REPORT

Differential nitric oxide synthesis and host apoptotic events correlate with bleaching susceptibility in reef corals

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Abstract Coral bleaching poses a threat to coral reefs worldwide. As a consequence of the temperature-induced breakdown in coral–dinoflagellate symbiosis, bleaching can have extensive effects on reef communities. However, our understanding of bleaching at a cellular level is limited, and this is particularly true regarding differential susceptibility among coral species. Recent work suggests that bleaching may represent a host innate immune-like response to symbiont dysfunction that involves synthesis of the signalling compound nitric oxide (NO) and the induction of host apoptotic-like cell death. In this study, we examined the activity of apoptosis-regulating enzymes alongside oxidised NO accumulation (a proxy for NO synthesis) in the reef corals *Acropora millepora*, *Montipora digitata*, and *Pocillopora damicornis* during experimental thermal stress. *P. damicornis* was the most sensitive species, suffering mortality (tissue sloughing) after 5 days at 33 °C but non-lethal bleaching after 9 days at 31.5 °C. *A. millepora* bleached at 33 °C but remained structurally intact, while *M. digitata* showed little evidence of bleaching. *P. damicornis* and *A. millepora* both exhibited evidence of temperature-induced NO synthesis and, after 5 days of heating, levels of oxidised NO in both species were fivefold higher than in controls maintained at 28.5 °C. These responses preceded bleaching by a number of days and may have occurred before symbiont dysfunction (measured as chlorophyll *a* degradation and oxidised NO accumulation). In *A. millepora*, apparent NO synthesis correlated with the induction of host apoptotic-like pathways, while in *P. damicornis,* the upregulation of apoptotic pathways occurred later. No evidence of elevated NO production or apoptosis was observed in *M. digitata* at 33 °C and baseline activity of apoptosis-regulating enzymes was negligible in this species. These findings provide important physiological data in the context of the responses of corals to global change and suggest that early events in the host may be important in the collapse of the coral–dinoflagellate symbiosis.

Keywords Thermal stress · Climate change · *Symbiodinium* · Cnidarian–dinoflagellate symbiosis

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Introduction

Tropical coral reefs face an uncertain future in a changing climate (van Hooidonk et al. 2013), and oceanic warming is already leading to declining reef health (Mydlarz et al. 2010). Reef corals are a complex symbiosis between a scleractinian host, photosynthetic dinoflagellate symbionts of the genus *Symbiodinium*, and a diverse microbial community (Muscatine and Porter 1977). The provision of organic carbon by the *Symbiodinium* cells enables corals to grow rapidly in nutrient-poor tropical seawater (Muscatine and Porter 1977). However, reef corals live close to their upper thermal thresholds and warming of only a few degrees can destabilise this symbiosis (Hoegh-Guldberg 1999). This often manifests as a "bleaching" of host tissue resulting from the expulsion or degradation of the symbionts and/or their photosynthetic pigments (Hoegh-Guldberg 1999).

Coral bleaching can lead to mortality (Jones 2008), depressed growth rates (Goreau and MacFarlane 1990), and a heightened susceptibility to pathogens (Mydlarz et al. 2010). While much is known about its ecological consequences (see Pratchett et al. 2009 for review), comparatively little is understood regarding the cellular physiology of bleaching (Weis 2008). This is especially true for the phenomenon of differential bleaching, where corals vary in their responses to elevated temperatures (Weis 2010).

The differential bleaching susceptibility of reef corals is an emergent property of the genotypic composition of the resident *Symbiodinium* population (Rowan et al. 1997; Sampayo et al. 2008), previous thermal history (Guest et al. 2012) and physiological and life-history characteristics of the host (Baird et al. 2008 for review; Fitt et al. 2009; Palmer et al. 2010; Putnam et al. 2012). The latter in particular is becoming recognised as important in determining bleaching susceptibility; corals that associate with multiple *Symbiodinium* genotypes and acquire these symbionts from the environment are more sensitive to environmental stress than are species with maternal symbiont transmission and high symbiotic specificity (Putnam et al. 2012). However, this recognition of the host's role is a comparatively recent development as the coral bleaching field has been dominated by studies examining the role of the dinoflagellate symbiont (Jones et al. 1998; Warner et al. 1999; Baker 2001; Berkelmans and van Oppen 2006; Fisher et al. 2012). This is partly the result of a hypothesis that bleaching is "adaptive" for reef corals (Buddemeier and Fautin 1993; Baker 2001) and partly a consequence of our rapidly improving knowledge of symbiont phylogenetics (nine *Symbiodinium* "clades" and numerous "subclades" are now recognised; LaJeunesse 2002; Pochon et al. 2006). Examination of *Symbiodinium* photophysiology (Jones et al. 1998; Warner et al. 1999) contributed to the development of a model of coral bleaching as a consequence of symbiont photosynthetic dysfunction. Overproduction of reactive oxygen species (ROS), the onset of oxidative stress, and subsequent cellular damage were proposed as the proximate causes of this symbiotic collapse (Downs et al. 2002). Temperature- and light-induced ROS generation have been experimentally confirmed in *Symbiodinium* (Lesser 1996; Lesser 2006 for review; Suggett et al. 2008; Saragosti et al. 2010), but damage to host tissue in the absence of symbiont dysfunction (Gates et al. 1992; Nii and Muscatine 1997; Dunn et al. 2004, 2012; Paxton et al. 2013) and the release of photosynthetically competent and viable symbionts from bleaching corals (Ralph et al. 2001; Hill and Ralph 2007) have also been reported. Clearly, not all thermal bleaching in corals is symbiont-driven (Baird et al. 2008; Tolleter et al. 2013).

