

## Multi-omics determination of metabolome diversity in natural coral populations in the Pacific Ocean

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Coral reefs are considered one of the most emblematic ecosystems in our oceans, but their existence is increasingly threatened by climate change. In this study, natural populations of two reef-building coral genera, *Pocillopora* spp. and *Porites* spp., and one hydrocoral *Millepora* cf. *platyphylla* from two different marine provinces in the Pacific Ocean were investigated using a multi-omics approach as part of the *Tara* Pacific expedition. Here, we propose a standardised method consisting of a biphasic extraction method followed by metabolomics analysis using mass spectrometry for the lipidome and <sup>1</sup>H nuclear magnetic resonance for hydrophilic metabolites. Our study assessed a broad range of the metabolome and is the first to identify and add 24 compounds by NMR and over 200 lipids by MS analyses for corals. Metabolic profiles were distinct among genera but not within genotypes of the cnidarian corals. Although endosymbiotic dinoflagellates of the family Symbiodiniaceae are known to play a central role in the metabolomic signature of the coral holobiont, they did not account for all differences. This suggests that a combined effect by different members of the coral holobiont and an interaction with the environment might be at play. Our study provides foundational knowledge on the coral holobiont metabolome.

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Contemporary warm-water coral reefs have dominated shallow-water marine environments on Earth for over 500,000 years<sup>1</sup>. These biodiversity hotspots are some of the most productive ecosystems in the world, providing essential goods and services and high socio-economic returns to coastal nations<sup>2</sup>. With the turn of the 21<sup>st</sup> century, a clearer picture of the intimate association between corals, their endosymbiotic dinoflagellate (photosymbiont), and their microbiome began to emerge, and the co-operative functioning of the coral holobiont became widely accepted<sup>3</sup>. Over the past three decades, marked increases in seawater temperature and acidification have far exceeded average projections. These rapid changes have, in turn, presented new environmental challenges for coral holobionts, such as more frequent exposure to heat stress<sup>4</sup>. Heat stress is detrimental to the underlying symbiotic relationship necessary for the overall functioning of the coral holobiont as a whole<sup>5</sup>. Studies have shown that extended periods of temperature stress may ultimately lead to the expulsion of the photosymbiont by the coral host in a process referred to as coral bleaching. Coral bleaching remains the leading threat to the future persistence of coral reef ecosystems<sup>6</sup>. However, the impact of environmental stress on individual corals is still not fully understood.

The -omic sciences have become fundamental in systems biology and offers new insights into genotypic and phenotypic variability. This coupled with environmental data is a powerful approach in unravelling the complexity of how organisms function and respond to environmental change. Metabolomics provides information on thousands of metabolites naturally present in living organisms<sup>7</sup>. These metabolites are products of various biochemical processes involved in growth, development, or reproduction, among other essential cellular processes, and therefore provide a broad overview of the physiological state of an organism. Since the coral host lives in symbiosis with many other organisms, any metabolomic analyses will collectively reflect the physiological state of the coral, its photosymbionts and associated microbiome (the coral holobiont)<sup>8,9</sup>. However, essential changes in phenotypic traits of the coral holobiont or individual members of the holobiont can be traced through laboratory studies<sup>10</sup>. These studies offer essential insights into the functional response of coral holobionts exposed to environmental stress such as temperature<sup>8,11</sup>, disease<sup>12,13</sup>, bleaching history<sup>14</sup>, or biochemical defense<sup>15</sup>. More recently, different genotypes in the same species of coral have been shown to express different metabolic profiles under induced stress<sup>8</sup>, and such data are currently being used to inform coral reef conservation<sup>16</sup>. However, much of what is known about the metabolome of coral holobionts represents an incomplete picture of the full extent of their chemical diversity. Unlike genomics or proteomics, there is no single technique able to provide a complete picture of the metabolome of an individual.

At present, several extraction methods have been used to study the metabolome of the coral holobiont, and three analytical techniques have been applied, GC-MS or LC-MS and <sup>1</sup>H NMR, each with their own advantages and limitations<sup>17</sup>. In the past, GC-MS was typically used over NMR for the analysis of polar metabolites<sup>18–20</sup>, however, the former is not universal as it requires an initial derivatisation step before analysis.

The first study on the coral metabolome was published in 2012 and used a modified Folch extraction method (biphasic CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O) and LC-MS/MS<sup>21</sup>. This method has also been recently used to study the lipodome of corals by Imbs et al.<sup>22</sup>. In 2013, Gordon compared different lipid extraction methods and recommended a single phase 70% aqueous methanol extraction followed by either NMR or LC-MS analyses<sup>17</sup>. The Gordon extraction method has been widely used for coral metabolomics. For example, Sogin et al. analysed polar metabolites from corals

with <sup>1</sup>H NMR<sup>23</sup>, Quinn et al. 2016 studied the lipids of corals using LC-MS/MS<sup>15</sup>, Hillyer et al. and Matthews et al. used GC-MS to study the primary metabolism of the coral holobiont<sup>20,24</sup> and more recently Lohr et al. 2019 used <sup>1</sup>H NMR for the analysis of polar metabolites and LC-MS/MS for the lipids of their corals, but with limited annotation of metabolites<sup>8</sup>. Andersson et al. posit that a biphasic extraction modified from the Bligh and Dyer method is more efficient and robust for the analysis of small polar metabolites compared to the single-phase extraction method by Gordon et al.<sup>12</sup>. Other extractions methods have also been used, for example Farag et al. 2018 applied a biphasic extraction method developed by Sogin in 2016 and analysed polar metabolites of soft corals by GC-MS and lipids by LC-MS/MS<sup>25</sup>. Interestingly, Sogin et al. used a modified Matyash method and the nontoxic solvent methyl *tert*-butyl ether (MTBE) for the biphasic extraction of lipids which were analysed with GC-MS<sup>13</sup>. A brief historical account of methods and chemical analyses used in coral metabolomics, highlights the need for a more unified approach (Table S2). The lack of standardised methods for coral metabolomics together with limited publicly available chemical reference libraries and the general lack of knowledge on how metabolites function in nature represents major bottlenecks in non-targeted metabolomics.

The impact of climate change on coral holobionts has been well studied under laboratory conditions<sup>10,11</sup>. Therefore, much of our current knowledge on functional responses in coral holobionts is based on laboratory experiments<sup>26</sup>, with only a few studies combining ex-situ experiments with in-situ studies<sup>14</sup>. Even fewer studies have investigated the variability in the metabolomic profiles of populations of coral holobionts across a broad geographic range or in distinct environments such as bioregions. With the rising threat of climate change on coral reef ecosystems, the *Tara* Pacific expedition aimed to explore the biological and chemical diversity of natural populations of coral holobionts across the Pacific Ocean<sup>27</sup>. Three dominant coral holobionts, two scleractinian genera, *Pocillopora* spp. and *Porites* spp. and one species of hydrocoral *Millepora* cf. *platyphylla*, were sampled across an ecological and geographic gradient encompassing distinct marine provinces<sup>28</sup>. This extensive collection of more than 2600 samples provided a rare opportunity to study patterns of genetic, proteomic and metabolomic variability in relation to environmental differences and biogeography. In this study, we optimise a standard, scalable metabolomic approach on a subset of samples using a biphasic extraction followed by <sup>1</sup>H NMR and LC-MS to analyse a wide range of metabolites varying in polarity. Our metabolomic results were compared with environmental and genetic data generated as part of a larger collaborative effort of the *Tara* Pacific expedition and are discussed<sup>29</sup>.

