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The molecular ecophysiology of closely related pocilloporids of the South Pacific: a case study from the Austral and Cook Islands

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Abstract

In April-May 2013, the Khaled bin Sultan Living Oceans Foundation's research vessel, the *M.Y. Golden Shadow*, traversed a ~2,000-km, predominantly unexplored region of the South Pacific Ocean, a marine transect that incorporated reefs abutting the Austral Islands of French Polynesia and the Cook Islands. In addition to conducting traditional coral reef surveys on this four-week mission, molecular biological techniques were employed to attempt to assess the health of pocilloporid corals sampled at the majority of the 59 reefs visited. Ten molecular physiological response variables were measured in a 45-sample subset of the 122 colonies sampled, and, upon utilizing multivariate statistical approaches (MSA), it was found that *Pocillopora verrucosa* samples from Maria Atoll in the Austral Islands differed significantly from all other sampled colonies, particularly with respect to the *hsp90* mRNA expression signatures of their endosymbiotic dinoflagellate (genus *Symbiodinium*) communities. Additionally, a novel outlier detection technique employing heat maps and a variety of MSA identified five outliers (11% of the dataset); curiously, though, there was no effect of environment on outlier frequency. These five colonies were

hypothesized to be displaying aberrant behavior with respect to their cellular physiology at the time of sampling, and it is hoped that this MSA-based outlier discovery approach could be exploited to identify corals of compromised resilience in future coral reef monitoring endeavors.

Abbreviations

1. Analysis of similarity=ANOSIM
2. Ascorbate peroxidase (*apx1*)
3. Austral Islands=AI
4. Average live coral cover=ALCC
5. Biological composition=BC
6. Biological composition response variables=BCRV
7. Canonical axis=CA
8. Canonical correlation analysis=CCA
9. Cook Islands=CI
10. Discriminant analysis=DA
11. Environmental parameters=EP
12. French Polynesia=FP
13. Genome copy proportion=GCP
14. Global Reef Expedition=GRE
15. Heat shock protein=HSP and *hsp* for the protein and gene, respectively
16. Honestly significant difference=HSD
17. Living Oceans Foundation=LOF
18. Maximum=max.
19. Mitochondrial open reading frame=mORF
20. Molecular physiological response variables=MPRV
21. Multidimensional scaling=MDS
22. Multivariate ANOVA=MANOVA
23. Multivariate statistical approaches=MSA
24. National Museum of Marine Biology and Aquarium=NMMBA
25. Not assessed/applicable=NA
26. Not significant=NS
27. Photosynthesis-targeted gene=PTG
28. Photosystem I=*psI*
29. Physiological response variables=PRV
30. Principal component=PC
31. Principal components analysis=PCA
32. Reactive oxygen species=ROS
33. Real-time PCR=qPCR
34. Stress-targeted gene(s)=STG(s)
35. Surface area=SA
36. *Symbiodinium*=Sym
37. Temperature=temp.
38. Threshold cycle=Ct
39. Too few samples=TFS
40. Ubiquitin ligase=*ubiq-lig*
41. Upper control limit=UCL

Introduction

In recent years, there has been an urgent push to learn more about both the fundamental biology and ecophysiology of reef-building corals, which are threatened on a global scale by climate change and other anthropogenic pressures (Fabricius, 2005; Hoegh-Guldberg et al., 2007; Mayfield & Gates, 2007). Such

pervasive stressors necessitate the routine monitoring of coral reefs, a process that currently involves conducting SCUBA or snorkel surveys in which the number of diseased, dying, or dead corals are quantified and reported. Although such data are interesting and of use for certain reef management decisions, they are inherently acquired after the onset of whichever stressor(s) led to the demise of the resident corals (if such was indeed found to be the case). Furthermore, growth and reproductive output, which are ultimately the best predictors of fitness, cannot be measured in a single sampling event (i.e., without monitoring an organism over a multi-day timescale) and so are impractical health assessment metrics for most field-based projects, whereby reefs are typically survived only once or once every few months. Ideally, a prediction about coral health could be made from a biopsy sampled (only once) prior to visual manifestations of severe stress (*sensu* Downs et al., 2000), such as the breakdown of the anthozoan-dinoflagellate (genus *Symbiodinium*) endosymbiosis (i.e., bleaching). In humans, biomarkers measured within blood or biopsies, such as cholesterol, are coupled with whole-organism performance metrics (e.g., blood pressure) to attempt to gauge the health of an individual. Although by no means perfect, parameters such as blood sugar have well defined physiological consequences when their concentrations become too low or too high.

Currently, there are no well-validated biomarkers for the assessment of reef coral health, and a variety of methodological and logistical issues have thwarted efforts to develop such. For instance, although molecular biology-based approaches have been widely utilized to understand both the basic biology (Chen et al., 2015; Mayfield et al., 2010, 2012b, 2014c; Peng et al., 2011) and environmental physiology (Mayfield et al., 2011, 2012a, 2013a-d, 2014a-b, 2016; Putnam et al., 2013) of reef-building corals, only our own works have considered both compartments of the coral-dinoflagellate mutualism in attempting to make mechanistic constructions of cell biology (e.g., Mayfield et al., 2014d). As corals may reach 40% dinoflagellate in terms of their biological composition (BC), any approach for assessing the health of the coral "holobiont" (host+symbiont) that *only* features one member of the association will ultimately tell only part of the story. Therefore, any health/stress test designed for corals would ideally feature molecular physiological response variables (MPRV) spanning both compartments of the endosymbiosis. It is entirely possible, for instance, that one member of the association may be demonstrating early warning signs of stress while the other still appears healthy

from a cell physiology perspective.

An ideal biomarker for proactive coral health assessment would be a molecule (e.g., gene mRNA, protein, or metabolite) whose concentration and/or activity signifies a future physiological change. Therefore, it seems logical to target macromolecules involved in the cellular stress response, such as heat shock proteins (HSPs; Hochachka and Somero, 2002). However, *hsp* mRNAs in reef-building scleractinians and their endosymbiotic *Symbiodinium* populations do not show characteristic up-regulation in response to temperature increases (Mayfield et al., 2011); they appear instead to be constitutively expressed at high levels, thereby hindering their utility as biomarkers. Regardless, it is unlikely that a single macromolecule will accurately describe the health of any organism in a predictable fashion. Therefore, as mentioned above in the context of assaying MPRV across *compartments*, it is advisable to also measure a number of parameters across *biological scales*. Herein, a variety of physiological- and molecular-scale response variables were quantified in pocilloporid corals collected as part of a 2013 research cruise to the Austral Islands (AI) of French Polynesia (FP) and the Cook Islands (CI). Univariate and multivariate statistical approaches (MSA) were used to uncover corals displaying aberrant behavior with respect to 10 MRPV; such corals were hypothesized to be those that were either experiencing stress, or of compromised resilience, at the time of sampling.

Materials and methods

Sample and environmental data collection. The April-May 2013 cruise to the AI+CI aboard the Khaled bin Sultan Living Oceans Foundation's (LOF) *M. Y. Golden Shadow* is described in another work (Mayfield et al., 2015). Briefly, 30 and 29 sites were visited in the AI and CI, respectively, as part of the Global Reef Expedition (GRE), and pocilloporid corals were sampled from 21 and 27 of these sites, respectively. Maximum (max.) colony length (i.e., the distance spanning the two most distant points of the colony; cm; MPRV#1) and planar surface area (SA; cm^2; MPRV#2) were measured from images with ImageJ (National Institutes of Health, USA) by scaling to an object of known size included next to each colony when it was photographed (see Figure 1a-f.). These two proxies for coral size were measured in 60 and 62 coral colonies of the AI and CI, respectively. Color (MPRV#3) was determined subjectively for these 122 colonies and scored as 1=healthy, 2=pale, 3=very pale, or 4=bleached. Of these photographed and sampled colonies, 47 and 42, respectively, were genotyped (Mayfield et al., 2015), and 22 and 23, respectively, from 11 and 14 sites, respectively, were analyzed

herein for all MPRV. Max. length, planar SA, and color were deemed "physiological response variables" (PRV), though color was not included in most analyses due to its subjective nature.