One component of host physiology that is relevant during temperature stress involves nitric oxide (NO). The synthesis of NO, an antimicrobial and cell-signalling molecule (Wink and Mitchell 1998; Fang 2004; Wang and Ruby 2011), has been reported in symbiotic cnidarians (Perez and Weis 2006) and dinoflagellates (Trapido-Rosenthal et al. 2001; Bouchard and Yamasaki 2008; Hawkins and Davy 2012). In the host, NO generation during temperature stress is thought to form part of an innate immune-like response mediating the ejection of *Symbiodinium* cells from the symbiosis (Perez and Weis 2006; Weis 2008; Detournay and Weis 2011; Detournay et al. 2012). The negative effects of NO on *Symbiodinium* physiology (Hawkins and Davy 2012) and the links between host NO synthesis and host apoptotic-like pathway activation (Hawkins et al. 2013) support this hypothesis. Apoptosis is a highly conserved mechanism of programmed cell death (Kerr et al. 1972) that is regulated by caspase enzymes (Lasi et al. 2010). It is an important mechanism of symbiont loss during coral bleaching (Dunn et al. 2004; Weis 2008; Pernice et al. 2011) and is also a pathogen-removal strategy in metazoans (Carmen and Sinai 2007). The modification of apoptotic pathways has already been proposed as a mechanism for differential host cell survival *post*-bleaching (Tchernov et al. 2011), and the anemone *A. pallida* generates NO to differing degrees during heat stress depending on the dominant *in hospite* symbiont genotype (Perez 2007). An interesting question, therefore, concerns the extent to which differential regulation of NO synthesis and potential downstream processes such as apoptosis, for example, could influence the stability of different coral–dinoflagellate associations during stress.

This investigation aimed to test the hypothesis that the differential generation of nitric oxide might be involved in the differing susceptibilities of reef corals under thermal bleaching. Thus, the species selected for this study were chosen on the basis of their sensitivities to elevated temperature. We hypothesised that heating would result in the elevated synthesis of NO in host and symbiont and that host NO synthesis would correlate with the induction of apoptotic-like cell death pathways known to be involved in bleaching (Dunn et al. 2004; Pernice et al. 2011; Tchernov et al. 2011). We also hypothesised that these events (in the host) are consequences of symbiont photophysiological dysfunction (Perez and Weis 2006). Here, we provide physiological data that may have implications for our understanding of differential thermal bleaching in corals.

Materials and methods

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (Auckland, New Zealand) and were of the highest analytical grade available.

Collection and treatment of coral fragments

Fragments (ca. 5 cm length, 3 cm width) of *A. millepora* (cream morph), *P. damicornis* (pink morph), and *M. digitata* (green morph) were collected from Heron Island reef flat (Great Barrier Reef (GBR), Australia; 23°26′43″S, 151°54′53″E) at low tide under the GBR Marine Park permit G12/34800.1. Fragments were transferred to flow-through aquaria and fixed to synthetic clay mounts using a small quantity (ca. 1 cm^2) of epoxy putty (Aqua Knead-It, Selleys, Padstow, NSW, Australia). Mounted fragments were then placed in experimental 60-L tanks under shade cloth (reducing incident midday irradiance from ca. 2,000 to 400–600 μmol photons m^{-2} s^{-1}). Four fragments were sampled from each of five replicate colonies, which were separated by at least 10 m on the reef flat in order to increase the likelihood of sampling genetically distinct individuals. A continuous flow system (ca. 1.5 L min^{-1}) ensured a constant turnover of seawater (from Heron Island reef flat), and fragments were acclimated for 6 d at 28.5 °C (±0.9 °C, SD), a temperature characteristic of summer daytime conditions on Heron Island reef flat (AIMS; http://data.aims.gov.au/aimsrtds/station.xhtml?station=130). Additional water flow was provided by a submersible pump (HX-6830, HAILEA, RaoPing, GuangDong, China). After the acclimation period, the temperature of two of the tanks was increased by approximately 1 °C per day (Electronic Supplemental Material ESM Fig. S1) and stabilised at either 31.5 °C (±0.6 °C) or 33 °C (±0.8 °C). Water temperatures were monitored every 5 min using HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA).

Fluorometric assessment of *in hospite* symbiont photosystem II (PSII)

Efficiency of symbiont PSII was measured using pulse amplitude modulation fluorometry (Diving-PAM, Walz, Effeltrich, Germany). Maximum (dark-adapted) quantum yields of PSII (F_v/F_m) were recorded daily, 30 min after sunset. Presented values are means of triplicate measurements of coral fragments designated for the final sampling point.

Coral fragment processing

Fragments were removed from experimental tanks shortly after noon on days 0, 5, 7, and 9 (ESM Fig. S1), wrapped in aluminium foil, flash-frozen in liquid nitrogen, and stored at −80 °C. All subsequent procedures were carried out in a 4 °C environment or on ice unless otherwise stated.

Tissue was removed from coral fragments by airbrushing (using the low pressure outlet of a SCUBA-diving regulator; Apeks Marine Ltd., Blackburn, Lancs., UK) into a zip-lock bag containing a known volume of 50 mM potassium phosphate, pH 7.8, 1 mM EDTA homogenisation buffer (HB; prepared using 18.3 MΩ water). Tissue suspensions were homogenised with a sawtooth homogeniser (Labserv D-130, Thermo Fisher Scientific, Albany, New Zealand) for 10 s at 20,000 rpm. Two 500-μL aliquots were removed for *Symbiodinium* cell counts (and to confirm the structural integrity of cells *post*-homogenisation) and photosynthetic pigment analysis. Samples for cell counting were fixed with 10 μL Lugols solution and stored at −20 °C. Pigment samples were centrifuged (16,000×*g* for 5 min), the supernatant discarded, and the pellet frozen and stored at −20 °C in the dark. The remaining tissue homogenate was centrifuged (1,500×*g* for 5 min) to separate remaining *Symbiodinium* cells from host material. The supernatant was removed and stored in 1-mL aliquots at −80 °C alongside the algal pellet.

Quantification of bleaching in coral fragments

Symbiodinium cell densities were quantified using haemocytometer counts (Improved Neubauer, Boeco, Germany), with at least six counts per sample (until coefficients of variability were <15 %). Cell density was normalised to coral fragment surface area, measured with the wax-dipping method (Stimson and Kinzie 1991). Symbiont chlorophyll *a* (Chl *a*) content was quantified by *N,N*-dimethylformamide (DMF) extraction carried out in darkness over 48 h at 4 °C. Extracts were centrifuged (16,000×*g* for 5 min) and triplicate 200-μL aliquots were measured in 96-well plates (UVStar, Greiner Bio-One GmbH, Germany) at 646.8, 663.8, and 750 nm using a microplate reader (Enspire® 2300, Perkin-Elmer, Waltham, MA, USA). Chl *a* concentrations were determined after optical path length correction (0.555 cm) using the equations of Porra et al. (1989).

