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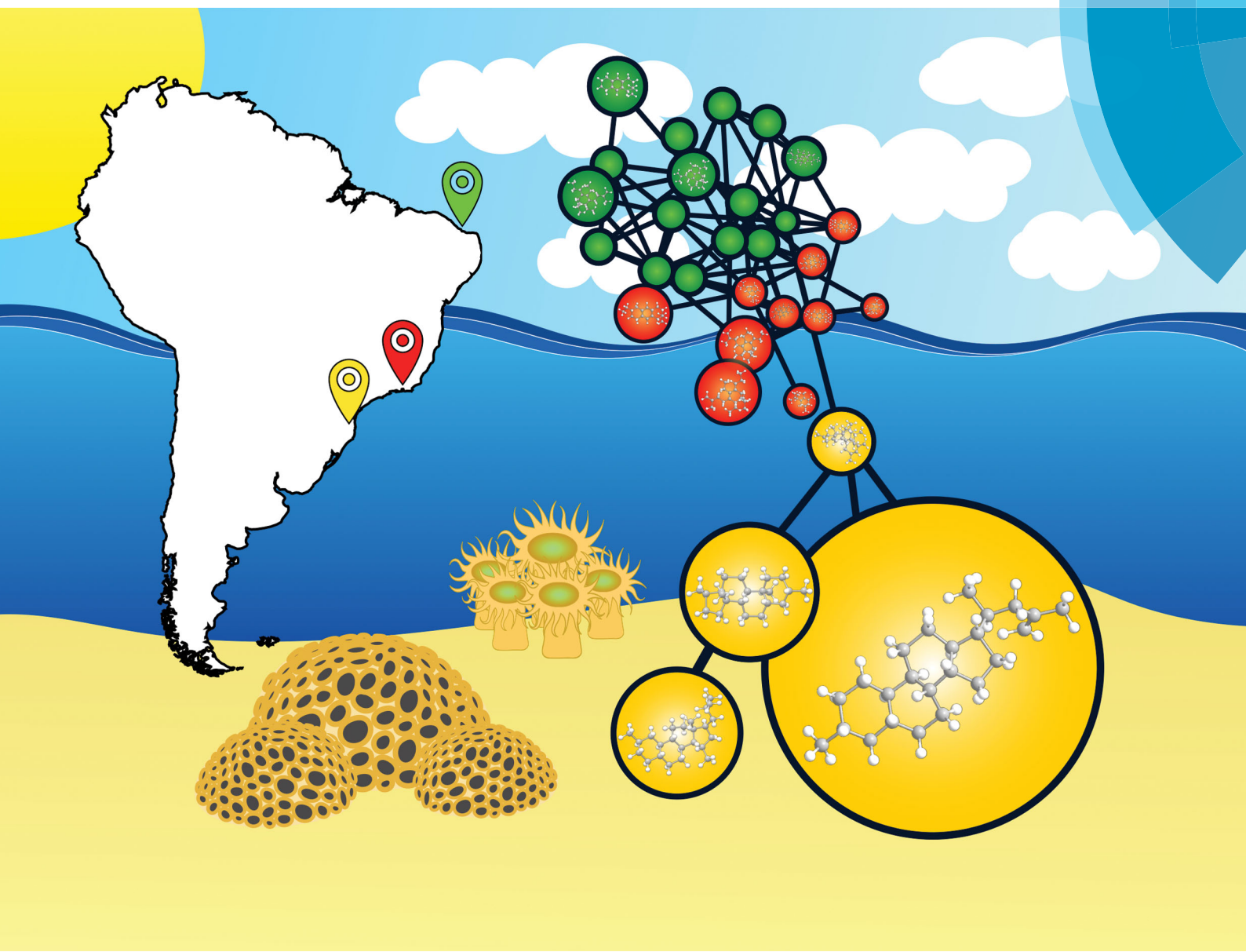

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Chemical profiling of two congeneric sea mat corals along the Brazilian coast: adaptive and functional patterns†

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Metabolomic profiles were explored to understand environmental and taxonomic influences on the metabolism of two congeneric zoanthids, *Palythoa caribaeorum* and *P. variabilis*, collected across distinct geographical ranges. Integrated mass spectrometry data suggested the major influence of geographical location on chemical divergence when compared to species differentiation.

