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PROGRAM

Assessment and interpretation of Symbiodiniaceae community composition in adult coral x Symbiodiniaceae pairings in mesocosms

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Stability of heat-evolved coral photosymbionts in a mesocosm system

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Title page image: Coral reef, Credit: Gary Cranitch, Queensland Museum

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We specifically acknowledge and thank the following Traditional Owners of sea Country that this report relates to:

Location	**Traditional Owner Group**
National Sea Simulator	Bindal
Davies Reef	Bindal
Falcon and Fantome Reef	Manbarra

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1 Executive Summary

Climate change is intensifying marine heatwaves, which pose a significant threat to reef-building corals by disrupting their symbiotic relationship with photosymbiotic microalgae, or photosymbionts. Elevated temperatures can lead to coral bleaching, where symbiosis degradation compromises coral health and survival.

A potential adaptive strategy is enhancing the thermal resilience of coral photosymbionts. At the Australian Institute of Marine Science experimental evolution has successfully been used to boost heat-resistance of photosymbionts, which confer improved coral resilience under temperature stress without growth trade-offs. However, the long-term stability of these heat-evolved symbionts under natural reef environments remains uncertain.

Here we report on the inoculation of two coral species (*Platygyra daedalea* and *Galaxea fascicularis*) with heat-evolved *Cladocopium* and *Durusdinium* and their long-term persistence in an experimental mesocosm system over nearly two years. The stability of the inocula differed according to the inoculum identity and the target coral. Heat-evolved *Cladocopium* persisted in both *G. fascicularis* and *P. daedalea*, albeit gradually retreating to background levels in *G. fascicularis*. Heat-evolved *Durusdinium* was acquired but failed to persist in *G. fascicularis,* however it was acquired by *P. daedalea* and persisted until at least 550 days post-inoculation (as did heat-evolved *Cladocopium*). This is the longest observation of persistence for these novel symbiont-coral pairings.

We discuss the importance of critical events during the experiment that impact the stability of coral photosymbiont communities, strategies to improve the scaling of this as an intervention, and directions for future research including small scale field studies.

2 Introduction

Climate change is increasing the frequency and severity of marine heatwaves. Reef building corals are particularly sensitive to temperature anomalies, whereby elevated temperatures for prolonged periods compromise the obligate symbiosis with photosymbiotic microalgae (hereafter photosymbionts). In extreme cases, elevated temperatures cause corals to bleach, which refers to the reduction or degradation of photosymbiont cells, revealing the white coral skeleton beneath. In this state corals are severely physiologically compromised, starving, and highly likely to suffer partial or complete mortality. During extreme climate anomalies, global mass coral bleaching events have been responsible for the reduction of up to 34% of coral cover in a single year (Hughes et al., 2018). Repeat occurrences and reduced recovery times between such events underpin predictions that coral reefs face severe ecological dysfunction by the end of century.

One pathway for corals to adapt to climate change is through the genetic and functional diversity of their photosymbionts (Baker et al., 2004; Berkelmans and van Oppen, 2006). There are estimated to be hundreds of such photosymbiont species that can associate with corals (Blackall et al., 2015, Thornhill et al., 2014), and it has been known for decades that variation (at taxonomic levels from genera to species to strains) in the thermal resilience of photosymbionts can impact the performance and/or survival of corals during marine heatwaves. Naturally thermo-tolerant symbionts, however, are expected to come with significant fitness trade-offs for corals (e.g., reduced growth, fecundity, Cunning et al., 2015). Such fitness trade-offs may be undesirable, for example, during intensive reef restoration practices that seek to produce many corals (e.g., under aquaculture settings) and deploy them on recovering reefs. Furthermore, recent marine heatwaves have been so severe that even corals known to harbour such resilient symbionts have exhibited bleaching (Palacio-Castro et al., 2023). As such, there is a need to explore strategies to enhance photosymbiont thermal resilience as a form of coral assisted evolution (van Oppen et al., 2015).

One such assisted evolution approach is experimental evolution, whereby, under laboratory conditions, photosymbiont cultures are subjected to selective agents that target a particular trait (Chakravarti et al., 2017). At the Australian Institute of Marine Science (AIMS), photosymbionts in pure culture at the Symbiont Culture Facility (SCF), specifically *Cladocopium proliferum* and *Durusdinium trenchi* (generalist symbiont species likely forming symbioses with > 100 species of coral on the Great Barrier Reef [GBR]) were subjected to temperatures ratcheted-up to 31 °C, at which some have been maintained for > 10 years (i.e., “selected strains”). Wildtype strains have been maintained at 27 °C for the same duration. *C. proliferum* has been an especially productive model species, and this approach has moved beyond proof of concept with heat-evolved strains performing significantly better than wildtype strains during temperature stress assays in culture (Chakravarti and van Oppen, 2018), and also conferring thermotolerance to coral larvae (Buerger et al., 2020), recruits (Quigley et al., 2023), and adults (Chan et al., 2023), without any clear trade-off against coral growth.

The aforementioned studies were conducted under controlled laboratory conditions. For example, Buerger et al., 2020 worked with symbiont-free coral larvae, which enables tight control of coral-photosymbiont relationships but does not reflect the complexity of natural symbiont communities on reefs, which include up to 11 genera of symbionts free-living in sediments, on the surfaces of macroalgae, or in the water column (Fujise et al., 2021). Furthermore, reef communities are diverse and there are many organisms that can be sources of photosymbionts (conspecific corals containing alternative photosymbionts, other reef building corals, soft corals, anemones, zoanthids, sponges, clams, etc). The long-term stability of heat-evolved symbionts within experimental corals in the presence of such genetic, functional, and environmental diversity is unknown.

3 Experimental Design and Methods

3.1 Experimental corals

We collected 10 genotypes (A-J) of *Galaxea fascicularis* and 10 genotypes (A-J) of *Platygyra daedalea* from Falcon Reef (October 2021). The *P. daedalea* and *G. fascicularis* colonies were divided into 2 x 2 cm fragments using a diamond-blade band saw (N = approx. 70 per colony for *G. fascicularis* and N = approx. 100 per colony for *P. daedalea*). Fragments were glued to aragonite propagation plugs (3 cm diameter) and transferred to indoor, 2800 L semi-recirculating aquaria (8 hour complete turnover) in the National Sea Simulator (27°C and approximately 100 umol photons m2 s-1 on a 12:12 h light:dark cycle with a 2 hour ramp from dark to maximum intensity at dawn and vice versa for dusk) for eight weeks. Corals were fed daily with a mix of Artemia and nutrient enriched rotifers. After acclimation, fragments were transferred to a controlled experimental room which provided the same lighting conditions but utilized a flow-through (rather than recirculating) system, providing 1 µM filtered seawater that was exchanged completely every hour.

