The Journal of Experimental Biology 214, 4240-4249

doi:10.1242/jeb.061267

RESEARCH ARTICLE

Patterns of coral ecological immunology: variation in the responses of Caribbean corals to elevated temperature and a pathogen elicitor

Caroline V. Palmer[1,2], Elizabeth S. McGinty[3], David J. Cummings[3], Stephanie M. Smith[3], Erich Bartels[4] and Laura D. Mydlarz[3,*]

[1]ARC Centre of Excellence for Coral Reef Studies and School of Marine and Tropical Biology, James Cook University, Townsville, QLD, 4811, Australia, [2]School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK, [3]Department of Biology, University of Texas at Arlington, Arlington, TX 76019, USA and [4]Mote Marine Laboratory–Tropical Research Laboratory, Summerland Key, FL 33042, USA

*Author for correspondence (mydlarz@uta.edu)

Accepted 19 September 2011

SUMMARY

Disease epizootics are increasing with climatic shifts, yet within each system only a subset of species are identified as the most vulnerable. Understanding ecological immunology patterns as well as environmental influences on immune defenses will provide insight into the persistence of a functional system through adverse conditions. Amongst the most threatened ecosystems are coral reefs, with coral disease epizootics and thermal stress jeopardizing their survival. Immune defenses were investigated within three Caribbean corals, *Montastraea faveolata*, *Stephanocoenia intersepta* and *Porites astreoides*, which represent a range of disease and bleaching susceptibilities. Levels of several immune parameters were measured in response to elevated water temperature and the presence of a commercial pathogen-associated molecular pattern (PAMP) – lipopolysaccharide (LPS) – as an elicitor of the innate immune response. Immune parameters included prophenoloxidase (PPO) activity, melanin concentration, bactericidal activity, the antioxidants peroxidase and catalase, and fluorescent protein (FP) concentration. LPS induced an immune response in all three corals, although each species responded differently to the experimental treatments. For example, *M. faveolata*, a disease-susceptible species, experienced significant decreases in bactericidal activity and melanin concentration after exposure to LPS and elevated temperature alone. *Porites astreoides*, a disease-resistant species, showed increased levels of enzymatic antioxidants upon exposure to LPS independently and increased PPO activity in response to the combination of LPS and elevated water temperature. This study demonstrates the ability of reef-building corals to induce immune responses in the presence of PAMPs, indicating activation of PAMP receptors and the transduction of appropriate signals leading to immune effector responses. Furthermore, these data address the emerging field of ecological immunology by highlighting interspecific differences in immunity and immunocompetences among Caribbean corals, which are reflected in their life-history characteristics, disease susceptibilities and bleaching-induced mortality.

Key words: *Montastraea faveolata*, *Stephanocoenia intersepta*, *Porites astreoides*, innate immunity, coral, disease resistance, prophenoloxidase, lipopolysaccaride, temperature stress.

INTRODUCTION

Ecological immunology examines why and how biotic and abiotic factors influence variation in immune function among taxa (Sheldon and Verhulst, 1996; Hawley and Altizer, 2011), and therefore will likely give rise to explanations of the frequently observed variation in disease susceptibility (Martin et al., 2011). Trade-offs, and the relative allocation of limited resources to costly functions, are among the primary principles of ecological immunology (Sheldon and Verhulst, 1996; Sadd and Schmid-Hempel, 2009). Given the global rise in disease epizootics (Harvell et al., 2002; Lafferty, 2009), the development of ecological immunology and its application to threatened keystone species is particularly timely. However, there are few investigations into variation in baseline immunity or immune responses of natural populations (Cornet et al., 2009; Palmer et al., 2010).

The rise of global sea surface temperatures, coincident with increased disease, has stimulated the intense investigation of the effects of a warming environment on marine ectothermic organisms (Harvell et al., 2009; Somero, 2010). Because the defense mechanisms of immunity, responsible for preventing infection and maintaining tissue integrity, are energetically costly, it is likely that under stressful conditions, during which nutrition is compromised, resource allocation to defense is also diminished (Moret and Schmid-Hempel, 2000). This hypothesis is consistent with observed increases in disease prevalence among both terrestrial and marine ecosystems (Harvell et al., 2002; Lafferty, 2009), and thus, given the trajectory of climatic change, is cause for concern. Corals provide an ecologically relevant study system because coral cover is declining globally, hindering their ability to build and sustain the structure and function of tropical coral reefs (Wild et al., 2011).

The increased incidence of coral disease following a thermal bleaching event is well documented within the Caribbean and globally (Harvell et al., 2001; Miller et al., 2006; Bruno et al., 2007; Brandt and McManus, 2009; Miller et al., 2009). However, the complex interplay between warmer water conditions, increased pathogen abundance, altered pathogen virulence and compromised

coral immunity remains to be established (Harvell et al., 2007; Mydlarz et al., 2009; Palmer et al., 2011a). On the Great Barrier Reef, Australia, levels of immunity directly relate to the susceptibility of coral families to both bleaching and disease (Palmer et al., 2010), which highlights the ecological importance of understanding coral immune responses. Despite several recent studies on elucidating coral immune responses to pathogens, the direct effects of elevated water temperature on specific immune pathways, especially during the initial stages of exposure, are less understood.

Recent studies into coral immunity demonstrate that corals possess many innate immune mechanisms similar to those of other invertebrates (for reviews, see Mydlarz et al., 2006; Dunn, 2009; Mydlarz et al., 2010; Reed et al., 2010). Prophenoloxidase (PPO) is the activating enzyme of the melanin-synthesis pathway, which is a key component of invertebrate immunity (Cerenius et al., 2008). In invertebrates, the melanin-synthesis pathway is responsible for providing cytotoxic defense (Nappi and Ottaviani, 2000) and for forming an impermeable melanin barrier between healthy host tissue and an invading organism (Nappi, 1973). The presence of the melanin-synthesis pathway has been demonstrated within reef-building corals, gorgonians and true soft corals from the Caribbean and Indo-Pacific (Mydlarz et al., 2008; Palmer et al., 2010; Mydlarz and Palmer, 2011). Furthermore, melanin-synthesis pathway activity is significantly upregulated in tissue naturally infected with parasites (Palmer et al., 2009a) and fungal pathogens (Mydlarz et al., 2008), as well as in visually compromised tissue (Palmer et al., 2008; Mydlarz et al., 2009; Palmer et al., 2011a). Additionally, as melanin is a UV-light-absorbing pigment (Meredith et al., 2006), its presence within coral epithelial cell layers may provide photoprotection for photosynthetic symbiotic algae that can become dysfunctional under high light conditions (Palmer et al., 2010).

In addition to the presence and upregulation of the melanin-synthesis pathway, corals also demonstrate antimicrobial activity as part of a defensive response. In other invertebrates, antimicrobial peptides and polypeptides are major components of antimicrobial defense mechanisms (Li and Karin, 2000; Silverman and Maniatis, 2001). Although antimicrobial peptides are likely produced by all multicellular organisms (Zasloff, 2002), their presence within corals has only been established for one species (Vidal-Dupiol et al., 2011). However, antimicrobial, or bactericidal, activity of secondary metabolites (Slattery et al., 1995; Mydlarz and Jacobs, 2006; Dunn, 2009) of the mucus layer (Brown and Bythell, 2005) and of symbiotic bacteria (Ritchie, 2006) has been established for several coral species. Furthermore, the release of bactericidal activity has been demonstrated in response to mechanical agitation of corals (Geffen and Rosenberg, 2005; Geffen et al., 2009) and tissue bactericidal activity has been shown to increase in infected corals (Gochfeld and Aeby, 2008; Mydlarz et al., 2009). These studies indicate that bactericidal activity is a key component of coral defense and therefore is a useful measurement for ascertaining levels of immunity.

Immune defenses that actively isolate and kill invading pathogens are the mainstay of a given immune response; however, equally important are the mechanisms in place to prevent damage to the host as a result of these responses. Invertebrate immune responses, such as activation of the melanin-synthesis pathway, produce cytotoxic radicals, such as reactive oxygen species (ROS), which can lead to oxidative stress and cause tissue damage (Nappi and Ottaviani, 2000). In addition to ROS produced during an immune response, coral symbiotic algae, *Symbiodinium* spp., produce ROS during thermal stress and may threaten the integrity of the host (Lesser, 1997; Mydlarz and Jacobs, 2004). Antioxidants, which readily scavenge oxygen radicals, are therefore vital for preventing self-damage and are frequently abundant in areas of tissue injury and pathogen infection (Halliwell and Gutteridge, 1999) as well as during thermal stress in corals (Lesser, 2006). Common enzymatic antioxidants within invertebrates include peroxidases, catalases and superoxide dismutases (Halliwell and Gutteridge, 1999). Additionally, corals may employ fluorescent proteins (FPs) to supplement enzymatic antioxidant activity within compromised tissue, although this is unlikely to be their primary function (Palmer et al., 2009a; Palmer et al., 2009b).

