Identifying metabolic alterations associated with coral growth anomalies using ^{1}H NMR metabolomics

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Abstract: Coral growth anomalies (GAs) are tumor-like protrusions that are detrimental to coral health, affecting both the coral skeleton and soft tissues. These lesions are increasingly found throughout the tropics and are commonly associated with high human population density, yet little is known about the molecular pathology of the disease. Here, we investigate the metabolic impacts of GAs through ^{1}H nuclear magnetic resonance (NMR) metabolomics in *Porites compressa* tissues from a site of high disease prevalence (Coconut Island, Hawaii). We putatively identified 18 metabolites (8.1% of total annotated features) through complementary ^{1}H and ^{1}H–^{13}C heteronuclear single quantum correlation NMR data that increase confidence in pathway analyses and may bolster future coral metabolite annotation efforts. Extract yield was elevated in both GA and unaffected (normal tissue from a diseased colony) compared to reference (normal tissue from GA-free colony) samples, potentially indicating elevated metabolic activity in GA-afflicted colonies. Relatively high variation in metabolomic profiles among coral samples of the same treatment (i.e., inter-colony variation) confounded data interpretation, however, analyses of paired GA and unaffected samples identified 73 features that differed between these respective metabolome types. These features were largely annotated as unknowns, but 1-methylnicotinamide and trigonelline were found to be elevated in GA samples, while betaine, glycine and histamine were lower in GA samples. Pathway analyses indicate decreased choline oxidation in GA samples, making this a pathway of interest for future targeted studies. Collectively, our results provide unique insights into GA pathophysiology by showing these lesions alter both the absolute and relative metabolism of affected colonies and by identifying features (metabolites and unknowns) and metabolic pathways of interest in GA pathophysiology going forward.

Introduction

46

47 Coral reefs contain a disproportionately large amount of the ocean's biodiversity and productivity,
48 making them one of the most ecologically and economically important ecosystems in the world
49 (Odum and Odum 1955; Moberg and Folke 1999). Nonetheless, the status of these ecosystems has
50 declined in recent decades due to a combination of local and global challenges (Hughes et al.
51 2017). Increasing atmospheric carbon dioxide levels provide a ubiquitous threat to coral reefs due
52 to the associated increase in oceanic temperatures and ocean acidification (Hoegh-Guldberg et al.
53 2007), while chemical pollution, eutrophication, fishing pressure, and other localized stressors also
54 contribute to coral reef degradation (Knowlton 2001; van Dam et al. 2011). Coral disease
55 occurrence is commonly linked to human activity (Green and Bruckner 2000) and is influenced by
56 both local and global stressors, which may shift host-pathogen interactions in favor of disease
57 (Lesser et al. 2007; Burge et al. 2014). Disease outbreaks can devastate impacted coral populations
58 (e.g., Aronson and Precht 2001), and the rising prevalence and impact of coral diseases make them
59 a substantial threat to coral reef health worldwide (Bruckner 2016). Coral diseases often share
60 similar visual indicators, and any given disease may not originate from a single causative agent,
61 thus complicating efforts to define disease pathology (Work and Aeby 2006; Lesser et al. 2007).
62 However, emerging molecular techniques have the potential to improve diagnostic ability by
63 investigating new aspects of coral biology as well as disease pathophysiology and etiology
64 (Pollock et al. 2011).

65 Coral growth anomalies (GAs) are generally characterized by irregular and accelerated
66 growth of a less dense skeleton resulting in a tumor-like mass on a coral colony, with overlying
67 tissues having fewer polyps, fewer endosymbiotic dinoflagellates (family Symbiodiniaceae), and
68 reduced reproductive potential (Work et al. 2016). Histological descriptions of GAs have shown

69 irregular polyp structure, thickened calicoblatic layer, and increased cell proliferation with
70 suppressed apoptosis (Domart-Coulon et al. 2006; Yasuda and Hidaka 2012). These lesions affect
71 many coral species and are common across the Indo-Pacific but less common in the Caribbean
72 (Work et al. 2016). GAs do not typically lead directly to coral mortality, but their abnormal
73 characteristics lower the fitness of impacted colonies and therefore pose an ecological threat to
74 coral populations where prevalence is high (Stimson 2011).

75 Although the biological and ecological impacts of these lesions have been studied using a
76 wide range of methods (e.g., Domart-Coulon et al. 2006; Burns and Takabayashi 2011; Kelly et
77 al. 2016; Palmer and Baird 2018; Preston and Richards 2021), the cause and pathogenesis of GAs
78 remain unknown. Early theorized causes include ultraviolet radiation (Coles and Seapy 1998,
79 Stimson 2011) and pathogenic microorganisms (Kaczmarsky and Richardson 2007). More recent
80 molecular investigations have produced contradictory results regarding the involvement of
81 oncogenes in GA pathology (Spies and Takabayashi 2013; Zhang et al. 2017; Frazier et al. 2017).
82 To date, conclusive evidence to support any of these hypotheses is lacking, however, the
83 correlation between human population and GA prevalence (Aeby et al. 2011a, 2011b) strongly
84 indicates localized human activities influence GA formation.