Coral reefs and other sensitive marine ecosystems are under major threats related to human activities. In this scenario, it is of utmost relevance to understand reef organisms in a broader context. Over the past few decades, a new paradigm has emerged regarding the association of macro and microorganisms within holobionts. Nevertheless, understanding the complex patterns of interactions in holobionts remains challenging. The adaptive implications of these close relationships are pervasive in nature in diverse organisms, including plants and humans.[1,2] Although there are species-specific associations and regulation of symbiosis may be intimately connected to health and disease states, many of the functional aspects involved in such ties remain unclear.[3,4] Therefore, exploring omics patterns offers a crucial strategy for building a comprehensive record of an organism's chemical response emerging from different biochemical and environmental drivers.[5–7]

To further unravel the complex chemical variability among holobionts and their response to environmental elements, this work compared the metabolic profile of the two zoanthid species, *Palythoa caribaeorum* and *P. variabilis* (Fig. 1A), across distinct geographic ranges in the Brazilian coast. *Palythoa* species have been extensively studied since Moore & Scheuer described the occurrence of palytoxin in *P. toxica*.[8] Healthy specimens were collected from five locations (Fig. 1B), considering spatial scales from regional (tens of kilometers) to continental distances (thousands of kilometers). Paracuru and Taíba, northern locations in the state of Ceará (CE), are typically equatorial with consistently warm waters and sandstone substrates. Búzios and Arraial do Cabo, in the state of Rio de Janeiro (RJ), are tropical locations under the influence of upwelling, with colder waters. Arvoredo Island, in the state of Santa Catarina (SC), is near the southern distribution limit for both *Palythoa* species and represents an environmental extreme for these sea mats.

Due to the limited chemical coverage of each analytical method,[9] the full-range metabolomic fingerprinting of 27 zoanthid extracts was obtained through GC-MS, LC-DAD-IT, LC-DAD-TOF and MALDI-TOF/TOF, providing a comprehensive panel of coral's chemical composition (Fig. 1C). The multisource metabolome data were individually evaluated by multivariate analyses (Fig. S1, S2, S11 and S12, ESI†), and then merged using data fusion (Fig. 1D, ESI methods†). Notably, data fusion improved class separation compared with individual datasets.

The score plot suggested the influence of geographical location on chemical divergence (Fig. 1D). Additionally, BIOENV analysis using metabolic and environmental variables that drive marine species' distribution, as suggested in the Bio-ORACLE global dataset,[10] showed a strong correlation (coefficient of correlation (r^2) = 0.7464, $p < 0.001$) between nitrate, cloud cover, chlorophyll a and calcite variation with the metabolomic profile, emphasizing the role of environmental parameters in metabolomic expression (Fig. 1E).

The GC-MS analysis showed predominance of fatty acids and cholesterol analogues (Table S4, ESI†). Identification of the polar metabolome, assisted by MS/MS-based fragmentation