Identification of dominant *in hospite Symbiodinium* genotypes

Symbiodinium aliquots were washed three times in HB (2,000×*g* for 5 min) and cells were processed following the methods of Logan et al. (2010). PCR amplification of the internal transcribed spacer-2 (ITS2) region of the nuclear ribosomal DNA was carried out using the forward primer ITS-D (5′-GTGAATTGCAGAACTCCGTG-3′) and reverse primer ITS2-Rev2 (5′-CCTCCGCCTACTTA-TATGCTT-3′). All PCR amplicons were checked for correct size via gel electrophoresis (1.5 % [w/v] agarose gel), purified (ExoSAP-IT; GE Healthcare, Life Sciences), and sequenced in both directions by Macrogen Korea (Seoul, South Korea). Sequences were identified using the NCBI and GeoSymbio databases (Franklin et al. 2012).

Preparation of *Symbiodinium* cell lysates

Frozen *Symbiodinium* pellets were washed four times with HB by repeated centrifugation ($800 \times g$ for 5 min) to remove host material. The cell suspension was transferred to a 1.5-mL tube containing 50 mg acid-washed glass beads (710–1,180 μm; Sigma-Aldrich, Auckland, New Zealand). Cells were lysed in a bead-mill (Tissuelyser LT, Qiagen Inc., Hilden, Germany) for 10 min at a frequency of 50 Hz. Visual counts (haemocytometer) confirmed cellular disruption, and the cell lysate was immediately analysed.

Quantification of the proxies for NO production

Flash-freezing corals in the field limits the live, real-time quantification of NO (with confocal microscopy and fluorescent dyes, for example; Perez and Weis 2006). However, NO in biological systems is quickly oxidised to either nitrite (NO_2^-) or nitrate (NO_3^-) (Bryan and Grisham 2007), and these biomarkers have been used to successfully quantify cnidarian NO synthesis (Detournay and Weis 2011; Detournay et al. 2012; Hawkins et al. 2013). The relative contributions of NO_2^- and NO_3^- to total oxidised NO (NO_x^-) depend on the immediate cellular environment at the sites of NO synthesis (Bryan and Grisham 2007), so overall assessments of NO synthesis benefit from measurements of total oxidised NO (NO_x^-; the sum of NO_2^- and NO_3^-).

Nitrite (NO_2^-) assay

Sample NO_2^- content was calculated using the fluorescent indicator 2,3-diaminonaphthalene (DAN, Invitrogen, Auckland, New Zealand; Nussler et al. 2006) according to the methods of Hawkins et al. (2013). Host homogenates and symbiont cell lysates were centrifuged ($16,000 \times g$ for 20 min) prior to processing, and DAN fluorescence (excitation: 365 nm, emission: 410 nm) was measured using a microplate reader (Enspire® 2300, Perkin-Elmer, Waltham, MA, USA). Negative controls (using HB instead of sample) were prepared in order to control for unreacted DAN fluorescence (a false positive), and NO_2^--dependent fluorescence was calculated relative to that of a sample blank in order to control for the significant autofluorescence of reef coral homogenates (Palmer et al. 2010). A calibration curve of 10–10,000 nM $NaNO_2$ in HB was used to determine final sample NO_2^- concentrations.

Total oxidised NO (NO_x^-) assay

Quantification of total NO_x^- with DAN requires the reduction of nitrate to nitrite, and this was achieved by adding 66 μL 0.1 M vanadium (III) chloride (VCl_3) solution in 1 N HCl (Miranda et al. 2001) to the sample (centrifuged as above) prior to the addition of the DAN reagent. Addition of DAN solution to *Symbiodinium* cell lysates in the presence of VCl_3 promoted the development of a persistent and interfering blue colour that quenched DAN fluorescence and could not be removed; these samples were therefore not included in the analysis. Host homogenates were incubated as described previously (Hawkins et al. 2013) and DAN fluorescence was quantified using a microplate reader (see above) and a calibration curve of 10–10,000 nM $NaNO_3$ in HB. Again, negative controls (using HB instead of sample) were prepared in order to control for unreacted DAN fluorescence (a false positive), and final NO_x^--dependent fluorescence was calculated relative to autofluorescence-only sample blanks.

As positive controls, homogenates of untreated (Day 0) corals from each species were incubated for 4 h with 1 mM of the NO donor sodium nitroprusside (SNP).

Assessment of caspase-like enzyme activity

The activity of enzymes resembling mammalian caspase-3 (an "executioner" caspase; Lasi et al. 2010) and caspase-9 (an "initiator" caspase in the mitochondrial pathway; Lasi et al. 2010) was quantified using a colorimetric assay kit (Sigma-Aldrich, Auckland, New Zealand) with the substrates Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA) and Ac-Leu-Glu-His-Asp-pNA (Ac-LEHD-pNA), respectively (Hawkins et al. 2013). Caspase-like enzymes cleave these tetrapeptide substrates at the terminal aspartic acid residue with a specificity determined by the identity of the preceding three amino acid residues. Briefly, coral host homogenates were thawed on ice and centrifuged ($16,000 \times g$ for 20 min). Aliquots of the supernatant were removed for protein quantification [Bradford assay with bovine serum albumin (BSA) as a standard (Bradford 1976)], and duplicate aliquots of the remainder were removed for caspase-like enzyme analysis. Briefly, ten microlitres XXXX-pNA substrate (2 mM) was mixed with 40 μL "assay buffer" [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 2 mM EDTA, 0.1 % 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT)], and 50 μL coral homogenate. After gentle shaking for 10 s, samples were incubated according to the manufacturer's instructions. Sample blanks (minus caspase substrates) were prepared in order to control for sample absorbance, and to control for false positives caused by substrate autolysis, reagent blanks (using HB instead of sample) were measured alongside. To further determine the rate of non-specific substrate hydrolysis by coral homogenates, three untreated replicate samples from each coral species were pre-incubated for 30 min with 20 μM DEVD- or

LEHD-aldehyde (-CHO), inhibitors of caspase-3 and caspase-9, respectively.

Statistical analyses

Data analysis was carried out with PASW Statistics 18.0 (IBM, Armonk, NY, USA). Where appropriate, data were analysed using repeated measures analysis of variance (RMANOVA) with Greenhouse-Geisser correction used whenever Mauchly's test returned a significant result. RMANOVA post hoc tests represent pairwise comparisons between treatments at certain time points with Bonferroni correction. Multivariate ANOVA (MANOVA) was used to examine baseline caspase-like enzyme activity, and additional analyses were carried out using one-way ANOVA. Data were log-transformed (or arcsine square root-transformed, in the case of F_v/F_m) prior to parametric analysis.

