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ORIGINAL ARTICLE

Intraspecific variation in polar and nonpolar metabolite profiles of a threatened Caribbean coral

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Abstract

Introduction Research aimed at understanding intraspecific variation among corals could substantially increase understanding of coral biology and improve outcomes of active restoration efforts. Metabolomics is useful for identifying physiological drivers leading to variation among genotypes and has the capacity to improve our selection of candidate corals that express phenotypes beneficial to restoration.

Objectives Our study aims to compare metabolomic profiles among known, unique genotypes of the threatened coral *Acropora cervicornis*. In doing so, we seek information related to the physiological characteristics driving variation among genotypes, which could aid in identifying genets with desirable traits for restoration.

Methods We applied proton nuclear magnetic resonance (^{1}H-NMR) and liquid chromatography-mass spectrometry (LC-MS) to identify and compare metabolomic profiles for seven unique genotypes of *A. cervicornis* that previously exhibited phenotypic variation in a common garden coral nursery.

Results Significant variation in polar and nonpolar metabolite profiles was found among *A. cervicornis* genotypes. Despite difficulties identifying all significant metabolites driving separation among genotypes, our data support previous findings and further suggest metabolomic profiles differ among various genotypes of the threatened species *A. cervicornis*.

Conclusion The implementation of metabolomic analyses allowed identification of several key metabolites driving separation among genotypes and expanded our understanding of the *A. cervicornis* metabolome. Although our research is specific to *A. cervicornis*, these findings have broad relevance for coral biology and active restoration. Furthermore, this study provides specific information on the understudied *A. cervicornis* metabolome and further confirmation that differences in metabolome structure could drive phenotypic variation among genotypes.

Keywords Coral restoration · Mass spectrometry · Metabolomics · Lipidomics · *Acropora cervicornis* · Coral reefs

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1 Introduction

Coral reefs are one of the ecosystems most at risk as Earth enters the Anthropocene (Hughes et al., 2017), an era in which human activity has become a major force impacting ecological and environmental processes (Crutzen, 2006; Lewis & Maslin, 2015). Changes, including increases in sea surface temperature (Levitus et al., 2000; Rayner et al., 2003), disease (Aronson & Precht, 2001a, b; Harvell et al., 2007, Maynard et al., 2017), pollution (Loya & Rinkevich, 1980, Donovan et al., 2020), and extreme weather events (Emanuel, 2005, 2013), are increasingly impacting coral reefs (Goreau & Hilbertz, 2005; Young et al., 2012). In the Western Atlantic, stony coral cover has decreased by greater than 80% in the last 40 years (Gardner 2003, Maynard et al., 2017; NOAA, 2018). The decline of stony corals throughout the Western Atlantic was heavily driven by the loss of Caribbean staghorn coral, *Acropora cervicornis*, and its congener, *A. palmata* (Gardner et al., 2003). The decline of these species, once spatially dominant on fore reefs throughout the region (Aronson & Precht, 2001a, b; Pandolfi & Jackson, 2006), is largely attributed to disease (Aronson & Precht, 2001a, b; Gardner et al., 2003). As a large, open-branching species, *A. cervicornis* is essential for creating three-dimensional structure (Bellwood et al., 2004; Young et al., 2012). This species has declined by 97% over the last 40 years throughout South Florida and parts of the Caribbean (Acropora Biological Review Team, 2005) and therefore became one of the first corals listed under the U.S. Endangered Species Act (NMFS, 2006). Subsequently, *A. cervicornis* has become one of the species most commonly cultured for restoration efforts (Young et al., 2012, Boström-Einarsson et al., 2020) and is a focal species for ongoing research related to coral restoration.

Concurrent with remediation of environmental stressors leading to coral decline, recovery of *A. cervicornis* would benefit from the development of novel tools to enhance the capacity for, and efficiency of, restoration (Baums, 2008; Harris et al., 2006; Van Oppen et al., 2017). Natural variation in phenotype is well-documented within coral populations (Granados-Cifuentes et al., 2013; Suggett et al., 2019) and specifically *A. cervicornis*. Previous studies have suggested intraspecific variability exists with regard to disease resistance (Vollmer et al., 2008; Bock, 2018), growth, and thermal tolerance (Lirman et al., 2014; Drury et al., 2017; Lohr & Patterson, 2017) in this species. Understanding intraspecific variation in regard to phenotypes of interest could substantially improve the long-term success of coral restoration efforts. Genotypes possessing key phenotypes could be strategically integrated in restoration, selective breeding, and adaptive management strategies. Metabolomic profiling is a tool that can potentially aid in the process of identifying these genotypes for use in restoration.

Metabolomic profiling provides a mechanism to identify and measure an organism's full complement of metabolites, including those that may underlie key phenotypes (Patti et al., 2012). Exploring the metabolome of corals presents particular challenges due to the complex interactions between corals, the symbiotic zooxanthellae living their tissues, and their environment (Gordon & Leggat, 2010). Although these interactions complicate our understating of coral physiology (Rohwer et al., 2002), metabolomics can provide valuable insight into the anabolic and catabolic pathways that drive variation between genetically different corals of the same species and potentially identify metabolite biomarkers that indicate how a coral responds to its environment (Sogin et al., 2014).

