

Opposing host–symbiont transcriptional responses associated with menthol-induced destabilization of coral symbiosis

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Abstract

Experimental induction of coral bleaching is widely used to investigate the molecular and physiological mechanisms underlying coral–algal symbiosis breakdown. However, a major challenge is achieving controlled destabilization of coral–algal symbiosis without simultaneously inducing widespread systemic damage in the coral holobiont. For example, thermal bleaching models often trigger extensive secondary stress responses that can obscure molecular processes specifically associated with early symbiosis destabilization. Chemical bleaching methods such as menthol exposure may provide a more controlled framework for studying symbiosis destabilization, yet their transcriptomic consequences remain poorly characterized. Here, we used dual RNA-seq to investigate the molecular responses of the coral host *Stylophora pistillata* and its dominant symbiont *Symbiodinium microadriaticum* following menthol-induced bleaching. Transcriptomic analyses were integrated with KEGG functional profiling and oxygen respirometry measurements to characterize the molecular and physiological consequences of controlled symbiosis disruption. Notably, functional enrichment analyses revealed opposing directional shifts between host and symbiont, indicating marked functional decoupling following menthol exposure. The host exhibited extensive transcriptional remodeling, including enrichment of pathways associated with proteostasis, protein turnover, catabolism, and cellular remodeling, whereas the symbiont showed a comparatively limited transcriptional response with substantially fewer differentially expressed genes and weaker functional enrichment patterns. Despite extensive transcriptomic changes, oxygen respirometry measurements remained relatively stable between control and menthol-treated nubbins, indicating preserved short-term metabolic functionality. Consequently, menthol bleaching may provide a useful experimental framework for investigating host-associated molecular mechanisms underlying early coral–algal symbiosis destabilization, while potentially being less suitable for studying symbiont-specific processes involved in natural bleaching responses.

Introduction

Coral reefs are sustained by the mutualistic endosymbiosis between scleractinian corals and photosynthetic dinoflagellates of the family Symbiodiniaceae. This association supports reef productivity in oligotrophic marine environments through the exchange of nutrients and photosynthetically derived carbon between host and symbiont (Davy et al. 2012; LaJeunesse et al. 2018). Disruption of this symbiosis, commonly referred to as coral bleaching, results in the loss, degradation, or dysfunction of symbiotic algae and represents one of the major drivers of coral reef decline under climate change (Hughes et al. 2018; Grottoli et al. 2025). Consequently, understanding the cellular and molecular mechanisms underlying coral–algal symbiosis destabilization has become a central challenge in coral biology (Weis 2008; Helgoe et al. 2024). In this context, the ability to generate aposymbiotic colonies reliably and reproducibly is a fundamental prerequisite for comparative studies of symbiotic and non-symbiotic physiological states, and for investigating the molecular events associated with symbiosis onset, maintenance, and collapse (Wang et al. 2012; Baumgarten et al. 2015; Matthews et al. 2016). Nevertheless, achieving controlled destabilization of coral–algal symbiosis without

simultaneously inducing widespread systemic damage remains a major challenge in experimental coral bleaching research.

In this context, most experimental coral bleaching studies rely on thermal stress to induce symbiosis breakdown. Although thermal bleaching has provided fundamental insights into oxidative stress, photoinhibition, and host–symbiont interactions, the thermal tolerance thresholds of reef-building corals are often extremely narrow and remain difficult to predict a priori, as they are highly context-dependent and can vary among species, geographic locations, acclimatization histories, and experimental conditions. (Hughes et al. 2017; Savary et al. 2021; Denis et al. 2024; Bubb et al. 2026). Consequently, even relatively small increases in temperature or exposure time may rapidly shift coral responses from symbiosis destabilization to widespread physiological impairment. Such responses include oxidative imbalance, mitochondrial dysfunction, apoptosis, and tissue necrosis, complicating the identification of molecular processes specifically associated with destabilization of the symbiotic state rather than generalized cellular stress responses (Desalvo et al. 2010; Rådecker et al. 2021; Savary et al. 2021). Moreover, considerable variation exists among thermal bleaching protocols used across studies, and sublethal thresholds capable of consistently inducing bleaching without severe host impairment remain poorly defined (Grottoli et al. 2021; Bubb et al. 2026). Consequently, many experimental studies may reflect late-stage stress responses rather than the early molecular mechanisms governing symbiosis destabilization.

Chemical bleaching approaches offer an alternative strategy for investigating coral endosymbiosis destabilization under more controlled and potentially less destructive conditions. Several chemical agents have been employed for this purpose, including the photosystem II inhibitor diuron (DCMU), the herbicide glyphosate, and the monoterpene menthol (Wang et al. 2012; Scharfenstein et al. 2022; Zhou et al. 2022). Among these, menthol has emerged as a particularly reproducible bleaching agent capable of efficiently removing Symbiodiniaceae from coral tissues while maintaining relatively stable host physiological performance and without inducing extensive tissue degeneration (Wang et al. 2012; Matthews et al. 2016). Despite its increasing use as an experimental bleaching method, the transcriptomic consequences of menthol exposure remain largely unexplored in reef-building corals. In particular, the molecular processes associated with menthol-induced destabilization of coral–algal symbiosis remain poorly understood. Accordingly, elucidating how coral hosts and their algal symbionts respond to menthol exposure may help disentangle the early molecular events underlying symbiosis disruption under controlled experimental conditions.

To investigate the molecular consequences of menthol-induced bleaching, we employed the *Stylophora pistillata*–*Symbiodinium microadriaticum* holobiont as an experimental model system. *S. pistillata* has been extensively used as a model organism in coral biology and represents one of the most studied reef-building coral species in stress response experiments (Meziere et al. 2022). In addition, the availability of a sequenced and annotated reference genome for *S. pistillata* (Voolstra et al. 2017), combined with the published genome of its dominant symbiont *S. microadriaticum* (Aranda et al. 2016), makes this holobiont particularly well-suited for dual transcriptomic investigations. Together, these genomic and

transcriptomic resources position the *S. pistillata*–*S. microadriaticum* holobiont as a well-characterized experimental system for investigating the molecular basis of coral endosymbiosis.

Using this experimental framework, we integrated dual RNA-seq, KEGG functional profiling, superpathway-level transcriptomic organization, and oxygen respirometry measurements to characterize the molecular and physiological consequences of menthol-induced destabilization of coral endosymbiosis and to compare the responses of the coral host and its algal symbiont.

Materials and Methods

The experiment described below was conducted within the curatorial facilities of the Tropical Department of the Aquarium of Genoa (Genoa, Liguria, Italy).

Molecular Identification of Donor Colonies and Symbionts

A total of six adult colonies of *Stylophora pistillata* maintained in six different indoor tanks (3 × 1 × 0.7 m; 2100 L) were used as donor colonies for all subsequent analyses. Molecular identification of both the donor colonies and the associated dominant symbiont was performed using small coral fragments (~ 2 cm in length) excised from each donor colony (n = 6) and immediately preserved in molecular-grade ethanol. Total genomic DNA was extracted from ethanol-fixed tissues using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

For *S. pistillata* identification, the mitochondrial cytochrome c oxidase subunit I (COI) region was amplified using primers LC01490 and HCO2198 (Folmer et al. 1994). PCR reactions were carried out in 25 µL volumes containing Phusion High-Fidelity PCR Master Mix 2X (Thermo Fisher Scientific), primers at a final concentration of 10 µM, and 2.5 µL of genomic DNA. Thermal cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 46°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 7 min (Folmer et al. 1994).