Results

Symbiodinium genotypes

Dominant *in hospite* ITS2 genotypes were as follows: *Acropora millepora*—C3; *Montipora digitata*—C15; and *Pocillopora damicornis*—C42 (type 2). All colonies of each species harboured the same ITS2 type and the nature of returned ITS2 sequences suggested the presence of a single symbiont genotype within individual colonies.

PSII fluorescence and bleaching of reef corals at elevated temperature

Exposure to elevated temperature for 9 days led to significant declines in the dark-adapted PSII quantum yields (F_v/F_m) of all three species (Table 1), and these responses differed significantly between species (RMANOVA, time × species × temperature, $F_{4.4,48.6} = 56.86$, $P < 0.001$). F_v/F_m in *A. millepora* and *P. damicornis* (initially 0.68 ± 0.01 and 0.64 ± 0.01 [both ± SEM], respectively) declined to 0.34 ± 0.01 and 0.49 ± 0.02, respectively (Fig. 1a–c). F_v/F_m in *M. digitata* remained at the control level until late in the experiment when a slight but statistically significant decrease became apparent (Fig. 1b). Data are not available for *P. damicornis* after 6 days at 33 °C when fragments suffered 100 % mortality (visible as tissue sloughing); physiological analyses were therefore carried out on fragments incubated at 31.5 °C.

Baseline Chl *a* content differed between ITS2 types (one-way ANOVA, $F_{2,28} = 23.67$, $P < 0.001$); the C42 (type 2), symbionts of *P. damicornis* had the highest amount per cell ($6.9 \text{ pg} \pm 0.1$, Tukey HSD post hoc, $P < 0.01$ for both comparisons), and the C3 symbionts of

Table 1 Statistical analysis (RMANOVA, time × temperature interaction) of symbiont physiological parameters in reef corals exposed to control (28.5 °C) and elevated temperatures (31.5 °C for *Pocillopora damicornis*; 33 °C for *Acropora millepora* and *Montipora digitata*)

Species (ITS2 type)	Parameter	F value	P value
Acropora millepora (C3)	F_v/F_m	$F_{1.4,8.5} = 186.22$	<0.001*
	Chlorophyll *a* content	$F_{3,21} = 1.834$	0.172
	Nitrite (NO_2^-) content	$F_{3,15} = 6.771$	0.043*
Montipora digitata (C15)	F_v/F_m	$F_{3.1,26.7} = 9.99$	<0.001*
	Chlorophyll *a* content	$F_{3,24} = 4.18$	0.016*
	Nitrite (NO_2^-) content	$F_{3,15} = 0.407$	0.75
Pocillopora damicornis (C42)	F_v/F_m	$F_{1.7,13.7} = 30.23$	<0.001*
	Chlorophyll *a* content	$F_{3,21} = 0.84$	0.487
	Nitrite (NO_2^-) content	$F_{3,18} = 1.137$	0.361

* Statistical significance ($P < 0.05$)

A. millepora had the lowest amount per cell ($3.3 \text{ pg} \pm 0.2$, Tukey HSD post hoc, $P < 0.05$ for both comparisons). C3 was the only type to display declining Chl *a* content at elevated temperature and, while there was no significant time × treatment interaction (Table 1), type C3 cells at 33 °C contained 58 % less Chl *a* after 9 days than did the equivalent controls (Fig. 1d).

Pre-treatment symbiont densities differed significantly between species (one-way ANOVA with Welch correction, $F_{2,10.8} = 39.29$, $P < 0.001$), with *P. damicornis* having 80 % fewer *Symbiodinium* cells per cm² than the two other species (Tukey HSD post hoc, $P < 0.01$ for both comparisons). When incubated at elevated temperature (31.5 °C for *P. damicornis* or 33 °C for *A. millepora* and *M. digitata*) for 9 days, symbiont densities declined significantly in *A. millepora* and *P. damicornis* (to ca. 10 and 50 % of initial densities, respectively) and no significant changes were observed in *M. digitata* (Table 2; Fig. 1g–i).

Nitrite and total oxidised NO in reef corals at elevated temperature

Nitrite (NO_2^-) levels (nmol per unit soluble protein) in the type C3 *Symbiodinium* cells of *A. millepora* increased significantly at elevated temperature (Table 1; Fig. 2a), but this was only apparent on day 9 of the treatment. An increasing trend was apparent in the symbionts of *P. damicornis* but no significant time × temperature interaction was observed (Table 1; Fig. 2c). No changes in
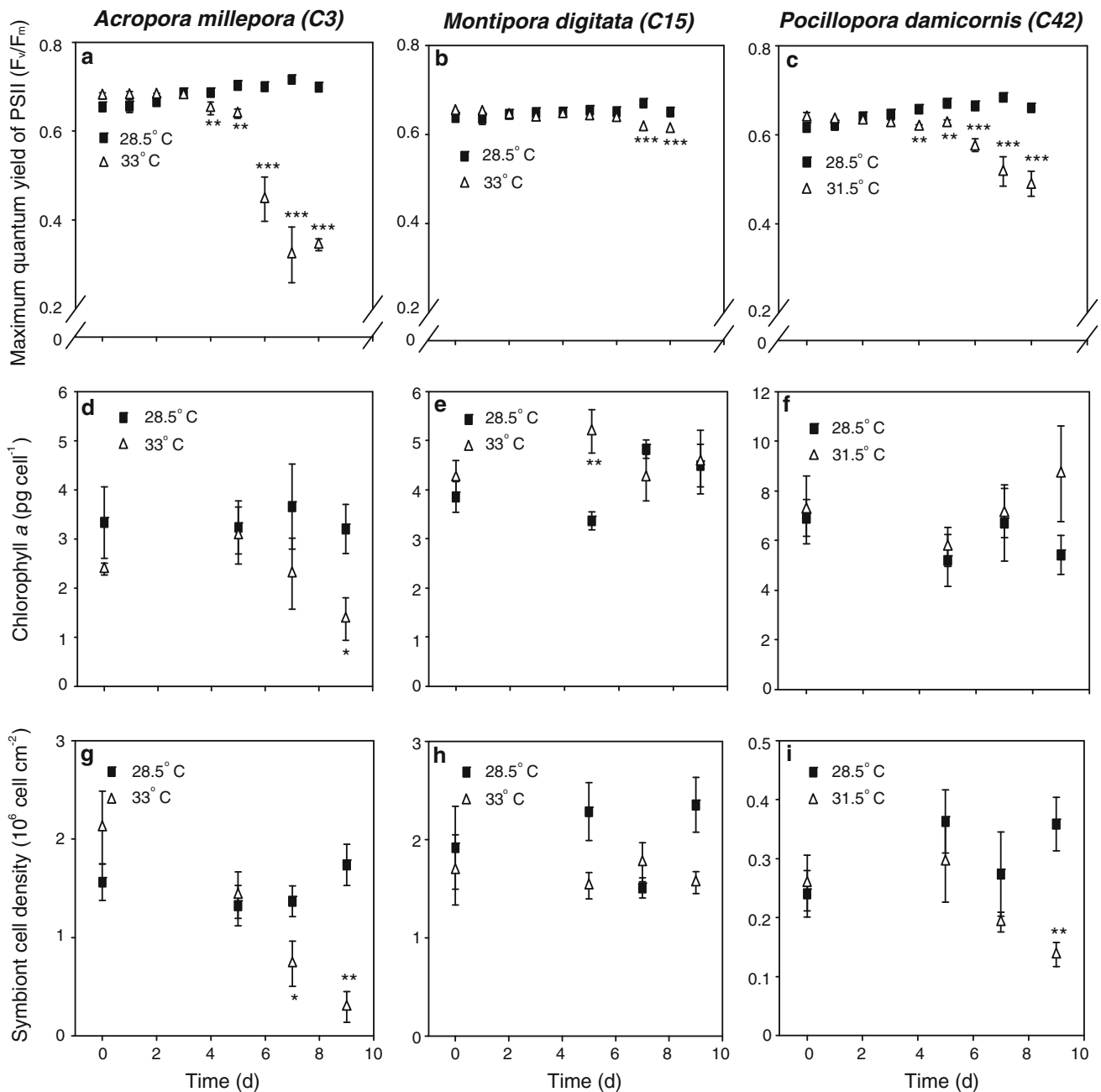
Fig. 1 Quantum yields of photosystem II, symbiont chlorophyll *a* content and density in *Acropora millepora*, *Montipora digitata* and *Pocillopora damicornis*. **a–c** Maximum quantum yield of photosystem II (F_v/F_m); **d–f** Chlorophyll *a* content (pg per cell); **g–i** Symbiont cell density per cm^2 coral fragment surface. Values are mean ± SEM and *asterisks* denote significant differences relative to controls at each time point (RMANOVA, pairwise post hoc with Bonferroni correction, $n = 5$ independent colonies per time point except *A. millepora* after 7 d at 33 °C where $n = 4$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Note the different *y*-axis scales in **d–f** and **g–i**