As constituent levels of immunity and the induction and maintenance of an immune response are energetically costly processes (Armitage et al., 2003), they may be traded-off against other important life-history characteristics, such as growth and reproduction (Sadd and Schmid-Hempel, 2009). A coral that invests primarily in immunity may be slow growing, but will likely demonstrate higher levels of constituent immunity and/or induce greater immune responses than a species that invests primarily in growth or reproduction. As such, baseline immunity and/or immune responses of different coral species are likely to vary depending upon their different life-history characteristics (Palmer et al., 2008; Palmer et al., 2010). Therefore, although all healthy corals are likely to be immunocompetent, and thus able to induce a proficient immune response, the relative immunocompetence (i.e. the magnitude of a response) (Adamo, 2004) among different coral species is likely to vary. Establishing the relative immunocompetence of various coral species will provide insights into ecological patterns, such as disease prevalence, and better establish the ability of different corals to deal with adverse conditions.

In this study we investigated the immunological responses of three Caribbean coral species, *Montastraea faveolata*, *Stephanocoenia intersepta* and *Porites astreoides*, to the presence of thermal stress followed by exposure to pathogen-associated molecular patterns (PAMPs). These molecular patterns are recognized by pattern recognition receptors, such as lipopolysaccharide (LPS)-binding proteins and peptidoglycan recognition protein, and elicit invertebrate immune responses, including the melanin-synthesis pathway (Ratcliffe et al., 1991; Wittwer et al., 1997). In coral studies, the use of LPS bypasses well-documented difficulties in infecting coral with live bacteria (Lesser et al., 2007) and can test for the induction or suppression of immune pathways by PAMP receptors (Xian et al., 2009; Liu et al., 2011).

The three Caribbean coral species chosen for this experiment demonstrate disparate life-history characteristics and are documented to vary in susceptibility to stressors, such as disease and bleaching (e.g. Cróquer and Weil, 2009a; Cróquer and Weil, 2009b; Edmunds, 2010). *Montastraea faveolata* colonies are the primary framework builders of Caribbean coral reefs; however, because of an apparent high susceptibility to thermal stress and coral disease (Cróquer and Weil, 2009b), abundances of this genus are in decline (Miller et al., 2006; Edmunds and Elahi, 2007). Conversely, *P. astreoides* displays weed-like life-history characteristics and has increased in relative abundance within the Caribbean (Green et al., 2008; Edmunds, 2010), despite a high percentage becoming bleached during the widespread Caribbean bleaching in 2005 (Oxenford et al., 2008; Brandt, 2009; Cróquer and Weil, 2009b). *Stephanocoenia intersepta* is considered a disease-susceptible hard coral, and has total disease prevalence similar to that of *M. faveolata* and other *Montastraea* species (Cróquer and Weil, 2009a), but the lowest percentage bleaching of the three coral species (Cróquer and Weil, 2009b).

Identifying how these coral species respond to the threat of thermal stress and pathogens will help determine the mechanisms behind the observable patterns of disease and bleaching, and begin to establish how Caribbean reefs will continue to be affected by climate change.

MATERIALS AND METHODS

Coral collection

Eight visually distinct colonies of each of the three Caribbean species, *Montastraea faveolata* (Ellis and Solander 1786), *Stephanocoenia intersepta* (Lamarck 1816) and *Porites astreoides* (Lamarck 1816), were collected from the National Oceanic and Atmospheric Administration Florida Keys National Marine Sanctuary (FKNMS) Coral Nursery in Key West, FL, in July 2008. The corals used in these experiments were all resident at the FKMNS Coral Nursery for approximately 1 year prior to use. Furthermore, the use of coral from the FKMNS Coral Nursery enabled the inclusion of *M. faveolata*, a species considered endangered by the International Union for Conservation of Nature Red List (Aronson et al., 2008), within our study. At the FKNMS Coral Nursery, the coral colonies were maintained as distinct non-related colonies because of their visual appearance, e.g. round and with no straight broken edges, indicating that it was unlikely they were all pieces of the same original larger colony. Corals demonstrated no visibly discernable signs of stress while at the FKNMS Coral Nursery. The colony pieces (approximately 50–75 cm^2) were transferred to Mote Marine Laboratory–Tropical Research Laboratory (TRL), Summerland Key, FL, under the specifications of research permit number FKNMS-2007-050. All the corals appeared healthy during the transfer to TRL, and in the following days prior to experimentation. The corals were placed into a large semi-shaded (with 70% light-reducing shadecloth) water table with flow-through seawater at ambient temperature. The coral colonies were held in these conditions for 2 weeks before each individual coral colony was fragmented into at least four pieces, representing colonial replicates to be used across treatments, each approximately 15 cm^2, and returned to the water table to recover for 3 days.

Experimental design

The experimental setup consisted of six large plastic containers that were placed in two large water tables and supplied with a slow constant flow of 20 µm-filtered seawater at ambient temperature from the open pressure seawater system at TRL. The water tables were under a canopy of 70% light-reducing shadecloth. To control the temperature, one submersible heater and one submersible water pump were placed into each of the plastic containers. These large containers served as temperature-controlled water baths. Three of the large containers were then haphazardly chosen as elevated temperature treatment water baths in which the water temperature was raised to the target 30.5°C. The water temperatures for the three ambient containers ranged from a mean (±s.e.m.) of 26.7±0.12°C in the morning to 28.5±0.15°C at noon and 28.04±0.15°C in the late afternoon over the course of the 4 day experiment. The elevated temperature treatment containers ranged from a mean of 28.9±0.14°C in the morning to 30.5±0.15°C at noon and 29.5±0.15°C in the late afternoon. The variances of the six container temperatures were equal and there were no statistical differences among the means of each of the three ambient containers or among the means of each of the elevated temperature containers. In the three elevated temperature containers, daily means were significantly higher, by approximately 2°C, than those of the three ambient temperature containers (ANOVA, N=13, F=18.07, P<0.001).

Six smaller plastic aquaria holding 1.5–2 l seawater, equipped with individual airstones to aerate and mix the water, were submerged within each of the six larger plastic containers, for a total of 36 small aquaria. Three coral fragments of the same species were placed 5–10 cm away from each other into the smaller aquaria, and thus did not come into contact with each other. Our experimental design allowed for a maximum of 108 coral fragments, although this experiment only used 96 fragments. The smaller aquaria were haphazardly designated for one of four treatments: (1) ambient water temperature (mean 27.7±0.13°C) and control (no LPS); (2) ambient water temperature with 5 µg ml^{-1} LPS, lyophilized from *Escherichia coli* 0127:B8 (Sigma-Aldrich, St Louis, MO, USA) and dissolved in sterile filtered seawater; (3) elevated water temperature (mean 29.7±0.15°C) and control (no LPS); and (4) elevated water temperature with 5 µg ml^{-1} LPS. All eight colonies of the three species were represented in each treatment with clonal replication across treatments (four fragments from each of the eight colonies per species for a total of 32 fragments from each coral species). The coral fragments were exposed to a constant flow (from the larger containers) of 20 µm-filtered seawater at either ambient or elevated water temperature for 2 days as a pre-treatment. To begin the LPS exposure, the system was closed by stopping the water circulation in the small plastic aquaria by raising each container's rim slightly above the waterline of the larger water bath using 4 cm blocks. The existing water in each of the small aquaria was carefully exchanged with appropriate-temperature 0.2 µm sterile filtered seawater, and 5 µg ml^{-1} LPS dissolved in sterile filtered seawater was added to those designated for the LPS treatment. The small aquaria were kept slightly raised in the water baths throughout the LPS or control (no LPS) treatments, so that the water within each small aquaria remained isolated while water temperatures continued to be maintained at a mean of 27.7±0.13°C for the ambient containers and 29.7±0.15°C for the elevated temperature treatments. LPS exposure occurred over a period of 12 h beginning at 19:30 h, sunset occurred at 20:18 h and sunrise at 06:48 h. Water flow during the 12 h closed incubation was accomplished by the airstone in each aquaria, which was flowing vigorously throughout the entire experiment. None of the coral fragments demonstrated any tissue necrosis, lightening or change in color and appeared healthy throughout the entire experiment. The experiment was ended after the 12 h exposure to LPS and the coral fragments were snap-frozen in liquid nitrogen immediately after removal from the aquaria and shipped on dry ice to the University of Texas at Arlington where they were stored at –80°C.