For *Symbiodinium* identification, the ITS2 region was amplified using primers SYM_VAR_5.8S2 and SYM_VAR_REV (Hume et al. 2018) under similar reaction conditions. Thermal cycling consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 10 s, 56°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min (Hume et al. 2018).

PCR products were verified by electrophoresis on 1.5% agarose gels and sequenced by Sanger sequencing at BMR Genomics (Padua, Italy). Raw chromatograms were inspected and edited using FinchTV (v1.4.0), aligned in MEGA11 (Tamura et al. 2021), and taxonomically assigned using MEGABLAST searches against the NCBI database.

Nubbins Preparation and Experimental Design

Coral nubbins were generated as previously described (Isa et al. 2026). Briefly, eighteen *S. pistillata* nubbins (~ 7 cm in length) were obtained by fragmenting the aforementioned six donor colonies (three nubbins per colony; n = 18). Each nubbin was affixed to a 12 mm screw anchor using a two-part epoxy adhesive resin (Veneziani, Italy; Isa et al., 2024) and transferred to an indoor recirculating tank system for a four-week recovery period. The tank system consisted of a 2100 L tank connected to a recirculating aquaculture system (RAS) and continuously supplied with seawater through a pump (AstralPool Victoria Plus) operating at 10 m³ h⁻¹, ensuring complete water turnover approximately every 30 min. Water was recirculated into the tank after passing through a filtration system. The filtration system consisted of a sand filter (Astralpool Artic), filled with coral sand as filtration medium, coupled with a UV sterilization unit capable of filtering particles ranging from 0.4 to 2 mm, and a UV filter (Panaque 750 S AB equipped with four 40 W lamps). Nubbins were fed daily according to the internal husbandry protocol of the Aquarium of Genoa. Specifically, 2 L of a suspension composed of the marine algae *Tetraselmis suecica* (3 × 10⁶ cells mL⁻¹), mixed with cultured rotifers *Brachionus plicatilis* at a density of approximately 600 ind mL⁻¹ (Martino et al. 2023), were directly administered to the recovery tank.

Water parameters of the tank were maintained at 25 ± 0.5°C, 35 ± 1 PSU salinity, and ~ 200 µmol photons m⁻² s⁻¹ under a 12 h:12 h light–dark photoperiod.

Following the recovery period, nubbins were randomly assigned to two experimental groups: (i) 8 nubbins were subjected to menthol-induced bleaching, whereas (ii) 8 additional nubbins were maintained under control conditions. The remaining two nubbins were retained as backup specimens and were not included in downstream analyses.

Menthol-Induced Bleaching Procedure

Menthol bleaching was achieved following established protocols for corals (Wang et al. 2012; Matthews et al. 2016). Briefly, menthol (M2772, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile seawater (UV-sterilized, sand-filtered, and boiled for 40 min) to a final concentration of 0.38 mM. The eight nubbins assigned to the bleaching treatment were incubated for 8 h in 10L transparent polycarbonate containers (Ferplast; 35 × 20 × 23 cm) filled with menthol-enriched seawater, followed by a 16 h recovery period in fresh menthol-free sterile seawater. This cycle was repeated daily for three consecutive days to induce the loss of more than 95% of resident algal symbionts (Isa et al. 2026). Water quality parameters in the 10 L Ferplast, including salinity, pH, ammonium, and nitrite, were monitored daily throughout the experiment. Water chemistry remained stable, with salinity ranging between 35.5 ± 1 PSU, pH ranging from 8.0 ± 0.1, and ammonia and nitrite concentrations remaining below 0.01 mg L⁻¹.

Following the bleaching treatment, small coral fragments (~ 3 cm) five nubbins per treatment (bleached and control; total n = 10) were immediately preserved in RNAlater solution (Thermo Fisher Scientific) for subsequent RNA-seq analysis. Samples were maintained at 4°C for 24 h to facilitate tissue penetration and subsequently stored at – 80°C until RNA extraction (Toniolo et al. 2026).

Oxygen Respirometry Measurements

Following the three-day menthol exposure, the three nubbins per treatment that were not sampled for RNA-seq analyses were used for oxygen respirometry measurements to assess net photosynthesis (Pn) and dark respiration (R_Dark).

Measurements were performed using a FireStingO₂ oxygen respirometry system (PyroScience, Germany) equipped with optical oxygen sensor spots (optodes) and a temperature probe. Sealed 330 mL glass incubation chambers filled with sterile seawater (UV-sterilized, sand-filtered, and boiled for 40 min) were partially submerged in a temperature-controlled water bath to minimize external thermal fluctuations. Prior to each measurement, incubation chambers were maintained in the water bath for approximately 25 min to equilibrate internal and external temperatures. Following thermal equilibration, individual nubbins were introduced into the chambers and subjected to a light–dark–light incubation sequence consisting of three consecutive 15 min phases. Incubation times were selected based on preliminary assays conducted prior to the experiment, which indicated rapid and reproducible oxygen responses while minimizing prolonged confinement stress. Illumination during the light phases was provided by an LED lamp (~ 20–30 W) corresponding to moderate reef-lighting conditions, whereas darkness during the dark phase was achieved by covering chambers with black plastic bags.

Dissolved oxygen concentration ($\mu\text{mol L}^{-1}$) and temperature ($^{\circ}\text{C}$) were recorded at 1 s intervals using FireStingO₂ software. Net photosynthesis and dark respiration were estimated as changes in dissolved oxygen concentration measured between the beginning and the end of each incubation phase. Dissolved oxygen fluxes were normalized to the total surface area of each nubbin. Coral surface area was estimated using a cylindrical approximation based on height and diameter measurements, including branch surfaces when present (Table 1; Isa et al., 2026). Blank incubations containing sterile seawater only were performed under identical conditions to monitor background oxygen fluctuations unrelated to coral–algal metabolism.

Differences in surface-normalized oxygen fluxes between healthy and menthol-bleached corals were assessed using non-parametric Wilcoxon rank-sum tests (Mann–Whitney tests). Statistical analyses were performed in RStudio (R v4.4.2).

RNA Extraction, Library Preparation, and Sequencing

For transcriptomic analyses, five nubbins per treatment (menthol-bleached and control) were sampled following the three-day experimental period. Coral tissues were removed using filtered compressed air connected to a sterile 1000 μL pipette tip (Savary et al. 2021) and subsequently mechanically homogenized using 1mm beads and the TissueLyser II instrument (Qiagen, Hilden, Germany). Homogenization consisted of two disruption cycles at 30 Hz for 30 s separated by a 1 min incubation on ice to minimize heat-induced RNA degradation and preserve RNA integrity. Total RNA was extracted using the NucleoSpin miRNA kit (Macherey-Nagel) following the manufacturer's protocol. RNA samples

were shipped on dry ice to BMK (Germany) for library preparation using the Illumina TruSeq Stranded mRNA kit. Libraries were multiplexed and sequenced on an Illumina NovaSeq6000 platform to generate 150 bp paired-end reads, targeting approximately 40 million paired-end reads (~ 6 Gb) per sample.

Read Processing, Mapping, and Count Matrix Generation

Raw sequencing reads were quality-checked using FastQC (v0.11.9; Andrews, 2010), and summary reports were generated using MultiQC (v1.27.1; Ewels et al., 2016). These analyses revealed the absence of significant adapter contamination or quality deterioration; therefore, adapter trimming was not performed, as previous studies have shown that aggressive trimming may introduce biases in RNA-seq quantification and affect transcript abundance estimates (Williams et al. 2016; Liao and Shi 2020; Mohamed et al. 2020).