For this study, we employed proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and liquid chromatography mass spectrometry (LC-MS) to compare metabolomic profiles among seven unique genotypes of *A. cervicornis* from a common garden. ^{1}H-NMR is less sensitive compared to LC-MS, but is valuable for identifying unknown compounds (Markley et al., 2017). Conversely, LC-MS can detect smaller compounds and those occurring at lower concentrations (Emwas, 2015). This investigation builds upon two previous studies characterizing intraspecific variability among a set of *A. cervicornis* genotypes in a common garden (Lohr & Patterson, 2017; Lohr et al., 2019). Data from this study provide further insight into intraspecific variation in coral metabolomes.

2 Material and methods

2.1 Sample collection and extraction

This study used corals collected from a Coral Restoration Foundation (CRF) nursery in Tavernier, Florida. Coral genotypes were previously determined using microsatellites developed by Baums et al. (2009). Seven genotypes of interest were selected (Table 1) based on past research that suggested differences in growth, bleaching resistance, and disease resistance (Bock, 2018; Lohr & Patterson, 2017). Research by Lohr and Patterson (2017) was carried out in an ocean nursery, whereas Bock (2018) was performed in ex situ aquaria. A subset of these seven genotypes was previously profiled in Lohr et al. (2019): U25, U41, and U44. In an effort to assess whether differences in these three genotypes were also present in the current study, we analyzed the three genotypes both separately and in concert with the

Table 1 Phenotypic characteristics of the seven *Acropora cervicornis* genets examined in this study

Genotype	Trait
K1	Slow growth, moderate bleaching susceptibility (Lohr & Patterson, 2017)
K2	Rapid growth, moderate bleaching susceptibility (Lohr & Patterson, 2017), low disease resistance (Bock, 2018)
K3	Moderate growth, moderate bleaching susceptibility (Lohr & Patterson, 2017)
U25	Slow growth, high bleaching susceptibility (Lohr & Patterson, 2017), low disease resistance (Bock, 2018)
U41	Rapid growth, moderate bleaching susceptibility (Lohr & Patterson, 2017)
U44	Moderate growth, bleaching resistant (Lohr & Patterson, 2017)
U77	Slow growth, moderate bleaching susceptibility (Lohr & Patterson, 2017), high disease resistance (Bock, 2018)

other four. For each of the seven genotypes, we collected six nubbins (two fragments from three different coral colonies per genotype).

In January 2018, coral colonies from the CRF nursery were brought to the surface intact. All collections occurred on a single day in the winter season. During the winter, water temperatures are lower in the Florida Keys, and thus, metabolomic alterations due to thermal stress were not expected. Furthermore, in order to minimize seasonal variability, the sampling period was selected to match a previous study (Lohr et al., 2019) with which this study seeks to compare results. While still submerged at the surface, ~ 3-cm nubbins were clipped from actively growing branch tips, wrapped in aluminum foil, and immediately frozen in liquid nitrogen. Following transportation to the lab, nubbins were ground to a powder in an ice-chilled mortar and pestle in 10 mL of 2:1 (v/v) chloroform/methanol solution. The chloroform/methanol and ground tissue solution was then transferred into a 20 mL glass vial and vortexed for 10 s. The glass vial was then placed in an ice bath for 10 min. Two mL of 0.9% NaCl were then added to that same glass vial and vortexed for an additional 10 s. Samples were then placed on ice for 30 min to allow for phase separation. After the allotted time, the polar phase (methanol, water and metabolites) was isolated and placed in a separate 20 mL glass vial labeled "aqueous" and the nonpolar phase (lipids and chloroform) was left in the original vial and labeled "organic." The aqueous phase samples were dried overnight using a lyophilizer (ThermoScientific, Dallas, USA). The resulting aqueous phase dry powder was re-dissolved in 1 mL of water, and the pH was adjusted to 7.0 for each sample. After pH adjustment, the mixture was centrifuged at 13,200 rpm at 4 °C (for 30 min) and the resulting supernatant was dried via lyophilization. The nonpolar phase samples were dried using inert nitrogen gas. Both polar and non-polar dried powder samples were stored at − 80 °C until processing for ^{1}H-NMR and LC-MS.

2.2 ^{1}H-NMR spectroscopy

Metabolomic analyses were performed at the Southeast Center for Integrated Metabolomics (SECIM) at the University of Florida. Dried powder of aqueous phase samples acquired from methanol/chloroform/water extraction were dissolved in 50 mM sodium phosphate buffer with 0.5 mM D6-deuterated sodium trimethylsilylpropanesulfonate (DSS-d6). NMR spectra were measured using the first slice of a NOESY pulse sequence (tnnoesy) (Ravanbakhsh et al., 2015) using 14.1 T Bruker Avance II NMR system with a CP TXI CryoProbe. The acquisition parameters used in Lohr et al. (2019) and Myer et al. (2020) were utilized to acquire proton spectra. All spectra were processed and the integrated areas were extracted using MestReNova 11.0–17,609 (Mestrelab Research S.L.). Before Fourier transformation, baseline correction and phase correction were applied with a line-broadening factor of 0.22 Hz and spectra were normalized with respect to a DSS signal at 0.0 ppm. Concentrations were assessed by integration of peak areas and comparison to the DSS standard intensity.

