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Modeling environmentally-mediated variation in reef coral physiology

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ABSTRACT

Increases in seawater temperature associated with global climate change are causing the mutualistic relationship between reef-building corals and the symbiotic dinoflagellates (genus *Symbiodinium*) that reside within their cells to break down. There is consequently an urgent need to develop tools for modeling coral biology in response to environmental shifts, an enterprise that is complicated by the fact that no pristine reefs remain on Earth. This work sought to 1) uncover the environmental factors that contribute most to observed spatio-temporal variation in coral physiology and 2) devise means of detecting anomalous behavior in field corals by analyzing a dataset from the Austral (French Polynesia) and Cook Islands of the South Pacific with a multivariate statistical approach. Upon employing this multi-tiered analytical platform, host genotype was found to be the most significant driver of variation in physiology of the pocilloporid coral colonies sampled across the two archipelagos. Furthermore, those colonies demonstrating the most extensive variation across the seven response variables assessed tended to deviate most significantly from the global mean response calculated across all samples, suggesting that high within-sample physiological variability may be one means of delineating aberrant coral behavior in the absence of data from pristine control reefs.

1. Introduction

Earth's coral reefs are threatened by a multitude of anthropogenic stressors, most notably global climate change (GCC; Hoegh-Guldberg et al., 2007; Mayfield and Gates, 2007; Hughes et al., 2018). Some reefs, though, have proven resilient to prolonged exposure to elevated temperatures (Palumbi et al., 2014; Krueger et al., 2017). Although whether these resilient reefs or, in contrast, those found to be markedly compromised, should be prioritized for conservation is beyond the scope of this article, there is nevertheless an urgent need to develop the capacity to predict future coral behavior such that we may, for instance, identify both stress-tolerant and stress-prone reefs in the near future (Cinner et al., 2016; Putnam et al., 2017).

Ideally, an estimate of the physiological condition of a coral could be made from a single biopsy sampled at only one time point. In humans, in which there is a much greater body of knowledge on health and stress, we possess well-validated biomarkers, such as blood cholesterol and sugar, whose concentrations are tightly associated with later declines in health. Unfortunately, no such biomarkers have been rigorously validated for assessment of coral health, and a plethora of both methodological and logistical issues have thwarted progress in this field (Louis et al., 2017). For instance, the antibodies of Downs et al. (2000, 2002, 2005) lack specificity to either the coral host or their endosymbiotic dinoflagellate (genus *Symbiodinium*) populations; these antibodies (barring those targeting photosynthesis-related processes) bind proteins of both compartments of the symbiosis. This issue, though, could be remedied by the development of compartment-specific antibodies (sensu Chen et al., 2015), as well as the use of a biological composition control (Mayfield et al., 2009); with the latter approach, a host/*Symbiodinium* ratio of biological material (e.g., RNA, DNA, protein, or lipid) is calculated for each sample such that macromolecular concentration data can be normalized in a manner that controls for variation in the relative quantities of host anthozoan and dinoflagellate biological material, both of which can vary significantly across samples, over time (Mayfield et al., 2010), and/or in response to experimental treatment (Mayfield et al., 2014a).

In addition to proteins, gene mRNAs have also been measured in reef corals in an attempt to make conjectures about their health (Kenkel et al., 2011; Mayfield et al., 2013a; Barshis et al., 2013). Unfortunately, genes encoding well-studied stress proteins, such as heat shock proteins

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(HSPs; Feder, 1996) and ubiquitin ligases (ubiq-lig), are expressed at high levels at all times by reef corals (Mayfield et al. 2011 and 2014d, respectively), even in some of the most remote regions of the South Pacific (Mayfield et al., 2016a); this precludes the ability to use their mean expression levels as indicators of environmental stress. Furthermore, even were differentially regulated mRNAs identified in corals exposed to unfavorable conditions (e.g., elevated temperatures) in the laboratory (sensu Mayfield et al., 2013c, 2014b), it is ill-advised to compare coral responses across experiments carried out in different locations due to the varying effects of environmental history on coral physiology (Mayfield et al., 2012a,b); a "control" coral used in an experiment carried out in heavily impacted locations like Southern Taiwan or the Great Barrier Reef may, for instance, actually possess a similar phenotype as a conspecific hypothesized to be stressed in a relatively more pristine location (e.g., the Line Islands; Sandin et al., 2008). In other words, even the control corals in such experiments are likely to be physiologically compromised to some degree, given that they were sampled from a reef threatened by GCC alone (in a remote location) or GCC plus local, anthropogenic assaults (e.g., water pollution; Fabricius, 2005; Huang et al., 2011) in impacted locations, such as Taiwan (Liu et al., 2012). One solution to this quandary would be to carry out environmental challenge experiments with corals from each reef site of interest to gain insight into how corals at diverse locales respond to environmental change. However, undertaking controlled tank studies may be infeasible in many remote locations, and, of more concern, such a great length of time would be required to acquire such data that many reefs may have already deteriorated by the time an accurate diagnosis of their health is made.

We have recently posed an alternative that acknowledges the fact that a healthy coral may no longer exist (Mayfield et al., 2017a,b); barring the acquisition of coral health data derived from samples acquired prior to the Industrial Revolution, it may be more pragmatic to instead document normalcy (or lack thereof) in a particular region (typically at the country or island-scale). Not only will such baseline data allow us to track the continued responses of these corals to environmental change (*sensu*Anderson and Thompson, 2004), but coral colonies found to be displaying statistically aberrant behavior may ultimately be found to be of compromised health (or, alternatively, of marked resilience). In this work, our goal was to outline not only how to delineate corals displaying deviant behavior, but, more generally, to investigate the environmental factors contributing most significantly to variation in coral physiology. The latter aim stems from the need to address the high inter- and intra-specific variation in coral physiology typically observed *in situ* (Gates and Edmunds, 1999; Manzello et al., in press), as well as in the laboratory (Mayfield, 2016; Parkinson et al., 2018). Specifically, we utilized two common, information theory-based statistical approaches, stepwise regression (univariate) and distance-based linear modeling ("DistLM;" multivariate), to identify environmental parameters (EP) that contributed most significantly to variation in coral physiology using a dataset from the Austral Islands (AI) of French Polynesia and the Cook Islands (CI) generated during the Khaled bin Sultan Living Oceans Foundation's (LOF) "Global Reef Expedition" (GRE), the largest coral reef survey ever undertaken. Specifically, we chose the AI + CI dataset because of the low degree of human impact at these sites; by uncovering environmental drivers of variation in coral physiology in areas not appreciably affected by local anthropogenic stressors, we hypothesized that we could more confidently attribute the physiological variability documented in coral colonies to natural environmental heterogeneity (rather than to local human activities).