Because RNA was extracted from intact coral tissue containing both coral host and algal symbiont compartments, transcriptomic analyses were performed using a dual RNA-seq approach based on simultaneous mapping against a combined *Stylophora pistillata*–*Symbiodinium microadriaticum* reference genome. To enable host–symbiont read assignment, a custom chimeric reference genome was generated by merging the *S. pistillata* (NCBI assembly accession: GCF_002571385.2) and *S. microadriaticum* (NCBI assembly accession: GCA_001939145.1; see Results) reference genomes and annotations. Host and symbiont genome assemblies (FASTA files) and corresponding annotations (GFF files) were separately merged to generate a unified reference genome and annotation set for read alignment and gene quantification.

High-quality reads (Phred score > 30) were simultaneously mapped against the chimeric host–symbiont reference genome using STAR (v2.7.11b; Dobin et al., 2013) to reduce potential cross-mapping artifacts compared to sequential mapping approaches, as recommended for dual RNA-seq analyses of interacting biological systems (Aprianto et al. 2016; Wolf et al. 2018; Espindula et al. 2019). Gene-level quantification was performed using featureCounts (Subread v2.0.6; Liao et al., 2014), with exon-level features summarized at the gene level. Read pairs (from R1 and R2) were counted as single sequencing fragments rather than as independent reads. Resulting count matrices were subsequently partitioned into host and symbiont datasets based on gene identifiers and used for downstream analyses.

Transcriptomic and Functional Analyses

Transcriptomic analyses were conducted in R (v4.4.2) using DESeq2 (v1.46.0; Love et al., 2014), edgeR (v4.4.2; Robinson et al., 2009), and vegan (v2.7.3; Oksanen, 2013). Differential expression analyses were performed separately for host and symbiont datasets using DESeq2, fitting negative binomial generalized linear models with condition as the sole explanatory variable (~ condition; control versus menthol-treated samples). Genes with fewer than 10 counts in fewer than five samples were excluded prior to analysis. Log2 fold changes were estimated and shrunk using the apegglm (v1.28.0; Zhu et al., 2019) method. Genes with adjusted p-values < 0.05 were considered significantly differentially

expressed. For selected downstream analyses and visualizations, an additional $|\log_2 \text{ fold change}| \geq 1$ threshold was applied.

For exploratory multivariate analyses, genes with zero counts across all samples were removed and implemented in edgeR to retain genes with sufficient counts across biological replicates for reliable multivariate analyses. Variance stabilizing transformation (VST) implemented in DESeq2 was then applied to normalized count data. Principal Component Analysis (PCA) was performed on the 500 most variable genes using the `prcomp` function. Differences between control and menthol-treated transcriptomes were assessed using PERMANOVA (`adonis2`, `vegan` package) based on Euclidean distance matrices computed from variance-stabilized expression values. Statistical significance was evaluated using 4,999 permutations. Homogeneity of multivariate dispersion was assessed using `betadisper` followed by permutation testing (`permutest`, 4,999 permutations). To evaluate the robustness of the symbiont response, supplementary analyses were repeated after temporary exclusion of one extreme sample (M2) identified based on PCA clustering patterns.

Heatmaps were generated using `pheatmap` (v1.0.13; Kolde, 2019) based on variance-stabilized counts of significantly differentially expressed genes. Expression values were scaled by gene (row-wise z-score), and hierarchical clustering was performed using Euclidean distance.

Functional annotation was performed using KEGG Orthology (KO) assignments generated with GhostKOALA. KO terms were mapped to KEGG pathways using a custom KO-to-pathway reference table and grouped into higher-level KEGG functional categories and superpathways. For superpathway-level profiling, only significantly differentially expressed genes (adjusted p-value < 0.05) were retained. Gene-level log₂ fold changes were aggregated within each superpathway to calculate summary statistics including mean and median log₂ fold changes. Superpathways represented by fewer than two genes were excluded.

KEGG pathway over-representation analysis (ORA) was performed separately for up- and down-regulated differentially expressed genes using a one-sided Fisher's exact test (alternative hypothesis: greater). The background universe included all tested genes with at least one KEGG annotation. Only pathways with a total annotated gene set size between 10 and 500 genes were considered for analysis. P-values were corrected for multiple testing using the Benjamini–Hochberg method. Pathways with an adjusted p-value < 0.05 and a minimum overlap of at least 3 differentially expressed genes were considered significantly enriched. For visualization, up to 15 pathways per organism and direction were displayed, ranked by adjusted p-value, gene count, and rich factor. Bubble plots were generated using `ggplot2` (v4.0.2; Wickham, 2016), where point size represented the number of contributing differentially expressed genes, point color represented the adjusted p-value, and the x-axis represented the rich factor, calculated as the ratio of differentially expressed genes to the total number of annotated genes in each pathway.

Results

MEGABLAST analysis of the COI consensus sequence generated from all individual coral sequences ($n = 6$) revealed the highest similarity with sequences assigned to *Stylophora pistillata*. Top matches showed 98–100% query coverage, sequence identities up to 99.85%, and E-value of 0.0, confirming the taxonomic identity of the analyzed coral colonies. No nucleotide substitutions were detected among individual coral sequences, indicating that all colonies belonged to the same mitochondrial lineage.

Similarly, MEGABLAST analysis of the ITS2 consensus sequence generated from all individual symbiont sequences ($n = 6$) revealed the highest similarity with sequences assigned to *Symbiodinium microadriaticum*. Top matches showed 100% query coverage, sequence identities up to 98.23% and, E-value $4e^{-135}$, indicating *Symbiodinium microadriaticum* as the dominant symbiont associated with the analyzed coral colonies under the experimental conditions. Sequence alignment of individual ITS2 sequences revealed the presence of two closely related haplotypes differing by four nucleotide substitutions. This intra-specific variability was not investigated further, as it was beyond the scope of the present study.

Menthol-Induced Bleaching and Oxygen Respirometry

As expected, menthol treatment induced a pronounced bleaching phenotype in *Stylophora pistillata* nubbins, characterized by a marked reduction in tissue pigmentation compared to control colonies (Fig. 1). Although symbiont cell density was not directly quantified in the present experiment, the same bleaching protocol previously resulted in approximately 95–98% loss of *Symbiodiniaceae* cells in *S. pistillata* (Isa et al. 2026), supporting the effectiveness of the implemented bleaching procedure.

Despite the pronounced symbiont loss, dissolved oxygen fluxes did not significantly differ between healthy and menthol-bleached *S. pistillata* nubbins during either light or dark incubations (Wilcoxon rank-sum tests, $p > 0.05$ for all comparisons). Both net photosynthesis and dark respiration showed substantial overlap between treatments (Table 1), indicating that menthol-induced bleaching did not produce detectable short-term alterations in surface-normalized oxygen dynamics under the experimental conditions tested. These observations are consistent with previous studies reporting limited physiological differences between symbiotic and menthol-induced aposymbiotic *S. pistillata* colonies despite effective disruption of the symbiotic state (Wang et al. 2012).

Read Mapping and Transcriptomic Responses to Menthol Treatment

Dual RNA-seq mapping against the combined *Stylophora pistillata*–*Symbiodinium microadriaticum* reference genome revealed a marked shift in host–symbiont read composition following menthol treatment (Table 2). Control samples exhibited a relatively balanced distribution of reads between host and symbiont ($\sim 48\%$ host and $\sim 52\%$ symbiont of total mapped reads), whereas menthol-treated samples were dominated by host-derived reads ($\sim 90\%$), with a corresponding reduction in symbiont-

derived reads (~10%). Despite this substantial decrease in symbiont representation, each menthol-treated sample retained between 3.1 and 4.6 million reads assigned to *S. microadriaticum*, enabling independent transcriptomic analyses of both partners.

