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RESEARCH ARTICLE

Heat-evolved algal symbionts enhance bleaching tolerance of adult corals without trade-off against growth

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Abstract

Ocean warming has caused coral mass bleaching and mortality worldwide and the persistence of symbiotic reef-building corals requires rapid acclimation or adaptation. Experimental evolution of the coral's microalgal symbionts followed by their introduction into coral is one potential method to enhance coral thermotolerance. Heat-evolved microalgal symbionts of the generalist species, *Cladocopium proliferum* (strain SS8), were exposed to elevated temperature (31°C) for ~10 years, and were introduced into four genotypes of chemically bleached adult fragments of the scleractinian coral, *Galaxea fascicularis*. Two of the four coral genotypes acquired SS8. The new symbionts persisted for the 5 months of the experiment and enhanced adult coral thermotolerance, compared with corals that were inoculated with the wild-type *C. proliferum* strain. Thermotolerance of SS8-corals was similar to that of coral fragments from the same colony hosting the homologous symbiont, *Durusdinium* sp., which is naturally heat tolerant. However, SS8-coral fragments exhibited faster growth and recovered cell density and photochemical efficiency more quickly following chemical bleaching and inoculation under ambient temperature relative to *Durusdinium*-corals. Mass spectrometry imaging suggests that algal pigments involved in photobiology and oxidative stress were the greatest contributors to the thermotolerance differences between coral hosting heat-evolved versus wild-type *C. proliferum*. These pigments may have increased photoprotection in the heat-evolved symbionts. This is the first laboratory study to show that thermotolerance of adult corals (*G. fascicularis*) can be enhanced via the uptake of exogenously supplied, heat-evolved symbionts, without a trade-off against growth under ambient temperature. Importantly, heat-evolved *C. proliferum* remained in the corals in moderate abundance 2 years after first inoculation, suggesting long-term stability of this novel symbiosis and potential long-term benefits to coral thermotolerance.

KEYWORDS
coral bleaching, experimental evolution, reef restoration, spatial metabolomics, Symbiodiniaceae, symbiosis, thermotolerance, trade-off

1 | INTRODUCTION

Tropical coral reefs are among the most biodiverse ecosystems on Earth. The survival of reef-building corals depends on nutrient exchange between the coral host and its microalgal photosymbionts (family: Symbiodiniaceae) (Davy et al., 2012; Muscatine et al., 1984; Muscatine & Porter, 1977; Quigley et al., 2018). These corals cannot survive without the carbon and nitrogen translocated from Symbiodiniaceae (Muller-Parker et al., 2015), and symbiodiniacean physiological properties largely determine their coral host's thermotolerance (Berkelmans & van Oppen, 2006; Palacio-Castro et al., 2023; Quigley et al., 2018). However, climate change increases sea surface temperatures and the frequency, duration, and intensity of marine heatwaves (Bindoff et al., 2019), causing the dissociation of this obligate partnership (i.e., coral bleaching) that results in coral starvation and often death (Hoegh-Guldberg, 1999). Globally, marine heatwaves are linked to coral population decline in the last two to three decades (Hughes et al., 2018).

Introducing heat-tolerant Symbiodiniaceae into corals may enhance coral climate resilience. Many members of the symbiodiniacean genus *Durusdinium* are naturally heat tolerant and are often found in higher abundance in corals from extreme or fluctuating environments, or after mass bleaching events (Baker et al., 2004; Boulotte et al., 2016; Camp et al., 2019; Oliver & Palumbi, 2011; Palacio-Castro et al., 2023; Quigley et al., 2022; Stat & Gates, 2011). Higher thermotolerance is generally seen in corals hosting *Durusdinium* sp. when compared to those hosting members of the genus *Cladocopium* (Berkelmans & van Oppen, 2006; Palacio-Castro et al., 2023; Silverstein et al., 2015), the most widespread symbiont genus in Indo-Pacific scleractinian corals (LaJeunesse, 2005).

However, physiological trade-offs are often associated with *Durusdinium* sp. under ambient temperatures, making it a less ideal candidate for reef restoration (Ortiz et al., 2013). Compared to conspecifics hosting *Cladocopium* in the wild, field studies have shown that corals hosting *Durusdinium* had slower coral growth (Little et al., 2004), lower amounts of stored lipids and smaller eggs (Jones & Berkelmans, 2011), and less photosynthate translocation to the host (Baker et al., 2013). These trade-offs against coral growth (Cunning et al., 2015) and photosynthate translocation (Allen-Waller & Barott, 2023; Cantin et al., 2009) have also been demonstrated in laboratory studies. For instance, coral juveniles harboring *Cladocopium* C1 had double the amount of ^{14}C photosynthate (Cantin et al., 2009), 22% more ^{15}N acquisition (Baker et al., 2013) and higher relative electron transport rate of photosystem II (Cantin et al., 2009) than those dominated by *Durusdinium* sp. Growth is an important fitness trait, as corals with slower growth may be poor competitors for space and take longer to reach reproductive maturity (Randall et al., 2020).

Experimental evolution has successfully enhanced the thermotolerance of multiple marine microalgal species in vitro (Chan et al., 2021), including *Cladocopium proliferum* (Butler et al., 2023; Chakravarti et al., 2017). Recent studies have shown that some heat-evolved *C. proliferum* strains can enhance coral bleaching tolerance in larvae and juveniles (Table 1). Buerger et al. (2020) inoculated

TABLE 1 Summary of the results and conditions of previous studies inoculating coral larvae or juveniles with heat-evolved Symbiodiniaceae.

Coral species	Life stage	Environment	*Cladocopium proliferum* strains[a]	Heat-evolved duration	Treatment	Results	Reference
Acropora tenuis	Juvenile (10 months old)	Laboratory	Heat-evolved: SS1 and SS8 Wild-type: WT10	~8 years at 31°C	31°C, 41 days	Under elevated temperature, WT10-juveniles bleached and showed reduced respiration, while SS1/SS8-juveniles did not. Under ambient temperature, SS8-juveniles grew two times larger than SS1- and WT10-juveniles	Quigley et al. (2023)
Acropora tenuis	Juvenile (2 months old)	Laboratory	Heat-evolved: SS1 Wild-type: WT10	~8 years at 31°C	32°C, 58 days	Juveniles hosting heat-evolved SS1 had higher survival than those hosting wild-type WT10	Quigley and van Oppen (2022)
Acropora tenuis	Larvae	Laboratory	Heat-evolved: SS1–SS10 Wild-type: WT10, WT15	~4 years at 31°C	31°C, 7 days	Larvae hosting 3 of the 10 heat-evolved strains (SS1, SS7, SS8) had higher bleaching tolerance (i.e., stable or increased cell density during heat stress) than those hosting WT10. Larvae hosting SS1 also had higher bleaching tolerance than those hosting WT15	Buerger et al. (2020)

[a]Note that these studies utilized the same *Cladocopium proliferum* wild-type strain as this study (WT10, culture ID SCF 055-01.10), although it was referred with different names ('WT1' in Buerger et al., 2020; "*C. goreaui* C1" in Quigley & van Oppen, 2022). WT15 was referred to as WT2 in Buerger et al. (2020).

Acropora tenuis larvae with 10 different strains of heat-evolved *C. proliferum* (SS1–10), three of which (SS1, 7, 8) enhanced larval bleaching tolerance compared to those inoculated with wild-type *C. proliferum* (WT10) in the laboratory. Furthermore, *A. tenuis* juveniles (2 or 10 months old) inoculated with heat-evolved strains (SS1 or SS8) survived better or had less bleaching than those inoculated with WT10 under elevated temperatures (31 or 32°C) in the laboratory (Quigley et al., 2023; Quigley & van Oppen, 2022). While these findings are promising, it is unclear whether they can be extrapolated to other coral species and to the adult life phase, given larvae/juveniles and adults differ significantly physiologically. Importantly, long-term monitoring has not been conducted in previous studies and only one has examined potential holobiont trade-offs in juveniles (Table 1).

While sexually produced coral larvae and juveniles have been explored as reef restoration resources (Randall et al., 2020) and some small-scale successes have been achieved (e.g., dela Cruz & Harrison, 2017), most reef restoration programs to date rely on adult coral fragments (aka "coral gardening") (Lirman & Schopmeyer, 2016; Rinkevich, 2021; Schmidt-Roach et al., 2020). Therefore, methods that can enhance adult thermotolerance and evidence that this enhancement is not limited to one species (*A. tenuis*) have major implications to reef restoration programs worldwide.

Furthermore, the nature of coral-Symbiodiniaceae partnership differs significantly between larvae/juveniles and adults. Coral larvae are mostly aposymbiotic (i.e., free of algal symbionts), and juveniles have more diverse and flexible Symbiodiniaceae community than adults (Chan et al., 2019; Gómez-Cabrera et al., 2008; Little et al., 2004). In contrast, adult corals cannot be rendered fully aposymbiotic with thermal (Coffroth et al., 2010) or chemical bleaching (Puntin et al., 2023; Scharfenstein et al., 2022; Wang et al., 2012). It is not known whether heat-evolved Symbiodiniaceae can establish a population in adult corals in the presence of remnant native Symbiodiniaceae as potential competitors. Except for the recent study by Scharfenstein et al. (2022), there is no unequivocal evidence that adult corals can acquire exogenously supplied Symbiodiniaceae. Furthermore, only wild-type Symbiodiniaceae was tested in that study. Following a thermal treatment that reduced >98% of the corals' native Symbiodiniaceae population and the inoculation with heterologous strains of *Symbiodinium*, *Breviolum*, and *Durusdinium*, only *Breviolum* was detected in some adult *Porites divaricata* 3 weeks post-inoculation (Coffroth et al., 2010). This inoculum was no longer detected 5 weeks post-inoculation. In another study, thermally bleached *Acropora millepora* (naturally dominated by Cspc, C3k) was inoculated with heterologous *Durusdinium trenchii* and *C. proliferum* cultures and the inoculation was also unsuccessful (Morgans et al., 2020).

This study tests whether heat-evolved *C. proliferum* can form a symbiosis with adult *G. fascicularis* and if so, whether the novel symbiosis can enhance adult coral bleaching tolerance and whether this comes trade-offs. The coral *G. fascicularis* was chosen because it is known to host both *Cladocopium* (C1, C21a) and *Durusdinium* (D1–4) naturally (Puntin et al., 2023; Wepfer et al., 2020). The Great Barrier Reef (GBR) *G. fascicularis* colonies of this study host *D. trenchii*, *D. glynnii*, and/or *Cladocopium* C40 in the wild (see Section 3). We hypothesized that (1) heat-evolved and wild-type *C. proliferum* can co-exist with the remnant native *Durusdinium* within a coral host, (2) corals hosting wild-type *C. proliferum* will be the least resilient coral group under a simulated heat stress event, and (3) the metabolite profile will differ between resilient and susceptible coral groups.

To study the biomolecules that are potentially linked to coral resilience/susceptibility, high-resolution mass spectrometry imaging (MSI) was used for holobiont metabolite profiling. Mass spectrometry provides the highest coverage of metabolite/lipid detection and identification compared to any other techniques. The conversion of this method into 2D analysis through imaging enables the separation between host and symbiont metabolites relevant to host anatomical features, which would be otherwise not measurable in conventional bulk tissue metabolomics (liquid/gas-chromatography mass spectrometry) (Chan et al., 2023). Additionally, MSI uses significantly less biomass than bulk tissue metabolomics. This recently developed method utilizes intact coral tissue sections and can provide the relative intensity of hundreds of metabolites (e.g., different classes of lipids, pigments, signaling/structural biomolecules) at a spatial resolution of 50 μm in situ (Chan et al., 2023).

