Shifting Baselines: Repeat bleaching drives coral physiotypes through environmental legacy and cellular memory

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Abstract

Global climate change is altering coral reef ecosystems. Notably, marine heat waves are producing widespread coral bleaching events with increased frequency, with projections for annual bleaching events on many reefs by mid-century. The response of corals to elevated seawater temperatures is, however, influenced by a combination of environmental legacies, the biology of the host, and the resident symbiont community, each of which have the capacity to modulate resistance and resilience to environmental stress. It is critical, therefore, to evaluate the potential for shifting physiological and molecular baselines driven by these factors in back-to-back *in situ* bleaching (and recovery) events given the potential for mortality of corals in response to episodic bleaching episodes and increased ocean warming. Here, we use the regional bleaching events of 2014 and 2015 in the Hawaiian Islands and subsequent recovery periods to test the hypothesis that coral multivariate responses (physiotypes) differed in back-to-back bleaching events, modulated by both environmental histories and symbiotic partnerships (Symbiodiniaceaea). Bleaching severity was greater in the first-bleaching event, and concomitantly, environmental history effects were more pronounced. Melanin, an immune cytotoxic response, provided an initial defense during the first-bleaching event and primed antioxidant activity, which peaked in the second-bleaching event. While magnitude of bleaching differed, immune response patterns were shared among corals harboring heat-sensitive and heat-tolerant Symbiodiniaceae. This supports a pattern of increased constitutive immunity in corals during repeat bleaching events, demonstrated by greater specialized enzymes (catalase, superoxide dismutase, peroxidase) and attenuated melanin synthesis. This study demonstrates bleaching events trigger cumulative and interactive physiotypes driven by environmental legacy and cellular memory. Quantifying baseline and altered coral physiotypes (multivariate

phenotype) will be critical to understanding and forecasting the effects of increased bleaching frequency on coral biology and ecology in the Anthropocene.

1. Introduction

Environmental change at local and global scales is degrading coral reef ecosystems. Notably, long-term trends in ocean warming and punctuated marine heat waves are destabilizing the symbiosis between corals and their endosymbiont algae (Symbiodiniaceae) and increasing coral bleaching episodes (Hughes et al., 2018). The cumulative impacts of these episodic warming events not only shift ecological and functional baselines (Hughes et al., 2019; McWilliam et al., 2020), but may also have conditioning effects on coral biology, such that responses to bleaching events can be modulated by prior exposure (e.g., Brown et al., 2000). It is growing increasingly clear that susceptibility to bleaching in reef corals is based on the interaction of environmental histories (Ainsworth et al., 2016; Brown et al., 2000; Safaie et al., 2018) and holobiont traits, including endosymbiont communities and physiology of the coral host (Barshis et al., 2013; Palmer et al., 2010; Palumbi et al., 2014; Sampayo et al., 2008). Thermotolerant Symbiodiniaceae can confer bleaching resistance in corals (Sampayo et al., 2008), and the Hawaiian coral *Montipora capitata* exhibits reduced bleaching when dominated by *Durusdinium* as opposed to *Cladocopium* Symbiodinaceae (Cunning et al., 2016). However, corals are metaorganisms, and host properties such as constitutive immunity (Palmer, 2018b) and antioxidant capacity (Barshis et al., 2013) are also central to maintaining cellular homeostasis and preventing bleaching mortality (Palmer et al., 2010). As such, studies implicate immunological processes as targets for natural selection, integral to the future of reef-building corals in the face of climate change (Mydlarz et al., 2010; Palmer, 2018b; Pinzón C et al., 2014). The nexus of these influential properties of coral stress responses – symbiont community and host immunity – and their role in repetitive natural bleaching and recovery events remains to be fully understood, especially with respect to the role of environmental history (Palumbi et al.,

2014; Safaie et al., 2018). Given that global climate change will increase the frequency and severity of coral bleaching events (Hughes et al., 2018), understanding the state of the symbiotic, immune, and antioxidant physiotype of corals will reveal the cumulative and latent effects of bleaching on the function of reef corals through time and further enhance our capacity to project community responses to thermal stress events.

The widely documented recent major bleaching of the Great Barrier Reef (2016-2017) reduced coral cover by >50% (Stuart-Smith et al., 2018), altered coral (Hughes et al., 2018) and fish (Robinson et al., 2019) assemblages, and disturbed the stock-recruitment capacity of reef corals (Hughes et al., 2019). As climate change continues to intensify, it is now critical to track shifts in the biology and function of corals through examination of multivariate trait-spaces, or physiotype (Van Straalen, 2003). Trait-space approaches have recently been applied to track changes in reef coral communities and their representative trait diversity following bleaching (Hughes et al., 2018; McWilliam et al., 2020). Further tracking of multiple, interactive coral traits provides the opportunity to reveal the potential for beneficial acclimatization or negative legacy effects that may underly shifts in physiotype (e.g., Figure 1a) and testable mechanistic hypotheses underpinning resistance or susceptibility to repeated stress (Figure 1b).

An integrative multivariate approach to tracking coral performance is particularly informative when response variables are chosen in a targeted way to test specific hypotheses, such as the linkage between environmentally-driven physiological legacies, symbioses, and cellular mechanisms of bleaching (i.e., antioxidants, immunity) (Palmer et al., 2010; Palmer & Traylor-Knowles, 2012; Venn et al., 2008). For example, both the host and symbiont have enzymatic

defenses to mitigate oxidative stress (e.g., peroxidase, catalase, superoxide dismutase) (Venn et al., 2008) caused by host sources (e.g., heat-damaged mitochondrial membranes) and damage to the D1 protein of photosystem II (PSII) (Jones et al., 1998; Lesser, 1997) from heat and/or light-induced inhibition of the dark reactions of the Calvin Cycle. Ultimately, the net effect of oxidative stress on the holobiont leads to apoptosis and dysbiosis (Weis, 2008) (Figure 1b). Considering the multiple actions of the host and symbionts in the cascade of cellular responses leading to bleaching (Gardner et al., 2017; Palmer, 2018a), hypotheses focusing on initial as well as persistent effects of bleaching on the coral holobiont (i.e., acute and chronic stress, acclimatization) are particularly tractable within this multivariate integrative framework.

Thermal stress also triggers host immune responses (Mydlarz et al., 2009, 2010; Pinzón et al., 2015), and higher constitutive immunity (i.e., immune activity necessary for cellular homeostasis) reduces coral thermal sensitivity and disease susceptibility (Palmer et al., 2010). The melanin-synthesis pathway is an important component of host immunity, active in wound healing and pathogen invasion, in addition to serving as a photoprotectant that mitigates bleaching stress (Mydlarz & Palmer, 2011; Palmer et al., 2010) (Figure 1b). While melanin and antioxidants are active cellular responses to thermal stress, there is evidence that corals may rely on these mechanisms differently based on their environmental histories. For instance, greater melanin synthesis and lower antioxidant activity was observed in heat-stressed corals from a reef with lower pCO_2-variability compared to high pCO_2-variability (Wall et al., 2018). Considering the wide-ranging functions of melanin in host immunity, melanin synthesis may thus be an acute and broad-spectrum defense in physiologically stressed corals, with antioxidants having a more specialized response after chronic stress. Such different cellular mechanisms to combat

bleaching may be based on energetic requirements to mount immune responses (Fuess et al., 2018; Palmer, 2018a; Pinzón et al., 2015), variable in response to initial versus repeated stress (Ainsworth et al., 2016; Schoepf et al., 2015), or determined by the histories of biotic and abiotic challenges that modulate constitutive immunity (Mydlarz et al., 2009; Palmer, 2018a; Wall et al., 2018).