A number of environmental parameters (EP) were assessed at the survey sites, and these included: 1) latitude and longitude (i.e., GPS coordinates), 2) country (n=2: AI, FP vs. CI), 3) island (n=6: Raivavae [AI], Tubuai [AI], Maria Atoll [AI; Figure 1g-i], Rarotonga [CI], Aitutaki [CI], and Palmerston [CI]), 4) site (n=25 of the 59 total for the 45 samples discussed herein), 5) reef type (reef flat vs. fore reef), 6) reef exposure (windward vs. leeward), 7) depth of sampled colony (n=5 categorical groupings: <5 m, 5-10 m, 10-15 m, 15-20 m, and >20 m), 8) sampling date (n=16 separate sampling days), 9) sampling time (n=3 categorical groupings: <10:00, 10:00-14:00, and >14:00), 10) temperature (n=4 categorical groupings: 25-26, 26-27, 27-28, and 28-29°C), 11) salinity (n=5: 35.3, 35.4, 35.5, 35.6, and 35.7), 12) average live coral cover (ALCC; ranging from ~7-70%), 13) coral diversity, 14) fish abundance, 15) fish diversity, and 16) benthic composition. EP 1-8 (excluding survey/sampling dates for Tubuai and Maria Atoll, which were inadvertently excluded and are included herein instead [Table 2]) and 10-12 are presented in Mayfield et al. (2015), sampling time is discussed herein, and parameters 13-16 are not presented. As genotyping was conducted previously with 89 of the 122 sampled colonies (including all but one of the 45 samples discussed herein [#48 from Maria Atoll being the exception]), "host genotype/species" and *Symbiodinium* (Sym) assemblage were also considered as factors that could influence the MPRV discussed below. Latitude/longitude was not included in the statistical analyses, as "site" was considered to sufficiently capture the large-scale spatial variation. Similarly, the vast majority of reefs were fore reefs; therefore, reef type was excluded from the statistical analyses due to the fact that only 2 of the 45 samples were from reef flat environments (86.2 and 88.2 from Rarotonga site CIRR08).

RNAs and DNAs were extracted from the 45 samples, which had been preserved in RNALater® (Life Technologies) and transported under a CITES permit (see Mayfield et al., 2015.) from the CI to Taiwan's National Museum of Marine Biology and Aquarium (NMMBA). Upon extracting the nucleic acids (described in Mayfield et al., 2015), an RNA/DNA ratio was calculated (MPRV#4) to serve as a proxy for total gene transcription. The *Symbiodinium* genome copy proportion (GCP; Mayfield et al., 2009; MPRV#5) was calculated from 20 ng DNA as described previously (Putnam et al., 2013) in order to estimate the density of *Symbiodinium* within the sampled tissues.

These latter two parameters were deemed "biological composition" (BC) response variables (BCRV). It was hypothesized that the former of these two BCRV would undergo increases in stressed corals, as environmental change could lead to elevated expression levels of a variety of gene mRNAs encoding proteins involved in the stress/acclimation response. In contrast, low levels of the *Symbiodinium* GCP were hypothesized to be indicative of stress, as corals require relatively high (10^6 cells/cm^2), stable concentrations of their endosymbiotic dinoflagellates in order to maintain homeostasis with respect to cellular energy budgets (Furla et al., 2005; Gates, 1990).

Despite attempting to sample only the model coral for research, *Pocillopora damicornis* (Traylor-Knowles et al., 2011), a number of cryptic species were nevertheless collected inadvertently due to their similar appearance *in situ* (see Table 2 herein and Mayfield et al., 2015.). Therefore, host coral gene expression was not assessed because it was unclear whether the *P. damicornis* real-time PCR (qPCR) assays developed in our prior works (e.g., Mayfield et al., 2014d) would efficiently bind cDNAs of congeneric species. Instead, only *Symbiodinium* mRNAs were targeted, as our primers have routinely been used successfully to assess endosymbiotic dinoflagellate gene expression in samples across the Pacific Ocean (e.g., Mayfield et al., 2010). Expression of six genes was measured: *hsp40*, *hsp70*, *hsp90* (MPRV#6-8), ascorbate peroxidase (*apx1*; MPRV#9), ubiquitin ligase (*ubiq-lig*; MPRV#10), and photosystem I (*psI*; subunit III). The former three stress-targeted genes (STGs) are molecular chaperones involved in protein processing and quality control and are therefore of critical importance in the eukaryotic stress response (Feder, 1996). *apx1* encodes a protein, APX1, that detoxifies reactive oxygen species (ROS), which are generated during photosynthesis (Lesser, 1996, 1997) and can reach detrimental levels when photoinhibition occurs due to environmental stress (Jones et al., 1998). The final STG, *ubiq-lig*, tags denatured or otherwise non-functional proteins to be digested by the proteasome and is therefore also important in cells experiencing stress (Welchman et al., 2005). The lone photosynthesis-targeted gene (PTG), *psI*, was only measured in a sub-sample of specimens; therefore, it is not discussed further herein. It was hypothesized that the former five genes would undergo up-regulation in response to environmental stress and could thereby serve as mRNA-level biomarkers for assessment of coral health on a proactive, pre-bleaching timescale (*sensu* Downs et al., 2005).

Data analysis I-univariate statistics. The data were first analyzed with univariate ANOVAs (performed with JMP® [ver. 12]) to uncover the effects of the following 10 EP: country, island, site, reef exposure, sampling date, sampling