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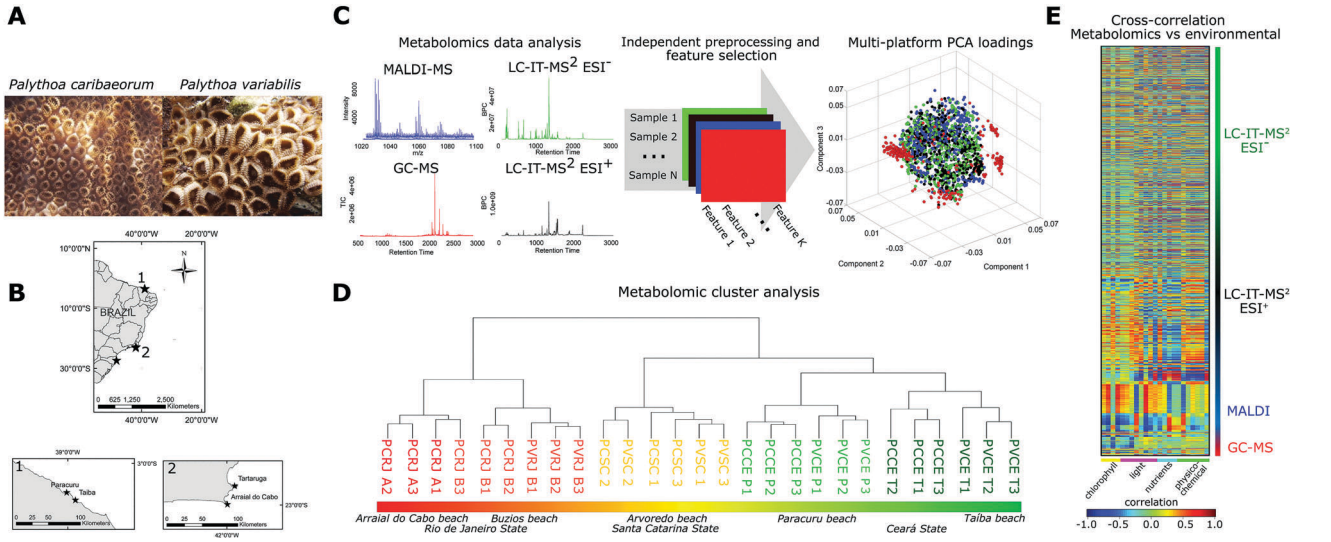

Fig. 1 Zoanthid sampling and metabolomics. (A) Images of zoanthids *Palythoa caribaeorum* and *Palythoa variabilis* (B) sampling sites. (C) Metabolomics analysis workflow showing representative metabolic profiles obtained from the distinct analytical methods and the multisource PCA loadings containing variables from GC-MS (red dots), MALDI-TOF MS (blue dots), ESI positive LC-MS (black dots) and ESI negative LC-MS (green dots). (D) Unsupervised hierarchical clusters of the multisource augmented data matrix from *Palythoa* species. Colour in the PLS score plot indicates the sampling location as Rio de Janeiro (red), Ceará (green) or Santa Catarina (yellow). (E) Correlations of individual ions from metabolomic profiles with environmental variables obtained from the BIO-ORACLE database. Environmental variables were grouped according to their biotic and abiotic nature.