Fragments were randomly allocated among four treatments. Three of four treatments require chemical bleaching to reduce the symbiont densities. Briefly, feeding with Artemia and rotifers was paused and three rounds of menthol (0.38 mM, Chan et al., 2023) were applied using three days of exposure followed by three days rest (round one), or four days exposure with three days rest (rounds two and three). During exposure to menthol, a small aquarium pump and aerator was used to prevent anoxic conditions. On the final day of bleaching, feeding was resumed as above. Overall, bleaching was judged to be effective based on strong reduction in pigmentation (Figure 1). Bleached corals were randomly allocated among 30 L treatment tanks (N = 4 tanks per treatment) and inoculated with their assigned treatment.

Two treatments consisted of inoculation with experimentally evolved, thermotolerant strains of *Cladocopium proliferum* (SCF055-01.08, hereafter referred to as SS8) and *Durusdinium trenchii* (SCF086-01.04, hereafter referred to as HSD), a 're-inoculation control' (RIC), which comprised freshly isolated homologous symbionts from conspecific corals randomly sampled from the 10 genotypes, and a Control treatment (not chemically bleached nor inoculated). Inoculation occurred across three phases: phase one - three days with 1500 cells/mL (final concentration), followed by four days rest; phase two – three days with 500 cells/mL (day two) or 750 cells/mL (days two and three), followed by five days rest; and phase three – 1,000 cells/mL (day one) or 500 cells/mL (day two). This phasing was required as culture flasks grew at different rates and densities (e.g., SS8 vs HSD) and, achieving desired final concentrations for an experiment of this scale was challenging (N = 8 inoculation tanks per treatment across both species). Corals recovered for 100 days in this system, at which point (June, 2022) they were ramped down from 27°C to 25°C (0.25°C day) in preparation for transfer to an outdoor mesocosm system on an annual profile.

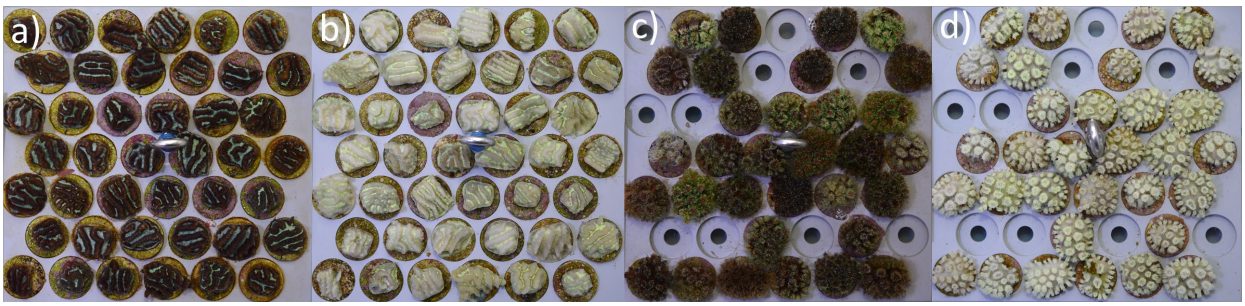

Figure 1: a) Platygyra daedalea fragments prior to chemical bleaching. b) P. daedalea fragments after chemical bleaching. c) Galaxea fascicularis colonies prior to chemical bleaching. d) G. fascicularis colonies after chemical bleaching.

3.2 Mesocosm design

The experimental mesocosms (N = 3) were 2800 L, semi-recirculating aquaria (8-hour turnover) that were pre-conditioned with diverse crustose coralline algae communities. Because of this pre-conditioning, we connected the circulation of all three systems for 24 hours to allow for the exchange of any free-living Symbiodiniaceae populations already present. The mesocosms were situated outdoors under 30% shade cloth. Constant PAR and temperature logging was performed within the systems. Water temperature was programmed to follow an annual cycle representative of Davies Reef at 5m (data averaged from 1998-2015 from the AIMS weather station; Figure 2). We designed the mesocosms to be Symbiodiniaceae-centric, containing hosts (Table 1) known to harbour diverse Symbiodiniaceae species. Corals (N = 4 colonies per species) from Davies Reef (mid-shelf) and Fantome Reef (in-shore) were collected, divided in three, and distributed evenly among the three mesocosms (all colonies present in all systems). From within the National Sea Simulator long term holdings, giant clams (*Tridacna*), anemones (*Actiniaria* and *Exaiptasia*), zoanthids, and foraminfera were acquired and distributed across the mesocosms (Table 1). Each mesocosm was also seeded with 10 L of raw sediment from Fantome Reef. All collections were performed under Great Barrier Reef Marine Park Authority AIMS general permit (G21/38062.1) or project specific permits (G21/45605.1 superseded by G22/46479.1).

The mesocosms were assembled for the long-term holding of experimental *P. daedalea* and *G. fascicularis* fragments. The experimental corals on 3 cm aragonite plugs were inserted into 6 cm x 6 cm x 2 cm (width x length x height) PVC devices during transfer from the experimental room to the mesocosm systems into which they were randomly allocated. Feeding was performed daily as above. Corals were maintained in the system from this point (June, 2022) until peak summer (February, 2023) at which point a simulated heat-wave event was conducted.

Symbiodiniaceae Sources		
Collected From	**Symbiodiniaceae Source**	**Replicates**
Fantome Reef	*Montipora aequituberculata*	4 Colonies
Fantome Reef	*Psammocora sp.*	4 Colonies
Fantome Reef	*Porites lobata*	4 Colonies
Fantome Reef	*Sediment*	NA
Davies Reef	*Acropora spathulata*	4 Colonies
Davies Reef	*Acropora kenti*	4 Colonies
Davies Reef	Diploastrea heliopora	4 Colonies
Davies Reef	Pocillopora sp.	4 Colonies
Davies Reef	Stylophora pistillata	4 Colonies
National Sea Simulator	*Actiniaria*	2 Species
National Sea Simulator	*Zoantharia*	2 Species
National Sea Simulator	*Exaiptasia Pallida*	Many individuals
National Sea Simulator	*Tridacna*	3 Individuals
National Sea Simulator	*Foraminifera*	Numerous species
National Sea Simulator	CCA	Numerous species
National Sea Simulator	Seawater	NA

Experimental Corals		
Collected From	**Species**	**Replicates**
Falcon Reef	*Platygyra daedalea*	10 Colonies
Falcon Reef	*Galaxea fascicularis*	10 Colonies

Table 1: (left) List of Symbiodiniaceae sources including their source location, and the number and type of replicates used. (right) List of experimental corals including their source location and the number of colonies used.