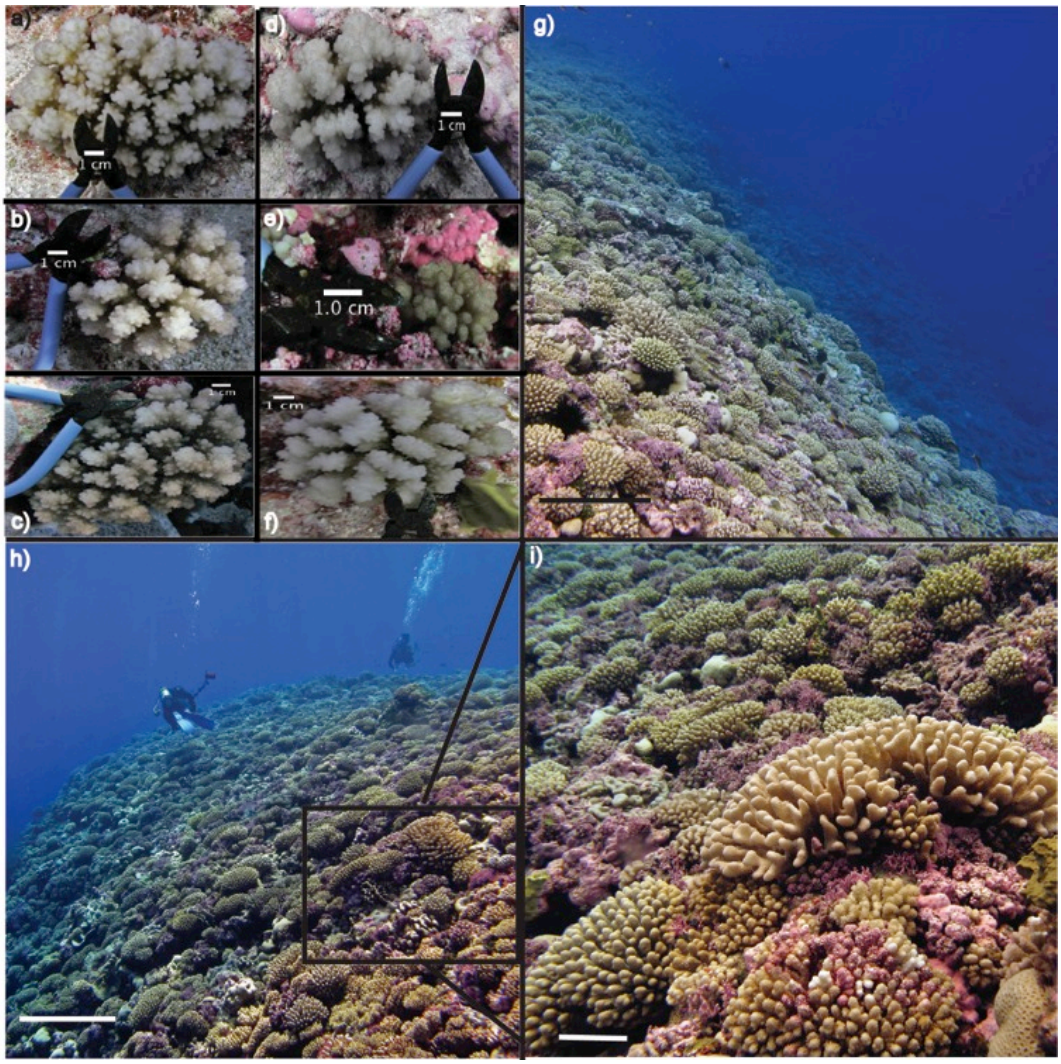

Fig 1. *Pocillopora verrucosa* samples and habitat images of Maria Atoll, Austral Islands. The six corals depicted in (a), (b), (c), (d), (e), and (f) represent samples 43, 47, 49, 50, 51, and 55, respectively; all except 43 were analyzed for all MPRV discussed herein. One sample analyzed from Maria (48) was not genotyped, though it is likely to be *P. verrucosa* based on morphological analysis alone. The reef habitat images of (g), (h), and (i) are of Maria Atoll site AUMA30 (Mayfield et al., 2015), and the final panel is a magnified image of the inset in (h). The scales bars in these latter three panels represent 50, 50, and 10 cm, respectively.

time, colony depth, temperature, salinity, and ALCC. For this analysis, host genotype and *Symbiodinium* assemblage were also considered as factors that could influence the following 10 MPRV: colony color, max. colony length, colony planar SA, RNA/DNA ratio (unit-less), *Symbiodinium* GCP (unit-less), and expression of the five *Symbiodinium* mRNAs (raw, inverse threshold cycle [Ct] values normalized to the *Symbiodinium* GCP). Host coral species frequency (freq.) was also tested as a response variable, in which case likelihood ratio-based chi-squared tests were instead used to determine if the relative proportions of the five species (see Table 2.) differed across the 10 EP. There were too few samples (TFS) with mixed-clade *Symbiodinium* assemblages to consider the effect of this parameter on host genotype freq. (Table 1). Since 128 comparisons (see Table 1.) were performed, a Bonferroni adjustment of 11.4 ($\sqrt{128}$) was made to the *a priori*-established α level of 0.05, resulting in a multiple comparisons-adjusted α of 0.004.

Two additional univariate statistical approaches were used to uncover MPRV and EP that contributed significantly to the overall variation between samples. First, JMP's predictor screening function was used to rank the MPRV in terms of their proportional contribution to the cumulative difference between three EP that were found to significantly influence a number of MPRV in the preliminary, univariate ANOVA-based analysis (Table 1): 1) island, 2) temperature, and 3) host genotype. Heat maps were then created with JMP to visually depict variation in nine of the MPRV; color was excluded due to its semi-quantitative nature, and host species/genotype was considered as an EP in this analysis. Furthermore, there were too few samples with mixed-clade *Symbiodinium* assemblages to plot this parameter on the heat map.

A heat map score was then generated for each of the 45 samples. A score of "1" was given when a sample's *Z*-score was less than -2 (extreme low outlier; see scale of Figure 2c.) or greater than 4.5 (extreme high outlier) for any MPRV. For instance, a sample with *Z*-scores of -2 (the lightest shade of green in Figure 2c) for one MPRV and +5 (the lightest shade of red in Figure 2c) for another would be given a heat map score of 2. This method was the first of three used to identify systematic outliers. Max. length and planar SA were included in the heat map but excluded from this scoring analysis, as large/old corals are not necessarily healthier than small/young colonies. Therefore, only the two BCRV (*Symbiodinium* GCP and RNA/DNA ratio) and the five STGs were considered in the calculation of the heat map score.

Data analysis II-multivariate statistics. Three MSA were used to understand the relationship between environment and the molecular physiology of the 45 samples using

Z-score-transformed data: 1) principal components analysis (PCA), 2) canonical correlation analysis (CCA)-based multivariate ANOVA (MANOVA) conducted with JMP's discriminant analysis (DA) function, and 3) multidimensional scaling (MDS) performed with PRIMER (ver. 5). The former and latter were used to depict overall variation in the dataset, as well as identify outliers (those samples falling outside of the core [i.e., interior] region of the dataspace), and ANOSIM was performed with PRIMER to determine the effect of the following eight EP on the composite phenotype: country, island, reef exposure, site temperature, site salinity, host genotype, host colony depth, and sampling time. Of the 45 samples, all but 3 were comprised of clade C *Symbiodinium* only; therefore, the sample size for determining the effect of *Symbiodinium* assemblage on coral molecular physiology was too small. ANOSIM *p*-values were considered significant at an α of 0.05, and at least 999 permutations were performed. DA was performed to determine the effects of the two EP that were found to most greatly influence variation in the dataset (discussed below), island and host genotype/species, and Wilks' lambda was considered significant when $p<0.01$. DA was also used to determine the MPRV that best separated the multivariate means (i.e., centroids) within the 2-dimensional dataspace encompassed by the first two canonical axes (CA) for these two comparisons.

In addition to uncovering outliers 1) with heat maps and 2) via PCA/MDS, a third type of outlier analysis was performed with JMP to identify samples whose multivariate means deviated significantly from the global centroid, and this analysis considered two of the three PRV (excluding color), the two BCRV, and the five STGs. Samples with Mahalanobis distances significantly higher than the upper control limit (UCL) calculated by JMP were deemed "Mahalanobis outliers." When a sample was 1) considered a Mahalanobis outlier and 2) given a heat map score of 1 or greater, said sample was considered to have displayed aberrant behavior and deemed a "tier 1 outlier." When a sample was considered a Mahalanobis outlier but did not have a heat map score greater than 0, it was deemed a "tier 2 outlier." When a sample was given a heat map score of 1 but was not a Mahalanobis outlier, it was deemed a "tier 3 outlier;" only tier 1 and 2 outliers were considered in the total outlier count for the dataset. Therefore, the Mahalanobis distance was given more weight than the heat map score for determining a true (i.e., tier 1) outlier. This is because the Mahalanobis distance considers all MPRV simultaneously and is therefore more conservative than the heat map score, in which the seven MPRV used for calculating the score were considered individually.