Menthol treatment strongly affected the host *S. pistillata* transcriptome, resulting in a clear separation between control and treated samples along PC1, which explained 89.8% of the total variance (Fig. 2A). PERMANOVA analysis confirmed a highly significant effect of treatment on host transcriptomic profiles ($R^2 = 0.886$, $p = 0.0072$). In contrast, *S. microadriaticum* exhibited a weaker but still significant transcriptomic response to menthol treatment (PERMANOVA: $R^2 = 0.212$, $p = 0.0238$), characterized by greater within-group variability and partial overlap between conditions (Fig. 2B). PC1 explained 45% of the total variance in the symbiont dataset, indicating a less coordinated and more heterogeneous response compared to the coral host. Notably, the treatment effect remained significant even after removal of the most extreme sample (M2; PERMANOVA: $R^2 = 0.285$, $p = 0.018$), indicating that the observed transcriptomic shift was not driven by a single outlier.

No significant differences in multivariate dispersion were detected between host treatment groups (PERMDISP: $F = 0.76$, $p = 0.504$), indicating comparable within-group variability. In contrast, multivariate dispersion differed significantly between symbiont treatment groups (PERMDISP: $F = 6.77$, $p = 0.015$), indicating greater transcriptomic heterogeneity among menthol-treated *S. microadriaticum* transcriptomes.

Differential expression analysis further highlighted the contrasting transcriptomic responses of the coral host and its symbiont. In the host *S. pistillata*, a total of 4,073 genes were significantly differentially expressed between control and menthol-treated samples (adjusted p -value < 0.05 , $|\log_2FC| \geq 1$), indicating extensive and coordinated transcriptional reprogramming. Hierarchical clustering and heatmap visualization of differentially expressed genes revealed a clear separation between experimental conditions, with highly consistent expression profiles among biological replicates (Fig. 3A). In contrast, the symbiont *S. microadriaticum* exhibited a markedly weaker transcriptional response. Only 128 genes were significantly differentially expressed at an adjusted p -value < 0.05 , of which 111 also satisfied the additional $|\log_2FC| \geq 1$ threshold. Consistent with PCA analyses, heatmap visualization showed weaker clustering patterns and greater variability among menthol-treated symbiont samples, indicating a less coordinated and more heterogeneous transcriptional response (Fig. 3B).

Functional Profiling of Host and Symbiont Responses

Functional profiling analyses further highlighted the contrasting biological responses of the coral host and its algal symbiont following menthol-induced bleaching (Figs. 4–5). Differentially expressed genes were functionally annotated using KEGG and summarized at two levels: broad KEGG functional categories/superpathways and individual KEGG pathways by over-representation analysis (ORA). Across nearly all major KEGG functional categories, the directional shifts observed in *S. pistillata* were opposite to those detected in *S. microadriaticum* (Fig. 4). For example, pathways associated with Genetic

Information Processing, including Translation and Folding, sorting and degradation, showed strong positive shifts in the host but negative shifts in the symbiont. A similar opposing pattern was observed across most remaining KEGG categories. Within Metabolism, notable exceptions included Carbohydrate metabolism, which showed a positive shift in the host but a negative shift in the symbiont, and Glycan biosynthesis and metabolism, for which the opposite pattern was observed (Fig. 4). Membrane transport and Lipid metabolism represented a further exception, showing negative shifts in both organisms.

In *S. pistillata*, functional analyses revealed extensive transcriptional reorganization across multiple biological categories. Among up-regulated genes, ORA identified significant enrichment of pathways associated with protein turnover and energy metabolism, including Proteasome (> 100 genes), Ribosome (~ 100 genes), Protein processing in the endoplasmic reticulum (~ 50 genes), Oxidative phosphorylation, and Citrate cycle (TCA cycle) (Fig. 5A). Consistently, superpathway-level profiling showed strong positive shifts in Translation and Folding, sorting and degradation categories, together with increased representation of Energy metabolism-related functions (Fig. 4A). Conversely, several pathways associated with signaling, lipid metabolism, extracellular interactions, and nitrogen metabolism were significantly enriched among down-regulated genes, including Neuroactive ligand–receptor interaction (> 100 genes), ECM–receptor interaction, Biosynthesis of unsaturated fatty acids, Steroid hormone biosynthesis, Nitrogen metabolism, and multiple amino acid metabolism pathways including Arginine and proline metabolism, Glycine, serine and threonine metabolism, and Tryptophan metabolism (Fig. 5A). Superpathway-level analyses similarly revealed pronounced negative shifts in Lipid metabolism, Signaling molecules and interaction, and Membrane transport categories (Fig. 4A). Notably, despite an overall positive mean delta at the Amino acid metabolism superpathway level, specific sub-pathways within this category were significantly enriched among down-regulated genes, reflecting heterogeneous directional changes within this functional category.

In contrast, *S. microadriaticum* exhibited a substantially weaker functional response (Figs. 4B, 5B). Only two pathways remained significantly enriched after multiple-testing correction: Nitrogen metabolism among up-regulated genes and Fructose and mannose metabolism among down-regulated genes, both involving a limited number of genes (Fig. 5B). Despite the reduced statistical signal, superpathway-level profiling revealed functional shifts in several metabolic categories. In contrast to the coral host, metabolic superpathways including Energy metabolism, Carbohydrate metabolism, and Amino acid metabolism showed predominantly positive pathway-level shifts, whereas Translation and Folding, sorting and degradation displayed negative shifts relative to controls (Fig. 4B). The strongest functional signal detected in the symbiont corresponded to the Environmental adaptation superpathway, which showed the largest positive mean pathway delta across all categories (Fig. 4B).

Discussion

Opposing host and symbiont transcriptional responses during menthol-induced bleaching

In this study, dual RNA-seq analysis revealed that menthol-induced bleaching elicited markedly distinct and largely opposing transcriptional responses in the coral host *Stylophora pistillata* and its dominant algal symbiont *Symbiodinium microadriaticum*. Indeed, functional superpathways showing positive shifts in the host generally showed negative shifts in the symbiont, and vice versa, highlighting a marked functional decoupling between the two partners in response to menthol-induced bleaching. Opposing transcriptional responses between coral hosts and algal symbionts have previously been documented at the level of individual genes (Leggat et al. 2011). However, a near-systematic divergence across major KEGG functional superpathway categories, as observed here, has not been previously reported and may reflect fundamentally distinct physiological priorities adopted by each partner following destabilization of the symbiotic state. In addition to this directional divergence, the two partners also differed in the magnitude of their transcriptional response, with *S. pistillata* exhibiting 4,073 differentially expressed genes compared to only 111 in *S. microadriaticum*.