Galaxea fascicularis

Platygyra daedalea

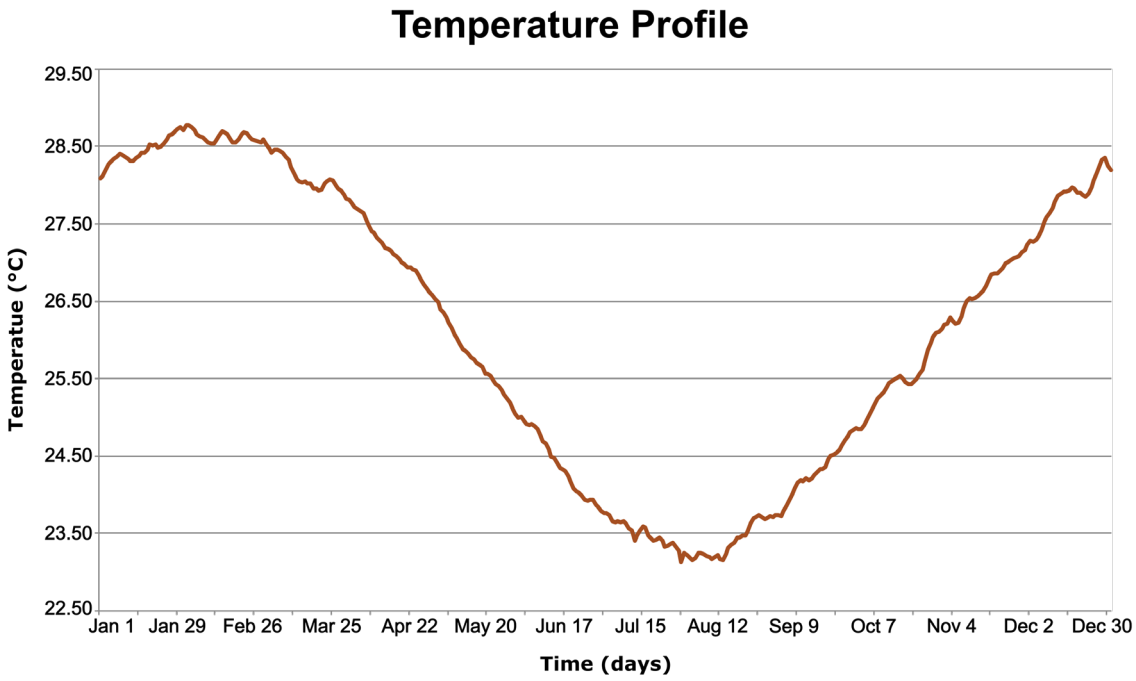

Figure 2: Yearly temperature profile used in the mesocosms based on the 1998-2015 average temperature of Davies Reef at 5 m depth.

3.3 Simulated heat-wave event

We subjected all *P. daedalea* and *G. fascicularis* experimental corals to a simulated heatwave experiment at the end of February 2023, which coincides with the annual maximum monthly mean temperature for Davies Reef (Figure 2). After 263 days in the mesocosm system, the experimental corals from the three replicate tanks were divided in half and equally distributed across two temperature treatments: Ambient (3 replicate tanks) and Elevated (max temperature of 32.25°C, 3 replicate tanks). The temperature of the Ambient treatment was set to the Davies Reef profile (as in Figure 2) and continued following this seasonal profile for the duration of the heat stress experiment. The Elevated treatment temperature was ramped upwards over 3 weeks (0.25°C/day) to +3°C above the maximum monthly mean temperature (29.2 °C for Falcon Reef – the reef from which *P. daedalea* and *G. fascicularis* were collected from) was reached. The maximum temperature was maintained for 20 days, resulting in an accumulation of 11.57 Degree Heating Weeks. Lighting levels were maintained as previously described (approx 100 µmol photons m^2 s^{-1}) with a one-hour ramp from dawn to full intensity and vice versa for dusk. Following heat stress, the corals were ramped down to the Ambient temperature profile and all corals were returned to the mesocosms for recovery.

3.4 Sampling time points

To quantify the symbiont community present within each experimental coral, small tissue biopsies (approx. 3 x 3 mm) were collected using cuticle clippers, rinsed in 0.22 uM filtered seawater to reduce any epi-biotic Symbiodiniaceae, blot-dried with paper towel, and then snap frozen in liquid nitrogen. Samples were transferred to a -80 °C freezer until subsequent DNA extraction (see below) and further genetic analysis. This sampling was performed at nine timepoints for *P. daedalea* and *G. fascicularis* (Figure 3), and a subset of timepoints for the other mesocosm community members. Sampling was designed to coincide with major events in the experiment, including immediately prior to chemical bleaching (Pre-CB), after inoculation/prior to transfer to the mesocosms (T0), throughout winter to summer (T1, T2, T3), at the end of the heat stress experiment (T4), and recovery from heat-stress after return to the mesocosms (T5, T6). Finally, a subset of corals that were to be deployed in the field (see final section on Future Work) were sampled prior to deployment (T7). See Figure 3 for a summary of this timeline.

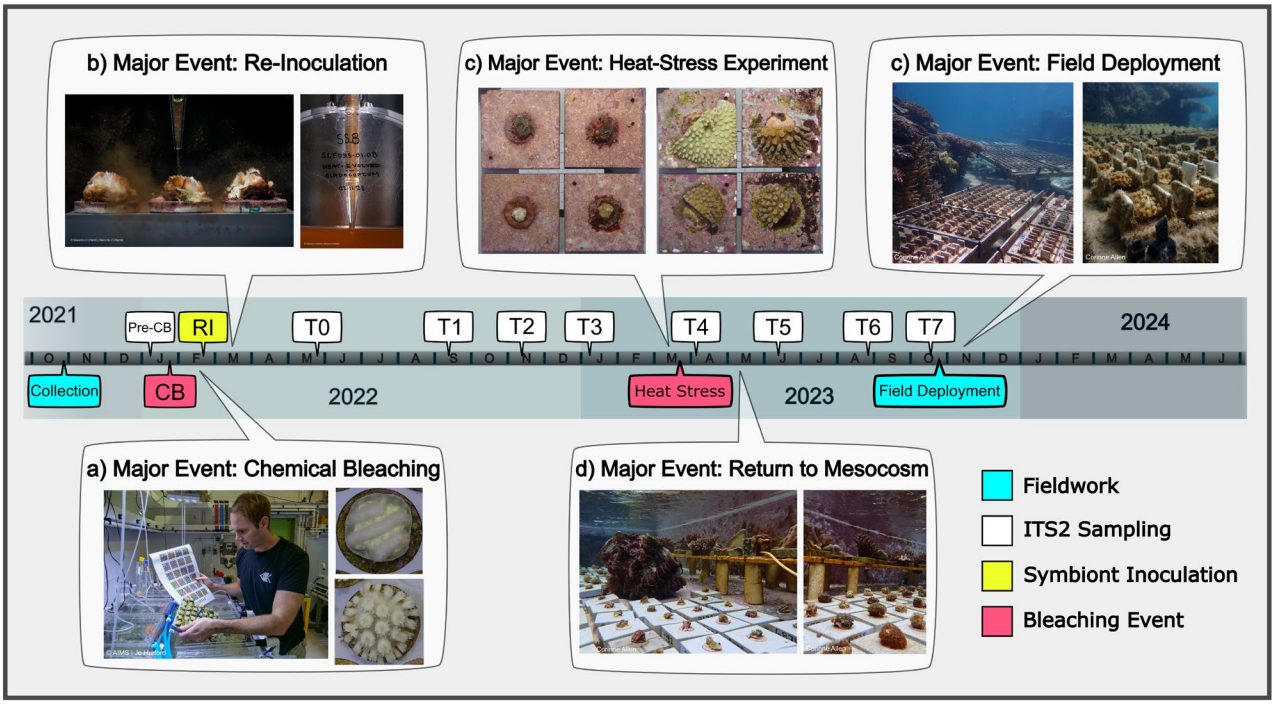

Figure 3: Timeline of the experiment, from colony collection to field deployment. Important events and sampling timepoints are listed on the timeline to clarify when these different activities took place. a) Experimental corals were subjected to three rounds of chemical bleaching. The corals expelled the majority of their native symbiont communities, allowing for greater uptake of the inoculum. b) Experimental corals were divided into three re-inoculation treatments. Each treatment underwent three rounds of inoculation. Corals were given ~100 days to recover before being moved to their respective Mesocosms. c) Experimental corals underwent a thermal stress treatment where each fragment was divided in half. One half remained at ambient temperature while the other was exposed to elevated temperatures. d) Experimental corals were returned to their respective mesocosms allowing for further investigation of the stability of their symbiotic communities. e) Experimental corals were deployed to their point of origin, Falcon Reef.