2. Materials and methods

2.1. Description of the dataset

Details of the April–May 2013 research expedition to the AI and CI can be found in Mayfield et al. (2015, 2016a), and the target coral for both missions was the model reef coral *Pocillopora damicornis* (Traylor-Knowles et al., 2011). We focused our efforts on this coral species not only because of our extensive experience with it in the laboratory (Mayfield et al., 2013a,b), but also because of its wide distribution (Veron, 2000), especially in the territorial waters of those countries that fund coral reef research to a significant degree: the United States, Taiwan, Japan, Australia, and Israel. We consequently hypothesized that it would be found at the majority of the reef sites surveyed (unlike other proposed model corals [e.g., *Orbicella faveolata* and *Acropora millepora*], whose ranges are much more limited).

Readers interested in the geology of these understudied regions of the South Pacific should consult Chub (1927; AI) and Woodroffe et al. (1990; CI). For convenience, we have reiterated key findings from our prior works in the AI and CI in an online appendix. Briefly, 30 reef sites were surveyed in each archipelago, and pocilloporid corals were sampled from 21 (n = 60 colonies) and 27 (n = 62 colonies) of these sites, respectively. Of these 122 colonies from the AI and CI, 47 and 42 were genotyped (Mayfield et al., 2015), respectively, and 22 and 23, respectively (from 11 and 14 sites, respectively), were analyzed for the molecular-physiological response variables (MPRV) discussed below and in Table 2. A variety of EP (Table 1) were assessed at each of the 60 reefs surveyed to attempt to uncover which factors are most important in driving 1) physiological differences between coral colonies and 2) transcriptional variability within samples. As outlined in Table 1, only 9 of the 15 EP assessed were generally considered in the multivariate statistical analyses (MSA) discussed below; reasons for the exclusion of the other 6 can be found in Table 1. Likewise, of the 10 MPRV assessed in each of the 122 sampled colonies, only 7 were generally considered in the MSA featured herein; reasons for exclusion of the remaining 3 can be found in Table 2.

Since most MSA are highly sensitive to missing data, only those 43 coral samples for which no data were missing were included in the statistical analyses outlined below; this included 21 and 22 samples from the AI and CI, respectively. Although the target species was *P. damicornis*, *P. acuta* was synonymized with *P. damicornis* at the time of surveying; the two species were not formally distinguished until the publication of Schmidt-Roach et al. (2014). Likewise, although most sampled colonies appeared as *P. damicornis in situ*, such was not always confirmed upon genotyping (Mayfield et al., 2015), and, of this 43-sample subset, 1, 6, 8, 11, and 17 were *Pocillopora* sp. haplotype 8a (2.3%), *P. verrucosa* (14%), *P. meandrina* (18.6%), *P. acuta* (25.6%), and *P. damicornis* (39.5%), respectively. For this reason, and because we hypothesized that physiological differences may exist between these closely related species, "host species" was included as an EP (Table 1). Since only one *Pocillopora* sp. haplotype 8a colony was genotyped, it was excluded from the analysis; the resulting final sample size was 42.

2.2. Data analysis overview, PERMANOVA, and PERMDISP

In addition to providing baseline data for an under-surveyed region of the South Pacific, we were interested in identifying the EP (Table 1) that are most important in driving variation in the physiological response (Table 2) of pocilloporid corals, and a variety of both univariate and multivariate statistical approaches were employed to achieve this goal. For all MSA, response variable data were standardized (i.e., converted to z-scores) prior to analysis such that all would be on the same scale. As discussed in the online appendix, ANOVA, multivariate ANOVA (MANOVA; Mayfield et al., 2016a), analysis of similarity (ANOSIM; Mayfield et al., 2016a), recursive partitioning (RP; herein), and multiple regression (herein) were initially used to analyze the data, though they were ultimately found to be inappropriate (ANOVA and MANOVA) or inferior (ANOSIM, RP, and multiple regression) methods for testing effects of environment on coral physiology. Given such issues with these parametric approaches, PERMANOVA (permutational ANOVA of [raw] similarity) was carried out with PRIMER 6 with the PERMANOVA + plug-in (Anderson et al., 2008) to test for the effects of

Table 1
Environmental parameters (EP) assessed at each survey site. Reefs of two atolls surveyed in the Austral Islands, Rurutu and Rimatara, had been decimated by crown of thorns seastars at the time of surveying (April 2013) and so very few corals were sampled there (see Mayfield et al., 2015 for details.). In total 9 of the 15 EP were incorporated into the statistical models discussed in the manuscript text. In other research cruises, we also measured light (as photosynthetically active radiation) and included it as an EP that could influence coral physiology (e.g., Mayfield et al., 2017b). It should be mentioned that the average live coral cover (ALCC) represents the benthic cover of all scleractinian corals, not just the target pocilloporids. NA = not applicable. STG = stress-targeted gene.

EP	#Bins/categories	Included in analysis?	Reason for exclusion	Hypothesis
1. Country	2 countries: French Polynesia (Austral Islands [AI]) vs. Cook Islands (CI)	No	Island hypothesized to best capture large spatial scale variation.	NA
2. Island	6 islands: Raivavae, Tubuai, Maria (AI), Rarotonga, Aitutaki, or Palmerston (CI)	Yes	NA	Effect of island[a]
3. Site	48 sites (corals were not sampled from 12 of the 60 sites surveyed.)	No	Generally too few colonies were sampled from a single site to include this EP.	NA
4. Reef exposure	2 types: leeward (protected) vs. windward (exposed)	Yes	NA	Effect of reef exposure[b]
5. Reef zone	2 zones: fore reef vs. reef flat	No	Generally too few colonies were sampled from reef flats to include this EP.	NA
6. Reef type	2 types: barrier reef vs. fringing reef	No	Reef type generally co-varied with island.	NA
7. Colony depth	6 bins: 5–10, 10–15, 15–20, 20–25, 25–30, or > 30 m	Yes	NA	Effect of depth[b]
8. Sampling date	16 days: see Mayfield et al. (2015)	No	Generally too few colonies were sampled on the same day to include this EP.	NA
9. ALCC (%)	6 bins: 10–20, 20–30, 30–40, 40–50, 50–60, or > 60%	Yes	NA	Effect of ALCC[a]
10. Temperature (temp; °C)	4 bins: 25–26, 26–27, 27–28, or 28–29 °C	Yes	NA	Effect of temp.[a]
11. Salinity (unitless)	3 bins: 35.5, 35.6, or 35.7	Yes	NA	Effect of salinity[a]
12. Sampling time	3 bins: < 10:00, 10:00–14:00, or > 14:00	Yes	NA	Effect of time[b]
13. Coral host genotype[c]	5 species: *P. damicornis*, *P. acuta*, *P. verrucosa*, *P. meandrina*, or *Pocillopora* sp. (haplotype 8a)[d]	Yes	NA	Effect of host[a]
14. Coral colony color[c]	4 colors: normal, pale, very pale, or bleached	Yes	NA	Higher STG expression in bleached corals[b]
15. *Symbiodinium* assemblage[c]	3 assemblages: clade C only, clades A + C, or clades C + D	No	Nearly all colonies hosted clade C exclusively.	NA

[a] confirmed by certain statistical approaches (Table 3).
[b] not statistically supported by the majority of statistical approaches (Table 3).
[c] technically a property of the sampled colony (rather than the environment) but nevertheless hypothesized to influence coral physiology.
[d] The lone *Pocillopora* sp. haplotype 8a colony sampled was excluded from the statistical analysis (see main text.).

