

## Temporal patterns in innate immunity parameters in reef-building corals and linkages with local climatic conditions

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**Abstract.** Extremes in seasonal environmental conditions can significantly impact the health and physiological functioning of reef corals, underscoring the need for knowledge of seasonally specific baselines from which to monitor and forecast impending stress. Increases above summertime means in seawater temperature, sunlight intensity, turbidity, or sedimentation may reduce coral immunocompetency and increase disease and bleaching susceptibility. We analyzed temporal patterns in innate immunity parameters over nine time points throughout one year to establish baseline levels from which anomalies might be detected for representative species from three major reef-building coral families (Acroporidae, Faviidae, and Poritidae). Temporal patterns in both phenoloxidase activity and expression of green fluorescent protein-like proteins varied among the three families, as did overall constitutive levels. For example, *Porites cylindrica* had 2.8-fold higher yearly average levels of phenoloxidase activity than *Acropora millepora*, which had the lowest levels. In contrast, mean fluorescence was lowest in *Acropora millepora* and highest in *Echinopora mammiformis*. Relationships between the potential physical drivers (seasonal variation in seawater temperature, rainfall, salinity) and temporal patterns in these parameters also differed among the three species. For example, phenoloxidase activity was positively correlated with seawater temperature in *A. millepora*, but negatively correlated in both *E. mammiformis* and *P. cylindrica*. Distinctions in constitutive levels and temporal patterns in these parameters among species suggest that corals from these three families have evolved different strategies for investing resources into innate immune parameters. Such differences highlight the need for species-specific baselines and long-term assessments to accurately predict coral reef trajectories in rapidly changing environments.

**Key words:** chromoprotein; coral; fluorescence; green fluorescent protein-like proteins; immunity; indicator; phenoloxidase; prophenoloxidase; salinity; temperature.

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## INTRODUCTION

Global declines in coral reef ecosystem function is a critical concern, both for vertebrates and invertebrates dependent on reefs for food and shelter, and for the millions of people who draw extensively on them for food, coastal protection, tourism income, and cultural values (Burke et al. 2011). Increasing levels of environmental and anthropogenic pressures facing corals worldwide (Hughes et al. 2003) highlight the urgent need to establish reference baselines for coral physiological parameters that could be used as tools for evaluating, monitoring, and forecasting coral health to preempt and manage activities that might cause further declines. As a competent immune system is integral to maintaining an organism's health, parameters associated with the coral innate immune system, such as activity of the prophenoloxidase (proPO) system and expression of green fluorescent protein-like (GFP-like) proteins, have been suggested as potential indicators of stress based on their detectable responses to the variations in local environmental conditions (D'Angelo et al. 2008, Palmer et al. 2010, Roth and Deheyn 2013).

Both parameters vary in response to warm seawater temperature anomalies, which are known to significantly affect the health of the coral holobiont. In particular, warm temperature anomalies cause disruptions to the coral-*Symbiodinium* endosymbiosis leading to bleaching (Douglas 2003), as well as shifts in coral-associated microbial communities toward potential pathogenic microbes (Littman et al. 2010) and increases in the virulence of coral pathogens (Ben-Haim et al. 2003) that may be linked to increased coral disease prevalence in summer. Heavy monsoonal rainfall, which results in reduced salinity and increased terrestrial runoff leading to increased levels of turbidity and agricultural pollutants, is also a major stressor for corals. Warm seawater temperatures and changes in salinity and agricultural pollutants have all been shown to compromise the functioning of the innate immune systems of a range of marine invertebrates, thereby increasing disease susceptibility and mortality (Tseng and Chen 2004, Ellis et al. 2011, Lin et al. 2012). In addition, both low salinity (Kerswell and Jones 2003, Jones and Berkelmans 2014) and cold sea surface temperatures (Saxby

et al. 2003) have been implicated in coral bleaching. Extremes in seasonal environmental conditions can therefore significantly affect coral health, fitness, and disease susceptibility. Although targeted, short-term studies are beginning to explore how environmental stressors affect the proPO-activating system and GFP-like protein expression, an understanding of what constitutes seasonal baselines in healthy levels is needed to explore these parameters as potential indicators of immune-compromising environmental conditions.

The prophenoloxidase (proPO)-activating system is part of the melanin synthesis pathway and forms an important, highly conserved component of the innate immune system of invertebrates (Cerenius et al. 2010), including both gorgonian and scleractinian corals (Mydlarz et al. 2008, Palmer et al. 2008). In corals, higher levels of immune parameters associated with this system, particularly phenoloxidase activity and melanin content, have been linked to higher resistance to both bleaching and disease (Palmer et al. 2010). The proPO system is activated when components of microbial cell walls are detected, leading to the activation of a protease cascade that cleaves proPO into PO (Cerenius et al. 2010). In turn, PO oxidizes phenolic compounds into cytotoxic quinone intermediates, which non-enzymatically form melanin. Whereas melanin forms a barrier and immobilizes microbes, cytotoxic quinones and reactive oxygen species (ROS) formed eliminate invading organisms. In corals, melanin and PO have been found to play a significant role in wound healing (Palmer et al. 2011b, D'Angelo et al. 2012, van de Water et al. 2015a) and in anti-microbial (Mydlarz et al. 2008, Palmer et al. 2011a, van de Water et al. 2015b) and anti-parasite (Palmer et al. 2008, Burge et al. 2013) immune responses. Additionally, melanin deposits have been implicated in bleaching mitigation via photoprotection (Palmer et al. 2010). However, knowledge of baseline levels of PO activity characteristic of healthy corals and of the additional capacity for activation represented by the stored inactive proenzyme proPO is currently lacking. Studies of baseline levels of these parameters and how they vary seasonally are essential first steps in evaluating the natural variation in immune parameters of reef corals.

Expression of GFP-like proteins is thought to contribute significantly to the cellular-based stress responses of corals, ameliorating bleaching by absorbing and dissipating solar radiation that could otherwise trigger photoinhibition (Baird et al. 2009). In contrast to their photoprotective role at high light intensities (Salih et al. 2000, Dove et al. 2001), fluorescent proteins (FPs) may also augment light for photosynthesis at low light intensities (Kawaguti 1969, Schlichter et al. 1986), although this role appears minimal (Gilmore et al. 2003). Similarly, there is strong evidence that non-fluorescent chromoprotein (CP) has a photoprotective role (Salih et al. 2000, Dove et al. 2001) for *Symbiodinium* photosystems (Smith et al. 2013). Increased expression of GFP-like proteins has been found in areas of high tissue proliferation, such as at branch tips and in regenerating tissues during wound healing (D'Angelo et al. 2012, van de Water et al. 2015a), potentially protecting both coral and *Symbiodinium* cells from light stress in areas with low levels of *Symbiodinium* pigmentation. GFP-like proteins may also exhibit antioxidant properties (Bou-Abdallah et al. 2006, Palmer et al. 2009a) by mitigating the effects of ROS produced by defective *Symbiodinium* photosystems (Lesser 1996), and via the proPO-activating system and the oxidative burst as part of an innate immune response (Palmer et al. 2008, 2009b, D'Angelo et al. 2012, van de Water et al. 2015a).