cellular NO_2^- were observed in the C15 symbionts of *Montipora digitata* (Fig. 2b).

Host tissue NO_2^- (pmol per unit soluble protein) differed significantly between species at the start of the experiment (one-way ANOVA with Welch correction, $F_{2,12.5} = 28.55$, $P < 0.001$), with *A. millepora* having significantly (approximately twofold) higher host tissue NO_2^- than the other two species (Tukey HSD post hoc,

$P < 0.01$ for both comparisons). However, tissue NO_2^- content did not respond to temperature in any of the coral species (Table 2, ESM Fig. S2). Baseline total oxidised NO (NO_x^-) concentrations in the host also differed between species (one-way ANOVA with Welch correction, $F_{2,12.7} = 4.01$, $P = 0.045$), but in this case *A. millepora* and *M. digitata* had similar NO_x^- levels while those of *P. damicornis* were lower (≤35 %; Tukey HSD post hoc,

Table 2 Statistical analysis (RMANOVA, time × temperature interaction) of host physiological parameters in reef corals exposed to control (28.5 °C) and elevated temperatures (31.5 °C for *Pocillopora damicornis;* 33 °C for *Acropora millepora* and *Montipora digitata*)

Species	Parameter	F value	P value
Acropora millepora	Tissue nitrite (NO$_2^-$)	$F_{3,18} = 0.89$	0.465
	Tissue total oxidised NO (NO$_x^-$)	$F_{3,18} = 6.614$	0.031*
	LEHDase (caspase-9-like) activity	$F_{3,21} = 3.82$	0.025*
	DEVDase (caspase-3-like) activity	$F_{3,21} = 7.313$	0.002*
	Symbiont density	$F_{3,18} = 6.572$	0.003*
Montipora digitata	Tissue nitrite (NO$_2^-$)	$F_{3,18} = 0.232$	0.873
	Tissue total oxidised NO (NO$_x^-$)	$F_{1.6,9.5} = 1.23$	0.321
	LEHDase (caspase-9-like) activity	$F_{3,24} = 0.085$	0.968
	DEVDase (caspase-3-like) activity	$F_{3,24} = 0.464$	0.633
	Symbiont density	$F_{3,18} = 1.426$	0.268
Pocillopora damicornis	Tissue nitrite (NO$_2^-$)	$F_{3,18} = 1.672$	0.209
	Tissue total oxidised NO (NO$_x^-$)	$F_{3,12} = 3.920$	0.037*
	LEHDase (caspase-9-like) activity	$F_{3,24} = 5.416$	0.005*
	DEVDase (caspase-3-like) activity	$F_{1.5,11.7} = 4.71$	0.04*
	Symbiont density	$F_{3,21} = 4.779$	0.011*

* Statistical significance ($P < 0.05$)

$P < 0.05$). Tissue NO$_x^-$ in *A. millepora* and *P. damicornis* responded significantly to temperature over the experimental period (Table 2), with heightened NO$_x^-$ levels observed on day 5 for both species (Fig. 2d, f). No significant changes in NO$_x^-$ content were seen in *M. digitata* (Table 2; Fig. 2e).

Responses of host caspase-like enzymes to elevated temperature

Baseline host caspase-like enzyme activity was highly variable between the three coral species (Fig. 2g–i), with DEVDase (caspase-3-like) activity lower in *M. digitata* than in the other two species (one-way ANOVA, $F_{2,29} = 42.58$, $P < 0.001$, Fig. 2h). Further examination revealed no significant difference in substrate cleavage between *M. digitata* homogenates treated with and without caspase-like enzyme inhibitors (MANOVA, Pillai's Trace, $F_{3,12} = 0.89$, $P = 0.495$; Tukey HSD post hoc DEVDase: $P = 0.867$,

LEHDase: $P = 0.253$). Effects of heating on host caspase-like enzyme activity differed depending on the species and the duration of treatment (Table 2; Fig. 2g–i). No changes in DEVDase or LEHDase (caspase-9-like) activity were seen in *M. digitata* (Table 2; Fig. 2h). LEHDase and DEVDase activity in *A. millepora* and *P. damicornis* responded to increasing temperature (Table 2; Fig. 2g–i), and after 5 days at 33 °C in *A. millepora* and 7 days at 31.5 °C in *P. damicornis*, their activities were at least twofold higher than in control samples. This was followed by a slight decline in both species, although caspase-like enzyme activity remained significantly elevated in *A. millepora* throughout the treatment period.