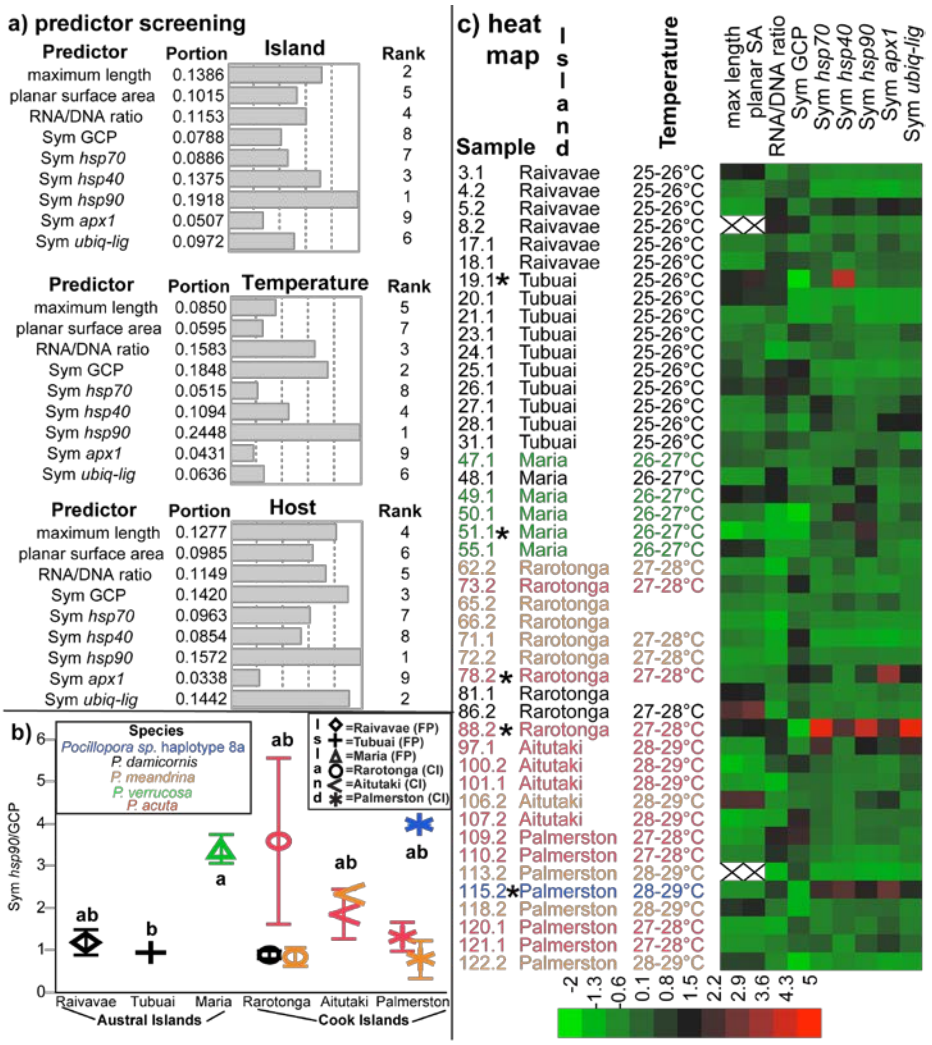

Fig 2. Univariate statistical analysis of the AI+CI dataset. Various univariate statistical approaches were used to analyze the 45 samples. (a) JMP's predictor screening function was first utilized to identify MPRV contributing the greatest proportions of the cumulative differences between islands (n=6), temperatures (n=4 categorical groupings), and host (n=5 species) with *Z*-score-transformed data. It should be noted that the portion plots are not all of the same scale. Expression of the *Symbiodinium hsp90* mRNA was plotted across island and host coral species (b), and error bars represent standard deviation of the mean. For certain data points, error bars do not extend beyond the icon and so are not evident. There were significant effects of island, host, and temperature on *hsp90* expression (Table 1), and the letters above error bars represent Tukey's *post-hoc* groups ($p<0.05$) between islands. The species color code in the legend also applies to (c). In (c), the heat map was scaled uniformly across all nine MPRV using non-transformed data, and the five tier 1+2 outliers (Table 2) have been denoted by asterisks (*).

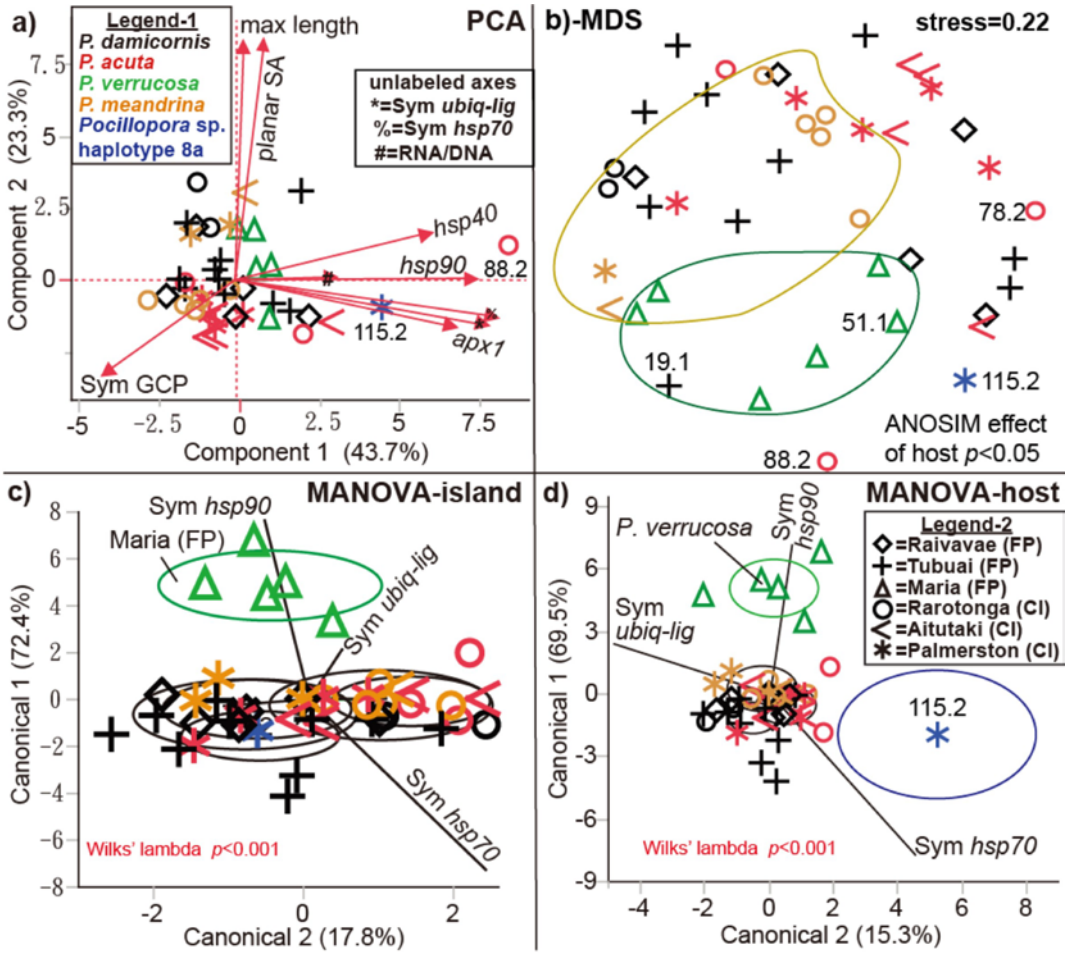

Fig 3. MSA of the AI+CI dataset. PCA (a), MDS (b), and MANOVA-based DA (c-d) were used to uncover variation within the dataset and determine which MPRV best partitioned samples within it. The host coral species legend in (a) and the island legend in (d) apply to all other panels. In the PCA and MDS plots, two outliers, 88.2 and 115.2, have been labeled, and all five outliers have been labeled in the latter. In (b), the *P. meandrina* and *P. verrucosa* samples were encircled by hand to emphasize clustering. In the DA CCA plots, only the dominant vectors have been shown, and centroids represent 95% confidence. It is clear from panels c-d that *P. verrucosa* samples from Maria Atoll, which were sampled at temperatures between 26 and 27°C, were distinct from all other samples, and this is due in part to their higher *hsp90* mRNA expression levels.

