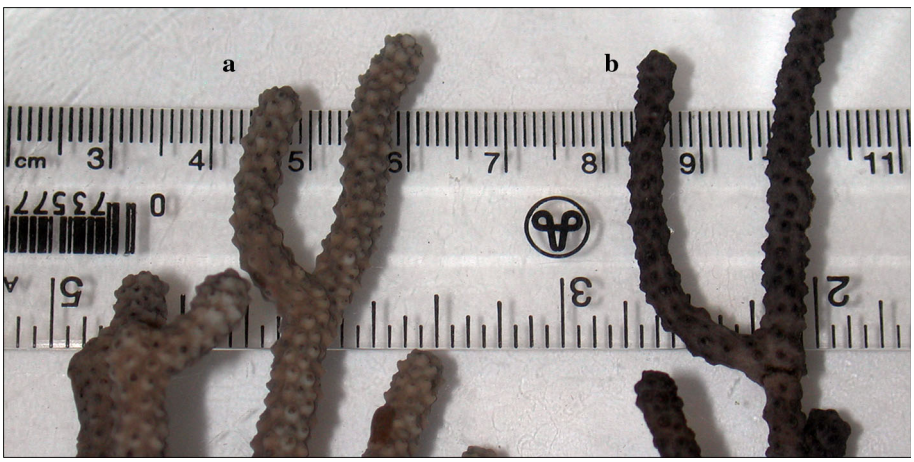

Fig. 2 NO induced bleaching in the soft coral *E. fusca*. **a** Colony of *E. fusca* incubated for 10 h at 25 °C with the NO donor SNP (5 mM). **b** Control colony maintained in seawater at 25 °C. A fivefold increase in *Symbiodinium* loss (~5 % *Symbiodinium* loss), and pronounced discoloration of host tissue was observed in treated samples when compared to controls