2 | MATERIALS AND METHODS

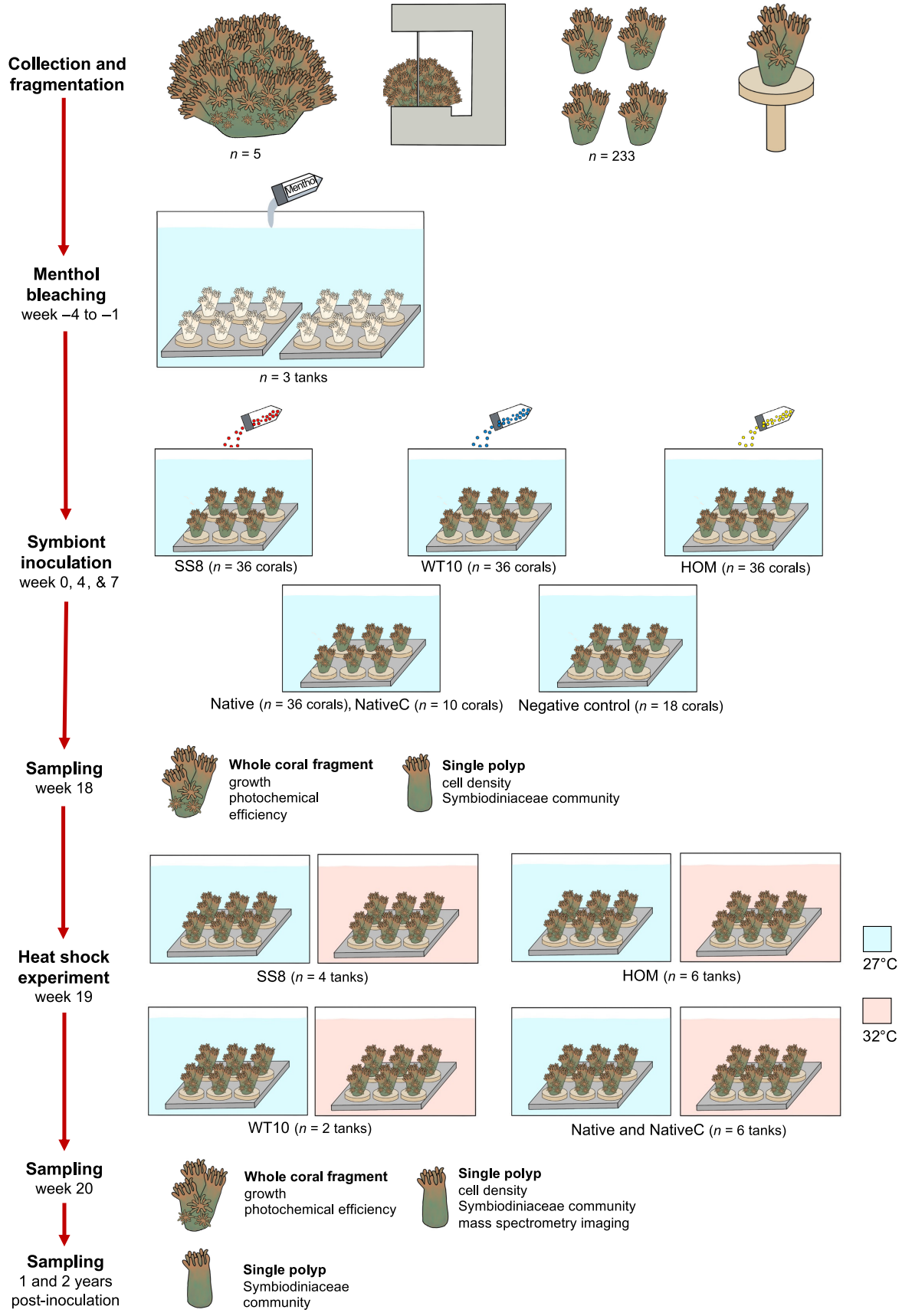
2.1 | Coral inoculation experiment

2.1.1 | Coral colonies

Five colonies of *Galaxea fascicularis* (genotype G1–5) were obtained from Falcon Reef (18°45.989′ S, 146°32.130′ E), the GBR (collection permit: G12/35236.1). Four of the colonies (genotype G1, 3–5) were dominated by *D. trenchii* and *D. glynnii*, and one (G2) was dominated by *Cladocopium* C40 (see Section 3.1.2; Figure S1; Table S2). Coral colonies were cut into ~4-polyp fragments and secured onto a 22 mm diameter ceramic plug with glue (Seachem Reef Glue™) (Figure 1; Table S2). The fragments were returned to the aquaria for a 10-day recovery period to allow the cut surfaces to heal prior to menthol bleaching. The remaining part of the colonies were kept in the recirculating system to obtain homologous Symbiodiniaceae for inoculation. See Supplementary Methods S1 for details on coral collection and aquarium conditions; Figure 1; Table S1 for experimental timeline.

2.1.2 | Menthol bleaching and Symbiodiniaceae cell density

Menthol bleaching was used to remove the coral's native Symbiodiniaceae following our pilot study adapted from Matthews et al. (2016), Puntin et al. (2023), and Wang et al. (2012) (Supplementary Methods S2). A total of 233 coral fragments (40–50 per coral genotype, Tabel S2) were distributed randomly and evenly across three 9 L tanks and dosed with 0.38 mM of menthol for 8 h



(08:30–16:30) under light. The recirculating pumps stayed on to ensure even mixing. After 8 h, the corals were removed from the tanks, rinsed in seawater and placed in the 120 L recirculating system to rest overnight (16:30–08:30). The procedure was repeated for four consecutive days and the corals were not fed. On the 5th day, the corals were fed and left to rest in the 120 L system for 3 days. This

FIGURE 1 Simplified experimental design and timeline. The abbreviation of the inocula and coral groups refer to: heat-evolved *Cladocopium proliferum* (SS8-corals), wild-type *C. proliferum* (WT10-corals), freshly isolated, homologous Symbiodiniaceae (a mix of ~80% *Durusdinium* sp. and ~13% *Cladocopium* C40; see Section 3) from *Galaxea fascicularis* (HOM-corals), as well as control corals with no menthol treatment and no inoculation that were natively dominated by *Durusdinium trenchii* and *D. glynnii* (Native-corals) or *Cladocopium* C40 (NativeC-corals). Note that NativeC-corals were only used for mass spectrometry imaging due to their low number of replicates. See Table S1 for full details on the experimental timeline.

TABLE 2 Details of the Symbiodiniaceae inocula of this study.

Symbiodiniaceae treatment	Species	Details	Culture ID	°C[a]	Original host	Host origin
WT10	*Cladocopium proliferum*	Wild-type, cultured	SCF 055-01.10	27	*Acropora tenuis*	Magnetic Island, Australia
SS8	*Cladocopium proliferum*	Heat-evolved, conferring[b], cultured	SCF 055-01.08	31	*Acropora tenuis*	Magnetic Island, Australia
HOM	*Durusdinium trenchii*, *D. glynnii* and *Cladocopium* C40	Wild-type, freshly isolated	NA	27	*Galaxea fascicularis*	Falcon Reef, Australia

[a]Indicates the temperature at which the cultures (WT10 and SS8) or the corals for homologous Symbiodiniaceae isolation (HOM) were maintained at.
[b]The heat-evolved strain SS8 was derived from the same mother culture as WT10. It was experimentally evolved under an elevated temperature of 31°C (Chakravarti et al., 2017) and it conferred enhanced bleaching tolerance to *Acropora tenuis* coral larvae (Buerger et al., 2020).

weekly schedule was repeated for 4 weeks, until the corals were visually bleached as assessed with high-power stereomicroscopy (Leica M205 FA). A total of 46 coral fragments were not exposed to menthol and were kept as controls; of these, 36 and 10 fragments were dominated by *D. trenchii* and *D. glynnii* (Native-corals), and *Cladocopium* C40 (NativeC-coral), respectively (Figure 1). To quantify the number of remaining Symbiodiniaceae cells in the corals, five single polyps per genotype, with or without menthol treatment were sampled at the end of the 4-week treatment (5 replicates × 4 genotypes × 2 treatments, $n = 40$) (Supplementary Methods S2).

2.1.3 | Symbiodiniaceae culture and inoculation

The chemically bleached corals were subjected to three Symbiodiniaceae treatments: inoculation with heat-evolved *C. proliferum* (SS8), wild-type *C. proliferum* (WT10) and freshly isolated, homologous Symbiodiniaceae (a mix of ~80% *D. trenchii*, *D. glynnii*, and ~13% *Cladocopium* C40; see Section 3) from *G. fascicularis* (HOM) (Table 2). The corals from these treatments are referred to as "SS8-corals", "WT10-corals", or "HOM-corals". SS8 was developed via experimental evolution (Chakravarti et al., 2017); it has better photochemical efficiency, growth rate, and lower levels of extracellular reactive oxygen species (ROS) than WT10 under elevated temperature of 31°C in vitro (Chakravarti et al., 2017). SS8 was chosen because it was one of the three heat-evolved strains that conferred enhanced bleaching tolerant to *A. tenuis* larvae relative to WT10 (Buerger et al., 2020). Cultures of SS8 and WT10 were grown under their respective temperatures (Table 2) at 30–60 μE m^{-2} s^{-1} (12:12 h, light:dark) in 1% IMK culture medium. The HOM Inoculum was freshly isolated from a ~5 cm diameter fragment of each *G. fascicularis* genotype. The Symbiodiniaceae were isolated via water-piking with ~750 mL of filtered seawater, and the blastate was centrifuged at 3000 *g* for 5 min and resuspended in filtered seawater three times to remove residual coral tissue and mucus before coral inoculation.

Coral inoculation was carried out in four replicate tanks per Symbiodiniaceae treatment and with nine coral fragments per tank (i.e., 3 Symbiodiniaceae treatments × 4 tanks × 9 coral fragments = 108 total) (Figure 1). Three inoculations were conducted over the course of 7 weeks (week 0, 4, and 7; Figure 1; Table S1). On the day of inoculation, the circulating pumps were stopped, and the water volume of the tanks was reduced to 4 L to allow high densities of the algal inocula. The inocula were added to yield a final density of ~10^4 cells mL^{-1} in the tanks; Shellfish Diet 1800® was added at 5000 cells mL^{-1} to encourage phagocytosis. After 5 h, the water volume was increased to 8 L and the circulating pumps were restarted. The normal water volume (13.5 L) was resumed the following day. Eighteen coral fragments were kept as negative control (i.e., chemically bleached but no inoculation). The corals were allowed to recover for 18 weeks after the first inoculation to regain the Symbiodiniaceae population prior to a short-term heat stress experiment (Figure 1). Due to the relatively low light intensity that the cultured Symbiodiniaceae were exposed to (30–60 μE photons m^{-2} s^{-1}), the light intensity of the aquaria was reduced to 45–55 μE m^{-2} s^{-1} at full sun until the completion of the last inoculation; thereafter light levels were gradually increased to 80–85 μE m^{-2} s^{-1} at full sun (Table S3). Corals were fed three times a week and 50% of the seawater was changed weekly.

2.1.4 | Assessment of inoculation outcomes via DNA metabarcoding

Four sets of coral samples, each containing one polyp, were taken to verify the inoculation outcomes via metabarcoding.

These included (1) each coral genotype shortly after collection from the field (1 replicate × 5 genotypes, $n = 5$), (2) corals at the end of menthol treatment ($n = 4$), (3) corals at 7 weeks after the first inoculation (4 replicates × 4 tanks × 4 coral groups + 8 negative control, $n = 72$), and (4) corals at 18 weeks after the first inoculation (6 replicates × 4 coral groups, $n = 24$) (Table S1). In addition, 1 mL of each inoculum was sampled (10^4 cells mL^{-1}, 3 replicates × 3 inocula, $n = 9$) and three 50:50 *Cladocopium*:*Durusdinium* mock communities were made (Supplementary Methods S5). See Supplementary Methods S6 for DNA extraction, PCR amplification, and library preparation procedures. Samples were sequenced at the Walter and Eliza Hall Institute with Illumina MiSeq v3.

2.2 | Heat stress experiment

2.2.1 | Experimental design

The design of the heat stress experiment was based on the results of the Symbiodiniaceae community analysis post-inoculation (see Section 3.1.2) and included five coral groups: (1) SS8-corals, (2) WT10-corals, (3) HOM-corals, (4) Native-corals, and (5) NativeC-corals (Figure 1; Table 3). A total of six to seven coral fragments were randomly and evenly distributed into each of the ambient (27°C) and elevated (32°C) treatment tanks (replicate tank $n = 4$ for SS8-corals, $n = 2$ for WT10-corals, $n = 6$ for HOM-corals, $n = 6$ for Native-corals and NativeC-corals) (Table 3). WT10-corals had lower replication since only one coral genotype (G3) acquired this inoculum. NativeC-corals were only used for metabolite profiling as only 10 fragments total were available. For the elevated treatment, the temperature was ramped up 1°C day^{-1} until reaching 32°C, which was then maintained for 8 days. This duration and temperature were chosen based on a pilot experiment which showed that certain *G. fascicularis* colonies began to exhibit signs of stress under these conditions. Since corals may exchange Symbiodiniaceae when in close proximity with each other, corals of different Symbiodiniaceae treatments were maintained in their respective tanks. The seawater temperature and carbonate chemistry conditions are shown in Table S4.

2.2.2 | Symbiodiniaceae community and physiological measurements

Sampling for Symbiodiniaceae community, Symbiodiniaceae cell density, photochemical efficiency, coral survival and size was conducted at the beginning (i.e., before temperature ramping, at week 18 after the first inoculation) and the end (i.e., after 8 days under 32°C, at week 20) of the experiment (Figure 1; Table S1). One coral polyp each was sampled for Symbiodiniaceae community analysis (6 replicates × 4 coral groups × 2 temperatures, $n = 48$) and cell density measurement (5 replicates × 4 coral groups × 2 temperatures, $n = 40$). The four coral groups here refer to SS8-, WT10-, HOM-, and Native-corals. Because the relative abundance of SS8 in corals was highly variable at week 18, six extra samples were collected under elevated temperatures. To better capture variability at the population level, all sampling was conducted randomly, instead of tracking specific coral fragments over time.