2.3 LC–MS global metabolomics

2.3.1 Summary of procedures (polar metabolites)

Polar extracts were diluted 10 × with 0.1% formic acid in water. Global metabolomic profiling was performed on a Thermo Q-Exactive Oribtrap mass spectrometer with Dionex UHPLC and autosampler (Thermo, San Jose, CA). All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at *m/z* 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 × 2.1 mm, 2 µm column (Mac-MOD Analytical, Chadds Ford, PA) with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. This polar embedded stationary phase provides improved coverage, but does have some limitation in coverage of very polar species such as sugars. The flow rate was 350 µL/min with a column temperature of 25 °C. 4 µL was injected for negative ions and two µL for positive ions.

All subsequent data analyses were normalized to the sum of metabolites for each sample. MZmine (freeware) was used to identify features, deisotope, align features, and perform gap-filling to fill in any features that may have been missed

in the first alignment algorithm. All adducts and complexes were identified and removed from the data set. Data were searched against the University of Florida's Southeast Center for Integrated Metabolomics' (SECIM) internal retention time metabolite library (level 1 annotation (Blaženović et al., 2018)). In addition, data were searched against METLIN, the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Global Natural Products Social Molecular Networking (GNPS) libraries (level 3 annotation). Thresholds used for searches were 10 ppm in the negative ion mode and 5 ppm in the positive ion mode.

2.3.2 Summary of procedures (nonpolar metabolites)

Samples that were extracted and dried for NMR analysis were reconstituted with 200 µL 2-propanol for LC-MS analysis. Global lipidomic profiling was performed using a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler. All samples were analyzed in positive and negative heated electrospray ionization with the same mass resolution for polar metabolites. Separation was achieved on an Acquity BEH C18 1.7 µm, 50 × 2.1 mm column with mobile phase A as 60:40 acetonitrile:10 mM ammonium formate with 0.1% formic acid in water and mobile phase B as 90:8:2 2-propanol:acetonitrile:10 mM ammonium formate with 0.1% formic acid in water. The flow rate was 500 µL/min with a column temperature of 50 °C. 5 µL was injected for negative ions and 3 µL was injected for positive ions.

Data from positive and negative ion modes were separately analyzed using LipidMatch software (Koelmel et al., 2017). First, all MS2 raw files were converted to.ms2 and MS raw files to.zXML using MSConvert. A peak list was generated after running MzMine on all.mzXML files. An input folder that included all.ms2 files and the peak list were used to run LipidMatch to identify features.

2.4 Statistical analysis

All statistical tests were conducted at a significance level of $\alpha = 0.05$. Statistical analysis was performed separately on the positive and negative ion data. Concentrations of compounds identified by ^{1}H-NMR and LC-MS were compared among genotypes (K1, K2, K3, U25, U41, U44, and U47) using ANOVA. False Discovery Rate (FDR) was used for multiple comparison correction for both polar and nonpolar metabolites and only *p* values of 0.05 or less were reported. Polar and nonpolar metabolomic profiles were compared among genotypes using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). PCA is an unsupervised multivariate analysis that can be used to determine similarities and differences among multiple samples (Want et al., 2011). PCA can be further utilized to simplify, predict, and model data (Wold et al., 1987). Conversely, PLS-DA is a supervised multivariate analysis that attempts to maximize separation among groups (Want et al., 2011) and determine the group a sample most likely belongs to (Brereton & Lloyd, 2014). PLS-DA models were validated using permutation tests based on separation of samples. The combination of PCA and PLS-DA allows for a comprehensive analysis of metabolomic profiles among the genotypes of interest. Variable Importance in Projection (VIP) was used to summarize the importance of each variable (i.e. metabolite) in driving separation among treatments (i.e. genotypes) in the PLS-DA models. Compounds with VIP values > 1 are generally considered to be influential in PLS-DA models. The present study used a conservative cutoff value of > 2 to identify highly important compounds driving separation.

3 Results

3.1 LC–MS polar metabolites

LC–MS detected a total of 5866 features in the positive mode (POS) and 4207 features in the negative mode (NEG). ANOVA identified polar metabolites that differed significantly among genotypes ($p < 0.05$) in the positive ion mode ($n = 149$) and in the negative ion mode ($n = 527$). Combined, PCA components 1 and 2 described 39.9% (POS) and 41.4% (NEG) of the total variance. PLS-DA results are shown in Fig. 1. The PLS-DA model was validated, as the permutation test yielded observed statistics of $p = 0.03$ (POS) and $p = 0.03$ (NEG). There were 74 (POS) and 124 (NEG) features with a VIP score of two or greater, and thus considered to be driving the separation among groups in the PLS-DA model. PLS-DA showed relatively separate clustering of genotypes in both positive and negative modes, particularly in the negative mode (Fig. 1). Features were annotated by searching against an internal retention time metabolite library of 1100 compounds as well as the Human Metabolome Database (https://www.hmdb.ca). All PCA and PLS-DA plots, along with a full list of polar features identified through LC-MS (including those driving separation among genotypes), are provided as Supplementary Data. Variation in a subset of polar metabolites among genotypes is illustrated in Fig. 2.