3.5 ITS2 Sequencing and data analysis

The ITS2 marker carries significantly phylogenetic information and resolves Symbiodiniaceae taxa (LaJeunesse, 2002). DNA extraction was performed following a modified version of Wayne's method (Boulotte et al., 2016). Briefly, diluted DNA (1:20) was then used to generate triplicate PCR reactions of the ITS2 region using the protocol and primers detailed in Hume et al. (2018). Pooled triplicate PCR reactions were used to generate sequencing libraries following Illumina's protocol and libraries were sequenced on an Illumina MiSeq sequencer with 2 x 300bp PE chemistry at the Walter and Eliza Hall Institute (Melbourne, Australia). The raw sequence data were submitted to SymPortal (Hume et al., 2019) for Symbiodiniaceae ITS2 profiling.

Reference intragenomic ITS2 sequence variants (i.e., Defining Intragenomic Variants - DIVs) from the SCF cultures were used to create a diagnostic profile indicating the presence of the inoculum (Figure 4). For SS8 (SCF055-01.08), a total of 18 diagnostic DIVs were identified, in descending order of abundance: C1, C1b, C42.2, C1bh, C1c, C1br, C1cb, C3, C1w, C3ju, C72k, C1ge, C42ao, C1al, C42ca, C3sa, C1jx, C1cu. These DIVs have been observed across multiple sequencing runs (e.g., Scharfenstein et al., 2022; Chan et al., 2023). Only the C3 is omitted from this approach as it is a sequence shared with wild *Cladocopium* naturally occurring in *P. daedalea* and *G. fascicularis*. For the HSD culture (SCF086-01.04), a total of 11 DIVs are consistently retrieved, in order of decreasing abundance: D1, D4, D6, D4c, D4f, D1c, D1l, D4au, D6c, D4d, D1ih. One sequence, D4au, is unique to SCF086-01.04, and its presence can be used to identify samples that contain the inoculum. DNA standards from SCF055-01.08 and SCF086-01.04 cultures were included in this study across multiple sampling time points as internal references.

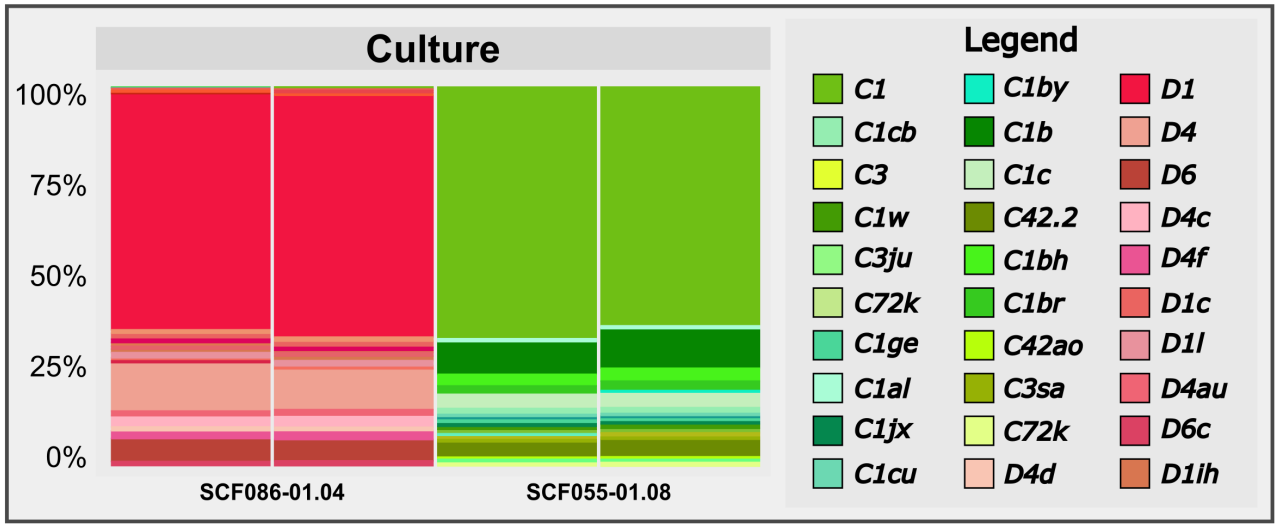

Figure 4. Relative abundance of ITS2 DNA sequences for both heat-selected cultures. Each individual bar represents a biological replicate and the colours within those bars represent the abundance of each DIV present in the sample.

4 Results and Discussion

4.1 Symbiodiniaceae diversity across mesocosms

The symbiont communities of hosts and free-living Symbiodiniaceae (sediment, seawater, and epiphytic on crustose coralline algae) in the three mesocosms were analysed (Figure 5), and these results are discussed in the following sections.

Figure 5: Relative abundance of Symbiodiniaceae genera based on ITS2 DNA sequences for all hosts and environmental sources in the mesocosms. Each individual bar represents a biological replicate and the colours within those bars represent the sequence abundance of each genus present in the sample. Source locations of all material are listed above the graphs. The graphs are faceted by host genus/species and individual bars are ordered by the timepoint where the sample was taken and the colony it came from. Environmental samples (i.e. sediment samples) are labelled by the mesocosm they were sampled from.

4.1.1 Falcon Reef

The experimental corals (*P. daedalea* and *G. fascicularis*) were sourced from Falcon Reef. At T0, Control colonies (those not subjected to chemical bleaching) of *G. fascicularis* were dominated by *Durusdinium* (D1) and *Cladocopium* (C40 & C21). Additionally, at T3 *G. fascicularis* exhibited *Symbiodinium* (A2) at background levels that was not initially present and was likely acquired from the environment. At T0, Control colonies of *P. daedalea* were dominated by *Cladocopium* (C21 & C3) and all samples contained a background level of *Durusdinium* (D1). These host-symbiont pairings were expected based on prior studies (Scharfenstein et al., 2022, Chan et al., 2023).