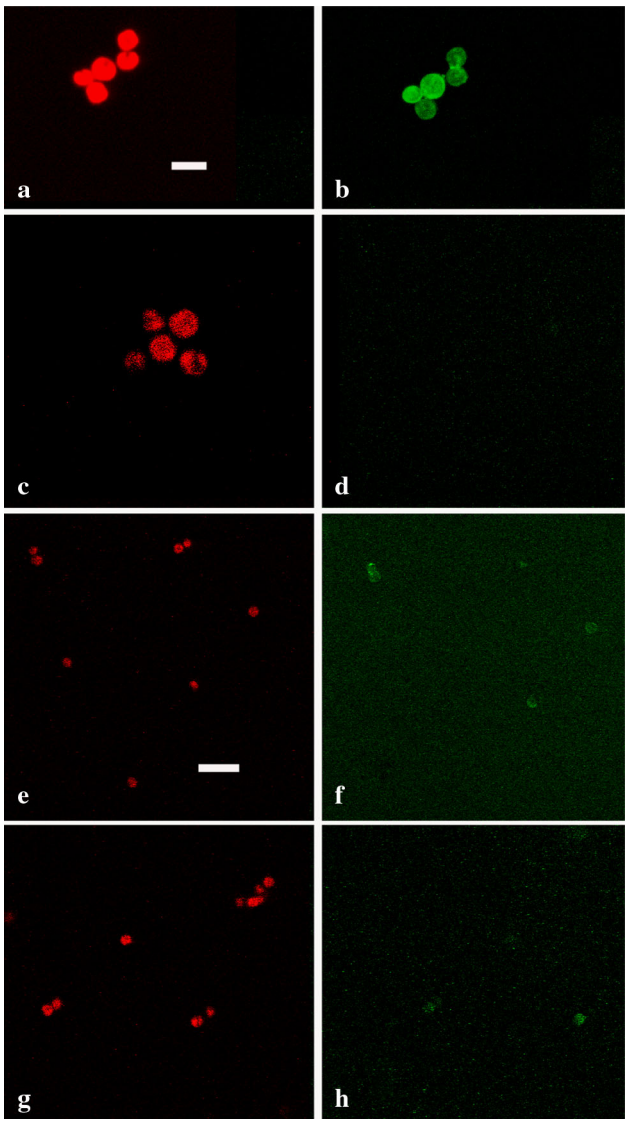

Fig. 3 Detection of NO with DAF-FM diacetate by CLSM. Chlorophyll autofluorescence is presented in red (ex 488 nm, em 580 nm), and the localization of NO is denoted in green (ex 488 nm, em 525 nm). **a**, **b** *Symbiodinium* cells exposed to 31 °C. **c**, **d** *Symbiodinium* cells pre-incubated in 20 mM cPTIO, then exposed to 31 °C. **e**, **f** *Symbiodinium* cells pre-incubated in 5 mM L-NMMA, then exposed to 31 °C. **g**, **h** *Symbiodinium* controls maintained at 25 °C. (*Scale bar* 20 μm for **a**–**d**. *Scale bar* 85 μm for **e**–**h**)

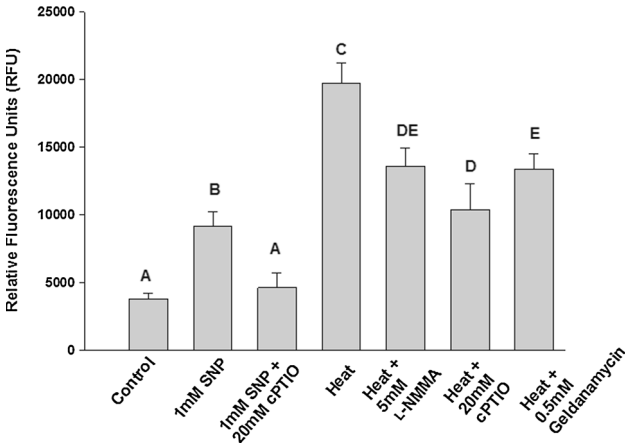

Fig. 4 Fluorometric quantification of NO associated with *Symbiodinium* cells following a 10-h temperature treatment. (*Bars* represent mean +1 SE; $n = 5$; one-way ANOVA, $p = 0.006$). The *same letters* above the *bars* indicate means that are not significantly different as determined by a Tukey's post hoc test

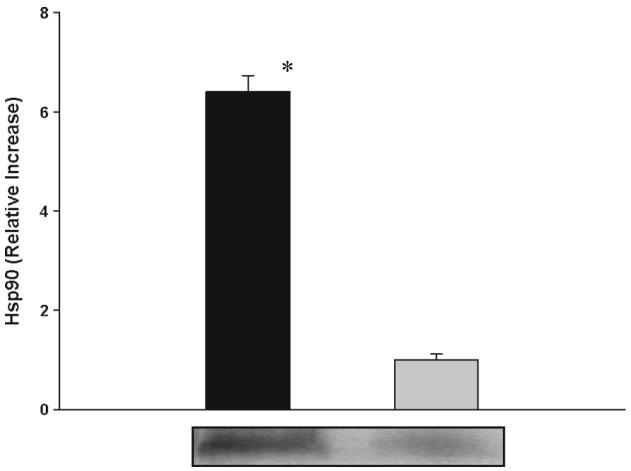

Fig. 5 Elevated temperature (heat = 31 °C; control = 25 °C) increases the relative expression of Hsp90 in *Symbiodinium* isolated from *E. fusca*. A representative Western blot (300 μg total soluble protein per *lane*) depicting the upregulation of Hsp90 as shown with corresponding band intensity quantified using ImageJ software. Data points represent the mean band intensity values of three replicates (+1 SE). Statistical difference was calculated using a Student's *t* test ($p < 0.050$). The *asterisk* indicates difference between groups

First, the presence of NO was shown to promote *Symbiodinium* cell loss; however, the presence of NOS and Hsp90 inhibitors (L-NMMA and GDA, respectively) reduced the effects of this temperature-induced response. Second, GDA was capable of attenuating temperature-induced NO production. Third, the interaction between Hsp90 and NOS was shown to occur in response to elevated temperature. It should also be emphasized that there was a strong significant positive correlation between *Symbiodinium* cell loss and NO production in all treatments examined.