Table 2
Molecular-physiological response variables (MPRV) assessed in the sampled pocilloporid coral colonies. Of the 10 MPRV assessed in each of the 122 sampled coral colonies, only 7 were generally included in the suite of statistical analyses described in this article. It should be noted that, because a mix of host coral species were sampled, no host coral target genes were analyzed because it was unclear whether our previously designed *Pocillopora damicornis* real-time PCR assays (e.g., Mayfield et al., 2014d) were functional with orthologs of the closely related congenerics (e.g., *P. acuta*). GCP = genome copy proportion. NA = not applicable.

MPRV	Unit/abbreviation	Proxy	Included in statistical analyses?	Reason for exclusion	Hypothesis
Size and biological composition response variables (n = 4)					
1. Maximum (max.) length	cm	Size	No	Size not reflective of coral health	NA
2. Planar surface area	cm^2	Size	No	Collinear with max. length	NA
3. RNA/DNA ratio	Unitless	Total gene expression	Yes	NA	High or low values indicative of aberrant behavior.
4. *Symbiodinium* GCP	Unitless	*Symbiodinium* density	Yes	NA	Low values indicative of aberrant behavior (bleaching).
***Symbiodinium*gene expression (n = 6)**		**Function**			
5. Heat shock protein 40	*hsp40*	Molecular chaperone	Yes	NA	High values indicative of aberrant behavior.
6. Heat shock protein 70	*hsp70*	Molecular chaperone	Yes	NA	High values indicative of aberrant behavior.
7. Heat shock protein 90	*hsp90*	Molecular chaperone	Yes	NA	High values indicative of aberrant behavior.
8. Ubiquitin ligase	*ubiq-lig*	Cellular stress response/proteolysis	Yes	NA	High values indicative of aberrant behavior.
9. Ascorbate peroxidase	*qpx1*	Oxidative stress response	Yes	NA	High values indicative of aberrant behavior.
10. Photosystem I (subunit III)	*psI-III*	Photosynthesis	No	Only measured in half of samples	NA

each of the target EP (Table 1) on the similarity among samples after first creating a Euclidean distance matrix (EDM) with the standardized data. Additionally, the PERMDISP feature of PRIMER 6 (Anderson, 2006) was utilized with this same EDM. PERMDISP is a distance-based test for homogeneity of multivariate dispersions, and we hypothesized that the dispersion in the coral physiological response might differ across environmental gradients, particularly ALCC levels. For both similarity-based approaches, an alpha level of 0.05 was set.

2.3. Modeling the coral physiological response with stepwise regression

When dealing with a large number of predictor variables (i.e., the EP of Table 1), hypothesis-driven approaches such as ANOVA may lead to type I errors, even when dramatically adjusting the alpha level as in Mayfield et al. (2016a, 2017a,c; from 0.05 to 0.004). Furthermore, hypothesis testing is poorly suited for identifying the optimal model for explaining a dataset, even when a multivariate similarity approach like PERMANOVA is used (Anderson et al., 2000). For these reasons, information theory has been increasingly used in ecology (Anderson and Burnham, 2002), either in isolation, or in conjunction with hypothesis testing, in order to develop the most appropriate, parsimonious best-fit model for explaining the behavior of a particular response variable (Mazerolle, 2006). Information theory is used routinely in coral reef ecology (Jørgensen et al., 2005) and ichthyology (Conover et al., 2006), but it has not been widely adopted in coral physiology research (but see Kenkel et al., 2015), in which hypothesis testing is traditionally more common (e.g., Mayfield et al., 2013c,d; Putnam et al., 2013; Mayfield et al., 2014c).

Herein we used an information theory-based stepwise regression program in JMP® (ver. 13) to select the best-fit model for each of eight response variables: maximum (max.) colony length, the *Symbiodinium* genome copy proportion (GCP), the RNA/DNA ratio, and expression of five *Symbiodinium* stress-targeted genes (STGs; Table 2). Backwards model selection was used such that all EP were initially considered, and a stopping rule was enacted based on minimizing the "Bayesian information criterion" (BIC). Finally, a "combine" rule was employed such that, when necessary to minimize the BIC, certain EP were partitioned heuristically into sub-categories/bins. To depict the stepwise regression data in graphical form, the relative weight of each EP model term (when there were multiple) was scaled based on its *F* statistic such that the sum of all "strength of effect" scores equaled 100%. For instance, if stepwise regression included two EPs whose *F* statistics were 2 and 4, the size of the former's bubble (33%) would be half that of the latter (67%) in Fig. 3.

The aforementioned, information theory-based stepwise regression analyses sought to identify the EP (or combinations thereof), that best accounted for variation in each of eight response variables. However, we were also interested in the suite of EP that best modeled variability in the multivariate coral response. Therefore, as a distance-based analog to this information theory + linear modeling analysis, PRIMER's "DistLM" (distance-based linear modeling; Clarke et al., 2014) program, was used with the "best" selection procedure and a minimum Akaike's information criterion (AICc). In other words, the EP(s) best accounting for between-sample differences in the underlying EDM was/were identified.

2.4. Outlier analysis

A detailed treatise on identification of outliers in the AI + CI dataset, which was first presented in Mayfield et al. (2016a), has been summarized in the online appendix. Briefly, the Mahalanobis distance (MD) was calculated across the seven MPRV to serve as the primary means of identifying outliers, and those samples with MD values > 7 and whose heat map scores (HMS; see online appendix for details.) were ≥ 1 were considered outliers. Briefly, a sample featuring a MPRV with a *z*-score > 2 would be given an HMS of 1. To corroborate this outlier

assignment, the principal coordinate from the primary axis of a principal coordinates ordination (PCO) analysis carried out by PRIMER 6 was calculated and regressed against the MD. It should be noted that, because this PCO was carried out with an EDM, it is comparable to the more commonly employed principal components analysis (PCA). The MD and PCO1 score are collectively referred to as "multivariate variability terms" throughout the manuscript. We also calculated a second (after the HMS) "univariate variability term" known as the "variability index" (VI), which was first described by Mayfield et al. (2017a,b, 2018b). This term is simply the standard deviation of the standardized data across all response variables for an individual coral biopsy. For instance, if the *z*-scores for the seven MPRV measured herein were 1, 2, 3, 4, 5, 6, and 7 for a particular coral sample, its VI would be 2.2.