85 The development of molecular biology –omics techniques has facilitated the investigation
86 of biological questions that were previously inaccessible (Joyce and Palsson 2006), and these
87 techniques have recently emerged to supplement more traditional approaches in attempting to
88 determine disease causative agents (Madsen 2005). One such molecular tool is metabolomics, a
89 developing field dedicated to the study of low-molecular-weight compounds (here we refer
90 specifically to the water-soluble polar metabolites) in biological samples under predetermined
91 physiological conditions. These compounds are essential products and intermediates in

92 biochemical pathways that fluctuate with gene expression and enzymatic activities, making the
93 metabolome closely tied to the biochemical phenotype and thus excellent for closely monitoring
94 organismal response to perturbations such as disease (Goodacre 2007; Viant 2008; Bundy et al.
95 2009). Targeted approaches are generally used in cases where specific, predetermined metabolites
96 are of particular interest (Roberts et al. 2012). In other cases, an untargeted approach is common,
97 where as many metabolites as possible are measured and biological interpretations are made based
98 on the composition and relative abundances of the entire metabolomic profile (Alonso et al. 2015).
99 These studies do not necessarily rely on traditional hypotheses to generate valuable data and
100 biological information. In fact, many untargeted metabolomics studies naturally result in
101 hypothesis formation when unexpected metabolic effects and activities are revealed (Kell and
102 Oliver 2004; Bundy et al. 2009). These characteristics make metabolomics a potentially strong
103 method for progressing our understanding of coral disease pathogenesis.

104 Applications of metabolomics to study stony corals have been limited by challenges
105 associated with fitting common metabolomic methods to these unique organisms. The coral
106 holobiont presents a complex biological matrix consisting of coral tissues and contiguous skeleton,
107 symbiotic dinoflagellates, and associated microbial communities. Therefore, typical best practices
108 for metabolomics cannot be applied automatically. Accordingly, many coral metabolomics studies
109 have emphasized methods development and optimization (Gordon et al. 2013; Andersson et al.
110 2019) or demonstrating the utility of metabolomics in coral research by establishing foundational
111 properties of the coral metabolome (Parkinson and Baums 2014; Sogin et al. 2014; Hartmann et
112 al. 2017; Sogin et al. 2017; Lohr et al. 2019b; Vohsen et al. 2019). Nevertheless, metabolomics
113 methods have been used to investigate the impacts of thermal stress and bleaching history (Sogin
114 et al. 2016; Hillyer et al. 2017; Hillyer et al. 2018; Lohr et al. 2019a; Roach et al. 2021; Williams

115 et al. 2021), ocean acidification (Putnam et al. 2016; Sogin et al. 2016), coral/non-coral interactions
116 (Quinn et al. 2016; Matthews et al. 2020; Roach et al. 2020), and chemical pollutants (Stien et al.
117 2019; Stien et al. 2020) on the coral metabolome. However, metabolomics analyses have not been
118 used to investigate the molecular impacts and pathophysiology of coral diseases such as GAs.

119 The finger coral *Porites compressa* is an important reef-building coral in the Hawaiian
120 Islands and is common to the reefs of Kaneohe Bay, Oahu (Bahr et al. 2015). The bay has been a
121 site of high GA occurrence since the 1990s when they were first observed in *P. compressa*
122 (Domart-Coulon et al. 2006; Stimson 2011). Despite research efforts to characterize the disease,
123 the causative agents and specific biochemical impacts of GAs in *P. compressa* are still poorly
124 understood. As a part of a larger endeavor to characterize GAs, *P. compressa* samples from
125 Kaneohe Bay were previously characterized using morphological, elemental and boron isotope
126 analyses (Andersson et al. 2020). These efforts identified a novel GA lesion morph and
127 demonstrated that the pH was lower in the internal calcifying fluid of GAs compared to healthy
128 samples. Here, we expand on this previous work by using an untargeted ^{1}H nuclear magnetic
129 resonance (NMR) metabolomics approach to examine GAs from the same *P. compressa* samples
130 in order to further elucidate the metabolic impacts these lesions have on affected colonies.

131

132 # Materials and methods

133

134 **Sample collection and processing**

135 Coral samples were collected according to methods from Andersson et al. (2020), a conjoining
136 study analyzing the elemental composition of skeletons from the same samples described here. In
137 brief, samples (approximately 4 cm in diameter) were collected by hammer and stainless-steel
138 chisel directly north of Coconut Island at depths less than 3 m within a 1 hr period in March 2014

139 (State of Hawaii Division of Aquatic Resources Special Activity Permit 2011–1). Samples of three
140 visually determined treatment groups were collected from individual diseased and healthy *P.*
141 *compressa* colonies: coral fragments exhibiting GAs (GA, n = 15), apparently normal fragments
142 directly adjacent to (i.e., touching) GA lesions on the same colony (unaffected, n = 15), and
143 apparently normal fragments from the nearest, distinct, adjacent colony free of GAs
144 (approximately 0.05–2 m away; reference, n = 15) (Fig. 1). Each GA sample was further
145 categorized based on the morphology of the GA lesion as either a traditional, bulbous GA (Form
146 1) or a less protuberant, novel GA morph (Form 2) that was first described during our
147 morphological characterization of these samples (Fig 1b-c) (Andersson et al. 2020). In this way, a
148 total of 45 samples were collected from 30 individual coral colonies. Samples were collected into
149 Teflon bags and brought directly to the surface where the seawater was poured from the bags, the
150 bags were sealed with zip ties, and frozen in liquid nitrogen (LN_2) to preserve the physiological
151 state of the samples.

152 Samples were generally processed following procedures recommended for metabolomics
153 analysis of reef-building corals outlined by Andersson et al. (2019). Frozen samples were
154 transferred from LN_2 and freeze-dried in a VirTis Genesis OX lyophilizer with a Wizard 2.0
155 controller (SP Industries Inc., Warminster USA). Subsequent sample handling time and exposure
156 to air were limited to reduce contamination and partial rehydration of the soft tissues. In some
157 cases, the GA lesion could not be cleanly separated from the surrounding tissues during sample
158 collection underwater. In these cases, the GA and unaffected samples were therefore collected
159 simultaneously as a single fragment (e.g., Fig 1b) and the GA lesions were separated from
160 surrounding unaffected coral by hammer and stainless-steel chisel after lyophilization. Firm-
161 bristled plastic brushes were then used to collect the dry, soft tissue powders from the coral samples

162 by evenly brushing the entirety of each fragment (~10 s per fragment area). These powders served
163 as the primary materials for ^{1}H NMR analysis and were comprised of various parts of the holobiont
164 including the coral soft tissues, symbiotic dinoflagellates, associated microbial communities, and
165 skeletal powder incidentally removed during brushing.

