Multivariate statistical approaches for uncovering spatio-temporal and treatment-derived differences in the molecular physiology of a model coral-dinoflagellate mutualism: a meta-analysis

Anderson B Mayfield Corresp., [1, 2, 3] , **Chii-Shiarng Chen** [2, 3, 4, 5]

[1] Living Oceans Foundation, Landover, Maryland, United States of America
[2] National Museum of Marine Biology and Aquarium, Checheng, Pingtung, Taiwan
[3] Taiwan Coral Research Center, Checheng, Pingtung, Taiwan
[4] Department of Marine Biotechnology and Resources, National Sun Yat-Sen University, Kaohsiung, Taiwan
[5] Graduate Institute of Marine Biotechnology, National Dong Hwa University, Checheng, Pingtung, Taiwan

Corresponding Author: Anderson B Mayfield
Email address: andersonblairmayfield@gmail.com

Background: Multivariate statistical approaches (MSA), such as principal components analysis and multidimensional scaling, seek to uncover meaningful patterns within datasets by considering multiple response variables in a concerted fashion. Although these techniques are readily used by ecologists to visualize and explain differences between study sites, they could theoretically be employed to differentiate organisms within an experimental framework while simultaneously identifying response variables that drive documented experimental differences.

Methods: A meta-analysis employing various MSA was conducted to re-analyze data from two studies that sought to understand the response of the common, Indo-Pacific reef coral *Seriatopora hystrix* to temperature changes.

Results: Gene expression and physiological data partitioned experimental specimens by time of sampling, treatment temperature, and site of origin upon employing MSA.

Discussion: These findings 1) signify that *S. hystrix* and its dinoflagellate endosymbionts display physiological and molecular signatures that are characteristic of sampling time, site of colony origin, and/or temperature regime and 2) promote the utility of MSA for documenting biologically meaningful shifts in the physiological and/or sub-cellular response of marine invertebrates exposed to environmental change.

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[1]National Museum of Marine Biology and Aquarium, Checheng, Pingtung, Taiwan
[2]Living Oceans Foundation, Annapolis, MD, USA
[3]Taiwan Coral Research Center, Checheng, Pingtung, Taiwan
[4]Graduate Institute of Marine Biotechnology, National Dong-Hwa University, Checheng, Pingtung, Taiwan
[5]Department of Marine Biotechnology and Resources, National Sun Yat-Sen University, Kaohsiung, Taiwan
*corresponding author: andersonblairmayfield@gmail.com. Tel. 1-337-501-1976
mailing address: National Museum of Marine Biology and Aquarium
2 Houwan Rd.
Checheng, Pingtung 944
Taiwan, R.O.C.

Abstract

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Abbreviations

1. analysis of similarity=ANOSIM
2. ascorbate peroxidase=*apx1*
3. α-tubulin=*tuba*
4. ß-actin=*actb*
5. canonical axis=CA
6. canonical correlation analysis=CCA
7. chlorophyll a=chla
8. cytoskeleton-targeted genes=CTGs
9. discriminant analysis=DA
10. genome copy proportion=GCP
11. heat shock protein=HSP/*hsp* for protein and gene, respectively
12. Houbihu=HBH
13. Houwan=HWN
14. long-term ocean acidification experiment= LTOAE
15. maximum quantum yield of photosystem II=Fv/Fm
16. messenger RNA=mRNA
17. metabolism-targeted genes=MTGs
18. multidimensional scaling=MDS
19. multivariate ANOVA=MANOVA
20. multivariate statistical approaches=MSA
21. National Museum of Marine Biology and Aquarium=NMMBA
22. nitrate transporter-2=*nrt2*
23. not applicable=NA
24. ocean acidification=OA
25. organic anion transport=*oatp*
26. osmoregulation-targeted genes=OTGs
27. parts per million=ppm
28. phosphoglycolate phosphatase=*pgpase*
29. phospholipase-α2=*cplap2*
30. photosynthesis-targeted genes (PTGs)
31. photosystem I (subunit III)=*psI*
32. *Pocillopora damicornis* high temperature x pCO_2 study= PD*p*CO2
33. *Pocillopora damicornis* short-term temperature experiment=PDSTTE
34. principal component=PC
35. principal components analysis=PCA
36. real-time PCR=qPCR
37. ribulose-1,5-bisphosphate carboxylase/oxygenase=RBCL/*rbcL* for protein and gene, respectively
38. *Seriatopora hystrix* short-term temperature experiment=SHSTTE
39. *Seriatopora hystrix* variable temperature study=SHVTS
40. site of origin=SO
41. stable=stab
42. *Symbiodinium*=Sym
43. temperature=temp

44. temperature treatment=TT
45. threshold cycle=Ct
46. transient receptor cation channel=*trcc*
47. tropomyosin=*trp1*
48. variable=var

Introduction

In recent years, a concerted effort has been made to better understand the basic biology of reef-building corals (Peng et al., 2011; Chen et al., 2012; Mayfield et al., 2012b; Chen et al., 2015), as well as their response to changing environments (DeSalvo et al., 2008; Bellantuono et al., 2011; Mayfield et al., 2013c); the latter topic is especially pertinent given the extent of the anthropogenic pressures currently facing the high biodiversity ecosystems constructed by these cnidarian-dinoflagellate (genus *Symbiodinium*) endosymbioses (Hoegh-Guldberg et al., 2007; Huang et al., 2011). The impact of changing environments on corals is undoubtedly complex, and many species have been shown to acclimate to extreme abiotic regimes previously hypothesized to compromise the integrity of these calcium carbonate-accreting mutualisms. As an example, although most scleractinian coral-*Symbiodinium* associations readily dissociate when exposed to even small changes in their aquatic milieu, particularly with respect to temperature (Gates, 1990; Gates & Edmunds, 1999), those of Southern Taiwan have proven to be markedly resilient to an array of laboratory-simulated environmental challenges (Table 1).

For instance, the common, Indo-Pacific reef-builder *Seriatopora hystrix* showed no mRNA-level molecular chaperone response when exposed for two days to 30°C (Mayfield et al., 2011), a temperature hypothesized to ultimately elicit bleaching in this species based on observations made in Japan and elsewhere (Loya et al., 2001). In fact, the expression of only 2 genes out of the 14 targeted (6 from *Symbiodinium* and 8 from the coral host), the cytoskeleton-targeted genes (CTGs) β-actin (*actb*) and α-tubulin (*tuba*), were determined by real-time PCR (qPCR) to be affected by temperature (Mayfield et al., 2014a). Mayfield et al. (2011) hypothesized that such a lack of a molecular chaperone response, in particular, in either compartment of this holobiont ("host+endosymbiont") may have been due to mRNA "front-loading" (*sensu* Barshis et al., 2013) in samples of this "*S. hystrix* short-term temperature experiment" (SHSTTE). Briefly, corals of Southern Taiwan inhabit environments characterized by episodic upwelling, whereby temperatures may change by up to 9°C in a matter of several hours (Jan & Chen, 2008). Therefore, they could be predicted to exhibit high expression levels of heat shock proteins (HSPs) and other stress-targeted genes (STGs) and proteins even during ambient conditions in order to have the molecular machinery requisite for a temperature change-induced stress response at the time temperatures begin to fluctuate due to upwelling.

As an unexplored counter-hypothesis, it is also plausible that concerted, biologically meaningful changes in expression of multiple gene mRNAs and other molecular physiological response variables were simply overlooked due to having used univariate statistical approaches only. Multivariate statistical approaches (MSA), such as principal components analysis (PCA), canonical correlation analysis (CCA), and multidimensional scaling (MDS), can theoretically uncover treatment-derived and spatio-temporal differences *not* revealed by univariate statistics-based approaches employing standard ANOVA models by instead looking at the relationships or

correlations between various combinations of response variables simultaneously. Specifically, MSA can differentiate samples and treatments by integrating data across multiple parameters and so can partition samples within an experimental datascape in a holistic manner. Another advantage of MSA, such as multivariate ANOVA (MANOVA) and discriminant analysis (DA), is that such techniques are more statistically conservative when analyzing datasets featuring a large number of response variables; by assessing all parameters (e.g., 17 in the SHSTTE) in an integrated, single-step model, the chances of making a type I error are substantially reduced.

Given these merits, MSA were used to ascertain whether the *S. hystrix*-*Symbiodinium* holobiont was truly unresponsive to a short-term exposure to a temperature treatment (TT) hypothesized to elicit stress (*sensu* Downs et al., 2000). As a comparison in this meta-analysis, the dataset of the "*S. hystrix* variable temperature study" (SHVTS), which was also conducted at Taiwan's National Museum of Marine Biology and Aquarium (NMMBA), was re-explored, as corals of this study showed clear physiological and gene expression differences across both TT (stable vs. variable) and site of origin (SO; Mayfield et al., 2012a). Regarding the latter factor, unlike the SHSTTE, in which all corals were from an upwelling site within Nanwan Bay (Taiwan's southernmost embayment), Houbihu (HBH), half of those corals of the SHVTS were from a non-upwelling site, Houwan (HWN), which abuts NMMBA and is characterized by low coral cover and poor water quality due to coastal agricultural runoff (Liu et al., 2012). It was predicted that MSA could be used to conclusively demonstrate the lack of a gene expression effect on high temperature samples of the SHSTTE and, similarly, further verify both SO and TT differences in the molecular physiology of samples of the SHVTS. MSA were also employed to define characteristic phenotypes for samples of the SHVTS by identifying molecular physiological (gene expression+physiology) parameters that best separated samples by TT; the response variables underlying such TT-partitioned phenotypes would be those most likely to be involved in the response of this widely distributed coral to temperature changes.