We hypothesized that samples with high VI (within-sample variation) would also tend to be characterized by relatively high MD (between-sample variation; i.e., a greater distance from the global centroid), and linear regression analysis was used to determine the significance of this relationship across the 42-sample dataset. We also regressed the MD against the standardized values of the individual MPRV in order to determine which response variables were the most important drivers of a sample being characterized as an outlier. To confirm the results of this analysis, two approaches were taken. First, JMP's "predictor screening" program was used to calculate the relative effects of the MPRV on the MD. Then, partial least squares (PLS) analysis was used to create a "variable importance plot" (VIP) in which response variables were scored relative to their predictive influence on the MD. JMP's recommended VIP threshold of 0.8 was set *a priori*(i.e., MPRV with VIP $>$ 0.8 were considered to best model the MD.). Finally, multiple nominal logistic regression was used with outlier status (yes vs. no) as the response variable in order to create a best-fit model featuring EP as predictors of whether or not a sample would be considered an outlier. For all MSA, an alpha level of 0.05 was established *a priori*.

3. Results

3.1. Environmental variation

The overall variability in several continuous EP can be found in Fig. 1, and a detailed treatise on the environmental data can be found in the online appendix. Briefly, a canonical correlation analysis (CCA)

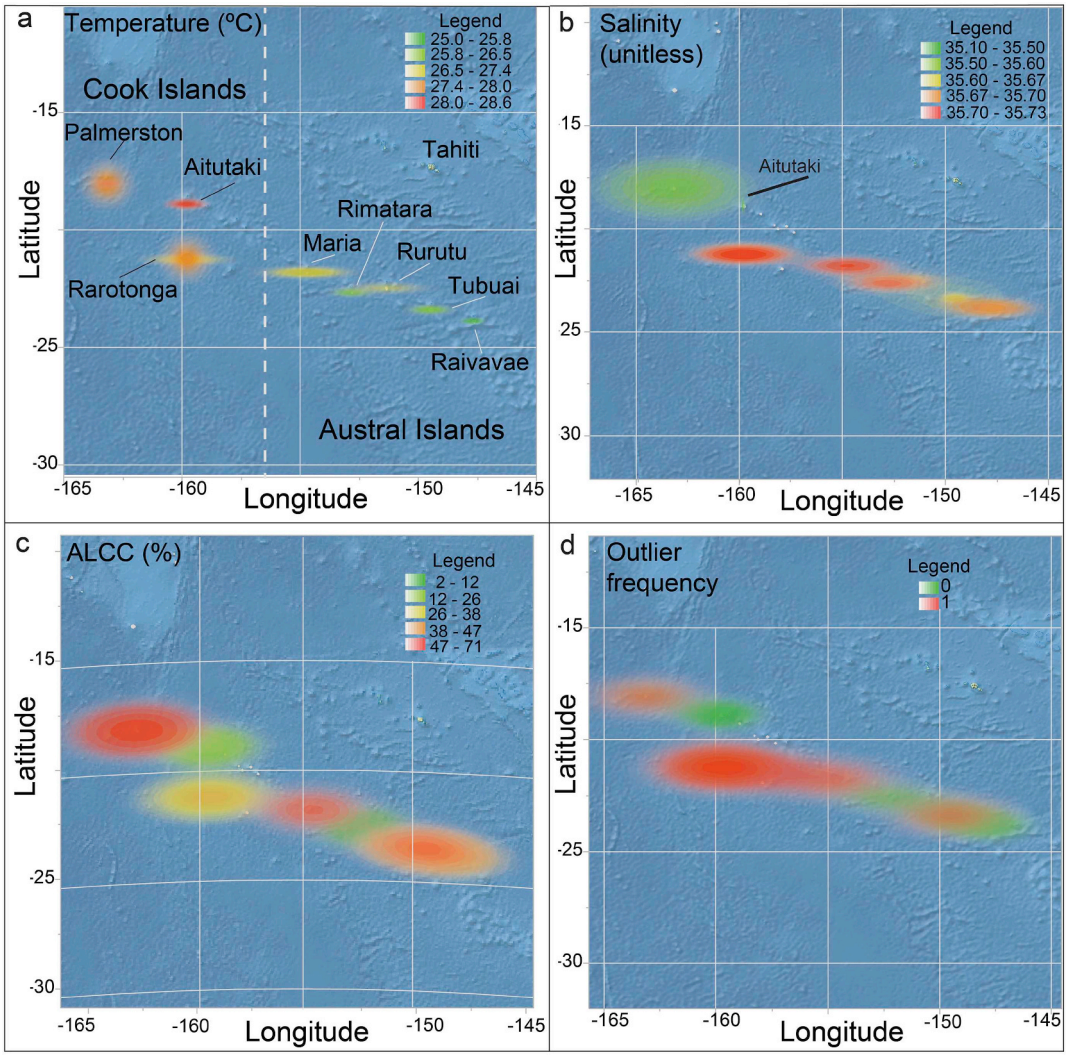

Fig. 1. Contour plots of environmental and outlier frequency data in the Austral Islands (AI) of French Polynesia and the Cook Islands (CI). The sizes of the data clouds are proportional to the data variability; certain islands in close proximity to each other (e.g., Raivavae and Tubuai in [b-d]) tend to be masked. In other instances, environmental parameters (EP) were homogeneous within an island, in which case clouds are essentially absent (e.g., salinity [b] at Aitutaki). In [a] only, the islands have been labeled, and a grey, dotted line has been used to approximate the territorial divide between the CI (left-side) and AI (right-side). For a data-free map of the AI and CI, please see Mayfield et al. (2015). For a canonical correlation analysis (CCA) plot of the environmental data across islands, please see Fig. A1.