## Results

**Untargeted LC-MS and <sup>1</sup>H NMR metabolomics and annotation of coral metabolites.** Specimens of two genera of scleractinian corals, *Pocillopora* spp. and *Porites* spp. and one hydrocoral, *Millepora* cf. *platyphylla* were collected by SCUBA from various sites across the Pacific Ocean<sup>27</sup>. For this study, three biological replicates were collected for each species from two sites in two islands located in distinct marine provinces, the Gambier archipelago (Southeast Polynesia) and Samoa (Central Polynesia) (Fig. S1, Table S1)<sup>28</sup>. Among other factors, these islands are subject to different temperature regimes: with the average sea surface temperature (SST) for Gambier (island 06) reaching 25.07 °C (std 1.64 °C) and 24.89 °C (std 1.70 °C) at each site, and reaching 28.35 °C (std 0.92 °C) and 28.68 °C (std 0.89 °C) at each site in Samoa (island 10)<sup>26</sup>.

Our metabolomic approach was designed to be easily scalable to analyse thousands of samples collected during the *Tara* Pacific expedition and was initially tested on a subset of 36 samples. A rapid and reproducible extraction method was developed by modifying the Matyash lipid extraction<sup>30</sup>, followed by LC-HRMS/MS and <sup>1</sup>H NMR analyses of the non-polar and polar metabolites, respectively (Fig. S2)<sup>30,31</sup>. Extraction yields were maximized by liquid-liquid partitioning of the freeze-dried material using a biphasic solvent mixture MTBE/MeOH/H<sub>2</sub>O (7:4:4). The analytical methods, LC-MS/MS for the upper organic fraction and <sup>1</sup>H NMR for the lower aqueous fraction, were chosen to limit the number of downstream analyses. For each biological replicate, three technical replicates were analysed by LC-MS in order to validate our methodology (Fig. S3).

This extraction method allowed for the annotation of a large part of the lipidome using the most recent tools in molecular networking by LC-HRMS/MS and the comprehensive database LIPID MAPS and the Metabolomics Workbench (<https://www.metabolomicsworkbench.org/>). Lipids were initially annotated by comparison with available public databases using the Global Natural Product Social Molecular Networking platform. Positive identities were assigned to metabolites based on cosine scores higher than 0.7 in the molecular networks (MN). The annotation of some lipids were expanded from the GNPS platform using the LIPID MAPS classification by combining structural similarities obtained from the molecular network with accurate masses and fragment similarity<sup>32</sup>. A list of 14 clusters was identified using HRMS and MS/MS data available for various lipids. This included a large diversity of glycerolipids such as 1,2-diacyl-sn-glycerol (DG), triacyl-sn-glycerol (TG), 1,2-diacyl-3-monoglycosyl-sn-glycerol (MGDG), and 1,2-diacyl-3-diglycosyl-sn-glycerol (DGDG). Betaine lipids such as 1,2-diacyl-3-(6'-sulfo- $\alpha$ -D-quinovosyl)-sn-glycerol (SQDG), 1-mono- (MGCC) and 1,2-diacylglyceryl-3-O-carboxyhydroxymethylcholines (DGCC), 1,2-diacylglyceryl trimethylhomoserines (DGTS), 1,2-diacyl-sn-glycero-3-phosphate (PA), 1-mono- (LPC) and 1,2-diacyl-sn-glycero-3-phosphocholine (PC) were also identified in other clusters together with the phospholipids Platelet-Activating Factors (PAF) and their deacetylated analogues referred to as lyso-PAF (Supplementary Data 1). Finally, known fatty acids and amide derivatives such as hexacanamide and erucamide were also identified at the center of several large clusters of the MN (Fig. S4 and S5).

<sup>1</sup>H NMR spectroscopy revealed several major metabolites in the polar metabolome of each genus. Despite the utility of NMR in metabolomics as a universal method, it has been applied less often than LC-MS for studying coral holobionts<sup>12,13,26</sup>. Among the major metabolites, 24 were detected and 18 were annotated collectively for all three coral genera using synthetic standards, and six remained unknown. The list of the metabolites is indicated in Figure S9 and includes amino acids, formate, acetate, choline derivatives, and small betaines such as GlyB, AlaB, ProB, HProB, homarine and trigonelline.

**Metabolome variability among coral holobiont species/genotypes.** Genetic analyses were used to identify different lineages in the cnidarian hosts and endosymbiotic dinoflagellates (Table S1) for each of the 36 individuals studied here. Five genotypes were recovered for *Pocillopora* and two genotypes for *Porites*<sup>33</sup>. Lastly, a single genotype was identified for *Millepora cf. platyphylla*. The scleractinian corals hosted endosymbiotic dinoflagellates belonging mainly to the genus *Cladocopium* at both islands, while the hydrocoral hosted Symbiodiniaceae predominantly from the genera *Symbiodinium* in Gambier (Island 06) and *Cladocopium* in Samoa (Island 10), but also some genotypes of Clade G, and Clade F. Except for one specimen of *Pocillopora*, all