Table 1. **Summary of eight environmental challenge studies performed at Taiwan's NMMBA between 2009 and 2014.** Please see the "Abbreviations" page for the full names of the experiments. In general, only corals exposed to 31.5°C for 2-4 weeks (PDSTTE#2) were found to bleach, die, and/or, more generally, display a significantly different phenotype that could be detected with the molecular physiological approach employed. OA=ocean acidification. *dataset re-analyzed herein with MSA.

Experiment	Year	Species	Sample material	Time-scale	Temp. (°C)	pCO_2 (ppm)	Major finding(s)	Reference(s)
SHSTTE*	2009	*S. hystrix*	Colony	2 d	27 vs. 30	NA	No response to elevated temp.	Mayfield et al., 2011, 2014a
SHVTS*	2010	*S. hystrix*	Nubbin	7 d	26 vs. 23-29 (var.)	NA	Corals can acclimate to variable temp., even if they had never before been exposed to such temp. regimes *in situ*.	Mayfield et al., 2012a, 2014a, in press Mayfield, Fan & Chen, 2013b
PDpCO_2-larvae	2010	*Pocillopora damicornis*	Larvae	10 d	25 vs. 29	400 vs. 630	No response to OA. Mild response to elevated temp. No interaction effect of OA and high temp.	Putnam et al., 2013
PDpCO_2-adult	2010	*P. damicornis*	Nubbin	2 wk	25 vs. 29	400 vs. 850	No response to OA. Mild response to elevated temp. No interaction effect of OA and high temp.	
PDLTTE	2010-2011	*P. damicornis*	Nubbin	9 mo	27 vs. 30	NA	No significant response to elevated temp., albeit *Symbiodinium* affected more strongly than host.	Mayfield, Fan & Chen, 2013a; Mayfield, Chen & Liu 2014; Mayfield et al., 2014c
PDSTTE#1	2011	*P. damicornis*	Nubbin	4 wk	Up to 32 (var.)	NA	Corals can acclimate to high temp. if temp. decreases to ambient at night.	Mayfield et al., 2013
PDSTTE#2	2011	*P. damicornis*	Nubbin	4 wk	27 vs. 31.5	NA	Exposure to 31.5°C for ~10 d elicits bleaching.	Mayfield et al., 2013
LTOAE	2014	*P. damicornis* *S. hystrix*	Nubbin	6 mo	25 vs. 31	400 vs. 1,000	Corals can acclimate to OA on a multi-month timescale.	

Materials and methods

The experiments

The SHSTTE (Mayfield et al., 2011, 2014a) and SHVTS (Mayfield et al., 2012a, in press) are described in prior works. Briefly, whole *S. hystrix* colonies from HBH were exposed to either the control (27°C) or elevated temperature (30°C) for 48 hr in the former, and RNAs, DNAs, and proteins were extracted from triplicate colonies housed within each of three replicate tanks at each of the two TT at each of four sampling times (6, 12, 24, and 48 hr; 18 samples/sampling time). A tank average was calculated across the three pseudo-replicates within the same tank sampled at each time, resulting in 24 data points that were analyzed by the MSA discussed below (n=3 biological replicates/sampling time/TT x 4 sampling times x 2 TT). All coral colonies were collected under Kenting National Park permit 0992900398 issued to Dr. Tung-Yung Fan (2009). Univariate repeated measures ANOVAs were used previously (Mayfield et al., 2011, 2014a) to assess the effects of time, TT, and their interaction on the 17 response variables described below.

In the SHVTS, six corals were sampled from each of the two aforementioned SO, and 48 nubbins were generated from the 12 colonies, all of which were of the same genotype (Mayfield et al., 2014a). Half of the nubbins from the six colonies of each SO were randomly assigned to stable TT aquaria maintained at 26°C, whereas the other half were placed into those aquaria of the variable TT, which ranged from 23-29°C over a 6-hr period. The 12 tanks (n=3 for each SO x TT interaction group) each contained four nubbins, two of which were sampled at time 0 (while all tanks were still at the acclimation temperature of 26°C) and two of which were sampled after 7 d of TT exposure; only the later 24 samples are discussed herein. In the case of the physiological response variables (discussed below), a tank average was calculated across the two pseudo-replicates sampled at the same time, resulting in a total sample size of 12 only. The molecular-scale data (n=17 parameters) were left unpooled for DA, but not for PCA. MDS was used with the 12 pooled samples after having incorporated all 23 response variables (described below). These 23 parameters were previously assessed individually with two-way ANOVAs to determine the effects of SO, TT, and their interaction (Mayfield et al., 2012a, 2014a, in press).

Response variables

The same 17 molecular response variables were assessed in the samples of each experiment and included three biological composition parameters: 1) the *Symbiodinium* genome copy proportion (GCP; a proxy for cell density; Mayfield, Hirst & Gates 2009), 2) the RNA/DNA ratio (a proxy for total transcription), and 3) the protein/DNA ratio (a proxy for total translation). Expression of 6 *Symbiodinium* of 8 host mRNAs was also quantified in each sample. The *Symbiodinium* target genes spanned three cellular processes: photosynthesis, metabolism, and the stress response. The photosynthesis-targeted genes (PTGs) included ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*), photosystem I (subunit III; *psI*), and phosphoglycolate phosphatase (*pgpase*). The lone metabolism-targeted gene (MTG) was nitrate transporter-2 (*nrt2*), and the two STGs were ascorbate peroxidase (*apx1*) and *hsp70*. The host genes also spanned three cellular processes: the cytoskeleton, the stress response, and transport processes involved in osmoregulation. The four CTGs were *actb*, *tuba*, tropomyosin (*trp1*), and

ezrin. The three osmoregulation-targeted genes (OTGs) were transient receptor cation channel (*trcc*), organic anion transporter (*oatp*), and phospholipase-α2 (*cplap2*). The lone STG was *hsp70*. The SHVTS included six additional response variables for a total of 23 parameters assessed (Mayfield et al., 2014a). These included the *Symbiodinium* RBCL protein and five physiological response variables: growth, chlorophyll a concentration (chla; normalized two different ways [areal and per cell]), *Symbiodinium* density, and the maximum quantum yield of photosystem II (Fv/Fm).

Meta-analysis

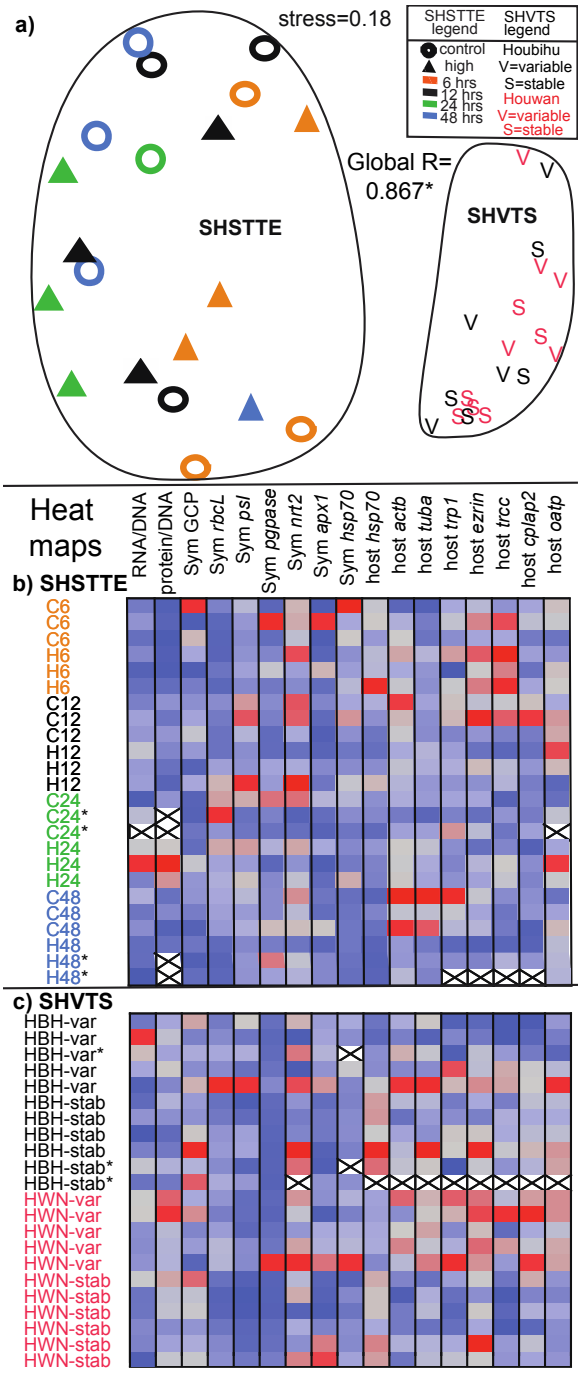
Both experimental datasets were considered in an initial MDS analysis performed with PRIMER (ver. 5) in order to both display the composite dataset and visualize inter-experimental variation. In this, and all other MDS analyses, Bray-Curtis similarity matrices were first created after having converted the data to *Z*-scores to account for the various parameters having different units. *Z*-score transformations were used for all other MSA, and all data in the supplemental Excel spreadsheet represent *Z*-scores, and not raw values. After constructing the MDS plot, which featured the 17 molecular-scale response variables only, analysis of similarity (ANOSIM) was conducted with PRIMER to determine the effect of experiment on the composite molecular phenotype (i.e., gene expression+biological composition) of the coral samples. Global R distribution *p*-values were considered significant at an α of 0.05 based on 999 permutations. Heat maps were created with JMP (ver. 12) to portray the relative levels of the 17 molecular response variables only. Except for MDS, JMP was used for all statistical analyses.