166

167 **Metabolite extraction for ^{1}H NMR metabolomics**

168 Samples were extracted and analyzed using ^{1}H NMR spectroscopy in four batches on consecutive
169 days, with each batch containing three or four randomly selected sets (paired GA and unaffected
170 samples from the same coral colony and corresponding reference sample from an adjacent colony)
171 of *P. compressa* tissue powders. Each batch also consisted of a coral homogenate control material
172 sample and blank sample to monitor for analytical reproducibility across batches and possible
173 method contaminants, respectively.

174 Metabolites were extracted from the tissue powders (all samples were standardized to 100
175 mg ± 3 mg prior to extraction) using methods modified from Bligh and Dyer (1959) and Wu et al.
176 (2008), as recommended for reef-building corals by Andersson et al. (2019). In short, a biphasic
177 solvent system consisting of chloroform, methanol, and water at a final ratio of 2:2:1.8 was used
178 to extract metabolites from the tissue powders (see Supplemental Text for full details). The polar
179 metabolite-containing fraction of each extract was transferred using a glass pipette into a
180 microcentrifuge tube and dried in a Vacufuge Concentrator 5301 (Eppendorf AG, Hamburg
181 Germany) at room temperature. The mass of the dried metabolite pellet from each sample was
182 recorded as the extract mass.

183 After extraction, the residual material was then placed in bleach (5.65–6% Laboratory
184 Grade Sodium Hypochlorite Solution) and rotated using a Roto-Shake Genie SI-1100 (SP

185 Scientific Inc., New York USA) for 24 h to oxidize and solubilize all remaining organics. After
186 bleach was removed, the remaining calcium carbonate powder was then rinsed with water, dried,
187 and weighed to record the mass of non-target skeleton that contributed to the tissue powder
188 extraction mass. The total amount of tissue extracted from each sample was then estimated by
189 subtracting the weight of skeletal contamination from the initial weight of the tissue powder. The
190 extract yield (extract mass by total mass of tissue extracted) was also calculated for each sample
191 as a normalized estimate of the extract mass.

192

193 **NMR spectroscopy data collection and processing**

194 Dried metabolites were rehydrated in 600 µL of deuterium oxide based 0.1 M sodium phosphate
195 buffer containing 1 mM of 3-trimethylsilylpropionic-2,2,3,3,-d, acid sodium salt (TMSP) as a
196 NMR chemical shift reference peak. Samples (550 µL) were then transferred to 5-mm NMR tubes
197 (NORELL, Inc., Morganton, North Carolina, USA) for ^{1}H NMR analysis. All NMR profiles were
198 obtained using a 700 MHz Bruker NMR spectrometer equipped with a TCI cryoprobe and a
199 SampleJet autosampler. One-dimensional (1D) ^{1}H NMR spectra were acquired (Topspin version
200 3.2) using a nuclear Overhauser effect spectroscopy pulse sequence consisting of 8 dummy scans
201 and 256 scans for 65,536 data points with a relaxation delay of 3 s. Spectra were produced from
202 the acquired free induction decay (FID) via Fourier transformation. Spectra were referenced,
203 phased and baseline-corrected automatically (Topspin version 3.2).

204 Two-dimensional (2D) NMR spectra were acquired using a ^{1}H–^{13}C heteronuclear single
205 quantum correlation (HSQC) experiment to resolve the spectral overlap in the 1D spectrum (Ross
206 et al. 2007; Markley et al. 2017) and confirm structural identification. The HSQC data were
207 acquired with 128 scans from 2048 data points from the 512 increments in the F1 dimension.
208 Sweep widths of 10.98 ppm (F2) and 180.0 ppm (F1) were used. A relaxation delay of 1.5 s

209 between acquisitions was used along with a refocusing delay of 30 ms. The FIDs were weighted
210 using a shifted sine bell function in both dimensions and chemical shifts were referenced to the
211 internal TMSP signal.

212

213 **Reproducibility and quality control**

214 Analytical and methodological reproducibility across batches were assessed by evaluation of inter-
215 batch control material samples. An in-house *Orbicella faveolata* control material was used for
216 these samples to conserve the limited quantities of the experimental coral tissue powders. A full
217 description of the *O. faveolata* control material and the reproducibility of control material and
218 experimental spectra can be found in supplementary materials (Supplemental Text; Fig. S1; Fig.
219 S2). Samples utilized for method optimization (sample identifications: 'unaffected-14' and 'GA-
220 14') (Andersson et al. 2019) were excluded from all statistical analyses. Samples where
221 methodological error prevented the measurement of the extract mass (sample identifications:
222 'unaffected-1' and 'GA-2') or the mass of tissue extracted (sample identifications: 'unaffected-4',
223 'reference-7', 'reference-9', 'reference-11' and 'reference-14') were also excluded from all
224 statistical analyses and analyses of extract data respectively (Table S1).

225

226 **Statistical analyses**

227 Metrics of metabolite extraction were compared between GA, unaffected and reference samples
228 using univariate analyses. Parametric one-way analysis of variance (ANOVA) models with Tukey
229 honestly significant difference (HSD) post-hoc tests were conducted to compare the mass of total
230 tissue extracted, extract mass and extract yield.