Table 1. Summary of univariate ANOVA and chi-squared test results. Host was considered as either a MPRV (left-most column) or as an EP (top row); the former (as frequency [freq.] data) was analyzed by likelihood ratio-based chi-squared tests to determine whether coral host species were equally represented across each of the 11 remaining EP while the latter was analyzed by one-way ANOVA for the individual comparisons of interest. For comparisons across ALCC, linear regression *t*-tests were used to determine if the slopes were significantly greater than 0, as the raw values, not categorical data, were used in the ANOVA model. As 128 separate comparisons were performed (12 EP x 11 MPRV minus all labeled as "too few samples" [TFS] or as "--"), a Bonferroni adjustment of 11.4 was made to the α level of 0.05, resulting in a multiple comparison-controlled, modified α of 0.004. Only comparisons highlighted in green fulfilled this criterion, with marginally significant *p*-values instead highlighted in yellow.

EP (top row)/ **MPRV** (left-most column)	country	island	site	exposure	date	time	depth	temp	salin-ity	ALCC	host	Sym assem-blage	Comparisons (Tukey's HSD)
host (freq.)	0.00	0.00	TFS	NS	TFS	NS	0.00	0.00	0.00	NS	--	NS	
color	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
max. length	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
planar SA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
RNA/DNA	0.02	NS	0.03	NS	NS	NS	NS	NS	NS	NS	0.01	NS	FP>CI, *P. damicornis>P. meandrina*
Sym GCP[a]	NS	NS	NS	NS	NS	NS	NS	0.04	NS	NS	NS	NS	27-28°C>26-27°C
hsp40[b]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
hsp70	NS	NS	NS	NS	NS	NS	0.04	NS	NS	NS	0.02	NS	
hsp90	NS	0.05[c]	NS	0.01	0.03	NS	NS	0.02	NS	NS	0.00[c]	NS	Maria Atoll>Tubuai, windward>leeward, 26-27°C>25-26°C, *P. verrucosa>P. damicornis+P. meandrina*
apx1	NS	NS	0.02	NS	NS	NS	NS	NS	NS	NS	0.02	NS	*Pocillopora* sp. haplotype 8a>*P. damicornis+P. verrucosa+P. meandrina*
ubiq-lig	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	

[a]significantly and positively correlated with color. [b]significantly and negatively correlated with color. [c]See Figure 2b.

Table 2. Sample information. GPS coordinates and maps of sites, as well as sample collection dates, NCBI accession numbers for sample mitochondrial open reading frame (mORF) sequences, sample Sym assemblages, site temperatures, site salinities, site ALCC values, site exposures, and reef types can be found in Mayfield et al. (2015). When the sample was deemed a Mahalanobis outlier and its heat map score was 1 or greater (highlighted in red), it was considered a tier 1 outlier and assumed to be a coral displaying aberrant behavior. The values for the MPRV that most significantly contributed to said sample being labeled as an outlier are italicized in bold. In contrast, one sample from Maria Atoll (51.1) and one from Palmerston (115.2) were deemed outliers by their Mahalanobis distance scores but were characterized by heat map scores of 0; these tier 2 outliers have been highlighted in yellow and may have been characterized by aberrant physiology at the time of sampling. The lone tier 3 outlier (heat map score>0 only), sample 50.1 from Maria Atoll, has been highlighted in green. There was not a statistically significant effect of island on outlier frequency (chi-squared likelihood ratio test, $p>0.05$).

Island	Host	Collection time	Color	Max. Length	Planar SA	Sym GCP (unit-less)	RNA/ DNA (unit-less)	*Symbiodinium* mRNA expression					Mahala-nobis outlier?	heat map score
Sample				(cm)	(cm^2)			*hsp40*	*hsp70*	*hsp90*	*apx1*	*ubig-lig*		
Raivavae (FP; 6/18)														
3.1	*P. damicornis*	16:15	3	13	108	0.27	0.34	82	130	22	3.8	2.3		0
4.2	*P. damicornis*	9:30	3	7	24	0.31	0.6	27	35	7.3	0.64	0.69		0
5.2	*P. damicornis*	16:15	3	5.2	14	0.22	0.95	***440***	290	52	***11***	9.0		0
8.2	*P. damicornis*	16:40	3	NA	NA	0.37	1.1	240	160	33	6.6	3.3		0
17.1	*P. damicornis*	11:35	3	7.1	31	0.19	0.65	260	140	22	5.6	2.9		0
18.1	*P. damicornis*	11:40	3	5.4	17	0.31	0.83	130	340	47	8.2	7.2		0
	Raivavae avg.±std. dev.		3.0±0.0	7.5±3.2	39±39	0.28±0.07	0.75±0.27	200±150	180±110	31±17	6.0±3.6	4.2±3.2	**Freq.**	**0/6**
Tubuai (FP; 10/19)														
19.1[a]	*P. damicornis*	8:45	2	13	125	0.07	0.83	320	69	8.0	1.6	1.3	**Yes**	2
20.1[a]	*P. damicornis*	8:50	4	13	85	0.14	0.84	23	18	2.3	0.69	0.10		0
21.1[a]	*P. damicornis*	9:00	3	6.2	21	0.15	0.51	104	21	3.3	0.81	0.20		0
23.1[a]	*P. damicornis*	10:35	4	7.2	45	0.34	0.36	270	390	38	5.3	10.1		0
24.1[a]	*P. damicornis*	10:45	4	9.1	42	0.19	0.17	203	150	18	2.5	2.6		0
25.1[a]	*P. damicornis*	10:55	3	11	39	0.43	0.86	64	160	27	3.0	2.9		0
26.1[a]	*P. damicornis*	14:55	3	12	69	0.36	1.1	94	260	42	6.5	7.5		0
27.1[a]	*P. damicornis*	15:00	4	6.5	28	0.23	0.86	240	460	30	5.0	9.4		0
28.1[a]	*P. damicornis*	15:10	3	6.4	19	0.18	0.57	170	280	21	8.0	8.4		0
31.1[b]	*P. damicornis*	9:10	3	9.1	41	0.28	0.79	190	180	43	6.5	1.6		0
	Tubuai avg.±std. dev.		3.0±0.7	9.4±2.7	51±33	0.24±0.11	0.69±0.28	170±95	200±150	23±15	4.0±2.6	4.4±4.0	**Freq.**	**1/10**