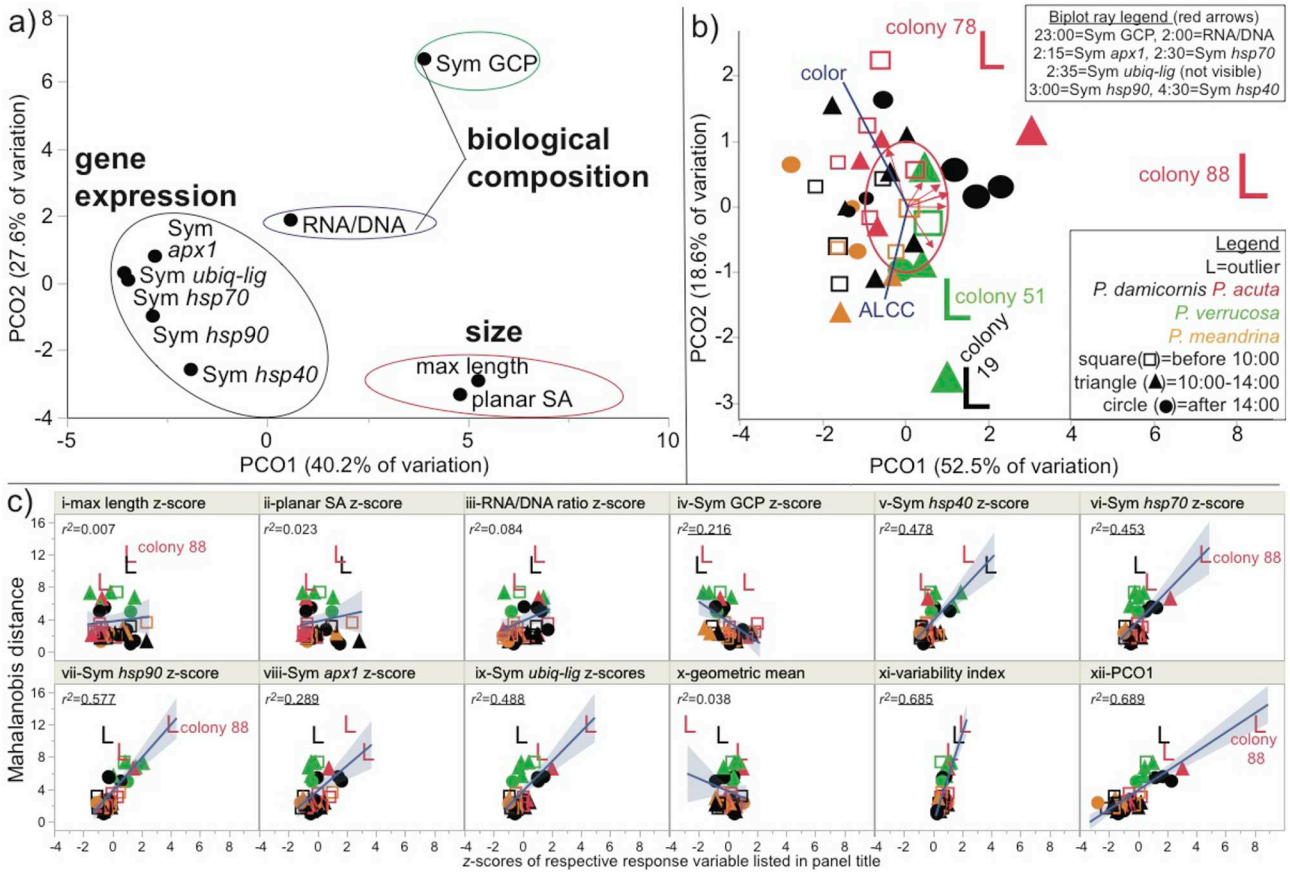

Fig. 2. Multivariate analysis of the Austral and Cook Islands dataset. A principal coordinates ordination (PCO) analysis of a between-response variable (RV) Euclidean distance matrix (EDM) was performed with nine RV [a], and the circles were drawn by eye (i.e., they do not connote statistical clustering.). PCO (instead using a between-sample EDM) was also used to depict similarity between samples (n = 42) over two dimensions [b], as well as identify 1) outliers ("L;" all four have been labeled, and the sizes of all 42 sample icons are proportional to their Mahalanobis distances [MD].) and 2) RV best modeling variation in coral physiology (seven molecular physiological RV only [as red arrows]; see "Biplot ray legend" in upper right corner, in which numbers on left of legend correspond to approximate positions on a clock.). Vectors (blue) for environmental parameters whose Pearson correlation coefficients (r; against the coordinate axes) were > 0.3 have been included. The bottom-right legend of [b] also applies to [c], in which the MD has been plotted against each of the nine RV (i-ix), as well as their geometric mean (x), the variability index (xi), and the PCO primary axis coordinates (PCO1; xii); r^2 values of statistically significant associations ($p < 0.01$) have been underlined, and shading around the best-fit lines represents 95% confidence. The most aberrantly behaving colony (#88) has been labeled in [b] and certain sub-panels of [c].

revealed that the abiotic environment (Table 1) varied significantly across islands (Fig. A1). For descriptions and images of the 60 survey sites, please see Mayfield et al. (2015) and http://coralreefdiagnostics.com/french-polynesia, respectively; the latter website also features images of all sampled coral colonies, with hyperlinks to 1) data files (JMP or Excel format) and 2) DNA sequences (hosted on the NCBI server) overlaid onto the images.

3.2. Variation in coral physiology

Nine response variables (those of Table 2 with the exclusion of *Symbiodinium psI*/III mRNA expression) were assessed in each of 42 coral colonies sampled from the AI and CI in order to uncover relationships between coral physiology and environment (Fig. 2). First, a PCO analysis of these nine response variables was carried out with a between-response variable EDM in order to uncover the relationships among them (Fig. 2a). Max. colony length and planar colony SA fell close to each other in the plot given their collinearity. These size-based EP were not generally considered in any additional statistical analyses, as coral colony size was not hypothesized to be indicative of coral health (i.e., larger colonies are not necessarily healthier than small ones, although coral physiology indeed differs across colony age/size [Elahi and Edmunds, 2007]). The *Symbiodinium* gene mRNAs tended to cluster together in the plot, whereas the two biological composition parameters (RNA/DNA ratio and the *Symbiodinium* GCP) were less similar to each other. The first axis of a PCO carried out with a between-*sample* EDM (Fig. 2b) was dominated by the five *Symbiodinium* genes, whereas the second and third (not shown; 13%) featured *Symbiodinium* density (GCP) and the RNA/DNA as the dominant loading factors, respectively. Furthermore, those samples farther from the core region of the plot were characterized by larger MD (icon sizes in Fig. 2b are proportional to their MD.), and the four outliers uncovered (discussed in more detail below) were found near the plot's perimeter.