231 To facilitate statistical analysis of the ^{1}H NMR metabolomics data, peaks were aligned
232 using the Least Square method with a max shift of 0.05 ppm to correct for small variations in

233 chemical shift across samples using NMRProcFlow v1.2 (Jacob et al. 2017). An adaptive,
234 intelligent binning procedure was subsequently performed on the spectra from 0.2–10 ppm with a
235 signal-to-noise threshold equal to 3:1 (De Meyer et al. 2008). This procedure assigned spectral
236 features (peaks) into bins, thereby excluding spectral noise (non-peaks) from downstream
237 analyses. Residual water (4.7–5.0 ppm) and other contaminants detected in the blank sample
238 spectra (Table S2; Fig. S3) were precluded from binning and bins that contained only noise were
239 removed manually. Remaining bins (330 total) were exported as data tables and normalized by
240 extract mass (Fig. S4) to enable relative comparisons of metabolomic profiles. For annotated
241 features that spanned multiple bins (i.e., two bins containing separate peaks of a putative doublet),
242 statistical results were presented from a selected representative bin.

243 Multivariate analyses were conducted using Metaboanalyst 4.0 (Xia and Wishart 2016)
244 where bins were mean-centered and Pareto-scaled in order to decrease the dominance of bins with
245 the largest intensities (van den Berg et al. 2006) prior to principal component analysis (PCA).
246 PCAs were used to visually assess the quality of the data and to evaluate trends within and among
247 treatment groups. Partial least squares discriminant analysis (PLS-DA) models were used as a
248 supervised multivariate approach to help determine bins that differed between treatment groups
249 that displayed divergence in PCA.

250 Predictive ability of PLS-DA models (Q^2) was evaluated with a 10-fold cross validation of
251 the first two components, and model quality was further assessed with permutation tests by group
252 separation distance with 1000 permutations (Bijlsma et al. 2006; Syzmanska et al. 2012). Twenty
253 sub-models were created for each PLS-DA model to evaluate reproducibility of validation metrics,
254 and the averages were reported as iterative Q^2 (cross-validation) and p-values (permutation tests).

255 Variable importance in projection (VIP) scores were used to rank important bins and were
256 calculated for each bin by averaging the scores of the first two components.

257 Each set of unaffected and GA samples were collected from the same coral colony;
258 therefore, additional multivariate analyses were used in order to increase resolution by focusing
259 on unaffected-GA pairs to discount inter-colony variation. An average metabolic change vector
260 (AMCV) was calculated from a PCA of intact unaffected-GA pairs by averaging of the difference
261 in PCA scores between each unaffected-GA pair. The AMCV was multiplied by the PCA loadings
262 of each bin to give a modified loadings score indicating its contribution towards the AMCV
263 (Southam et al. 2008). The bins that provided the largest contributions to the AMCV were
264 determined as the inflection point of the plotted cumulative sum of the AMCV loadings (Fig. S5).

265 Univariate analyses were subsequently used to further elucidate patterns from multivariate
266 approaches. Parametric one-way ANOVA models were used to compare relative intensities in ^{1}H
267 NMR spectra (normalized by extract mass) for all bins between all three treatments. Additionally,
268 paired t-tests were conducted to compare all bin intensities between intact pairs of GA and
269 unaffected samples (n = 24). Corrections to account for multiple statistical tests were used to
270 separately adjust the p-values from the ANOVA and paired t-tests of the spectral bins, using the
271 false discovery rate method (Benjamini and Hochberg 1995) for both corrections. Distribution
272 normality and homoscedasticity of variables for all univariate analyses were evaluated graphically.

273

274 **Metabolite identification**

275 A full spectrum annotation was conducted using a representative 1D ^{1}H NMR spectrum and 2D
276 ^{1}H–^{13}C HSQC spectrum from each of the three treatment groups in order to identify as many
277 metabolites as possible in the *P. compressa* extracts. Metabolites were annotated using Chenomx
278 NMR Suite (v8.31; Edmonton, Alberta, Canada) 700 MHz spectral libraries to match peak shape

and intensity from the 1D ^{1}H NMR spectra. Additionally, 2D ^{1}H–^{13}C HSQC data were used to match resonances from the Human Metabolome Database (Wishart et al. 2007) and the Biological Magnetic Resonance Bank (Ulrich et al. 2008) metabolite databases to complement putative metabolite identifications from 1D annotations. Spectral features that could not be assigned a metabolite identification were annotated as unknowns. Splitting pattern, ^{1}H and ^{13}C chemical shifts were also recorded for identified and unknown spectral features. Annotated features were matched to the spectral bins used for statistical analyses to facilitate biological interpretations.

Results and discussion

Metabolome annotations

The full spectrum annotation resulted in the identification of 18 (8.1% of total annotated features) putative metabolites, 12 (5.6% of total annotated features) of which were supported by 2D HSQC resonance chemical shifts (Table 1; Fig. S6), in addition to 204 unknown spectral features (Table S3). All putatively identified metabolites were present in all three treatment groups. Previous annotations of *P. compressa* metabolomes resulted in only two putatively identified compounds (Sogin et al. 2014), both of which (alanine, glucose) were also found in our spectra. In both cases, glucose was present in low concentrations compared to other features in the metabolomic profile of *P. compressa* (Fig. S6) (Sogin et al. 2014). Glucose is an energetically and physiologically important coral metabolite (Burriesci et al. 2012; Ochsenkuhn et al. 2017; Hadaidi et al. 2019) that is commonly identified in metabolomic profiles of other coral species (e.g., Putnam et al. 2016; Sogin et al. 2016; Hillyer et al. 2017). In contrast to *P. compressa*, glucose is one of the most abundant metabolites measured in other coral species such as *Acropora aspera* (Hillyer et al. 2017). Furthermore, known coral-associated metabolites such as trehalose (Hagedorn et al. 2015),

303 floridoside (Ochsenkuhn et al. 2017), and arabinose (Hadaidi et al. 2019) were not visible in our
304 *P. compressa* spectra at all. Reasons for such species-specific differences in these metabolites are
305 unclear but will be of interest going forward.