Island / Sample	Host	Collection time	Color	Max. Length (cm)	Planar SA (cm^2)	Sym GCP (unit-less)	RNA/ DNA (unit-less)	*Symbiodinium* mRNA expression					Mahala-nobis outlier?	heat map score
								hsp40	*hsp70*	*hsp90*	*apx1*	*ubig-lig*		
Maria (FP; 6/16)														
47.1[c]	*P. verrucosa*	9:55	3	9.6	53	0.20	0.47	160	140	56	2.8	3.7		0
48.1[c]	unknown	10:10	1	5.4	16	0.18	1.0	***406***	77	30	2.0	2.5		0
49.1[c]	*P. verrucosa*	10:20	4	14	89	0.28	1.0	240	260	109	4.0	8.0		0
50.1[c]	*P. verrucosa*	11:50	1	7.9	37	0.08	0.13	***220***	58	23	1.6	1.4		1
51.1[c]	*P. verrucosa*	12:00	4	2.8	4.6	0.12	0.13	130	150	***56***	3.4	4.4	**Yes**	0
55.1[c]	*P. verrucosa*	16:30	1	13	86	0.18	0.29	140	160	54	4.8	4.0		0
	Maria avg.±std. dev.		2.3±1.5	8.8±4.3	48±35	0.17±0.07	0.50±0.04	200±103	140±72	55±30	3.1±1.2	4.4±2.2	**Freq.**	**1/6**
Rarotonga (CI; 10/34)														
62.2	*P. meandrina*	15:10	3	6.1	23	0.27	0.12	307	120	20	5.0	2.5		0
73.2	*P. acuta*	9:10	3	9.3	44	0.40	0.42	220	170	28	3.4	4.3		0
65.2	*P. meandrina*	9:00	1	7.3	29	0.23	0.30	290	170	33	6.3	2.5		0
66.2	*P. meandrina*	9:15	3	5.7	15	0.26	0.11	120	120	18	2.4	1.4		0
71.1	*P. meandrina*	14:40	4	7.4	27	0.43	0.20	33	38	6.8	0.59	0.13		0
72.2	*P. meandrina*	15:00	3	5.5	18	0.32	0.22	190	160	37	4.6	3.3		0
78.2	*P. acuta*	11:25	4	6.3	26	0.41	0.45	170	670	109	***34***	19	**Yes**	1
81.1	*P. damicornis*	14:25	4	14	100	0.28	0.39	106	160	27	5.9	3.8		0
86.2	*P. damicornis*	11:20	4	17	162	0.32	0.23	200	150	25	4.8	2.2		0
88.2	*P. acuta*	11:55	3	13	108	0.10	0.91	***340***	***500***	***77***	***6.5***	12	**Yes**	4
	Rarotonga avg.±std. dev.		3.2±0.9	9.2±4.1	55±50	0.30±0.10	0.34±0.23	200±96	230±200	38±31	7.3±9.5	5.1±5.8	**Freq.**	**2/10**

Island / Sample	Host	Collection time	Color	Max. Length (cm)	Planar SA (cm^2)	Sym GCP (unit-less)	RNA/ DNA (unit-less)	*Symbiodinium* mRNA expression					Mahala-nobis outlier?	heat map score
								hsp40	*hsp70*	*hsp90*	*apx1*	*ubig-lig*		
Aitutaki (CI; 5/13)														
97.1	*P. acuta*	10:10	3	5.8	14	0.20	0.89	450	***900***	73	7.3	***19***		0
100.2	*P. acuta*	12:00	4	3.2	4.7	0.38	0.66	240	390	45	4.7	8.0		0
101.1	*P. acuta*	12:15	3	5.6	8.0	0.26	0.11	140	220	30	3.7	6.8		0
106.2	*P. meandrina*	9:30	2	17	142	0.24	0.34	120	210	54	9.4	2.2		0
107.2	*P. acuta*	9:45	4	3.8	7.1	0.45	0.26	220	430	65	9.0	8.4		0
	Aitutaki avg.±std. dev.		3.2±0.9	9.2±4.1	55±50	0.30±0.10	0.34±0.23	200±96	230±200	38±31	7.3±9.5	5.1±5.8	**Freq.**	**0/5**
Palmerston (CI; 8/16)														
109.2	*P. acuta*	10:00	4	5.2	17	0.47	1.1	240	400	73	11	4.6		0
110.2	*P. acuta*	10:20	4	6.7	29	0.29	0.89	86	190	14	2.8	3.8		0
113.2	*P. meandrina*	16:00	4	NA	NA	0.32	0.12	11	11	2.7	0.43	0.062		0
115.2	*Pocillopora* sp. haplotype 8a	9:40	3	6	20	0.13	0.80	***430***	***340***	52	***9.2***	5.6	**Yes**	0
118.2	*P. meandrina*	11:45	4	12	98	0.13	0.47	110	62	21	3.0	1.2		
120.1	*P. acuta*	9:00	2	5.2	12	0.30	0.19	260	150	33	8.0	3.2		0
121.1	*P. acuta*	9:20	3	5.8	18	0.27	0.42	60	310	57	10	5.5		0
122.2	*P. meandrina*	11:10	4	11	76	0.09	0.36	33	15	5.4	0.41	0.038		0
	Palmerston avg.±std. dev.		3.5±0.8	7.4±2.9	39±34	0.25±0.13	0.54±0.35	150±140	190±150	32±26	5.6±4.4	3.0±2.3	**Freq.**	**1/8**

[a]Sampled on 2013-4-14. [b]Sampled on 2013-4-15. [c]Sampled on 2013-4-20.

Results and discussion

Univariate statistical results. Although environmental datasets featuring a large number of both EP and MPRV are better analyzed by the MSA presented below, a preliminary, univariate statistics-based approach was used to uncover MPRV that demonstrated significant variation across various EP. It is clear from Table 1 that most comparisons yielded negative results except for host species freq., which varied significantly across country, island, depth, temperature, and salinity. This result was unsurprising given that Mayfield et al. (2015) found a degree of spatial niche partitioning within the *Pocillopora* genus in this same sample set. Specifically, *P. damicornis* was much more likely to be found in the AI than in the CI, where its closely related sister species *P. acuta* was more common.

Only one other MPRV, the *Symbiodinium hsp90* mRNA, varied significantly after the Bonferroni adjustment was made; specifically, *Symbiodinium* populations within the five host coral species expressed significantly different levels of this gene (Table 1). To a lesser extent, expression of this gene also varied across island and temperature. For that reason, JMP's predictor screening function was used to rank the MPRV in terms of their influence on the cumulative difference between islands, temperatures, and hosts (Figure 2a), and, indeed, *hsp90* contributed most significantly to the collective difference between 1) the six islands (19% of the cumulative difference), 2) the four temperature groupings (24.5%), and 3) the five host species (16%). The island and host effects were evident upon plotting (Figure 2b), and the *hsp90* mRNA expression of *Symbiodinium* populations within corals of Maria Atoll (AI) was significantly higher than that of *Symbiodinium* within corals of Tubuai (AI; Tukey's honestly significant difference [HSD] $p<0.05$).

When looking at the heat map of the nine MPRV across the 45 samples (Figure 2c), it is evident that several samples displayed aberrant behavior/physiology. Specifically, sample 19.1 displayed an anomalously low *Symbiodinium* GCP (i.e., *Symbiodinium* density) and anomalously high *hsp40* mRNA expression, resulting in a heat map score of two (Table 2). Sample 78.2 from Rarotonga expressed the *apx1* mRNA at a slightly anomalous level, resulting in a heat map score of one. Sample 88.2 (also from Rarotonga) had the highest heat map score (Table 2): four. This was due to an anomalously low

Symbiodinium GCP (+1) and anomalously high *hsp70*, *hsp90*, and *ubiq-lig* mRNA expression (+3 summed across all three mRNA targets).