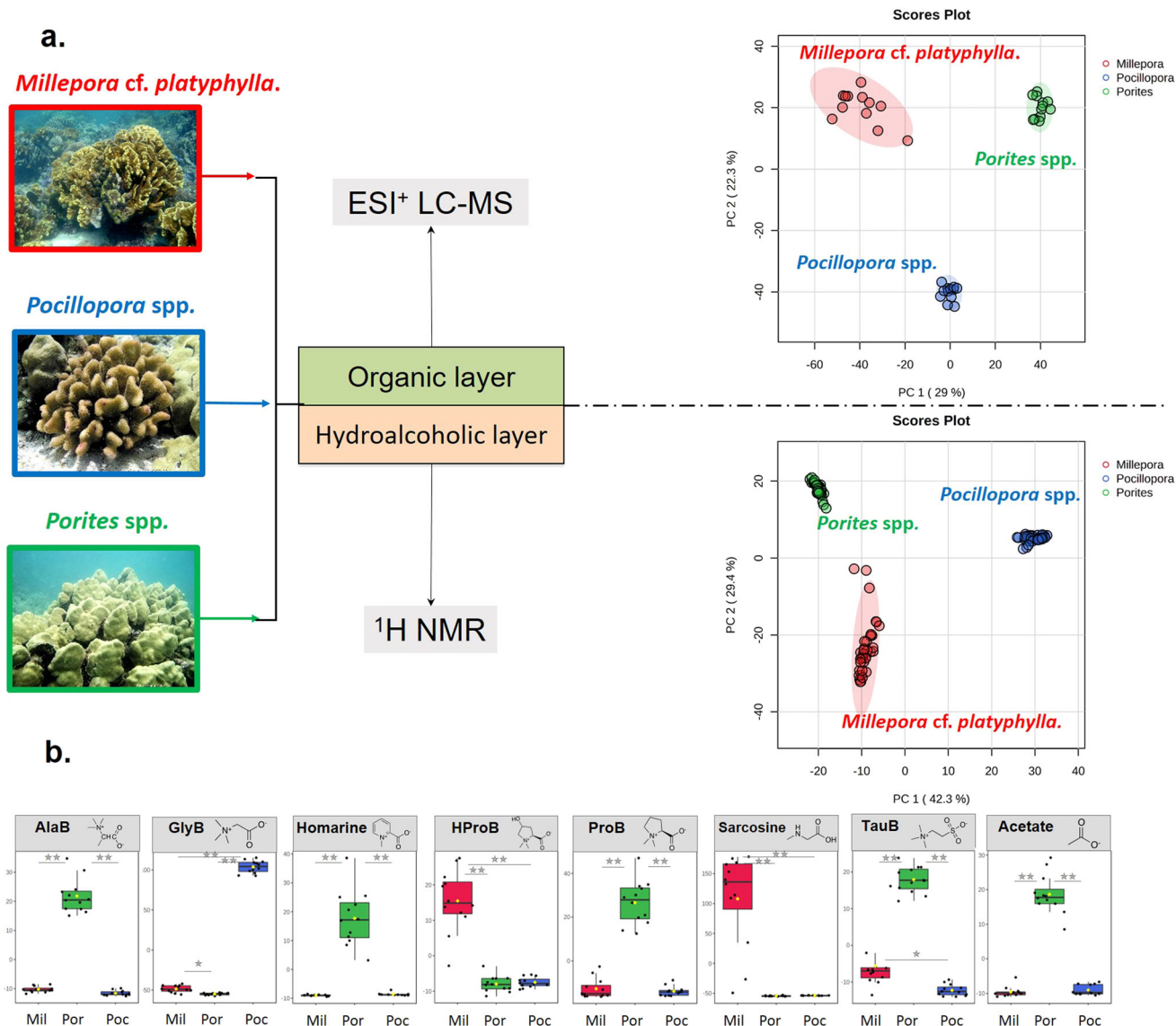
other endosymbiotic dinoflagellates were specific to their scleractinian host genera<sup>33</sup>. Overall, among the 36 specimens of the three studied genera, a higher diversity of Symbiodiniaceae was observed in the cooler island 06 (Gambier), while a single genus of Symbiodiniaceae (*Cladocopium*) was present at the warmer island 10 (Samoa).

The LC-MS analyses of the non-polar (lipidome) extracts were significantly different among all three coral host genera (Fig. 1a). However, the same families of lipids were generally found in all three genera but at different concentrations and with structural differences in their fatty acid chains. Of the 14 clusters, a large cluster of polar betaine lipids namely, MGCC (also referred to as lyso-DGCC) and diacylglycerylcarboxyhydroxymethylcholine (DGCC) was identified in all three genera (Fig. S7). The identity of these metabolites were further confirmed by comparison of MS/MS data with similar metabolites identified from the coral *Montipora capitata*<sup>14</sup>. The distribution of these betaine lipids was distinct among genera with DGCC 38:6 (16:0/22:6) being the central metabolite together with its lyso derivatives MGCC 16:0 and MGCC 22:6. While the diacylated derivatives were the major DGCC in *Millepora cf. platyphylla* and *Porites* spp., the monoacylated derivatives were highly present in *Pocillopora* spp. (Fig. S7). Two large clusters of platelet activating factor (PAF) and lyso-PAF were also identified in all three species, but varied in their relative abundance among host genera (Fig. S8)<sup>15,34</sup>. Lyso-PAF C16 and C18 were also present in all genera from both islands, but the bioactive PAF C16 and C18 were only detected in specimens of *Pocillopora* spp. Interestingly, a putatively new sulfolipid cluster prevalent on island 06 was detected using HRMS and MS/MS data in *M. cf. platyphylla*. Further comparison with well-known lipids led to the tentative identification of a betaine lipid derived from cysteine. However, the identity of these compounds will need to be confirmed by isolation and further characterisation by <sup>1</sup>H NMR (Fig. S6).

The <sup>1</sup>H NMR profiles of the polar extracts were highly distinct among the three host genera with only a few shared signals (Fig. S9). A large majority of the identified metabolites were amino acids or their betaine derivatives, which appeared to be highly genus specific. For example, the major metabolites present in *Millepora cf. platyphylla* were HProB and sarcosine, while GlyB was the major metabolite found in *Pocillopora* spp. and acetate, TauB, AlaB and homarine were present at high concentrations in all specimens of *Porites*. (Fig. 1b).

**Metabolome variability with geography.** The variability in the metabolome of all three coral host genera was assessed in relation to their environment (two sites on two islands), using unsupervised and supervised statistics applied to <sup>1</sup>H NMR and LC-MS data. Significant differences were observed in the lipidomes of specimens of the same host genus and among the two studied islands for all three genera in the non-supervised PCA analyses, and to a lesser extent among sites within islands but with some degree of overlap for some genera (Fig. 2a). Betaine lipids were the main driver of genus-level differences at both islands (Fig. S7). For *Millepora cf. platyphylla* and *Pocillopora*, the diacyl derivatives DGCC were highly concentrated in specimens from island 06 (Gambier) and the lyso derivatives MGCC in specimens from island 10 (Samoa) but this trend was less apparent for *Porites*. (Fig. S4).

The <sup>1</sup>H NMR data largely supported differences between islands for *Porites* but not for the other two coral host genera. A large variability in the metabolome of *Millepora cf. platyphylla* was observed for samples collected from site 01 in island 06 (Gambier) using both methods.