The exogenous addition of NO led to *Symbiodinium* cell loss in *E. fusca* as has been reported in several other symbiotic cnidarian systems. Hawkins et al. (2013) found a 20 % increase in symbiont loss in the anemone *Aiptasia pulchella* when incubated with NO (derived from 1 mM *S*-nitrosoglutathione) over 48 h. Similarly, Perez and Weis (2006) found a 17 % increase in symbiont cell loss in *A. pallida* when exposed to 1 mM SNP over a 24-h period. While there is consistent evidence that NO can induce bleaching in these systems, the origin and temporal dynamics of heat-induced NO production are different than

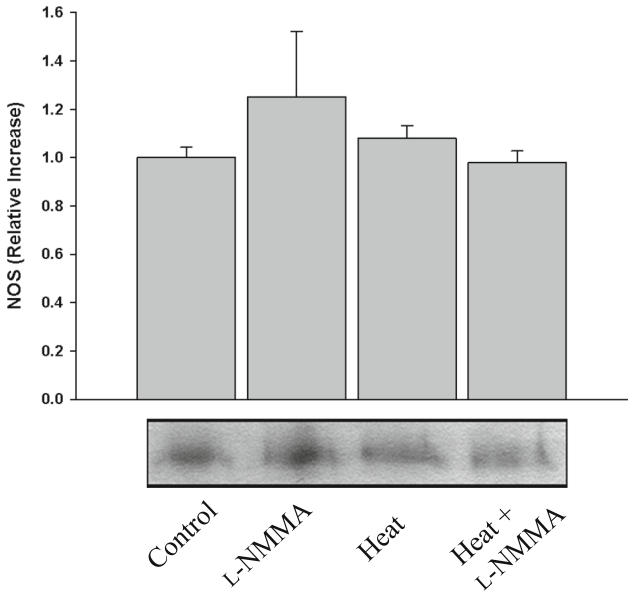

Fig. 6 Elevated temperature (heat = 31 °C; control = 25 °C) or presence of L-NMMA (5 mM) does not affect the relative expression of NOS in *Symbiodinium* isolated from *E. fusca*. A representative Western blot (500 µg total soluble protein per *lane*) depicting the absence of NOS upregulation is shown with corresponding band intensity. Data points represent the mean band intensity values of three replicates. *Bars* represent mean +1 SE ($n = 3$; one-way ANOVA, $p = 0.386$)

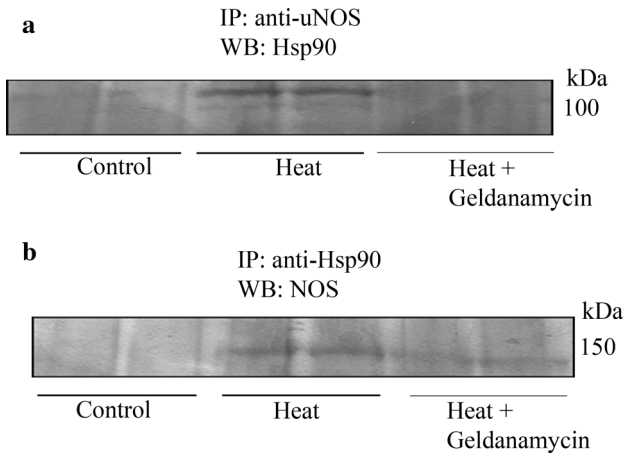

Fig. 7 Elevated temperature (heat = 31 °C; control = 25 °C) promotes the association between Hsp90 and NOS in *Symbiodinium*. **a** Lysates from *Symbiodinium* (500 µg total soluble protein per *lane*) were immunoprecipitated with polyclonal antibody against u-NOS. Immunoprecipitates (IP) were Western blotted (WB) for Hsp90. **b** Lysates from *Symbiodinium* (500 µg total soluble protein per *lane*) were immunoprecipitated with polyclonal antibody against Hsp90. Resulting immunoprecipitates were Western blotted for NOS. The presence of 0.5 mM GDA attenuated the effect of thermal stress on the formation of the Hsp90–NOS heterocomplex. All samples were run in duplicate