Extract preparation

For all three coral species, tissue was removed from the frozen samples with a Paansche airbrush (Chicago, IL, USA) using a coral extraction buffer (50 mmol l^{-1} phosphate buffer, pH 7.8, with 0.05 mmol l^{-1} dithiothreitol) over ice. Tissue at the edge of each sample (approximately 1 cm) was not removed to reduce the effects of the fragmentation injury being included within this study. The coral tissue slurry was homogenized with a Power Gen 125 tissue homogenizer with medium sawtooth generator (Fisher Scientific, Pittsburgh, PA, USA) for 20 s and 0.5 ml of five of the eight replicates per coral species was removed for melanin analysis (see below). The remaining tissue slurry was snap-frozen in liquid nitrogen. To extract the coral proteins from intact cells, tissue slurries were thawed over ice and homogenized again using the Power Gen 125 tissue homogenizer with medium sawtooth generator and left on ice for 5 min to allow proteins to go into solution. Samples were then vortexed with a spatula of glass beads for 20 s and left on ice

for 5 min further. Samples were centrifuged at 4°C at 2400 ***g*** for 5 min, and the supernatant (coral protein extract) was removed from the cellular debris and stored at –80°C until use.

All colorimetric measurements were calculated using a Synergy 2 multi-Detection microplate reader with Gen5 software (BioTek, Winooski, VT, USA). All assays were run in duplicate or triplicate on separate 96-well microtiter plates. Total protein concentration of each coral extract was determined using the Quick Start Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and used to standardize all the protein activity assays described below. Boiled extracts were used as a negative control in all enzyme assays; in all cases activity was strongly diminished or completely inhibited (data not shown).

PPO and melanin bioassays

PPO activity was determined for each sample using duplicate 20 µl aliquots of the protein extract in clear 96-well microtiter plates, with 40 µl of 50 mmol l^{-1} phosphate buffer (pH 7.5). To each well, 25 µl of trypsin (0.1 mg ml^{-1} in deionised water) was added and the reaction was initiated by the addition of 30 µl of 10 mmol l^{-1} L-1,3-dihydroxyphenylalanine (L-DOPA; Sigma-Aldrich). The absorbance at 490 nm was measured over 15 min (during the linear portion of the reaction) and the change in absorbance was normalized to mg protein for each sample.

To determine the concentration of melanin within each sample, the freeze-dried aliquots of tissue slurry were gently vortexed with 0.3 ml of 10 mol l^{-1} sodium hydroxide (NaOH) and left overnight. Samples were vortexed for 10 s and then centrifuged at 7000 ***g*** for 5 min. For each sample, 30 µl of supernatant were aliquoted in duplicate into a 96-well ½-area microtiter plate (Greiner Bio-one, Monroe, NC, USA). The plates were read at an absorbance of 410 nm and the concentration of melanin was determined using a standard curve of commercial melanin (Sigma-Aldrich) dissolved in 10 mol l^{-1} NaOH and treated the same as the samples on each microtiter plate. Data presented are converted to mg melanin normalized to g tissue.

Bactericidal activity bioassays

Bactericidal activity was tested using a bacteria turbidity assay conducted in sterile 96-well flat-bottom microtiter plates (Greiner Bio-one). Twenty microliters of each coral extract were diluted in 50 µl Difco™ marine broth (Becton, Dikinson and Co., Le Pont de Claix, France) and 100 µl bacteria stock was added into each well. The bacteria stock consisted of overnight cultures of *Vibrio alginolyticus* (generously provided by K. Ritchie, Mote Marine Laboratory, Sarasota, FL, USA) in exponential growth, diluted to 0.2 optical density at 600 nm (OD_{600nm}). The extracts and bacteria were gently mixed and read at OD_{600nm} every hour with a Biotek Synergy 2 spectrophotometer (BioTek) for 6 h at 29°C. Assays were run in triplicate wells along with the following controls: bacteria alone and bacteria + coral homogenate buffer. Growth inhibition of bacteria cultures was measured by comparing the growth rate of bacteria with coral extracts to bacteria and buffer controls (no different from bacteria alone) with the following formula: $3.3\times\log(t_f/t_i)/n$, where, t_f is the final OD_{600}, t_i is the initial OD_{600} and $n=t_f-t_i$. The linear portion of logarithmic growth (0–2 h of growth) was used to select the initial and final hour and was kept standard between runs.

Antioxidant and fluorescent protein bioassays

Peroxidase activity was determined for each sample using 10 µl aliquots in duplicate within 96-well microtiter plates, with 35 µl of 10 mmol l^{-1} phosphate buffer (pH 6.0). To each well, 40 µl of 25 mmol l^{-1} guaiacol was added and the reaction was initiated with the addition of 25 µl of 20 mmol l^{-1} high purity 30% hydrogen peroxide (H_2O_2; Sigma Aldrich). The absorbance was read at 470 nm every min for 15 min and the change over time was calculated for the linear part of the reaction (0–8 min) and normalized to mg protein in each sample (ΔAbs_{470nm} mg^{-1} protein min^{-2}).

H_2O_2 scavenging activity for each sample was determined using 20 µl of protein extract with 80 µl of 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) in duplicate wells of a UV-transparent 96-well microtiter plate. The assay was activated by the addition of 50 µl of 50 mmol l^{-1} H_2O_2. The absorbance was measured at 240 nm over 10 min and the change in absorbance over time was calculated for the linear part of the reaction (0–3 min) and converted to mmol l^{-1} H_2O_2 using a standard curve of known concentrations, normalized per mg protein and time (mmol l^{-1} H_2O_2 scavenged mg^{-1} protein s^{-1})

To obtain fluorescence emission spectra, aliquots of 30 µl of each sample were added to triplicate wells in a black, clear-bottom 384-well microtiter plate with parallel aliquots of extraction buffer to control for independent effects. Each well was excited at 280 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). This wavelength was used as wavelengths in the UV range are optimal for exciting FPs across the spectrum, whilst 280 nm is far enough away from the emission peak of the cyan FP to avoid crossover of the excitation and emission peaks (Shagin et al., 2004). Emission spectra were measured in 5 nm increments from 400 to 650 nm. The fluorescence of each sample was standardized to the sample's total protein concentration (Palmer et al., 2009b). The mean total fluorescence per mg protein for each sample was calculated by summing the standardized relative fluorescence units between 465 and 600 nm and a mean was then taken of total fluorescence for each sample.

Data analysis

The variance between temperature treatments was equal and there was no statistical difference within the three ambient containers or within the three elevated temperature containers. The data were therefore analyzed using a two-way multivariate ANOVA (MANOVA) for each coral species, which included mean activities of immunity parameters (PPO, bactericidal activity, peroxidase, H_2O_2 scavenging and FP) with temperature and LPS as factors. Assumptions of normality and homoscedasticity (Shapiro–Wilks and Levene's tests, respectively) were satisfied with log-transformed data. Melanin concentration was excluded from the MANOVA and analyzed using a two-way ANOVA because the replication was less (N=5) than the rest of the immune measures (N=8). One-way ANOVAs with Tukey's honestly significantly different (HSD) *post hoc* tests were used to further identify significant differences between treatments when MANOVA analysis indicated significant model effects. All statistical analysis was performed using JMP statistical discovery software version 6.0.2 (SAS Institute, Cary, NC, USA).

RESULTS

Between-species comparisons

Each of the three Caribbean reef-building coral species used in this study, *M. faveolata*, *S. intersepta* and *P. astreoides*, demonstrated differing levels of constituent immunity, as indicated by the immune parameter activities under ambient temperature and no LPS treatment. For example, mean PPO activity (Fig. 1A) varied significantly among coral species ($F_{2,22}$=153.64, P<0.001), with *P. astreoides* having the highest PPO activity at 1.55, which was

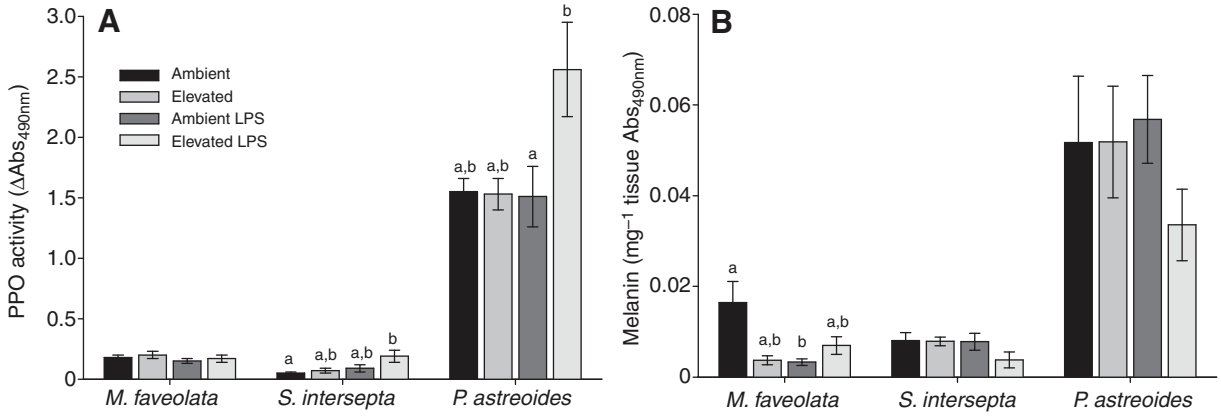

Fig. 1. Mean (±s.e.m.) melanin-synthesis immune responses of *Montastraea faveolata*, *Stephanocoenia intersepta* and *Porites astreoides* to elevated water temperature and lipopolysaccharide (LPS) treatments. (A) Prophenoloxidase (PPO) activity; (B) melanin concentration. Letters indicate significant differences at $P<0.05$.