4.1.2 Fantome Reef

Three coral species were collected from Fantome Reef, a reef in the same inshore system as Falcon Reef (the 'Palm Islands' group in Manbarra Sea Country). The symbiont composition of *Montipora aequituberculata* was stable throughout the experiment and across mesocosm systems with all corals dominated by *Cladocopium* (C26). The C26 symbionts of *M. aequituberculata* from in-shore central GBR have previously been studied and were expected (Kenkel and Bay, 2018). At T0, *Porites lobata* corals were also dominated by *Cladocopium* (C15), a commonly observed host-symbiont partnership across the entire Indo-Pacific (Smith et al., 2008; Levas et al., 2013). The symbionts of *Psammocora sp.*, which are

significantly less studied, present a challenge as they appear closely related to the culture inoculum (*C. proliferum*, SCF055-01.08, SS8) and are from the same 'C1 radiation' (Butler et al., 2023). However, slight variations in the ITS2 sequence composition of these related taxa can reliably be used to delineate them (Figure 6). Similar C1-like symbionts were also observed in anemones (see below).

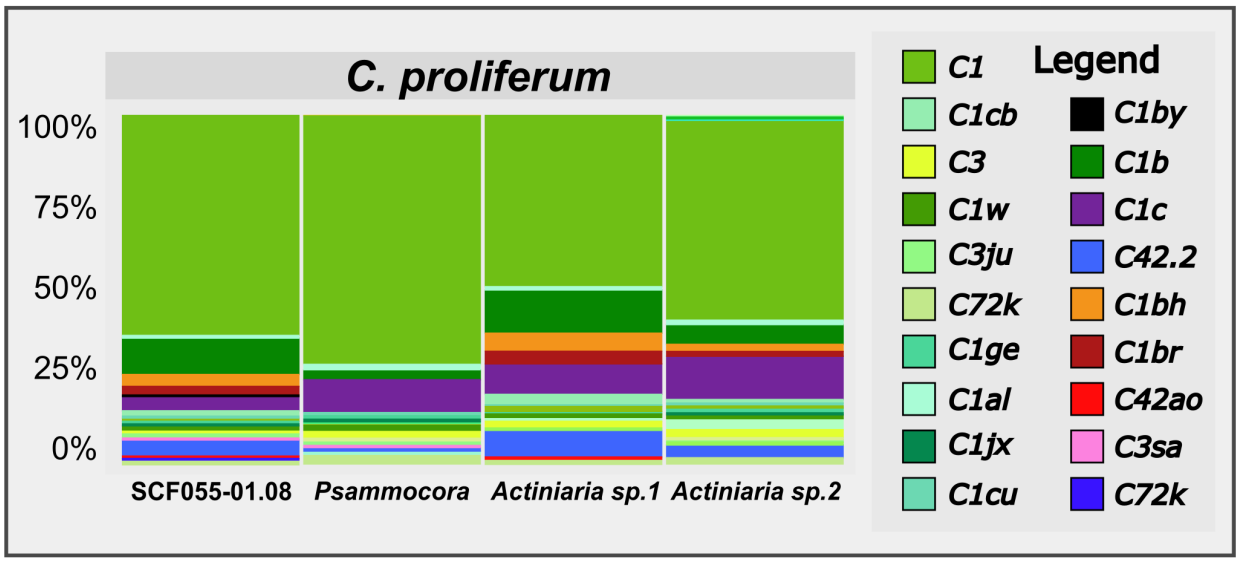

Figure 6: Relative abundance of ITS2 DNA sequences including representative samples from all hosts containing Cladocopium proliferum, and a sample of the SCF055-01.08 culture. While these samples contain many of the same sequences, the proportions of those sequences vary. There are also two sequences in S88, C1by and C72k, that are not present in Psammocora or Actiniaria.

4.1.3 Davies Reef

Davies Reef is a mid-shelf reef (approx. 75 km from shore) and five coral species were collected from this location. *Acropora spathulata* was dominated by *Cladocopium* (C3 & C50), as was *Acropora kenti* (C3 & C21). The community composition of *Diploastrea heliopora* was highly dependent on the colony, with two colonies dominated by *Durusdinium* (D6) and two colonies dominated by *Cladocopium* (C40, C3). *Pocillopora sp*. were dominated by *Cladocopium* (C1d, a well-studied symbiont formally described as *Cladocopium pacificum,* Turnham et al. 2021). *Stylophora pistillata* was dominated by *Cladocopium* (C8). These associations are typical for GBR corals, though the symbionts of *D. heliopora* are little studied.

4.1.4 The National Sea Simulator

Both species of Actiniaria were dominated by *Cladocopium* from the C1 radiation, as for *Psammocora* (Figure 6). The two zoanthid species were dominated by *Cladocopium* (C3, C20, C39 & C62). *Foraminifera* were dominated by Cladocopium (C15). *Exaiptasia pallida* was the only species dominated mostly by *Breviolum* (B1), with *Cladocopium* (C3) dominating one sample, and being present at background levels in others. Likewise, *Tridacna sp.* was the only species dominated by *Symbiodinium* (A3). *Durusdinium* (D4 & D5) was dominant in some *Tridacna* and made up a large proportion of others.

The environmental samples were distinct from any one host. For example, the C50 sequence was highly abundant in seawater, sediment, and CCA, however, and this abundance does not feature in any of the sampled hosts. Most CCA samples were dominated by *Cladocopium* (C50), with *Durusdinium* (D1) and *Breviolum* (B1) occasionally representing a larger proportion of the sample. Seawater samples were dominated by *Cladocopium* (C50, C17 & C3) and contained smaller proportions of *Durusdinium* (D1), and *Breviolum* (B1). The sediment samples were the most diverse by far, with some samples dominated by *Cladocopium* (C50) and some by *Durusdinium* (D1), with *Symbiodinium* (A2 & A1) as a close third.

4.1.5 Patterns of Background Symbiodiniaceae

The background symbionts (i.e., those at < 5% relative abundance) found in this experiment were shared across most host species. *Symbiodinium* was one of the most prevalent background genera, with most corals (and some of the CCA) containing A3 sequences. The only exceptions to this were *A. kenti*, which also contained A2 (present in the CCA as well), and *Pocillopora*, which contained the A1 sequence that was found in some seawater and CCA samples. Background abundances of *Breviolum* (B1), *Fugacium* (F4), and *Gerakladium* (G3) were also near ubiquitous. The sediment samples contained some unique sequences such as F3 and F5 (although also found in the CCA), and I2 and I4 from the Clade I. *Cladocopium*, while a dominant genus across the system, was background in *Tridacna* (C21, C8, C20 and C3).

This ubiquitous nature of these background symbionts and their unknown role in symbioses (Boulotte et al., 2016) raises some interesting questions. Are they simply present in the system as free-living symbionts (i.e., sourced from the sand or CCA) and then, for example, colonise the mucus layer or the gastric cavity of corals, but ultimately fail to achieve an intracellular state? In addition, given how ubiquitous *Symbiodinium* is as a background symbiont in this system and its dominance in the giant clam, are the clams (with their significant biomass) expelling many *Symbiodinium* cells into the system (the same would apply to Aiptasia and *Breviolum*) which subsequently are detected when taking tissue biopsies? Such questions cannot be answered here but highlight the challenges of working within systems of diverse host-symbiont and free-living Symbiodiniaceae communities (Fujise et al., 2021).