Cell count, DNA extraction, PCR amplification, and library preparation were conducted as before. Coral size and survival were determined by polyp count using all corals (SS8-corals $n = 24$, WT10-corals $n = 14$, HOM-corals $n = 36$, Native-corals $n = 36$). A polyp was counted as "dead" when >50% of its tissue was lost, and a new grown polyp was counted as "alive" when its six primary septa were fully formed. All corals were used to measured photochemical efficiency (dark-adapted or maximum quantum yield of photosystem II; F_v/F_m; Supplementary Methods S4).

2.3 | Statistical analysis

2.3.1 | Symbiodiniaceae community

All raw data and R codes for statistical analysis generated in this study are available on https://doi.org/10.5281/zenodo.8336616.

TABLE 3 Details of the coral groups in the heat stress experiment.

Coral group	Dominant Symbiodiniaceae species	Menthol treatment	Coral inoculation	Coral genotype	Tank, *n*	Fragment, *n*	Polyp, *n*
SS8-corals	*Cladocopium proliferum* C1	Yes	Yes—heat-evolved, cultured	G3, G4	4	24	312
WT10-corals	*Cladocopium proliferum* C1	Yes	Yes—wild-type, cultured	G3	2	14	168
HOM-corals	*Durusdinium trenchii* and *D. glynnii* D1	Yes	Yes—freshly isolated from *G. fascicularis*	G3, G4	6	36	396
Native-corals	*Durusdinium trenchii* and *D. glynnii* D1	No	No	G3, G4	6	36	306
NativeC-corals	*Cladocopium* C40	No	No	G2	N/A[a]	10	85

[a]Due to the low number of replicates of NativeC-corals, Native-corals and NativeC-corals were in the same treatment tanks. Also note that NativeC-corals were only used for mass spectrometry imaging.

Raw sequences were submitted to SymPortal (Hume et al., 2019) for Symbiodiniaceae ITS2 taxa and profile analysis. Data visualization and statistical analysis was conducted in R (version 4.1.2) with a customized pipeline. Samples with low read count (four samples, <500 reads each) were excluded from the analysis. PERMANOVA (Oksanen et al., 2016) based on unweighted UniFrac distance and 999 permutations was applied to compare the Symbiodiniaceae communities of the coral groups between ambient and elevated temperatures. Multivariate homogeneity of variances was tested and confirmed using *betadisper* (Anderson et al., 2006).

2.3.2 | Cell count, coral growth, survival, and photochemical efficiency

For Symbiodiniaceae cell count, coral growth and photochemical efficiency, the effect of coral group and temperature treatment (fixed factors with interaction) and genotype (random factor) was analyzed using generalized linear mixed-effects models (GLMER) with the package *lme4* (Bates et al., 2015) or *glmmTMB* (Brooks et al., 2017). Negative binomial (Symbiodiniaceae cell count and coral growth data) and beta family with a logit link (photochemical efficiency data) were selected, and model fit was confirmed with *DHARMa* (Hartig, 2022). Since all coral fragments had three to four polyps at the time of inoculation (week 0), the number of polyps at week 18 (coral size) can be directly used for size comparison, with no normalization required.

For photochemical efficiency, the three technical replicate measurements of each sample were first merged before statistical analysis. Coral survival was calculated based on the number of dead polyps at the end of the heat stress experiment, offset by growth (if any). The effect of coral group and temperature treatment was analyzed separately using the non-parametric Kruskal–Wallis test, followed by Dunn's test (Dunn, 1964) for pairwise comparisons. The *p*-values were corrected using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995). A Kruskal–Wallis test was chosen over GLMER because the under-dispersion nature of the data violates model assumptions. The percentage of change was used for visualization (i.e., number of polyp change divided by the starting number of the polyps).

2.4 | High-resolution MSI

2.4.1 | Sample collection and Fourier transform ion cyclotron resonance matrix-assisted laser desorption/ionization MSI

Three samples of each coral group, each with two technical replicates, were collected for metabolite profiling with MSI after 8 days under 27 and 32°C (3 biological replicates × 2 technical replicates × 5 coral groups × 2 temperatures = 60 total). To better identify host metabolites, three samples of chemically bleached corals were collected immediately after 4 weeks of menthol treatment. Each sample consisted of one coral polyp that was removed with a bone cutter. Sample preparation, cryosectioning, matrix spray and MSI were conducted following the methods in Chan et al. (2023) (Supplementary Methods S7). Briefly, coral samples were anaesthetized with $MgCl_2$, rinsed with MQ water, embedded and snap frozen (Figure 2). The samples were cryosectioned (Leica CM 1860, −25°C) until a substantial amount of the frozen body and tentacles were exposed. A thin layer (~2 μm) of the embedding medium was applied on the exposed sample to strengthen its integrity before collecting a section. Four consecutive sections were collected per sample on a cryofilm at 12 μm thickness, freeze-dried, mounted on a stainless-steel sheet, and sprayed with α-cyano-4-hydroxycinnamic acid.

Matrix-assisted laser desorption/ionization MSI (MALDI-MSI) analysis was conducted on the Bruker SolariX (7T XR hybrid ESI–MALDI–FT–ICR–MS) (150–2000 *m/z*, 50 μm spatial resolution, positive ion mode). SCiLS was used for noise removal and peak picking, and the final peak list consists of 418 metabolites. Their intensities (average peak area) were normalized with root mean square and with a section's total surface area. See Supplementary Methods S8 for metabolite annotation details.

2.4.2 | Statistical analysis

The normalized data were analyzed in MetaboAnalyst 5.0 with log transformation and no data scaling, and data normality and homogeneity confirmed visually. First, the data were divided into ambient and elevated temperatures to examine the effect on coral groups within a temperature treatment. The top 60 most significant metabolites were shown on a heatmap, with Euclidean distance and Ward clustering algorithm applied. Next, the effect of temperature within a coral group was examined using *t*-tests. All significant metabolites detected were visualized on a heatmap. A metabolite was considered significant when p_{adj} (Benjamini & Hochberg, 1995) was <.05 and fold change (FC; calculated based on the non-log transformed data) was >1.3 (i.e., >30% difference). The localization and relative intensities of metabolites with significant differences were then visualized on SCiLS.

2.5 | Long-term monitoring

To track the long-term stability of symbiosis with SS8, SS8-corals were kept under ambient temperature (27°C) and sampled at 1 year ($n = 4$) and 2 years ($n = 4$) after the first inoculation, for Symbiodiniaceae community analysis. WT10-coral had no survivors by year one.

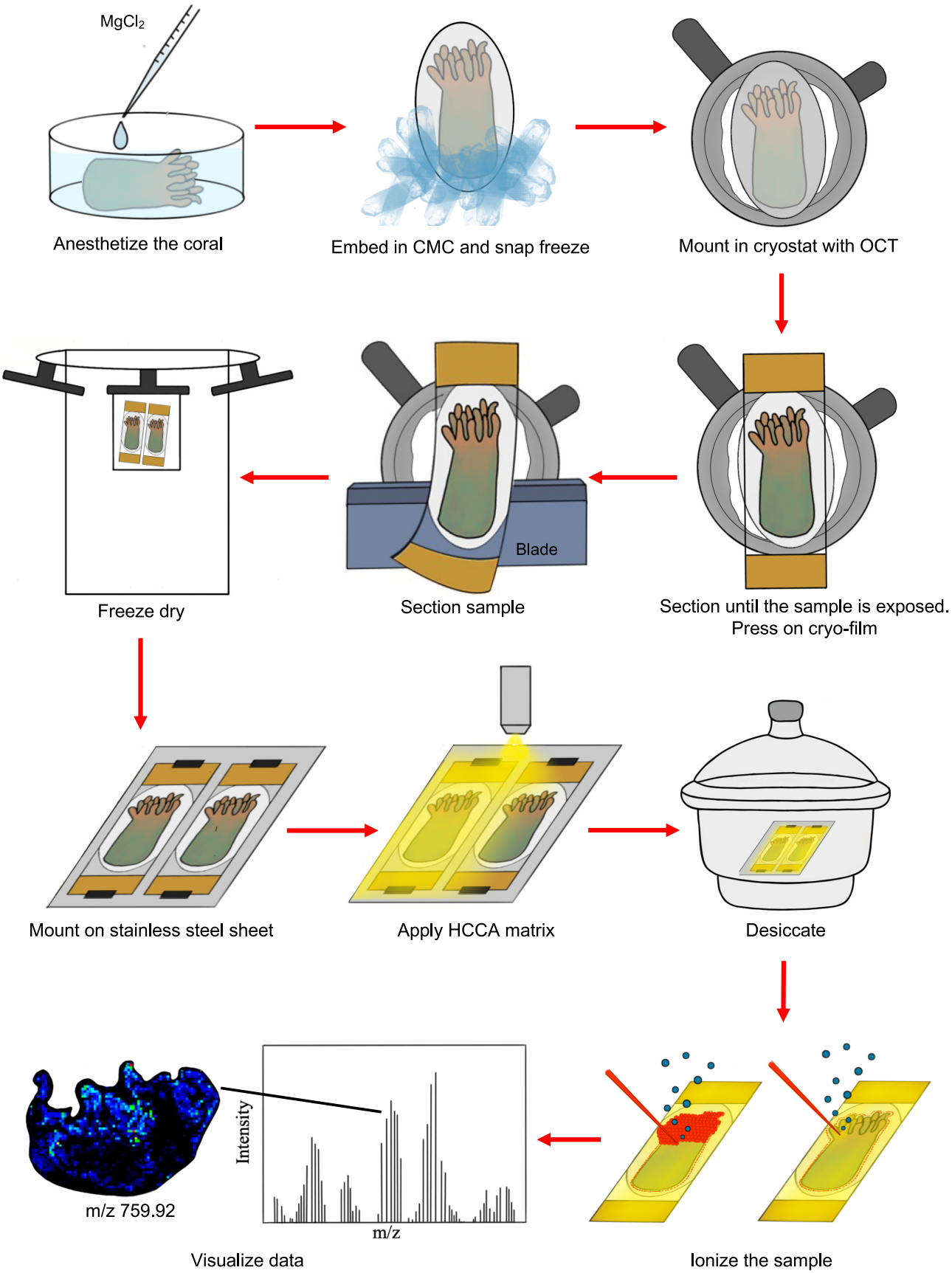


FIGURE 2 Fourier transform ion cyclotron resonance matrix-assisted laser desorption/ionization mass spectrometry imaging workflow of the coral samples. CMC, 2% carboxymethyl cellulose with gelatin; HCCA, α-Cyano-4-hydroxycinnamic acid; OCT, optimal cutting temperature compound.

3 | RESULTS

3.1 | Coral inoculation experiment

3.1.1 | Efficacy of menthol treatment

Four weeks of menthol treatment removed ~98.8% of *G. fascicularis*' native Symbiodiniaceae. On average, control corals (non-menthol-treated) contained ~2,730,000 Symbiodiniaceae cells per polyp, whereas menthol-treated corals had ~33,000 cells per polyp (i.e., 121 times fewer cells compared with the control) (Figure 3). Menthol treatment resulted in little coral mortality (0%–4.2%), except in one coral genotype (G2, 75% mortality), which was excluded from downstream physiological measurements due to a limited number of replicates remaining (Table S2). Coral genotype had a significant effect on Symbiodiniaceae cell density in control corals (Kruskal–Wallis, $\chi^2 = 10.79$, df = 3, $p = .013$) and menthol-treated corals (Kruskal–Wallis, $\chi^2 = 12.49$, df = 3, $p = .005$). In both cases, genotypes G3, G4, and G5 had similar cell densities while G1 had a higher cell density than G3 and G4 (Figure 3; Table S5). Menthol treatment removed 97.8% of G1's Symbiodiniaceae population, but removed 99.1%–99.2% of G3, G4, and G5's Symbiodiniaceae population.

3.1.2 | Inoculation outcome

ITS2 metabarcoding was used to assess the Symbiodiniaceae community composition in experimental corals (average read depth: ~16,000 per sample). No ITS2 profile was identified in DNA extraction and PCR negative controls (average <55 reads), indicating there was no contamination. The native Symbiodiniaceae community of *G. fascicularis* was dominated by *D. trenchii* and *D. glynnii* (87%–97%; ITS2 profile: D1–D4–D4c–D1h–D1c, D1–D4–D4c–D1c–D1cj, D1–D4–D4c–D6–D1c–D2, or D1–D4–D2–D4c–D6), with the exception of genotype G2 being dominated by *Cladocopium* C40 (72%; ITS2 profile: C40–C3–C115–C40h) (Figure S1). These native Symbiodiniaceae populations are hereafter referred to as "native *Durusdinium*" and "native *Cladocopium*". The ITS2 profile of the inocula SS8 and WT10 (C1–C1b–C1c–C42.2–C1bh–C1br–C1cb–C3) was distinct from the native Symbiodiniaceae communities of *G. fascicularis*, therefore detection of this profile is a reliable indicator of successful inoculation (Figure 4). The freshly isolated Symbiodiniaceae from *G. fascicularis* (HOM inoculum) contained mixed native *Durusdinium* (75%–85%) and native *Cladocopium* (9%–18%) (Figure 4).