Separate multivariate analysis for genotypes U25, U41, and U44 detected a total of 5866 features in the positive mode and 4207 features in the negative mode. ANOVA identified polar metabolites that differed significantly among genotypes ($p < 0.05$) in the positive ion mode ($n = 92$) and in the negative ion mode ($n = 236$). PCA components 1 and 2 described 45.7% (POS) and 54.2% (NEG), respectively, of the total variance. A PLS-DA permutation test was not

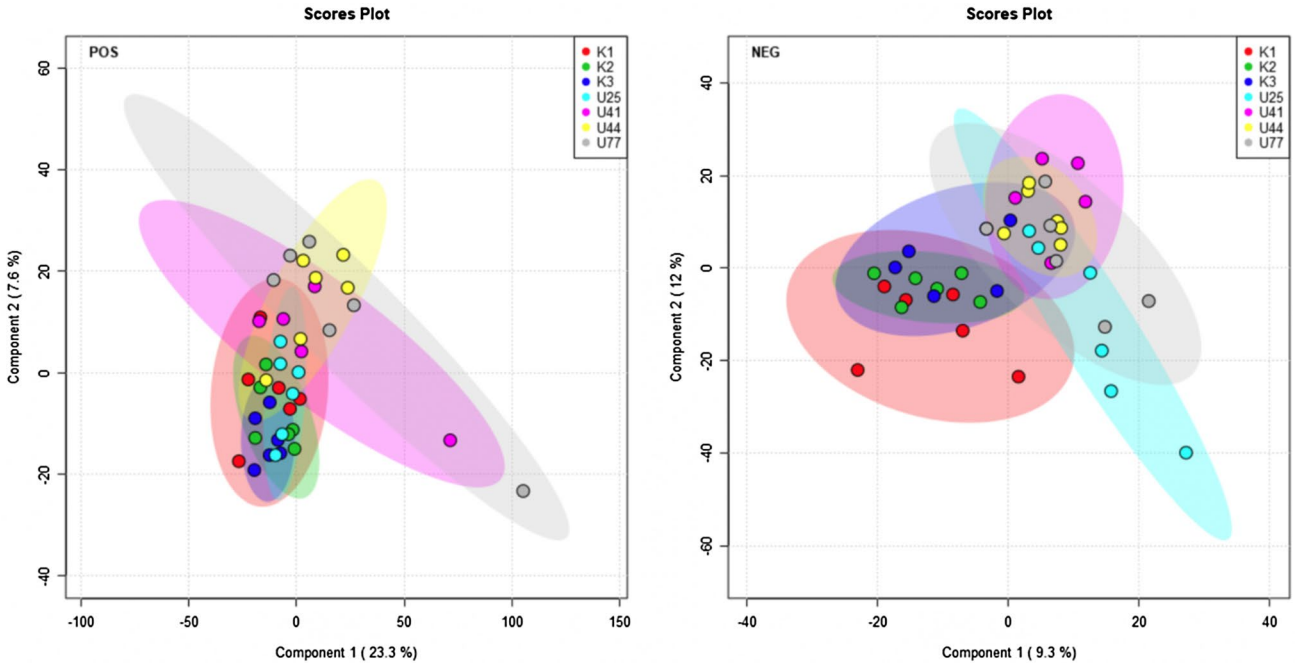

Fig. 1 PLS-DA model comparing LC–MS metabolomic profiles for the positive (left) and negative (right) mode among seven unique genotypes of *A. cervicornis*. The amount of variance explained is shown in parentheses on each axis

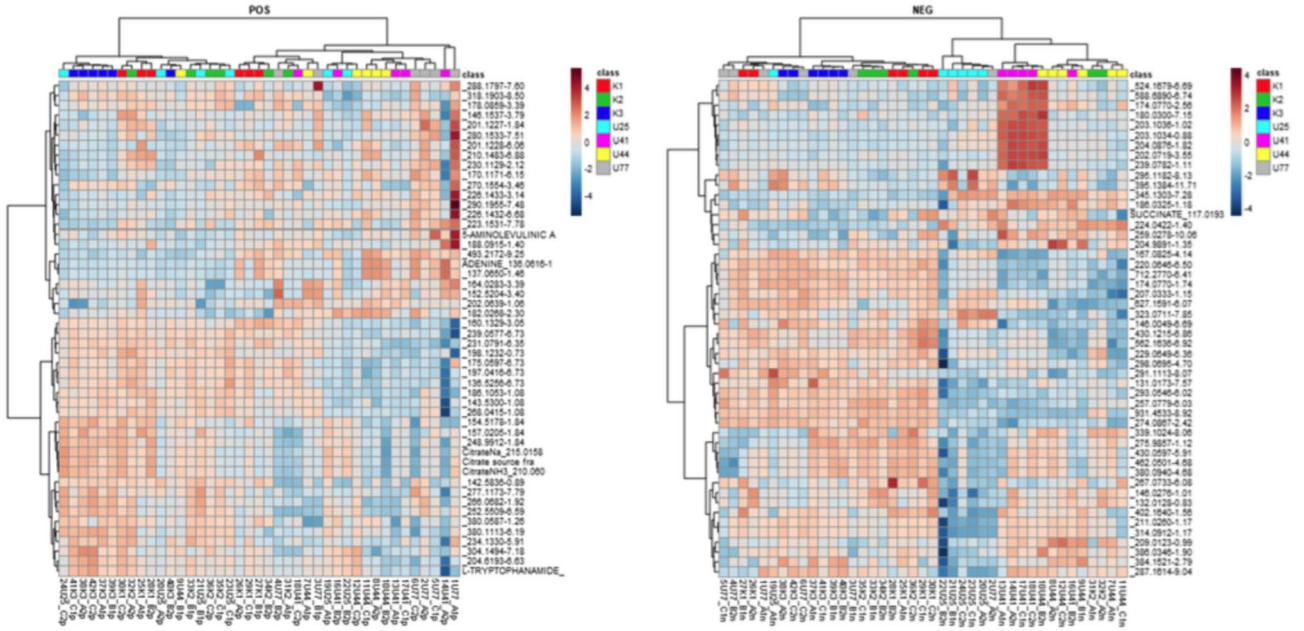

Fig. 2 Heat maps of top 50 polar metabolites from ANOVA for the positive (left) and negative (right) mode among seven unique genotypes of *A. cervicornis*