The Hawaiian Islands, once thought to be a bleaching refuge for corals, experienced severe bleaching in 2014 and 2015 across the populated Main and remote Northwestern Hawaiian Islands (Bahr et al., 2017; Couch et al., 2017). In Kāneʻohe Bay, Oʻahu, Hawaiʻi, 7.1 degree heating weeks (DHW) were observed in 2014 and 10.2 in 2015, beginning in August and July, respectively (*see* Methods), resulting in 45±2% (mean±SE) bleaching in the first event and 30±4% bleaching in the second (Bahr et al., 2017). An ecologically dominant reef coral, *Montipora capitata* (Dana 1846), showed significant bleaching in both events (Figure 2a), with differences in bleaching responses observed at the site and event level.

Using the opportunity presented by these natural bleaching events (Figure 2), we tracked coral physiotypes using a multivariate trait-based approach. We quantified the response of *M. capitata* in Kāneʻohe Bay to the back-to-back bleaching events of 2014 and 2015 (i.e., bleaching in October 2014, recovery in February 2015, bleaching in October 2015, and recovery in February 2016) at two Kāneʻohe Bay reefs (Lilipuna and Reef 14) with contrasting environmental histories (*see* Methods) (Wall et al., 2018). We predicted that: (H1) immunity and antioxidant contributions to the bleaching response are influenced by site environmental histories and symbiont communities, and (H2) immunity and antioxidant activity will not occur with the same

magnitude or trajectory after repeated bleaching events due to the accumulation of physiological stress, or triggering of acclimatory mechanisms. Further, while the thermal tolerance of symbiont communities is vital to coral bleaching sensitivity, evidence for different immunity or antioxidant capacities in coral holobionts hosting thermally tolerant symbiont species is lacking. Given that the coral *M. capitata* is known to be dominated by two genetically and physiologically contrasting Symbiodiniaceae, *Cladocopium* sp. and *Durusdinium glynnii* (Cunning et al., 2016; Wall et al., 2020), we further predicted (H3) that holobionts harboring heat-tolerant *D. glynnii* would show immune and antioxidant responses linked to greater bleaching resistance. We measured coral physiotypes as represented by: physiology (symbiont density, areal- and cell-specific chlorophyll *a*, total protein and biomass) as measurements of bleaching magnitude, along with mechanisms contributing to bleaching resistance through antioxidant capacity (peroxidase, catalase, superoxide dismutase), and the immune response of the melanin cascade (prophenoloxidase, melanin). This approach allows for a more holistic picture of the cumulative and interactive physiological outcomes driven by environmental legacies and cellular memory.

2. Materials and Methods

2.1 Site description

Coral bleaching and recovery at two reef systems: a fringing reef (hereafter, 'Lilipuna' [21°25′36.8″N, 157°47′24.0″W]) in southern Kāneʻohe Bay adjacent to the Hawaiʻi Institute of Marine Biology (HIMB) on Moku o Loʻe, and an inshore patch reef (hereafter, 'Reef 14' [21°27′08.6″N, 157°48′04.7″W]) in central Kāneʻohe Bay. These reefs have been previously described (Wall et al., 2018). Briefly, these reef sites were chosen due to their unique

environmental history of seawater pCO_2 and hydrofynamics (Drupp et al., 2013). Seawater pCO_2 adjacent to Lilipuna and Reef 14 is comparable (ca. 450 μatm), however, diel pCO_2 flux is significantly higher (196 – 976 μatm pCO_2) in central Kāneʻohe Bay near Reef 14 relative to the Bay's southern basin proximate to Lilipuna (225 – 671 μatm) (Drupp et al., 2011; Drupp et al., 2013).

2.2 Environmental monitoring

Temperature (Hobo pendants, ± 0.53 °C accuracy, 0.14 °C resolution, Onset Computer Corp., Bourne, MA) and photosynthetic active radiation (PAR) loggers (Odyssey, Dataflow Systems Limited, Christchurch, New Zealand) were placed at Lilipuna and Reef 14 at a depth of 1 m. Temperature and PAR were recorded continuously at 15 min intervals from October 2014 - February 2016, however, gaps in environmental data do exist as a consequence of instrument failure and loss. Gaps in logger temperature data were supplemented with NOAA temperature data from the Moku o Lo'e station at HIMB (NOAA, 2019). PAR loggers were calibrated against a LI-1400 quantum meter (Li-Cor, Lincoln, Nebraska, USA) attached to a cosine LI-192 underwater quantum sensor. Temperature loggers were calibrated with a certified digital thermometer (5-077-8, +/- 0.05 °C accuracy, Control Company, USA) and cross-calibrated against each other for standardization. Kāneʻohe Bay degree heating weeks (DHW) were calculated in R using *in situ* Moku o Loʻe temperature data (NOAA, 2019), with the difference between mean half-week temperatures (i.e., mean hourly temp over 3.5 d) and the maximum monthly mean temperature (27.7 °C, Jokiel & Brown, 2004) to determine 'hotspots'. DHW were determined as the number of hotspots >1 across a rolling 12 weeks window (i.e., 24 half-weeks) (NOAA, 2020).

2.3 Benthic surveys and coral collections

Four sampling periods were identified as corresponding to a 'bleaching period' following the point of maximum thermal stress (10 October 2014 and 12 October 2015) and a post-bleaching 'recovery period' approximately 4 months after peak seawater warming (11 February 2015 and 26 February 2016). In each time period, benthic surveys were conducted at each reef site using a 20 m transect and a line-point-intersect at 1 m intervals. Transects were positioned parallel to natural contours of the reef, being the north-south axis of the fringing reef (Lilipuna) and the east-west axis of patch reef (Reef 14). At each reef, transects (n = 2) were placed along the reef crest (1 - 2 m), and benthic community cover was recorded at the species level for reef corals (*Montipora capitata, Pocillopora* spp. [*P. acuta*, *P. damicornis*], *Porites compressa*), and either crustose coralline algae (CCA), macroalgae, or sand/bare/turf. For corals, bleaching state was quantified categorically, being either non-bleached (i.e., appearing fully pigmented), or bleached (i.e., exhibiting degrees of tissue paling/pigment variegation or being wholly white). *M. capitata* cover was calculated as the proportion of total benthic cover, and *M. capitata* bleaching extent was calculated as proportion of total *M. capitata* colonies bleached.

In each sampling period, forty coral branch tips (< 4 cm length) of *M. capitata* were collected from corals along the reef crest at a depth of ca. 1 m (State of Hawai'i Department of Land and Natural Resources, Special Activity Permit 2015-17 and 2016-69). Immediately post collection, corals were snap frozen in liquid nitrogen and returned to HIMB and stored at -80 °C. Subsequently, coral samples were photographed on dry ice. While remaining frozen, each colony was split in half along its longitudinal axis. One-half of each coral fragment was stored at HIMB

(-80 °C) until processing for physiological assays and qPCR. The corresponding fragment-halves were used for immunological assays and were shipped to the University of Texas at Arlington using a dry-shipper charged with liquid nitrogen. Additional samples collected from the same sites in February 2014 (Wall et al., 2018) were used to contextualize changes in coral physiology prior to the onset of bleaching. February 2014 samples used for "initial" context of the response variables in this study were from exposed to seasonally ambient temperatures (24.5 °C) in the laboratory for two weeks, previously reported in (Wall et al., 2018). Immunity samples were collected, frozen, and analyzed without laboratory exposure (see below).