4.2 Symbiont communities within experimental *Galaxea fascicularis*

As described above, Control *G. fascicularis* are dominated by *Cladocopium* and *Durusdinium,* with many individual fragments harbouring mixtures of these two symbiont genera. For those maintained at ambient temperature across the entire study, the average proportion of *Cladocopium* was 45% versus *Durusdinium at 55%.*

Figure 7: Relative abundance of Symbiodiniaceae genera and the two inocula as assessed by ITS2 DNA sequences for experimental Galaxea fascicularis. Each individual bar represents a biological replicate and the colours within those bars represent the sequence abundance of each genus present in the sample. The graphs are faceted by timepoint (days 'post RI' refer to days since re-inoculation with treatment symbionts and days 'post HS' refer to days following the completion of the heat stress experiment). Individual bars are ordered by the colony it came from. Heat-stress and post heat-stress timepoints(T4-T7) are also ordered by the temperature treatment they were exposed to. The heat-evolved inoculum has been given different shades than the rest of their genus to show where uptake was successful.

4.2.1 *G. fascicularis*: Uptake and Persistence of the inocula

Following chemical bleaching and inoculation, we found that 47.8% of corals (22 out of 46) had taken up SS8 (Fig. 7, SS8-T0). Investigating the change in SS8 abundance from timepoint to timepoint showed that while uptake was significant (0.0% to 5.2% relative abundance at T0, p = 0.007), there was a significant decline in the relative abundance of SS8 (p = 0.013) when comparing T0 (5.2%) to T3 (0.3%). However, no

significant decline in SS8 abundance was observed between timepoints T0 and T1, T1 and T2, or T2 and T3. This suggests that the reduction of SS8 was gradual following deployment of corals to the mesocosm. Uptake of HSD was lower, with only 13.7% of corals (7 out of 51) demonstrating presence of the inoculum. While the uptake of HSD was significant (0.0% to 9.7% relative abundance, $p = 0.011$) the complete loss of HSD in the subsequent timepoint was also significant (9.7% to 0.0%, $p = 0.011$) and, in contrast to SS8, this loss was rapid, immediately following deployment to the mesocosm. Successful uptake of SS8 in *G. fascicularis* was expected based on prior research, but its persistence was expected to be longer term as it remained stably associated with this species for the length of a laboratory experiment of 2 years (42% mean relative abundance; Chan et al., 2023). However, this is the first test of HSD in *G. fascicularis*. Because *G. fascicularis* has a different *Durusdinium* as a dominant symbiont (according to comparisons with SCF086), and *Durusdinium* is generally resistant to chemical bleaching, it is possible that HSD is not compatible or is simply out-competed by the native *Durusdinium*.

4.2.2 *G. fascicularis*: Notable Chemical Bleaching Effect

G. fascicularis that underwent chemical bleaching had a significantly lower proportion of *Cladocopium* than those that did not (8.4% vs 31.4%, $p < 0.001$). This difference was maintained ($p < 0.05$) at every timepoint and the chemically bleached corals did not revert to their former compositions, even in the RIC treated corals which received their native *Cladocopium* (alongside their native *Durusdinium*) symbionts as inoculum. This indicates that the effects of chemical bleaching were felt far beyond the initial event and may have altered the symbiotic communities of *G. fascicularis* in favor of *Durusdinium*.

4.3 *G. fascicularis*: Community Stability with a Thermal Stress Event

The reduction of SS8 from T0 to T3 meant this treatment had minimal heat-evolved inoculum going into the heat stress experiment, and following heat stress, SS8 was near eliminated (0.3% ambient vs 0.0% elevated, $p = 0.015$). When the experimental corals from the control treatment entered the heat stress experiment (2 months after timepoint T3), we saw a significant loss of native *Cladocopium* in corals experiencing heat stress (55.4% at ambient vs 17.7% at elevated, $p = 0.0001$). This decline became even more pronounced during the following two timepoints, where the proportion of *Cladocopium* in ambient corals was approximately 50% but dropped to 6% in those at elevated temperatures ($p < 0.001$). The differences in other treatments were less pronounced, presumably because they had already experienced major community restructuring that favoured *Durusdinium* over *Cladocopium*. These results (thermally induced shifts from *Cladocopium* to *Durusdinium*) corroborate the chemically induced shifts described above but differed for the two inoculum treatments. For example, in SS8-inoculated corals (regardless of presence of SS8 at this timepoint) there were no significant differences in the proportion of *Cladocopium* across ambient and elevated temperatures. However, by this timepoint HSD-inoculated corals, despite no-longer having any HSD symbionts, did show several significant differences between temperature treatments. For example, ambient corals had significantly more *Cladocopium* than those at Elevated temperatures (4% vs 0.4%, $p < 0.0001$) at T4 (during the heat stress experiment). This difference was maintained in the following two timepoints ($p < 0.05$). The same pattern was seen in RIC corals at T4 (4.7% vs 0.8%, $p = 0.0002$) and T5 (2.8% vs 0.3%, $p = 0.03$). This suggests that the inoculum can impact the symbiont community composition, even if its integration into the community lacks stability. Such phenomena have also been observed in bacterial inoculation studies (Santoro et al., 2021). For example, a study by Zhang et al., 2021, found that the addition of a BMC (beneficial micro-organism for corals) consortium had the ability to change a corals microbial community without the inoculum being stably incorporated into the coral microbiome.

4.4 Symbiont communities within experimental *Platygyra daedalea*

The chemical bleaching of the 10 *P. daedalea* genotypes in this experiment, while effective at removing homologous symbionts (Figure 8), had a strong negative effect on the health of the colonies (Figure 9). This was unexpected given our success with this protocol for this same species in two prior experiments (Scharfenstein et al., 2022, Nitschke et al., in prep). Many corals exhibited signs of starvation (e.g., tissue retreating) during the period of recovery following chemical bleaching and inoculation with symbionts. Uncertainty around whether *P. daedalea* would recover in the mesocosms meant that we delayed sampling

until the recovery outcome was clear (as such, there is no T0 or T1 timepoint for *P. daedalea* subject to chemical bleaching).

As described above, all *P. daedalea* were dominated by *Cladocopium* (C21) but with consistent background levels of *Durusdinium* at approximately 1-5% (Figure 8).