306 Previous NMR-based coral identifications have typically relied on matching 1D ^{1}H shifts
307 to metabolite databases, resulting in level 2 (putative) identifications as classified by the
308 Metabolomics Standards Initiative (Sumner et al. 2007). Level 2 identifications are generally
309 susceptible to misidentification because they do not utilize authentic chemical standards, and
310 identification of coral metabolites is further limited by the scarcity of metabolite database
311 resources devoted to non-model organisms. Therefore, to increase annotation rigor, we annotated
312 as many features as possible from the entire spectrum while implementing complementary 2D ^{1}H–
313 ^{13}C HSQC data, in addition to 1D ^{1}H spectra.

314 Although still producing level 2 identifications, this approach increased the confidence of
315 metabolite identity by providing crucial information regarding atom connectivity. The 18 (8.1%
316 of total annotated features) putative metabolites we identified are comparable in number to other
317 NMR-based coral metabolomics studies (e.g., 3 – 26 identifications (11.1 – 28.6% of total
318 annotated features, 1D only) (Sogin et al. 2014; Putnam et al. 2016; Lohr et al. 2019b). NMR-
319 based studies typically seem to report fewer putative metabolite identifications compared to their
320 mass spectrometry-based counterparts, which generate high feature numbers despite similar
321 putative identification capacity for both analytical methodologies (e.g., 99 – 271 identifications
322 (11.2 – 22.3% of total annotated features) (Sogin et al. 2016; Hillyer et al. 2017; Lohr et al. 2019b).
323 Annotation results are also influenced by differences in the number and type of coral species
324 analyzed, ionization and detection strategy of the measurement tool, and metabolite identification
325 rigor. In comparison, 204 features from our spectra were annotated as unknowns, including many

326 of the features with the highest relative intensities (e.g., Unknowns #72, 73, 58, 82, 68, 71) (Table
327 S3; Table S4), further demonstrating the need for improved metabolite databases for non-model
328 organisms such as coral.

329 While all levels of metabolite identification can be useful for preliminary probes of coral
330 metabolism, increased identification confidence is necessary to establish foundational knowledge
331 of coral metabolomes and to generate high-quality metabolic pathway hypotheses to be tested.
332 Therefore, our more rigorously annotated putative metabolites provide increased confidence to the
333 metabolic pathway analyses discussed in the following sections. Furthermore, our annotation
334 efforts bolster the amount of high-quality metabolite data available in this developing field, which
335 can be used as a reference to aid annotation efforts in future studies. This will help the field
336 transition from utility-based to more experimental studies, with critical biological interpretations
337 and future research directions (e.g., targeted studies) depending largely on such identifications.

338

339 **GA impacts on coral metabolism**

340 We used two approaches to assess differences in metabolism between *P. compressa* samples. (1)
341 The extract yield was used to evaluate the overall metabolic output of the samples. Additionally,
342 (2) the composition and relative abundance of measured metabolites (i.e., metabolomic profiles)
343 in GA, unaffected and reference samples were used to assess GA impacts on coral metabolism and
344 specific metabolic pathways. The use of the extract data provided complementary metabolic
345 information regarding the absolute metabolism of these samples that would be overlooked if only
346 the relative comparisons of normalized metabolomic profiles. It should be noted that these masses
347 consist of the entire suite of metabolites (and potentially other process carryover contaminant
348 molecules) contained in the extract polar fraction. Therefore, while differences may provide a
349 general overview of metabolic activity, discerning the effects of specific metabolites or metabolite

350 classes is not possible using this method alone. Furthermore, this method is confounded by any
351 inherent differences in extraction efficiency between GA and healthy tissues.

352 The extract yield was nearly identical for unaffected and GA samples (Tukey HSD post-
353 hoc p = 0.983); however, for reference samples it was approximately 66% and 70% that of
354 unaffected and GA samples respectively (ANOVA $F_{2,33} = 5.106$, p = 0.012) (Fig. 2a; Table S5). In
355 order to sustain their elevated skeletal and tissue growth (Domart-Coulon et al. 2006), GAs rely
356 on nutrient imports from surrounding unaffected tissues (Stimson 2011) as well as local resources
357 re-allocated from other vital biological functions such as reproduction, energy storage in the form
358 of lipids, and internal pH regulation (Domart-Coulon et al. 2006; Palmer and Baird 2018; Sale et
359 al. 2019; Andersson et al. 2020). Our results seemingly indicate an increase in overall metabolic
360 activity in both GA lesions and apparently healthy (unaffected) areas from GA-afflicted colonies
361 compared to GA-free colonies. This increased metabolic activity in GAs may be linked to the
362 energy burden the lesions impose on affected colonies and further demonstrates that the metabolic
363 impacts of GAs are not limited to the lesions themselves.

364 Metabolomic profile differences were evaluated through PCA of all samples, which
365 showed large amounts of overlap between GA, unaffected, and reference treatments, with
366 particularly high overlap between unaffected and reference samples. Therefore, PCA scores alone
367 were not useful for distinguishing to which treatment group a given sample belongs (Fig. 2b).
368 Isolated comparisons across treatments (GA–unaffected, unaffected–reference, GA–reference)
369 again showed high similarity of samples in PCA (Fig. S7a-c), indicating the extent of GA impacts
370 on metabolomic profiles is low compared to other sources of variation, such as differences between
371 individual coral colonies. These results contrast with the clear morphological, histological, and
372 physiological differences previously documented in *P. compressa* GAs (Domart-Coulon et al.

373 2006). Furthermore, other stressors such as temperature and coral-neighbor interactions generally
374 do result in group separation of coral metabolomes in PCA or principal coordinates analysis plots
375 (Quinn et al. 2016; Hillyer et al. 2017).