For the SHSTTE and SHVTS datasets individually, PCA was first used to depict variation in two dimensions, and it was hypothesized that meaningful groupings of samples might be unveiled with this approach alone. Unlike MANOVA, PCA does not generate multivariate means (i.e., centroids) and only gives a visual representation of the dataspace in multiple dimensions. It can also be used to find combinations of response variables that account for a large proportion of the variation within a dataset. PCA was conducted with a variety of different combinations of samples and response variables (Table 2) to attempt to find the minimum number of parameters that could visibly partition samples by TT or time in the SHSTTE and by TT or SO in the SHVTS. Next, a DA based on MANOVA and CCA was used to attempt to determine if the experimental sample centroids could be quantitatively separated within the dataspace. When sufficient replicates existed for the comparison of interest, Wilk's lambda values were calculated, and p values < 0.05 were considered to represent significance. When data points were missing for the time x TT interaction groups in the SHSTTE, Roy's max root values were instead calculated.

For the SHSTTE, discriminations by TT alone, time alone, and the interaction of TT and time were tested (n=17 parameters), and for the SHVTS, discriminations by TT alone, SO alone, and the SO x TT interaction were tested (n=23 parameters). DA was also performed for subsets of response variables in each experiment: molecular parameters only (n=17 parameters), the *Symbiodinium* molecular response only (n=6-7), the host coral mRNAs only (n=8), the physiological variables only (SHVTS only; n=4), and photosynthesis parameters only (n=3-6; Table 2). For both experimental datasets, PRIMER was used to perform MDS using the Bray-Curtis similarity matrix on *Z*-score-transformed data, and ANOSIM was performed to determine

time, TT, and time x TT effects in the SHSTTE and SO, TT, and SO x TT effects in the SHVTS. Finally, JMP's "predictor screening" function was used to rank the response variables in terms of their proportional contribution to the cumulative difference between sampling times and TT in the SHSTTE and between SO and TT in the SHVTS; only those parameters contributing to greater than 10% of the cumulative difference are discussed herein.

Figure 1. MDS plot and heat maps of the SHSTTE and SHVTS datasets. The MDS analysis (a) was conducted with the 17 molecular response variables only since physiological parameters were not measured in samples of the SHSTTE. *p<0.01. For the SHSTTE (b) and SHVTS (c) heat maps, the relative scale extends from dark blue (very low) to dark red (very high), and "x's" denote missing data. Samples marked by asterisks (*) were excluded from the MDS and most other MSA. Likewise, one sample from each of the HBH-variable (var) and HWN-var groups was excluded from the heat maps themselves due to the respective RNA extractions having failed. Please see the main text for full names of the target genes.



Results

Overview of the dataset

Prior to assessing variation and uncovering patterns within each of the two experiments, a collective MDS analysis was first conducted with 20 and 19 data points of the SHSTTE and SHVTS, respectively (Figure 1a); briefly 4 and 5 samples, respectively, out of the 24 total in each experiment were excluded from most of the MSA due to missing data. Some such samples are evident from heat maps of the SHSTTE (Figure 1b) and SHVTS (Figure 1c), and the

associated data were generally lacking due to failed nucleic acid extractions. It is clear from the MDS plot and the corresponding ANOSIM *p*-value (0.002) that samples of the two experiments were well separated when looking at all 17 molecular response variables. It is also apparent that the SHSTTE demonstrated greater overall variation than did the SHVTS. Although there was some overlap, the stable and variable TT samples of the SHVTS were somewhat separated from each other, and those samples sacrificed after 6 hr in the SHSTTE were slightly shifted to the right of the plot (i.e., away from those points of the other three sampling times). Both of these patterns are described in detail below using MSA specific to each dataset.

SHSTTE-PCA

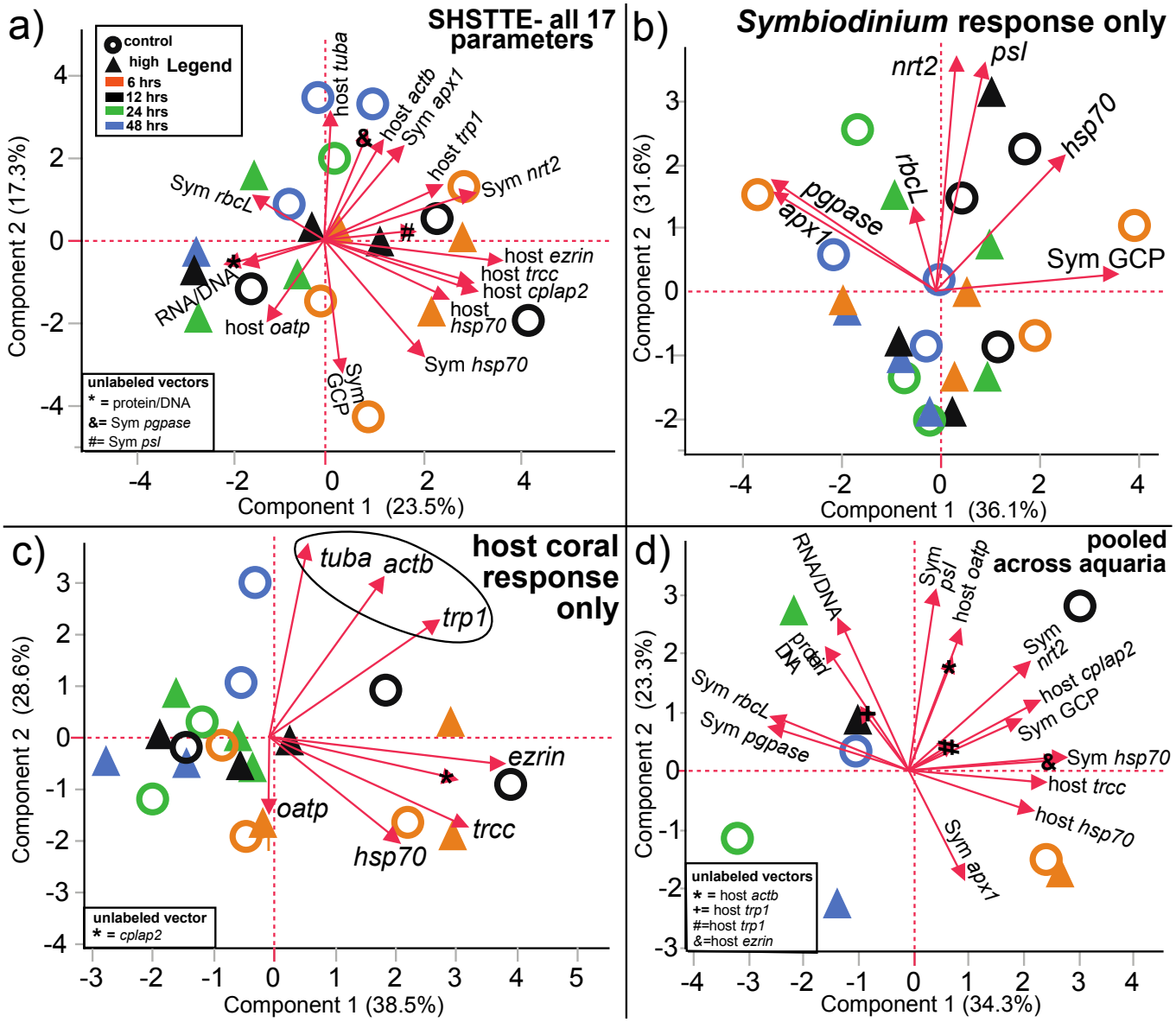
PCA (on correlations) was first performed on all 17 molecular response variables across 20 of the 24 samples (Figure 2a), and the first two eigenvectors captured only ~40% of the variation. However, it is clear that those corals sacrificed at the 6-hr sampling time tended to partition away from the others. It was hypothesized that a reduction in the number of parameters could lead to eigenvectors collectively encompassing a greater percentage of the variation in the dataset. When looking only at the seven *Symbiodinium* parameters (GCP+ 6 mRNAs), the first and second principal components (PC) encompassed ~67% of the variation (Figure 2b), and the response variable contributing the loading score with the highest positive value in PC1 was the *Symbiodinium* GCP. The second PC was dominated by the PTGs (excluding *rbcL*), meaning *Symbiodinium* density was negatively correlated with PTG expression. PCA of the *Symbiodinium* response variables only did not appear to be able to partition the data by TT or time (Figure 2b), and the data of both TT and all four sampling times were inter-mixed (i.e., panmixia).