what was observed in *E. fusca*. Perez and Weis (2006) demonstrated that symbiotic *A. pallida* exposed to elevated temperatures for 24 h produced NO while aposymbiotic anemones or isolated *Symbiodinium* did not. Similarly, Hawkins et al. (2013) reported that when symbiotic *A. pulchella* were exposed to 33 °C for 24 h, host gastrodermal tissue underwent a marked increase in NO production. The authors also showed that isolated *Symbiodinium* cells were capable of generating enhanced levels of NO, but this was after 10 d of elevated temperature treatment. The fact that considerable levels of bleaching were noted after 48 h implies that the dominant source of NO was host-derived. In my study, NO and NOS were detected in *Symbiodinium* cells that were freshly isolated from *E. fusca* following a 10-h incubation at 31 °C. Similar findings were reported by Trapido-Rosenthal et al. (2001) who noted that NOS-like activity could be found in *Symbiodinium* cells that were freshly isolated from *A. pallida*. However, the authors noted that NOS-like activity was absent in an independent monoculture of *Symbiodinium*. This would support the notion presented by Perez and Weis (2006) that either algae produce NO in response to host-derived factors or freshly isolated algae are not 100 % clean of host cell contamination (host gastrodermal cells that have the capability of producing NO). The ability for cultured *Symbiodinium* to produce NO upon exposure to elevated temperature cannot be ruled out, as Hawkins and Davy (2012) and Bouchard and Yamasaki (2008) have both demonstrated that *Symbiodinium* can generate NO independent of a cnidarian host. Hawkins and Davy (2012) provided evidence that selected subclades could up-regulate NO production as early as 24 h when incubated at 32 °C. Bouchard and Yamasaki (2008) demonstrated that pure cultures of *Symbiodinium microadriaticum* could significantly increase NO production over 2 h at 34 °C. While it was not possible to determine whether aposymbiotic *E. fusca* had the ability to produce NO, the fact that *Symbiodinium* preparations were washed repeatedly and NOS could be localized to *Symbiodinium* extracts strongly suggests that NO was originating from the symbiont source.

The physiological roles of Hsp90 chaperone complexes in photoautotrophs are not well understood, yet several reports have documented their involvement in terrestrial plant disease resistance, genetic buffering, and the regulation of transcription factors in response to abiotic stress (Sangster and Queitsch 2005; Zhao and Houry 2007; Kadota and Shirasu 2012). To my knowledge, this study represents the first report describing the functional role of Hsp90 in a marine algal–coral symbiosis. Even though the expression of *Symbiodinium* Hsp90 was shown to be upregulated in this study, corals and their symbionts show diverse patterns of Hsp expression in response to thermal stress. For example, in some corals, elevated temperature has been shown to increase Hsp levels (Fang et al. 1997; Sharp et al. 1997; Kingsley et al. 2003; Robbart et al. 2004; Desalvo et al. 2008, 2010; Fitt et al. 2009). However, other

studies reported no change, or even a decrease, in Hsp content following thermal stress (Edge et al. 2005; Voolstra et al. 2009; Mayfield et al. 2011). Specifically regarding Hsp90, Polato et al. (2010) detected a down-regulation of Hsp90α 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 Hsp90 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 Hsp90 in *Acropora millepora* larvae at 28 and 31 °C for 3 h. However, these transcriptional responses were reduced by 10 h. Similar discrepancies in Hsp expression patterns have been reported in dinoflagellates as well, as Rosic et al. (2011a, b) reported reduced expression of Hsp90 in *Symbiodinium* cultures and *in hospite* when exposed to elevated temperatures. Interestingly, Leggat et al. (2011) reported a differential response of Hsp90 in the coral *Acropora aspera* (upregulation) and its dinoflagellate symbiont *Symbiodinium* (downregulation) when exposed to thermal stress. Conversely, Guo and Ki (2012) noted a significant upregulation in Hsp90 expression in *Prorocentrum* in response to elevated temperature. The experimental conditions and detection techniques employed (i.e., immunoblot versus gene expression) in these studies likely influenced the variability of Hsp expression reported. Overall, it is clear that reports demonstrating the presence/absence of Hsp expression in cnidarians are not lacking. However, data identifying the physiological roles of Hsps in corals are scarce compared to the wealth of knowledge that is available characterizing the specific regulatory roles of Hsps in mammalian systems (Li and Buchner 2013). Future work examining the biological consequences and signaling pathways influenced by variability in Hsp expression would greatly assist our understanding of coral stress physiology.