To further explore the relationships among 1) the MD, 2) the response variables, and 3) three variability terms, 12 scatterplots were produced (Fig. 2c). Although the MD did not correlate significantly with the size parameters (Fig. 2c-i-ii) or RNA/DNA (Fig. 2c-iii), it was negatively associated with the *Symbiodinium* GCP (Fig. 2c-iv); corals with lower *Symbiodinium* density tended to be more different from the average coral. In contrast, the MD was positively and linearly associated with expression of the five *Symbiodinium* STGs (Fig. 2v-ix). The geometric mean calculated across the seven molecular response variables did not correlate significantly with the MD (Fig. 2c-x), though there was a strong, positive, linear correlation between the VI and the MD (Fig. 2c-xi); those samples characterized by the most variability across the seven MPRV (i.e., high intra-sample variability) also tended to be the most different from the mean coral (i.e., high sample-centroid distance). Finally, as is evident from Fig. 2b and c-xii, the MD correlated

Table 3
Summary of the effects of seven environmental parameters (EP) on the univariate and multivariate coral physiological response. For the univariate (stepwise regression) and multivariate statistical approaches (MSA; seven molecular-scale response variables only), significantly affected response variables (*F*-test, $p < 0.05$) and multivariate *p*-values have been included in the cells, respectively, except for the case of distance-based linear modeling (DistLM), in which those EP "included" in the model characterized by the minimum Akaike information criterion value have instead been inserted. The 1) variability index (VI), 2) Mahalanobis distance (MD), and 3) principal coordinate ordination (PCO) primary axis (PCO1) values were also treated as response variables in the stepwise regression analysis. Since no significant differences were uncovered for reef exposure or coral sampling time, these EP have been excluded from the table. ALCC = average live coral cover. NS = not statistically significant. Sym = *Symbiodinium*. Temp. = temperature.

	Univariate statistical approach	Multivariate statistical approaches		
EP	Stepwise regression (information theory)	PERMANOVA	DistLM	Conclusion(s)
Island	Sym *hsp90*, MD	NS	Excluded	Island featured in best-fit models for MD and Sym *hsp90*.
ALCC	RNA/DNA, MD, Sym GCP	NS	Included	Variance in coral physiology differed across coral cover levels (discussed in text).[a]
Temp.	PCO1	NS	Excluded	Temp. significantly affected between-sample similarity (principal coordinates).
Salinity	Sym *apx1*	NS	Included	Salinity influenced Sym *apx1* mRNA expression and multivariate distance.
Depth	Sym GCP	NS	Included	Depth affected *Symbiodinium* density and multivariate distance.
Host species	Sym GCP, Sym *hsp70*, Sym *ubiq-lig*, MD, PCO1, VI	0.04	Included	Most significant driver of variation in coral physiology of all nine EP analyzed.
Colony color	Sym *hsp40*	NS	Included	Colony color only affected Sym *hsp40* mRNA expression and multivariate distance.

[a] Significant PERMDISP effect ($p < 0.05$).

significantly, positively, and linearly with the coordinates of the first PCO axis (see the online Mendeley data file for exact MD values and PCO coordinates.).

Both hypothesis testing and information theory approaches were used to determine the EP that best modeled variation in the physiology of the sampled coral colonies, and the data have been summarized in Table 3. The similarity matrix-based PERMANOVA only found host species to affect similarity among samples (albeit marginally), as is somewhat evident in the PCO of Fig. 2b. When using an information theory approach to model multivariate similarity (all seven MPRV) between samples with PRIMER's DistLM function (Table 3), a model featuring colony color, salinity, depth, host species, and ALCC resulted in the minimum AICc of 80 ($r^2 = 0.30$).

For the response variables analyzed individually, the information theory-based stepwise regression yielded best-fit models whose adjusted r^2 averaged 0.31, and three EP were included in the average model (Table 4). The best-fit models generated by stepwise regression are shown in Table 4, detailed in the online appendix, and the relative weights (i.e., strength of influence) of the model terms have been plotted in Fig. 3a-b. When considering only the statistically significant best-fit model EP terms (Fig. 3b and underlined and capitalized EP in Table 4), host was found in more models than any other EP (Fig. 3c). In contrast, exposure and sampling time were not statistically significant EP in any model; these EP are therefore not depicted in Fig. 3. Temperature was only a significant factor in the best-fit model for the first PCO axis, which is not shown in Fig. 3a-b. Colony color only featured as a statistically significant term in the best-fit model for *Symbiodinium hsp40*. For a detailed treatise on the stepwise regression analysis, please consult the online appendix.

3.3. Outlier analysis

By virtue of their MD and HMS, 5 of the 43 samples for which no data were missing were considered to be outliers; one of these was the lone *Pocillopora* sp. haplotype 8a colony sampled and is not considered further. In general, outliers were characterized by lower *Symbiodinium* densities (Fig. 2c-iv), though this difference was not statistically significant (student's *t*-test of outliers vs. non-outliers, $p > .05$). In contrast, outliers expressed higher levels of *Symbiodinium hsp90* (3-fold; Wilcoxon rank-sum test, $p < .05$), *hsp70* (3-fold; Wilcoxon rank-sum test, $p < .01$), *apx1* (2.5-fold; Wilcoxon rank-sum test, $p < .05$), and *ubiq-lig* (3-fold; Wilcoxon rank-sum test, $p < .05$). None of the other response variables differed significantly between outliers and non-outliers. Not surprisingly, then, *Symbiodinium* gene expression contributed most significantly to the MD, as determined by a predictor screening analysis (Fig. A2a) and PLS (Fig. A2b). The VI was approximately 2.5-fold higher in outliers than in non-outliers (Wilcoxon rank-sum test, $p < .01$); those samples that demonstrated greater variability across response variables were also characterized by a greater distance from the overall mean response centroid (discussed above in the context of the correlation between the VI and the MD). When performing a stepwise nominal logistic regression (minimum BIC/forward selection) of outlier frequency (yes vs. no), a best-fit model ($r^2 = 0.80$; $p < .01$) featuring the following EP resulted in the minimum BIC of 28: salinity ($p < .001$), host ($p = .01$), colony color (NS), and ALCC (NS). It is worth noting that none of these EP in isolation significantly affected outlier frequency (X^2 tests, $p > .05$). Additional details of the outlier analysis can be found in the online appendix.

4. Discussion

4.1. Host species effects

The multivariate, distance-based alternative to stepwise regression, DistLM, included the host term in the best-fit, minimum-AICc model, in addition to colony color, salinity, depth, and ALCC. Given that these same EP were generally also found in the individual response variable stepwise regression models, we conclude that these five EP, in addition to island, are the most important of all 15 originally assessed in terms of their influence on coral physiology. Of these six EP, host contributed most significantly to the variation between colonies (Table 5). Specifically, host was the most important driver of variation for *Symbiodinium* density (i.e., the GCP), as well as *Symbiodinium ubiq-lig* and *hsp70* mRNA expression. Furthermore, host species was a significant EP in the best-fit models for two of the three variability terms: MD and VI. It was also the only EP for which PERMANOVA documented a statistically significant effect.