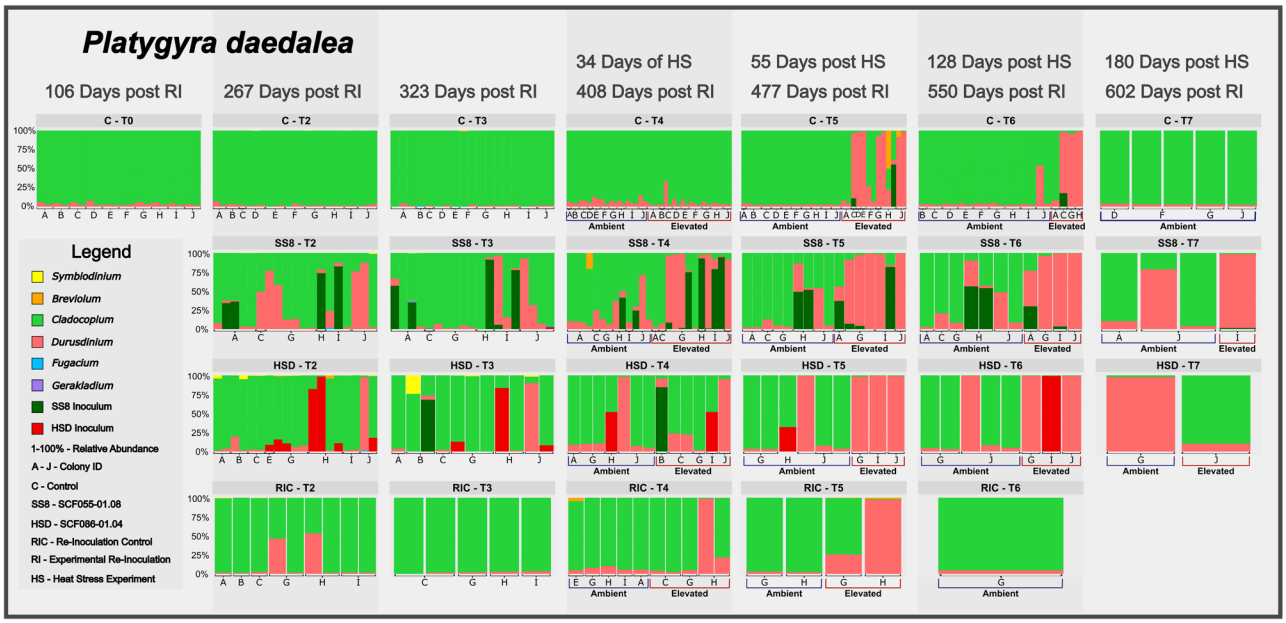

Figure 8: Relative abundance of Symbiodiniaceae genera and the two inocula as assessed from ITS2 DNA sequences for experimental Platygyra daedalea. Each individual bar represents a biological replicate and the colours within those bars represent the sequence abundance of each genus present in the sample. The graphs are faceted by timepoint (days 'post RI' refer to days since re-inoculation with treatment symbionts and days 'post HS' refer to days following the completion of the heat stress experiment), and individual bars are ordered by the colony it came from. Heat-stress and post heat-stress timepoints (T4-T7) are also ordered by the temperature treatment they were exposed to. The heat-evolved inoculum has been given different shades than the rest of their genus to show where uptake was successful.

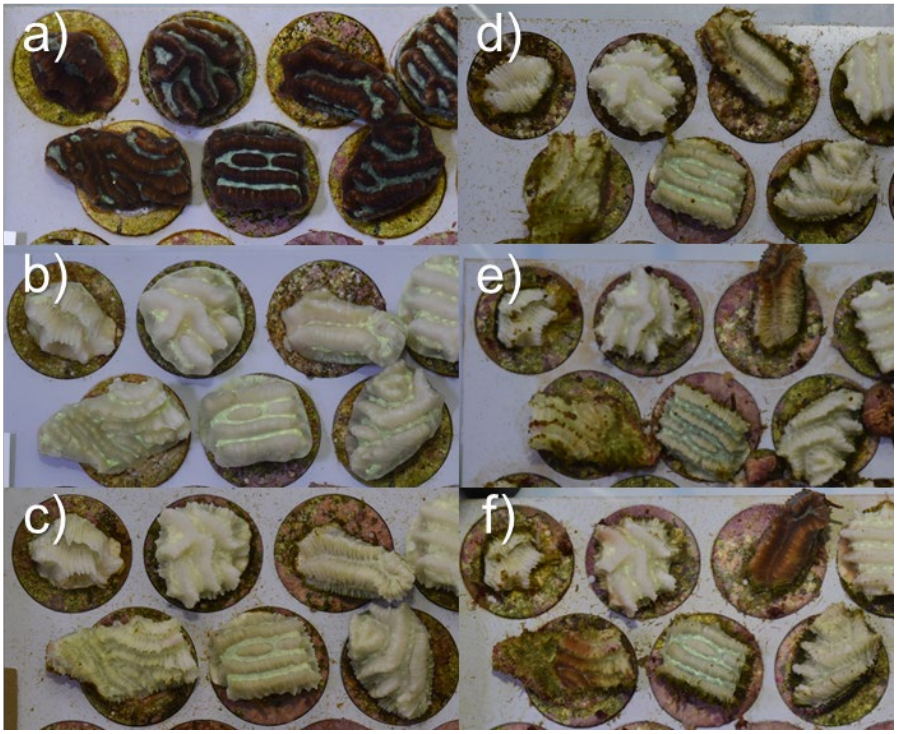

Figure 9: Time series of the effects of chemical bleaching on Platygyra daedalea. a) Pre-CB. b) 2 rounds of CB. c) 40 days post-CB. d) 69 days post-CB. e) 82 days post-CB. f) 97 days post-CB.

4.4.1 *P. daedalea*: Uptake and Persistence of the Inocula

Assessing the uptake success of the inocula in *P. daedalea* is more challenging, as it was not healthy enough to sample until 297 days post re-inoculation (T2). As such, the results below are a minimum estimate of uptake success. Regardless, both SS8 and HSD were present in all timepoints up to T6 (550 days post inoculation). At T2, 21% of corals (4 out of 19) inoculated with SS8 contained SS8 and 37% of corals (7 out of 19) inoculated with HSD contained HSD. Throughout the experiment there were no significant shifts in SS8 or HSD abundance across timepoints. This indicates that, regardless of its uptake success, once successfully established, the likelihood that SS8 and HSD persist is greater for *P. daedalea* than for *G. fascicularis.*

4.4.2 *P. daedalea*: Chemical Bleaching Effect

The effects of chemical bleaching on native *Cladocopium* were evident at the first point of sampling (T2), where corals that underwent chemical bleaching had significantly ($p < 0.0001$) less *Cladocopium* (75%) than those that did not (99%), with native *Durusdinium* increasing from background to dominant in 17% of corals sampled. In fact, this effect of shifts to *Durusdinium* were maintained ($p < 0.05$) at every timepoint, with chemically bleached corals retaining altered compositions. This corroborates results from *G. fascicularis* that the effects of chemical bleaching are long lasting and that *Durusdinium* symbionts, given the chance to proliferate, do so rapidly and reach a stable state.

4.4.3 *P. daedalea*: Community Stability during a Thermal Stress Event

The Control *P. daedalea* fragments (those not subjected to chemical bleaching) when moved from the mesocosms to ambient temperatures (for the heat-stress phase of the experiment) showed a slight but significant shift in community composition, decreasing their *Cladocopium* proportions (99% to 94%, $p < 0.0001$). The next timepoint was after the corals had returned to the mesocosms and showed that corals in the ambient temperature treatment significantly reverted to their former *Cladocopium* abundance (94% to 98%, $p < 0.0001$). This indicates that even minor changes in the environment, such as being moved from an outdoor tank to an indoor tank (temperature was maintained but lighting quantity and quality would have changed), can induce changes in photosymbiont community composition. This may contribute to differences in results among experiments (i.e., controlled lab conditions vs outdoor mesocosms).