patterns and Global Molecular Networking analysis (GNPS),[11] revealed the presence of mycosporine and related amino acid derivatives (**1–7**), zoanthid alkaloids (**8–11**), ecdysteroids (**12–26**), phosphatidylcholine (PC) derivatives (**27–37**), indole diterpenes (**38–39**) and sulphonoceramides (**40–41**), as outlined in Fig. 2, Fig. S4–S10 and Table S5 (ESI†). The metabolic identification was supported by the isolation of major metabolites and validation by a combination of ^{1}H NMR, ^{13}C NMR, UV and HRMS analyses (Fig. S13–S18, ESI†). Overall, the geographic grouping was mainly explained with lipids (fatty acids and steroids) detected by GC-MS, phosphatidylcholine detected by MALDI-TOF-MS, along with phosphatidylcholine derivatives and polyhydroxy steroids detected by LC-MS analysis (Fig. S11 and S12, ESI†). The influence of associated microbial communities on metabolite patterns of the holobiont was revealed by the detection of bacterial compounds **1–7**, **38** and **39** (Table S5, ESI†). In the soft coral holobiont *Sinularia capillosa*, sterols, terpenes and *N*-containing compounds grouped specimens were collected from different locations.[12] Thus, the use of metabolomic profiling as a complementary tool for the taxonomy of zoanthids as proposed by Cachet *et al.*[13] should be carefully considered. Recently, Torda *et al.*[14] discussed the mechanisms that support the capacity of reef-building corals to acclimatize and adapt to climate change under different environmental conditions, such as seawater temperature. Transgenerational plasticity of holobionts is pondered as an adaptive benefit in coral responses to climate changes, however the authors make a strong point to improve understanding of the underlying processes and propose strategies to discriminate other forms of plasticity. We collected two species inhabiting different water temperatures over the course of several years, allowing a detailed analysis of the major metabolites. The detection of polar metabolites by HPLC-IT-MS yielded several notable observations in metabolite distribution. Mycosporine and other amino acid derivatives, detected RT between 2 and 5 min, were observed in higher proportion and chemical variability in samples from the RJ state. Metabolites such as methyl-palythine and palythene were not detected in samples from CE. Aromatic amino acids of mycosporine-type strongly indicate microalgae association in marine environments with intense sunlight.[15] Zoanthamine alkaloids increased in relative proportion from SC, CE and RJ, respectively. Interestingly, ion *m*/*z* 510 (zoanthenamine) had a higher intensity in samples from CE and lower values in samples from RJ and SC, while ion *m*/*z* 524 (zoanthamide) showed an opposite influence, with higher peaks in SC, trailed by RJ and CE. Based on their structures, zoanthamide and zoanthenamine differ upon epoxidation and γ-lactone cyclization. Indole diterpene derivatives were observed in all samples and distinct distribution depending on the isomer. This is the first time these indole diterpenes are found in *Palythoa*, likely biosynthesized by *Ascomycota* fungus, previously isolated from microbial communities associated with *Palythoa* sp.[16,17] Terpendole E and G were found in higher abundance in samples from RJ, whereas samples from SC showed lower relative amounts. Isomers related to these compounds that were not fully characterized displayed the opposite distribution. This disparity could be related to variations in indole terpene biosynthesis, as these metabolites are of microbial origin. PC derivatives detected by LC-MS showed similar distribution to the MALDI-TOF-MS analysis, with a higher phosphatidylcholine content in *Palythoa* species collected from the SC state (Table S6, ESI†).

Ecdysteroids showed a major influence (higher relative proportions) on samples from CE and SC, with higher intensities observed in Taíba beach, when compared to Paracuru beach. Ecdysteroid showed similar distribution when compared to