Although nearly all corals possessed *Symbiodinium* of clade C only (see online Mendeley data file.), it is possible that sub-cladal *Symbiodinium* diversity contributed to the differences in *Symbiodinium* density and gene expression among the four predominant host corals genotyped (*P. damicornis*, *P. acuta*, *P. verrucosa*, and *P. meandrina*). Unfortunately, markers commonly used to genotype *Symbiodinium* (e.g., its2) are intragenomically variable; in a coral biopsy in which many thousands, or even millions, of *Symbiodinium* cells may be present, it is therefore not possible to tease apart intra- from inter-genomic variation (Wilkinson et al., 2015). Upon the analysis of the growing number of *Symbiodinium* genomes (e.g., Shoguchi et al., 2013), it will soon be possible to identify molecular markers that will allow us to confidently assess *Symbiodinium* diversity in complex, endosymbiotic samples such as those obtained and analyzed herein. Then, it could be known, for instance, whether differences in physiological properties of the (host) pocilloporid coral

Table 4
Stepwise regression best-fit models. Statistically significant EP ($p < .01$) have been underlined in all CAPITAL letters, and all error terms represent standard deviation. The relative weights of the best-fit model environmental parameters (EP) uncovered for each response variable have been depicted graphically in Fig. 3a-b (in which *F* statistics have been scaled relative to 100%). adj. = adjusted. BIC = -Bayesian information criterion. MD = Mahalanobis distance. PCO = principal coordinates ordination. Temp. = temperature. VI = variability index. *statistically significant *F*-test *p*-value for entire model (< 0.05).

Response variable	Stepwise regression model terms	adj. r^2 (BIC)
Physiological response variables		
Max. length[a]	Host	0.12 (54)*
RNA/DNA[b]	ALCC > host > depth	0.44 (328)*
Sym density[c]	HOST > DEPTH ≥ ALCC > color	0.47 (310)*
Symbiodinium gene expression		
Sym *apx1*[a]	SALINITY > color > depth > temp. > host > island > alcc	0.37 (127)[c]
Sym *ubiq-lig*[b]	HOST > time	0.28 (330)*
Sym *hsp70*[a]	HOST > alcc > time	0.25 (105)*
Sym *hsp40*[a]	COLOR	0.21 (107)*
Sym *hsp90*[a]	ISLAND	0.21 (104)*
Variability terms		
MD[b]	ISLAND > HOST > alcc	0.40 (326)*
PCO (axis 1)[b]	TEMP. ≥ HOST > ALCC > salinity	0.37 (330)*
VI[a]	HOST > alcc > island	0.28 (56)*
mean adj. r^2		0.31 ± 0.11
mean# EP in best-fit model		2.9 ± 1.8

[a] log-transformed data.
[b] rank-transformed data.
[c] used minimum *p*-value stopping rule and mixed model selection instead of minimum BIC rule with backwards selection.

colonies sampled (e.g., tissue thickness and corallite morphology), or, alternatively, the identity of the *Symbiodinium* cells *in hospite*, were more important contributors to the inter-host variation observed in algal cell density and gene expression. This observation that *Symbiodinium* gene expression patterns vary across congeneric host species is novel and may have implications for predicting how each species will respond to future environmental change. A more detailed treatise on molecular plasticity and heterogeneity with respect to acclimation potential (*sensu* Kenkel and Matz, 2016) can be found below.

4.2. High STG expression levels

The virtual absence of localized anthropogenic influence in the sparsely populated AI and CI had led us to hypothesize that *Symbiodinium* STG expression levels would be lower than in conspecifics from our field sites in Southern Taiwan (Mayfield et al., 2012a); such was not confirmed. In fact, the expression levels of *Symbiodinium* gene mRNAs were relatively high for all genes (threshold cycle [Ct] = 22–25 for the *hsps* and *ubiq-lig* and Ct = 28 ± 0.3 [std. dev. for this and all error terms henceforth] for *apx1*). In the case of *Symbiodinium hsp90* mRNA expression in particular, mean values (25.1 ± 1.7) were statistically similar to those of *Symbiodinium* populations within corals from heavily impacted reefs of Southern Taiwan (24.8 ± 2.1; Mayfield et al., 2013a). Expression levels of the other four *Symbiodinium* STGs were generally comparable to those of Taiwanese *in hospite Symbiodinium* (clade C) populations, as well (data not shown).

Although HSPs and ubiq-ligs aid in protein homeostasis and degradation, respectively (Welchman et al., 2005), protein turnover does not only occur during episodes of environmental stress (Hochachka and Somero, 2002); rather, it occurs to some degree at all times. Therefore, constitutively high expression levels of these four genes do not, in and of themselves, signify that the sampled coral colonies were stressed. It is also worth noting here that, given their clustering in the between-response variable PCO and their relative multicollinearity in the between-sample PCO, it may be superfluous/redundant to target all five of these *Symbiodinium* STGs in future studies. Such collinearity also points to the utility of PLS, which was employed herein for assessing the relative influence of response variables on the MD, in analyzing coral-*Symbiodinium* mRNA expression data (in which it is likely that at least some pairs of genes will co-vary; Mayfield et al., 2014b).

Furthermore, it was recently found that there is no congruency between mRNA expression and protein concentration in reef corals (Mayfield et al., 2016c); although gene mRNAs could still potentially serve as biomarkers (provided that aberrant expression levels ultimately lead to a compromised phenotype), their concentrations cannot be used to reconstruct cellular physiologies unless protein data are simultaneously acquired (Mayfield et al., 2018a). Therefore, it cannot be currently stated whether, for instance, the expression levels measured for *Symbiodinium apx1*, which encodes a protein involved in the oxidative stress response, are actually indicative of baseline levels of reactive oxygen species formation (which is to be expected in photosynthetic organisms [Lesser, 1997; Jones et al., 1998; Lesser, 2006]). All proteins co-extracted from the samples whose DNAs and RNAs were analyzed herein have been archived (precipitated in acetone at −80 °C); concentrations of the respective stress-targeted proteins should therefore be assessed in the future to determine whether corals of the AI and CI were simultaneously synthesizing high concentrations of proteins involved in protein turnover and the stress response.

4.3. Variability in coral response versus mean coral response

As mentioned in the Introduction, simply using absolute gene expression or protein concentration levels as a proxy for the degree of stress in an individual coral colony is likely unfounded; because we do not know what "healthy" concentrations are for any analyte given that all coral reef research has been undertaken in the post-Industrial era, how can we then claim that the concentration of any particular biomarker is indicative of a decline in coral health in the absence of tank experiments carried out with the colonies of interest? Unfortunately, there are no pristine coral reefs left on Earth given the wide-reaching effects of GCC; for instance, coral reefs of the uninhabited, extremely remote Chagos Banks (aka British Indian Ocean Territory) are now bleaching annually, even in the absence of all other anthropogenic stressors (Sheppard et al., 2017). Additionally, although there is a good chance that ancient coral DNAs could be recovered such that they might be sequenced (Baker et al., 2013), RNAs, proteins, and other functionally important molecules will almost surely have degraded in coral fossils; we are unlikely, then, to ever acquire a knowledge of the cellular