Coral families differ in their susceptibilities to disease (Willis et al. 2004, Raymundo et al. 2005, Lamb and Willis 2011) and bleaching (Marshall and Baird 2000, Loya et al. 2001); thus, evidence that taxonomic (family-level) trends in disease susceptibility are inversely correlated with constitutive levels of PO activity (Palmer et al. 2010) underscores the need to examine baseline levels that are at least family- or preferably species-specific. On the Great Barrier Reef (GBR), coral species in the family Acroporidae are among the most vulnerable to coral diseases, whereas species in the family Poritidae are among the most resistant; species in the family Faviidae tend to be intermediate in their susceptibility to disease (Willis et al. 2004, Palmer et al. 2010). Similarly, there is a hierarchy in bleaching susceptibility among coral taxa, with massive taxa (e.g., Poritidae) being more resistant than fast-growing branching taxa (e.g., Acroporidae)

(Marshall and Baird 2000, Loya et al. 2001). However, within-species susceptibility can be significantly influenced by various other physiological and environmental factors, such as thermal history (Guest et al. 2012, Howells et al. 2013), *Symbiodinium* endosymbiont composition (Howells et al. 2012) and density (Cunning and Baker 2013). Such variations in bleaching and disease susceptibility highlight the need to investigate temporal patterns in baseline levels of parameters that may influence the stress susceptibility among coral families.

In this study, we investigated the seasonal patterns in activity of the proPO system and the expression levels of GFP-like proteins to establish the baseline levels for a common species in each of three major reef-building coral families. Species selected span the range from resistant to highly susceptible to bleaching and disease. Seasonal patterns in sea temperature, cloud cover, rainfall, and salinity were documented over the corresponding year-long period to identify the potential links between the variation in environmental factors and the immune parameters investigated. For example, we evaluated whether corals upregulate GFP-like proteins and the proPO system during summer months, potentially to cope with the increased environmental stressors that can lead to bleaching and disease.

## MATERIALS AND METHODS

### *Field sampling design and sample collection*

This study was conducted in Pioneer Bay (coordinates: 18°36'24.9114" S, 146°29'20.205" E), situated on the sheltered, western side of Orpheus Island within the Great Barrier Reef Marine Park. Samples of corals in the families Acroporidae (*Acropora millepora*—red color morph), Faviidae (*Echinopora mammiformis*), and Poritidae (*Porites cylindrica*) were collected from similar-sized tagged colonies over the course of one year, from October 2009 to September 2010 (Fig. 1). Numbered plastic cattle tags and cable ties were used to tag 10 colonies of *E. mammiformis* located at 2–4 m depth and eight colonies of each of *A. millepora* (2–3 m depth) and *P. cylindrica* (4–5 m depth). Colonies were sampled and photographed at nine time points: October 2009 (late austral spring); December 2009 and January, February, and March



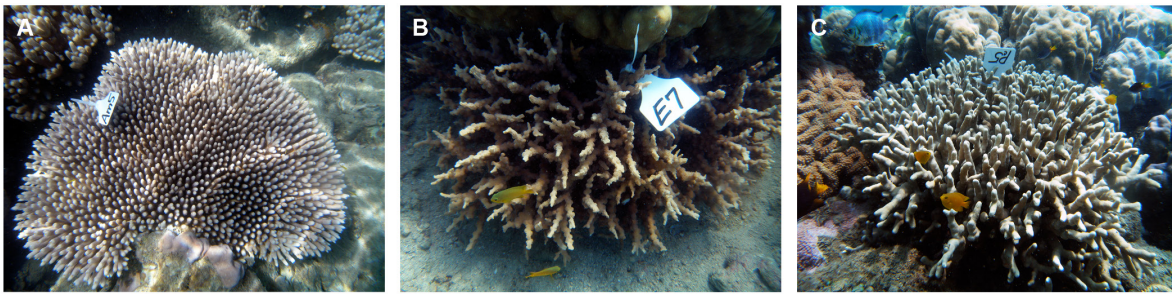


Fig. 1. Study species from three important reef-building coral families. (A) *Acropora millepora* (Acroporidae), (B) *Echinopora mammiformis* (Faviidae), and (C) *Porites cylindrica* (Poritidae).

of 2010 (austral summer); May, July, and August of 2010 (austral winter); and September 2010 (early austral spring). At each time point, one branch (approximately 5 cm in length) was sampled from the middle of each tagged colony using surgical bone cutters, placed in a plastic bag underwater, transferred to a cryogenic tube and snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until processed.

Although sampling generates lesions and elicits an immune response in the coral through the increased activity of the proPO system and GFP-like protein expression, this response is localized to the lesion area (Palmer et al. 2011b, D'Angelo et al. 2012, van de Water et al. 2015a). Sealing of lesions and recovery of tissue structures is accomplished within  $\sim 48$  h (Palmer et al. 2011b). Given the localized response and the sufficient recovery time between sampling time points, our sampling regime is not expected to have had an effect on the immune parameters analyzed here in other locations within the coral colonies, and to have influenced our results.

#### Sample preparation

To prepare the tissue lysates, frozen fragments were thawed on ice and approximately  $4\text{ cm}^2$  of coral tissue was removed from the fragment using an airbrush into 10 mL of ice-cold extraction buffer (50 mM Tris-HCl, pH 7.8, with 50 mM dithiothreitol). Each tissue sample was homogenized for 45 s (IKA T10 Basic homogenizer, IKA-Werke GmbH & Co. KG, Staufen, Germany). The resulting slurry was centrifuged at 3500 rpm for 5 min to remove the cell debris and *Symbiodinium*, and the supernatant was collected and stored at  $-30^{\circ}\text{C}$ . Total protein content of each

sample was determined using the DC Protein Assay kit (Bio-Rad, Hercules, California, USA) following the manufacturer's instructions, and the endpoint absorbance was read using a SpectraMax M2 (Molecular Devices, Sunnyvale, California, USA).

#### Phenoloxidase activity

Phenoloxidase activity was assayed according to the procedures outlined in Palmer et al. (2011a), with some modifications. Both total potential (trypsin-activated) phenoloxidase (tpPO) activity (as defined in van de Water et al. 2015a) and active phenoloxidase (PO) activity were measured to analyze the total capacity and the active fraction of the proPO system, respectively, in each sample. To analyze tpPO activity, 20  $\mu\text{L}$  of coral tissue lysate was loaded in triplicate into wells of a 96-well plate, to which Tris-buffered saline (50 mM, pH 7.8; 40  $\mu\text{L}$ ) and trypsin (25  $\mu\text{L}$  0.1 mg/mL) were added. Reaction mixtures were incubated for 20 min to allow for activation of prophenoloxidase by trypsin, and then, 30  $\mu\text{L}$  of 10 mM dopamine hydrochloride (Sigma-Aldrich, St. Louis, Missouri, USA) was added to each mixture. As a blank, 20  $\mu\text{L}$  of extraction buffer was used. The same procedure was followed to analyze PO activity, except that 25  $\mu\text{L}$  double-distilled water was substituted for the trypsin solution. Absorbance was measured at 490 nm every 5 min for 45 min using the SpectraMax M2 (Molecular Devices). Data for each sample were independently obtained in triplicate. Phenoloxidase activity was calculated as the change in absorbance using the linear portion of the reaction curve over time and standardized to the total protein content of each sample.