The symbiont community response to heat stress lagged behind the actual stress event. Immediately after heat stress there was no significant decrease in *Cladocopium* abundance. However, there were significant differences between ambient and elevated corals at the following timepoint (98% vs 34% *Cladocopium*, $p < 0.0001$). This likely suggests that heat stress does not result in the preferential expulsion of *Cladocopium* over *Durusdinium*, but that *Durusdinium* was competitively superior at re-colonising the vacant host tissue during the recovery period (after temperatures were returned to ambient). It is possible that the remaining *Durusdinium* may have been less compromised by exposure to elevated temperature, which is consistent with the reputation of this genus as comprising thermotolerant or rather, 'extremophile' species (Nitschke et al 2022). At T5 there was a significant drop in *Cladocopium* abundance in the elevated treatment (76% vs 0.8%, $p = 0.005$). The significant difference between temperature treatments was retained at T6 (77% vs 0.5%, $p = 0.016$).

SS8 had an immediate and strong significant response ($p < 0.01$) to the elevated temperatures, with the proportion of *Cladocopium* in the elevated treatment dropping to 29% while remaining at 78% in the ambient treatment. Unlike the SS8-inoculated corals, HSD-inoculated corals showed no significant change in *Cladocopium* abundance between temperature treatments at T4, though sample sizes were low.

5 Conclusions, Recommendations, and Future Work

5.1 Conclusions

- The stability of the inocula differed according to the inoculum identity and the target coral. SS8 persisted in both *G. fascicularis* and *P. daedalea*, albeit gradually retreating to background levels in *G. fascicularis*. SS8 has been maintained at high densities in *G. fascicularis* for up to two years in prior studies, though this was under static laboratory conditions. HSD was acquired but failed to persist in *G. fascicularis,* however it was acquired and persisted in *P. daedalea* until at least 550 days post-inoculation. This, plus the observations of SS8 in *P. daedalea*, is the longest observation of persistence for these novel symbiont-coral pairings. This stability is noteworthy when considering the compromised health of the *P. daedalea* caused by chemical bleaching.
- With relatively little effort, photosymbiont-centric mesocosms with significant genetic diversity (at least seven genera were retrieved in the present study) can be established within which to test the stability of experimental coral-symbiont pairings.
- While not explored in detail here, the symbionts of mesocosm taxa were stable through time, but also likely contributed to apparent 'background symbiont' signals detected within *P. daedalea* and *G. fascicularis* (such as *Symbiodinium* and *Breviolum* from *Tridacna* and Aiptasia, respectively). Other background Symbiodiniaceae, including *Gerakladium*, are unaccounted for and should be explored in greater detail.
- Different taxa from the C1 radiation (such as our SCF055.08/SS8 inoculum and the C1 in *Psammocora* and Actinaria) were retrieved and can be delineated using unique DIVs and differential DIV abundances. These may be distinct genotypes of *C. proliferum* (or closely related species), but this requires future study. The same is true for the SCF086 *Durusdinium* inoculum and wild *Durusdinium* in *P. daedalea* and *G. fascicularis*. Overall, our inocula were novel taxa (relative to those present in the system) introduced to via the experimental corals.
- Community shifts from chemical bleaching (primarily favouring wild *Durusdinium*) are retained after the event has passed, indicating its effects are long lasting. Proliferating native *Durusdinium* likely contest the uptake of the inocula and this presents a challenge for investigations of the impact of heat-evolved photosymbionts in adult corals (though see recommendations below).
- As for chemical bleaching, heat stress induces significant changes in community composition (also favouring *Durusdinium*), and these changes can be maintained upwards of 100 days after the stressor has subsided.
- Community stability is disrupted when moving from one experimental system to another. This was evidenced by a consistent reversible change in community in control *Platygyra* at ambient temperatures when moved from the mesocosm, to the experimental room (favouring *Durusdinium*), and back to the mesocosm.

5.2 Recommendations

- From experiment to experiment we have found variation in susceptibility (i.e., negative health outcomes) to chemical bleaching. It should not be assumed that coral genotypes of the same species will respond equally to standard chemical bleaching protocols and where possible (though with significant time investment), genotype-specific dose-response optimisation should be piloted prior to experimentation.
- Uptake success appears variable at the level of technical replicates (i.e., fragments of an individual genotype). The reason for this is unclear but a likely contributor is competition between the inoculum

and residual symbionts that remain post-bleaching. For this reason, inoculum density should not be compromised as it was in the present study (i.e., 1,500 cells mL vs e.g., 5,000 cells mL used in other experiments) when upscaling, noting this is (to date) the largest experimental inoculation of adult corals with heat-evolved photosymbionts.

- Shifting experimental corals among systems was necessary to achieve the various phases of the experiment (chemical bleaching, long term holdings, heat-stress experiments, recovery, etc). These environmental changes impact the photosymbiont community stability and potentially the integration of the inoculum. Where possible, future experiments should likely be conducted in one consistent system (e.g., entirely outdoors or entirely indoors).

5.3 Future work

- Since the inception of this long-term mesocosm experiment, a small-scale field trial permit (G22/46479.1) was granted for the deployment of Falcon Reef corals containing heat-evolved *Durusdinium* and *Cladocopium* back to Falcon Reef. Such a deployment is underway including juvenile and adult *P. daedalea* (inoculated in different experiments), but also included a subset of the experimental *G. fascicularis* fragments from this mesocosm experiment. While these *G. fascicularis* were unlikely to contain much (if any) inoculum, ongoing monitoring of the deployed *P. daedalea* yield promising results on the stability and performance of SS8 and HSD under field settings, and these results are in preparation for publication.
- Interactions among *Cladocopium* and *Durusdinium* are a central feature of the GBR coral-Symbiodiniaceae system. Rather than trying to substitute wild *Cladocopium* or *Durusdinium* for heat-evolved counterparts, mixed-inoculations of heat-evolved *Cladocopium*+*Durusdinium* as a single treatment should be explored in future work.
- A high-throughput system for menthol dose-response curves that yields effective symbiont reduction while minimizing the trade-off against coral health would be a valuable tool. This would minimize recovery time (reducing experiment duration) but also enable quantitative investigations of how bleaching extent/severity impacts uptake and persistence of heat-evolved symbionts and subsequent enhancements.
- Another multiplier on the path to achieving scale will be to the miniaturisation of sampling protocols (i.e., integrating multiple small biopsies across the surface of colonies) and maximising throughput of genetic identification techniques. It is likely that we under-estimate uptake success because only small, minimally invasive biopsies are taken from each coral and therefore observations here should be considered minimum estimates of success. These biopsies in the future should be distributed across the colony surface. Furthermore, ITS2 sequencing is laborious and costly, and while it yields high resolution data, efforts should be made to develop diagnostic, quantitative probes of the major symbionts in the system (e.g., C21, wild *Durusdinium*, C1, etc).

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