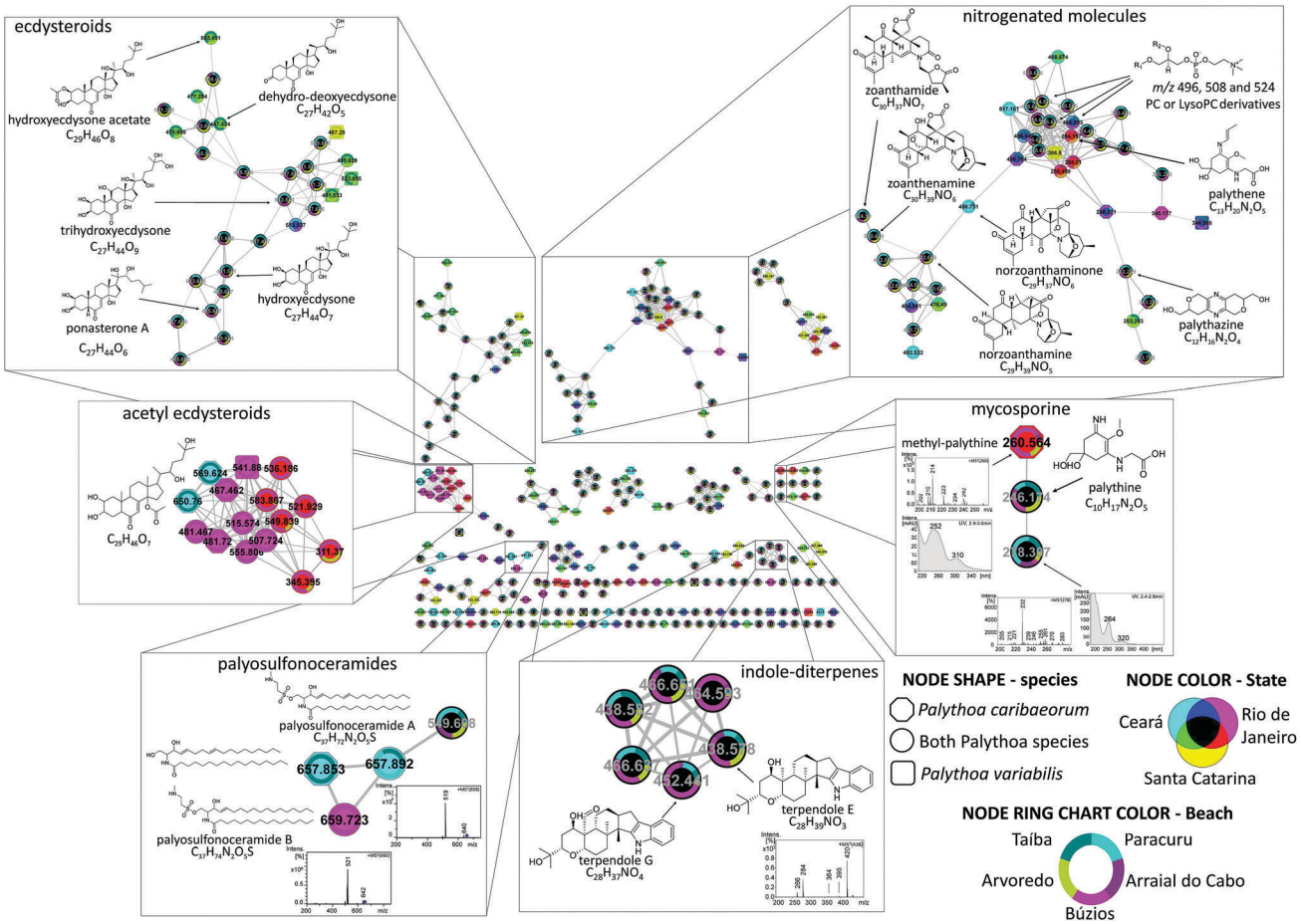

Fig. 2 Palythoa holobiont molecular networks. Related precursor ions were networked based on MS/MS fragmentation pattern similarities. The node central color reflects the Brazilian state where the samples were collected, node ring charts show the proportion of spectra for each locality, and node shape indicates the distribution of the parent ion among the two species.

GC-MS steroids variation (with a higher influence on CE and SC samples), however the intensities were opposite in samples from Taíba and Paracuru. Since the LC-MS showed polar steroids, the results suggest more intense steroid oxidation in samples from Taíba beach, compared with Paracuru beach (CE). Some ecdysteroid derivatives, with changes in the oxidation pattern, were not observed in samples from RJ (ions *m*/*z* 447, 477, 479, 503, 495, and 523). Nevertheless, both positional isomers of hydroxyecdysone (*m*/*z* 481) showed an opposite distribution among zoanthid samples, with higher intensities on *P. caribaeorum* collected on Arraial do Cabo beach, RJ. Molecular networks also clustered acetyl ecdysteroids, mainly from RJ samples. Although the chemical structures could not be completely elucidated, the fragmentation pattern was similar to that previously described for this class of metabolites, in conjunction with the common neutral loss of 60*u*, consistent with the loss of CH_2OH from an acetyl substituent.[13] Although the environment played a major role in phenotype manifestation, metabolic profiling also revealed minor chemical divergences between the zoanthids. *P. caribaeorum* from Búzios was characterized by higher abundance of fatty acids and phosphatidylcholine derivatives, while *P. variabilis* was characterized by a higher content of amide fatty acids and mycosporine derivatives. In samples from CE, *P. caribaeorum* also showed higher amounts of phosphatidylcholine derivatives, whereas *P. variabilis* presented more types of ecdysteroids.