approximately ninefold higher than *M. faveolata* and 31-fold higher than *S. intersepta* (Tukey's HSD, $P<0.001$). Conversely, the concentration of melanin did not vary significantly among the coral species under ambient temperature and control (no LPS) conditions ($F_{2,12}=10.86$, $P=0.08$), although *P. astreoides* had sixfold more melanin than *S. intersepta* and threefold more than *M. faveolata* (Fig. 1B). All three species demonstrated bactericidal activity under ambient temperature and control (no LPS) conditions (Fig. 2). *Porites astreoides* demonstrated the highest bactericidal activity at approximately twofold higher percent inhibition than *M. faveolata* and *S. intersepta* ($F_{2,22}=9.23$, $P<0.001$; Tukey's HSD, $P<0.001$), which had approximately equivalent levels of activity at 20% bacterial inhibition. Peroxidase activity was present in all three coral species, and varied significantly among them under ambient and control conditions ($F_{2,22}=21.49$, $P<0.001$). *Porites astreoides* and *M. faveolata* had approximately equivalent peroxidase activity, at a level of $0.75\,\Delta Abs_{470nm}\,mg^{-1}$ protein min^{-1}, whereas *S. intersepta* had significantly lower activity (Tukey's HSD, $P<0.001$) at $0.04\,\Delta Abs_{470nm}\,mg^{-1}$ protein min^{-1} (data not shown). The rate of H_2O_2 scavenging under ambient and control conditions was approximately constant among the coral species, between 1 and $1.5\,\Delta Abs_{240nm}\,mg^{-1}$ protein s^{-1} (Kruskal–Wallis, $K_2=5.46$, $P=0.07$; Fig. 3A). The levels of total fluorescence under ambient temperature and no LPS treatment varied significantly among the coral species ($F_{2,22}=10.86$, $P<0.001$), with *S. intersepta* having the lowest levels with just 20% of the fluorescence of *M. faveolata* and *P. astreoides* (Tukey's HSD, $P<0.001$; Fig. 3B).

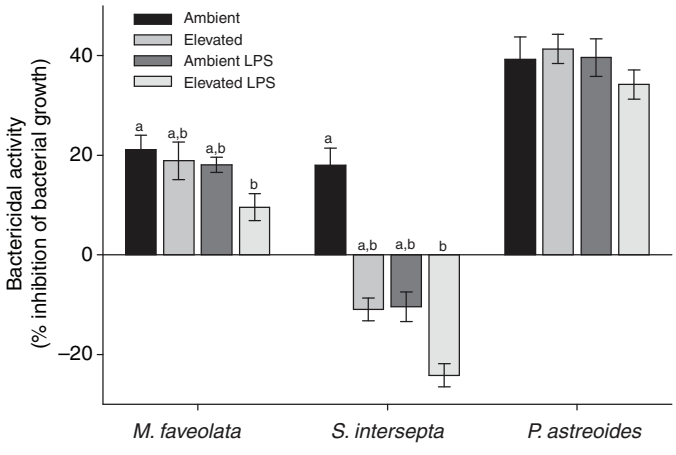

Fig. 2. Mean (±s.e.m.) bactericidal activity of *Montastraea faveolata, Stephanocoenia intersepta* and *Porites astreoides* against *Vibrio alginolyticus* in response to elevated water temperature and LPS treatments. Letters indicate significant differences at $P<0.05$.

Within-species effects of temperature and LPS

All three of the studied Caribbean reef-building corals demonstrated changes in the activities or concentrations of the measured immunity parameters in response to elevated temperature and elicitor (LPS) treatments (Wilks' lambda whole model, $P<0.001$; Tables 1–3). Peroxidase activity did not vary within species among the treatments and so data are not shown.

Montastraea faveolata

PPO activity of *M. faveolata* did not differ significantly among treatments (Fig. 1A, Table 1), with activities at approximately $0.2\,\Delta Abs_{490nm}\,mg^{-1}$ protein for each treatment. However, *M. faveolata* demonstrated a significant variation in melanin concentration, driven by temperature stress alone, with an overall decrease in melanin in elevated temperature treatments (Fig. 1B). Bactericidal activity of *M. faveolata* varied significantly among treatments ($F_{3,27}=3.52$, $P=0.03$; Table 1), demonstrating a decrease from 21% bactericidal activity to 9% effective against *V. alginolyticus* under the combined elevated temperature and LPS treatment (Tukey's HSD, $P<0.05$; Fig. 2). The rate of H_2O_2 scavenging and total fluorescence did not vary significantly among the treatments for *M. faveolata* (Fig. 3).

Stephanocoenia intersepta

PPO activity of *S. intersepta* varied significantly among treatments (PPO whole model, $F_{3,27}=3.93$, $P=0.02$; Table 2). This significant variation was primarily driven by the LPS treatment effect (LPS effect, $F_{1,27}=6.97$, $P=0.01$; Table 2). However, the PPO activity of *S. intersepta* samples treated with the combination of elevated temperature and LPS was 2.5-fold higher than samples under the ambient control treatment (Tukey's HSD, $P<0.05$; Fig. 1A). Melanin concentration did not vary significantly among treatments, although the lowest concentration occurred within the combined elevated temperature and LPS treatment (Fig. 1B). Bactericidal activity of *S. intersepta* varied significantly among treatments ($F_{3,27}=33.10$, $P<0.001$), driven by both temperature and LPS effects, resulting in a significant decrease in bactericidal activity in all treatments (Fig. 2).

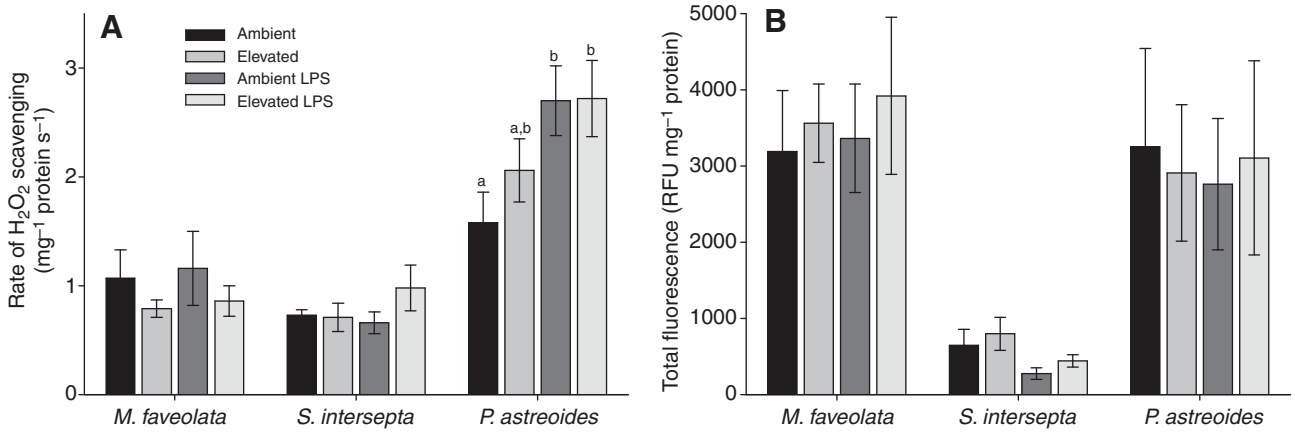

Fig. 3. Mean (±s.e.m.) antioxidant immune responses of *Montastraea faveolata, Stephanocoenia intersepta* and *Porites astreoides* to elevated water temperature and LPS treatments. (A) H_2O_2 scavenging; (B) total fluorescence. Letters indicate significant differences at $P<0.05$.