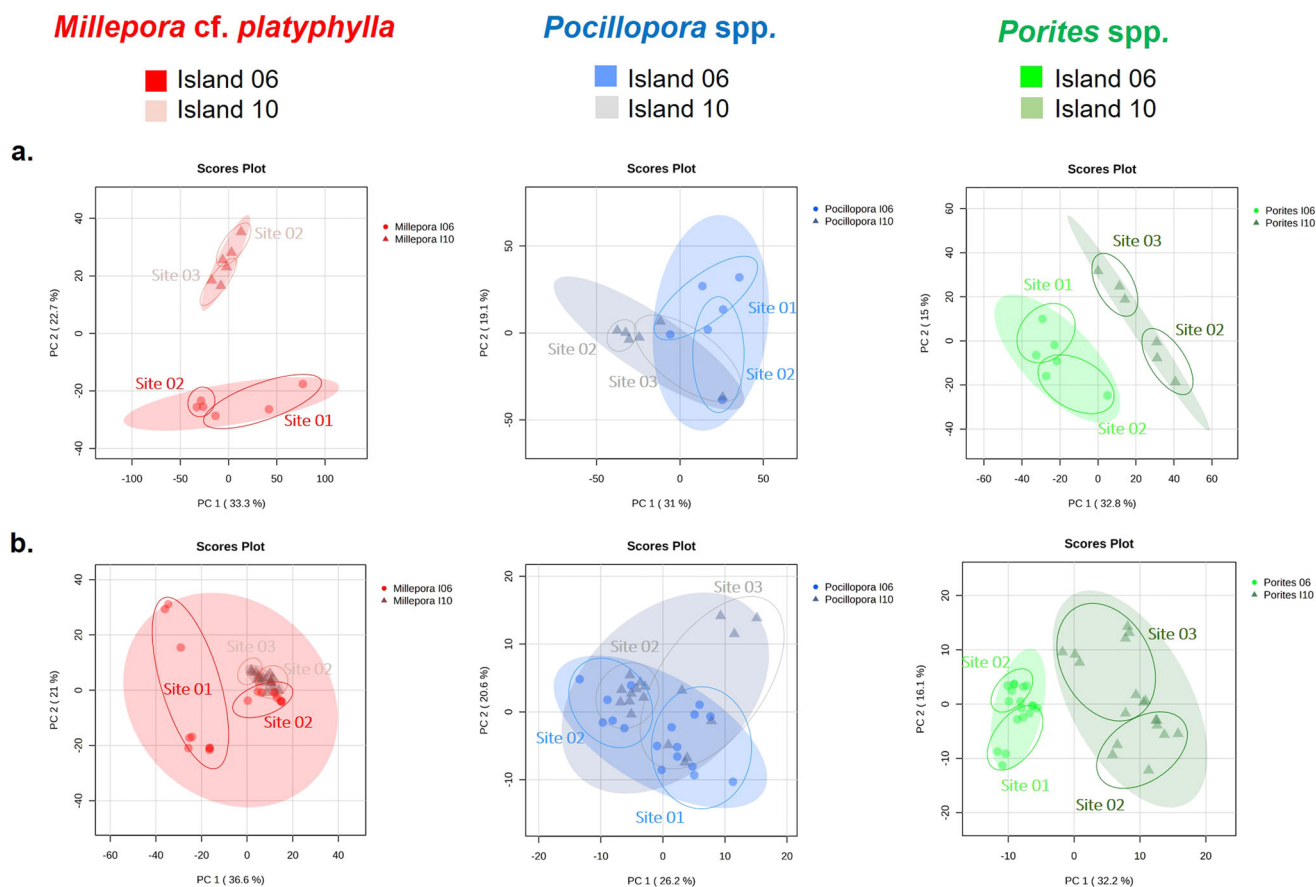
**Fig. 1** Metabolomic approach and metabolome variability among the three studied coral hosts. **a** PCA score plot based on LC-MS (non-polar metabolome) and  $^1\text{H}$  NMR (polar metabolome) data matrix. **b** Boxplot of relative concentrations for some significantly variable metabolites in the three different genera (*Millepora* in red, *Pocillopora* in blue, *Porites* in green) studied by NMR. Y axes are represented as relative units. Data were normalized to the total spectral area. The bar plots show the normalized values (mean  $\pm$  standard deviation). Medians are indicated by horizontal lines within each box. (\*\*  $p$  value  $< 0.001$ ; \*  $p$  value  $< 0.01$ , post hoc analysis: Fisher's LSD).

## Discussion

The *Tara* Pacific expedition provided a unique opportunity to study the -omics of natural populations of coral holobionts across the Pacific Ocean. Such information will serve as an important baseline for future studies.

**A standardized method for the corals metabolomics.** We demonstrate here, on a small subset of samples collected during the *Tara* Pacific expedition, an efficiency biphasic liquid/liquid partitioning method followed by  $^1\text{H}$  NMR for analysis of the hydrophilic extract coupled with MS for analysis of the organic extract to gain a more complete metabolic overview of our studied coral holobionts. We chose MS analysis for the organic fraction because lipid signals tend to overlap in  $^1\text{H}$  NMR spectra and  $^1\text{H}$  NMR for analysis of the polar extract because polar metabolites have been largely overlooked in MS metabolomics which targets medium to low polarity metabolites. To our

knowledge, only one other study has employed both NMR and LC-MS analyses for coral metabolomics, but their study differs from ours in that they use a one phase solvent extraction which limited the annotation of the metabolome<sup>8</sup>. Our method differs from Lohr et al. in several other aspects. First, we used a biphasic extraction using MTBE (Matyash) which allowed for the easy collection of the organic phase on the top layer and avoided the use of non-eco-friendly solvents. Furthermore, the percentage of the three solvents were adjusted to expedite the two phase separation as recently demonstrated<sup>35</sup>. In addition, a phenylhexyl column was used instead of the commonly used C18 column in order to expand the analysis of less polar lipids by LC-MS/MS. A single extraction method for both polar and non-polar metabolites was not only cost effective and less time consuming but also limited the number of downstream analyses that would otherwise be needed. Furthermore, this extraction method allowed for the accurate annotation of compounds by NMR using standards synthesised in the laboratory for several known small betaines or



**Fig. 2 Metabolome variability with geography.** PCA score plots for the three host genera located in sites O1 and O2 of island O6 (Gambier) and sites O2 and O3 of island O10 (Samoa) from (a) LC-MS/MS (ESI<sup>+</sup>), and (b) <sup>1</sup>H NMR.

amino acid derivatives. In this way we obtained the highest level of confidence (level 1) when annotating compounds, many of which were annotated for the first time, and followed the prescribed standards for metabolomic analyses<sup>36</sup>.