Seven weeks after the first inoculation, two of the four *G. fascicularis* genotypes (G3, G4) contained detectable levels of SS8 or WT10 (Figure S2), hence only these genotypes were retained for the experiment. The proportion of SS8 or WT10 accounted for ~1%–15% of the corals' Symbiodiniaceae communities. By 18 weeks after the first inoculation, SS8 accounted for an average of 56% (ranged 4%–87%) in SS8-corals, and WT10 accounted for an average of 69% (ranged 54%–81%) in WT10-corals (Figure 5). HOM-corals had a similar Symbiodiniaceae community to the Native-corals (which was not bleached nor inoculated), both being dominated by native *Durusdinium*. *Cladocopium* C40 had minimal relative abundance in

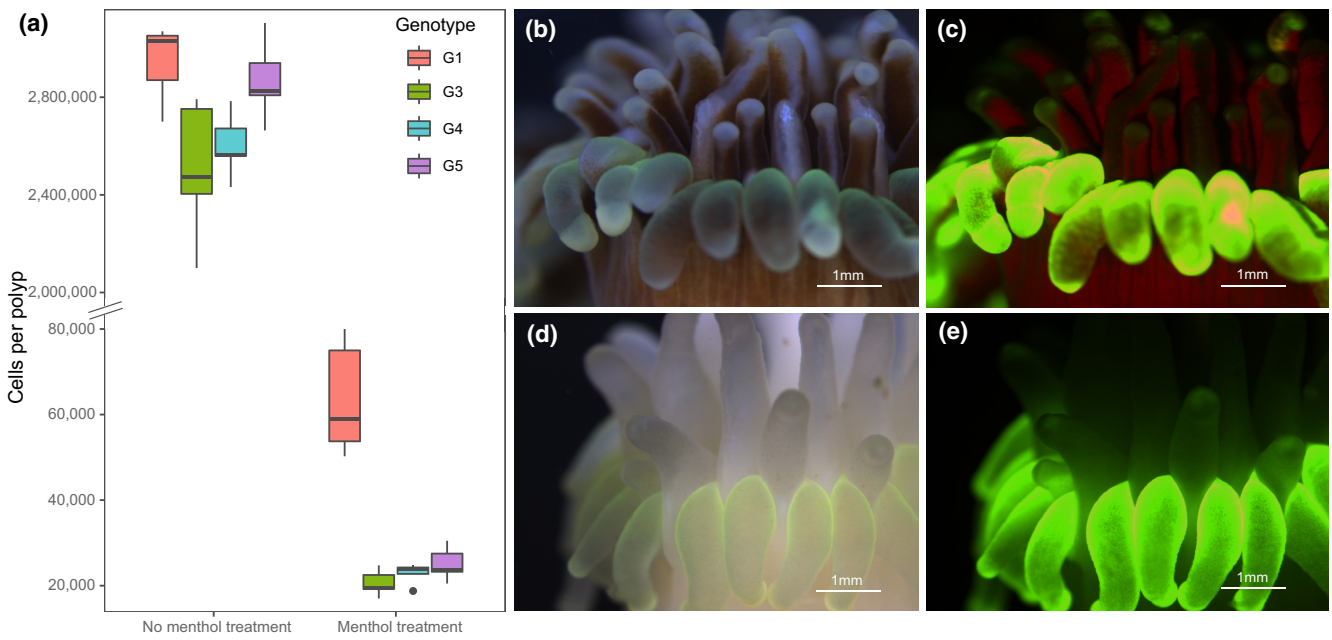


FIGURE 3 Symbiodiniaceae cell densities and images of control (no menthol treatment) and menthol-treated fragments of *Galaxea fascicularis*. (a) Cell density, (b) bright field image, and (c) fluorescent image of a control *G. fascicularis* polyp (non-menthol-treated). (d) Bright field image and (e) fluorescent image of a *G. fascicularis* polyp after 4 weeks of menthol treatment. Note that there is a *Y*-axis break in (a) due to the very large cell density differences between control and menthol-treated groups. The images represent coral genotype G3 and were taken with a Leica M205 FA using a green fluorescence protein filter.

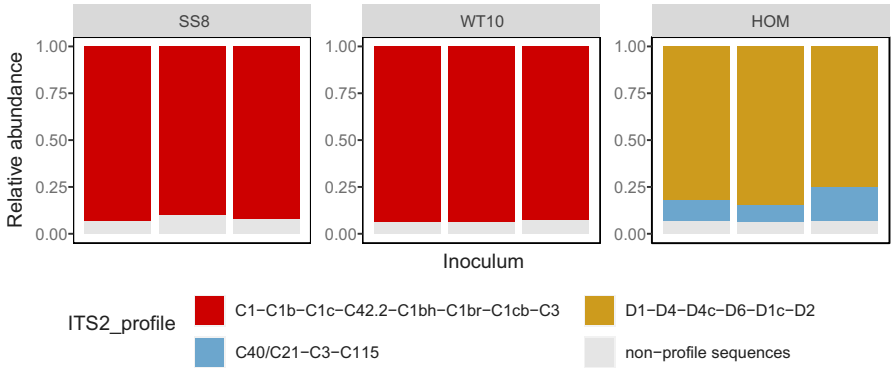


FIGURE 4 ITS2 profile of the inocula. HOM, homologous inoculum freshly isolated from *Galaxea fascicularis*; SS8, heat-evolved *Cladocopium proliferum*; WT10, wild-type *C. proliferum*. Each bar represents a biological replicate.

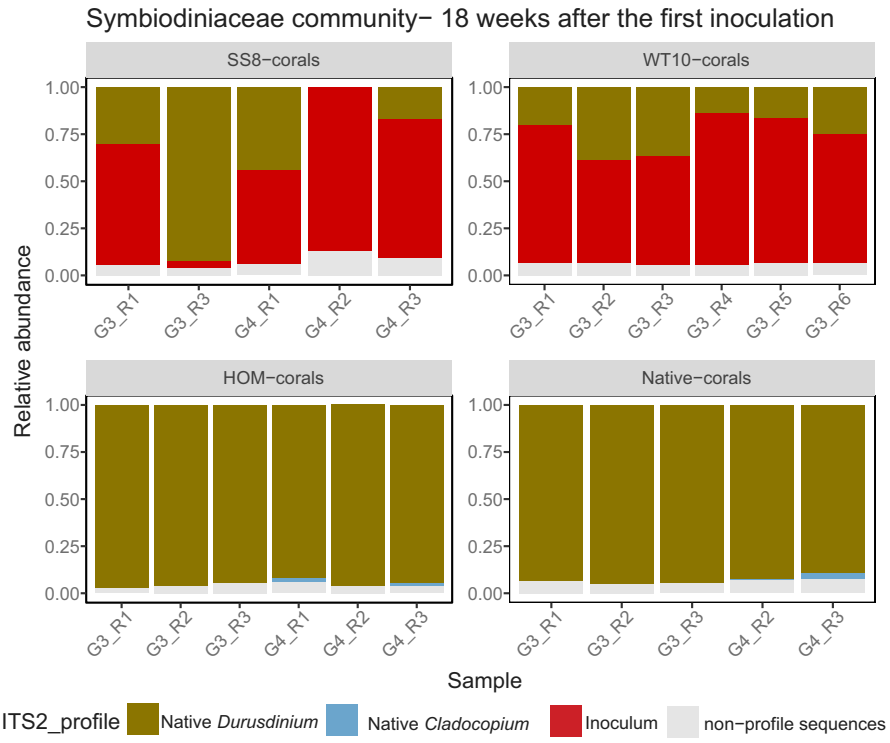


FIGURE 5 Coral-associated Symbiodiniaceae communities 18 weeks after the first inoculation. The Symbiodiniaceae treatments of the corals included: heat-evolved *Cladocopium proliferum* (SS8-corals), wild-type *C. proliferum* (WT10-corals), freshly isolated, homologous Symbiodiniaceae from *Galaxea fascicularis fascicularis* (HOM-corals). Control corals with no menthol treatment nor inoculation are also included (Native-corals). All corals were grown under ambient temperature during this period. "G" refers to the sample's genotype, "R" refers to the sample's replicate number. Note the sampling was conducted randomly to capture population-level variability, and specific individual fragments were not tracked over time. Since only two *G. fascicularis* genotypes (G3, G4) contained detectable levels of the inoculum SS8 or WT10, other genotypes were excluded from the heat shock experiment and were not reported here. Note that one SS8- and Native-coral sample were excluded due to low read numbers. Also note that the same red color is used for inocula SS8 and WT10—they were originally derived from the same mother culture and cannot be distinguished by ITS2 metabarcoding (see Figure 4).

HOM-corals, suggesting the C40 component of the HOM inoculum (9%–18%) had no or little infection success.

3.2 | Heat stress experiment

3.2.1 | Symbiodiniaceae community

At the end of the heat stress experiment (i.e., 8 days under 32°C), the Symbiodiniaceae communities of WT10-corals under ambient and elevated temperatures were significantly different (PERMANOVA, $p = .004$). The proportion of WT10 in coral fragments at ambient temperature (79%) was on average 21% higher compared to that at elevated temperature (58%) (Figure 6). In contrast, the Symbiodiniaceae communities of SS8-, HOM-, and Native-corals remained unchanged under the two temperatures (PERMANOVA, $p = .50$, .83, .97, respectively; Figure 6). In SS8-corals, a high level of variability in SS8 relative abundance (ambient: 43%–80%; elevated: 24%–73%) was observed, whereas limited variability was found in WT10-corals (ambient: 73%–82%; elevated: 50%–64%). The Symbiodiniaceae

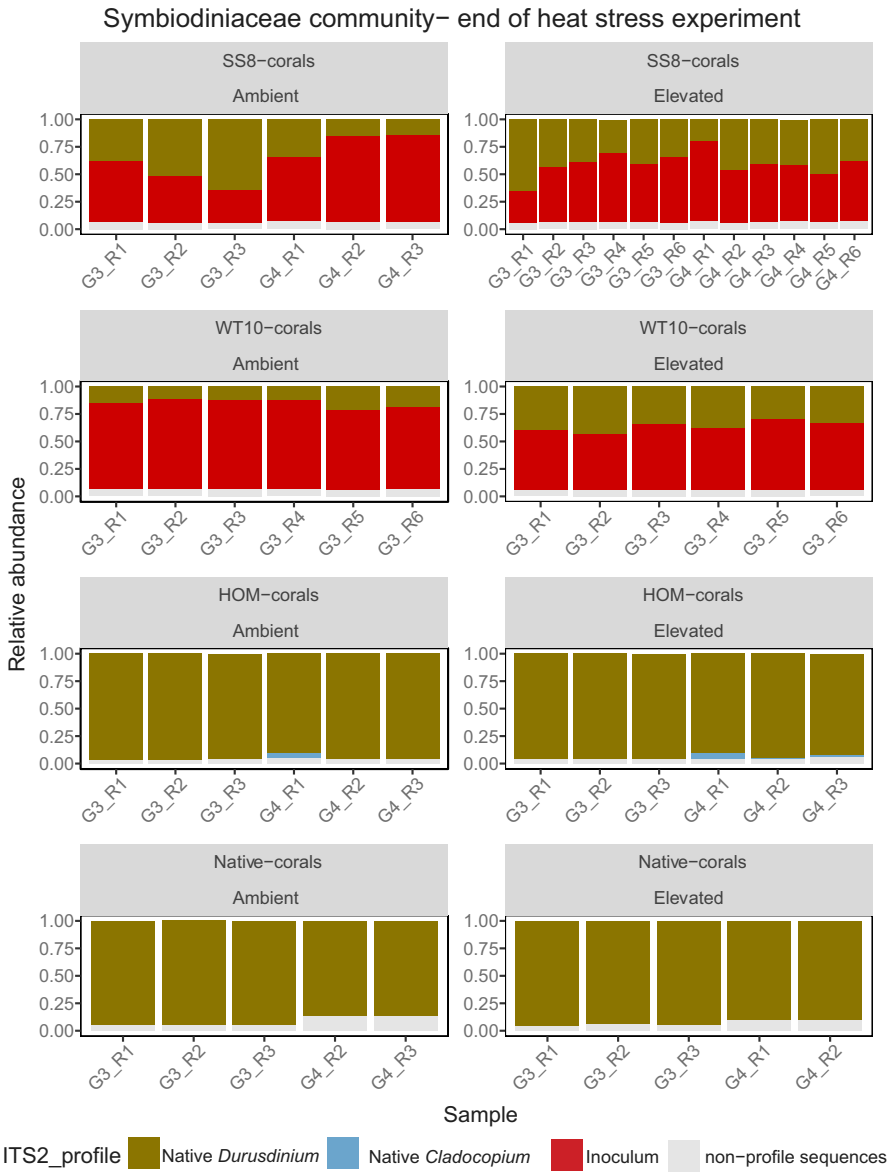


FIGURE 6 Coral-associated Symbiodiniaceae communities at the end of the short-term heat stress experiment (8 days, 32°C), 20 weeks after the first inoculation. The abbreviations of the experimental groups refer to corals that were inoculated with and dominated by heat-evolved *Cladocopium proliferum* (SS8-corals), wild-type *C. proliferum* (WT10-corals), freshly isolated, homologous Symbiodiniaceae from *Galaxea fascicularis* (HOM-corals); as well as control corals with no menthol treatment nor inoculation (Native-corals). "G" refers to the sample's genotype, "R" refers to the sample's replicate number. Note the sampling was conducted randomly to capture population level variability, and specific individual fragments were not tracked over time. An extra six samples were collected for SS8-corals under elevated temperature due to high level of variability in SS8 relative abundance observed in week 18 (Figure 5). Only coral genotypes that contained detectable levels of SS8 or WT10 (G3, G4) were retained in the heat shock experiment, hence other genotypes were not reported here. Note that one Native-coral sample under ambient and elevated temperatures was excluded due to low read number.

community of HOM- and Native-corals comprised of nearly 100% native *Durusdinium* under both temperatures.