2.4 DNA extraction and symbiont community analysis

Symbiodiniaceae DNA was extracted by adding an isolate of coral tissue (500 μl) to 500 ul DNA buffer (0.4 M NaCl, 0.05 M EDTA) with 2 % (w/v) sodium dodecyl sulfate, following a modified CTAB-chloroform protocol (Cunning et al., 2016) (dx.doi.org/10.17504/protocols.io.dyq7vv). Following DNA extraction, the composition of the symbiont community in *M. capitata* was assayed using quantitative PCR (qPCR) (Cunning et al., 2016) by quantifying specific actin gene loci corresponding to internal transcribed spacer (ITS2) region of rDNA for Symbiodiniaceae genera *Cladocopium* and *Durusdinium* (namely, ITS2 types C31, with C17 and C21 and D1-4-6 [*Durusdinium glynnii* (Wham et al., 2017)]) known to be the numerically dominant symbionts of *M. capitata* in Kāneʻohe Bay (Cunning et al., 2016). Specificity of genus-level primers have been previously validated using a combination of Symbiodiniaceae ITS2 and actin gene sequencing (Cunning & Baker, 2013). Duplicate qPCR reactions (10 μl) were run for each coral sample using a StepOnePlus platform (Applied Biosystems) set to 40 cycles, a relative fluorescence (ΔR_n) threshold of 0.01, and internal cycle

baseline of 3 - 15. Symbiont genera detected in only one technical replicate were considered absent. The relative abundance *of Cladocopium and Durusdinium* symbionts (i.e., C:D ratio) in each sample was determined from the ratio of amplification threshold cycles (C_T) for *Cladocopium* and *Durusdinium* (i.e., C_T^C, C_T^D) using the formula C:D = 2^(C_T^C - C_T^D), where genus-specific C_T values are normalized according to gene locus copy number and fluorescence intensity (Cunning et al., 2016). Coral colonies were determined to be *Cladocopium*- or *Durusdinium*-dominated based on numerical abundance (>0.5 proportion) of each genus from qPCR analysis (Innis et al., 2018).

2.5 Physiological metrics

The extraction and processing of coral and symbiont tissues was performed following established methods (Wall et al., 2018). Briefly, coral tissue was removed from the skeleton using an airbrush filled with filtered seawater (0.2 μm), yielding 10 - 30 ml of tissue slurry. Extracted tissues were briefly homogenized and subsampled for the following physiological metrics: symbiont cell densities, chlorophyll *a* concentrations, protein biomass, and the total organic biomass determined from as the ash-free dry weight (AFDW) of coral + algae tissues. Both the archival tissue slurry and tissue aliquots were stored at -20 °C.

All physiological metrics were normalized to the surface area (cm^2) of coral skeleton using the paraffin wax-dipping technique (Stimson & Kinzie, 1991). Symbiont cell counts were obtained measured by replicate cell counts (n = 6 - 10) on a haemocytometer, and expressed as symbiont cells cm^{-2}. Chlorophyll *a* was quantified by concentrating algal cells through centrifugation (13,000 rpm × 3 min) and extracting pigments in the algal pellet in 100 % acetone for 36 h in

darkness at -20 °C. Spectrometric absorbance were measured (λ = 630 and 663 nm) using a 96-well quartz plate with two technical replicates; chlorophyll concentrations quantified using the equations for dinoflagellates (Jeffrey & Humphrey, 1975), normalized for path length and expressed as ug chlorophyll *a* cm^{-2} and pg chlorophyll *a* symbiont $cell^{-1}$. Total protein concentration (soluble + insoluble) was quantified using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Pierce Biotechnology, Waltham, MA). Protein solubilization was achieved by adding 1 M NaOH and heating (90 °C) for 60 min, followed by the neutralizing to ca. pH 7.5 with 1 N HCl. Protein was measured spectrophotometrically (λ = 562 nm) in a 96-well plate with three technical replicates against a bovine serum albumin standard and expressed as mg protein cm^{-2}. Total fraction of organic biomass was measured by drying a subsample of coral tissue at 60 °C (48 h) in pre-burned aluminum pans followed by burning in a muffle furnace (450 °C) for 4 h. The difference between the dried and burned masses is the AFDW and expressed as mg cm^{-2}.

2.6 Oxidative stress and immunity metrics

Oxidative stress and immunology metrics were determined using previously published protocols (Mydlarz et al., 2009; Mydlarz & Palmer, 2011; Palmer et al., 2011; Wall et al., 2018). A 3 - 4 ml aliquot of coral tissue slurry was obtained by airbrushing with coral extraction buffer (100 mM TRIS buffer + 0.05 mM dithiothreitol). Tissue was homogenized for 1 min on ice (hand held homogenizer, Powergen 125, Fisher Scientific, Waltham, MA), and 1 ml of the resulting slurry was freeze-dried for 24 h (VirTis BTK freeze-dryer, SP Scientific, Warminster, PA) and used for melanin concentration estimates. The remaining slurry was centrifuged at 4 °C at 2,500 × *g* (Eppindorf 5810 R centrifuge, Hamburg, Germany) for 5 min to remove cellular debris and

achieve a cell free extract. Antioxidant and prophenoloxidase assays were performed using cell free extract. Protein concentrations of cell free extract were determined using the RED660 protein assay (G Biosciences, Saint Louis, MO) with a bovine serum albumin standard curve. Melanin concentration estimates were determined by melanin extraction from freeze-dried tissue slurry. All assays were run in duplicate on separate 96-well microtitier plates using a Synergy HT multidetection microplate reader using Gen5 software (Biotek Instruments, Winooski, VT).

Oxidative stress was determined by measuring the activity of the antioxidants catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD). CAT and POX were monitored as a change in absorbance over time. SOD activity was determined by endpoint analysis comparing sample absorbance to a positive and negative standard. CAT activity is expressed as mM hydrogen peroxide (H_2O_2) scavenged mg protein^{-1} min^{-1}. Activity was monitored as a change in absorbance over 15 min after 10 μl 25 mM H_2O_2 was added to 10 μl cell free extract and 50 μl of 10 mM phosphate buffered saline (PBS) (pH 6.0). POX activity is expressed as activity mg protein^{-1} min^{-1}. Activity was monitored by guiacol oxidation and is represented by a change in absorbance over 15 min after 12.5 μl of 25 mM H_2O_2 was added to 25 μl of 25 mM guiacol in 10 mM PBS (pH 6.0), 10 μl cell free extract and 50 μl PBS (pH 6.0). SOD activity is expressed as activity mg protein^{-1}. Activity was determined using a commercially available kit (SOD determination kit #19160; Sigma-Aldrich, Saint Louis, MO) following manufacturer's instructions.

The melanin synthesis pathway was studied by prophenoloxidase (PPO) activity and melanin (MEL) concentration per sample. PPO activity is expressed as activity mg protein^{-1} min^{-1}.

Activity was monitored by L-DOPA oxidation and is represented by a change in absorbance over 30 min. 20 μl of cell free extract and 50 μl of 10 mM phosphate buffered saline (PBS) (pH 7.0) was incubated at room temperature with 20 μl of trypsin (0.2 mg ml^{-1} concentration). After incubation, 20 μl of 25 mM L-DOPA (Sigma Aldrich, Saint Louis, MO) was then added as a substrate and change in absorbance was monitored. MEL is expressed as mg melanin mg $tissue^{-1}$. Concentration was determined after melanin was allowed to extract from freeze-dried tissue for 48 h in 400 μl of 10 M NaOH after a brief period of bead-beating with 1 mm glass beads. A 65 μl aliquot of extracted melanin was used to determine endpoint absorbance at 495 nm and resulting values were estimated using a standard curve of commercial melanin (Sigma-Aldrich, Saint Louis, MO) and calibrated to μg melanin mg $tissue^{-1}$.