Prior to this study, only a few metabolites were collectively reported from our studied coral holobionts using both MS and NMR analyses. This included, three small betaine derivatives (homarine<sup>37</sup>, TauB and ProB<sup>38–40</sup>) and various fatty acid derivatives from *Porites* spp.<sup>41</sup>, as well as a comprehensive list of symbiotic lipids from *Millepora* cf. *platyphylla*<sup>42,43</sup>. An additional eight compounds were reported from *Porites* spp. (lactate, alanine and trigonelline)<sup>22</sup> and *Pocillopora* spp. (Glycine betaine (GlyB), dimethylsulfoniopropionate (DMSP), taurine, ProB and dimethyltaurine)<sup>40</sup> based on NMR analyses. The first three compounds reported in the latter study were shown to be major compounds produced by the algal photosymbiont. To our knowledge, this is the first study to identify 24 metabolites unambiguously by <sup>1</sup>H NMR in corals. One of the major compounds identified in this way were various osmolytes obtained in the polar phase. Osmolytes have been shown to play a key role in the functioning of coral holobionts. Similar studies have only reported putative annotations (18 metabolites in ref. 12, 4 in ref. 22, 14 in ref. 23, and 10 in ref. 8) at a lower confidence (level 2) by comparison with the literature. Dimethylsulfoniopropionate (DMSP) which is often associated with heat stress has been widely reported in various hard corals such as *Porites*, *Pocillopora*, *Acropora* and *Stylophora* based on LC-MS or GC-MS<sup>40,44</sup>. However, our <sup>1</sup>H NMR analysis did not detect DMSP in any of our studied coral holobionts. Whether this is due to the absence of DMSP in the corals or a lack of sensitivity of NMR

remains to be further tested. A similar level of confidence could not be attained for our MS data as standards for lipids are not widely available commercially, especially in a pure form. Nevertheless, the annotation of entire clusters of lipids was possible by comparison with MS/MS data available in online databases and annotation based on similarity through molecular networking and LIPID MAPS. This allowed for the putative annotation of more than 210 lipids. Overall, our study adds several major, polar betaines based on NMR analyses and a comprehensive list of lipids based on MS analyses to the ever-growing chemical reference library of coral holobionts. We believe that such data on a diverse set of metabolites with varying polarity will serve as a baseline for future metabolomic studies on coral holobionts.

**Metabolomic variability among coral holobionts.** The intricate and often complex association between cnidarian corals, their photosymbiont and associated microbiota makes it difficult to tease apart the functional role of different members of the coral holobiont. Laboratory studies are typically used to determine the functional role of either the photosymbiont or associated microbiota while the residual effects are often attributed to the coral host. However, determining and quantifying the functional role of different members of the coral holobiont under laboratory conditions is not always easy. Not to mention the stress induced by the transport of corals to an aquarium and their acclimation to artificial conditions. Therefore, the study of natural populations is important to assess the contribution of both the environment and the genetic basis underpinning adaptation. -Omics approaches have been incredibly useful in this regard. The overall

metabolome (non-polar and polar) variability in our study was highly structured among different genera of coral holobionts for both MS and NMR analyses. Similar results were found for two species of *Acropora* studied under in-situ and ex-situ scenarios<sup>26</sup>. Species-specific patterns were also observed for four reef building corals using <sup>1</sup>H NMR<sup>22</sup>, as well as for five deep-sea corals using LC-MS<sup>45</sup>. A large variety of lipids in our analyses, particularly the glycerolipids, were shared across all three coral holobionts. Interestingly, a new family of sulfolipids was tentatively identified in *Millepora* cf. *platyphylla* by comparison of LC-MS/MS fragmentation patterns. However, the confirmation of their structure will require further isolation and NMR analyses but nevertheless illustrates the potential application of MS analyses in highlighting unique families of lipids. The potential of lipidomics in bio-discovery has recently been explored in macroalgae and may have a similar potential for studies on microalgae and the coral holobiont<sup>46</sup>.

Polar metabolites are typically involved in growth, development, and reproduction while non-polar metabolites are closely linked to changes in the environment or evolutionary adaptation. Interestingly, we found distinct metabolic profiles for both NMR (major compounds) and MS analyses emphasizing the intricate relationship between different members of the coral holobiont and a collective metabolic response related to the environment. Although this was expected for MS analyses, as different genera are known to produce a unique lipidome, this has not been well documented for NMR analyses. Most hydrophilic metabolites obtained from <sup>1</sup>H NMR analyses were amino acid derivatives such as polar betaines. These compounds are known to play a key role in regulating osmolytes and have been previously reported in several marine invertebrates but rarely in corals<sup>38,40,47,48</sup>. The role of polar betaines in corals is still poorly understood but are likely to be involved in osmoregulation similar to other marine invertebrates<sup>40</sup>. They are also thought to have a photoprotective role<sup>38</sup>. Importantly, different polar betaines were differentially present in different coral holobionts in our study. Alanine betaine, taurine betaine and choline were highly concentrated in *Porites* spp., while sarcosine and hydroxyproline betaine were highly concentrated in *Millepora* cf. *platyphylla* and glycine betaine was highly concentrated in *Pocillopora* spp. The pyridinium derivative homarine was identified as a major metabolite in *Porites* spp. but was absent in the other two genera. Trigonelline, an isomer of homarine, was also present at lower concentrations in *Porites* spp. but absent in the other two genera. Homarine has been shown to act as a deterrent in *Porites*<sup>37</sup>, while trigonelline has mainly been studied through its degradation pathway in corals<sup>49</sup>, and has only been reported in *Porites* spp.<sup>22</sup>. Other amino acid derived betaines, such as proline betaine (ProB), and glycine betaine (GlyB) which have been reported from various species of corals<sup>40</sup>, were also present in both our scleractinian coral genera. Studies on the metabolic and catabolic pathways of GlyB indicate that this metabolite is typically the primary osmolyte produced in some species of coral<sup>48</sup>. All betaines are known to play a key role in osmoregulation and since the composition of such compounds appear to be genus specific, it suggests that different modes of regulation are employed by different genera of corals. These differences may therefore account for the wide metabolomic variability observed among the studied corals.

Laboratory studies indicate that distinct metabolomic profiles are produced in response to environmentally induced stress for several coral holobionts<sup>9,11,13,26</sup>. This range of metabolic plasticity observed in the laboratory is likely a reflection of the adaptive capacity of coral holobionts to environmental gradients in nature. In our study, we assessed the metabolomic variability of three coral holobionts in two distinct marine provinces, which

differ primarily in average SST, among other environmental variables<sup>29</sup>. The cooler but less temperature stable (higher temperature range) Gambier harboured higher levels of genetic diversity particularly in the photosymbiont (different genera of Symbiodiniaceae) as well as for the scleractinian corals themselves. We believe that the higher diversity of endosymbiotic dinoflagellates is due to the selection of different species of Symbiodiniaceae in response to heat stress, which was most apparent in site 01. Except for one individual, all three coral holobionts in Samoa hosted *Cladocopium* while both *Symbiodinium* and *Cladocopium* were present in Gambier. The selection of species of *Cladocopium* only in individuals in Samoa could be due to the consistently warmer and stable temperature environment (higher average mean temperature but lower range in temperature) with fewer days of heat stress. The selection of heat tolerant Symbiodiniaceae following a bleaching event or due to heat stress has been shown before, but their influence on the overall metabolic profile of coral holobionts in nature is less well known. The lipidome studied here by LC-MS was largely influenced by the environment and was distinct in both marine provinces for all three coral holobionts. Although many of the same lipids were present at the different islands, some differed in concentration. An example of which is the family of sulfolipids identified in our study and was highly concentrated in individuals of *Millepora* cf. *platyphylla* in Gambier. We believe that different environmental conditions closely linked to temperature at the different islands had a significant effect on various metabolic rate processes which ultimately led to varying concentrations and types of lipids (storage vs structural lipids) being produced in the different islands. For *Millepora* cf. *platyphylla*, different genera of Symbiodiniaceae were present in Gambier (*Symbiodinium*) and Samoa (a single clade of *Cladocopium*) which may be responsible for the different metabolic profiles observed, particularly in the lipidome which may be linked to higher heat stress tolerance<sup>50</sup>. However, *Porites* spp. harboured a single genotype C15 of *Cladocopium* in Gambier and Samoa but also exhibited different metabolic profiles in different islands for both polar (NMR) and less polar metabolites (LC-MS). Furthermore, *Millepora* cf. *platyphylla* and *Porites* spp. containing symbionts of the same genus *Cladocopium* (C66 and C15 respectively) at Samoa exhibited different metabolic profiles at both sites (sites 02 and 03). This suggests that the endosymbiotic dinoflagellate alone cannot account for all metabolic differences but rather a combined effect of the coral holobiont and an interaction with the environment might be at play. It also suggests that different genera employ different strategies to cope with changes in the environment.