376 PCA of only GA and reference samples showed group separation along the second
377 principal component (PC 2; 25.6% explained variance (EV)) (Fig. S7c) and comparison of these
378 two sample types therefore warranted further investigation using supervised PLS-DA. Validation
379 of the PLS-DA model comparing GA to reference samples (Fig. S7d) indicated an effect of
380 treatment ($p = 0.034 \pm 0.001$; mean ± SE), but the predictive power ($Q^2 = 0.394 \pm 0.013$; mean ±
381 SE) of the model was low (Syzmanska et al. 2012). Therefore, a conservative VIP threshold (VIP
382 > 2) (i.e., Lohr et al. 2019b) was used to identify 13 important features from the model, all of
383 which were annotated as unknowns (Table S6).

384 PCA plots were also used to assess differences in metabolomic profiles between bulbous
385 Form 1 GAs and the less protuberant Form 2 GAs. Form 1 samples generally had less metabolic
386 variation and appeared to be slightly different compared to Form 2 samples in PC 2 (22.0% EV)
387 (Fig. S8). However, small sample size and imbalanced replication of Form 1 ($n = 9$) and Form 2
388 ($n = 3$) lesions precluded the use of meaningful univariate or supervised multivariate analyses here.
389 Therefore, despite consistent macro-morphological differences and preliminary evidence for trace
390 elemental and metabolomic differences (Andersson et al. 2020), more research is necessary to
391 confirm these two morphs as distinct *P. compressa* GA lesions.

392 Subsequent univariate comparisons across GA, unaffected, and reference samples showed
393 average spectral intensities between groups were similar for nearly all features and did not
394 highlight any new features of interest. However, Unknown #86 at 3.37 ppm did have higher
395 intensities in both unaffected and reference compared to GA samples (ANOVA $F_{2,38} = 13.321$, p

396 = 0.014) (Table S4), supporting the importance of this feature in the PLS-DA model (VIP = 2.34)
397 (Table S6). Full statistical results for all 330 bins are presented in Table S4. Collectively, these
398 results showed that any detectable, systematic differences GAs imposed on coral metabolomes
399 were largely confounded by relatively high inter-colony variation among the samples.

400 Individual coral species generally have distinct and relatively consistent metabolomic
401 profiles (Sogin et al. 2014; Putnam et al. 2016; Andersson et al. 2019), yet variation among
402 individuals of the same species is sufficient to allow for the distinction of unique genotypes using
403 PCA (Lohr et al. 2019b). As we have demonstrated above, this variation can obscure systematic
404 differences in the presenting phenotypes. A similar phenomenon was observed during our previous
405 elemental characterization of these samples, where differences in certain trace elements (e.g.,
406 Mg/Ca, U/Ca, Va/Ca, Mo/Ca) and internal pH were only apparent once paired analyses of
407 unaffected and GA samples were implemented (Andersson et al. 2020). Therefore, we continued
408 our characterization of GA metabolomic profiles below by capitalizing on the unique paired status
409 of the GA and unaffected samples collected from the same colony (genotype), which allowed for
410 more explicit investigation of disease-related metabolic shifts by controlling for inter-colony
411 variation.

412

413 **Analysis of paired metabolomic profiles**

414 Visual inspection of the PCA for only paired GA and unaffected samples (combined 57.2% EV
415 for PC 1 and PC 2) again showed considerable group overlap (Fig. 3). However, a consistent
416 pattern can be observed between each paired set of samples, with all unaffected samples (except
417 sample 7U) having a lower PC 2 (20.9% EV) score than their respective GA sample. This indicated
418 a consistent metabolic shift between unaffected and GA samples, which was obscured by the
419 relatively larger inter-colony variation. The observed variation between individual unaffected–GA

420 pairs could be due to a variety of genetic, environmental, or disease-progression factors. Therefore,
421 collecting additional genetic data (e.g., DNA or RNA) or controlling environmental variables (e.g.,
422 diet, neighbor interactions) may help interpret this variability in future GA studies.

423 An AMCV was calculated to determine which features were primarily responsible for
424 driving the constant shift between paired samples in the PCA scores plot (Fig. 3). Modified
425 loadings scores were created to determine the contribution of each feature towards the AMCV
426 (Fig. S9) and the 41 features with largest contribution to the AMCV (see Fig. S5) were highlighted
427 in Table 2, while loadings for all features were listed in Table S4. Paired t-tests separately
428 identified a total of 61 features that differed between unaffected and GA samples (paired t-test $p <$
429 0.05) (Table S4). Of the 41 most influential AMCV features, 29 differed according to univariate
430 analyses as well ($p < 0.05$) (Table 2). A combined total of 73 unique features were identified by
431 either multivariate or univariate analyses and these trends were confirmed visually using the raw
432 data (e.g., Fig. 4; Fig. S10; Table S4). These results highlight the benefits of collecting and
433 analyzing paired samples in coral metabolomics research when possible, where inherent
434 metabolome variation between individual coral colonies can be a challenge.

435 Of these features, 1-methylnicotinamide, trigonelline (GA high), betaine, glycine, and
436 histamine (GA low) were putatively identified (Table 2). Betaine and glycine were influential in
437 the AMCV, while univariate analyses provided evidence of differences between GA and
438 unaffected samples for betaine ($t_{11} = -2.685$, $p = 0.060$), glycine ($t_{11} = -3.403$, $p = 0.032$), histamine
439 ($t_{11} = -2.966$, $p = 0.046$), trigonelline ($t_{11} = 3.077$, $p = 0.041$) and 1-methylnicotinamide at 8.86
440 ppm ($t_{11} = 3.342$, $p = 0.032$) and 8.95 ppm ($t_{11} = 3.203$, $p = 0.036$) (Table 2). It should be noted
441 that glycine and Unknown #102 overlapped in the same statistical bin, although both features

442 appeared to be low in GA compared to unaffected samples by direct spectral comparison (Fig.
443 S10).