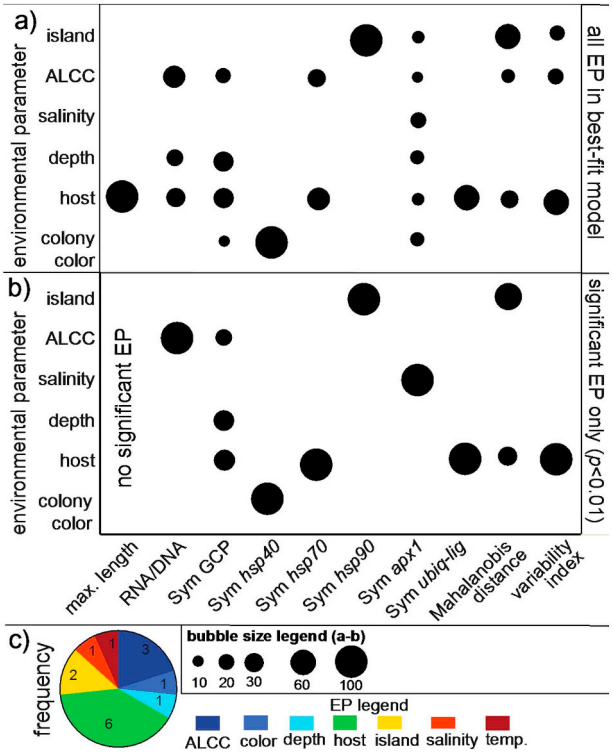

Fig. 3. Environmental parameters (EP) included in best-fit models for coral molecular-physiological response variables generated by stepwise regression. In [a], all EP for each model have been shown, whereas in [b], only statistically significant (individual *F*-test, $p < .01$) EP have been included. In both, bubble size is proportional to the strength of effect for each EP included in the model (i.e., larger bubbles represent a stronger effect of the respective EP; see text for details of calculation). The breakdown of the 15 significant EP identified by stepwise regression has been shown as a pie graph in [c], in which case the values inside the wedges reflect the number of times each of the seven EP featured was included as a significant term in a best-fit model (Table 4). Please note that temperature (temp.) was included because it was found to be a significant term in the best-fit model of the principal coordinates ordination (PCO) axis 1 (PCO1) coordinate values, which was not depicted in [a-b]; the three significant EP found in the PCO1 model (Table 4) account for the difference in significant EP found in [b] ($n = 12$) and [c] ($n = 15$).

concentrations of functionally important macromolecules from corals whose abiotic environments have not been dramatically altered by humankind.

Instead, the variability among response variables might actually be of greater interest with respect to gauging coral performance than the mean concentrations of the respective analytes (sensu Kovács et al., 2014). Herein we documented a statistically significant, positive correlation between the MD and the VI. This means that those coral colonies that demonstrated the most variation among response variables also tended to be those whose mean physiological performance deviated most from the norm (i.e., outliers). It is currently premature, however, to speculate whether these outliers characterized by high MD and VI were of diminished resilience compared to those whose physiology approximated that of the average colony. In humans, high variability across genes is a hallmark of many cancers (Cleophas et al., 2006; Han et al., 2016; Sharma et al., 2018), nearly all of which result from a loss of transcriptional control. In corals, such transcriptional "noise" could therefore reflect a deviation from homeostasis that may be indicative of compromised health; alternatively, the capacity to exhibit large shifts in molecular biology could simply be construed as evidence of phenotypic plasticity (Kenkel and Matz, 2016) and therefore signify an advantageous property of these corals. Whether or not high within-sample variation across response variables is prognostic of stress or resilience should, then, be directly tested in future experiments carried out either in the laboratory or on the coral reef.

Regardless of the ultimate fate of these colonies, the transcriptional variation in particular may attest to the degree of genetic material available for selection (Parkinson et al., 2018). We therefore further advocate that, when such outliers are uncovered within a dataset, they not be discarded; although their inclusion may thwart the ability to detect a significant difference in statistical models, their exclusion will result in the loss of information about colonies that actually be most interesting from a physiological perspective. Indeed, we recommend the MD-based "aberrancy detection system" outlined herein be used as a means of generating a list of coral colonies to monitor most closely. For individuals working on transcriptomic and proteomic technologies (e.g., Mayfield et al., 2016b), this outlier selection approach may allow for the identification of the most informative subset of samples in the all-too-common event in which it is prohibitively expensive to analyze all samples generated in an experiment (or collected during a field season).

Only return visits to these same study sites will allow us to conclusively determine whether those outliers with high MD and variability across gene expression, in particular, are more or less likely to succumb to environmental change, and multivariate "control charts" (Anderson and Thompson, 2004) could be used to detect significant deviations from the molecular-physiological baseline established in this work. Future trips to these remote, South Pacific field sites will also allow us to determine whether the fact that the mean dispersion (as

Table 5
Major conclusions from the Austral Islands-Cook Islands pocilloporid coral dataset. Please see Fig. A2c for a plot of the dispersion data across average live coral cover (ALCC) levels.

Finding	Statistical approach(es) supporting observation	Reference
P. damicornis is more likely to be found at depths > 15 m and in higher coral cover areas relative to *P. acuta*.	1. X^2 test 2. nominal logistic regression	Mayfield et al. (2015)
Pocillopora verrucosa colonies from Maria Atoll (Austral Islands) differ from other colonies, mainly by virtue of their higher *Symbiodinium hsp90* mRNA expression levels.	MANOVA	Mayfield et al. (2016a)
Five of the forty-two colonies analyzed in detail (12%) are outliers.	1. Mahalanobis distance 2. HMS 3. PCO	Mayfield et al. (2016a)
Host species is the most important driver of variation in coral physiology in this 42-sample dataset.	1. PERMANOVA 2. DistLM 3. stepwise regression	Herein
Samples with high Mahalanobis distances (i.e., outliers) are characterized by relatively higher inter-response variable variation (i.e., high variability indices).	1. Outlier analysis 2. Multiple correlation 3. Linear regression	Herein
Significant effect of ALCC on dispersion of coral response.	PERMDISP	Herein

determined by PERMDISP; Fig. A2c) was highest in sites with 20–30% coral cover (Table 5) signifies that those corals of the low ALCC sites are more likely to display aberrant behavior (as had been hypothesized previously by Mayfield et al., 2017a, 2017b). In fact, mean multivariate data dispersion of the lowest coral cover sites (10–20%) was significantly *lower* than for the 20–30% coral cover sites; clearly, then, there is not a simple linear relationship between coral cover and inter/intra-sample data dispersion. Furthermore, there was no relationship between ALCC and the MD, nor were outliers more frequently found on reefs with low coral cover. This means that the use of coral cover alone as a proxy for the mean behavior of a reef's resident corals may, as hypothesized by Wooldridge (2014), be unjustified.

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Competing interests statement

The authors state that no competing interests exist.

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