Discussion

This study provides evidence that host-level responses such as nitric oxide synthesis and apoptotic-like cell death may be involved in the differential thermal bleaching of reef corals. We report marked differences in physiological responses to heating, even between two "temperature-sensitive" coral species. Furthermore, heightened host NO synthesis (measured as oxidised NO accumulation) and apoptotic pathway activation appeared to be initiated simultaneously in *A. millepora*. Perhaps most importantly, however, we observed substantial interspecific variability in the baseline activity of apoptotic-like pathways in the host. Together with recent investigations, these findings could contribute to a developing physiological framework describing the differing sensitivities of corals to warming oceans.

Photophysiological dysfunction, symbiont NO synthesis, and coral bleaching

Exposure to heating of 1 °C per day to 3–5 °C above ambient revealed significant differences in symbiont physiology and the overall bleaching susceptibility of the reef corals *A. millepora*, *M. digitata,* and *P. damicornis*. As observed previously, the *M. digitata*-C15 association was comparatively robust in its response to heating, while the *A. millepora*-C3 and *P. damicornis*-C42 (type 2) symbioses proved much more unstable (Fisher et al. 2012; Guest et al. 2012).

Consistent with the absence of chlorophyll *a* degradation and the modest decline in F_v/F_m in the C15-type symbiont cells of *M. digitata*, there was no evidence of upregulated symbiont NO synthesis—a thermal stress response (Bouchard and Yamasaki 2008)—in this species. In contrast, the symbionts of *A. millepora* and *P. damicornis* both displayed evidence of heightened NO production (accumulation of cellular nitrite) after 9 days of
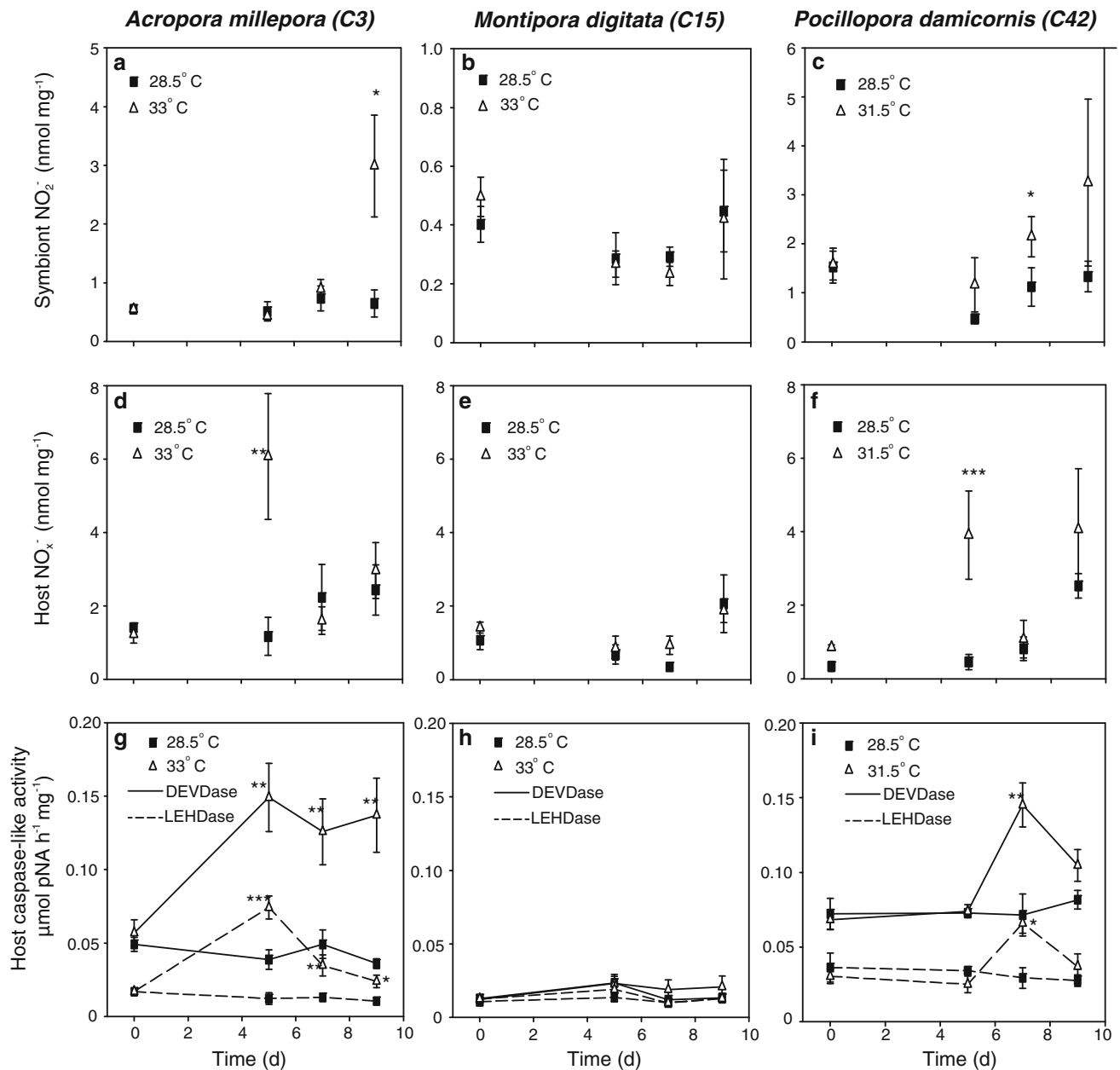
Fig. 2 Oxidised NO accumulation and apoptotic events in the reef corals *Acropora millepora*, *Montipora digitata* and *Pocillopora damicornis*. **a–c** Symbiont nitrite (NO_2^-) content (nmol per mg protein). **d–f** Host total oxidised NO (NO_x^-) content (nmol per mg protein). **g–i** Host caspase-like enzyme activity (rate of substrate cleavage per mg protein). Values are mean ± SEM and *asterisks* denote significant differences relative to controls at each time point. (RMANOVA, pairwise post hoc with Bonferroni correction, $n = 5$ independent colonies per time point except *A. millepora* after 7 d at 33 °C where $n = 4$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Note the different *y*-axis scales in **a–c**