validated, and the PLS-DA model was therefore not used for analysis. PCA indicated no separation between the polar metabolomes of U25, U41 and U44.

3.2 LC–MS nonpolar metabolites

LC–MS detected a total of 922 features in the positive mode and 340 features in the negative mode. ANOVA identified nonpolar metabolites that differed significantly among genotypes ($p < 0.05$) in the positive ion mode ($n = 446$) and in the negative ion mode ($n = 178$). Combined, PCA components 1 and 2 described 37.3% (POS) and 35.9% (NEG) of the total variance. The PLS-DA model was validated for the negative mode, but not for the positive mode, as the permutation test yielded observed statistics of $p = 0.01$ (NEG) and $p = 0.12$ (POS) (Fig. 3). There were 14 (NEG) features from PLS-DA with a VIP score of two or greater, and thus were considered to drive separation among groups. PLS-DA showed relatively separate clustering of genotypes in the negative mode (Fig. 3). Features were annotated by searching against an internal retention time metabolite library of 1100 compounds as well as the METLIN, KEGG, HMDB, and GNPS Libraries. All PCA and PLS-DA plots, along with a full list of nonpolar features identified through LC-MS (including those driving separation among genotypes), are provided as Supplementary Data. Variation in a subset of nonpolar metabolites among genotypes is illustrated in Fig. 4.

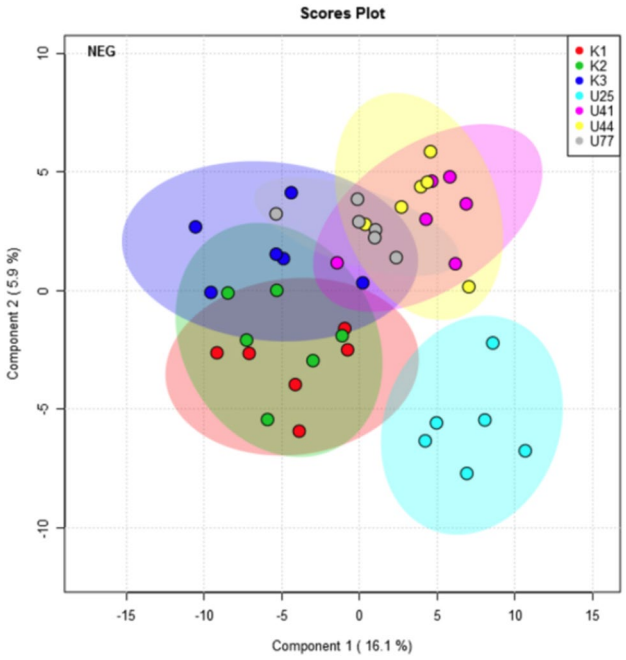

Fig. 3 PLS-DA model comparing LC–MS negative mode nonpolar metabolite profiles among seven unique genotypes of *A. cervicornis*. The amount of variance explained is shown in parentheses on each axis

Separate multivariate analysis of the lipidome for only genotypes U25, U41, and U44 detected a total of 922 features in the positive mode and 340 features in the negative mode. ANOVA identified nonpolar metabolites that differed significantly among genotypes ($p < 0.05$) in the positive ion mode ($n = 286$) and in the negative ion mode ($n = 94$). PCA components 1 and 2 described 46.6% (POS) and 45.8% (NEG), respectively, of the total variance. A PLS-DA permutation test was not validated, and the PLS-DA model was therefore not used for analysis. PCA indicated overlap between the nonpolar metabolomes of U41 and U44, however the nonpolar metabolome of U25 was generally distinct from those of the other two genotypes in both the positive and negative modes (Fig. 3).

3.3 ^{1}H-NMR spectroscopy

No chemical shift showed significant differences among the genotypes ($p < 0.05$) in False Discovery Rate (FDR) corrected ANOVA. No separation was observed among genotypes in either PCA or PLS-DA analysis. PCA and PLS-DA score plots are presented in Supplementary Data (Figs. 4 and 5).

4 Discussion

The current and projected future threats to coral reefs demand a better understanding of the complex physiological drivers that lead to intraspecific differences in response to environmental conditions. Metabolomics has been used in a variety of ways to study corals (Hillyer et al., 2018; Lohr et al., 2019; Quinn et al., 2016; Sogin et al., 2014). LC-MS indicated differences in polar and nonpolar metabolite profiles among seven genotypes of the threatened coral *Acropora cervicornis*. Although ^{1}H-NMR results were inconclusive, our data generally support previous findings (Lohr et al., 2019) and further suggest metabolomic profiles differ among various genotypes of the threatened species *A. cervicornis* sampled from a common garden.