### Chromoprotein and fluorescent protein expression

Twenty microliters of tissue lysate was added to each well of a black, clear bottom 384-well plate in triplicate for each sample. Expression of chromoprotein was analyzed by measuring the absorbance at 588 nm using a SpectraMax M2. The fluorescence spectrum was analyzed by measuring the emission wavelengths between 400 and 700 nm, with a 5-nm resolution, emitted upon the excitation of FPs at 280 nm. The 280-nm excitation wavelength was used, as it is optimal for the excitation of FPs across the spectrum and is sufficiently distant from the cyan fluorescent protein (CFP) emission peak to prevent interference with its excitation and emission (Shagin et al. 2004). All data were normalized to the total protein content. Fluorescence spectra and FP expression levels were calculated as described in van de Water et al. (2015a). In summary, the exponentially decaying background scatter was subtracted from each spectrum between 445 and 645 nm and multiple regression models based on the purified FP spectra were fitted to the data to calculate the proportions of the individual FPs (cyan, green, and red fluorescent protein) present using the formula  $B = F450/\exp(k \times (w - 450))$ , where F450 is the fluorescence reading at 450 nm (no FP emission),  $w$  is wavelength in nanometers, and  $k$  is an empirically determined coefficient that provides a good fit of the model. Parameter  $k$  was set at 0.1, 0.12, and 0.25 for *A. millepora*, *E. mammiformis*, and *P. cylindrica*, respectively. The relative proportions of FPs were multiplied by the coefficient reflecting the relative excitability of purified *A. millepora* FPs at 280 nm (for *A. millepora*: CFP: 0.85, GFP: 0.92, RFP: 1; for *E. mammiformis* and *P. cylindrica*: CFP: 1, GFP: 1, RFP: 1). Samples where the  $R^2$  of the model fit was less than 0.9 were excluded from the analysis.

### Environmental parameters

Daily environmental data (seawater temperature, rainfall, and salinity) were obtained at a depth of 3 m from the Australian Institute of Marine Science Orpheus Island Platform and Sensor Float 1 (Integrated Marine Observing System [IMOS]) located in Pioneer Bay (see <http://www.aims.gov.au>). Daily cloud fraction data (Level 2, MOD06) were acquired from the Moderate Resolution Imaging

Spectroradiometer (MODIS) on board the Terra satellite (downloaded via [ladsweb.nascom.nasa.gov](http://ladsweb.nascom.nasa.gov); algorithm description in King et al. 1997). As cloud cover is variable on local scales and MODIS data result from a single snapshot (or, at most, two snapshots) around 10:30 local time, cloud fraction conditions across each daytime period were inferred by considering the data from pixels in the vicinity of the study site. The radial extent of cloud influence was estimated using local wind information. Near-surface wind speeds in the IMOS data during the study period had a mean value of 19.8 km/h, which for an 8-h daytime period corresponds to a distance of 158.4 km. However, upper-level wind speeds are typically greater than those near the surface, plus the geometry of sun angle at the location and wind direction through each day confer the additional variability affecting spatial values to be included in the daily cloud fraction estimate. Accordingly, the distance was reduced (by half) to 79.2 km, which is approximately 0.7 arc degrees, and cloud fraction values within a radius of 0.35 arc degrees of Pioneer Bay were averaged for each day. All environmental parameter data are displayed in Appendix S1: Fig. S1.

### Statistical analysis

Differences in immune parameters between the yearly average and sampling times were analyzed using a linear mixed-effects (LME) model. Pairwise comparisons on the temporal patterns between the consecutive months were made using LME models, and the Bonferroni correction was applied to obtain the critical  $P$ -value based on  $\alpha = 0.05$ . Immune parameters were added to the model as dependent fixed factors and sampling time and/or species as independent fixed factors. In addition, colony was included as a random factor to account for the repeated sampling of the same individual colony. A  $t$ -test was used to test for the differences in the fluorescence levels between summer and winter for each species. Correlations between immune and environmental parameters (average of 10 d prior to sampling) were analyzed using Pearson's  $r$ . All analyses were conducted using the statistical software package S-PLUS 8.0. Differences were considered significant when  $P < 0.05$ , or  $P < 0.05/n$ , in the case of