exposure. The only previous study of NO in clade C *Symbiodinium* (Hawkins and Davy 2012) reported a temperature-induced downregulation of NO synthesis. This could be due to the use of cultured *Symbiodinium* cells rather than those sampled *in hospite*, as their physiology is likely to be different outside the host (Gates et al. 1995). The nature of the treatments—rapid heating in the previous study *versus* a 1 °C d^{-1} rate here—may also have

contributed, as could sub-cladal differences in symbiont physiology. However, our data support the hypothesis that symbiont NO synthesis may contribute to their in situ degradation during thermal stress (Hawkins and Davy 2012). Its influence on the eventual intensity of bleaching is less clear, however, and might depend on the time at which host cellular pathways are activated (Hawkins et al. 2013). In any case, further methodological advances would

be invaluable for investigating *Symbiodinium* NO synthesis and may confirm the extent to which this phenomenon can affect the bleaching responses of reef corals.

Cellular events in the coral host

Our findings support the hypothesis that host NO synthesis is involved in coral bleaching (Perez and Weis 2006; Weis 2008), but suggest that the situation in reef corals may be much more complex than in laboratory model systems. For example, host tissue NO_2^- content—which responds to temperature in *Aiptasia pulchella* (Detournay and Weis 2011)—changed little in the species examined here. Only when total oxidised NO (NO_x^-) was quantified did responses become apparent. This was most obvious in *A. millepora* and less so in *P. damicornis*. *M. digitata*, the only species of the three not to bleach, showed little evidence of temperature-induced NO_x^- accumulation. While it must be acknowledged that there are multiple sources of dissolved inorganic nitrogen in the coral–dinoflagellate association, the interspecific differences in NO_x^- and the strong positive effect of the NO donor SNP (ESM Fig. S3) support the suitability of this parameter for assessing tissue NO synthesis.

The corals in this study displayed a range of stress responses, from the limited reaction of *M. digitata*, to the classic photoinhibition/bleaching of *A. millepora*, to the rapid host mortality of *P. damicornis* at 33 °C. The time at which some of the interspecific differences became apparent is of particular interest, and the early nature of host NO_x^- accumulation and apoptotic events in *A. millepora*, specifically, raises questions about the importance of symbiont photophysiological dysfunction in initiating the bleaching cascade. The leakage of ROS from compromised symbionts is thought to be a primary mechanism by which symbiont dysfunction stimulates bleaching (Lesser 2006; Tchernov et al. 2011), and our observation of apparent host apoptosis following declining F_v/F_m in *A. millepora* is consistent with this model. However, the level of photosynthetic dysfunction necessary to elicit oxidative stress in *Symbiodinium* remains unclear, and the evidence for *in hospite* ROS leakage from symbionts is limited. Most studies that have observed ROS generation/leakage in *Symbiodinium* employed light-stresses (e.g., Lesser 1996; Suggett et al. 2008; Saragosti et al. 2010) or rapid heat-shock treatments (McGinty et al. 2012) significantly more intense than those used here. For example, the temperature-induced ROS production reported by Suggett et al. (2008) required an irradiance of 1,000 μmol photons m^{-2} s^{-1} and occurred when F_v/F_m had declined to <30 % of control levels. In our study, host apoptotic events in *A. millepora* (on Day 5) occurred at with symbiont F_v/F_m > 90 % of control values. Tchernov et al. (2011) observed that ROS

generation was heightened in heat-stressed coral fragments containing symbionts, relative to pre-bleached specimens, and therefore proposed the symbiont as the source of the ROS. However, host ROS production as a response to the presence of symbionts could have produced the same result. Given the early nature of host responses in this study, and the limited empirical support for the symbiont ROS-leakage hypothesis, we propose that our observations may result from stimuli other than symbiont-derived ROS.

Our data support the hypothesis that host-derived NO—perhaps alongside any ROS present in the system—could play a role in the responses of reef corals to thermal stress (Perez and Weis 2006). The transient increase in NO_x^- content of *A. millepora* host tissue correlated with increasing activity of enzymes associated with apoptotic cell death, and NO (especially in the presence of ROS) is known to influence apoptosis by interacting with components of the mitochondrial apoptosis pathway (Wink and Mitchell 1998; Hawkins et al. 2013). A rapid increase in host-derived NO could also adversely affect symbiont physiology (Hawkins and Davy 2012). However, the situation appears to be different in *P. damicornis*, where timing of apparent host NO synthesis (NO_x^- accumulation) and declining symbiont F_v/F_m did not correlate with host caspase-like enzyme activation. This suggests that alternative apoptotic pathways (perhaps less dependent on NO) exist in *P. damicornis*, but with little known about the regulation of cell death in scleractinians it is difficult to make specific hypotheses at this stage.