MSA results. Normal quantile plots and heat maps can only focus on one response variable at a time, while MDS, PCA, and CCA can consider multiple MPRV in a concerted, integrated fashion. Herein three different MSA were used to portray the dataspace in a holistic manner. First, PCA was employed for the nine MPRV (excluding color, host genotype, and Sym assemblage). PCA did not appear to distinguish samples by EP to any great extent, despite the fact that the first two eigenvectors captured nearly 70% of the variation. Interestingly, the first three principal components (PC) were comprised of 1) the five *Symbiodinium* STGs (44%), 2) the two PRV (23%), and 3) the two BCRV (12.5%), respectively, suggesting that the three groupings of MPRV show contrasting patterns. As was documented in another study (Mayfield 2016), *Symbiodinium* gene expression was negatively correlated with *Symbiodinium* density. This negative correlation between gene expression and the *Symbiodinium* GCP was statistically significant for *hsp40* and *hsp90* and may be due to the fact that, as *Symbiodinium* densities decrease due to, for instance, elevated temperatures (i.e., bleaching), the remaining cells are likely simultaneously undergoing a stress response necessary to restore homeostasis in light of said environmental stressor. In contrast, when *Symbiodinium* densities are high, the coral holobiont is likely healthy, and so there is possibly a decreased chance that cells are experiencing stress; therefore, high *Symbiodinium* density could indeed be hypothesized to be associated with lower levels of expression of the five STGs.

Despite the fact that PCA failed to partition samples by island, host, or any other EP, it did identify two outliers: Rarotonga 88.2 and Palmerston 115.2. As mentioned above, the former exhibited the highest heat map score, suggesting that PCA can be used to corroborate univariate statistics-based findings. PRIMER's MDS function was also able to separate sample 88.2, and, to a lesser extent, 115.2, from the other 43 samples in the plot (Figure 3b). Furthermore, sample 19.1, which was a *P. damicornis* sample from Tubuai, more closely clustered with the *P. verrucosa* samples from Maria Atoll; indeed, this sample was found to be an outlier in terms of both its heat map score (2) and its Mahalanobis distance value. As with PCA, it appears that MDS could be a means of identifying outliers, albeit instead via the

use of the Bray-Curtis similarity matrix. One other notable trend stands out in the MDS plot; the *P. verrucosa* samples seemed to cluster together, as did the *P. meandrina* samples. This suggests an effect of host on the composite molecular physiology, which was indeed verified by ANOSIM, (albeit marginally; p=0.05). However, as the stress of the MDS plot was above 0.2, this finding should be interpreted with caution.

Unlike the univariate approaches (Table 1), neither PCA nor MDS were able to verify a significant island effect, and the data from the six islands appear intermixed in Figure 3a-b. To determine quantitatively whether island was indeed a factor significantly influencing the composite molecular physiology of the 45-sample dataset, JMP's DA function, which is based on MANOVA and CCA, was used to generate multivariate island centroids within a two CA dataspace (Figure 3c). From the Wilks' lambda p-value, it is clear that island significantly influenced the multivariate MPRV response, and *hsp90* mRNA expression appears to be the MPRV that best partitioned those samples of Maria Atoll from the other samples along CA1. Given that the predictor screening function found *hsp90* to be the MPRV that contributed most greatly to the cumulative difference between islands, this is an unsurprising result.

It was actually the negative relationship between *Symbiodinium hsp90* and *hsp70* mRNA expression that accounted for the separation of the *P. verrucosa* samples from Maria Atoll from other samples. Given that *P. verrucosa* was only sampled at Maria Atoll, the DA of host (Figure 3d) yielded similar information: *P. verrucosa* samples were distinct from the others, and this was also due to an inverse relationship between *hsp70* and *hsp90*. Temperature also partitioned *P. verrucosa* samples of Maria Atoll away from all others (data not shown), as this was the only island visited whose temperature was between 26 and 27°C. Therefore, it cannot currently be stated which was more important in driving the differences between the *P. verrucosa* samples of Maria Atoll (Figure 1) and all other samples: the temperature, the atoll, or the host. All that can currently be stated is that *P. verrucosa* samples of this atoll have unique gene expression profiles, and, furthermore, the majority of them were quite pale (Figure 1a-f); only upon the analysis of conspecifics sampled at similar temperatures elsewhere can it be conclusively determined whether such signatures show fidelity to host (i.e., *P. verrucosa*), temperature (26-27°C), or island (Maria Atoll), if any of the above.

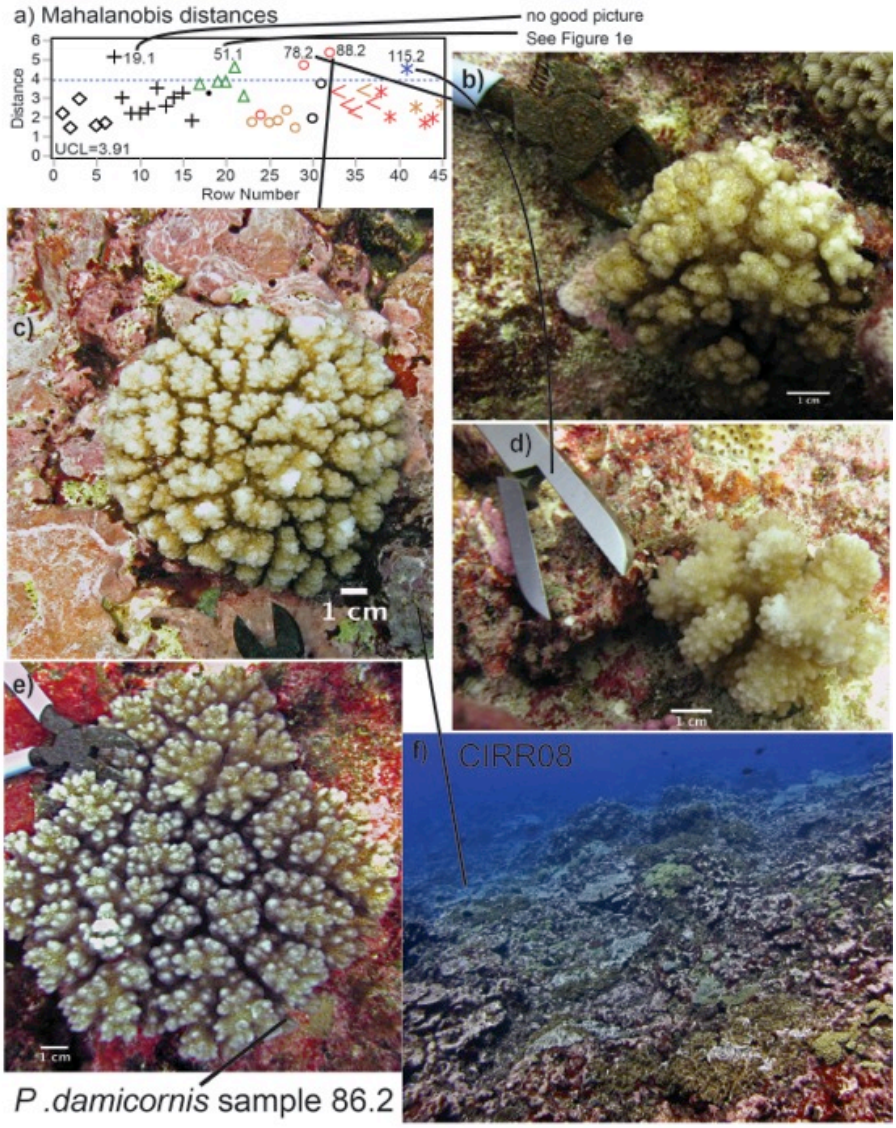

Fig 4. Mahalanobis distances and images of the outlier samples. For panel a, please see Figures 2-3 for the species and island legends. The five Mahalanobis outliers (i.e., those falling above the UCL of 3.91) have been labeled in not only this figure, but also in Figures 2 and 3. The sample from Maria Atoll represented by the filled circle was not genotyped. There were no good quality pictures of sample 19.1 from Tubuai, and sample 51.1 from Maria Atoll is depicted in Figure 1e. The remaining tier 1 (78.2 [b] and 88.2 [c]) and tier 2 (115.2 [d]) outliers have been shown, in addition to a coral colony (86.2 [e]), sampled from the same reef (site CIRR08 [f]) as the most significant outlier in the dataset: sample 88.2 (c).