Our study was explicitly designed to build upon previous work by Lohr et al. (2019). Consistent with Lohr et al. (2019), we found unique metabolomic signatures among three genotypes of *A. cervicornis*: U25, U41, and U44. Variation among these three genotypes was observed in nonpolar, but not polar, metabolite profiles. In the present study, the nonpolar metabolite profile for U25 grouped separately, whereas in Lohr et al. (2019), U44 was distinct. However, it is important to note that different collection and extraction methods used in these two studies resulted in resolution of different sets of metabolites. Specifically, Lohr et al. (2019) initially preserved samples in chilled methanol and used a methanol extraction, which resolves only polar metabolites.

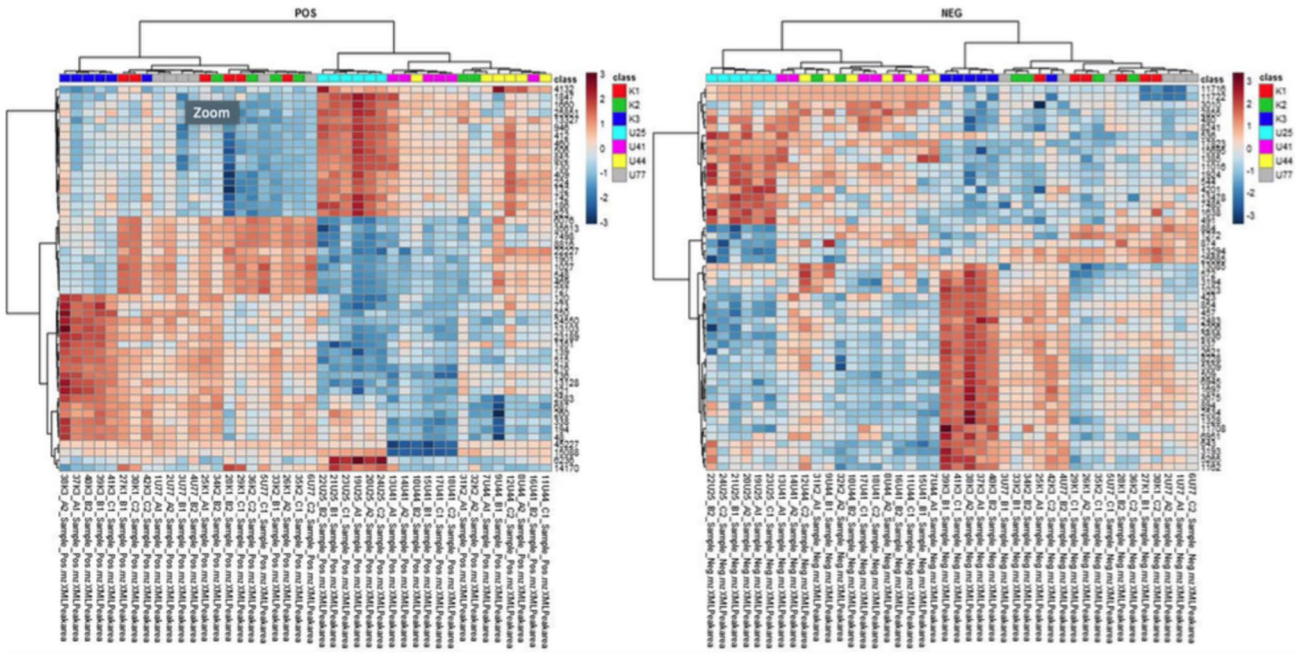

Fig. 4 Heat maps of top 50 nonpolar metabolites from ANOVA for the positive (left) and negative (right) mode among seven unique genotypes of *A. cervicornis*