In fact, only the ambient control samples exhibited bactericidal activity. The elevated temperature treatment, LPS treatment and the combination of the two resulted in a positive effect on the growth of *V. alginolyticus* and no measurable bactericidal activity. H_2O_2 scavenging activity and peroxidase did not vary significantly among treatments (Table 2), although the combined temperature treatment and LPS demonstrated the highest H_2O_2 scavenging activity overall (Fig. 3A). Total fluorescence was significantly negatively affected by LPS treatment and significantly decreased by 57 and 44% in both ambient and elevated temperature LPS treatments, respectively, as compared with ambient controls with no LPS (LPS effect, $F_{1,27}=5.42$, $P=0.03$, Fig. 3B).

Porites astreoides

PPO activity of *P. astreoides* was stable among the first three treatments (ambient temperature and no LPS, elevated temperature and no LPS and elevated temperature with LPS) at approximately $1.5\,\Delta Abs_{490nm}\,mg^{-1}$ protein (Fig. 1A). However, the addition of LPS at elevated water temperature led to a 1.7-fold increase in PPO activity (LPS×temperature, $F_{1,27}=4.21$, $P<0.05$; Table 3). Melanin concentration did not vary statistically significantly among the treatments (Table 3); however, it was lower in the combined elevated temperature and LPS treatment (Fig. 1B). Bactericidal activity of *P. astreoides* extracts remained high and unaffected by any treatment (Table 3, Fig. 2). The rate of H_2O_2 scavenging varied with LPS treatment ($F_{3,27}=2.88$, $P=0.049$; Table 3), as demonstrated by an increase of 1.7- and 1.3-fold when exposed to LPS under ambient and elevated water temperature treatments, respectively, as compared with the ambient no LPS treatment (Fig. 3A). Total fluorescence did not vary significantly among the treatments (Table 3, Fig. 3B).

DISCUSSION

The three species of Caribbean coral investigated, *M. faveolata*, *S. intersepta* and *P. astreoides*, all demonstrated activity of each of the immunity parameters measured. The activities of the immune parameters are consistent with recent studies that document the presence of the melanin-synthesis pathway, including phenoloxidase activity and melanin-containing granular cells, bactericidal activity, FPs and antioxidants within numerous hard coral species (Palmer et al., 2008; Mydlarz et al., 2009; Palmer et al., 2010). Furthermore, all three of the coral species tested showed a significant response (in at least one measure) to exposure to the PAMP – commercial LPS – used in this study. This is the first experimental evidence that demonstrates the likely presence and activation of pattern recognition receptors in Caribbean reef-building corals, which complements previous genomic and biochemical evidence (Miller et al., 2007; Kvennefors et al., 2008; Dunn, 2009; Voolstra et al., 2011). As an immune response elicitor, it is unsurprising that LPS exposure alone changed a total of five immune measures (across all species), as it is well known as a potent immune modulator across many phyla (Wittwer et al., 1997; Liu et al., 2011).

Table 1. *Montastraea faveolata* whole-model MANOVA and ANOVA results for each immunity parameter and each treatment

	Whole model			Temperature effect			LPS effect			Temperature × LPS		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
MANOVA												
Wilks' lamda	**59.1**	**5, 24**	**<0.001**	**3.03**	**5, 24**	**0.029**	2.25	5, 24	0.082	0.43	5, 24	0.83
Univariate tests												
PPO activity	0.78	3	0.513	0.57	1	0.46	1.72	1	0.20	0.06	1	0.81
Bactericidal activity	**3.52**	**3**	**0.028**	**4.53**	**1**	**0.04**	**4.54**	**1**	**0.04**	1.51	1	0.23
Peroxidase activity	2.35	3	0.09	3.84	1	0.06	3.09	1	0.09	0.12	1	0.73
H_2O_2 scavenging	0.48	3	0.702	1.35	1	0.26	0.08	1	0.79	0.00	1	0.99
FP concentration	0.25	3	0.863	0.609	1	0.44	0.61	1	0.83	0.09	1	0.77
ANOVA												
Melanin	2.3	3	0.115	**6.22**	**1**	**0.026**	0.43	1	0.52	0.01	1	0.97

FP, fluorescent protein; LPS, lipopolysaccharide; PPO, prophenoloxidase.
Results in bold are significant at the $\alpha=0.05$ level.

Table 2. *Stephanocoenia intersepta* whole-model MANOVA and ANOVA results for each immunity parameter and each treatment

	Whole model			Temperature effect			LPS effect			Temperature × LPS		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
MANOVA												
Wilks' lamda	**2682**	**5, 24**	**<0.001**	**11.4**	**5**	**<0.001**	**16.12**	**5**	**<0.001**	0.337	5	0.885
Univariate tests												
PPO activity	**3.93**	**3**	**0.02**	3.84	1	0.06	**6.97**	**1**	**0.01**	0.99	1	0.33
Bactericidal activity	**33.10**	**3**	**<0.001**	**49.52**	**1**	**<0.001**	**48.56**	**1**	**<0.001**	1.21	1	0.28
Peroxidase activity	0.73	3	0.55	1.92	1	0.18	0.00	1	0.96	0.26	1	0.62
H_2O_2 scavenging	0.71	3	0.56	0.34	1	0.56	0.29	1	0.60	1.49	1	0.23
FP concentration	2.37	3	0.09	1.66	1	0.21	**5.42**	**1**	**0.03**	0.03	1	0.87
ANOVA												
Melanin	1.33	3	0.30	1.515	1	0.24	1.634	1	0.22	1.304	1	0.271

Results in bold are significant at the α=0.05 level.

Melanin-synthesis pathway

The melanin-synthesis pathway is considered a key component of invertebrate immunity (Söderhäll and Cerenius, 1998) and, as such, all three coral species showed changes in this pathway. PPO activity was significantly induced by LPS exposure in *S. intersepta* and by the combination of elevated temperature stress and LPS in *P. astreoides*, whereas melanin concentration was negatively affected by elevated temperature in *M. faveolata*. These results unequivocally demonstrate the ability of various coral species to induce melanin-synthesis pathway activity in the presence of pathogen elicitors, as documented for numerous other invertebrates (e.g. Asokan et al., 1997; Bidla et al., 2008; Xian et al., 2009). Consistent with the findings for *S. intersepta* and *P. astreoides*, invertebrates induce PPO activity during the invasion of foreign organisms, as the cytotoxic properties of the melanin-synthesis pathway aid in the elimination of pathogens (Nappi and Ottaviani, 2000). Similarly, an upregulation of phenoloxidase activity has been documented at white-syndrome disease lesions in an Indo-Pacific coral (Palmer et al., 2011a). However, PPO did not increase in response to LPS in *M. faveolata*, consistent with findings of yellow-band-infected colonies of this species (Mydlarz et al., 2009), indicating that conditions were not adverse enough to warrant this immune response, it was unable to be upregulated or other immunity parameters (outside the scope of this study) were preferentially used.

The decrease in melanin concentration in *M. faveolata* and the lack of variation of melanin among treatments for *S. intersepta* and *P. astreoides* may be due to the release of melanin *via* degranulation (Xian et al., 2009; Palmer et al., 2011b). The degranulation of redox-active melanin (Nappi and Ottaviani, 2000) from melanin-containing cells to aid during phagocytosis and barrier formation (Foley and Cheng, 1977; Xian et al., 2009) would therefore lead to a reduced concentration within the host tissue. In addition, melanin degranulation may be triggered to mitigate thermal stress (Palmer et al., 2010) by forming a photo-protective layer.

Bactericidal activity

Both *M. faveolata* and *S. intersepta* exhibited the same trend of decreasing bactericidal activity with all treatments, but this was only statistically significant in the combined elevated temperature and LPS exposure treatment. In fact, only the ambient temperature controls of *S. intersepta* extracts actually demonstrated any bactericidal activity; the rest of the treatments promoted growth of *V. alginolyticus*, which has been reported previously for another coral species (Gochfeld and Aeby, 2008). Although the significant decrease in bactericidal activity by both *M. faveolata* and *S. intersepta* in response to the LPS and elevated temperature treatments appear counter-productive to an effective immune response, antibacterial compounds may be released upon recognition of an immune challenge (e.g. Geffen and Rosenberg, 2005; Xian et al., 2009), thus killing potentially harmful bacteria within the immediate surroundings. Conversely, there is also evidence that within-tissue bactericidal activity is increased in corals naturally infected with yellow band disease (Mydlarz et al., 2009) and white syndrome relative to unaffected parts of the same colony (Gochfeld and Aeby, 2008). These studies of within-tissue bactericidal activity of naturally infected corals may not accurately compare with our study because the effects of the infecting bacteria themselves and the coral mucus may play a large role in observed antimicrobial activity (Ritchie, 2006). Because our study measured the initial response to a pathogen elicitor within 12 h of exposure, the

Table 3. *Porites astreoides* whole-model MANOVA and ANOVA results for each immunity parameter and each treatment

	Whole model			Temperature effect			LPS effect			Temperature × LPS		
	F	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
MANOVA												
Wilk's lamda	**215.5**	**5, 23**	**<0.001**	0.94	5	0.48	1.99	5	0.134	1.06	5	0.41
Univariate tests												
PPO activity	**3.58**	**3**	**0.03**	3.92	1	0.06	2.32	1	0.14	**4.21**	**1**	**0.049**
Bactericidal activity	0.68	3	0.57	0.130	1	0.72	0.77	1	0.39	1.08	1	0.31
Peroxidase activity	1.09	3	0.37	1.45	1	0.24	0.89	1	0.35	0.83	1	0.37
H_2O_2 scavenging	2.88	3	0.05	0.88	1	0.36	**7.36**	**1**	**0.01**	0.77	1	0.39
FP concentration	0.02	3	1.00	0.00	1	0.99	0.00	1	0.99	0.01	1	0.94
ANOVA												
Melanin	0.42	3	0.74	0.56	1	0.47	0.139	1	0.715	0.506	1	0.488

Results in bold are significant at the α=0.05 level.

downregulation of the bactericidal activity observed in our experiments may be caused by suppression of the pathway, but could also be due to the release of antibacterial compounds into the surrounding water, and thus represents an important immune response (Geffen and Rosenberg, 2005; Geffen et al., 2009).