When looking at the host coral mRNAs only (Figure 2c), the first two PC explained a similar percentage of the variation (~65%) as did the first two *Symbiodinium* PC (Figure 2b); furthermore, as when looking only at the *Symbiodinium* response variables, samples did not appear well separated by TT or time. However, the 6-hr data appear to be somewhat distinct from the others, with PC1 accounting for this apparent separation. The dominant loading factors for PC1 were two CTGs (*ezrin* and *trp1*) and two OTGs (*trcc* and *cplap2*). The CTGs co-varied, as evidenced by the similar trajectory of their biplot axes (circled for emphasis in the figure itself), and three of the four CTGs (excluding *ezrin*) contributed most significantly to PC2 in terms of eigenvector loading scores.

To determine whether extensive variation within treatments (i.e., a tank effect) accounted, in part, to the failure to document significant partitioning by TT, data were pooled across triplicate tanks within each of the eight TT x time interaction groups (Figure 2d). However, the first two PC accounted for less than 60% of the variation, and it is clear that the four data points of each TT are essentially mixed with those of the other TT. However, it does seem as if the time 6- and 24-hr data are well separated, as was somewhat evident when all data were considered (Figure 2a). A more quantitative approach was therefore utilized to investigate these temporal changes in the molecular signatures of samples of the SHSTTE.

Figure 2. PCA of the SHSTTE dataset. All 17 response variables (a), including three biological composition parameters (RNA/DNA ratio, protein/DNA ratio, and the *Symbiodinium* GCP), expression of 6 *Symbiodinium* mRNAs, and expression of 8 coral mRNAs, were assessed

across 20 of the 24 total samples (four samples were omitted due to missing data points [see Figure 1.]). All 24 samples were included in the PCA of the *Symbiodinium* response variables only (GCP+6 genes; [b]). For the eight host coral genes (c), the same four samples as in (a) were omitted, and the ends of the vectors representing three CTGs have been encircled to emphasize their co-variation. Data were also analyzed as pooled across aquaria for all 17 response variables for each of the eight TT x time interaction groups (d). The legend in (a) applies to all panels.



SHSTTE-DA, MDS, and predictor screening

When looking at the interaction of TT and time (Figure 3a) using data from all 17 response variables in a MANOVA/CCA-based DA analysis, Roy's max root was statistically significant, and this is likely due to the wide separation of samples of times 6 and 24 hr along canonical axis (CA) 1. This partitioning was driven by a negative relationship between *Symbiodinium pgpase* and *apx1* mRNA expression (Table 2). Within the 6- and 24-hr centroids, the high TT samples were reasonably well separated from the control samples, demonstrating the

interaction of TT and time. It should be noted, though, that only one sample comprised the control-24-hr group due to missing data. When looking at discrimination by TT alone (Figure 3b), it appears that the control and high TT groups were well separated along CA1; however, the Wilk's lambda value was not statistically significant. When looking at temporal discrimination only (Figure 3c), the 6-hr and 24-hr 95% centroids do not overlap, and are, furthermore, well separated across CA1. However, the Wilk's lambda was not significant, potentially due to the significant degree of overlap between the 12- and 48-hr centroids.

When looking at the MDS plot (Figure 3d), samples were significantly sorted by time (ANOSIM Global R p=0.002), but not by TT. Regarding the former factor, while the 12- and 48-hr samples were intermixed, the 6- and 24-hr times appear well separated, as was also evidenced by DA (Figure 3a, c), and, to some extent, PCA (Figure 2a, c). PCA, DA, and MDS all appear to suggest, then, that time, and not TT, was more important in accounting for variation in the SHSTTE dataset. Therefore, the predictor screening function of JMP was used to identify the response variables that explained the greatest proportion of the differences between sampling times (Figure 4a), and these were found to be the protein/DNA ratio (21% of the cumulative difference), *Symbiodinium pgpase* mRNA expression (14%), and the RNA/DNA ratio (13%). DA (Figure 3a) also found *Symbiodinium pgpase* to contribute to the separation of samples by time, specifically by distinguishing those samples of the 24-hr sampling time (Table 2).

Regarding TT (Figure 4a), only three response variables contributed to 10% or more of the documented cumulative difference in the molecular physiology of the control and high temperature samples: host *trp1* (17%), *Symbiodinium apx1* (14%), and the RNA/DNA ratio (13%). However, the expression of neither gene, nor the RNA/DNA ratio, differed significantly between TT despite the fact that the expression of *trp1* was 4-fold less in high temperature samples. When looking at the interaction of time and TT (Figure 4a), the RNA/DNA ratio and *Symbiodinium apx1* were the only factors that contributed to greater than 10% of the total difference between the four time x TT groups (22 and 12%, respectively); neither showed an interaction effect when analyzed by repeated measures ANOVA (p>0.05; Mayfield et al., 2014a).

SHVTS-PCA

PCA, DA, and MDS were all able to separate the samples by TT in the SHVTS, and some approaches were able to separate the four TT x SO groups (Table 2). First, PCA was able to distinguish a variety of informative groupings (Figure 5a). When looking at all 23 response variables for data pooled across pseudo-replicates for each of the 12 aquaria, there was a clear separation of the stable and variable TT samples along both PCs. However, the total variation encompassed by these two PCs was less than 55%. PC1 was comprised of a mix of host and *Symbiodinium* genes (Table 2), while the second PC consisted mainly of *Symbiodinium* PTGs. There was some degree of partitioning by SO within each TT, though still some overlap. Clearly, the effect of TT on the molecular physiology of *S. hystrix* was greater than that due to SO.

Figure 3. Discriminant and MDS analysis of the SHSTTE dataset. JMP's DA function was used to test for the effect of temperature x time (a), temperature alone (b), and time alone (c), and 4 of the 24 total samples were omitted due to missing data points (see Figure 1.); therefore, Roy's max root, rather than Wilk's lambda, was calculated to test for a significant interaction

effect in (a). In (a), the C12 and H12 centroids (black circles; 95% confidence intervals for these and all centroids presented herein) lie within the H48 (blue circle) centroid and have been labeled with red lines. In (b), the high and control TT centroids are red with white lining and white only, respectively. In (a-c), not all axes have been presented or labeled due to spatial constraints in the panels themselves. MDS analysis of the same 20 samples with PRIMER (d). In this panel only, circles were drawn by hand and do *not* represent 95% confidence centroids. The legend for all four panels lies in the lower-right corner of (d). control temp.=C. high temp.=H. *p<0.05.