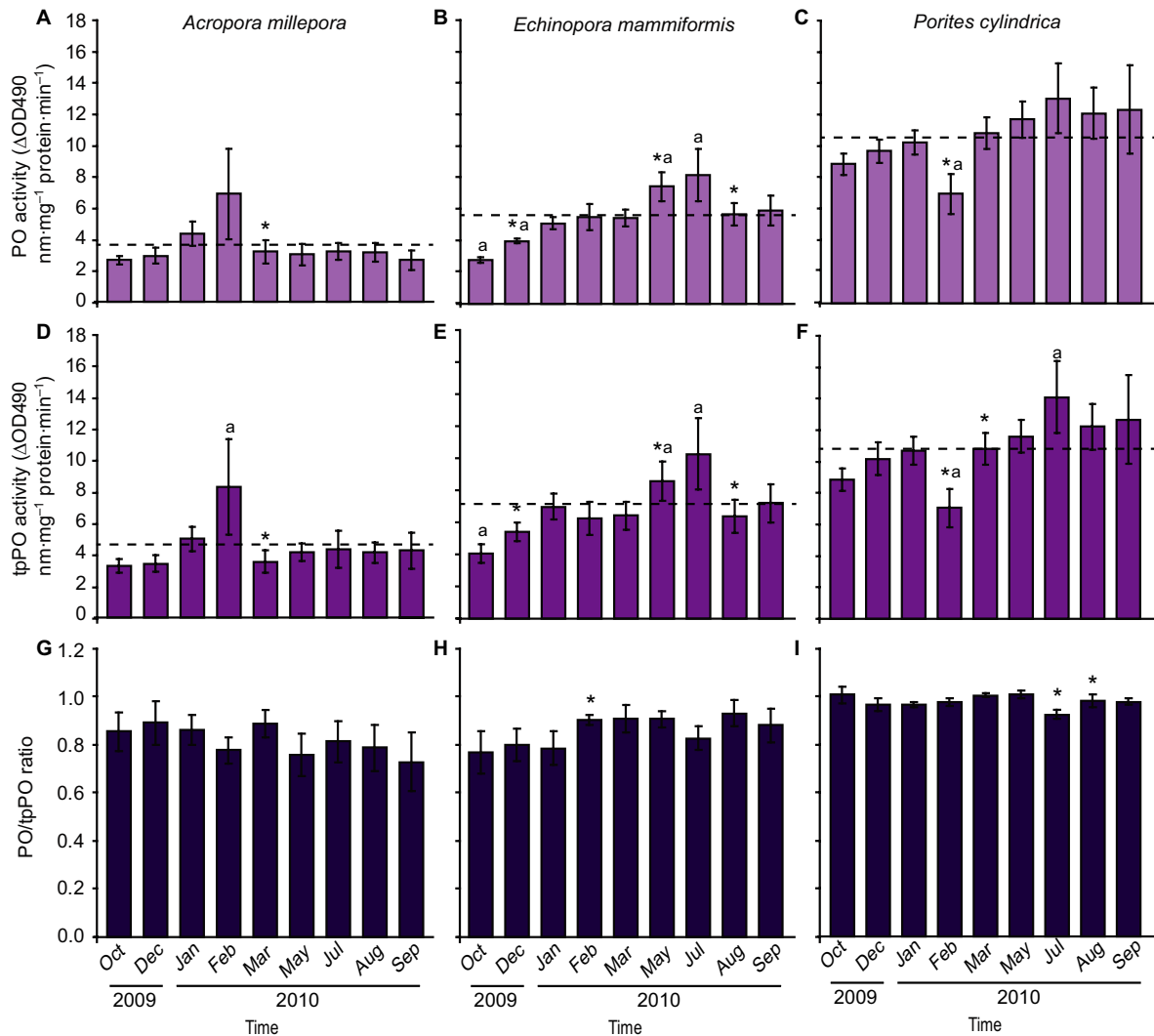


Fig. 2. Seasonal patterns in the proPO system. Temporal patterns in (A–C) active phenoloxidase (PO) activity; (D–F) the total potential phenoloxidase (tpPO) activity; and (G–I) ratio of PO activity and tpPO activity. Asterisk (\*) indicates the statistically significant difference ( $P < 0.05/n$ ) with previous time point. Letter “a” indicates the statistically significant difference ( $P < 0.05$ ) from the yearly average.

Bonferroni-adjusted  $P$ -values, with  $n$  being the number of pairwise comparisons.

## RESULTS

### Phenoloxidase activity

On average, mean constitutive levels of the two parameters associated with the proPO-activating system differed among the three coral species over the one-year duration of the study. The yearly average of active phenoloxidase (PO)

activity (measured as  $\Delta\text{OD } 490 \text{ nm}$  per mg protein per min) was more than twofold greater in *Porites cylindrica* ( $10.70 \pm 0.54$ ) than in *Acropora millepora* ( $3.73 \pm 0.48$ ), while *Echinopora mammiformis* ( $5.62 \pm 0.31$ ) had intermediate levels of PO activity (dashed lines in Fig. 2A–C). A similar pattern was found for yearly averages of the total potential phenoloxidase (tpPO) activity, which was more than twofold greater in *P. cylindrica* ( $10.89 \pm 0.55$ ) than in *A. millepora* ( $4.65 \pm 0.53$ ), and again intermediate in *E. mammiformis* ( $6.88 \pm 0.41$ )

(Fig. 2D–F). A strong correlation between tpPO activity (measured as  $\Delta OD$  490 nm per mg protein per min) and PO activity was detected for all three species (*A. millepora*:  $r = 0.95$ ; *P. cylindrica*:  $r = 0.99$ ; and *E. mammiformis*:  $r = 0.91$ ). The average percentage of tpPO activity present in the active form (PO) ranged from  $81.7\% \pm 2.6\%$  in *A. millepora*, to  $86.0\% \pm 1.9\%$  in *E. mammiformis* and up to  $98.0\% \pm 0.7\%$  in *P. cylindrica* (Fig. 2G–I), indicating that the majority of tpPO is present in its active state in all three species. Patterns in the ratios of PO to tpPO activities among species indicate that *A. millepora* stores the greatest proportion of its total potential phenoloxidase activity in the inactive proPO zymogen form and *P. cylindrica* the least.

Seasonal patterns in phenoloxidase activity also tended to differ among the three species. In *A. millepora*, both PO and tpPO activity remained constant for most of the year, except for an increase between the summer months of December and February (levels 2.3-fold higher in February than in March; PO  $P = 0.06$ ; tpPO  $P = 0.04$ ), which resulted in 1.8-fold higher activity levels in February compared with the yearly average (PO  $P = 0.06$ ; tpPO  $P = 0.04$ ) (Fig. 2A, D). *P. cylindrica* also displayed a generally stable level of phenoloxidase activity throughout the year, but for this species, both PO activity ( $P = 0.01$ ) and tpPO activity ( $P = 0.01$ ) were significantly reduced in February compared with January and March (Fig. 2C, F). Concomitantly, the highest levels occurred in winter for *P. cylindrica* (Fig. 2C, F). The most variable pattern of phenoloxidase activity was observed for *E. mammiformis* (Fig. 2B, E). Compared with the yearly average, PO activity was reduced in the late spring (October,  $P < 0.01$ ) and early summer months (December,  $P = 0.02$ ) and the activity of tpPO was reduced in October ( $P < 0.01$ ). Conversely, the activity was significantly higher than the yearly average in the early winter months of May (PO  $P = 0.01$ ; tpPO  $P = 0.03$ ) and July (PO  $P < 0.01$ ; tpPO  $P < 0.01$ ).

#### Green fluorescent protein-like proteins

Comparisons of the mean fluorescence spectra between summer and winter months revealed no major shifts in emission wavelength peaks in any of the three species (Fig. 3A–C). However, the overall mean levels of fluorescence were higher in summer (December–March) than in

winter (July–September) for *E. mammiformis* ( $P < 0.001$ ). In contrast, the overall mean levels of fluorescence did not differ significantly between summer and winter months for either *A. millepora* ( $P = 0.972$ ) or *P. cylindrica* ( $P = 0.195$ ) (Fig. 3A–C), although the peaks in total fluorescence in both the cyan FP and green FP regions were higher in summer for both species. This was particularly the case for *P. cylindrica*, which had a 1.5-fold higher peak in the cyan FP region (470 nm peak) in summer than in winter. More detailed analyses of temporal patterns in the total fluorescence at the level of month also differed among species (Fig. 3D–F). Overall, the mean total fluorescence throughout the year was 1.6-fold higher in *E. mammiformis* than in *A. millepora*, with mean levels intermediate in *P. cylindrica* (dashed lines in Fig. 3D–F). Total fluorescence levels in *A. millepora* were comparatively stable throughout the year (mean  $1264 \pm 158$  RFU/ $\mu$ g protein) (Fig. 3D), whereas they fluctuated significantly in the other two species (*E. mammiformis*,  $2024 \pm 708$  RFU/ $\mu$ g protein; and *P. cylindrica*,  $1760 \pm 426$  RFU/ $\mu$ g protein). Fluctuations in total fluorescence were particularly pronounced in summer months in *E. mammiformis*, reflecting the 8.1- to 13.5-fold reduction in February compared with the other summer months, and in both summer and winter months in *P. cylindrica* (Fig. 3E, F).

Yearly averages of chromoprotein (CP) expression tended to be the inverse of patterns found for total fluorescence, with mean levels more than twofold higher in *P. cylindrica* ( $0.81 \pm 0.08$ ) than in *E. mammiformis* ( $0.28 \pm 0.03$ ); mean levels were intermediate in *A. millepora* ( $0.58 \pm 0.08$ ) (Fig. 3G–I). Throughout the year, however, CP expression fluctuated significantly in all species. Temporal patterns in CP expression were roughly similar for all three species, with CP expression being upregulated by the greatest amount in January and May and consistently downregulated in July (Fig. 3G–I).