2.7 Statistical analysis

Permutation analysis of variance (PERMANOVA) and non-metric multidimensional scaling (NMDS) were performed using a matrix of all physiology and immunity responses in the package *vegan* (Oksanen et al., 2019). PERMANOVA used a scaled and centered matrix with Euclidean calculations of pairwise distances using *adonis2*. The NMDS data matrix was double standardized using a Wisconsin and square root transformation with a Bray-Curtis dissimilarity using *metaMDS*. Coral 'physiotypes' constituted convex hulls, with borders defined by the range of points in the multivariate trait space (e.g., NMDS1 and NMDS2). Physiotypes were grouped categorically by periods or the interactions of site, bleaching, and period. Physiotype centroids used in trajectory plots were calculated as the mean of NMDS1 and NMD2 for each category.

Ecological benthic data (*M. capitata* total cover and bleached cover) were tested in a linear model with Periods (two bleaching and two recovery events) and Sites (Lilipuna, Reef 14) as fixed effects. Physiology, antioxidant, and immunity response variables were analyzed using a linear model with Periods, Sites, and Symbiont community composition (*Cladocopium*- or *Durusdinium*-dominated) as fixed effects. Normal distribution and equal variance assumptions of ANOVA were examined by graphical representation of residuals and quantile:quantile plots. Where assumptions were not met, Box-Cox tests were performed (Box & Cox, 1964) and data transformations applied in the package *MASS* (Venable & Ripley, 2002). Analysis of variance tables for linear models were generated using type-II sum or squares with multiple comparisons using the package *emmeans* (Lenth, 2019). All analyses were performed in *R* version 3.6.1 (R Core Team, 2019). All data and code to generate figures and perform analyses are archived and openly available at Github (https://github.com/cbwall/Gates-Mydlarz-bleaching-recovery/releases/tag/ver.review).

3. Results

Montipora capitata benthic cover ranged from ~20-85% during the study period (Figure 2). Mean *M. capitata* cover was stable across time at Lilipuna (34-42%) but was much more variable at Reef 14, falling from 91±4 % (mean±SD) in October 2014 to 15±10% cover in January 2015. However, Reef 14 *M. capitata* cover increased to 28-51% from 2015-2016. In the two bleaching events (October 2014 and 2015) greater bleaching of *M. capitata* corals was observed at Reef 14, compared to Lilipuna ($p = 0.020$).

PERMANOVA results testing effects of Period (bleaching event), Site (Lilipuna *vs*. Reef 14) and dominant Symbiodiniaceae species (*Cladocopium* sp. or *D. glynnii*) revealed significant differences of all main effects ($p<0.001$) and the interaction of Period-by-Site ($p<0.001$) and Period-by-Symbiont community ($p<0.001$) (Figure 2 and 3, Table 1). In each of the four periods, coral physiotypes occupied unique positions in multidimensional trait space (Figure 2), with the location and trajectories of coral physiotypes in each period principally being a function of symbiont community, and to a lesser extent, site of collection (i.e., environmental legacy) (Fig 3, 4).

Physiotypes of bleached corals relative to recovered corals showed stronger separation in 2014-2015, which was apparent at both reef sites (Figure 3 *top row*) and paralleled patterns of greater bleaching prevalence in the first-bleaching event (Figure 2c). Similarly, site effects on coral physiotypes were most pronounced in the first bleaching event (Table 1, Figure 4). Despite greater bleaching at Reef 14 being observed in October 2014, shifts in bleaching and recovery performance envelopes were most distinct at Lilipuna (Figure 4). At the physiological level, separation of bleaching and recovery period physiotypes was most related to symbiont densities and chlorophyll concentrations (areal and per symbiont cell) (Figure 4 & S2, Table S1) ($p<0.001$). "Bleached" physiotypes observed in periods of temperature stress (October 2014 and 2015) generally aligned with colonies hosting the more thermally sensitive *Cladocopium* sp., although in the first event coral physiotypes at Reef 14 symbiont communities did not separate according to symbiont communities (Figure 4). Nevertheless, across all sampling periods *M. capitata* colonies dominated by *Durusdinium* symbionts had higher symbiont cell densities, less variable areal chlorophyll concentrations, and lower chlorophyll per symbiont cell compared to colonies dominated by *Cladocopium* symbionts ($p<0.001$) (Figure S2). Coral protein and total

biomass was variable across the study, but each showed positive correlations with *Durusdinium*-dominated physiotypes from Lilipuna in bleaching and recovery periods (Figure 4). Overall, protein was 9% higher in *Durusdinum*-dominated colonies (p=0.031) and 32% higher in Lilipuna corals during first bleaching (October 2014) but equivalent at all sampling points thereafter (p=0.002). Total biomass was lower during recovery periods (p<0.001) in addition to being 20-40% higher at Lilipuna compared to Reef 14 in all periods (except in February 2015 recovery) (p<0.001) and ~30% higher in *Durusdinium*-dominated colonies during the second bleaching period, but equivalent across holobionts in other periods (p=0.012) (Figure S2).

Immunity and antioxidant metrics (i.e., MEL, PPO, POX, CAT, SOD) differed through time in response to repeat bleaching and recovery (Figure 3, Figure 4) (p<0.001), while environmental legacies at the site-level and symbiont communities were less pronounced (Figure S2, S3, and Table S1, S2). In the first event, corals responded to thermal stress by increasing melanin synthesis (p<0.001) (with corresponding declines in PPO precursors) and increasing catalase (p<0.001). During recovery, prophenoloxidase (p<0.001) and superoxide dismutase increased (p<0.001) as melanin and catalase declined (Figure 4, 5, S2, & S3, Table S1, S2). Importantly, the melanin pathway increased in all corals in the 2014-bleaching event regardless of bleaching sensitivity (i.e., loss or retention of symbionts/chlorophyll), or symbiont community, and was a significant cellular response that shaped coral physiotypes in the first bleaching event (Figure 5, first row). In contrast, corals in the second bleaching event showed limited increase in melanin, despite reductions in prophenoloxidase. Correlated with the absence of melanin during second bleaching was a peak in catalase activity, which reached its highest level in 2015 bleaching (p<0.001) and was most pronounced in corals from Lilipuna compared to Reef 14 (p<0.001) (Table S2). The peak in catalase during 2015 bleaching corresponded with reductions in

peroxidase, particularly for *Cladocopium*-dominated colonies (p=0.002). Subsequently in the 2016 recovery period, catalase declined and peroxidase reached its highest values (p<0.001). Superoxide dismutase showed a unique trajectory, however, increasing progressively through time with each sampling period, being at its highest constitutive abundance after repeat bleaching in the 2016 recovery (p<0.001).

4. Discussion

Ocean thermal anomalies are reshaping the structure and function of coral reefs the world over. Many global reef systems have experienced three mass bleaching events since 2014 (Hughes et al., 2017), and climate models predict that by mid-century bleaching will be an annual phenomena (van Hooidonk et al., 2013). The stress responses of corals to ocean warming, however, is based on a network of interactions at biological and environmental levels, which can be dynamic in their response, which will influence how they respond to the physiological challenges posed by a warming planet (Suggett & Smith, 2019). Accordingly, there is a need for holistic measures of bleaching outcomes that incorporate the influence of environmental legacy (Palumbi et al., 2014), symbiont community (Suggett et al., 2017), and cellular memory (Brown et al., 2015) on coral holobionts during and after thermal stress, to test for shifting baselines that may ameliorate or exacerbate coral bleaching. Such physiotype tracking – or multivariate physiological and molecular time series data – provide the capacity for identification of the cellular mechanisms that contribute to resilience, acclimatization, resistance, allowing for better predictions of coral responses to intensifying climate change and other regional disturbances (Vercelloni et al., 2020).