3.2.2 | Symbiodiniaceae cell density

Under elevated temperature, WT10-corals had ~89% lower Symbiodiniaceae cell density when compared to their ambient counterparts (GLMER, $z = -39.81$, $p < .001$). Cell density in this group was the lowest of all experimental groups (Figure 7a; Table S6). Native-corals also had a ~11% decrease in cell density at elevated compared with ambient temperature (GLMER, $z = -2.45$, $p = .014$), whereas SS8- and HOM-corals showed no difference in cell density between temperatures (GLMER $p = .81$, .55, respectively) (Figure 7a). Under elevated temperature, the cell density of SS8-corals was similar to that of Native-corals which did not undergo menthol treatment (GLMER, $z = 1.59$, $p = .113$). Under ambient temperature, all coral groups that underwent menthol treatment and inoculation (SS8-, WT10-, HOM-corals) had lower cell densities than Native-corals (GLMER, $p < .001$ for all, Table S6), suggesting they had not fully recovered from chemical bleaching. By 20 weeks after the first inoculation, both SS8- and WT10-corals had recovered to ~82% of their original cell density prior to menthol treatment, while HOM-corals only recovered to ~65% (Figure 7b), despite these groups having been inoculated at the same cell density. Cell densities of SS8- and WT10-corals were higher than those of HOM-corals under ambient temperature (Figure 7a; Table S6). In all models, the variance component of the random effect (genotype) was estimated at, or very close to zero.

3.2.3 | Coral size and survival

All coral fragments had three to four polyps at the time of the first inoculation. They were grown under ambient temperature for an 18-week recovery period before the heat stress experiment. Over the recovery period, coral groups differed significantly in growth rate

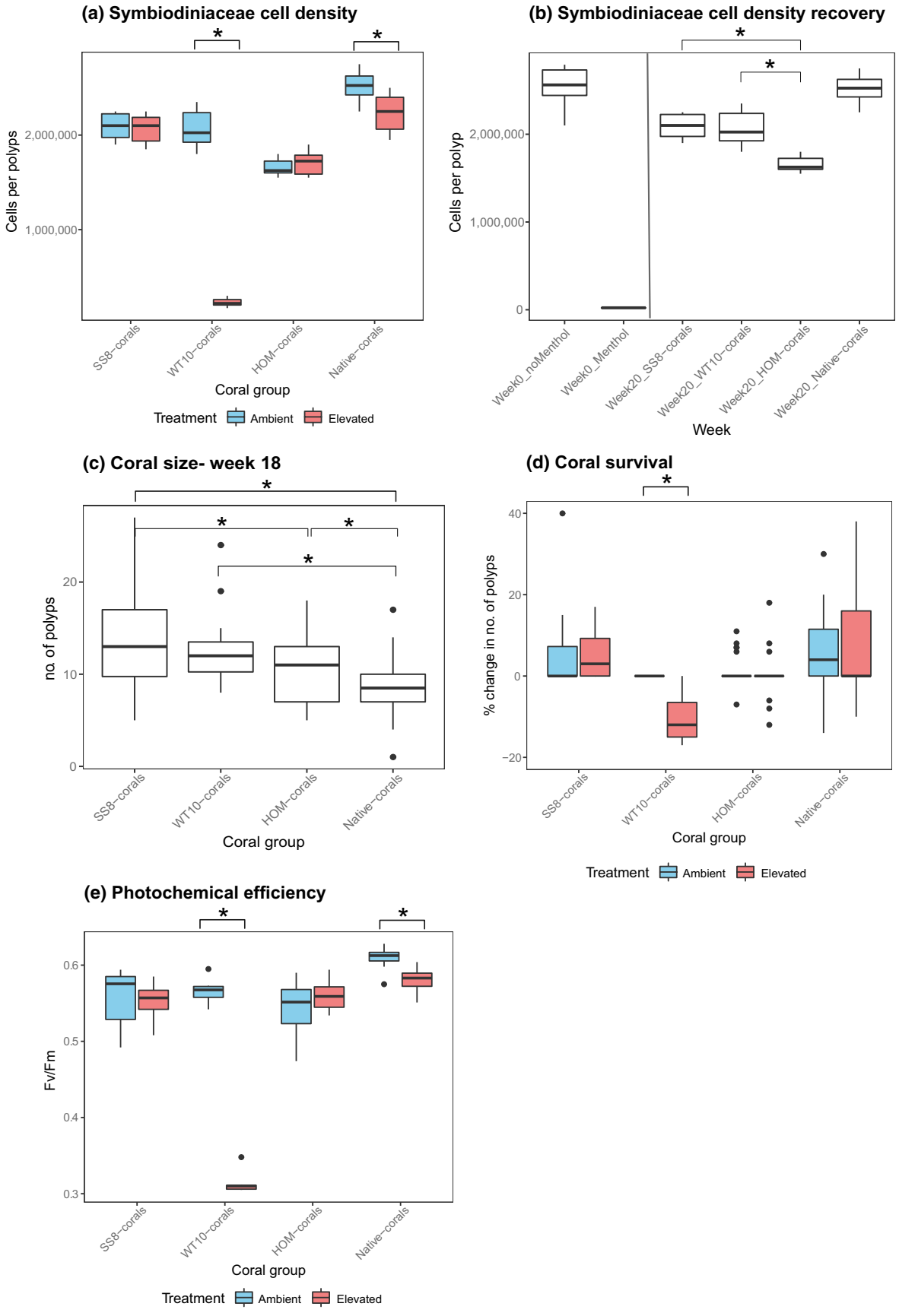


FIGURE 7 Physiological responses and coral size of the four coral groups. (a) Symbiodiniaceae cell density per coral polyp under ambient and elevated temperatures. (b) Symbiodiniaceae cell density per coral polyp before, immediately after, and 20 weeks after menthol treatment (at the end of the heat stress experiment). (c) Coral size (number of polyps) 18 weeks after first the inoculation; corals were exposed to ambient temperatures during this time. (d) Coral survival, presented as the percentage of change in the number of polyps. (e) Coral photochemical efficiency (maximum quantum yield, F_v/F_m). The Symbiodiniaceae treatment of the coral group included: heat-evolved *Cladocopium proliferum* (SS8-corals), wild-type *C. proliferum* (WT10-corals), freshly isolated, homologous Symbiodiniaceae from *Galaxea fascicularis* (HOM-corals). Control corals with no menthol treatment nor inoculation that were natively dominated by *Durusdinium* sp. were also included (Native-corals). "*" Indicates statistical significance. For simplicity, statistical significance is only shown for temperature effect in (a), (d) and (e) and not for coral group effect.

as assessed by their size at the end of this period. SS8-, WT10-, and HOM-corals grew more than Native-corals (GLMER, $p < .001$, <.001, =.028, respectively); SS8-corals also grew faster than HOM-corals (GLMER, $p = .003$) (Figure 7c; Table S7). The variance component of the random effect (genotype) was estimated at, or very close to zero in all cases. The median size after the 18-week period was 13 polyps (SS8-corals), 12 polyps (WT10-corals), 11 polys (HOM-corals), and 8.5 polyps (Native-corals) (Figure 7c). Survival over this period was 100% for all coral groups. At the end of the heat stress experiment, coral group had a significant effect on survival (Kruskal–Wallis, $\chi^2 = 20.07$, df = 3, $p < .001$), with WT10-corals having the lowest survival (Dunn test, $p < .001$, .015, <.001, respectively) (Figure 7d; Table S8). WT10-corals were the only group that differed in survival between ambient and elevated temperatures (12% mortality; Kruskal–Wallis, $\chi^2 = 9.02$, df = 1, $p = .003$).

3.2.4 | Photochemical efficiency

Under elevated temperature, photochemical efficiency of SS8- and HOM-corals did not differ from their ambient counterparts (GLM, $p = .56$, .06, respectively) (Figure 7e; Table S9). In contrast, both WT10- and Native-corals had lower photochemical efficiency under elevated temperatures than under ambient temperatures (GLM, $p < .001$ for both) (Figure 7e; Table S9). This pattern was already significant halfway through the heat stress experiment (day 4) (Figure S3). At the end of the heat stress experiment, WT10-corals exhibited a 44% difference in photochemical efficiency between ambient (F_v/F_m averaged 0.57) and elevated temperature (0.32), while Native-corals showed only 5% difference (ambient averaged 0.61; elevated 0.58). Under elevated temperature, SS8-, HOM-. and Native-corals had higher photochemical efficiency than WT10-corals (GLM, $p < .001$ for all, Table S9). Under ambient temperature, the photochemical efficiency of HOM-corals was slightly lower than that of WT10-corals (GLM, $p = .047$, difference = 0.02). The variance component of the random effect (genotype) was estimated at, or very close to zero in all models.

The history of menthol treatment had a small yet significant effect on coral photochemical efficiency under ambient temperature (GLM, $p < .001$ for all, F_v/F_m difference = 0.06) (Table S9). Despite the drop in photochemical efficiency in Native-corals under elevated temperature, Native-corals had higher photochemical efficiency than all coral groups (SS8-, WT10-, HOM-corals) that were treated with menthol and inoculated with Symbiodiniaceae, under both ambient and elevated temperatures (Table S9).

3.2.5 | Metabolite profiles

Using MSI, 418 metabolites were detected across samples following noise removal, 225 (61%) of which were annotated (Table S10, also see metabolite profile raw data on *zenodo*). Under elevated temperature, ANOVA detected 182 metabolites with significant differences in relative intensities between SS8-, WT10-, and HOM-corals (Table S11). A heatmap of the top 60 significant metabolites showed that half of the SS8-corals replicates clustered with HOM-corals, while the other half clustered with WT10-corals (Figure S4). *t*-Test showed that only 13 metabolites were significantly different between SS8- and WT10-corals under elevated temperature, with the majority (~40%) of these being algal pigments localized within the algal symbionts (Figures 8 and 9; Figure S5; Table S12). When these algal pigments were visualized, WT10- and SS8-corals displayed contrasting pigment profiles under elevated temperature, with SS8-corals having a similar profile to HOM-corals (Figure 8c). SS8- and HOM-corals did not differ in relative intensity in all algal pigments annotated (Figure 8c,d). One of these pigments (m/z 524.17) was completely absent in WT10-corals under elevated temperature, but was highly abundant in SS8- and HOM-corals (Figure 8d).

WT10-corals experienced the greatest difference in metabolite profile between ambient and elevated temperatures, with the intensities of 66 of 418 metabolites (~16%) being significantly different (Figure 10a; Table S13). In contrast, SS8-corals only had 12 significantly different metabolites (~3%) between temperatures (Figure 10b; Table S14). HOM- and Native-corals, both dominated by *Durusdinium*, showed no difference in metabolite profile between ambient and elevated temperatures. WT10-corals subjected to elevated temperature showed downregulation of three major metabolite classes: glycerophosphocholine (PC, 11 species), ceramide (Cer, 7 species), and betaine lipid (DGCC/MGDG/DGTA/DGTS, 10 species), with a FC ranging from 1.5 to 81 and with an average of 4.8 (Figure 10a; Table S13). SS8-corals exposed to elevated temperature also showed downregulation of the same three metabolite classes (PC, four species; Cer, two species; betaine lipids, three species), but the number of significantly different metabolites and their