The expression of *Symbiodinium* NOS was not affected by treatment suggesting this isoform is constitutively expressed. While L-NMMA is not known to regulate NOS expression, it is a well-known competitive inhibitor of both constitutive and inducible isoforms of NOS (Griffith and Kilbourn 1996). Based upon the results of this study, it is likely that NO is being produced via a NOS mechanism. In mammalian cells, endothelial and neuronal NOSs are constitutively expressed and are responsible for cardiovascular and neuronal signaling transmission, respectively (Yoshida and Xia 2003). The catalytic activity of these NOS isoforms was initially thought to be modulated by intracellular [Ca^{2+}] (Mittal and Jadhav 1994). However, it is now known that NOS activity is regulated by protein–protein interaction or protein phosphorylation (Fulton et al. 2001; Fleming and Busse 2003). Perez and Weis (2006) suggested that NO production in *Symbiodinium* may require exogenous host-derived factors. Trapido-Rosenthal et al. (2001) demonstrated that dinoflagellate symbionts from *A. pallida* contained low basal levels of NOS activity that could be enhanced by incubating the algae with an amino acid mixture that represented the free amino acid content of the host. In *Symbiodinium* obtained from *E. fusca*, NO production could be significantly reduced in the presence of GDA (ansamycin antibiotic that specifically blocks Hsp90 function by competitively binding to the ATP-binding site). This suggests that Hsp90 may be serving as an important posttranslational modulator of NOS activity. This same type of modulation has been reported as being responsible for regulating mammalian cardiac cell respiration (Ilangovan et al. 2004) and echinoid metamorphosis (Bishop and Brandhorst 2001). Mechanistically, it has been proposed that Hsp90 facilitates heme entry into the binding site of NOS (nitric oxide synthases are cytochrome p450-type hemoproteins that require a heme prosthetic group). The application of GDA may reduce the capacity of NOS to bind heme and therefore decrease NO production (Bender et al. 1999, 2000).

Aside from expanding upon the number of taxa in which NO and Hsp90 have a coordinated regulatory role, the significance of this study is that it describes a contributing mechanism toward the collapse of coral symbiosis (Fig. 8). Even though this study utilized a soft coral, a similar mechanism could be occurring in other ecologically important and related taxa such as scleractinians. The experimental evidence presented herein demonstrates that *Symbiodinium* produce NO by utilizing Hsp90 modulation, and this leads to symbiont expulsion. This Hsp90-

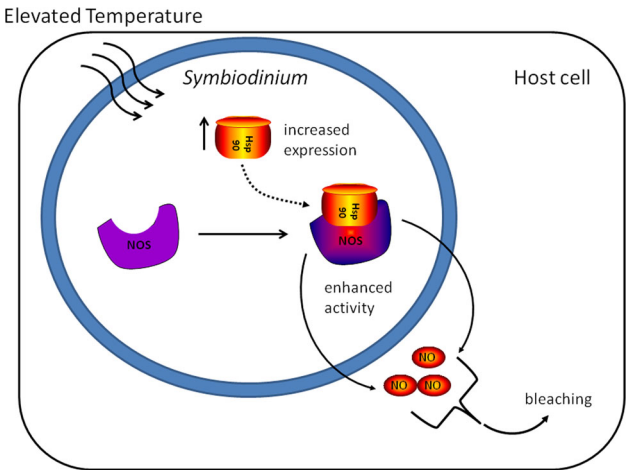

Fig. 8 Model of temperature-induced NO production in *Symbiodinium*. Elevated temperature causes an upregulation of Hsp90 which, through the formation of a heterocomplex, enhances the activity of a constitutively expressed isoform of nitric oxide synthase. The resulting production of NO constitutes a signaling molecule capable of inducing *Symbiodinium* expulsion from host tissue

dependent, NO-based regulatory mechanism shares similarities with the signaling pathways found in other taxa, but it has never before been demonstrated in a marine algae, suggesting that key elements of signal transduction responses are evolutionarily conserved.

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