Similar to the patterns in total fluorescence, levels of individual FPs, that is, cyan, green, and red fluorescent proteins (CFP, GFP, and RFP, respectively), expressed as a proportion of total fluorescence, did not differ significantly from the yearly average in *A. millepora*, in any of the 9 months (Fig. 4A, D, G). Proportional levels of each FP were also relatively stable in *P. cylindrica*,



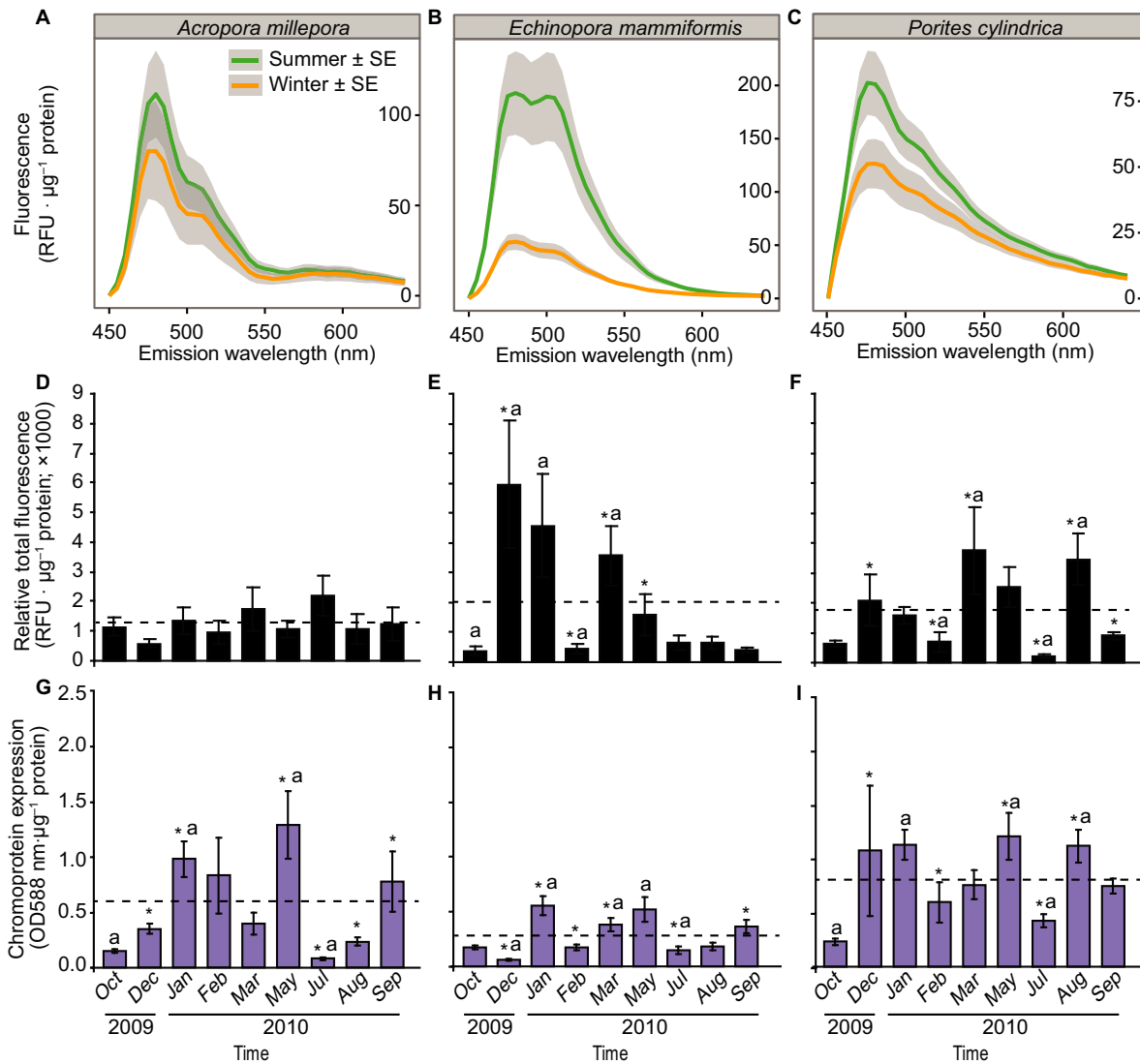


Fig. 3. Seasonal patterns in coral fluorescence. (A–C) Fluorescence spectra in summer and winter; shaded area indicates the SE of the mean (SEM); (D–F) total fluorescence levels; and (G–I) chromoprotein levels. Asterisk (\*) indicates the statistically significant difference ( $P < 0.05/n$ ) with previous time point. Letter “a” indicates the statistically significant difference ( $P < 0.05$ ) from the yearly average. RFU = relative fluorescence units; OD588nm = optical density at 588-nm wavelength.

with the exception of levels recorded in March, when the contribution of CFP to the total fluorescence was significantly increased and, concomitantly, the relative contribution of GFP and RFP was decreased (Fig. 4C, F, I). In contrast, individual FP proportions in *E. mammiformis* fluctuated significantly over time and around the yearly average, with no evident association with season (Fig. 4B, E, H).

#### Correlations between biological and environmental parameters

Based on the monthly average rainfall and sea-water temperatures from the five years prior to our study (Appendix S1: Fig. S2) as a reference, no anomalies in these two environmental parameters were observed during our sampling period (Appendix S1: Fig. S1). Between 2004 and 2009, the hottest monthly average temperature was