Because RNA was extracted from intact coral tissues containing both host and symbiont compartments, menthol-induced bleaching substantially altered the relative contribution of host- and symbiont-derived reads. Indeed, dual RNA-seq analyses revealed a reduction in symbiont-assigned reads from approximately 52% in control samples to approximately 10% in menthol-treated samples. Consequently, the reduced representation of symbiont transcripts may have lowered statistical power for detecting transcriptional responses in *S. microadriaticum*. However, host and symbiont datasets were analyzed independently following organism-specific filtering, normalization, and differential expression procedures, and each menthol-treated sample retained between 3.1 and 4.6 million reads assigned to *S. microadriaticum*. Together, these observations suggest that the comparatively weaker symbiont response is unlikely to be solely explained by reduced symbiont representation in menthol-treated samples. Notably, the asymmetry observed in this study is consistent with accumulating evidence from further studies showing that Symbiodiniaceae generally exhibit more limited transcriptional responses to environmental stress than their coral hosts (Barshis et al. 2014; Savary et al. 2021; Yuyama et al. 2022). More broadly, *Symbiodinium* and related genera have been suggested to rely on post-transcriptional regulatory mechanisms rather than large-scale gene expression reprogramming to respond to environmental perturbations (Gierz et al. 2017; Liew et al. 2017), which may partly explain the limited number of genes exceeding the $|\log_2FC| \geq 1$ threshold in *S. microadriaticum* observed in the present study. Accordingly, although reduced symbiont biomass following bleaching may have contributed to lower statistical power and increased variability among treated samples, the overall pattern observed here appears to reflect a genuine biological response.

Menthol-induced transcriptional reprogramming in the coral host

The contrasting regulation of protein synthesis and pathways involved in protein folding and degradation between host and symbiont was particularly pronounced. In *S. pistillata*, pathways associated with proteostasis and translational activity, including Proteasome, Ribosome, and Protein processing in the endoplasmic reticulum, were strongly enriched among upregulated genes, consistent with active cellular

remodeling and stress-associated proteostasis (Ruiz-Jones and Palumbi 2017; Yuyama et al. 2022). At the same time, enhanced representation of pathways related to transport and catabolism suggests activation of recycling and resource-mobilization processes. These responses are potentially associated with autophagy, lysosomal degradation, and intracellular turnover. Comparable activation of catabolic and proteostatic pathways has been widely documented during coral stress responses and is often interpreted as a compensatory mechanism supporting cellular maintenance under reduced symbiont-derived nutrient availability (Weis 2008; Desalvo et al. 2010; Kvitt et al. 2016). In contrast, several pathways associated with anabolic metabolism, including lipid metabolism, nucleotide metabolism, and metabolism of cofactors and vitamins, showed predominantly negative shifts, suggesting metabolic reprioritization away from biosynthetic investment and toward maintenance-associated functions (Helgoe et al. 2024). Together, these patterns portray a host undergoing extensive physiological restructuring following destabilization of the symbiotic relationship.

In addition, the strong downregulation of Glycan biosynthesis and metabolism pathways is particularly noteworthy given their central role in host–symbiont recognition and maintenance. Glycans are known to participate in adhesion, cell–cell recognition, and self/non-self-discrimination processes that contribute to the establishment and persistence of coral–algal symbiosis (Wood-Charlson et al. 2006; Tortorelli et al. 2021). The observed downregulation of glycan-associated pathways, together with reduced representation of Signaling molecules and interaction and Membrane transport categories, suggests disruption of the molecular communication networks required to sustain stable host–symbiont interactions.

Collectively, these findings may support the hypothesis that menthol-induced bleaching primarily arises through destabilization of host physiological and signaling processes rather than through direct transcriptional collapse or destruction of the algal symbiont (Wang et al. 2012; Matthews et al. 2016). This interpretation is further supported by the comparatively limited and heterogeneous transcriptional response observed in *S. microadriaticum*, which lacked evidence of broad coordinated stress-associated transcriptional remodeling.

Limited symbiont transcriptional responses to menthol-induced bleaching

Previous studies have proposed that menthol-induced bleaching may partly involve inhibition of *Symbiodinium* photosystem II activity (Wang et al. 2012; Matthews et al. 2016). However, the relatively limited and heterogeneous transcriptional response observed in *S. microadriaticum* in the present study, together with the absence of strong enrichment of photosynthesis-associated stress pathways, did not support widespread transcriptional disruption of photosynthesis-associated functions. Instead, our results were more consistent with a progressive destabilization of host–symbiont regulatory interactions preceding severe physiological degeneration of the symbiont. Nevertheless, future studies integrating transcriptomic analyses with direct measurements of photosynthetic performance, such as chlorophyll fluorescence and photosynthetic efficiency, will be necessary to determine whether the limited

transcriptional response observed here is accompanied by subtle functional impairments of the symbiont photosynthetic apparatus.

Although only a limited number of symbiont pathways reached significance in the ORA analysis, the enrichment of Nitrogen metabolism among upregulated genes may be noteworthy given the central role of nitrogen exchange in regulating coral–algal symbiosis (Rädecker et al. 2015, 2021). Altered nitrogen-associated metabolism in *S. microadriaticum* could therefore reflect changes in nutrient availability or metabolic coordination following destabilization of the symbiotic state. Conversely, the downregulation of Fructose and mannose metabolism may indicate localized alterations in carbohydrate-associated metabolic functions (Burriesci et al. 2012). However, given the small number of differentially expressed genes contributing to these enrichments, these pathway-level signals should be interpreted as preliminary rather than conclusive.

Despite the overall limited transcriptional response observed in the symbiont, selected metabolic superpathways, particularly Energy metabolism and Carbohydrate metabolism, together with the strong positive shift in the Environmental adaptation superpathway, suggested that *S. microadriaticum* retained evidence of active physiological adjustment under menthol exposure. These patterns may indicate that the relatively weak transcriptional response of the symbiont did not necessarily reflect complete physiological inactivity, but may have involved subtle and potentially heterogeneous adjustments in metabolic and stress-associated functions during symbiosis destabilization. At the same time, pathways associated with Translation and Folding, sorting and degradation showed predominantly negative shifts in the symbiont, possibly suggesting suppression of biosynthetic and proteostatic activity. This pattern was consistent with previous observations showing that *in hospite* Symbiodiniaceae often exhibit broad transcriptional repression relative to free-living states (Mohamed et al. 2020; Savary et al. 2021). It is therefore possible that under conditions of symbiosis collapse, this suppression could be further amplified by altered host cellular conditions rather than reflecting a fully autonomous symbiont-specific stress response.

Menthol bleaching as a controlled model of symbiosis destabilization

One of the most notable findings of the present study is that extensive transcriptomic remodeling occurred despite the apparent preservation of short-term metabolic performance. Menthol treatment induced a pronounced bleaching phenotype and extensive transcriptional reprogramming in the coral host, yet oxygen respirometry measurements remained largely unchanged between control and treated nubbins. At the same time, the algal symbiont exhibited only a comparatively limited and heterogeneous transcriptional response. Together, these observations suggest that substantial molecular reorganization can occur during menthol-induced bleaching without immediate collapse of bulk physiological functions.

This apparent decoupling between transcriptomic remodeling and physiological performance may provide important insights into the nature of menthol-induced bleaching. Rather than reflecting widespread degeneration of both partners, the observed responses are more consistent with a

progressive destabilization of the regulatory interactions sustaining coral–algal symbiosis. In particular, the strong host response, coupled with the comparatively limited transcriptional disruption detected in *S. microadriaticum*, suggests that menthol-induced bleaching may primarily affect host-associated processes involved in the maintenance and regulation of coral–algal symbiosis.

These observations further support the use of menthol bleaching as a useful experimental framework for generating aposymbiotic corals under controlled laboratory conditions. By effectively disrupting the symbiotic state while preserving short-term physiological functionality, menthol treatment may facilitate investigations of the molecular mechanisms associated with symbiosis maintenance and breakdown without the extensive secondary damage frequently associated with severe thermal stress. Nevertheless, menthol-induced bleaching should not be regarded as a direct proxy for natural bleaching events. Future studies integrating transcriptomic analyses with direct measurements of symbiont physiology, including photosynthetic efficiency and chlorophyll fluorescence, will be important to determine the extent to which menthol-induced bleaching recapitulates the functional responses observed during environmentally relevant bleaching processes.