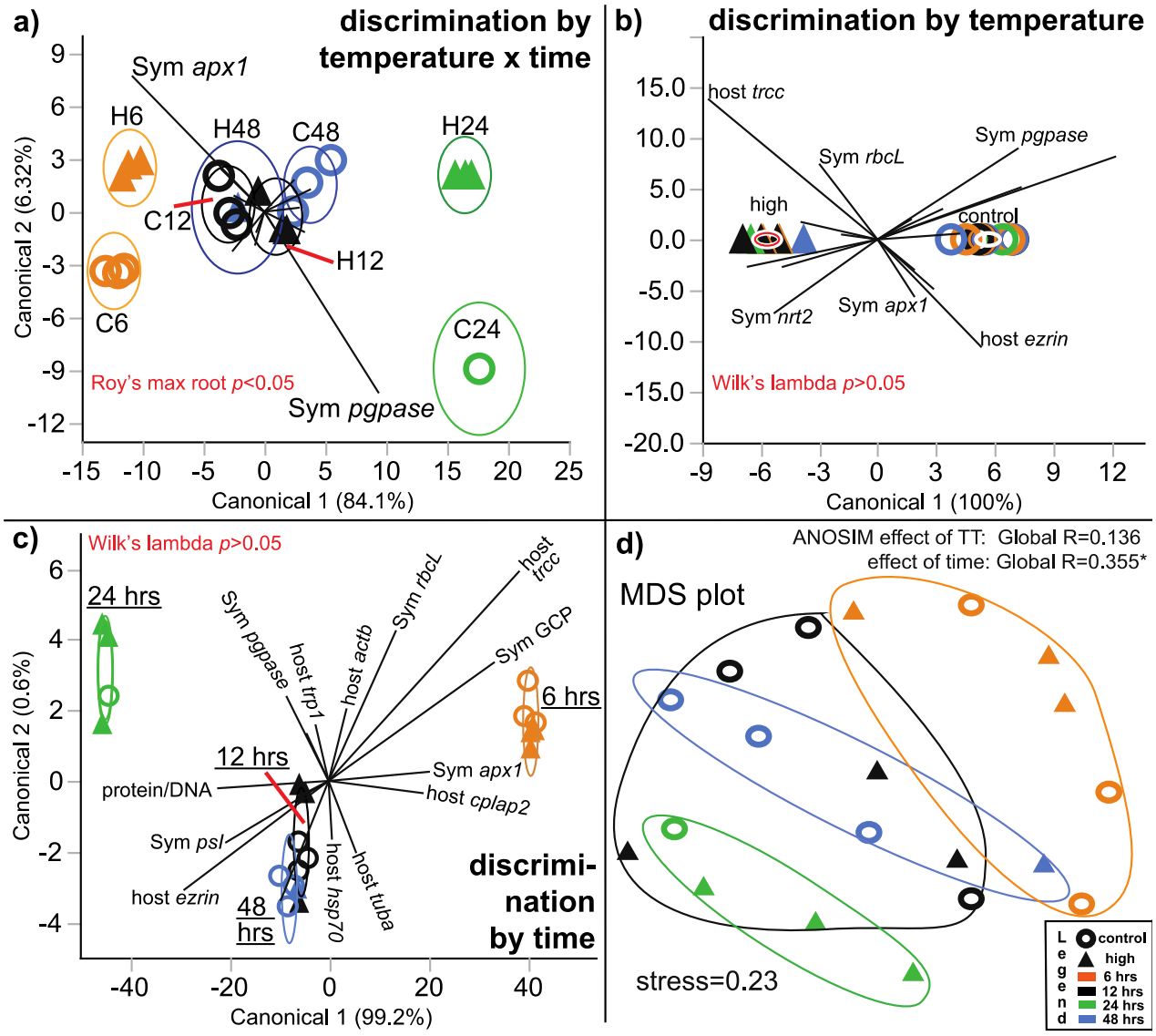
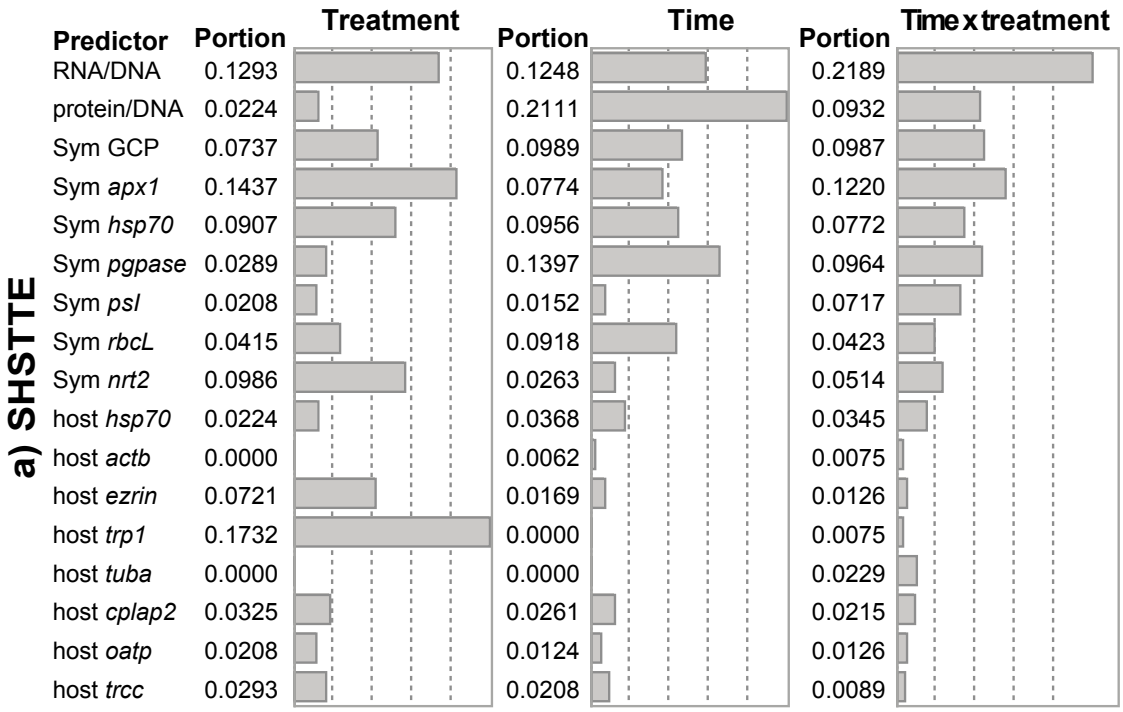


Figure 4. Predictor screening analysis of the SHSTTE and SHVTS. The predictor screening function of JMP was used to determine the response variables that accounted for the greatest proportions of the cumulative difference between TT, time, and TT x time in the SHSTTE (17 response variables; [a]) and between TT, SO, and SO x TT in the SHVTS (23 response variables; [b]). It should be noted that the relative scales differ between the proportion plots.