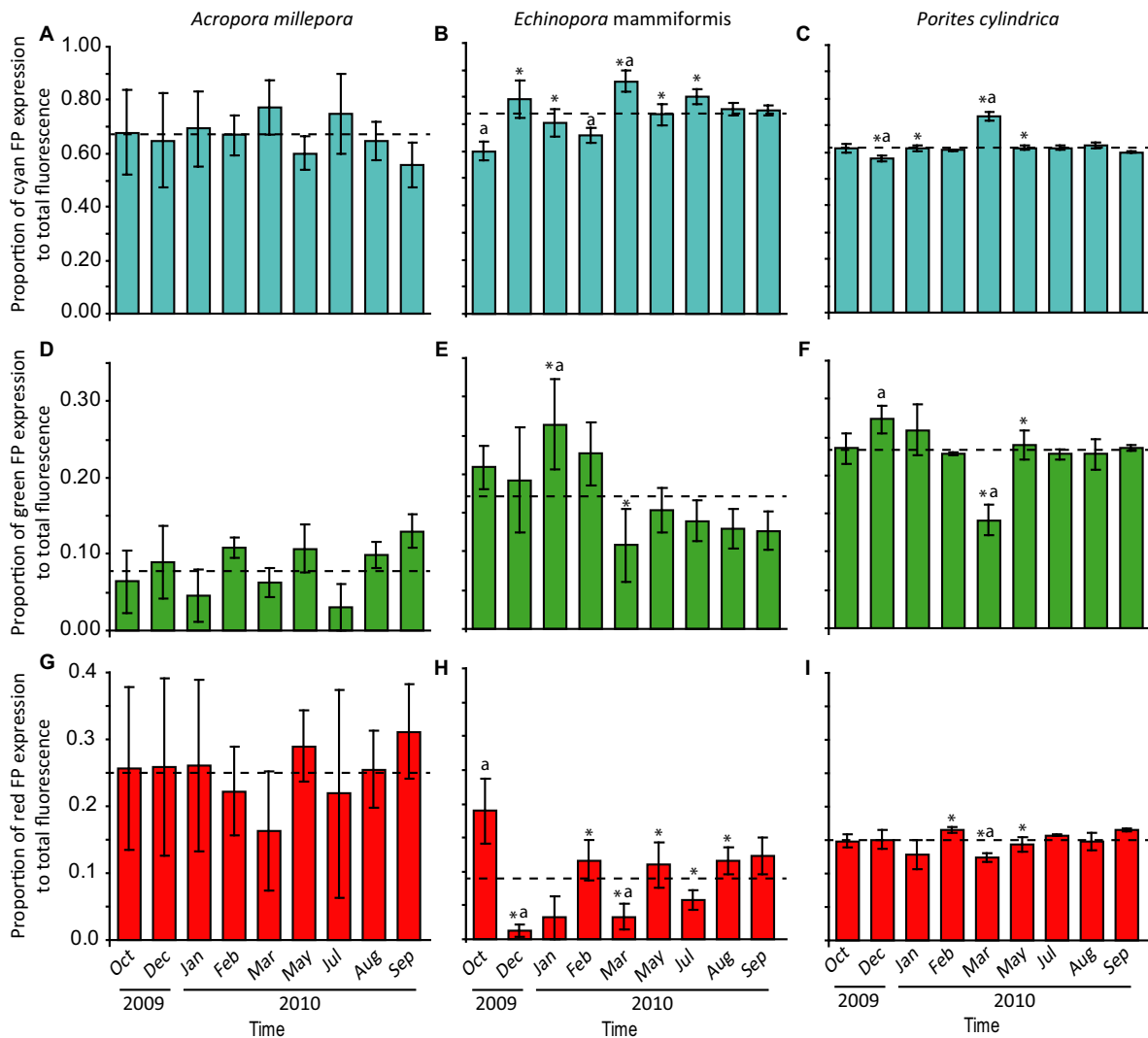


Fig. 4. Seasonal patterns in the proportion of individual fluorescent proteins (FP) to the total fluorescence. (A–C) cyan fluorescent protein; (D–F) green fluorescent protein; and (G–I) red fluorescent protein. An asterisk (\*) indicates the statistically significant difference ( $P < 0.05/n$ ) with previous time point. Letter “a” indicates the statistically significant difference ( $P < 0.05$ ) from the yearly average.

$29.2^{\circ} \pm 0.2^{\circ}\text{C}$  (mean  $\pm$  SEM), while the highest temperature recorded in our study was  $29.4^{\circ}\text{C}$ . Similarly, no anomalies in rainfall were detected, with a peak of 948 mm in January 2010, compared with peaks of 1068 and 1007 mm in October 2005 and January 2009, respectively ( $744 \pm 134$  mm (mean  $\pm$  SEM) rainfall in the wettest month in previous years).

Levels of PO activity were significantly correlated with temperature in all three coral species (Table 1). However, whereas PO activity was

positively correlated with temperature in *A. millepora*, it was negatively correlated with temperature in *E. mammiformis* and *P. cylindrica*. In *A. millepora*, PO and tpPO activities were also positively correlated with rainfall, but in contrast, in *P. cylindrica*, both parameters showed a negative correlation with rainfall (Table 1). No significant correlations between rainfall and any of the two biochemical parameters were observed in *E. mammiformis*.

Chromoprotein expression was correlated with one environmental parameter in one species;

Table 1. Correlations (Pearson's  $r$ ) between immune parameters (PO activity and GFP-like proteins) and environmental factors (temperature, rainfall, salinity, and cloud cover).

Species	Temperature	Rainfall	Salinity	Cloud cover
<b>PO activity</b>				
<i>Acropora millepora</i>	<b>0.26</b> ( $P = 0.049$ )*	<b>0.34</b> ( $P = 0.012$ )*	-0.18 ( $P = 0.240$ )	0.24 ( $P = 0.068$ )
<i>Echinopora mammiformis</i>	<b>-0.25</b> ( $P = 0.022$ )*	-0.01 ( $P = 0.949$ )	-0.01 ( $P = 0.947$ )	0.15 ( $P = 0.160$ )
<i>Porites cylindrica</i>	<b>-0.34</b> ( $P = 0.005$ )*	<b>-0.34</b> ( $P = 0.009$ )*	0.25 ( $P = 0.106$ )	-0.13 ( $P = 0.298$ )
<b>Total potential PO activity</b>				
<i>Acropora millepora</i>	0.21 ( $P = 0.1065$ )	<b>0.35</b> ( $P = 0.009$ )*	-0.19 ( $P = 0.249$ )	0.24 ( $P = 0.067$ )
<i>Echinopora mammiformis</i>	-0.20 ( $P = 0.068$ )	-0.06 ( $P = 0.639$ )	0.07 ( $P = 0.614$ )	0.11 ( $P = 0.315$ )
<i>Porites cylindrica</i>	<b>-0.36</b> ( $P = 0.003$ )*	<b>-0.35</b> ( $P = 0.006$ )*	0.29 ( $P = 0.055$ )	-0.13 ( $P = 0.314$ )
<b>Chromoprotein expression</b>				
<i>Acropora millepora</i>	<b>0.26</b> ( $P = 0.046$ )*	0.22 ( $P = 0.113$ )	-0.11 ( $P = 0.130$ )	0.13 ( $P = 0.308$ )
<i>Echinopora mammiformis</i>	0.10 ( $P = 0.343$ )	-0.001 ( $P = 0.989$ )	-0.12 ( $P = 0.391$ )	0.08 ( $P = 0.461$ )
<i>Porites cylindrica</i>	0.03 ( $P = 0.815$ )	-0.12 ( $P = 0.375$ )	0.22 ( $P = 0.161$ )	-0.19 ( $P = 0.112$ )
<b>Total fluorescence</b>				
<i>Acropora millepora</i>	-0.09 ( $P = 0.532$ )	0.01 ( $P = 0.975$ )	-0.10 ( $P = 0.591$ )	0.16 ( $P = 0.255$ )
<i>Echinopora mammiformis</i>	<b>0.30</b> ( $P = 0.009$ )*	0.05 ( $P = 0.707$ )	0.06 ( $P = 0.685$ )	-0.04 ( $P = 0.737$ )
<i>Porites cylindrica</i>	-0.03 ( $P = 0.781$ )	-0.09 ( $P = 0.513$ )	-0.08 ( $P = 0.615$ )	-0.10 ( $P = 0.427$ )
<b>CFP proportion</b>				
<i>Acropora millepora</i>	0.07 ( $P = 0.625$ )	0.05 ( $P = 0.755$ )	-0.09 ( $P = 0.573$ )	0.10 ( $P = 0.471$ )
<i>Echinopora mammiformis</i>	-0.16 ( $P = 0.181$ )	-0.04 ( $P = 0.745$ )	-0.06 ( $P = 0.684$ )	-0.12 ( $P = 0.295$ )
<i>Porites cylindrica</i>	0.05 ( $P = 0.714$ )	0.18 ( $P = 0.177$ )	<b>-0.57</b> ( $P = 0.0001$ )*	<b>0.39</b> ( $P = 0.001$ )*
<b>GFP proportion</b>				
<i>Acropora millepora</i>	-0.06 ( $P = 0.653$ )	0.09 ( $P = 0.539$ )	-0.08 ( $P = 0.670$ )	-0.03 ( $P = 0.827$ )
<i>Echinopora mammiformis</i>	<b>0.29</b> ( $P = 0.012$ )*	0.06 ( $P = 0.616$ )	0.10 ( $P = 0.519$ )	-0.01 ( $P = 0.911$ )
<i>Porites cylindrica</i>	0.03 ( $P = 0.808$ )	-0.20 ( $P = 0.122$ )	<b>0.52</b> ( $P = 0.0003$ )*	<b>-0.36</b> ( $P = 0.003$ )*
<b>RFP proportion</b>				
<i>Acropora millepora</i>	-0.06 ( $P = 0.652$ )	-0.08 ( $P = 0.582$ )	0.14 ( $P = 0.444$ )	-0.11 ( $P = 0.428$ )
<i>Echinopora mammiformis</i>	-0.15 ( $P = 0.203$ )	-0.02 ( $P = 0.861$ )	-0.07 ( $P = 0.645$ )	-0.15 ( $P = 0.209$ )
<i>Porites cylindrica</i>	-0.13 ( $P = 0.283$ )	0.09 ( $P = 0.504$ )	0.01 ( $P = 0.959$ )	0.03 ( $P = 0.834$ )