What is clear, however, is that the activity of apoptotic pathways varied significantly between coral species, and this supports a recent hypothesis that differential regulation of apoptosis may be a mechanism by which corals modify their responses to thermal stress (Tchernov et al. 2011). Host caspase-like enzyme activities in the bleaching-resistant *M. digitata* showed no response to temperature, and closer examination found that caspase inhibitors had no effect on the rates of substrate cleavage by *M. digitata* homogenates. This either indicates that caspase-like enzyme activity is almost non-existent in this species or it suggests that the enzymes that are present are so dissimilar to those in *A. millepora* or *P. damicornis* that they are unable to cleave the same substrate. The latter is highly improbable, as DEVDase activity has been successfully quantified in *Hydra* (Lasi et al. 2010), unicellular chlorophyte algae (Jimenez et al. 2009), and sponges (Wiens et al. 2003). We can therefore conclude that baseline caspase-like enzyme activity in *M. digitata* is negligible. By contrast, the two bleaching-susceptible species both displayed evidence of baseline caspase-like enzyme activity which responded to heating. Interestingly, the induction of host apoptotic pathways occurred days before any symbiont loss became apparent. Maybe this reflects a disruption
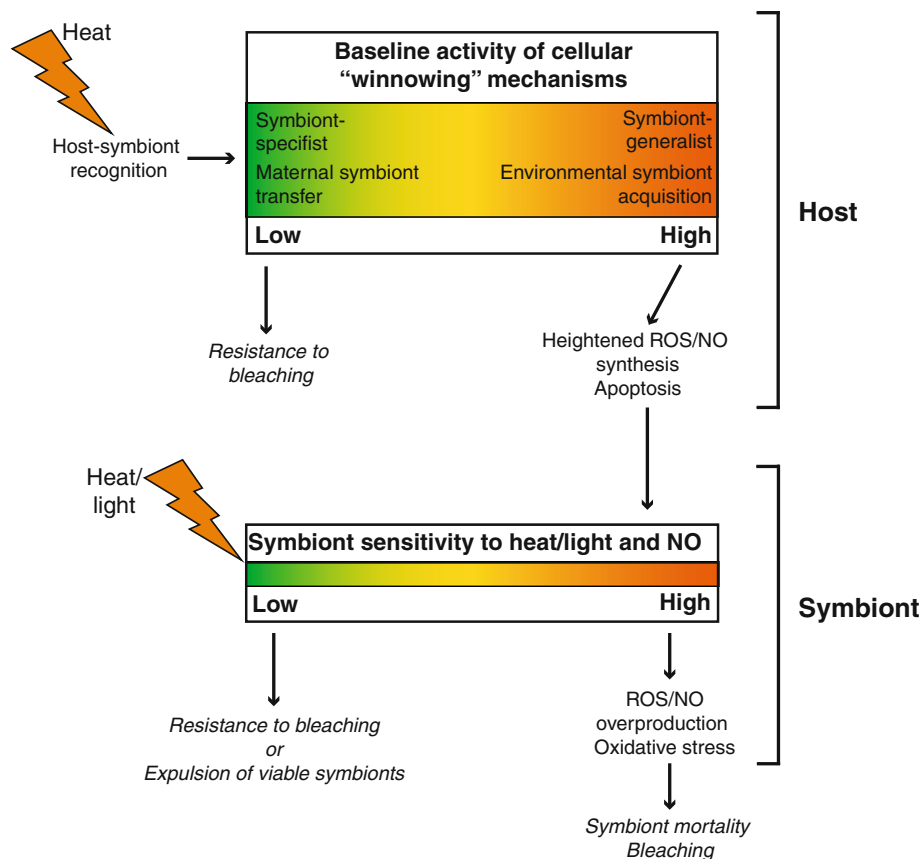
Fig. 3 Hypothetical model of differential bleaching susceptibility in reef corals, incorporating aspects of host-symbiont specificity and symbiont acquisition strategies at a cellular level. The presence of potentially temperature-sensitive "winnowing" pathways—beneficial for the regulation of genetically diverse *in hospite* symbiont populations—may increase the susceptibility of a coral species to thermal bleaching. Temperature-induced dysfunction of host-symbiont recognition mechanisms (see Vidal-Dupiol et al. 2009) might stimulate host-level "winnowing"-like responses such as nitric oxide (NO) synthesis and apoptosis. These responses, coupled with symbiont sensitivity to heat/light (Fisher et al. 2012), NO (Hawkins and Davy 2012) and oxidative stress (McGinty et al. 2012), could affect the bleaching outcomes (*italicised* in the figure) of a thermally challenged coral

of host-symbiont recognition pathways prior to symbiont degradation and bleaching (see Vidal-Dupiol et al. 2009 for examples). Further work is needed to fill in these gaps in our knowledge. While we cannot entirely discount symbiont-derived ROS as a stimulus for the host responses observed here, our data support a growing body of work suggesting that host-level mechanisms play an important role in determining the bleaching responses of corals (Nii and Muscatine 1997; Fitt et al. 2009; Palmer et al. 2010; Dunn et al. 2012; Paxton et al. 2013; Tolleter et al. 2013).

Towards a cellular basis for differential coral bleaching

To incorporate phenomena such as host NO synthesis and apoptosis into a discussion of differential bleaching susceptibility, one must also acknowledge their non-bleaching functions. As mentioned above, one such function (highly conserved in metazoans) is as a host response to infection (Fang 2004; Carmen and Sinai 2007; Wang and Ruby 2011).

Successful infection of *A. pallida* with homologous *Symbiodinium* (occurring naturally with that host) results in a downregulation of host NO synthesis (Detournay et al. 2012), and the selective removal of unsuitable symbionts ("winnowing") in *Fungia scutaria* larvae involves an apoptotic-like mechanism (Dunn and Weis 2009). These pathways, presumably advantageous for hosts that associate with multiple symbiont types (and must, therefore, "winnow"/regulate their intracellular symbiont population), appear highly sensitive to temperature (Perez and Weis 2006; Detournay and Weis 2011; Hawkins et al. 2013), and could potentially be disadvantageous during thermal stress. Many of the most bleaching-susceptible corals, including *A. millepora* and *P. damicornis*, associate with a wide range of symbiont types (Baird et al. 2009; Guest et al. 2012; Putnam et al. 2012) and, furthermore, *A. millepora* acquires its symbionts from the external environment (Baird et al. 2009). These species could benefit from a symbiosis "winnowing" mechanism. Indeed, the

dominant symbiont genotype in *A. millepora* on the northern GBR differs between larval and adult life stages (Cumbo et al. 2013), and expression of genes with potential "winnowing" roles has been observed in this species (Puill-Stephan et al. 2012). A heightened baseline activity of such pathways might leave symbiont-generalist corals such as *A. millepora* and *P. damicornis* more susceptible to bleaching (Fig. 3). Conversely, a species such as *M. digitata* that associates with very few symbiont types and relies solely on maternal symbiont transmission (Baird et al. 2009) would less frequently encounter (on a cellular level, at least) infective symbionts and would presumably receive little adaptive benefit from a "winnowing" mechanism. In concert with the physiological robustness of a C15-type symbiont (Fisher et al. 2012), the relative absence of these "winnowing" pathways could render *M. digitata* more resistant to thermal bleaching (Fig. 3).

This model is speculative, of course, but it does represent a testable hypothesis. To our knowledge, this study provides some of the first physiological data to support the findings of recent meta-analyses of coral–dinoflagellate associations and their susceptibilities to bleaching (Putnam et al. 2012). Further assessments of baseline NO-synthase/caspase activity and cell–cell recognition pathways in different coral–dinoflagellate associations—characterised by differing symbiont transmission modes and host-symbiont specificities—would be highly instructive. It is clear that the cellular responses of corals to elevated temperature are highly diverse. Elucidating the mechanisms underpinning this diversity is now a major challenge, especially given the environmental changes predicted for the coming century.

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