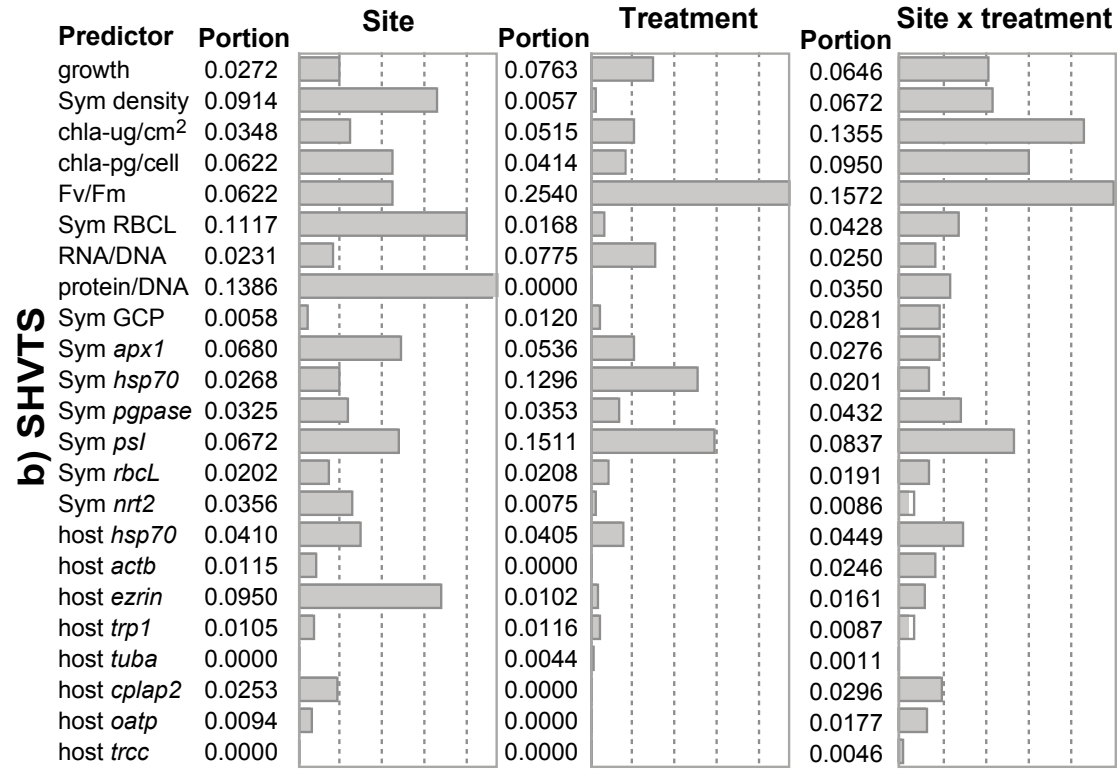


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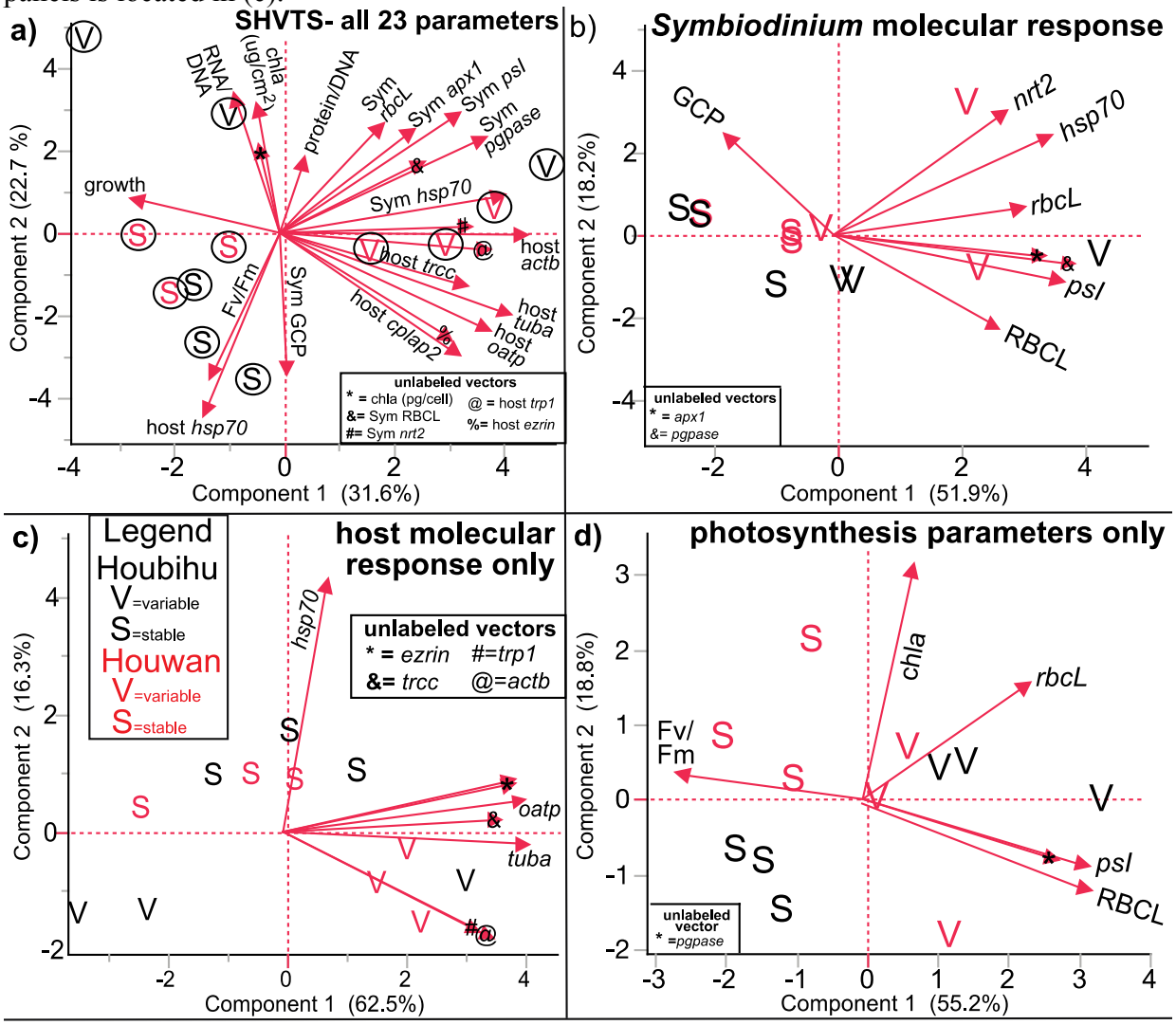
Table 2. **Summary of comparisons and major findings.** Dominant loading factors and canonical correlations are only included in the right-most column when the respective technique resulted in good partitioning of the data. "*Symbiodinium* molecular response" includes the GCP + 6 mRNAs for all comparisons except for PCA of the SHVTS, in which case the RBCL protein was also included. In certain cases, Wilk's lambda or comparable MANOVA-based statistics could not be calculated due to having a large number of response variables relative to observations. Only when the *Symbiodinium* data were included could temporal partitioning be achieved in the SHSTTE; in contrast, data from the eight host coral genes were required to separate samples by TT and SO x TT in the SHVTS. *statistically significant observation. When *p*-values approached significance (α=0.05), the exact values have been included. NA=not applicable.

Comparison-method	Fig.	# para-meters	# sam-ples	Conclusion(s)	Dominant loading factors/canonical correlations
SHSTTE vs. SHVTS *MDS*	1a	17	20 vs. 19	Experimental datasets are well separated*	*Symbiodinium hsp70*, host *oatp*, & RNA/DNA[c]
SHSTTE (Figures 2-3)					
PCA (Figure 2)					
All response variables	2a	17	20	Time=6-hr samples are somewhat separated	Mix of host & *Symbiodinium* genes
Symbiodinium molecular response	2b	7	24	panmixia	
Host coral genes	2c	8	20	Some separation of time=6-hr samples	Mix of CTGs, STG, & OTGs
All response variables (pooled)	2d	17	8	More separation by time than by TT	Mix of host & *Symbiodinium* genes
Photosynthesis parameters only		3[a]	20	panmixia	NA
DA (Figure 3)					
Discrimination by time and TT	3a	17	20	Times=6-hr & 24-hr are well separated*	Negative relationship between *Symbiodinium* *apx1* & *pgpase*
Host coral genes		8[a]	24	panmixia	
Symbiodinium molecular response		7[a]	22	panmixia	
Discrimination by TT only	3b	17	20	panmixia	
Host coral genes		8[a]	24	panmixia	
Symbiodinium molecular response		7[a]	22	panmixia	
Discrimination by time only	3c	17	20	Times=6-hr & 24-hr are well separated	Negative relationship between host *trcc* & *ezrin*
Host coral genes		8[a]	24	panmixia	
Symbiodinium molecular response		7[a]	22	Time=6-hr separated from other 3 times*	Negative relationship between *apx1* & *psI*
MDS	3d	17	20	Times=6-hr & 24-hr are well separated*	protein/DNA, *Symbiodinium hsp70*, & host *hsp70*[c]
SHVTS (Figures 5-6)					
PCA (Figure 5)					
All response variables	5a	23	12	Two TT are well separated	Mix of host & *Symbiodinium* genes
Symbiodinium molecular response	5b	8	12	Two TT are somewhat well separated	*Symbiodinium* genes & RBCL protein (PC1)
Host coral genes	5c	8	12	Two TT are well separated	Host *hsp70* (PC2)
Photosynthesis parameters only	5d	6	12	Two TT are well separated	*psI* & RBCL

DA (Figure 6)					
Discrimination by SO x TT Physiological response only	6a	4	12	Two groups are well separated (HBH-var & HWN-stab)*	Fv/Fm
Discrimination by SO x TT Molecular response only	6b	17	20	Four SO x TT groups are well separated	Negative relationship between host *actb* & *Symbiodinium psI*+protein/DNA
Discrimination by SO x TT *Symbiodinium* molecular response	6c	7	19	Moderate separation by SO x TT (p=0.059)	
Discrimination by SO x TT Host coral genes only	6d	8	21	Two TT are well separated*	Negative relationship between *hsp70* & *tuba*
Discrimination by SO x TT All response variables	6e	23	12	Four SO x TT groups are well separated[b]	Negative relationship between *Symbiodinium hsp70* & *nrt2*
Discrimination by SO only Physiological response only		4[a]	12	Two SO are somewhat separated	
Discrimination by SO only Molecular response only		17[a]	20	Two SO are well separated*	Negative relationship between *Symbiodinium pgpase* & *hsp70*
Discrimination by SO only *Symbiodinium* molecular response		7[a]	20	panmixia	
Discrimination by SO only Host coral genes only		8[a]	21	panmixia	
Discrimination by SO only All response variables		23[a]	12	Two SO are well separated[b]	Negative relationship between *Symbiodinium psI* & *hsp70*
Discrimination by TT only Physiological response only		4[a]	12	Two TT are well separated (p=0.052)	Fv/Fm
Discrimination by TT only Molecular response only		17[a]	20	Two TT are well separated (p=0.058)	Negative relationship between host *tuba* & *oatp*
Discrimination by TT only *Symbiodinium* molecular response		7[a]	20	Two TT are well separated (p=0.065)	Negative relationship between *rbcL+hsp70* & *apx1+pgpase*
Discrimination by TT only Host coral genes only		8[a]	21	Two TT are well separated*	Negative relationship between *hsp70* & *actb+tuba+trcc*
Discrimination by TT only All response variables		23[a]	12	Two TT are well separated[b]	Negative relationship between host *hsp70* & *actb*
MDS (Figure 6)	6f	23	12	Four SO x TT groups are well separated*	Physiological parameters & *Symbiodinium psI*[c]

412 [a]data not shown. [b]could not compute Wilk's lambda or Roy's max root. [c]determined by JMP's predictor screening function (Figure 4).

Figure 5. PCA of the SHVTS. Data from pseudo-replicate samples of the same tank were pooled, resulting in 12 data points in each plot. a) All 23 parameters. b) The *Symbiodinium* molecular response only (GCP+6 mRNAs+RBCL protein). c) The host molecular response only (8 mRNAs). In (c), the *cplap2* axis is below the *ezrin* one and is unlabeled. Likewise, the *trp1* and *actb* axes are overlapping. d) The six photosynthesis parameters only. The legend for all panels is located in (c).



In order to reduce the complexity of the dataset, PCA was also conducted only with the eight *Symbiodinium* molecular response variables: the GCP, the six mRNAs, and the RBCL protein (Figure 5b). It is clear that samples of the two TT, stable and variable, could be distinguished by PC1 (51.9%), which was dominated by the PTGs in terms of highest positive eigenvector loading factor scores (Table 2). The stable TT samples tended to cluster together, with the six variable TT samples showing greater variability and spread throughout the dataspace. Furthermore, the HWN variable TT samples showed greater dispersal than did the HBH ones. The *Symbiodinium* GCP was the most significant contributor to the second PC (18.7%); this indicates that *Symbiodinium* PTG expression was negatively correlated with *Symbiodinium* density, as was also the case in the SHSTTE. When looking at individual correlations of PTG

expression vs. *Symbiodinium* GCP (data not shown), all slopes were negative; however, these slopes were not significantly different from 0 (linear regression *t*-tests, $p>0.05$).