Antioxidants

Antioxidants are important during immune responses, which often induce oxidative stress conditions and can lead to self-harm (Halliwell and Gutteridge, 1999). In addition, in coral species that harbour symbiotic algae, oxidative stress conditions are readily induced during thermal bleaching (Lesser, 1997), necessitating the presence of antioxidants during thermal stress. However, peroxidase activity did not vary among the experimental treatments for any of the coral species. This suggests that baseline levels of this enzymatic antioxidant were adequate for preventing oxidative stress (Mydlarz and Harvell, 2007).

In most biological systems, H_2O_2 scavenging is most efficiently accomplished by catalase (Munoz-Munoz et al., 2009); however, coral FPs are also able to efficiently scavenge H_2O_2 (Palmer et al., 2009b). This additional antioxidant activity within coral tissue potentially supports that of enzymatic antioxidants during periods of extreme oxidative stress (Palmer et al., 2009b). Consistent with a lack of upregulation of the melanin-synthesis pathway, *M. faveolata* did not upregulate H_2O_2 scavenging or FP concentration in any experimental treatment. *Stephanocoenia intersepta* demonstrated a downregulation of FP concentration in response to LPS. Similar to a decrease in melanin concentration and bactericidal activity, a reduction in FP concentration may indicate that ROS may have been released for their antimicrobial properties, therefore necessitating the use of low levels of antioxidant activity. However, it is also likely that antioxidant scavenging is not the primary function of coral FPs (Palmer et al., 2009b).

In support of antioxidants preventing self-harm during an immune response, *P. astreoides* upregulated H_2O_2 scavenging in response to the presence of LPS. This is consistent with the induction of oxidative stress conditions during an immune response (Halliwell and Gutteridge, 1999) as a result of the oxidative burst and as a product of the upregulation of PPO activity, which are primary sources of oxidative stress during an invertebrate immune response (Nappi and Ottaviani, 2000; Sadd and Siva-Jothy, 2006). The lack of increased antioxidant activity for any of the coral species under elevated water temperature conditions suggests that a threshold of thermal stress was not induced. This is consistent with reports that a combination of sustained elevated water temperature and high UV light is required to induce the thermal stress that typically results in coral bleaching (Hoegh-Guldberg, 1999).

Ecological immunology of corals

There is a relationship between levels of baseline immunity and the relative susceptibility to both disease and bleaching in Indo-Pacific coral species that is likely a reflection on the level on resource investment into this key life-history trait (Palmer et al., 2010). The three Caribbean coral species used in this study were selected because of their disparate life-history characteristics and observed variation in susceptibility to disease and bleaching (Gardella and Edmunds, 2001; Cróquer and Weil, 2009a; Edmunds, 2010), and they consistently demonstrated significantly different baseline levels of immunity. In all six immunity measures, *P. astreoides* had considerably higher levels than both *M. faveolata* and *S. intersepta*. *Porites astreoides* is considered to be one of the more resilient coral species of the Caribbean, attributed largely to its weedy life-history characteristics (Green et al., 2008; Edmunds, 2010), which typically enable colonies to proliferate in unfavorable environments. Reported incidences of disease or bleaching-related mortality for *P. astreoides* are low relative to other Caribbean coral species, including *M. faveolata* and *S. intersepta* (Ward et al., 2006; Brandt and McManus, 2009; Cróquer and Weil, 2009a; Cróquer and Weil, 2009b; Weil and Cróquer, 2009). Conversely, *M. faveolata* and *S. intersepta* have relatively similar basal levels of most of the immune measures, with the exception of FPs, which were lower in *S. intersepta*, and both of these coral species are documented as being disease susceptible (Cróquer and Weil, 2009b; Mydlarz et al., 2009). However, *M. faveolata* is reported to also be susceptible to bleaching whereas *S. intersepta* does not bleach as readily (Brandt, 2009; Cróquer and Weil, 2009b). Overall, baseline levels of immune parameters and ecological data for these three Caribbean coral species support suggestions that coral immunity levels may be indicative of investment in immunity as a life-history trait (Palmer et al., 2010).

Of the three species, *S. intersepta* induced change in the highest amount of immune responses, showing decreases in bactericidal activity in response to elevated temperature and LPS separately, and a decrease in FP concentration and increase in PPO activity in response to LPS alone. *Montastraea faveolata* showed a decrease in bactericidal activity in response to both elevated temperature and LPS treatments independently, decreases in melanin concentration in response to temperature alone and, notably, was the only species not to upregulate any immune responses. Therefore, the primary defenses for *M. faveolata* and *S. intersepta* seem to involve release of cytotoxic compounds (melanin in *M. faveolata* and bactericidal activity in both *S. intersepta* and *M. faveolata*), which appear to be un-replenished, at least during the timeline of our experiment. Both *M. faveolata* and *S. intersepta* showed no changes in any immune factor under the elevated temperature and LPS combination treatment, even though both had immune responses to LPS at ambient temperature. This suggests that elevated water temperature does have a direct effect on specific immune mechanisms by preventing the changes in immunity that occurred as a result of LPS alone. The temperature challenge on the corals in this experiment (2°C increase for 2 days) was relatively mild compared with the temperature stress conditions associated with coral thermal bleaching events (Hoegh-Guldberg, 1999). Nonetheless, this may imply that these two coral species are highly susceptible to disease during the summer months and during bleaching events (Cróquer and Weil, 2009b).

The immune responses of *P. astreoides* are remarkably different from the other two species for several reasons. First, it was the only species to not show a decrease in activity of any immune response measured; the immune parameters induced by *P. astreoides* (PPO and H_2O_2 scavenging) were all upregulations. Second, *P. astreoides* was the only species to not induce any immune responses to elevated water temperature alone, and was only affected by elevated temperature in combination with LPS. For *P. astreoides*, maintaining both high constituent levels of immunity and the ability to increase PPO and H_2O_2 scavenging seems to benefit this coral in terms of disease and bleaching susceptibility. *Porites astreoides* generally has low disease prevalence (Cróquer and Weil, 2009a), and although this species experiences seasonal bleaching, bleaching-related mortality of massive *Porites* spp. is low (Stimson et al., 2002; Green et al., 2008).

The stimulation of immune responses with a bacterial product (LPS) as an elicitor, the potentially adverse effect of temperature on immune responses and the diversity in immune responses among the coral species are all key findings of this study. Our results are

consistent with hypotheses that increased disease prevalence during periods of warmer water temperature is partly due to a suppression of coral immunity (Harvell et al., 2002; Willis et al., 2004; Mydlarz et al., 2006; Harvell et al., 2007; Sato et al., 2009). Furthermore, our ecological immunology approach of multiple species and multiple immune measures provides a basis for developing an immunocompetence assessment for corals, and contributes to understanding natural patterns of disease and bleaching-related mortality. Collectively, our findings provide novel biological information on Caribbean coral species that can be used to better understand ecological patterns and, therefore, will likely increase the accuracy of reef response predictions to future climate-related events.

LIST OF ABBREVIATIONS

FP	fluorescent protein
H_2O_2	hydrogen peroxide
LPS	lipopolysaccaride
OD_{600nm}	optical density at 600 nm
PAMP	pathogen-associated molecular pattern
PPO	prophenoloxidase
ROS	reactive oxygen species

ACKNOWLEDGEMENTS

The authors thank J. Delaney and L. MacLaughlin (Florida Keys National Marine Sanctuary) for permitting and facilitating coral transfers under research permit number FKNMS-2007-050. We also thank L. Hunt [University of Texas at Arlington (UTA)], Y. Sato and B. Willis (James Cook University) for field assistance; J. Lima and S. Victor (UTA) and R. Jones and the UTA Genomics Core Facility for laboratory support; M. Matz (University of Texas at Austin) for the use of the fluorescent plate reader; and anonymous reviewers for comments that strengthened this manuscript.