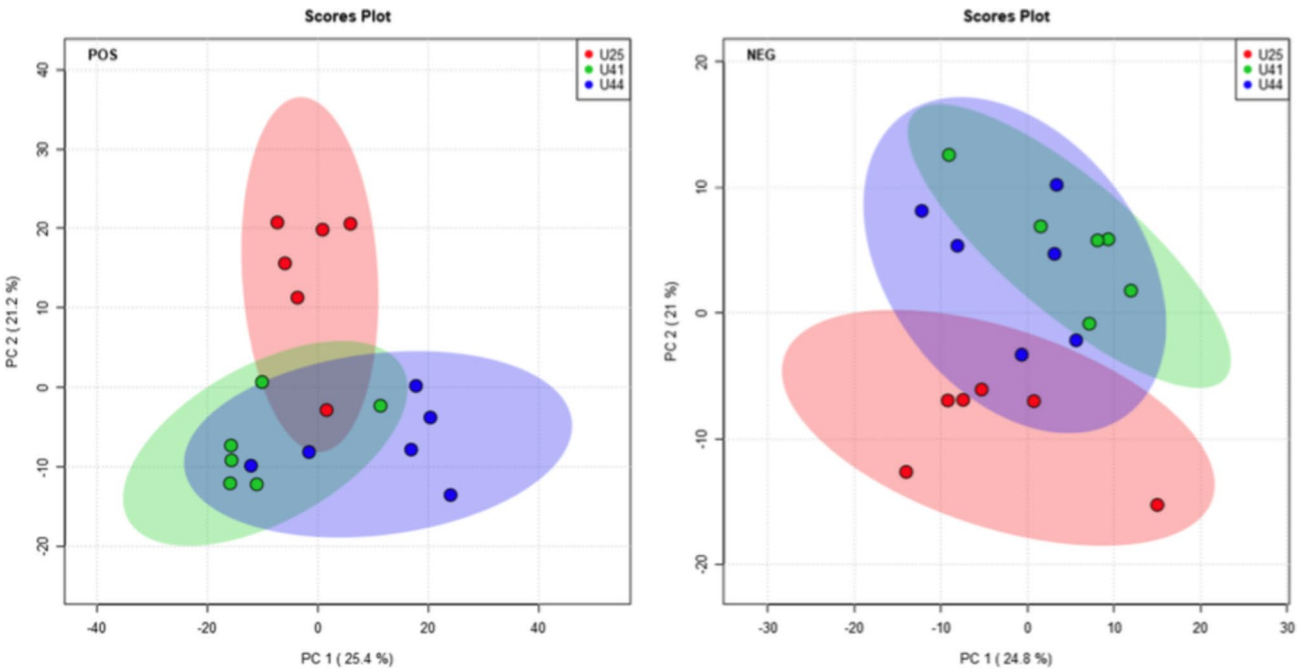

Fig. 5 PCA model comparing LC–MS nonpolar metabolite profiles for the positive (left) and negative (right) mode among the three unique genotypes of *A. cervicornis* also sampled in Lohr et al., 2019). The amount of variance explained is shown in parentheses on each axis

In the present study, samples were immediately flash frozen in liquid nitrogen and processed using a modified Folch extraction to identify polar and nonpolar metabolites. This variability in methods used is likely an important factor in the differences observed in these two studies. Additionally, differences in environmental conditions prior to and at the time of sample collection may have resulted in differences in metabolite profiles from year to year. Previous studies

have found that coral metabolite profiles shift in response to changes in environmental conditions, including temperature and partial pressure of carbon dioxide (Sogin et al., 2016).

The study of the coral metabolome is still relatively novel, and further standardization of methods is necessary to better compare results between studies. Our study used a methanol/chloroform/water extraction in an effort to examine both the polar and nonpolar metabolite structure of our samples. A wide variety of extraction methods have been used in metabolomic profiling studies, including those with which we sought to compare results. The optimal extraction solvent often depends on the goals of given study. For example, if lipids are a primary focus, a methyl tert-butyl ether (MTBE) extraction method may be beneficial, as it provides cleaner and faster lipid extraction due to the replacement of chloroform with a less dense non-polar solvent; this approach results in the non-polar fraction on top after phase separation (Matyash et al., 2008). In contrast, a study focused on non-targeted polar metabolite detection may benefit from a methanol only extraction. This approach has been shown to be more effective at minimizing the amount of lipids extracted and is capable of penetrating the cell walls of the symbiotic algae living in hermatypic coral tissue, allowing for detection of metabolites of interest from both the coral and its algal symbiont (Gordon et al., 2013). Ultimately, in order to mitigate variation caused by sampling methods, a modified Bligh and Dryer technique is generally considered standard for metabolite extraction from tissues and should be considered for biphasic sample extraction (Lin et al., 2007). A study performed by Anderson et al. (2019) concluded that a modified Bligh and Dryer extraction was the most reproducible for comparing metabolites across multiple coral species and a methanol extraction was best for feature detection when performing ^{1}H-NMR for hermatypic corals.

Although our methodology precludes a direct comparison of metabolites with the previous study, we did resolve a number of metabolites that varied among the genotypes compared in the present study. The primary polar metabolites driving separation among genotypes were largely unknown. However, hydroxyprolyl-valine and hydroxyguanosine were annotated as two of the dominant polar metabolites driving separation among genotypes. Hydroxyprolyl-valine is a dipeptide that is the product of incomplete protein digestion or catabolism (Wishart et al., 2018). Hydroxyproline may be important for protecting corals from stressful environmental conditions (Li et al., 2010). Valine is a neutral amino acid important to coral growth and tissue repair (Al-Moghrabi et al., 1993; Ramachandran & Natarajan, 2009); this primary metabolite may thus be of interest to coral restoration practitioners, and its role in *A. cervicornis* physiology warrants further investigation. Hydroxyguanosine is also a metabolite of interest due to its important role in the structure of DNA. Hydroxyguanosine is a purine nucleoside (Wishart et al., 2018), making it foundational to building DNA and thus to the genetic structure of the coral. The primary nonpolar metabolites driving separation among genotypes were in the class glycerophosphocholine (GPC). GPCs are glycerophospholipids, a class of lipids that contribute to cellular structure and the regulation of cellular processes (Coppens et al., 2014). GPCs are essential nutrients that function in major bio-metabolic pathways (Loening et al., 2005) and play a substantial role in the growth and metabolism of organisms (Carman & Zeimetz, 1996). Growth is an area of interest for restoration practitioners as they seek to select corals for outplanting to rebuild reef structure. However, tradeoffs are possible between growth and other traits, including skeletal density (Kuffner et al., 2017; Lohr & Patterson, 2017) and thermotolerance (Cunning et al., 2015; Ladd et al., 2017). Information on key GPCs involved in *A. cervicornis* growth are likely of interest and may prove useful in understanding these tradeoffs. Similarly, the role of GPCs in maintaining cellular integrity and the formation of protective boundary layers is another key function that warrants further investigation. The role of GPCs in cellular structure could make them important components of tissue regeneration during recovery from damage or disease. Future research to identify specific GPCs and link them to function in corals could benefit restoration efforts.