444 Many of the most influential features identified by both the unpaired (Table S6) and paired
445 (Table 2) analyses were annotated as unknowns, restricting pathway analyses to only a handful of
446 compounds (see Supplemental Text for in-depth comparison of unpaired and paired results). These
447 unknown features are of interest regarding GA pathophysiology and more effort is therefore
448 needed to identify important unknown compounds through isolation and spectroscopic techniques.
449 Nonetheless, using known biochemical activities of identified compounds can provide a useful
450 starting point for generating testable hypotheses regarding specific metabolite activity or metabolic
451 pathways that may be associated with GA pathophysiology in *P. compressa*. Although differences
452 in the five putative metabolites were difficult to detect without the advantage of paired unaffected
453 and GA samples, even subtle changes in tightly regulated metabolites may be physiologically
454 important. Therefore, the features contributing to this shift are good candidates for providing
455 insights into the molecular pathology of GAs, and preliminary pathway analyses of putatively
456 identified metabolites are discussed below.

457

458 **Metabolic pathway analyses**

459 Betaine is an important organic osmolyte (Ashraf and Foolad 2007) and has been shown to
460 accumulate in marine invertebrates in response to environmental stress (Liu et al. 2011; Cappello
461 et al. 2013), including in other reef-building coral species (Williams et al. 2021). However, we
462 observed low betaine levels in GA samples despite their abnormal condition that could be expected
463 to trigger such an accumulative stress response. Alternatively, this may be explained by betaine
464 accumulating in the unaffected tissues as a stress response to the directly adjacent GA lesions, or

465 by a decreased influx of symbiont-derived betaine in GAs due to their lower abundance of
466 symbiotic dinoflagellates (Domart-Coulon et al. 2006).

467 Glycine is an amino acid precursor (Amelio et al. 2014) that is highly abundant in the
468 skeletal organic matrix secreted by corals (Puverel et al. 2005). The organic matrix is thought to
469 act as a biological framework during skeleton formation, and the glycine composition of this
470 matrix differs between corals with distinct skeleton morphologies (Puverel et al. 2005). This
471 indicates that the amino acid composition of the organic matrix may affect skeleton structure
472 and/or morphology, thus the decreased levels of glycine we measure in GAs may be linked to their
473 irregular skeletal characteristics. Our previous elemental work on these samples indicates that GAs
474 allocate resources away from the pH regulation of their calcifying fluid to sustain their rapid
475 growth (Andersson et al. 2020). If this is the case, GAs may also allocate energy away from other
476 aspects of skeletogenesis, such as glycine synthesis for the organic matrix. Alternatively, it may
477 be that the faster extending skeletons of *P. compressa* GAs (Domart-Coulon et al. 2006) are
478 depleting glycine levels, thereby contributing to the low glycine we measure in GA compared to
479 unaffected samples.

480 Betaine and glycine together belong to a larger set of biochemical pathways involved in
481 glycine/serine metabolism, specifically the oxidation of choline to glycine, which can then be
482 interconverted with serine (Fig. 5). The decreased levels of betaine and glycine indicate a
483 downregulation in the choline oxidation pathway in GAs. In the cnidarian model *Aiptasia,* choline-
484 derived synthesis of glycine is upregulated in non-symbiotic individuals, a proposed indicator of
485 heterotrophic feeding (Cui et al. 2019). The lower choline oxidation we theorize in GA samples
486 would then indicate a decrease in choline-derived glycine (Fig. 5) and thus a decreased proportion
487 of heterotrophic feeding in GA lesions.

488 GAs in *P. compressa* contain fewer symbiotic dinoflagellates compared to unaffected
489 tissues (Domart-Coulon et al. 2006), so it might be expected that these lesions increase
490 heterotrophic feeding to compensate for the assumed decreased influx of photosynthates.
491 However, unlike some coral species, *P. compressa* does not increase heterotrophic feeding to
492 compensate for the loss of symbionts during bleaching (Grottoli et al. 2006), which may explain
493 why feeding is seemingly not increased in GAs. Additionally, the abnormal polyp characteristics
494 of GAs may restrict their ability to capture prey. For example, *P. compressa* GAs have fewer and
495 more dispersed polyps (Domart-Coulon et al. 2006; Andersson et al. 2020), which may reduce
496 heterotrophic feeding potential. If true, an inability for heterotrophic feeding to compensate for the
497 loss of symbiont-derived resources in GA lesions may contribute to the energetic burden that GA
498 tissues place on the rest of the coral holobiont to sustain their growth. Increased skeletal Mo/Ca
499 and V/Ca in these same GA samples may indicate decreased nitrogen fixation in the GA holobiont
500 as well (Andersson et al. 2020), which would only exacerbate GA reliance on external energy
501 sources.

502 The choline oxidation pathway is also linked to DNA methylation through the 1-carbon
503 cycle (Fig. 5). DNA methylation often functions to inhibit expression of unwanted genes
504 (Niculescu and Zeisel 2002) and is a known mechanism for phenotypic plasticity in corals as an
505 acclimatization response to environmental stress (Putnam et al. 2016). Moreover, metabolomics
506 analysis of benign hepatic tumors in flatfish revealed decreases in both choline and glycine
507 (Southam et al. 2008), and choline-deficient diets can induce hepatocellular carcinomas in rats
508 (Nakae et al. 1992). Although these results do not necessarily support the classification of GAs as
509 tumors (malignant or benign), the carcinogenic potential of this pathway in coral GAs warrants
510 further investigation. Based on these untargeted results, we hypothesize that heterotrophic feeding,

511 the oxidation of choline to glycine, glycine levels in the skeletal organic matrix, and epigenetic
512 DNA methylation are decreased in GA lesions relative to surrounding unaffected tissues. We
513 further theorize that these are concurrent results related to GA formation and/or growth, potentially
514 all stemming from decreased heterotrophic feeding, rather than mutually exclusive GA processes.
515 Therefore, metabolites belonging to these pathways are of interest for GA pathophysiology going
516 forward and could be the focus of future validation and targeted studies to further elucidate GA
517 impacts.