Symbiosis, via differential performance of genera and types (Sampayo et al., 2008; Stat et al., 2008) and the potential for switching and shuffling (Buddemeier & Fautin 1993; Baker et al., 2003), provide one clear example of how enviornmetnal history and shift performance baselines. In this study, the role of dominant Symbiodiniaceae on holobiont physiotype was present, but the symbiont community effects were often period- or site-dependent. The high symbiont and chlorophyll *a* densities in *M. capitata* colonies dominated by *D. glynnii* across periods aligns with the paradigm of thermotolerance within the genus *Durusdinium* and a resistance to expulsion despite incurring photodamage (Silverstein et al., 2017). Antioxidant and immunity metrics, however, were equivalent in colonies differing in dominant symbiont. Thus, dominant symbiont communities had negligible effects on coral host traits, which is counter to expectations that immunity would be modulated by symbiont-derived bleaching tolerance. In fact, the antioxidant and immunity responses of *Cladocopium* and *Durusdinium*-dominated colonies were similar, despite a greater loss of symbiont cells and photopigmentation in colonies dominated by *Cladocopium*. Yet, it was the interaction of Site, Period, and Symbiont that interacted most to shift physiotypes. For instance, Site effects were clear in the first event, possibly relating to greater bleaching in 2014, which reduced symbiont cells in both corals dominated by *Cladocopium* sp. and thermotolerant *D. glynii* at Reef 14. Lower bleaching prevalence in the second event, however, saw attenuated Site-by-Symbiont effects. Therefore, the symbiont community harbored by corals is integral to bleaching responses and coral physiotypes (Suggett et al., 2017), but the relative importance of symbiont effects are also tempered by present environmental conditions and site-specific environmental histories.

Coral physiotypes are influenced by the physiological state and integrity of the coral-Symbiodiniaceae symbiosis, which includes (but is not limited to) pigmentation and symbiont

densities. In our study, we observed symbiont loss/repopulation to drive changes in coral physiotypes between bleaching and recovery periods. In support of our hypothesis that prior events have cumulative impacts, coral physiotypes occupied distinct spaces in each sampling period and showed changing trajectorie through time (2014-2016). These trajectories may be driven by those responses active in either the first or second bleaching-recovery periods (such as melanin), or by changes in constitutive immune activity that are cyclical, or continuously increasing through time. For instance, changes in total biomass generally declined in the aftermath of bleaching, being lower in recovery periods, but did not follow patterns in symbiont or photopigmentation concentrations. Biomass also tended to increase through time and was highest at the end of the study (pooled across all samples). The progressive increases in constitutive antioxidant activity exemplified by superoxide dismutase, may also have contributed to reduced bleaching-induced tissue loss and contributed to overall coral colony survival (Thornhill et al., 2011). Therefore, the moderate increase in tissue biomass through time, despite repeat stress events, may be a result of greater resilience in corals surviving repeat bleaching events, in addition to seasonal and stress-dependent fluctuations in coral tissue (Fitt et al., 2000; Wall et al., 2019).

The observed differences in antioxidant and immune activity in corals during repeat bleaching and recovery reveal that shifts in cellular priorities and mechanisms for dealing with bleaching stress exist, which ultimately shapes the coral physiotypes and their trajectories through time and between stress states. In this case, increased catalase activity during bleaching may be linked to reactive oxygen and nitrogen species and increased host apoptosis-like pathways (Hawkins et al., 2014; Krueger et al., 2015). Conversely, the accumulation of superoxide dismutase appears decoupled from the onset and subsidence of thermal stress, as seen in catalase. This response

may reflect the cellular memory of corals to thermal stress, thereby acting as a buffer to future oxidative stress (Barshis et al., 2013; Brown et al., 2015) and reducing bleaching effects while also contributing to greater tissue retention and post-bleaching survival. Both corals and Symbiodiniaceae have a broad array of superoxide dismutase isoforms, which can be used to combat reactive oxygen species originating from damage to photomachinery and host mitochondria that can lead to apoptosis (Weis, 2008). The persistent increase of superoxide dismutase through time further suggests antioxidant "front-loading" (Barshis et al., 2013) is an important strategy used by corals during repetitive thermal stress to reduce damage.

Engagement of the melanin pathway (collectively here as the prophenoloxidase reservoir and the melanin product) is known to be an important generalized stress response for mitigating disease and thermal stress effects in stony and soft corals (Mydlarz et al., 2008; Palmer et al., 2010; Wall et al., 2018). Likewise, exposure to thermal stress increases antioxidant activity in corals and Symbiodiniaceae (Gardner et al., 2017). In the first-bleaching event, the melanin pathway was observed as the primary cellular response to warming in all corals, regardless of bleaching sensitivity or symbiont community. This initial peak in melanin synthesis further supports the central role of this pathway as a generalized cellular response to periodic stress, including wound repair, disease, photodamage, and bleaching, and/or a photoprotective role to reduce excess excitation energy (Mydlarz et al., 2008; Palmer et al., 2010; Palmer, Traylor-Knowles, et al., 2011; Wall et al., 2018). Its subsequent decline suggests the melanin cascade may i) prime the antioxidant response and no longer is required to act as a primary stress response role, or ii) had its capacity overwhelmed in the second-bleaching, resulting in corals primarily using antioxidant stress response mechanisms. Our findings are more in line with the former. Under this thermal-priming hypothesis, sublethal thermal stress and high variable temperature regimes can

contribute to protective mechanisms in the coral holobiont and bleaching resistance (Ainsworth et al., 2016; Barshis et al., 2013; Bellantuono et al., 2012; Safaie et al., 2018). However, recurring thermal stress past a threshold may overcome this acquired cellular resistance (Ainsworth et al., 2016).

In our study, the hierarchy of cellular stress responses shifts away from melanin-synthesis in favor of antioxidant activity, which may also indicate the specialized nature of antioxidants in mitigating cellular damage and maintaining coral holobiont homeostasis across a range of physiological processes and stress states (Murphy et al., 2019). Antioxidant proteins are central to cellular stress response network (Scandalios, 2002) and in corals there is clear evidence that antioxidant enzymes have a stress inducible role, driven by both environmental stress and pathogen elicitors (Mydlarz et al., 2008; Mydlarz & Harvell, 2007; Palmer et al., 2011). Indeed, *M. capitata* exposed to thermal stress showed greater antioxidant activity and lower immunity when primed by a history of high pCO_2-variability (Wall et al., 2018). The increasing role of catalase, peroxidase, and superoxide dismutase activity through time and in the second-bleaching event, compared to attenuated melanin-synthesis pathway, further demonstrates that specialized antioxidative enzymes are important for coping with chronic or persistent stress, as often reported in thermally stressed corals (Gardner et al., 2017; Lesser, 1997). Such constitutive upregulation of cellular defenses have also been observed in corals from areas of high thermal variance (Barshis et al., 2013) and those acclimatized to warmer conditions before the onset of bleaching stress (Bellantuono et al., 2012). In particular, the continued increases in superoxide dismutase and the persistence of high levels of peroxidase in the final 2016-recovery period indicate that increasing constitutive levels of select antioxidants is key to maintaining cellular homeostasis after repeated or chronic thermal perturbations. Such shifts in homeostatic

mechanisms through strategies like gene plasticity (Kenkel & Matz, 2016) or constitutive frontloading (Barshis et al., 2013) reveal the important role of immunity, antioxidants, and their interplay in shaping ecological and evolutionary trajectories of reef corals and the future of coral reefs.