Correlations between metabolite concentration and coral phenotype were observed (Table 1). Triacylglycerols, energy storage compounds, were more abundant in genotypes with slower growth rates (K1, U25 and U77) and less abundant in those with higher growth rates (K2 and U41). This may indicate that slow-growing genotypes allocate more energy to storage compared to fast-growing genotypes. Corals' algal symbionts have also been shown to play a role in both triacylglycerol levels (Zhukova & Titlyanov, 2003) and growth (Jones & Berkelmans, 2010). The relationship between coral host genotype, symbiont identity, growth, and triacylglycerol levels is likely complex and warrants additional study. Another correlation was found between coral phenotype and glycerolipid abundance. A study by Roach et al. (2021) found unsaturated betaine lipids, a family of glycerolipids, were more abundant in bleaching-susceptible corals. Our study also revealed that two unsaturated glycerolpids driving separation among genotypes (DG 18:0/22:4 and DG: 20:0/22:4) were more abundant in a bleaching-susceptible genotype (U25) compared to a bleaching-resistant genotype (U44). Although we were successful at annotating several metabolites that correlate with phenotype, more controlled lab studies and targeted metabolomic profiling will be necessary to explore the relationships between these metabolites and coral phenotypes of interest, including the metabolic pathways underlying phenotypic expression.

In order to improve the use of metabolomics both as a tool for identifying biomarkers and its application for coral

restoration, future research should focus on understanding internal and external factors driving changes in coral physiology. Multiple factors can change the metabolomic profile of a coral over time. Internally, the link between genome and phenome has been well studied, but the control of gene expression raises questions regarding whether similar genotypes will still express the same metabolomic signature. Changes in the metabolomic profiles of an organism can be the result of altered gene expression (Hollywood et al., 2006, Sogin et al., 2014) or post-transcriptional processes (Patti et al., 2012). Differences in coral-associated microbial communities has also shown to play a role in intraspecific variability in the metabolome of corals (Hartmanm et al., 2017). Miller et al. (2020) discovered significantly different microbial communities among a set of *A. cervicornis* genotypes sampled in an ocean-based nursery. These authors suggest that differences in microbiome composition may be influenced by the coral host. This supports the validity of classifying the holobiont metabolome by host genotype. Yet, it is important to recognize that we do not have a full understanding of how variable non-host holobiont components are across time, and this may hinder the reproducibility of metabolomics as a tool for phenotype exploration. Furthermore, environmental conditions, including changes in ultraviolet light exposure (Galtier d'Auriac et al., 2018), water temperature and pH, can impact a coral's metabolome (Sogin et al., 2016). The metabolome of corals has also been found to change due to non-self-competition with macroalgae (Barott et al., 2012; Quinn et al., 2016; Roach et al., 2020). Inconsistencies in the performance of corals in a nursery setting compared to an outplant setting also presents as an issue, as previous research has found that the performance of genotypes in a nursery setting does not necessarily reflect how the corals will perform once outplanted to a reef (O'Donnell et al., 2018). A coral nursery is a unique habitat and may not reflect the environment in which corals will be outplanted. Thus, when selecting genotypes of interest, consideration should be given to outplanting location by attempting to select a diverse group of genotypes well-matched to a given environment. Ultimately, when collecting samples for analysis or when seeking to compare results with other studies, special consideration must be given to the abiotic and biotic factors affecting the corals of interest.

Previous research and the present study indicate that future work identifying unknown metabolites is critical to the application of metabolomics for coral restoration (Lohr et al., 2019; Sogin et al., 2014). Without effective feature identification, the ability to discern biomarkers for key coral traits will be exceptionally difficult. Unfortunately, the extraction protocol used here suppressed some expected differences in metabolites that should have been detectable by ^{1}H NMR. Previous work with a methanol only extraction was more successful at detecting significant differences. Future studies should still consider using NMR, as it is known to be particularly useful for identifying the structure of unknown metabolites (Bingol & Brüschweiler, 2017).

The management and restoration of threatened coral populations will require mitigating environmental stressors and optimizing performance of corals used in restoration efforts. Restoration is a key strategy to slow the decline of reefs while global issues such as ocean warming and the emission of greenhouse gases are addressed (Randall et al., 2020). As these environmental stressors persist, it has become increasingly important to develop our understanding of the complex biological processes underlying a coral's response to its environment (Anderson et al., 2019). Understanding these processes can improve our insight into phenotypic variation in corals. Our findings are consistent with previous research on intraspecific variation in coral metabolomic profiles (Lohr et al., 2019; Sogin et al., 2016) and support the need for further investigation focused on metabolomic profiling as a tool for selecting corals with desirable characteristics.

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Author contributions JH, JP, RK, MM, JG and TG designed research. JH, KL and JP collected samples. JH and KL performed metabolite extraction. RK and MM processed and analyzed samples in ^{1}H-NMR. JC and TG processed and analyzed samples in LC-MS. JH, JP and KL analyzed data; JH, KL and JP wrote the manuscript. All authors read and approved the manuscript.

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