518

519 **Wider GA pathophysiology perspectives**

520 Our study is the first application of untargeted metabolomics analyses to study coral disease *in*
521 *situ*, and thus offers a unique perspective into the biochemical impacts of GAs in *P. compressa*.
522 These results are a part of a larger investigation of GA pathophysiology that includes
523 morphological descriptions and skeletal trace element measurements of these same coral samples.
524 Elemental and morphological results showed decreased pH of GA calcifying fluid and a porous
525 and fragile GA skeleton respectively, which we theorized to be the result of energy re-allocation
526 away from internal pH regulation to facilitate elevated GA tissue growth (Andersson et al. 2020).
527 Our metabolomics results expand on these findings by providing additional demonstrations of
528 abnormal energetics (i.e., increased unaffected and GA extract yield, metabolomic profile
529 differences between unaffected-GA samples) and by identifying specific metabolites and
530 metabolic pathways of interest that may be altered in this irregular GA metabolism. Combined
531 results from these studies seemingly indicate that GA metabolism is unbalanced towards
532 promoting rapid lesion growth, often at the expense of other essential biological processes and the
533 surrounding unaffected tissues, despite evidence that traditional autotrophic and heterotrophic
534 energy sources are compromised in GAs. Both studies also demonstrate the utility of analyzing

paired GA and unaffected samples when studying GAs. Overall, this work broadly advances our understanding of GA pathophysiology, particularly in *P. compressa*. Further work on GAs could aim to validate metabolomics results with targeted measurements of important metabolites and to extend GA characterization to additional analytical methods (e.g., mass spectrometry-based metabolomics, transcriptomics, stable isotope analyses to assess heterotrophic nutrition, DNA methylation assays, 16S rRNA gene sequencing) to provide a more comprehensive comparison of diseased and healthy holobiont functioning.

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Compliance with ethical standards

Competing interests

On behalf of all authors, the corresponding author states that there is no conflict of interest. Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedure adequately. Such identification is not intended to imply recommendation

560 or endorsement by the National Institute of Standards and Technology and the U.S. Government,
561 nor is it intended to imply that the materials or equipment identified are necessarily the best
562 available for the purpose.

563

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796 **Figure Legends**

797

798 **Figure 1.** Overview of *Porites compressa* sampling strategy and different sample types that were
799 collected. (a) Diagram depicting a *P. compressa* growth anomaly (GA) and unaffected sample pair
800 collected from a GA-afflicted colony (red box) and corresponding reference sample collected from
801 the nearest, distinct, adjacent *P. compressa* colony (green box). Representative photos of (b) Form
802 1 GA lesion and paired unaffected sample, (c) Form 2 GA lesion and paired unaffected sample,
803 and (d) reference coral sample taken in the laboratory after lyophilization. (b-d) Dashed red
804 outlines indicate GA lesions and unmarked areas show apparently normal (unaffected or reference)
805 coral; black bars indicate 1 cm.

806 **Figure 2.** (a) Average extract yield for growth anomaly (GA; n = 13), unaffected (n = 12) and
807 reference samples (n = 11). Points indicate group mean and error bars indicate standard error of
808 the mean. Displayed p-value is from one-way analysis of variance model, p-values for specific
809 post-hoc comparisons are listed in Table S5. (b) Principal component analysis scores plot of the
810 first two principal components (PC 1 and PC 2) for GA (n = 13), unaffected (n = 13), and reference
811 samples (n = 15). Ellipses indicate 95% confidence region of the multivariate t-distribution for
812 each group.

813 **Figure 3.** Principal component analysis (PCA) scores plot of the first two principal components
814 (PC 1 and PC 2) for paired growth anomaly (GA; n = 12) and unaffected samples (n = 12). Blue
815 lines indicate the difference in PCA scores between each unaffected–GA pair and the bold arrow
816 indicates the average metabolic change vector from unaffected to GA samples. Ellipses indicate
817 95% confidence region of the multivariate t-distribution for each group.

818 **Figure 4.** Boxplots showing relative intensity of growth anomaly (GA; n = 12) and unaffected (n
819 = 12) samples of selected features of interest based on average metabolic change vector loadings
820 (Table 2) with corresponding false discovery rate corrected p-values from paired t-tests. Boxes
821 indicate the group interquartile range (IQR) and the horizontal line within each box indicates the
822 group median. Whiskers extend above and below the upper and lower boundary of the boxes to
823 the group maximum and minimum (up to 1.5 × IQR) respectively. Relative intensities for the
824 individual samples in each group are shown as the points overlapping with the boxplots. Features
825 are listed in descending ^{1}H nuclear magnetic resonance (NMR) chemical shift (ppm) order.
826 Corresponding overlapped ^{1}H NMR spectra for these features are shown in Fig. S10.

827 **Figure 5.** Flow chart showing selected reactions relating to glycine and serine metabolism,
828 focusing on the oxidation of choline to glycine. Black arrows indicate metabolic reactions; blue
829 ovals indicate specified metabolites; green ovals indicate metabolites with decreased abundance
830 in growth anomaly relative to unaffected samples. Gray box indicates the enzyme betaine-
831 homocysteine S-methyltransferase (BHMT), which is responsible for catalyzing the conversion of
832 betaine and homocysteine to dimethylglycine and methionine. DMG = Dimethylglycine; SAM =
833 S-adenosylmethionine; SAH = S-adenosylhomocysteine.