In conclusion, we show coral physiotypes were distinct during each period of bleaching and recovery from 2014-2016, and this indicates a legacy effects driven by chronic stress or beneficial acclimation influenced both bleaching and recovery trajectories. Bleaching was strongly related to symbiont communities and their sensitivity to thermal stress, which was influenced by site environmental history. However, physiotypes were shaped by the cascade of immune and antioxidant activity in the bleaching and recovery periods irrespective of symbiont communities, providing a mechanistic hypothesis for the shifting coral physiotype baseline. Specifically, we observed changes in constitutive immunity from one year to the next in response to extreme stress events, indicating a fundamental change in the homeostatic mechanisms employed by corals. In an era of increasing frequency and magnitude of thermal stress, the ongoing study of mechanisms such as frontloading of genes like heat shock proteins and antioxidants to attenuate bleaching effects and support thermal resilience (Barshis et al., 2013) is of great importance. Additionally, the examination of acclimatization of populations to environmental stress that may be gained through gene expression plasticity (Kenkel & Matz, 2016), or epigenetic mechanisms (Durante et al., 2019; Eirin-Lopez & Putnam, 2019; Liew et al., 2018) will be critical. Our study provides details of cellular and physiological legacy effects in corals and evidence that environmental memory shapes the homeostatic strategies of corals, which ultimately dictates a coral's ability to respond to future stress events.

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Author contributions

C.B.W., C.A.R., L.D.M., R.D.G., and H.M.P. designed the project. C.B.W., C.A.R., L.D.M., and H.M.P. wrote the manuscript, and C.B.W. statistically analyzed the data. Coral collections were performed by C.B.W. and A.D.W. Laboratory analyses were performed by C.B.W., C.A.R., A.D.W., B.E.L., and D.E.K.

Competing interests

The authors declare they have no competing interests.

Data availability

All data and code to generate figures and perform analyses are archived and openly available at Github (https://github.com/cbwall/Gates-Mydlarz-bleaching-recovery/releases/tag/ver.review).

Table 1. Results of PERMANOVA testing the effects of repeated bleaching and recovery periods on *Montipora capitata* corals hosting two distinct symbiont communities at two reef locations.

Factor	*df*	*SS*	R^2	*F*	*p*
Period	3	881.552	0.310	50.870	**<0.001**
Site	1	61.448	0.022	10.638	**<0.001**
Symbiont	1	196.120	0.069	33.951	**<0.001**
Period × Site	3	65.488	0.023	3.779	**<0.001**
Period × Symbiont	3	55.435	0.020	3.199	**<0.001**
Site × Symbiont	1	5.250	0.002	0.909	0.474
Period × Site × Symbiont	3	20.822	0.007	1.202	0.228
Residual	269	1553.885	0.547		
Total	284	2840.000	1.000		

Period = sequential bleaching and recovery events from October 2014 – February 2016, *Site* = Lilipuna or Reef 14, *Symbiont* = *Cladocopium* sp. or *Durusdinium glynnii* dominated symbiont community. *SS* = sum of squares; *df* = degrees of freedom; bold *p* values represent significant effects ($p < 0.05$).

Figure legends

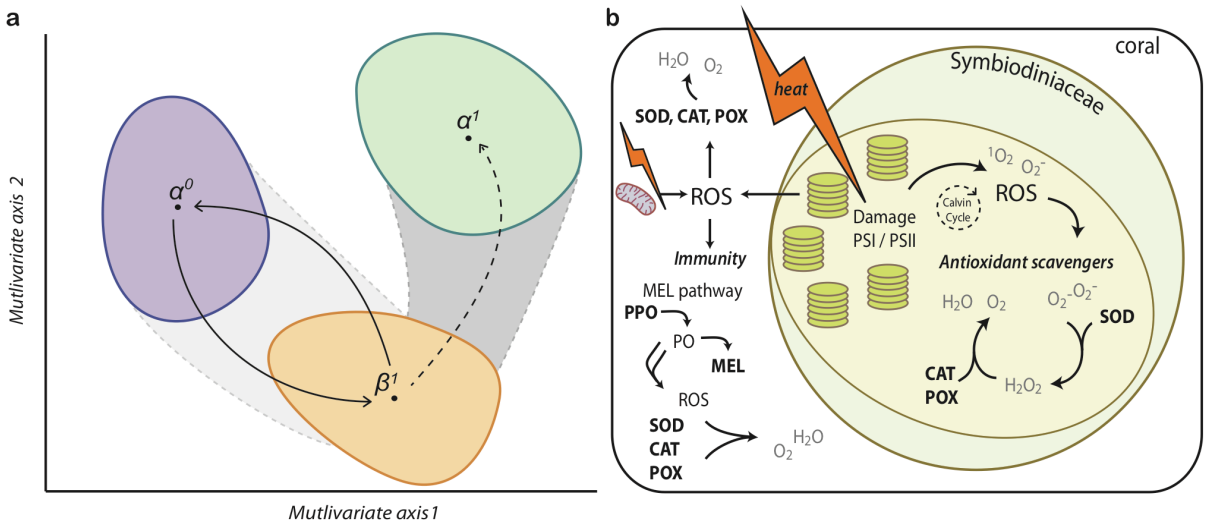

Figure 1. (a) Multivariate analyses identify changes in organism physiotype through space and time by accounting for the variation in multiple traits (Van Straalen, 2003). Transitions of organisms from initial (α^0) to stressed states (β^1), such as during bleaching in response to a heat wave, and their recovery to initial (α^0) or altered states (α^1). (b) Conceptual model of melanin and antioxidant activity in coral and Symbiodiniaceae under heat stress (indicated by lightning bolts). Reactive oxygen species (ROS), oxygen singlets ($^1O_2$) and superoxide (O_2^-), generated by symbiont photochemical dysfunction and host mitochondria membrane damage, are neutralized by a combination of antioxidant scavenger enzymes (e.g., superoxide dismutase [SOD], catalase [CAT], and peroxidase [POX]). Host immunity via the melanin-synthesis pathway can also scavenge ROS during intermediate steps that lead to the synthesis of melanin (MEL). Oxidative bursts during the melanin-synthesis pathway can also create ROS that act as antimicrobials, which may also lead to priming, or antioxidant enzymes up-regulation (shown in bold letters and dark lines).