We have also performed metagenomics on representative zoanthid specimens, including a total of nine samples and >621 Mb of high quality sequence data. As previously observed in soft corals,[16,17] approximately 14% of the data yield taxonomic signatures including hits associated with Cnidaria (30%) and Proteobacteria (55%) prevailing in eukaryotic and prokaryotic communities, respectively (Table S7, Fig. S19 and S20, ESI†). *De novo* cross-assembling and grouping of metagenome-shared entities (cross-contigs) using 'SHOT', 'minimum', 'Wootters' and 'reads' distance matrix methods recovered highly similar cladogram topologies, with samples grouping into species-specific clades (Fig. S19A, ESI†). According to the results, *P. variabilis* hosts more alphaproteobacteria and deltaproteobacteria, whereas gammaproteobacteria preferentially colonized *P. caribaeorum* (Fig. S19B–S20D, ESI†). These findings are consistent with recent reports on marine holobionts, in which the dynamic relationship between corals and their associated microbial communities has been more important than geographical conditions, suggesting a 'species-specialized' hologenome.[6,18–20]

From a macro perspective, environmental changes do not clearly influence the hologenome, as it is less important than interspecific divergence. However, the opposite effect can be assumed for the metabolome. Conservation of metagenome, despite geographical factors, may be ascribed to the establishment and maintenance of relationships with beneficial keystone microbes, possibly through gene transfer and chemical defence mechanisms. In contrast, variation in the metabolic profile as an adaptation to rapidly changing environmental stimuli reflects the actual condition of the reef-coral. Although *Palythoa* exhibited considerable metabolic structural diversity, most of the chemical variations were a consequence of skeleton specialization, and such metabolic diversification primarily results in biosynthetic functionalization of consistent classes of compounds. This hypothesis has recently been described and suggests stony coral holobionts modifying the same molecules in different ways to generate molecular diversity.[21] These findings led us to infer that the modifications of precursor chemical structures may constitute a key driving force of metabolite variability and adaptive competence. Considering this a likely positive correlation, distribution of polyhydroxylated ecdysteroids, the major secondary class and biomarker of several cnidarian holobionts,[13] was analyzed in terms of the oxidation pattern and geography. Ecdysteroids were found in all regions, but in higher abundance and greater structural variability in samples from CE, explaining the separation of this area in the partial least squares (PLS) plot (Fig. S21, ESI†). The PLS plot showed ecdysteroids with higher oxidation states ($C_{27}H_{45}O_8$ and $C_{29}H_{47}O_8$) in *P. variabilis* and *P. caribaeorum* samples from Taíba beach, whereas *P. variabilis* from Paracuru beach showed a higher content of ecdysteroids with lower oxidation states ($C_{27}H_{41}O_4$, $C_{27}H_{41}O_5$ and $C_{27}H_{43}O_5$); however, these groups of ecdysteroids were still more similar to each other than to all other groups. Environmental influences on steroid functionalization prompted questions regarding physiological function and endogenous metabolism. As cnidarians have not been shown to synthesize sterols *de novo*, these compounds are either derived from dietary sources or synthesized by associated microbes. Still, these organisms have steroid metabolizing enzymes that catalyse the reversible oxidation or reduction of 17-ketosteroids.[22] The microbiome selection also appears to be relevant to the evolution of important biochemical and physiological systems in corals.[23]

These findings suggest a strong influence of environmental factors on the metabolic variability of holobionts. Apparently, there is a consistent species-specific relationship amongst host–microbiota; nevertheless, these patterns do not translate into chemical correspondences across distinct spatial ranges.

Conflicts of interest

There are no conflicts to declare.

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