The key role of the photosymbionts in the functioning of the coral holobiont has been shown many times before but the differential production and concentration of metabolites in different environments analysed by <sup>1</sup>H NMR is less known. *Porites* spp. was the only coral holobiont in our study that hosted a single genus (*Cladocopium*) of endosymbiotic dinoflagellate. As mentioned earlier, many of the major compounds analysed by NMR have been shown to be of algal origin. Distinct metabolic profiles recovered for individuals of *Porites* spp. from different islands similarly indicated that metabolic profiles are not solely explained by differences in the endosymbiotic dinoflagellate but that the environment (plasticity of the endosymbiont or microbial contribution) also plays a key role. The suite of major metabolites (or analogues) produced in *Porites* spp. in different islands are likely driven by environmental differences closely linked to temperature and therefore key metabolic processes. Most polar metabolites in our study were polar betaines that are involved in osmoregulation and homeostasis. Metabolomic differences observed in *Porites* spp. within sites (similar average SST

temperature) however are probably more likely influenced by local environmental stresses other than temperature. This indicates a local adaptive capacity in *Porites* spp which is not observed for the other two coral holobionts. However it should be noted that the overlapping metabolomic profiles (polar metabolome) could also be due to co-occurrence of different genera of Symbiodiniaceae (*Cladocopium* or *Symbiodinium*) in the different islands and at different sites. For example, individuals of *Millepora* cf. *platyphylla* in Gambier (site 02) hosted *Symbiodinium* and in Samoa hosted *Cladocopium* which may have resulted in overlapping metabolomic profiles. This suggests that different genera of Symbiodiniaceae may produce similar polar metabolites which is probably associated with different physiological performance but that each genus also produces slightly different metabolic signatures in different environments. Our findings are consistent with a previous study where the same species of endosymbiotic dinoflagellate in a coral holobiont was shown to produce different metabolic profiles in different geographic locations<sup>51</sup>. Metabolic variability within a particular genus of the coral host is therefore primarily driven by the environment and rapid adaptation of the endosymbiont to new environments. Taken together, there is a high degree of plasticity of the metabolome of coral holobionts in different environments driven by the plasticity of the associated endosymbiont.

Although both scleractinian corals in our study were each characterised by different genotypes, distinct chemotypes were not recovered when the coral holobiont occupied the same or different islands. Metabolome variability among different genotypes of coral species collected from a single site has recently been demonstrated using LC-MS<sup>8</sup>, but has not been supported by later <sup>1</sup>H NMR analyses<sup>52</sup>. Interestingly, clustering analyses recovered two distinct groupings that were not consistent with the genotypes of the cnidarian host but could be due to differences in the environment and/or the endosymbiotic dinoflagellate. This was further supported by analyses of the hydrocoral *M.* cf. *platyphylla* which comprised a single species and exhibited different metabolic profiles in the two islands that were driven by either the presence of different Symbiodiniaceae (*Symbiodinium* in island 06 and *Cladocopium* in island 10) or the environment or both.

Similar patterns of metabolome variability related to geography have been shown for other marine organisms such as sponges<sup>53</sup>, and seaweeds using LC-MS<sup>54</sup>. It is well recognized that LC-MS analysis provides a broad overview of metabolites, of which a multitude of minor compounds probably originate from associated microorganisms and therefore patterns are understandably associated with the environment. However, our analyses based on NMR which assessed the major polar metabolites produced by the coral holobiont also showed a similar pattern of variation with the environment.

The metabolome variability of our studied coral holobionts corresponded to differences at the level of the genus followed by biogeographic differences. Major metabolites are often associated with specific and well-established metabolic pathways, and therefore it is reasonable to assume that an evolutionary or time-scaled effect on the production of metabolomes may be at play.

## Conclusions

Newly developed biphasic extraction method and combined metabolomic approach using LC-MS for the lipodome and <sup>1</sup>H NMR for the organic phase provided a broad overview of the metabolome diversity of natural populations of three coral holobionts in two marine provinces in the Pacific Ocean. The metabolome variability of our studied coral holobionts

corresponded to differences at the level of the genus followed by biogeographic differences. Major metabolites are often associated with specific and well-established metabolic pathways, and therefore it is reasonable to assume that an evolutionary or time-scaled effect on the production of metabolomes may be at play. Nevertheless, a collective response by different members of the coral holobiont and the influence of the environment are shown to be important. Our study provides essential foundational knowledge for coral metabolomics.

## Methods

**Chemicals.** Reference compounds (Ala, Lac, Val, Leu, Ile, taurine, formic acid, ProB, GlyB, TauB, trigonelline, choline, phosphocholine, glycerophosphocholine) for spiking experiments were purchased from Fluka (Sigma-Aldrich).

**Experimental design.** The sampling strategy was described elsewhere<sup>19</sup> and focused on sampling taxa from a wide distribution range across the Pacific Ocean. After collection by SCUBA, coral fragments were flash frozen on board using liquid nitrogen and preserved at  $-20^{\circ}\text{C}$  until subsequent analyses in the laboratory. For this study, 12 *Millepora* cf. *platyphylla*, 12 *Pocillopora* spp. and 12 *Porites* spp. fragments, collected from island 06 (Gambier archipelagos, September 2016), sites 01 ( $23^{\circ} 4' 29.280'' \text{S}$ ,  $135^{\circ} 1' 2.160'' \text{W}$ ) and 02 ( $23^{\circ} 9' 54.360'' \text{S}$ ,  $134^{\circ} 50' 52.727'' \text{W}$ ), and from island 10 (Samoa, December 2016), sites 02 ( $14^{\circ} 3' 41.207'' \text{S}$ ,  $171^{\circ} 25' 49.320'' \text{W}$ ) and 03 ( $13^{\circ} 55' 7.560'' \text{S}$ ,  $171^{\circ} 32' 29.640'' \text{W}$ ), were selected to develop the metabolomic approach described below (Table S1). Coral fragments were subsequently freeze-dried, ground into a powder, weighed and stored at  $-80^{\circ}\text{C}$  prior to the metabolomic analysis.