When looking at the host coral molecular response only (Figure 5c), it is clear that PCA was able to partition samples of the two TT. The first PC encompassed all three CTGs and four OTGs and explained 62.5% of the variation (Table 2). The second PC consisted of *hsp70* as the only positive loading score to the eigenvector, and this PC encompassed 16.3% of the variation. It is clear that the OTGs and CTGs tended to co-vary. As with the PCA of the *Symbiodinium* molecular response variables only (Figure 5b), the spread of the variable TT samples was greater than that of the stable TT samples. In contrast to the results of the PCA of the *Symbiodinium* molecular response only, the HBH samples showed more spread than those of HWN. Finally, when looking only at the six photosynthesis parameters (Figure 5d), it is clear that samples of the TT were well separated along PC1 (55.2%), in which *psI* gene and RBCL protein expression contributed the highest positive loading scores (Table 2). Samples of the two SO for the stable TT were separated along PC2, in which case chla content (pg/cell) contributed most significantly.

SHVTS-DA, MDS, and predictor screening

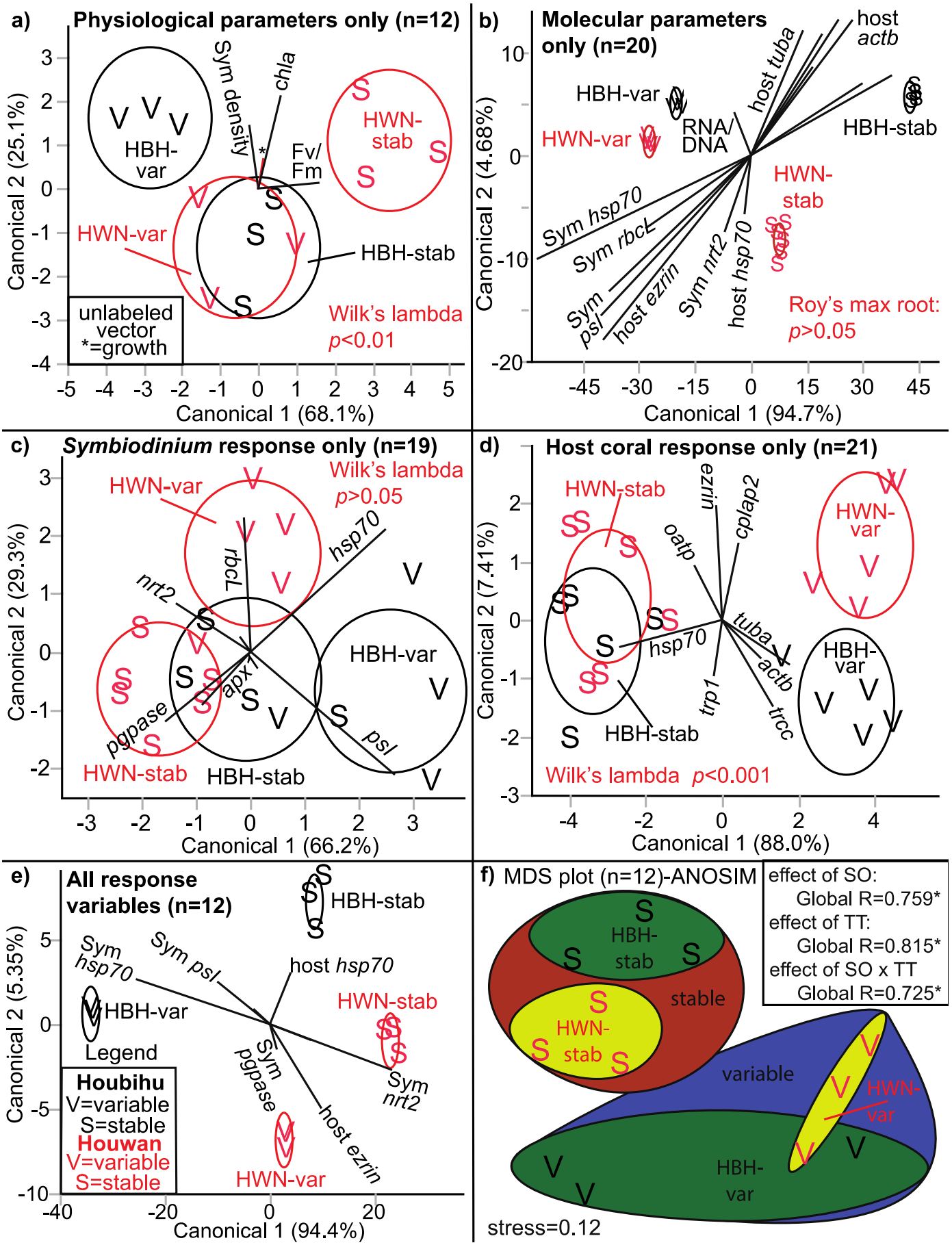
PCA was able to partition samples by TT and, to some extent, SO. Therefore, more quantitative MSA were used to verify these findings in a statistically rigorous manner. A variety of combinations of response variables were used to see which best modeled differences between the four SO x TT groups (Table 2). First, DA was performed with four of the five physiological response variables alone (areal chla was excluded in place of chla/cell; Figure 6a). Although Wilk's lambda for the interaction of SO and TT was significant, only two of the four groups appear well-separated: HBH-var and HWN-stab. HBH-stab and HWN-var appear inter-mixed. Fv/Fm was the most significant factor contributing to the separation of the former two groups (Table 2). When looking at all 17 molecular response variables with 20 of the 24 samples, all four SO x TT groups appear to be well separated (Figure 6b), though Wilk's lambda could not be computed due to the high number of response variables relative to the number of biological replicates (~1:1). A negative relationship between host *actb* and *Symbiodinium psI*+protein/DNA drove the partitioning of the four SO x TT groups (Table 2), though such partitioning was not considered statistically significant by Roy's max root.

After performing a DA of seven of the eight *Symbiodinium* molecular response variables only (RBCL protein expression was excluded since it was only assessed in 12 of the 24 samples; Figure 6c), it is clear that this sub-set was unable to differentiate the four SO x TT groups. In contrast, when looking at the eight host coral genes alone (Figure 6d), significant discrimination was achieved. However, the two SO were only well separated in the variable TT dataset and were intermixed for the stable TT samples. When looking at all 23 response variables, the four SO x TT groups were well separated (Figure 6e), though neither Wilk's lambda nor Roy's max root could be calculated due to the large number of response variables relative to observations.

In contrast to MANOVA-based DA, MDS can readily quantify relationships between samples even when the number of response variables is large relative to the number of observations, and MDS was able to distinguish the four SO x TT groups of the SHVTS (Figure 6f). ANOSIM found SO, TT, and SO x TT interaction effects to be significant, and, within the stable TT samples, it is clear that data points of the two SO were well separated. However, these

was some overlap between the HBH-var and HWN-var samples due to one HBH-var data point falling closer to those points of the latter group. JMP's predictor screening function (Figure 4b) found the five physiological parameters and *Symbiodinium psI* to contribute most significantly to the cumulative difference between the four SO x TT groups (Table 2). *psI* also contributed significantly to the cumulative difference between TT (Figure 4b), in conjunction with Fv/Fm and host *hsp70* (Table 2).

Figure 6. Discriminant and MDS analysis of the SHVTS. DA was used to model differences between the four SO x TT interaction groups with four of the five physiological parameters (excluding areal chla; [a]), all 17 molecular response parameters (b), seven of the eight *Symbiodinium* molecular response variables (excluding the RBCL protein; [c]), all eight host coral mRNAs (d), and all 23 response variables (e). The sample sizes displayed in the individual panels reflect the number of samples and not the number of response variables. Wilk's lambda could not be computed in (b) and (e) due to the large number of response variables relative to the number of samples. In (b), not all axes have been labeled. In (c), the *Symbiodinium* GCP axis falls between those of the mRNAs *nrt2* and *rbcL*. In (e), only the dominant axes/response variables have been shown due to spatial constraints in the panel itself. In (a-d) Wilk's lambda (a, c, d) and Roy's max root (b) values test the interaction of SO and TT. In (f), PRIMER's MDS function was used to portray the SHVTS dataspace, and the circles were hand-drawn to encompass the four SO x TT groups. All other circles represent 95% confidence centroids. The legend in (e) corresponds to all panels. *=$p<0.01$.