FUNDING

The authors would like to acknowledge National Science Foundation award no. IOS-1017458 and UTA startup for funding to L.D.M. to support this project as well as a Natural Environment Research Council studentship to C.V.P.

REFERENCES

Adamo, S. A. (2004). How should behavioural ecologists interpret measurements of immunity? *Anim. Behav.* **68**, 1443-1449.
Armitage, S. A. O., Thompson, J. J. W., Rolff, J. and Siva-Jothy, M. T. (2003). Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. *J. Evolut. Biol.* **16**, 1038-1044.
Aronson, R., Bruckner, A., Moore, J., Precht, B. and Weil, E. (2008). *Montastraea faveolata*. In *IUCN Red List of Threatened Species*, Version 2011.1. Available at www.iucnredlist.org.
Asokan, R., Arumugam, M. and Mullainadhan, P. (1997). Activation of prophenoloxidase in the plasma and haemocytes of the marine mussel, *Perna viridis* Linnaeus. *Dev. Comp. Immunol.* **21**, 1-12.
Bidla, G., Hauling, T., Dushay, M. S. and Theopold, U. (2008). Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. *J. Innate. Immun.* **1**, 301-308.
Brandt, M. E. (2009). The effect of species and colony size on the bleaching response of reef-building corals in the Florida Keys during the 2005 mass bleaching event. *Coral Reefs* **28**, 911-924.
Brandt, M. E. and McManus, J. W. (2009). Disease incidence is related to bleaching extent in reef-building corals. *Ecology* **90**, 2859-2867.
Brown, B. E. and Bythell, J. C. (2005). Perspectives on mucus secretion in reef corals. *Mar. Ecol. Prog. Ser.* **296**, 291-309.
Bruno, J. F., Selig, E. R., Casey, K. S., Page, C. A., Willis, B. L., Harvell, C. D., Sweatman, H. and Melendy, A. M. (2007). Thermal stress and coral cover as drivers of coral disease outbreaks. *PLoS Biol.* **5**, 1-8.
Cerenius, L., Lee, B. L. and Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol.* **29**, 263-271.
Cornet, S., Biard, C. and Moret, Y. (2009). Variation in immune defence among populations of *Gammarus pulex* (Crustacea: Amphipoda). *Oecologia* **159**, 257-269.
Cróquer, A. and Weil, E. (2009a). Spatial variability in distribution and prevalence of Caribbean scleractinian coral and octocoral diseases. II. Genera-level analysis. *Dis. Aquat. Org.* **83**, 209-222.
Cróquer, A. and Weil, E. (2009b). Changes in Caribbean coral disease prevalence after the 2005 bleaching event. *Dis. Aquat. Org.* **87**, 33-43.
Dunn, S. (2009). Immunorecognition and immunoreceptors in the Cnidaria. *Invert. Survival. J.* **6**, 7-14.
Edmunds, P. J. (2010). Population biology of *Porites astreoides* and *Diploria strigosa* on a shallow Caribbean reef. *Mar. Ecol. Prog. Ser.* **418**, 87-104.
Edmunds, P. J. and Elahi, R. (2007). The demographics of a 15-year decline in cover of the Caribbean reef coral *Montastraea annularis*. *Ecol. Monogr.* **77**, 3-18.
Foley, D. A. and Cheng, T. C. (1977). Degranulation and other changes of Molluscan granulocytes associated with phagocytosis. *J. Invert. Pathol.* **29**, 321-325.
Ganz, T. (2003). The role of antimicrobial peptides in innate immunity. *Integr. Comp. Biol.* **43**, 300-304.
Gardella, D. J. and Edmunds, P. J. (2001). The effect of flow and morphology on boundary layers in the scleractinians *Dichocoenia stokesii* (Milne-Edwards and Haime) and *Stephanocoenia michilini* (Milne-Edwards and Haime). *J. Exp. Mar. Biol. Ecol.* **256**, 279-289.
Geffen, Y. and Rosenberg, E. (2005). Stress-induced rapid release of antibacterials by scleractinian corals. *Mar. Biol.* **146**, 931-935.
Geffen, Y., Ron, E. Z. and Rosenberg, E. (2009). Regulation of release of antibacterials from stressed scleractinian corals. *FEMS Microbiol. Lett.* **295**, 103-109.
Gochfeld, D. J. and Aeby, G. S. (2008). Antibacterial chemical defenses in Hawaiian corals provide possible protection from disease. *Mar. Ecol. Prog. Ser.* **362**, 119-128.
Green, D. H., Edmunds, P. J. and Carpenter, R. C. (2008). Increasing relative abundance of *Porites astreoides* on Caribbean reefs mediated by an overall decline in coral cover. *Mar. Ecol. Prog. Ser.* **359**, 1-10.
Halliwell, B. and Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine.* Oxford: Oxford University Press.
Harvell, C. D., Kim, K., Quirolo, C., Weir, J. and Smith, G. (2001). Coral bleaching and disease: contributors to 1998 mass mortality in *Briareum asbestinum* (Octocorallia, Gorgonacea). *Hydrobiologia* **460**, 97-104.
Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S. and Samual, M. D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158.
Harvell, C. D., Jordan, E., Merkel, S., Raymundo, L., Rosenberg, E., Smith, G., Weil, E. and Willis, B. L. (2007). Coral disease: environmental change and the balance between coral and microbes. *Oceanography* **20**, 58-81.
Harvell, C. D., Altizer, S., Cattadori, I. M., Harrington, L. and Weil, E. (2009). Climate change and wildlife diseases: when does the host matter the most? *Ecology* **90**, 912-920.
Hawley, D. M. and Altizer, S. M. (2011). Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Funct. Ecol.* **25**, 48-60.
Hoegh-Guldberg, O. (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshw. Res.* **50**, 839-866.
Kvennefors, E. C. E., Leggat, W., Hoegh-Guldberg, O., Degnan, B. M. and Barnes, A. C. (2008). An ancient and variable mannose-binding lectin from the coral *Acropora millepora* binds both pathogens and symbionts. *Dev. Comp. Immunol.* **32**, 1582-1592.
Lafferty, K. D. (2009). The ecology of climate change and infectious diseases. *Ecology* **90**, 888-900.
Lesser, M. P. (1997). Oxidative stress causes coral bleaching during exposure to elevated temperatures. *Coral Reefs* **16**, 187-192.
Lesser, M. P. (2006). Oxidative stress in marine environments: Biochemistry and physiological ecology. *Annu. Rev. Physiol.* **68**, 253-278.
Lesser, M. P., Bythell, J. C., Gates, R. D., Johnstone, R. W. and Hoegh-Guldberg, O. (2007). Are infectious diseases really killing corals? Alternative interpretations of the experimental and ecological data. *J. Exp. Mar. Biol. Ecol.* **346**, 36-44.
Li, N. X. and Karin, M. (2000). Signaling pathways leading to nuclear factor-kappa B activation. *Methods Enzymol.* **319**, 273-279.
Liu, L., Yang, J. L., Qiu, L. M., Wang, L. L., Zhang, H. A., Wang, M. Q., Vinu, S. S. and Song, L. S. (2011). A novel scavenger receptor-cysteine-rich (SRCR) domain containing scavenger receptor identified from mollusk mediated PAMP recognition and binding. *Devel. Comp. Immunol.* **35**, 227-239.
Martin, L. B., Hawley, D. M. and Ardia, D. R. (2011). An introduction to ecological immunology. *Funct. Ecol.* **25**, 1-4.
Meredith, P., Powell, B. J., Riesz, J., Nighswander-Rempel, S. P., Pederson, M. R. and Moore, E. G. (2006). Towards structure-property-function relationships for eumelanin. *Soft Matter* **2**, 37-44.
Miller, D. J., Hemmrich, G., Ball, E. E., Hayward, D. C., Khalturin, K., Funayama, N., Agata, K. and Bosch, T. C. G. (2007). The innate immune repertoire in Cnidaria-ancestral complexity and stochastic gene loss. *Genome Biol.* **8**, R59.
Miller, J., Waara, R., Muller, E. and Rogers, C. (2006). Coral bleaching and disease combine to cause extensive mortality on reefs of the US Virgin Islands. *Coral Reefs* **25**, 418.
Miller, J., Muller, E., Rogers, C., Waara, R., Atkinson, A., Whelan, K. R. T., Patterson, M. and Witcher, B. (2009). Coral disease following massive bleaching in 2005 causes 60% decline in coral cover on reefs in the US Virgin Islands. *Coral Reefs* **28**, 925-937.
Moret, Y. and Schmid-Hempel, P. (2000). Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**, 1166-1678.
Munoz-Munoz, J. L., García-Molina, F., Varón, R., Tudela, J., García-Cánovas, F. and Rodríguez-López, J. N. (2009). Generation of hydrogen peroxide in the melanin biosynthesis pathway. *Biochim. Biophys. Acta* **1794**, 1017-1029.
Mydlarz, L. D. and Harvell, C. D. (2007). Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. *Comp. Biochem. Physiol. A* **146**, 54-62.
Mydlarz, L. D. and Jacobs, R. S. (2004). Comparison of an inducible oxidative burst in free-living and symbiotic dinoflagellates reveals properties of the pseudopterosins. *Phytochem.* **65**, 3231-3241.
Mydlarz, L. D. and Jacobs, R. S. (2006). An inducible release of reactive oxygen radicals in four species of gorgonian corals. *Mar. Freshw. Behav. Physiol.* **39**, 143-152.
Mydlarz, L. D. and Palmer, C. V. (2011). The presence of multiple phenoloxidases in Caribbean reef-building corals. *Comp. Biochem. Physiol. A* **159**, 372-378.
Mydlarz, L. D., Jones, L. E. and Harvell, C. D. (2006). Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates. *Annu. Rev. Ecol. Evol. Syst.* **37**, 251-288.