An additional limitation of the present study is the relatively small number of biological replicates used for transcriptomic analyses. The number of coral nubbins was intentionally constrained to minimize damage to donor colonies and to comply with animal husbandry and conservation requirements established by the Aquarium of Genoa. Accordingly, the primary objective of this study was to identify the major molecular trajectories associated with menthol-induced bleaching rather than to fully characterize inter-colony variability. Despite this limitation, transcriptomic responses were highly consistent among host replicates and revealed robust treatment effects across multiple analytical approaches. Future studies employing larger numbers of biological replicates and donor colonies will be valuable for further evaluating the generality of the patterns observed here.

Declarations

Competing interests

The authors declare that they have no competing financial or non-financial interests.

Ethics approval

Coral fragments were obtained and maintained in accordance with institutional and aquarium regulations. No field collection requiring governmental permits was conducted.

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Author Contribution

Experimental work was conducted by V.I., A.M., D.M., L.M.T., L.B., E.M., G.D.R., A.C., F.S. and F.C. Provision of infrastructure, aquarium facilities, and coral biological material was ensured by P.G., D.S., Y.D.L., M.M. and S.L. The first draft of the manuscript was written by V.I., A.M., and F.C. Funding acquisition was secured by D.M. and F.C. All authors contributed to the revision process, provided critical feedback, and approved the final version of the manuscript.

Data Availability

Data availability The raw RNA-Seq data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1467703. Sanger sequences used for taxonomic validation have been deposited in GenBank under accession numbers PZ477849 (*Symbiodinium microadriaticum* ITS2) and PZ477851 (*Stylophora pistillata* COI).

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Figures

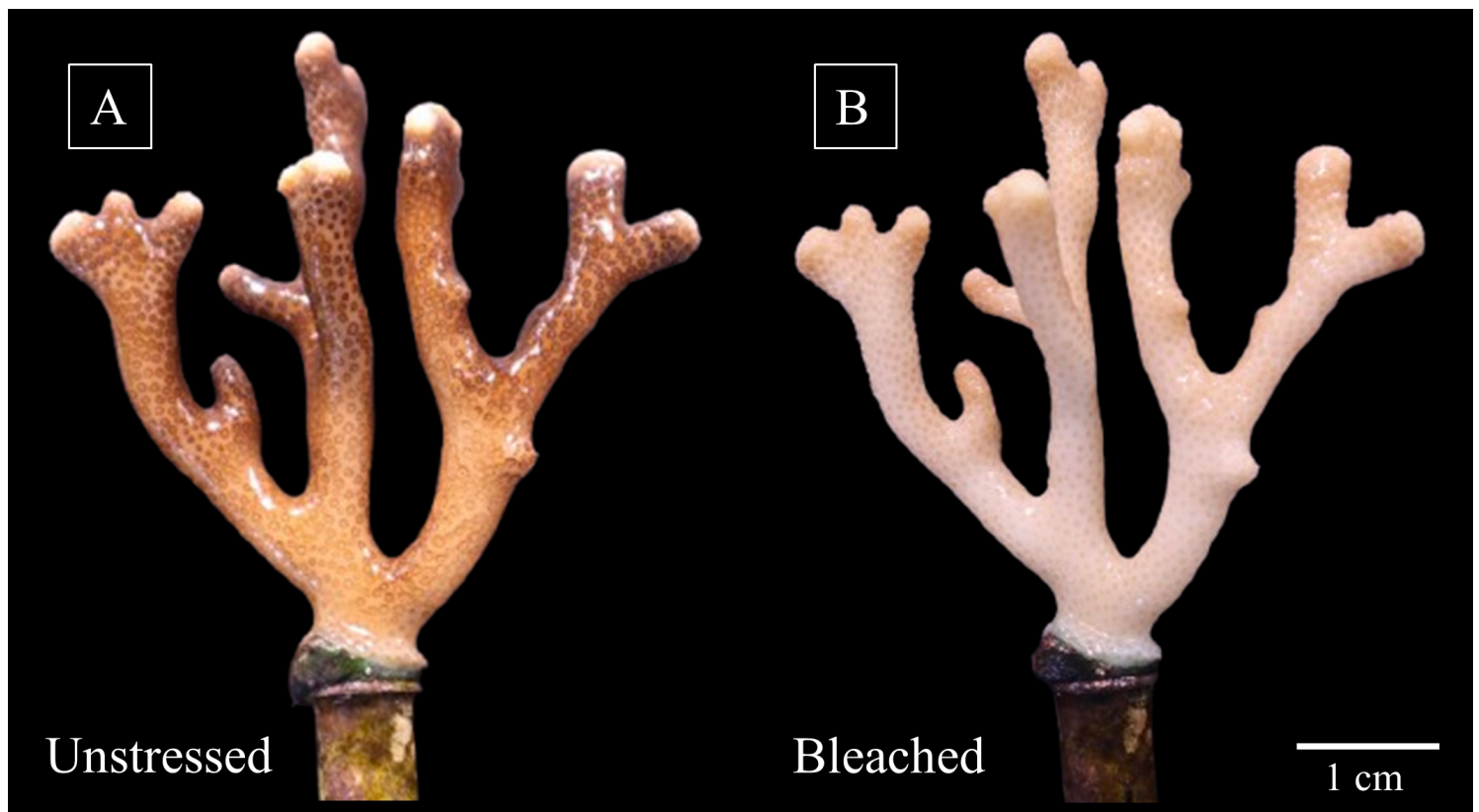


Figure 1

Representative appearance of control and menthol-bleached nubbins following the three-day bleaching treatment.