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Discussion

This represents the first work to exploit MSA for assessing molecular and physiological response variables spanning both compartments of an endosymbiotic organism, and the conceptual framework for doing so will ideally be of use to others interested in understanding how dual-compartmental organisms respond to changes in their environment. As mentioned above, corals of Southern Taiwan have proven to be resilient to a number of laboratory-induced environmental challenges, and MSA confirmed this univariate ANOVA-based observation for samples of the SHSTTE; specifically, there was no evident separation of samples between control and high temperatures. This leaves at least two hypotheses remaining: 1) the corals were truly unstressed upon a short-term exposure to 30°C or 2) non-responsive parameters were chosen. Given the well-documented photoinhibition that occurs when *Symbiodinium* are exposed to elevated temperatures (e.g., Jones et al., 1998), it seems likely that at least several PTGs should have undergone changes in mRNA expression. However, a recent work (Mayfield et al., in review) found virtually no congruency between gene and protein expression in *S. hystrix* or its endosymbiotic dinoflagellate populations. Therefore, it could be that the respective photosynthesis- and stress-targeted *proteins* indeed underwent changes in expression upon exposure to 30°C for 48 hr. Future work should, then, attempt to characterize the proteomes of pocilloporids, and other reef corals, exposed to theoretically stress-inducing temperatures to uncover the sub-cellular basis of the stress or acclimation response, whichever the case may be.

Despite the absence of a multivariate TT effect in the SHSTTE, there were some notable temporal differences, particularly when looking at the corals sampled after 6 hr of treatment exposure; when using DA, a mix of biological composition, host gene expression, and *Symbiodinium* gene expression data best separated the 6-hr samples from the others, and both DA and JMP's predictor screening function found the protein/DNA ratio to account significantly for this temporal difference. Indeed, the protein/DNA ratio was found previously to be temporally variable in these samples (Mayfield et al., 2014a). Furthermore, negative correlations between two *Symbiodinium* genes (the STG *apx1* and the PTG *pgpase*) and two host coral genes (the CTG *ezrin* and the OTG *trcc*) genes were found to partition samples of the 6-hr time from those of the 24-hr time (i.e., the two groups that were most distinct from one another); none of these genes were found to be temporally variable in expression by univariate repeated measures ANOVA (Mayfield et al. 2014a), demonstrating the utility of MSA in defining combinations of response variables that best explain patterns within a dataset.

From the DA, MDS, and, albeit less so, PCA, it is clear that corals of the four sampling times possessed unique gene expression+biological composition signatures, and this temporal change in the molecular phenotype of these samples may be related to the complex, dual-compartmental metabolism displayed by organisms, such as reef-building corals, that have acquired the capacity for photosynthesis via symbiosis (Mayfield & Gates, 2007; Mayfield et al., 2014b). Specifically, coral metabolism is temporally variable due to the periodic nature of light-driven photosynthesis (Mayfield et al., 2010, 2014b), and metabolic hysteresis driven by dinoflagellate photosynthesis as a function of the light cycle surely contributed to the temporal variation observed in the SHSTTE. Circadian rhythm may also have accounted, in part, for the separation of the 6- and 24-hr samples in the SHSTTE. The former were collected at 13:45, and, while stable, artificial light was used in this experiment, it is possible that the temporal gene

expression signatures were driven by an entrained response to high light levels that would normally be experienced at such times. The 12, 24, and 48-hr sampling times corresponded to 19:30, 7:30, and 7:30, respectively, times at which light levels would be relatively low *in situ*. However, the experimental corals had been reared under non-fluctuating, artificial light (12:12hr light-dark) for nearly one month at the time of sampling (including the pre-experimental acclimation period), and circadian rhythm can be abolished within two days of changing the light regime in endosymbiotic anthozoans (Mayfield et al., 2014b). Therefore, other factors besides metabolic hysteresis due to photosynthesis and circadian rhythm may have accounted for the unique molecular phenotype of corals sampled after 6 hr.

All MSA documented TT, and oftentimes SO, differences in the SHVTS. This is unsurprising given the distinct PTG expression profiles between stable and variable TT samples documented by Mayfield et al. (2012a). Despite such PTG expression variation, the host coral parameters were actually more likely to partition samples by TT in the SHVTS; this contrasts with the SHSTTE, in which the *Symbiodinium* response variables were relatively more important in the separation of samples across the dataspace. However, the predominant experimental factor leading to sample partitioning in the SHSTTE was time, rather than temperature. As *Symbiodinium* gene expression, and physiology in general, is known to be highly dynamic (Mayfield et al., 2014b), this finding was not unexpected. Importantly though, the fact that the *Symbiodinium* response variables more significantly contributed to temporal variation in the SHSTTE, while host coral parameters led to a relatively greater partitioning of samples by TT in the SHVTS, emphasizes that notion put forth by Mayfield et al. (2014c) that it is important to consider both compartments of the coral-*Symbiodinium* endosymbiosis when attempting to gauge the molecular physiological response of the composite holobiont to environmental change.

When performing PCA on the *Symbiodinium* molecular response only, the HWN variable TT samples showed greater dispersal than did the HBH ones. This could be because these HWN corals had never before experienced such variable temperature profiles *in situ*; as such, the variability in their response to fluctuating temperatures could be hypothesized to be greater than that of corals of HBH, which *do* routinely experience upwelling. Likewise, when looking at the MDS plot of the SHVTS dataset, the spread of the variable TT samples was greater than that of the stable ones, and a similar explanation could account for this observation. Indeed, variability in the physiological response to an environmental change has been predicted to be important in gauging marine animal health (Clarke & Warwick, 1994).

Curiously, though, the HBH samples exposed to variable temperatures showed a greater diversity in their molecular physiological response in the MDS plot than those of HWN exposed to this profile, in contrast to what was observed with PCA for the *Symbiodinium* response only. This variable reaction of HBH samples exposed to fluctuating temperatures may be due to differential acclimation strategies between the original colonies, which may have been from different micro-habitats within the HBH reef system. Although an effort was made to collect colonies at similar depths in a reasonably small area (~10-100 m of each other; Mayfield et al., 2012a), it is possible that the light environment *in situ*, for instance, may have differed between the colonies used to make the nubbins. Nubbins from the six colonies from HBH were mixed in a seawater table and randomly assigned to each TT. Therefore, the HBH-var MDS outlier may have represented a nubbin from a colony that experienced a different abiotic environment *in situ*

than the other two nubbins of that SO x TT group. In short, differing environmental histories of the colonies of HBH may have contributed to biological variation in the dataset, whereas the colonies removed from HWN may have been characterized by more similar environmental histories and thus behaved more similarly when exposed to a foreign temperature regime. Regardless of the explanation, it is clear the molecular physiology differed significantly between corals of the two SO and TT, suggesting that corals of the four SO x TT interaction groups displayed distinct behavior with respect to the 23 response variables assessed herein.

Although MSA were successfully used to define time-specific phenotypes in the SHSTTE and molecular physiologies with fidelity to both SO and TT in the SHVTS, there is not, as mentioned above, a significant degree of congruency between gene and protein expression in this reef coral (Mayfield et al., in review); therefore, although gene expression signatures may be used to partition corals from multiple SO and TT within an experimental dataspace in order to uncover intra- and inter-experimental differences, care should be taken before using such gene expression trends to enact mechanistic reconstructions of cell physiology, as has become standard in the field of coral biology (e.g., Barshis et al., 2013; Palumbi et al., 2014). Rather, the proteins that actually conduct cellular work are better molecular targets for those interested in making statements as to how corals respond to, for instance, changes in their abiotic environments. Such proteome-scale data could be analyzed in an analogous manner as was conducted herein in order to define protein expression signatures that underlie the sub-cellular capacity for reef corals to acclimate to global climate change. MDS is an especially well-suited means of displaying a molecular phenotype that integrates a number of different response variables and macromolecules as, unlike CCA, DA, and MANOVA, ANOSIM can still be calculated when the number of response variables is large relative to the number of samples. As such, it could hypothetically be used to screen for protein biomarkers of the coral response to environmental perturbation.

Conclusions

The MSA utilized defined molecular signatures across time in the SHSTTE dataset, which was largely found to feature negative findings (i.e., no significant change) when analyzed by traditional, univariate approaches alone (Mayfield et al., 2011; 2014a). Rather than the absolute expression level of a gene mRNA characterizing a sample group, relationships between multiple response variables and genes were instead found to better distinguish corals sampled at different times. In the SHVTS, multiple groupings of response variables (e.g., gene expression, physiological, and biological composition parameters) could partition samples by TT, and the molecular physiological phenotypes differed significantly between corals of these two TT. Unlike the SHSTTE, in which the *Symbiodinium* response was a greater contributor to the overall variation of the dataset, host coral response variables better partitioned data points of the two TT in the SHVTS. Furthermore, corals exposed to variable temperatures showed a greater range in their molecular physiological response relative to those exposed to stable temperature; however, depending on the MSA used, the spread in the dataspace was sometimes greater for samples of HBH, which experience both stable and variable temperatures *in situ*, and sometimes greater for those of HWN, which only experience stable temperatures *in situ*. This result is perplexing and could be driven by spatial heterogeneity in the abiotic environment of HBH, a

reef characterized by extensive temporal environmental variation due to upwelling (Jan & Chen, 2008).

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