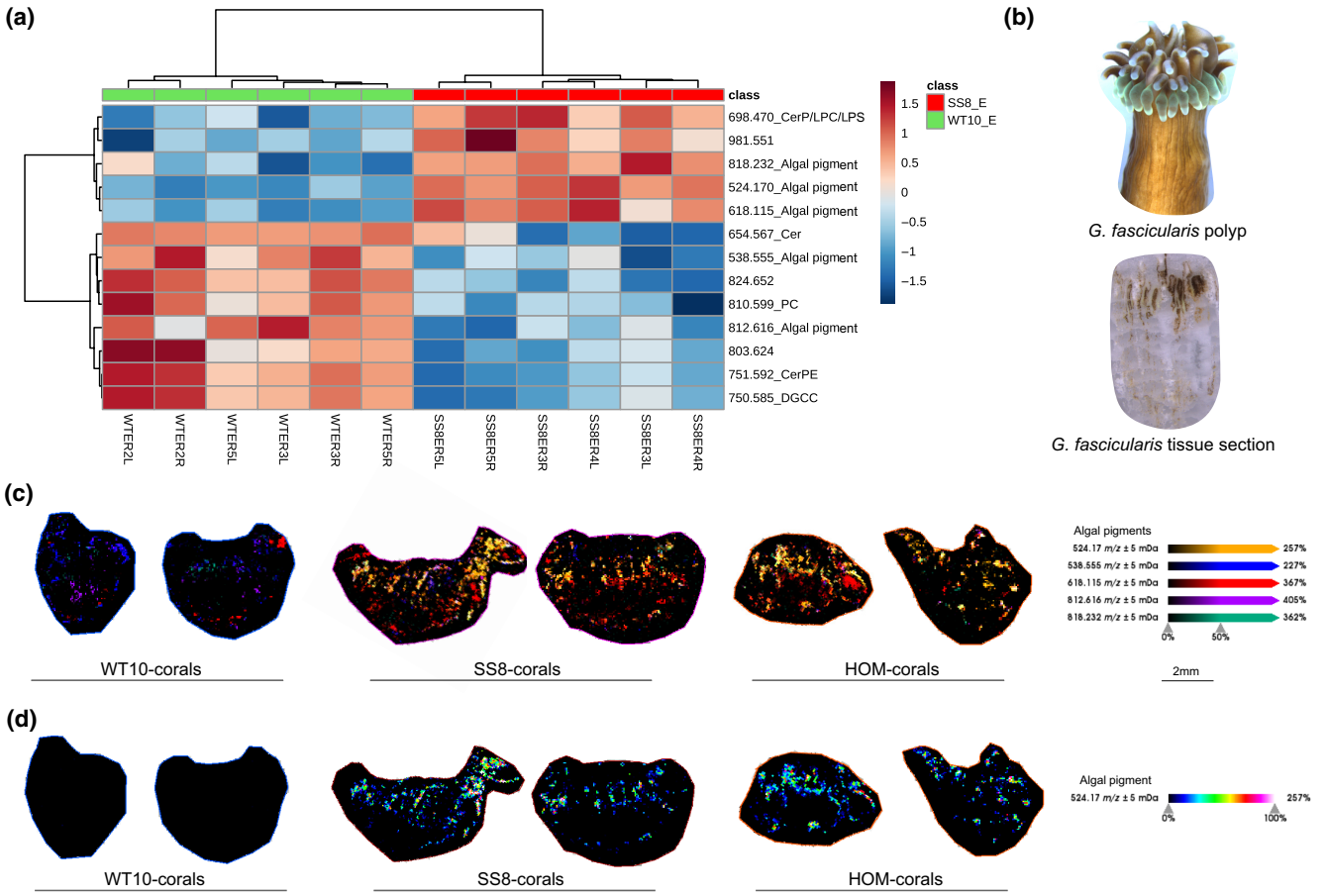


FIGURE 8 (a) Heatmap and annotations of the 13 metabolites with significant intensity differences between SS8- and WT10-corals under elevated temperature. The color scale indicates $\log_2$ fold change relative to the mean. The similarity between samples or between metabolites was calculated based on Euclidean distance and clustered with Ward clustering algorithm. (b) Images of a *Galaxea fascicularis* polyp and its tissue section at 12 µm thickness. (c) Algal pigment profile of WT10-corals (hosting wild-type *Cladocopium proliferum*), SS8-corals (hosting heat-evolved *C. proliferum*), and HOM-corals (hosting *Durusdinium trenchii* and *D. glynnii*) under elevated temperatures. (d) Localization and intensity of the algal pigment *m/z* 524.17. E = elevated temperature. The number after "R" refers to the sample's biological replicate number. L = technical replicate 1, R = technical replicate 2.

FC was much smaller (ranging from 1.9 to 4.5 and an average of 3) (Figure 10b; Table S14).

The localization of PC and betaine lipids is consistent with our previous study (Chan et al., 2023). PCs overlap completely with coral host, indicating that these are host signals (Figure 11a,b). Betaine lipids, in contrast, are concentrated in the coral tentacles, with little or no intensity along the tissue section boundary (Figure 11b,c). Along the coral tentacles, betaine lipids are found within the location of PCs, but always covering a smaller area than PCs, suggesting that betaine lipids are being packaged inside coral host tissues.

Under ambient temperature, ANOVA detected 308 significant metabolites among coral groups. A heatmap of the top 60 most significant metabolites showed no separation among the menthol-treated/inoculated groups (SS8-, WT10-, HOM-corals), however, clear clustering was observed between this group and control groups (non-menthol-treated; Native-, NativeG2-coral) (Figure S6). Menthol-treated/inoculated groups consistently showed an upregulation of six ceramides species, compared to the control groups, regardless of their dominant symbiont (*Cladocopium* or *Durusdinium*).

3.3 | Summary of coral physiological performance and metabolite profile

In summary, under ambient temperature WT10- and SS8-corals (both dominated by *C. proliferum*) had similar performance and performed better than HOM-corals (dominated by *D. trenchii* and *D. glynnii*) (Table 4). The highest resilience to elevated temperatures was seen for SS8- and HOM-corals, whereas WT10-corals showed signs of stress under elevated temperatures (Table 5).

3.4 | Long-term monitoring and mock community

Heat-evolved *C. proliferum* (SS8) was detected in all SS8-corals with moderate relative abundance 1 year (averaged 32%) and 2 years

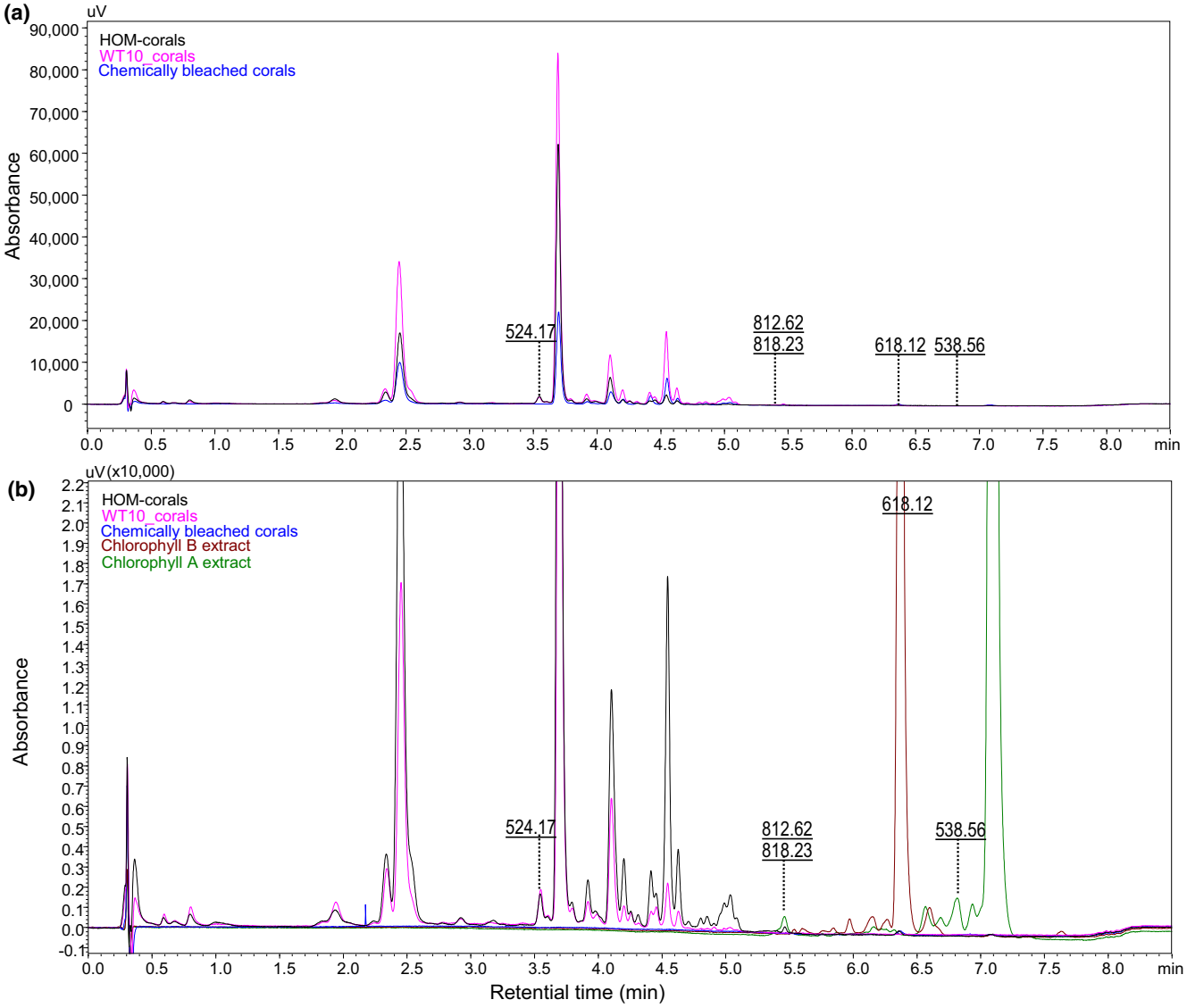


FIGURE 9 (a) High-performance liquid chromatography-ultraviolet chromatogram absorbing at 430 nm of the lipophilic pigment extracts of three coral samples, run in tandem with liquid chromatography–mass spectrometry to identify masses related to pigments. Each sample consisted of four coral polyps pooled from WT10-corals, HOM-corals, and chemically bleached corals. Note that these samples were for pigment annotation only and were not intended for quantitative comparison between experimental groups. (b) Overlay of the pigment profiles of the coral samples (black, pink, and blue traces) with chlorophyll B (brown trace) and chlorophyll A (green trace) extracts and their related pigments. The peaks of the algal pigments shown in Figure 8 are indicated here.

(averaged 42%) after first inoculation, suggesting long-term stability of this novel symbiosis (Figure 12).

4 | DISCUSSION

This study tested the potential of heat-evolved *C. proliferum* to establish symbiosis with adult corals and enhance their bleaching tolerance. We demonstrated that heat-evolved *C. proliferum* can indeed enhance adult coral thermotolerance without a trade-off against growth under ambient temperature, and that this novel symbiosis can persist long term (2 years).

4.1 | Heat-evolved *C. proliferum* can form a symbiosis with adult corals despite being a heterologous symbiont

While heat-evolved *C. proliferum* (SS8) has been experimentally evolved (Chakravarti et al., 2017) and maintained at 31°C for ~10 years, it can form a symbiosis with adult GBR *G. fascicularis*, to which it is a heterologous symbiont. SS8 can also colonize aposymbiotic coral larvae and juveniles of a taxonomically distant species (*A. tenuis*) (Buerger et al., 2020; Quigley et al., 2023; Quigley & van Oppen, 2022), indicating it can potentially be a reef restoration resource applicable to corals across taxa and life stages. Consistent