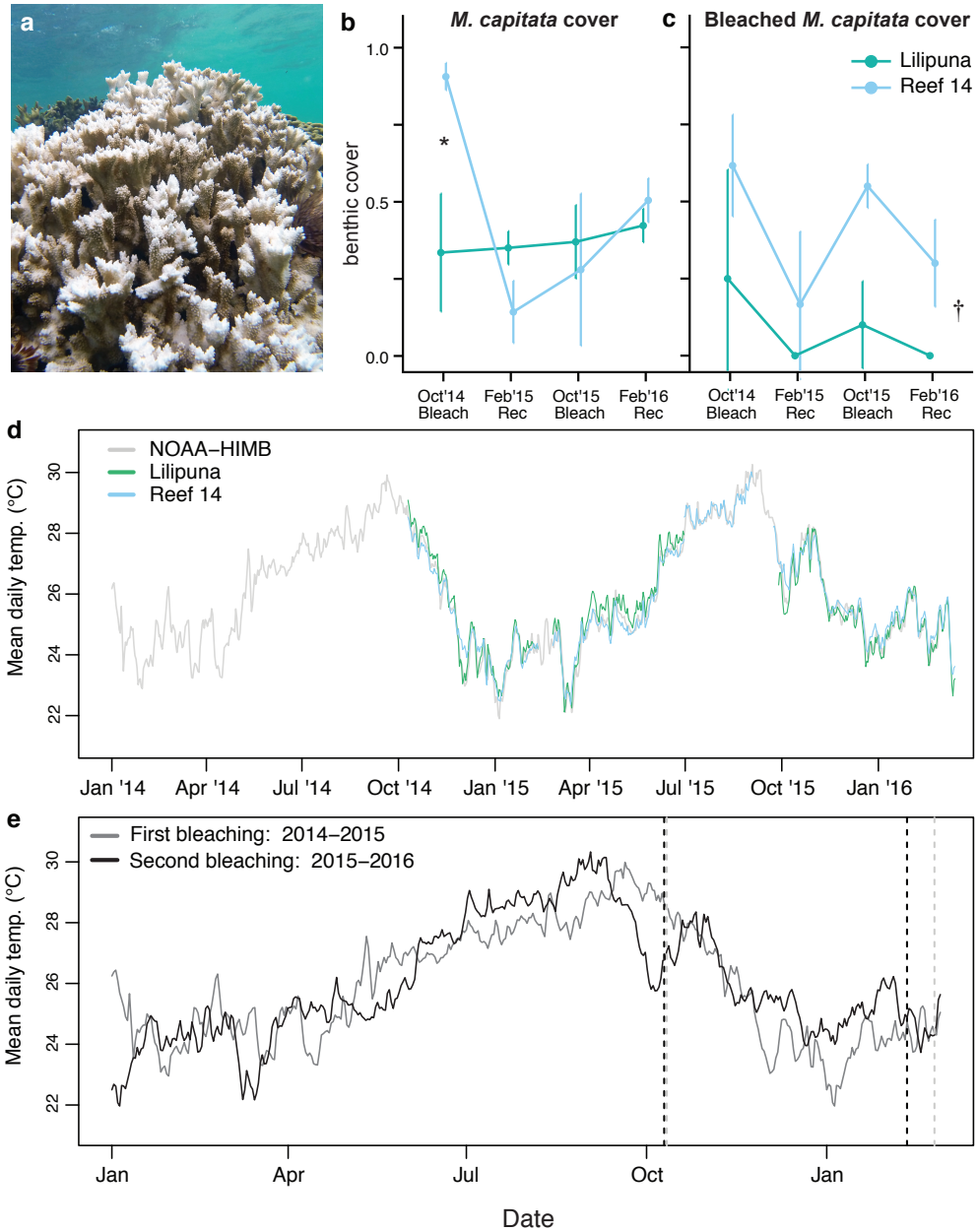

Figure 2. *Montipora capitata* cover and bleaching patterns at two reefs in relation to thermal regimes. (a) A bleached *M. capitata* colony and (b) benthic surveys of absolute *M. capitata* at two sites (Lilipuna and Reef 14) during and after thermal stress in 2014-2015 and 2015-2016. (c) The proportion of bleached *M. capitata* colonies scored during (October) and after (February) thermal stress (values are mean ± SD, *n* = duplicate transects). Symbols indicate differences among sites within a period (*) and among sites (†). (d) Overlay of mean daily temperatures at Lilipuna and Reef 14 and the NOAA-HIMB Moku o Lo‘e buoy from January 2014 - January 2016 and (e) a comparison of temperature ramping and cooling between the first and second bleaching events relative to our collection dates. Vertical dashed lines indicate coral collections during bleaching and recovery periods in the first (*black,* 2014-2015) and second (*gray*, 2015-2016) events.

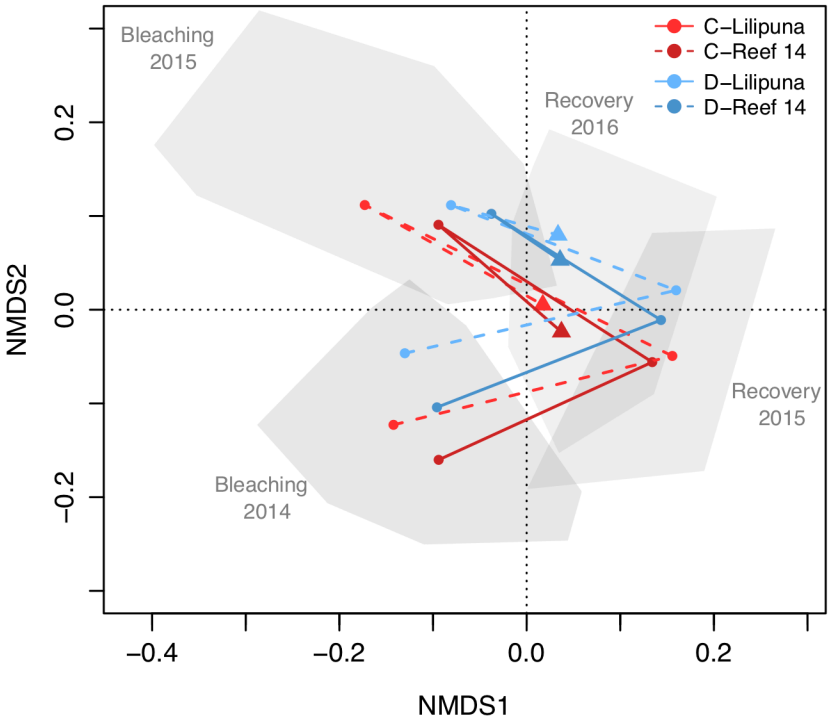

Figure 3. Non-metric multidimensional scaling (NMDS) analyses of coral performance envelopes during bleaching stress and post-bleaching recovery. *Montipora capitata* corals dominated by *Cladocopium* sp. (red) or *Durusdinium glynnii* (blue) symbionts from two sites (*solid lines* Lilipuna, *dashed lines* Reef 14). Convex hulls represent the coral physiotype (i.e., NMDS point clusters) of all corals in each time period, with points indicating mean centroids of physiotype polygon for each group. Lines show trajectories of mean centroids for each group across the four periods (Bleaching 2014, Recovery 2015, Bleaching 2015, Recovery 2016) and triangle arrowheads indicate trajectory termini.