Note: Significant correlations ( $P < 0.05$ ) are indicated with an asterisk (\*) and appear in boldface.

that is, CP was positively correlated with temperature in *A. millepora* (Table 1). Interestingly, a strong correlation was observed between CP and both PO ( $r = 0.72$ ,  $P < 0.01$ ) and tpPO ( $r = 0.67$ ,  $P < 0.01$ ) in *A. millepora*, but not in the other two species tested. With the exception of the correlation with temperature in *E. mammiformis*, total fluorescence did not correlate with any of the environmental parameters in any of the species examined (Table 1). In addition, we did not observe any correlation between rainfall and GFP-like protein expression (Table 1). The proportion of GFP showed a positive correlation with temperature in *E. mammiformis* (Table 1). In contrast, GFP was negatively correlated with cloud cover in *P. cylindrica*, whereas CFP was positively correlated with this parameter in this species (Table 1). Our findings also show that there is a strong positive correlation between salinity and the proportion of GFP in *P. cylindrica* and a strong

negative correlation with the proportion of CFP in this species (Table 1). An overview of the environmental parameters assessed can be found in Appendix S1: Fig. S1.

## DISCUSSION

### Seasonal patterns in the prophenoloxidase system

In this study, we show that there is high variability in mean levels of both activated and total potential PO activity over a year, and this occurs both within and among corals from three common reef-building families. The typically more than twofold higher levels of PO activity found in *Porites cylindrica*, a disease- and bleaching-resistant species, compared with the more susceptible *Acropora millepora*, suggest a significantly higher level of resource investment in innate immunity by *P. cylindrica*, which likely contributes to its greater stress resistance. With

one exception, year-long consistency in comparative levels of PO activity among the three species verifies and extends the taxonomic patterns in innate immunity reported by Palmer et al. (2010), which were based on samples collected at one point in time. In February, however, the pattern of typically greater PO activity in *P. cylindrica* than in *A. millepora* broke down. Upregulated PO activity above typically low constitutive levels in *A. millepora* and downregulated PO activity below typically high constitutive levels in *P. cylindrica* highlight the species-specific differences in investment in this immune parameter in response to summer environmental parameters.

In addition to a significant temporal variation in both active and total potential PO activity, the seasonal timing of peaks in these parameters differed among the three coral species. Peaks in PO and tpPO activities in *A. millepora* occurred during summer months, which is consistent with a report by van de Water et al. (2015b), and in combination with a positive correlation between PO activity and seawater temperature, this timing suggests that upregulation of these immune parameters could minimize stress that could lead to bleaching in this species, potentially by the antioxidant and UV-filtering mechanisms of the melanin produced (Meredith et al. 2006, Brenner and Hearing 2008). Increased PO activity in bleached colonies of *A. millepora* observed in an earlier study (Palmer et al. 2011a) and the increased numbers of melanin-containing granular amoebocytes in gorgonians during a bleaching event (Mydlarz et al. 2008) corroborate this hypothesis. Such upregulation could counteract changes in coral-associated bacterial communities induced by heat stress or provide photoprotection for coral tissues and symbionts (Palmer et al. 2010). In contrast, peaks in baseline levels of PO activity were found in winter months in both *P. cylindrica* and *E. mammiformis*, showing a negative correlation between this parameter and seawater temperature. However, the higher constitutive PO levels throughout the summer in these two species compared with *A. millepora* may have been sufficient to cope with summer levels of environmental parameters in the absence of seasonal anomalies. Potential differences in the allocation of energy resources to other components of the innate immune system by each coral

species may also explain the observed disparity among species. Variability in the temporal patterns in PO and tpPO activities within and among coral species highlights the importance of long-term baselines and the need for multiple assays to assess coral health, as outcomes are dependent on the species sampled and the seasonal timing of the sampling.

#### *Seasonal patterns in GFP-like protein expression*

Comparisons of the temporal patterns in GFP-like protein expression among corals revealed that both the total fluorescence and individual FP expression levels were also variable within and among coral species over time. In *A. millepora*, comparative stability in FP expression through time and the lack of correlation with normal seasonal variation for any of the four environmental parameters examined indicate that the constitutive levels of FP expression may be sufficient to fulfill the potential stress reduction function(s) in this coral species in the absence of seasonal anomalies. In contrast, 6.4-fold higher total fluorescence levels in summer compared with winter in *E. mammiformis* and a positive correlation with seawater temperatures highlight that coral species differ markedly in their strategies for investing in constitutive FP levels. Increasing investment in GFPs with increasing seawater temperature suggests that GFP may play a role in the heat stress response in *E. mammiformis*. The highly variable levels of total fluorescence in *P. cylindrica* over the year were not correlated with any of the four environmental parameters examined. Although total fluorescence was not correlated with salinity in *P. cylindrica*, increases in the proportion of CFP as salinity decreased and the concomitant decreases in GFP raise the possibility that CFP expression may be upregulated under prolonged low salinity stress in *P. cylindrica* at the expense of GFP expression. However, such an interpretation requires confirmation through experimental studies with a higher sampling resolution and raises additional questions about the function of FPs in corals.

Significant reductions in the total fluorescence levels in both *P. cylindrica* and *E. mammiformis* in February compared with the other summer time points, despite seawater temperatures being the highest recorded around any of our sampling

times, raise the possibility that fluorescence plays a role in mitigating stress induced by an environmental parameter other than temperature. Interestingly, the cloud cover signature at this time was distinctly different from all other time points, showing 13 consecutive days with more than 90% cloud cover in the study region, while cloud cover was below 90% at all other sampling times. Although solar radiation is increased in summer and heats up the seawater, clouds can reduce the amount of solar radiation that reaches corals. In accordance with a photoprotective function for FPs (Salih et al. 2000, Dove et al. 2001), it may be that these two species increased their fluorescence levels in summer in response to higher levels of solar radiation and then down-regulated levels when clouds reduced solar radiation to non-stress levels. These results would be consistent with the results of experimental studies that found strong positive correlations between GFP expression and light intensities (D'Angelo et al. 2008, Roth et al. 2010). Taken together, the total fluorescence levels appear to be upregulated in response to increased levels of solar radiation, providing corroborative evidence for a photoprotective function for FPs in corals.