**Pre-analytical treatment.** Each sample was divided into three different subsamples of 5 g for independent treatment and constituted the technical replicates. The subsamples were first extracted with 12 mL of MTBE/MeOH/H<sub>2</sub>O (7:4:1) in an ultrasonic bath for 5 min. After the addition of 3 mL of water, the tubes were placed in the ultrasonic bath for an additional 5 min. Extracts were vortexed and centrifuged at 3500 rpm for 20 min at  $10^{\circ}\text{C}$ . After centrifugation, 2 layers were obtained and each was transferred into a tube: 1 mL of the upper organic layer was aliquoted for LC-MS analyses, and approximately 6 mL of the lower hydroalcoholic layer were used for <sup>1</sup>H NMR analyses. Solvents were removed under *vacuum* using a centrifugal evaporator and the extracts were weighed. To assess instrument variability, data quality control quality (QC) samples were prepared, one per genus, and one with a mixture of the three genera. After optimization, QC samples were prepared by mixing 83  $\mu\text{L}$  of a re-suspended replicate 2 of each colony for the 3 genera (3 QC / mix of 12 extracts), with 30  $\mu\text{L}$  of the re-suspended replicate 1 of all the 36 extracts from the 3 genera (1 QC / 36 extracts). Blank samples were prepared using the same procedure for biological samples, but without the coral holobiont fragment. All the extracts were stored at  $-80^{\circ}\text{C}$  until analyses. Samples used for method optimization were excluded from all statistical analyses.

**Sample preparation for LC-MS, analytical methods and data acquisition.** The organic extracts analysed by LC-MS were prepared by adding 1 volume of ACN/MeOH (50:50) according to weight to reach a final concentration of 1 mg/mL for all samples. The supernatant was filtered on a  $0.22 \mu\text{m}$  ( $\phi$  13 mm) filter and transferred to analytical vials. The data acquisition was divided into four analytical batches: one by genus, and one for the mix of all three genera. Samples were randomized, and then organized in order of injection: extraction blanks, priming QC, randomized samples, QC, and extraction blanks. One QC was injected after analysis of every 8 vials. Up to 6 QC samples, 3 blanks, and 2 solvent injections were used to equilibrate the analytical system, and therefore subsequently removed from the data prior to data analysis. Principal Component Analysis (PCA) was performed to assess the technical variability (measured by replicate analysis of a pooled intrastudy QC sample) and biological variability as part of a quality control process. All samples were analyzed using a Vanquish Liquid Chromatography system using a Kinetex<sup>®</sup> 1.7  $\mu\text{m}$  Phenyl-Hexyl 100 LC column  $100 \times 2.1 \text{ mm}$ , coupled with an electrospray Q Exactive Plus Orbitrap mass spectrometer with a HESI source. The gradient used for the chromatographic separation consisted of two solvents, solvent A (0.1% formic acid in LC-MS grade water) and solvent B (0.1% formic acid in acetonitrile LC-MS grade). The flow rate was set at 0.40 mL/min with the following gradient:  $t = 0.0, 35\% \text{ B}$ ;  $t = 2.0, 35\% \text{ B}$ ;  $t = 10.0, 100\% \text{ B}$ ;  $t = 13.0, 100\% \text{ B}$ ;  $t = 14.0, 35\% \text{ B}$ ;  $t = 17.0, 35\% \text{ B}$ ; all changes were linear. The column temperature was set at  $30^{\circ}\text{C}$  and the injection volume was 5  $\mu\text{L}$ . Data were analysed in positive and negative ionization mode within the mass range of 120–1500  $m/z$  at resolution 35,000. Ion source parameters were set as follows: sheath gas = 45, aux gas = 15, sweep gas = 0, spray voltage = 3.2 kV, capillary temp. =  $320^{\circ}\text{C}$ , aux gas heater temp. =  $250^{\circ}\text{C}$ .

The raw data obtained in positive and negative modes were converted into mzXML files with the Proteowizard v3.0 19202 software. Data processing with W4M was performed in 7 steps: identification of peaks for each sample, clustering of similar peaks across all samples, retention time correction, re-clustering of

similar peaks across samples, peak integration followed by annotation, removal of ions from blanks and background, statistical analysis and finally data visualization<sup>50</sup>. A step verifying the quality of the generated matrix was performed at the end of the treatment. Among these steps, some fundamental parameters are listed as examples for the negative ionization mode: the interval of  $m/z$  value for peak picking was set to 0.001, the signal to noise ratio threshold was set to 10, the group bandwidth was set to 5 and the minimum fraction was set to 0.02.

The matrices generated for both ionization modes were exported as text files for file format modification. MetaboAnalyst 5.0<sup>51</sup>, and RStudio were used for statistical analyses. A Shepard diagram and Principal Component Analysis (PCA) were performed with Vegan, rgl and RVAideMemoire packages. These two analyses allowed for the detection of outliers. A supervised PPLS-DA (Powered Partial Least Square-Discriminant Analysis, (pls, car, tidyverse, ggplot2, mixOmics packages) analysis was performed and led to the Variable of Importance (VIP). A VIP score greater than 1.5 indicated a significant contribution to the model. A permutation test and a double cross-validation test (2CV) were performed to validate the PPLS-DA model. The variables of interest with VIP scores greater than 1.5 from the metabolomics study were statistically processed with the RStudio v2021.09.2 software by a non-parametric Wilcoxon test (agricolae package) to compare their distribution between populations.

In order to obtain relevant annotations on the VIPs, the MS and MS/MS spectra of the VIP were compared with the following databases: DRUGBANK, PubChem and MoNA (MassBank of North America). In addition, all VIPs were submitted to a putative annotation research study on the Compound Discoverer 2.1 software (Thermo Fisher Scientific).