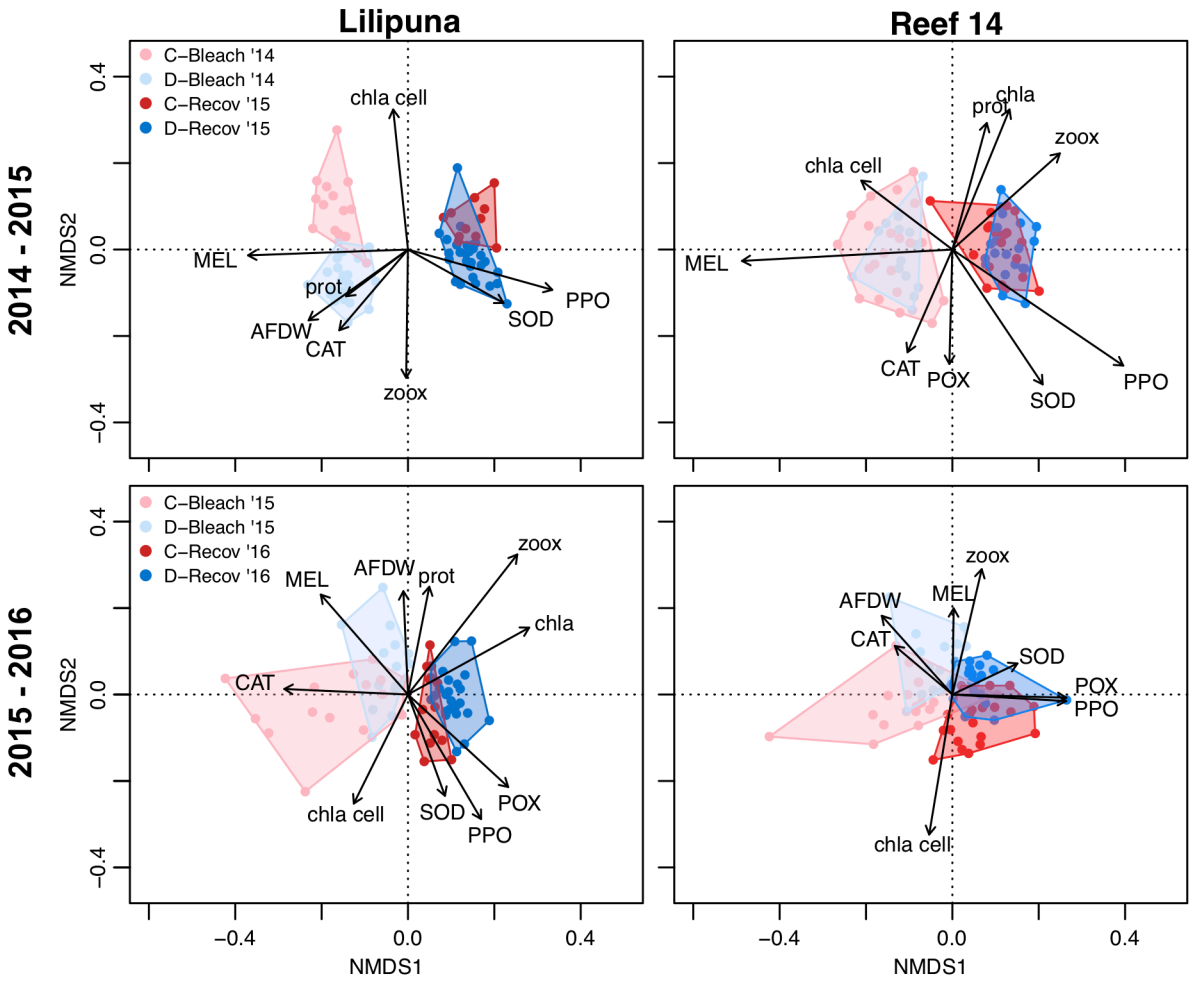

Figure 4. Non-metric multidimensional scaling (NMDS) analyses identify coral physiotypes separated by dominant symbiont type (colors), bleaching-recovery periods (lighter and darker shades), sites (columns), and events (rows). *Montipora capitata* corals dominated by *Cladocopium* sp. or *Durusdinium glynnii* symbionts from Lilipuna (left panel) and Reef 14 (right panel) during bleaching (Bleach) and recovery (Recov) periods. Biplot vectors represent significant physiology and immunity responses ($p < 0.05$) according to goodness of fit statistics. AFDW = ash-free dry weight biomass (mg gdw^{-1}), CAT = catalase, MEL = melanin, POX = peroxidase, PPO = prophenoloxidase, SOD = superoxide dismutase, chla = chlorophyll *a* μg cm^{-2}, chla cell = chlorophyll *a* per symbiont cell, prot = protein mg cm^{-2}, zoox = symbionts cm^{-2}.

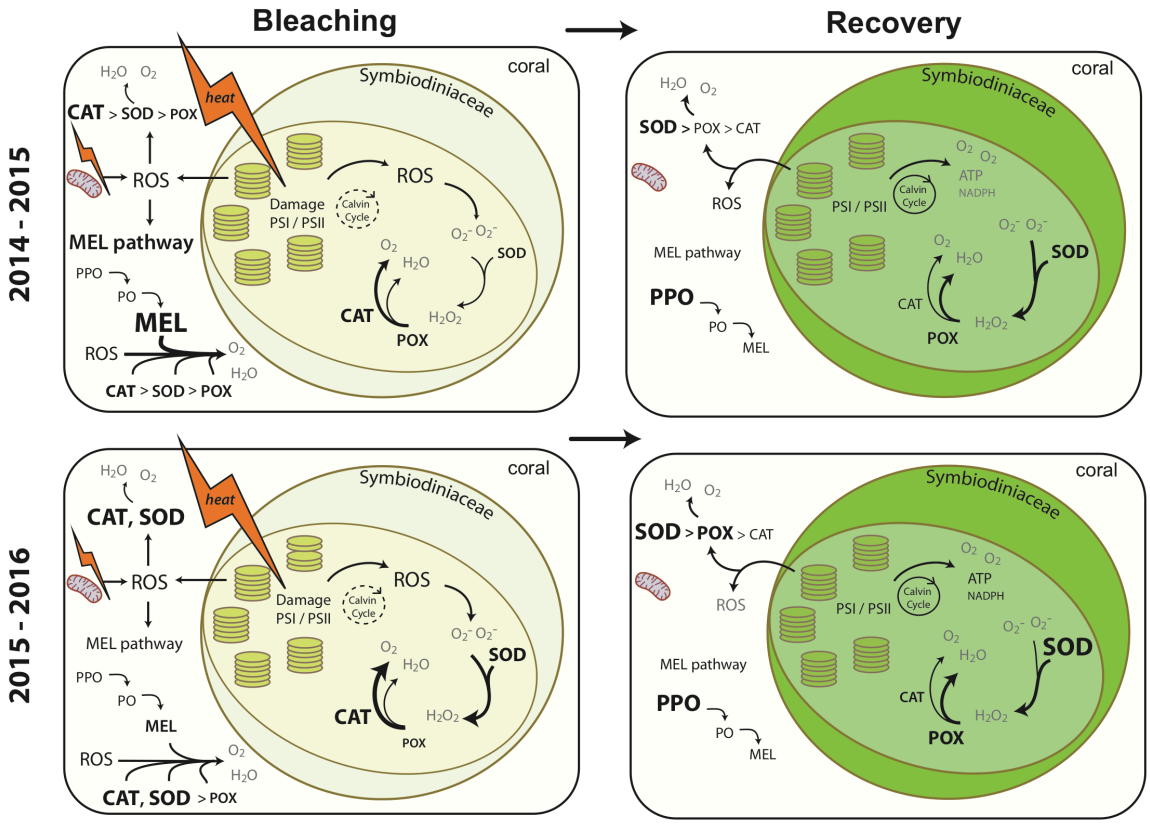

Figure 5. Schematic of mechanistic coral responses to repeated bleaching and recovery leading to shifted physiotypes. In the first event (*top row*) thermal stress leads to a substantial increase in MEL along with modest spikes in CAT and SOD. Corals in the first recovery showed increases in PPO (as the precursor to the melanin-pathway) as well as SOD and POX, while CAT activity declined. In the second event (*bottom row*), CAT and SOD spike with modest contributions of MEL and a general decline in POX. Corals in the second recovery had the highest levels of SOD across all time points, an increase in POX and a sharp decline in CAT (*see* Figure 2b for abbreviations).

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