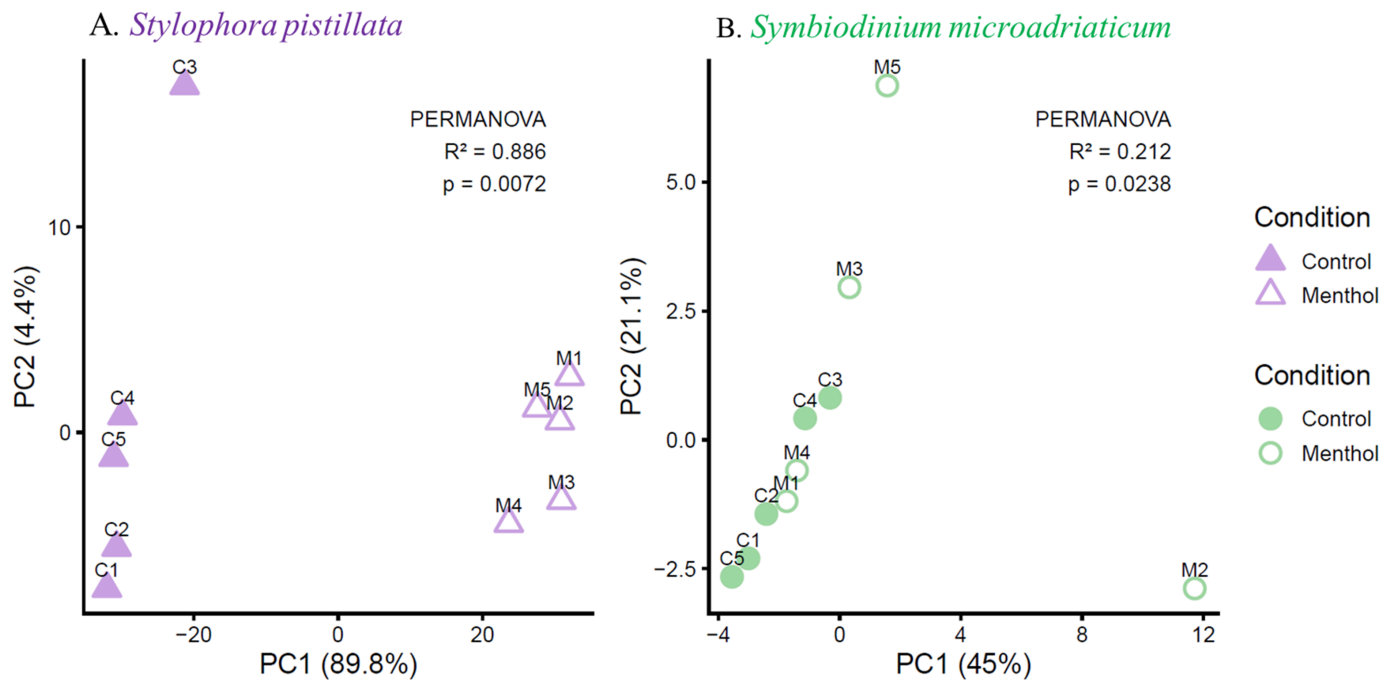


Figure 2

PCA of host and symbiont transcriptomic responses to menthol-induced bleaching. **(A)** shows the transcriptomic profiles between control (filled triangles) and menthol-treated (empty triangles) *S. pistillata* samples. **(B)** shows the transcriptomic response of control (filled circles) and menthol-treated (empty circles) *S. microadriaticum* samples. Values on the axes represent the percentage of variance explained by each principal component. PERMANOVA statistics are reported within each panel.

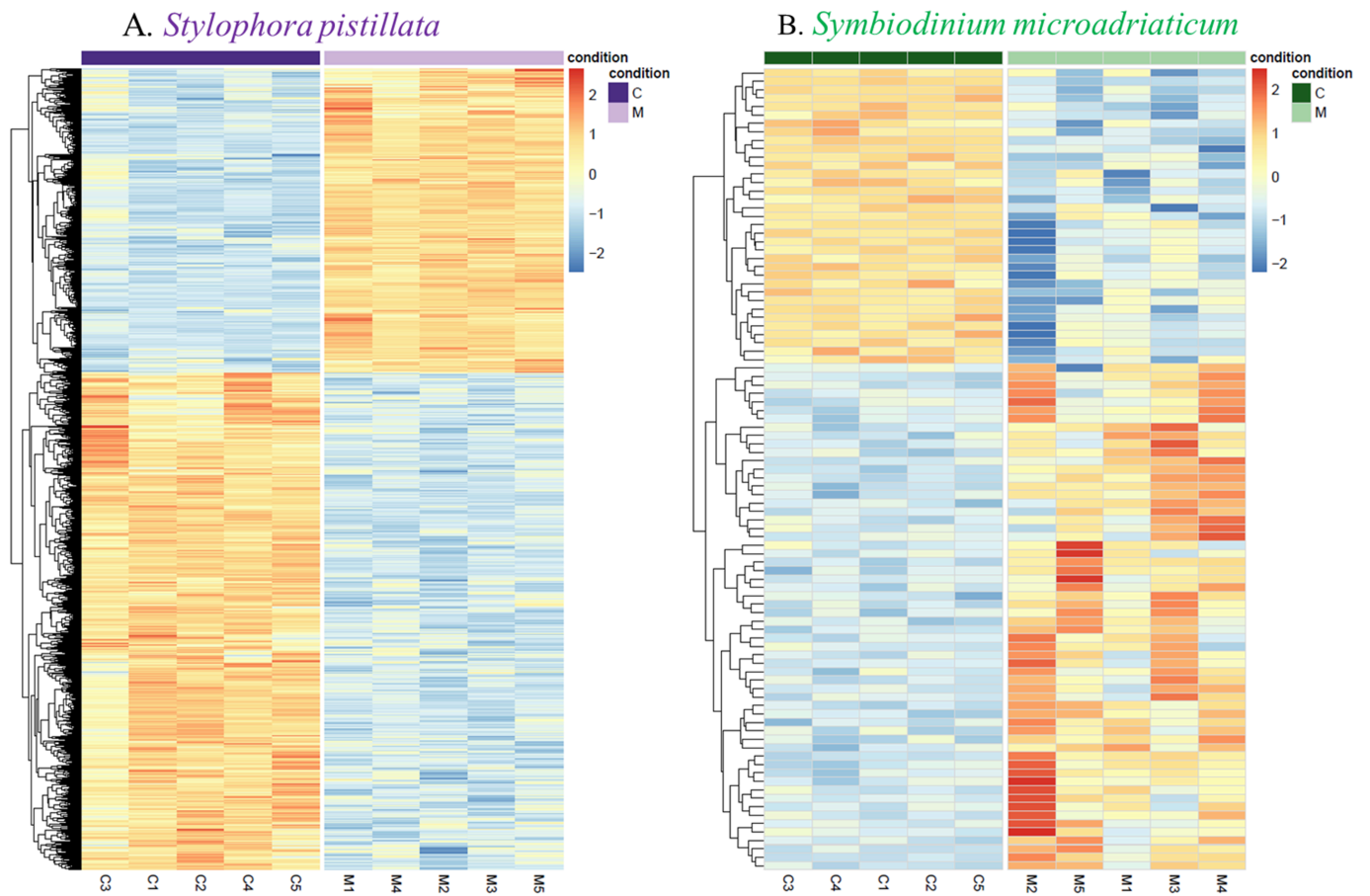


Figure 3

Heatmap visualization of differentially expressed genes in the coral host and algal symbiont following menthol-induced bleaching. Heatmap of significantly differentially expressed genes in (adjusted p-value < 0.05 , $|\log_2FC| \geq 1$), showing a clear separation between control and menthol-treated samples and highly consistent expression patterns among biological replicates. Heatmap of significantly differentially expressed genes in (adjusted p-value < 0.05), showing weaker clustering and greater variability among menthol-treated samples. Expression values represent variance-stabilized counts scaled by gene (row-wise z-scores). Hierarchical clustering was performed using Euclidean distance.