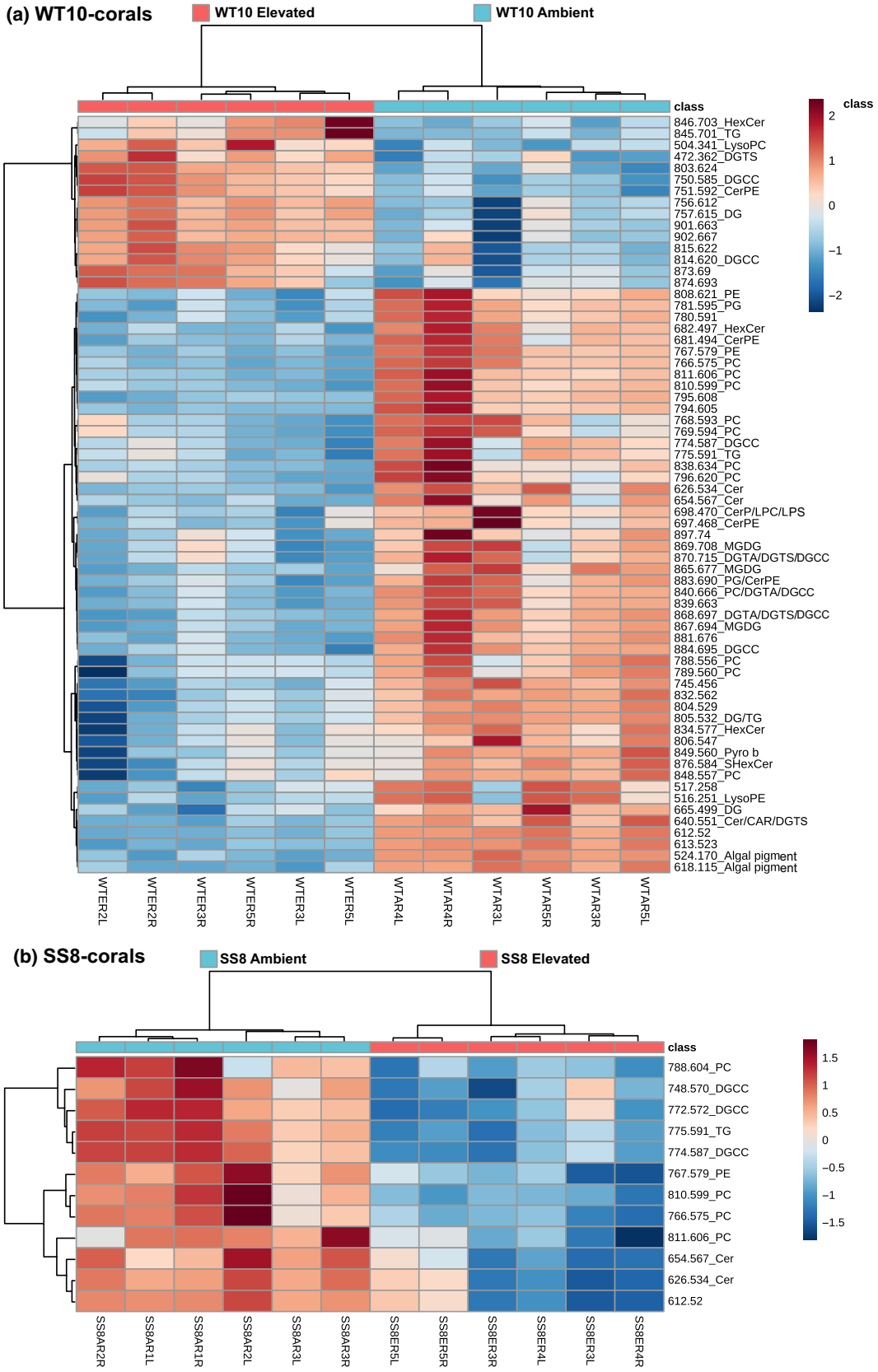


FIGURE 10 (a) Heatmap of the 66 metabolites with significantly different intensities between ambient and elevated temperatures in WT10-corals. The similarity between samples or between metabolites was calculated based on Euclidean distance and clustered based on Ward clustering algorithm. (b) Heatmap of the 12 metabolites with significantly different intensities between ambient and elevated temperatures in SS8-corals. The color scale indicates $\log_2$ fold change relative to the mean. A = ambient temperature, E = elevated temperature. The number after "R" refers to the sample's biological replicate number. L = technical replicate 1, R = technical replicate 2.

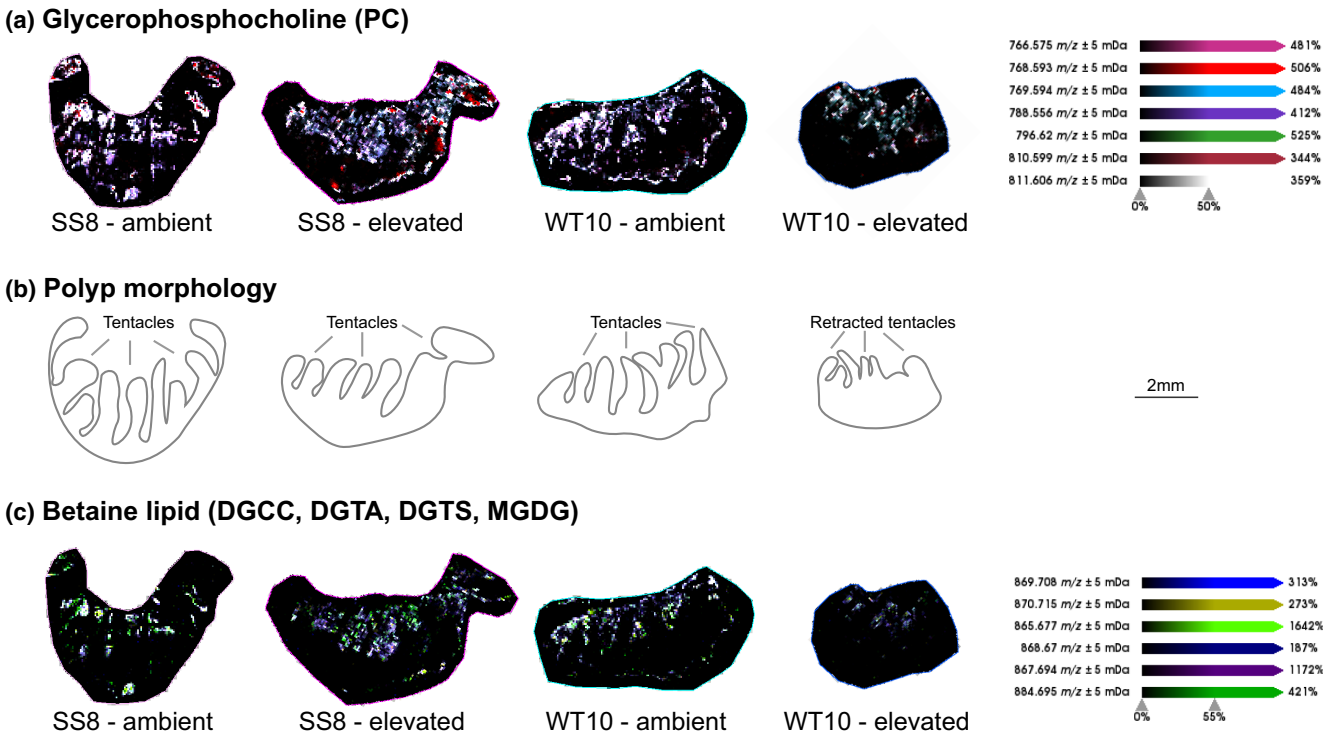


FIGURE 11 (a) Relative intensities and spatial distribution of glycerophosphocholine (PC) in SS8- and WT10-corals selected from the heatmaps (Figure 10). (b) Polyp morphology of the coral samples. Note the WT10-corals retracted their tentacles under elevated temperature. (c) Relative intensities and spatial distribution of betaine lipid (DGCC, DGTA, DGTS, MGDG; see Table S10) in SS8- and WT10-corals selected from the heatmaps (Figure 10). DGCC, diacylglycerylcarboxyhydroxymethylcholine; DGTA, diacylglycerylhydroxymethyltrimethylalanine; DGTS, diacylglyceryltrimethylhomoserine; MGDG, monogalactosyldiacylglycerol.

with previous studies (Puntin et al., 2023; Scharfenstein et al., 2022; Wang et al., 2012), menthol treatment was an effective way to produce corals with minimal native Symbiodiniaceae (<1%–2% of pre-bleaching densities), but did not produce fully aposymbiotic corals. Nevertheless, the presence of remnant native *Durusdinium* sp. did not prevent the uptake of heterologous *C. proliferum* in adult *G. fascicularis* (two of four coral genotypes for heat-evolved strain, one of four genotypes for wild-type strain). Although the inocula accounted for a small proportion of the endosymbiotic Symbiodiniaceae communities 7 weeks after the first inoculation, they continued to populate the corals, achieving a moderate to high relative abundance (43%–82%) by 20 weeks. This suggests that the generalist species, *C. proliferum*, is compatible with *G. fascicularis*. While SS8 and WT10 originated from the same mother culture from 10 years ago, SS8 was taken up by two of the four coral genotypes tested but WT10 was only taken up by one genotype. Thus, intraspecific host genotypic effects play a role in symbiont uptake.

The inoculation outcome of WT10 (Figure 5) was consistent with that of Scharfenstein et al. (2022) on six other adult coral species, all of which formed a symbiosis with WT10 at consistently high relative abundance. However, the above study only used one genotype per coral species, therefore host genotypic effects could not be evaluated. The proportion of WT10 in *G. fascicularis* was fairly homogeneous among replicates, but the relative abundance of SS8 varied across replicates from the same coral genotype under both temperatures (Figure 6). This contrasting pattern may have been a consequence of different growth rates between WT10 and SS8, which could have determined the competition outcome with native *Durusdinium* sp.

Under ambient temperature, in vitro growth rates are higher for *C. proliferum* than *D. trenchii* or *D. glynnii* (C. Alvarez-Roa, personal communication, June 16, 2023), and WT10 grows faster than SS8 (Buerger et al., 2020). Assuming growth rate differences persist *in hospite*, WT10 was likely able to colonize the corals with little competition from the remnant, more slowly growing native *Durusdinium* sp., resulting in a consistently high relative abundance among replicates. SS8, in contrast, had less of an advantage in growth rate and was therefore likely experiencing stronger competition with native *Durusdinium*. Given that each individual coral fragment varied slightly in Symbiodiniaceae cell density after menthol treatment (Figure 3a) and hence in competition intensity with native *Durusdinium* sp., SS8-corals were expected to have less predictable and less consistent inoculation outcomes even for the same coral genotype. In line with this, little replicate variability was found in aposymbiotic coral larvae inoculated with WT10 or SS8, where competition from native symbionts was absent (Buerger et al., 2020).

Despite the inoculation success observed here, a failure of symbiosis establishment between bleached adult corals and exogenously supplied, laboratory cultured, heterologous Symbiodiniaceae has been reported in several earlier studies (Coffroth et al., 2010;

TABLE 4 Summary of inoculation outcome and physiological performance of coral groups under ambient temperature, 20 weeks after the first inoculation.

Menthol treatment	Coral group	Dominant Symbiodiniaceae species	Symbiodiniaceae community-% of inoculum	Symbiodiniaceae cell density relative to pre-menthol level	Survival	Size (number of polyps)[a]	Photochemical efficiency
Yes	SS8-corals	*Cladocopium proliferum*	63%	82%	No diff.	13	0.56
Yes	WT10-corals	*C. proliferum*	79%	82%	No diff.	12	0.57
Yes	HOM-corals	*Durusdinium trenchii*, *D. glynnii*	N/A[b]	65%	No diff.	11	0.55
No	Native-corals	*D. trenchii*, *D. glynnii*	N/A[c]	99%	No diff.	8.5	0.61

Note: Dark red indicates a strong/moderate negative effect, whereas light red indicates a mild (≤10% change) negative effect.

[a]To utilize all coral replicates for size assessment, coral size was measured 18 weeks after the first inoculation instead of 20 weeks, before the replicates were split into ambient and elevated temperatures. All fragments consisted of three to four polyps at the start of the experiment.

[b]The inoculum had the same ITS2 profile as Native-corals, hence it was not possible to distinguish the proportion of *Durusdinium* acquired via the uptake of inoculum versus via the regrowth of remaining native Symbiodiniaceae after menthol treatment.

[c]Native-coral did not undergo menthol bleaching nor inoculation.

TABLE 5 Comparison of the Symbiodiniaceae community, physiological performance, and metabolite profile within coral groups under ambient versus elevated temperatures, at the end of the short-term heat stress experiment (i.e., 8 days exposure to 32°C).

Menthol treatment	Coral group	Dominant Symbiodiniaceae species	Symbiodiniaceae community change	Symbiodiniaceae cell density difference	Survival difference	Photochemical efficiency difference	No. of metabolites of significantly different intensities
Yes	SS8-corals	*Cladocopium proliferum*	No change	0%	+3%	No change	12
Yes	WT10-corals	*C. proliferum*	−21% of WT10	−89%	−12%	−44%	66
Yes	HOM-corals	*Durusdinium trenchii*, *D. glynnii*	No change	0%	No change	No change	0
No	Native-corals	*D. trenchii*, *D. glynnii*	No change	−10%	No change	−5%	0

Note: Dark red indicates a strong/moderate negative effect, whereas light red indicates a mild (≤10% change) negative effect.

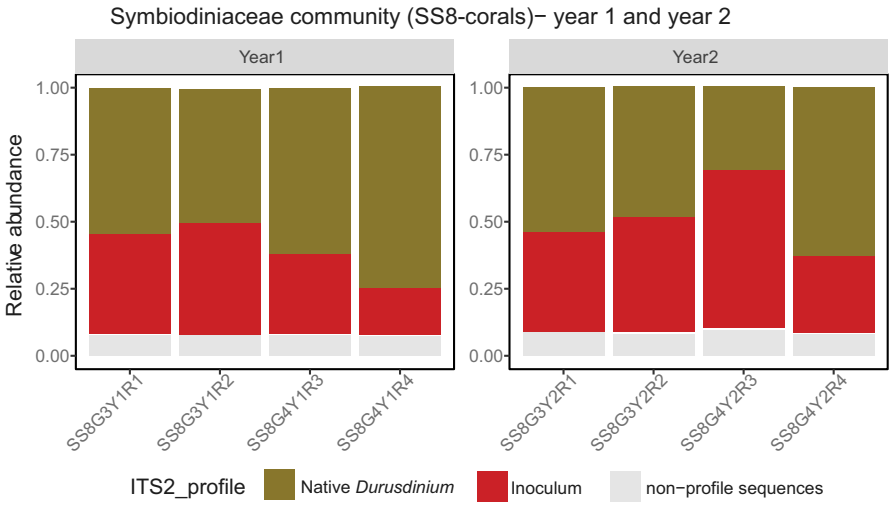


FIGURE 12 Coral-associated Symbiodiniaceae communities of SS8-corals 12 year and 2 years after the first inoculation of heat-evolved *Cladocopium proliferum* (SS8 strain). "G" refers to the sample's genotype, "R" refers to the sample's replicate number.