Mydlarz, L. D., Holthouse, S. F., Peters, E. C. and Harvell, C. D. (2008). Celluar responses in sea fan corals: granular amoebocytes react to pathogen and climate stressors. *PloS ONE* **3**, e1811.
Mydlarz, L. D., Couch, C. S., Weil, E., Smith, G. and Harvell, C. D. (2009). Immune defenses of healthy, bleached and diseased *Montastraea faveolata* during a natural bleaching event. *Dis. Aquat. Org.* **87**, 67-78.
Mydlarz, L. D., McGinty, E. S. and Harvell, C. D. (2010). What are the physiological and immunological responses of coral to climate warming and disease? *J. Exp. Biol.* **213**, 934-945.
Nappi, A. J. (1973). Hemocytic changes associated with encapsulation and melanization of some insect parasites. *Exp. Parasitol.* **33**, 285-302.
Nappi, A. J. and Ottaviani, E. (2000). Cytotoxicity and cytotoxic molecules in invertebrates. *Bioessays* **22**, 469-480.
Oxenford, H., Roach, R., Brathwaite, A., Nurse, L., Goodridge, R., Hinds, F., Baldwin, K. and Finney, C. (2008). Quantitative observations of a major coral bleaching event in Barbados, southeastern Caribbean. *Clim. Change* **87**, 435-449.
Palmer, C. V., Mydlarz, L. D. and Willis, B. L. (2008). Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals. *Proc. R. Soc. Lond. B* **275**, 2687-2693.
Palmer, C. V., Roth, M. S. and Gates, R. D. (2009a). Red fluorescent protein responsible for pigmentation in trematode-infected *Porites compressa* tissues. *Biol. Bull.* **215**, 68-74.
Palmer, C. V., Modi, C. K. and Mydlarz, L. D. (2009b). Coral fluorescent proteins as antioxidants. *PLoS ONE* **4**, e7298.
Palmer, C. V., Bythell, J. C. and Willis, B. L. (2010). Immunity parameters of reef corals underpin bleaching and disease susceptibility. *Fed. Am. Soc. Exp. Biol.* **24**, 1935-1946.
Palmer, C. V., Bythell, J. C. and Willis, B. L. (2011a). A comparative study of phenoloxidase activity in diseased and bleached colonies of the coral *Acropora millepora*. *Dev. Comp. Immunol.* **35**, 1096-1099.
Palmer, C. V., Traylor-Knowles, N. G., Willis, B. L. and Bythell, J. C. (2011b). Corals use similar immune cells and wound-healing processes as those of higher organisms. *PLoS ONE*, **6**, e23992.
Ratcliffe, N. A., Brookman, J. L. and Rowley, A. F. (1991). Activation of the prophenoloxidase cascade and initiation of nodule formation in locusts by bacterial lipopolysaccharides. *Devel. Comp. Immunol.* **15**, 33-39.
Reed, K. C., Muller, E. M. and van Woesik, R. (2010). Coral immunology and resistance to disease. *Dis. Aquat. Org.* **90**, 85-92.
Ritchie, K. B. (2006). Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar. Ecol. Prog. Ser.* **322**, 1-14.
Sadd, B. M. and Schmid-Hempel, P. (2009). Principles of ecological immunology. *Evol. Appl.* **2**, 113-121.
Sadd, B. M. and Siva-Jothy, M. T. (2006). Self-harm caused by an insect's innate immunity. *Proc. R. Soc. Lond. B* **273**, 2571-2574.
Sato, Y., Bourne, D. G. and Willis, B. L. (2009). Dynamics of seasonal outbreaks of black band disease in an assemblage of *Montipora* species at Pelorus Island (Great Barrier Reef, Australia). *Proc. R. Soc. Lond. B* **276**, 2795-2803.
Shagin, D. A., Barsova, E. V., Yanushevich, Y. G., Fradkov, A. F., Lukyanov, K. A., Labas, Y. A., Ugalde, J. A., Meyers, A., Nunez, J. M., Widder, E. A. et al. (2004). GFP-like proteins as ubiquitous metazoan superfamily: evolution of functional features and structural complexity. *Mol. Biol. Evol.* **21**, 841-850.
Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321.
Silverman, N. and Maniatis, T. (2001). NF-kappa B signaling pathways in mammalian and insect innate immunity. *Genes Dev.* **15**, 2321-2342.
Slattery, M., McClintock, J. B. and Heine, J. N. (1995). Chemical defenses in antarctic soft corals-evidence for antifouling compounds. *J. Exp. Mar. Biol. Ecol.* **190**, 61-77.
Söderhäll, K. and Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* **10**, 23-28.
Somero, G. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol.* **213**, 912-920.
Stimson, J., Sakai, K. and Sembali, H. (2002). Interspecific comparison of the symbiotic relationship in corals with high and low rates of bleaching-induced mortality. *Coral Reefs* **21**, 409-421.
Vidal-Dupiol, J., Ladriere, O., Destoumieux-Garzon, D., Sautiere, P., Meistertzheim, A. L., Tambutte, E., Tambutte, S., Duval, D., Foure, L., Adjeroud, M. et al. (2011). Innate immune responses of a scleractinian coral to vibriosis. *J. Biol. Chem.* **286**, 22688-22698.
Voolstra, C. R., Sunagawa, S., Matz, M. V., Bayer, T., Aranda, M., Buschiazzo, E., DeSalvo, M. K., Lindquist, E., Szmant, A. M., Coffroth, M. A. et al. (2011). Rapid evolution of coral proteins responsible for interaction with the environment. *PLoS ONE* **6**, e20392.
Ward, J. R., Rypien, K. L., Bruno, J. F., Harvell, C. D., Jordan-Dahlgren, E., Mullen, K. M., Rodriguez-Martinez, R. E., Sanchez, J. and Smith, G. (2006). Coral diversity and disease in Mexico. *Dis. Aquat. Org.* **69**, 23-31.
Weil, E. and Cróquer, A. (2009). Spatial variability in distribution and prevalence of Caribbean scleractinian coral and octocoral diseases. I. Community-level analysis. *Dis. Aquat. Org.* **83**, 195-208.
Wild, C., Hoegh-Guldberg, O., Naumann, M. S., Colombo-Pallotta, M. F., Ateweberhan, M., Fitt, W. K., Iglesias-Prieto, R., Palmer, C., Bythell, J. C., Ortiz, J. C. et al. (2011). Climate change impedes scleractinian corals as primary reef ecosystem engineers. *Mar. Freshw. Res.* **62**, 205-215.
Willis, B. L., Page, C. A. and Dinsdale, E. A. (2004). Coral disease on the Great Barrier Reef. In *Coral Health and Disease* (ed. E. Rosenberg and Y. Loya), pp. 69-104. New York: Springer.
Wittwer, D., Weise, C., Gotz, P. and Wiesner, A. (1997). LPS (lipopolysaccharide)-activated immune responses in a hemocyte cell line from *Estigmene acraea* (Lepidoptera). *Devel. Comp. Immunol.* **21**, 323-336.
Xian, J. A., Wang, A. L., Tian, J. X., Huang, J. W., Ye, C. X., Wang, W. N. and Sun, R. Y. (2009). Morphologic, physiological and immunological changes of haemocytes from *Litopenaeus vannamei* treated by lipopolysaccharide. *Aquaculture* **298**, 139-145.
Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395.