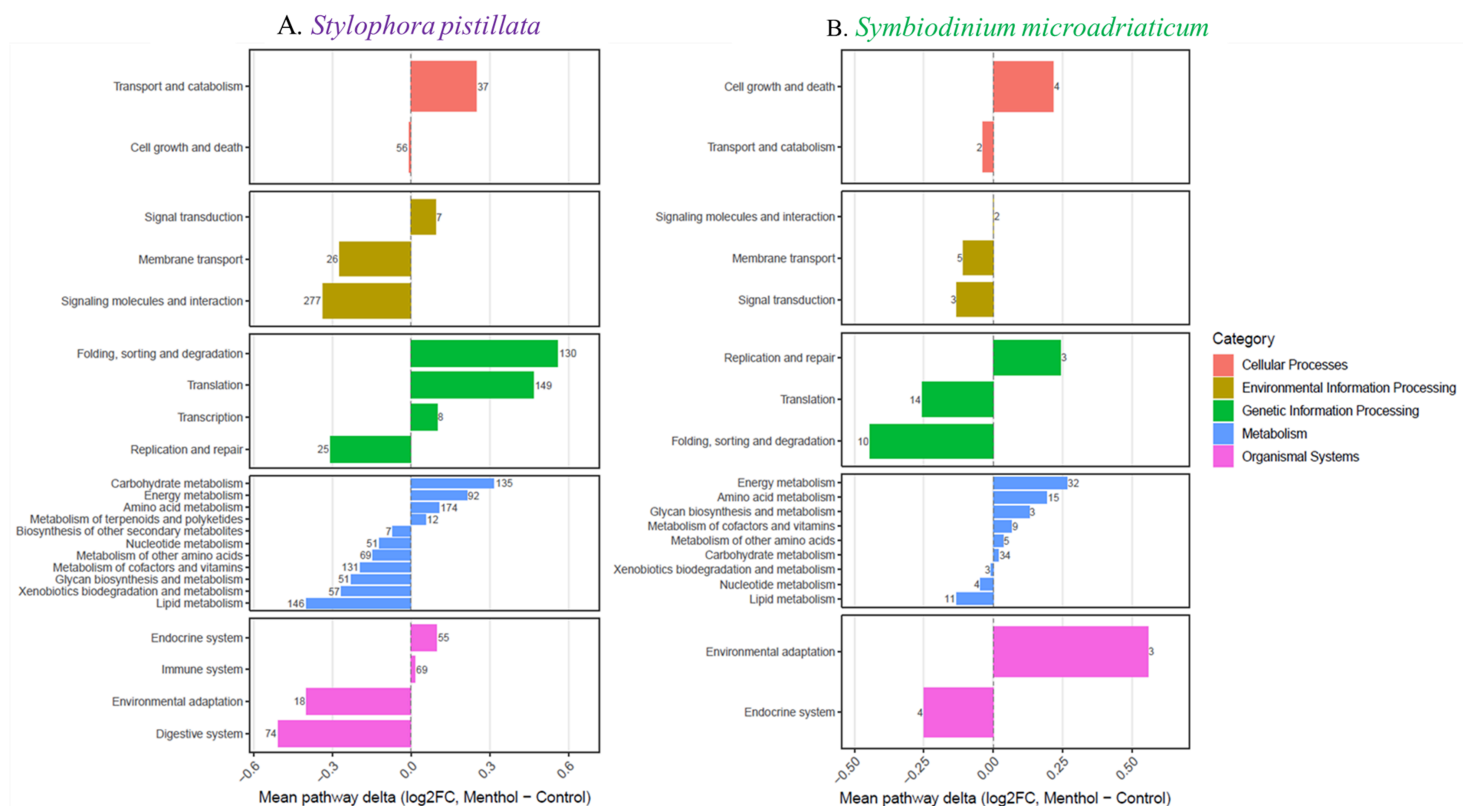


Figure 4

Superpathway-level functional profiling of differentially expressed genes in and following menthol treatment. Bars represent the mean log2 fold change (menthol – control) of significantly differentially expressed genes assigned to each KEGG superpathway. Positive values indicate a predominance of up-regulated genes, whereas negative values indicate a predominance of down-regulated genes within each superpathway. Numbers at the end of each bar indicate the number of DE genes contributing to each superpathway. Colors denote KEGG functional categories.

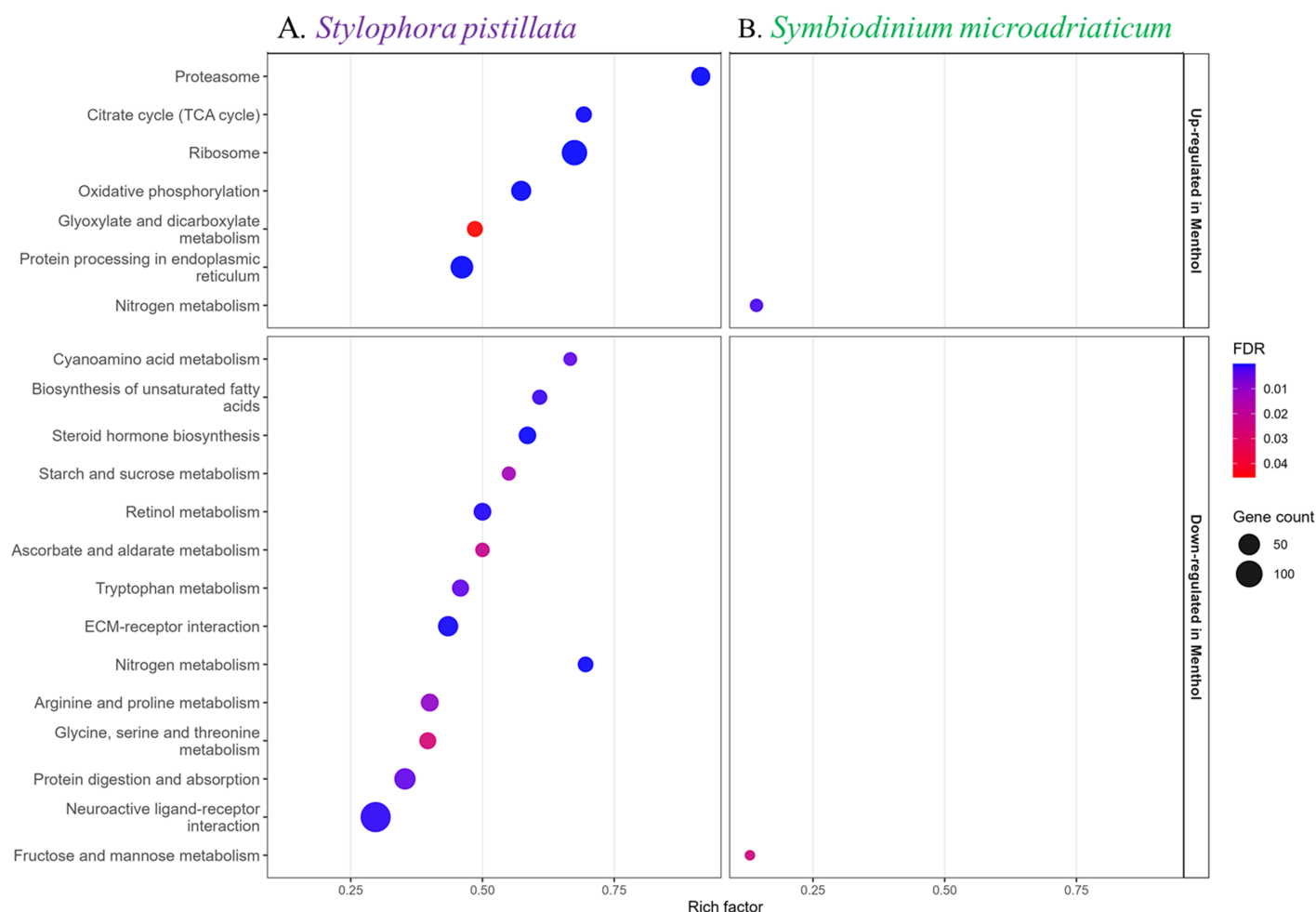


Figure 5

KEGG pathway enrichment analysis of differentially expressed genes in and following menthol treatment. Over-representation analysis (ORA) was performed separately for up- and down-regulated genes using one-sided Fisher's exact tests with Benjamini–Hochberg FDR correction. Bubble size represents the number of differentially expressed genes overlapping each pathway, whereas the x-axis indicates the rich factor (overlap/pathway size). Only significantly enriched pathways (FDR < 0.05) were retained. For visualization, top 15 pathways per organism and regulation category were selected.

Supplementary Files

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