Outlier analysis. Despite such a unique gene expression signatures with respect to the relationship between *hsp70* and *hsp90*, only one *P. verrucosa* sample from Maria Atoll, 51.1 (Figure 1e), was considered a Mahalanobis distance distance outlier (Figure 4a); interestingly, this sample did not display aberrant behavior for any MPRV in the heat map (Figure 2c), making it a tier 2 outlier. Similarly, sample 115.2 from Palmerston (Figure 4d), which was identified as an outlier by PCA, MDS, and its Mahalanobis distance score (Figure 4a), was also characterized by a heat map score of 0. This *Pocillopora* sp. haplotype 8a sample was the second of only two tier 2 outliers in the dataset. In contrast, sample 19.1, which was also a Mahalanobis distance outlier, was characterized by a heat map score of 2 and was therefore considered a tier 1 outlier. Sample 19.1 clustered with samples of *P. verrucosa*, rather than other *P. damicornis* samples, in the MDS plot; this signifies that it did not behave like conspecifics and supports its being labeled a tier 1 outlier.

There were only two additional tier 1 outliers, 78.2 (Figure 4b) and 88.2 (Figure 4c), both of which were *P. acuta* samples from Rarotonga. The former was characterized by a heat map score of only 1, whereas the latter had the highest heat map score of the dataset: 4. Furthermore, 88.2 was clearly identified as an outlier by PCA and MDS. Collectively, then, sample 88.2 demonstrated the most aberrant behavior with respect to the nine MPRV of the entire 45-sample dataset. This *P. acuta* sample was collected at site CIRR08 (Figure 4f) at 9 m depth and hosts *Symbiodinium* of clade C only. It was sampled close to midday, as was sample 78.2. Curiously, CIRR08 is the most distant site from Avarua, the main population center of Rarotonga, so it is unlikely that anthropogenic influence can be directly linked to the presumed level of stress experienced by this coral. It *did* represent one of only two corals sampled from a reef flat habitat in the entire dataset, the other being a *P. damicornis* sample (i.e., 86.2) from this same reef (16 m); however, sample 86.2 (Figure 4e) exhibited normal behavior.

There are myriad reasons why sample 88.2 may have exhibited aberrant behavior while a congeneric at the same site did not. It was the shallowest sample collected in Rarotonga amongst those processed for all nine MPRV, and high wave energy characterized the reefs during the survey and sampling periods; indeed, the reef was evidently scoured (Figure 4f). High wave energy would be more likely to influence shallower corals, and turbulent water may have caused rubble, sand, or other debris to coat the

coral colony. Although the coral (Figure 4c) was not exuding mucus, a sign that it was being smothered by sand or sediments, there were notable pale spots on the colony, and it is possible that such paled tissues were sampled. This would also explain why the *Symbiodinium* density was so low in this sample (Figure 2c). As explained above, low *Symbiodinium* density is typically indicative of environmental stress or even the beginnings of a bleaching response. As the coral appeared to be normally pigmented in general, yet the *Symbiodinium* density was low, this means the chlorophyll content/cell was relatively high. If this equated to high levels of photosynthesis, which could lead to elevated levels of ROS production, then this could explain why high levels of STG expression were measured in this sample.

The univariate-based statistical approaches (i.e., heat maps) agreed with MSA in identifying outliers in 3 of the 5 instances (60%; samples 19.1, 78.2, and 88.2), and these tier 1 outliers are the most likely of the 45-sample dataset to statistically represent those colonies that were displaying unusual molecular physiological phenotypes at the time of sampling. Regardless of the stringency (tier 1 [n=3] vs. tiers 1+2 [n=5] vs. tiers 1+2+3 [n=6]; Table 2), there was no effect of environment on outlier frequency. In other words, outliers appeared randomly distributed across islands, species, temperatures, etc., meaning their presumably aberrant behavior cannot be attributed to a single EP (e.g., high temperature). It is hoped that, by analyzing the approximately 1,000 *Pocillopora* sp. samples collected as part of the LOF GRE, a better grasp on the influence of environment on physiology will be obtained.

Recommendations for future coral health assessment. Although it was advocated above that both host coral and *Symbiodinium* MPRV be utilized in any reef coral stress test, only *Symbiodinium* gene mRNAs were targeted herein due to having inadvertently collected a mix of closely related host species; future work will, then, focus strictly on *P. damicornis*, as well as its closely related sister species *P. acuta*, such that both host coral and *Symbiodinium* macromolecules can be targeted. Then, it can be known which member of this association contributes more to the variation 1) between samples and 2) across the various EP assessed. In addition to the inclusion of molecular biomarkers spanning both compartments of the coral-dinoflagellate endosymbiosis, consideration should also be given to potentially unequally weighting the MPRV used in the aberrancy test. For instance, all MPRV analyzed herein were given equal weight in the calculation of the Mahalanobis distance despite the fact that *Symbiodinium hsp90* was clearly the one that best separated the *P. verrucosa*

samples of Maria Atoll from the other samples based on DA+CCA. Furthermore, it was the MPRV that contributed most to the cumulative difference between islands, hosts, and temperatures and so may be the most responsive MPRV to environmental change. If *hsp90* was given greater weight, then sample 88.2 would have been flagged as the *only* top tier outlier, given that it already had the highest heat map score *and* demonstrated an aberrant *hsp90* expression level.

Under ideal settings, it could be stated that the low outlier frequency documented (5 [tiers 1+2] of 45 colonies; 11%) signifies that the reefs surveyed were generally healthy (but see the discussion on the crown of thorns seastar-ravaged reefs of Rurutu [AI], Rimatara [AI], and parts of Aitutaki in Mayfield et al., 2015.), though this would entail the comparison to healthy control corals/reefs. Given that there are currently no reefs on Earth that are free of human impact, a control value for any MPRV cannot be currently obtained. Therefore, whether the aberrant behavior documented in corals, such as sample 88.2, actually signifies that these corals may soon bleach, or even die, cannot currently be predicted with confidence. Future work could, then, feature controlled tank experiments in which biomarkers that *do* directly predict near-future declines in coral health are identified. Although gene mRNAs could function as such molecular biomarkers, they could not be used to simultaneously make mechanistic reconstructions of cell physiology (as is commonplace in the coral biology field [e.g., Barshis et al., 2013]) due to the lack of congruency between gene and protein expression in reef corals (Mayfield et al., in press). However, even if such aquarium experiments are conducted, and protein biomarkers that predict consequent coral health decline are validated, it could be argued that a "control" coral used in a laboratory experiment in, for instance, Taiwan, would not be representative of a "healthy" coral in FP or CI, as the environmental histories are entirely different. It is hoped that, as mentioned above in conjunction with the attempt to explain which EP best accounts for variation between samples with respect to the MPRV, an assessment of a greater number of samples will ideally unveil patterns within or across MPRV that best signify future declines in coral health. Ultimately, though, it may be necessary to revisit certain sites to determine if the guesses about colony health made herein (i.e., that the outliers are more likely to be stressed) are actualized *in situ*. Upon

selection of an ideal suite of pre-validated molecular biomarkers that accurately assess coral health, the dual univariate+multivariate statistical approach employed herein may prove useful to other coral reef researchers looking to identify colonies displaying unusual behavior with respect to the cell physiology of the cnidarian hosts and their endosymbiotic dinoflagellate populations.

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