Non-fluorescent chromoprotein levels fluctuated throughout the year in all coral species, although a positive correlation with seawater temperature was found in *A. millepora*. This suggests a role for chromoprotein in the thermal stress response, which is consistent with the observation of elevated chromoprotein expression levels in naturally bleached colonies of this species (Seneca et al. 2010), where it could play a photoprotective role by reducing the levels of solar radiation reaching *Symbiodinium* (Smith et al. 2013). Chromoprotein may also exhibit antioxidant properties (Palmer et al. 2009a) and could therefore be upregulated in response to the increased PO activity at higher temperatures to mitigate the adverse effects of reactive oxygen produced by the proPO system. The strong correlations found between CP expression and PO activities in this species support this interpretation. Surprisingly, in *E. mammiformis* and *P. cylindrica*, there was no correlation between temperature and CP, or between CP and PO activities. Corals do, however, possess other more effective antioxidants, such as superoxide dismutase, catalase, and peroxidase (Mydlarz and Palmer 2011). Potentially,

these enzymes are the primary antioxidants in *E. mammiformis* and *P. cylindrica*, while in *A. millepora*, CP may play a more significant role in neutralizing ROS.

#### Comparative ecological immunology

Comparative differences in immune and stress response parameters among coral species representing three coral families strongly suggest that corals differ in the allocation of resources to constitutive levels of immune and stress response systems. Higher levels of PO activity in the resistant coral *P. cylindrica* compared with the intermediately resistant *E. mammiformis* and the susceptible *A. millepora* are consistent with the positive correlations between immune status and both disease and bleaching resistance, as found by Palmer et al. (2010). In addition, we also found that a significantly higher proportion of PO is in its active form in *P. cylindrica* than in *A. millepora*, which would further contribute to the differences in disease and bleaching susceptibilities between these species. *P. cylindrica* appears to have constitutively high levels of PO activity and is thereby capable of preventing microbial infection and responding immediately to disturbances. Due to the short half-life of activated PO, maintaining such high PO activity requires significant energy resources. *A. millepora*, on the other hand, has lower constitutive levels of PO activity, but does have the capability of inducing additional activation of PO in response to infections when and where necessary, by storing more PO in its inactive proPO form, likely in immune cells that migrate toward lesions (Palmer et al. 2011b). While this strategy may require fewer resources and thereby allows the allocation of more resources for other life history traits, such as colony growth (Palmer et al. 2010), these relatively low constitutive PO activity levels may provide a minimal protection against invading microbes, thus increasing the chance of infection and disease before a significant immune response can be orchestrated by the host. It should, however, be noted that none of the coral colonies followed in this study developed signs of disease or bleaching. Evaluating both PO and tpPO activity levels in corals may significantly increase our understanding of the ecological immunology of corals.

Differences in constitutive levels of fluorescence among the coral species studied also correlate



with life history differences. Fast-growing *A. millepora* generally maintained relatively stable high fluorescence levels across seasons compared with the other two species, which exhibited typically lower and more variable total fluorescence levels and generally have slower growth rates. Fluorescent proteins are known to be more highly expressed in growing tissues (D'Angelo et al. 2012) and positively correlate with coral growth rates (Roth et al. 2010, Roth and Deheyn 2013), possibly because of their photoprotective function, which may explain the relatively higher constitutive levels of fluorescence in the fast-growing species. Maintaining fluorescence is considered costly due to the relatively high FP expression levels, although studies of FP turnover suggest that due to their long half-life, maintaining high levels of FPs may be comparatively cheap (Leutenegger et al. 2007a). However, the apparent lack of induced FP expression in summer when solar irradiance and temperature, two important factors in coral bleaching, are highest was surprising. This could indicate that FP levels in *A. millepora* are not sufficiently adequate to mitigate the effects when these environmental factors reach stressful levels, or that summer temperatures and solar irradiance were not stressful. In contrast, *E. mammiformis* significantly upregulates FP levels, in particular GFP, in summer, which likely protects this coral from high irradiance and temperature and ameliorates bleaching (Baird et al. 2009). *P. cylindrica* may use its high constitutive levels of PO activity to produce photoprotective melanin, a function recently suggested for this compound (Palmer et al. 2010). Overall, these differences show the variability in strategies for investing resources among life history traits by different coral species.

#### **Biochemical tools for coral reef management**

An urgent need for tools to evaluate and monitor coral health to manage and preempt activities that might cause further declines has arisen over recent years due to the increasing environmental and anthropogenic pressures facing corals worldwide (Burke et al. 2011). In this study, we provide initial baseline references of various innate immunity parameters for three coral species belonging to three major reef-building coral families, which can be used to assess the suitability of PO activity and FP expression as potential indicators for

monitoring environmental stressors. During the 2009–2010 sampling period, no anomalies in either seawater temperature or rainfall occurred, making this a suitable period for the establishment of baseline immune parameter levels. A number of recent studies have suggested that these immune parameters may be suitable tools for reef managers (Palmer et al. 2010), such as using FP expression for monitoring coral health (Roth et al. 2010, D'Angelo et al. 2012). Generally, biomarkers should (1) be specific to a stressor and not other factors, (2) have a magnitude in change that reflects the magnitude of the stressor, and (3) have low background variability (Cooper et al. 2009). Our results show, however, that there is a significant variability over time in most parameters tested, although the degree of variability depended on the coral species. Temporal variability may be acceptable in cases where extensive baseline data are available. While the changes in GFP-like protein expression do not appear to be specific to particular stressors (e.g., responses to high solar irradiation [D'Angelo et al. 2008], heat stress [Dove et al. 2006], bleaching [Leutenegger et al. 2007b], and coral translocation [Bay et al. 2009]), the range of stressors that induce the changes in FP expression or in the ratio of individual FPs suggests that FP expression levels may function as a general stress biomarker for some corals. Although correlations are good indicators of relationships between immune parameters and environmental factors, experimental studies exploring how these potential biomarkers respond to stressors, as well as the magnitude of these responses, need to be addressed under controlled conditions. In addition, several key common indicator species should be identified and, for each of these, extensive baseline data for each biomarker should be obtained on both temporal and spatial scales. Overall, PO activity and FP expression can potentially be used as biomarkers for coral health, but additional research on baseline data and stressors is required before we can address their suitability and applicability.

#### **CONCLUDING STATEMENT**

Given the increasing environmental and anthropogenic pressures and stresses corals face worldwide, there is an urgent need to understand the coral stress responses to facilitate the

development of coral health monitoring tools and techniques. In this study, initial baselines of multiple ecological immune parameters were compared between three species from three major reef-building coral families. The significant differences observed in constitutive levels and the temporal patterns in PO activity and GFP-like protein expression among these species suggest that they have evolved different ecological immune strategies. Such variations underscore the need to obtain species-specific baseline levels and further long-term data before considering the suitability of these ecological immune parameters for routine coral health monitoring.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online at: <http://onlinelibrary.wiley.com/doi/10.1002/ecs2.1505/full>