Morgans et al., 2020). Possible factors that may have been responsible for these contrasting results include low inoculation cell densities, the absence of feeding and different temperatures during inoculation. This study and Scharfenstein et al. (2022) employed ~10^4 cells mL^{-1} (i.e., 10 times more than in Coffroth et al., 2010) and added food (*Artemia nauplii* or microalgae) during inoculation to encourage phagocytosis. Given that these adult corals contained remnant native Symbiodiniaceae, a high inoculation cell density and food supply may be vital for successful colonization by exogenous Symbiodiniaceae. In Morgans et al. (2020), inoculation was conducted under elevated temperature (32°C), and it has previously been shown that symbiont uptake is limited under elevated temperatures (Abrego et al., 2012). Given the significant potential for the use of adult coral inoculation experiments in designing tools for reef restoration, testing parameters to achieve optimal inoculation success is a priority area for future studies.

4.2 | *C. proliferum* accelerated recovery post-menthol treatment

While SS8-, WT10-, and HOM-corals underwent the same menthol treatment and inoculation process, the two groups dominated by *C. proliferum* (SS8- and WT10-corals) recovered faster than the group dominated by *D. trenchii* and *D. glynnii* (HOM-corals) (note that recovery occurred under ambient temperature). This is evidenced by higher Symbiodiniaceae cell density (SS8- and WT10-corals), coral size (SS8-corals) and photochemical efficiency (WT10-corals) compared to HOM-corals (Table 4; Figure 7b,c; Sections 3.2.2–3.2.4). While these corals were inoculated with the same Symbiodiniaceae cell density, SS8- and WT10-corals had recovered to ~82% of pre-menthol treatment level cell density, while HOM-coral only recovered to ~65% by 20 weeks after the end of menthol treatment. Several studies have also shown the advantages of symbiosis with *Cladocopium* under favorable, ambient temperatures on the GBR. For example, *A. tenuis* juveniles dominated by *Cladocopium* C1 grew two to three times faster in the field than those dominated by *Durusdinium* D1 (Little et al., 2004). Adult *A. millepora* dominated by *Durusdinium* sp. grew 29% and 38% slower than conspecifics dominated by *Cladocopium* C2 in the laboratory and field, respectively (Jones & Berkelmans, 2010). Based on these findings, the inoculation with laboratory cultured *C. proliferum* is a possible tool to accelerate coral recovery following mass bleaching events, as long as the seawater temperature has returned to and continues to stay at ambient. However, wild-type *C. proliferum* showed slower growth, lower photo-physiological performance, and more ROS production than heat-evolved *C. proliferum* under elevated temperature (31°C) in vitro (Buerger et al., 2020). This indicates that corals inoculated with wild-type *C. proliferum* may not perform as well as those inoculated with heat-evolved wild-type *C. proliferum* under a marine heatwave event.

4.3 | Heat-evolved Symbiodiniaceae conferred high bleaching tolerance to adult corals

Our findings showed corals hosting the wild-type *C. proliferum* were heat sensitive while corals hosting the heat-evolved strain SS8 were as tolerant to elevated temperature as HOM-corals. These results demonstrate that heat-evolved *C. proliferum* can confer a similar level of thermal tolerance to *G. fascicularis* as the naturally thermally tolerant *D. trenchii* and *D. glynnii*. In contrast, WT10-corals underwent 12% mortality, a 44% drop in photochemical efficiency and a 89% decline in Symbiodiniaceae cell density (Table 5). Under elevated temperature, WT10-corals shifted toward a more *Durusdinium*-dominated community and reduced the proportion of WT10 by 21%. *G. fascicularis* may have preferentially removed WT10 due to its high level of ROS released (Buerger et al., 2020) and its lower carbon acquisition ability than *Durusdinium* sp. under elevated temperature (Baker et al., 2013). Alternatively, WT10 may have had a higher mortality rate under elevated temperature.

Shuffling and/or switching to *Durusdinium* sp. is a commonly reported response to elevated temperatures and is interpreted as an adaptive response to warming (Berkelmans & van Oppen, 2006;

Boulotte et al., 2016; Chen et al., 2005; Mieog et al., 2007; Silverstein et al., 2015; Stat et al., 2013). Heat-evolved *C. proliferum* may be superior to wild-type *D. trenchii* and *D. glynnii* as it recovers to pre-bleaching densities faster when the temperature is benign and confers higher coral growth rates. The metabolite data confirmed that some of the SS8-coral replicates were metabolically more similar to *Durusdinium*-dominated coral under elevated temperature. The physiological data also align closely with the corals' metabolite profiles, with WT10-corals showing the most significant difference between ambient and elevated temperatures. No metabolite profile change was observed in *Durusdinium*-dominated corals under elevated temperature, as expected, and this was consistent with the physiological data where no (HOM-corals) or limited (Native-corals) negative effects were observed.

Importantly, the enhanced thermal tolerance of SS8-corals did not show a trade-off with coral growth under ambient temperature (Figure 7c; Section 3.2.3), which is typically found in *Durusdinium*-dominated corals from the GBR (Cunning et al., 2015; Jones & Berkelmans, 2010; Little et al., 2004). This makes heat-evolved *C. proliferum* a promising candidate for reef restoration.

4.4 | Implications of contrasting metabolite profiles between heat-evolved and wild-type holobionts

Nearly 40% of the metabolites that significantly differed in relative intensities between WT10- and SS8-corals under elevated temperature are pigments, co-localized with the algal symbionts. In contrast, SS8- and HOM-corals did not differ in relative intensity for all algal pigments annotated (Figure 8c,d), despite their dominant algal symbionts belonging to two different genera (*Cladocopium* vs. *Durusdinium*). Considering that WT10 and SS8 are both *C. proliferum*, such contrast in their pigment profile was unexpected. The detected pigments are minor carotenoid-like pigments and can be found co-varying with chlorophyll levels. Carotenoid pigments are generally biosynthesized by autotrophic marine organisms. They are known to protect the photosynthetic machinery in a number of ways, including by (1) limiting lipid peroxidation, (2) scavenging singlet oxygen ($^1O_2$), (3) preventing the formation of $^1O_2$ by reacting with chlorophyll in its triplet state (^{3}Chl), and (4) dissipating the excess excitation energy formed through the xanthophyll cycle (Das & Roychoudhury, 2014; Galasso et al., 2017). Carotenoid-like pigments mostly protect lipid membranes (by their associated lipophilicity). The contrasting algal pigment profiles of WT10- and SS8-corals suggest that a difference in photoprotective mechanisms may contribute to the enhanced thermal tolerance of SS8-corals.

Another notable difference is a downregulation of seven ceramide species in WT10-corals. Ceramides are intermediates for the biosynthesis and metabolism of sphingolipids. This lipid class is involved in cell signaling, and regulates the balance between apoptosis, cell survival, and proliferation (Bhattacharya, 2022; Rosset et al., 2021); several lines of evidence point toward its regulatory role in the cnidarian-Symbiodiniaceae symbiosis (Detournay & Weis, 2011; Kitchen et al., 2017; Kitchen & Weis, 2017; Rodriguez-Lanetty et al., 2006). Its downregulation in WT10-coral suggests that acute heat stress may have caused the corals to stop acquiring exogenous Symbiodiniaceae and to increase algal symbiont expulsion to avoid further cellular damage by ROS. Under elevated temperature, both *Cladocopium*-dominated groups (WT10- and SS8-corals) showed downregulation of PC, Cer, and betaine lipids, suggesting that these metabolite classes could be a *Cladocopium*-specific response to high temperatures. Nevertheless, compared with WT10-corals, SS8-corals experienced much smaller FCs and a much lower number of significantly different metabolites.

4.5 | Equilibrium symbiont levels were not reached over a 20-week period

Despite a long (20 weeks) recovery period following symbiont inoculation, none of the menthol-treated coral groups had recovered pre-treatment Symbiodiniaceae cell densities and had slightly lower photochemical efficiency than control (non-menthol-treated) corals. HOM-corals were inoculated with homologous Symbiodiniaceae and should achieve the same cell density and photochemical efficiency as Native-corals when fully recovered, whereas SS8- and WT10-corals may arrive at a different equilibrium level when fully recovered given they are a different species to the native symbionts. Nevertheless, it is worth noting that the dark-adapted yields of the menthol-treated coral groups (0.55–0.57) under ambient temperature were within the healthy range for corals.

While menthol is widely used to remove native Symbiodiniaceae in the coral model sea anemone, *Exaiptasia diaphana* ('Aiptasia') (Gabay et al., 2019; Matthews et al., 2016; Tsang Min Ching et al., 2022), and more recently in adult corals (e.g., Puntin et al., 2023; Scharfenstein et al., 2022), little is known of its potential long-term effect on cnidarians. Menthol is a cyclic terpene alcohol and has been hypothesized to cause coral bleaching via either photoinhibition of Symbiodiniaceae (Wang et al., 2017) or via Ca^{2+}-triggered exocytosis by binding to the transient receptor potential TRPM8 (Pang & Südhof, 2010). Matthews et al. (2016) reported no difference in survival, photosynthesis and respiration performance between menthol-treated and inoculated Aiptasia and untreated symbiotic anemones by 12 weeks. In the coral, *Isopora palifera*, a significant reduction (44%–50%) in glutamate dehydrogenase activity, total free amino acids and essential free amino acids, as well as a change in dominant free amino acid species, were found in adults 6–10 days after menthol treatment, reflecting major effects on the holobiont's nitrogen metabolism (Wang et al., 2012). In contrast, no such change was observed in *Stylophora pistillata* that underwent the same menthol treatment. However, these observations were based on freshly bleached corals and no longer-term monitoring has been conducted.

Our data detected the legacy of menthol treatment 20 weeks after its cessation, which could be a consequence of menthol effect and/or bleaching effect. MSI results showed a clear separation between the menthol-treated and control groups under ambient temperature. Among the top significant metabolites, ceramide species

stood out as one of the key contributing groups. Compared to control corals, all menthol-treated/inoculated groups had consistently higher relative intensity in these ceramide species. Higher ceramide relative intensity, together with the fact that these corals have not recovered to pre-treatment level cell density, suggests that these corals could still be in the active process of regulating and incorporating exogenous Symbiodiniaceae.

4.6 | Implications for reef restoration, limitations, and future directions

We have demonstrated for the first time that heat-evolved Symbiodiniaceae can form a symbiosis with adult corals (two of the four coral genotypes) and that this novel symbiosis persists for at least 2 years. Compared to the naturally heat tolerant *D. trenchii* and *D. glynnii*, heat-evolved *C. proliferum* (1) promoted faster coral recovery from chemical bleaching under ambient temperature, and (2) conferred thermal bleaching tolerance to adult corals to a similar extent as *Durusdinium* but without trade-off against coral growth. Since heat-evolved *C. proliferum* can also form a symbiosis with and confer thermotolerance to coral larvae and recruits of a taxonomically distant species, these symbionts are a potentially valuable resource for reef restoration applicable across coral species and life stages. Enhanced thermotolerance will "buy time" for reefs until carbon emission is curtailed and the climate is stabilized. The long-term stability of the symbiosis with heat-evolved *C. proliferum* offers hope that this symbiont population could self-sustain and may continue to provide benefits to the coral hosts for many years. However, it is also possible that heat-evolved *C. proliferum* could lose its thermotolerance after long-term exposure to ambient temperature, and this is an important avenue for future studies.

We acknowledge that this study was laboratory-based using only one coral species and one experimentally evolved Symbiodiniaceae strain and recommend future studies to: (1) expand to multiple coral species, (2) utilize multiple experimentally evolved Symbiodiniaceae strains, and (3) test the performance of corals hosting heat-evolved Symbiodiniaceae in the field. To better understand conditions that promote/impede stable establishment of heat-evolved Symbiodiniaceae, future studies can also track a subset of the same inoculated coral fragments over time and across conditions. To maximize the success of utilizing heat-evolved Symbiodiniaceae in reef restoration, the development of best practice protocols (e.g., optimal inoculum cell density, temperature, type of food supplement, and extent of menthol bleaching) is essential. If heat-evolved Symbiodiniaceae can improve thermotolerance of multiple coral species in the field without trade-offs against growth or other vital traits, options for large-scale deployment of heat-evolved Symbiodiniaceae culture directly into the reef environment can then be tested and considered.

AUTHOR CONTRIBUTIONS

Wing Yan Chan: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; supervision; visualization; writing – original draft; writing – review and editing. **Luka Meyers:** Data curation; formal analysis; investigation; visualization; writing – review and editing. **David Rudd:** Data curation; formal analysis; investigation; methodology; resources; software; validation; visualization; writing – review and editing. **Sanjida H. Topa:** Data curation; writing – review and editing. **Madeleine J. H. van Oppen:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All raw data and R codes for statistical analysis are available on https://doi.org/10.5281/zenodo.8336616. Details of each file are provided in Table S15. Metabolite annotations on MetaSpace are available at https://metaspace2020.eu/ (project name: heat-evolved algal symbionts enhance thermotolerance of adult corals without growth trade-off). Raw sequences of the ITS2 Symbiodiniaceae data sets are available in GenBank (project Accession nos.: PRJNA983409; SRR24910332-SRR24910491).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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