The molecular networks were generated in the positive and negative modes via the mzXML files, using the online workflow on the Global Natural Products Social molecular networking web-platform (GNPS)<sup>55</sup>. MS/MS spectra were window filtered by choosing only the top 6 peaks in the  $\pm 50$  Da window throughout the spectrum. For some analyses, data were clustered with MScluster with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. Edges between two nodes were included in the network if each of the nodes appeared in each other's respective top 10 most similar nodes. The library spectra were filtered in the same manner as the input data. All matches between network and library spectra were required to indicate a cosine score above 0.7 and at least six matched peaks. The generated similarity networks were compared with a set of databases proposed by GNPS.

mzXML files were directly processed using the MZmine 2.40.1<sup>56</sup>. The process was performed in both positive and negative ionisation modes. Chromatogram building was achieved using a minimum height of 2,000, and  $m/z$  tolerance of 0.005 Da (or 10 ppm). The local minimum search deconvolution algorithm was used with the following settings: S/N threshold = 10, minimum retention time range = 0.5 min, minimum ratio of coefficient/area threshold = 10, and peak duration range = 0.05 – 1.0 min. Chromatograms were de-isotoped using the isotopic peaks grouper algorithm with a  $m/z$  tolerance of 0.003 (or 10 ppm) and a RT tolerance of 0.2 min. Peak alignment was performed using the Joign aligner method ( $m/z$  tolerance at 0.005 or 10 ppm, absolute RT tolerance 1 min, absolute RT tolerance after correction of 0.5 min). The peak list was gap-filled with the peak finder module (intensity tolerance at 10%,  $m/z$  tolerance at 0.005 or 10 ppm, and absolute RT tolerance of 0.3 min). The library spectra were filtered with the same parameters as the input data. The generated data were compared to a set of databases proposed by Mzmine2 and MetGem 1.2.2 (LipidBlast, MASSBANK-EU, MetaboBASE, etc)<sup>57</sup>.

All spectra similarity networks generated with GNPS were imported into Cytoscape v3.7.2 for processing. For each mode (positive and negative ionization) all the metadata were merged to obtain a unique network of similarity. For each ionisation mode, all spectral similarity networks were cleaned when redundancy was observed. In such cases, each MS and MS/MS spectrum was compared among each other to validate that it corresponded to the same metabolite. Following this, the pie charts representing the proportion of each variable in the different samples were used to generate a consensus and an average value. The connections to the other nodes were represented by a mean cosine score derived from the two values obtained with GNPS.

**Sample preparation for NMR, analytical conditions and data acquisition.** NMR samples were prepared according to a published procedure<sup>58</sup>. Briefly, the frozen hydroalcoholic extracts were thawed at RT and data acquisition was performed immediately after NMR tube preparation. Factors that impact the spectra quality and comparability between samples include pH variation, differences in ionic strength and peak broadening due to the presence of paramagnetic and other metal cations. In order to reduce the chemical shift variation, the pH consistency was controlled by adjusting the sample using a  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  buffer. We screened ionic strength with three phosphate buffer concentrations (25, 50 and 100 mM) with pH 7.2–7.8, and presence (5–15 mM), or absence of EDTA. Optimal results were obtained using the following conditions: extract concentration = 15 mg/650  $\mu\text{L}$ , phosphate buffer concentration = 50 mM, no addition of EDTA, pH adjusted to 7.4 (data not shown).

The dried extracts were resuspended in 6 mL of  $\text{H}_2\text{O}$ . 1 mL of 50 mM phosphate buffer adjusted to pH 7.4 was added, and the solutions were then freeze-dried. The dried extracts containing phosphate buffer were resuspended in 1 mL of

$\text{D}_2\text{O}$  containing 3 % of 3-(trimethylsilyl)propionic-2,2,3,3- $d_4$  acid sodium salt (TMSP), vortexed and then centrifuged at 14,000 rpm for 20 min. A volume of 700  $\mu\text{L}$  of the supernatant, the particulate free extract, was transferred into a 5 mm Norell 500-7 NMR tube and analyzed.

$^1\text{H}$  NMR spectra were acquired for all samples using a 500 MHz ECZR JEOL (Tokyo, Japan) spectrometer equipped with a double resonance 5 mm multinuclear FG/RO Digital Autotune Probe S including a z axis gradient coil, and an auto sample changer 30 used at room temperature. Automation routines were carried using jaf scripts available upon request to A. Botana (JEOL UK).  $^1\text{H}$  NMR spectra were obtained using the Delta 5.3 software. Prior to measurement, samples were stored for 5 min inside the probehead for temperature equilibration at 25 °C. Automatic tuning and matching of the probe were carried out for each sample. A  $^1\text{H}$  NMR spectrum was acquired with water peak suppression using a standard proton single pulse experiment consisting of 2 dummy scans and 32 scans, for 65,536 data points, spectral width of 15 ppm, an acquisition time of 8.72 s, a relaxation delay of 10.64 s.

Spectral processing and post-processing steps: FID was multiplied by an exponential function equivalent to a 57 mHz line-broadening factor before applying a Fourier transformation. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated to the reference signal of TMSP at 0.00 ppm. Each spectrum in the region 10.00 – 0.2 ppm was segmented into intelligent bins, and the corresponding spectra areas were integrated using NMRProcFlow software. Statistical analysis was performed with MetaboAnalyst. Signals corresponding to the solvents were suppressed and a CSN normalization together with Pareto Scaling were used.

The annotation of metabolites in the hydroalcoholic extracts was performed by comparison of the major signals with those of the reference compounds (alanine, valine, leucine, isoleucine, threonine, glycine, lactate, formate, acetate, acrylic acid, trigonelline, glycine betaine, proline betaine, choline, phosphocholine, glycerophosphocholine, glucose, glycerol) assumed to be present (diagnostic peaks) or known to occur in reef-building corals. Betaine standards that were not commercially available (alanine betaine, hydroxyproline betaine, taurine betaine) were prepared following procedures already described<sup>41</sup>. The annotation was confirmed by spiking these reference compounds into one sample per genus. 2D NMR experiments (COSY, TOCSY, HSQC and HMBC) were used to characterize homarine. Sarcosine (N-methylglycine) was characterized by comparison with data bases and the Chemox identification software.

**Reporting summary.** Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## Data availability

The LC-MS and NMR raw data are stored in the platform Metabolights under the reference MTBLS7951.

The molecular networks were built on the platform GNPS and the corresponding link can be found here:

Millepora pos: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=6dede5cf7154454caec65460cd49925> Pocillopora pos: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=245c071ae17b4b9c9c1776538e7a5a6e> Porites pos: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7cd3f7743a7542fcb0ba2d9accf4a13> Millepora neg: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=3715a73f8c3e421094b14c5104e083dc> Pocillopora neg: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=712c15dc69d149b19b80e0cd0a3c74b7> Porites neg: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=68cca8c5d372401c9b09f64255be9d69>.

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### Author contributions

Members of the Tara Pacific Consortium, including D.F., S.A., Emi.B., Emm.B., Ch.B., C.d.V., E.D., M.F., P.F., P.E.G., E.G., F.L., St. P., Sa.R., M.B.S., S.S., R.T., R.V.T., P.W., D.Z., D.A., C.R.V., and Se.P., conceived of experiments and planned the sampling expedition. BB, OPT designed the study with input from FN and DR. BB and OPT directed the project. YZ prepared the samples. BB, DR and CG generated the data. MMR, CG, SC, BB and OPT analysed the data. MMR, BB and OPT wrote a draft of the article. MMR and OPT finalised the writing of the article. We would like to thank D.F., B.C.C.H and C.R.V. for providing the data and fruitful discussions about the genotypes of both the host and the endosymbiont.

### Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Additional information

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