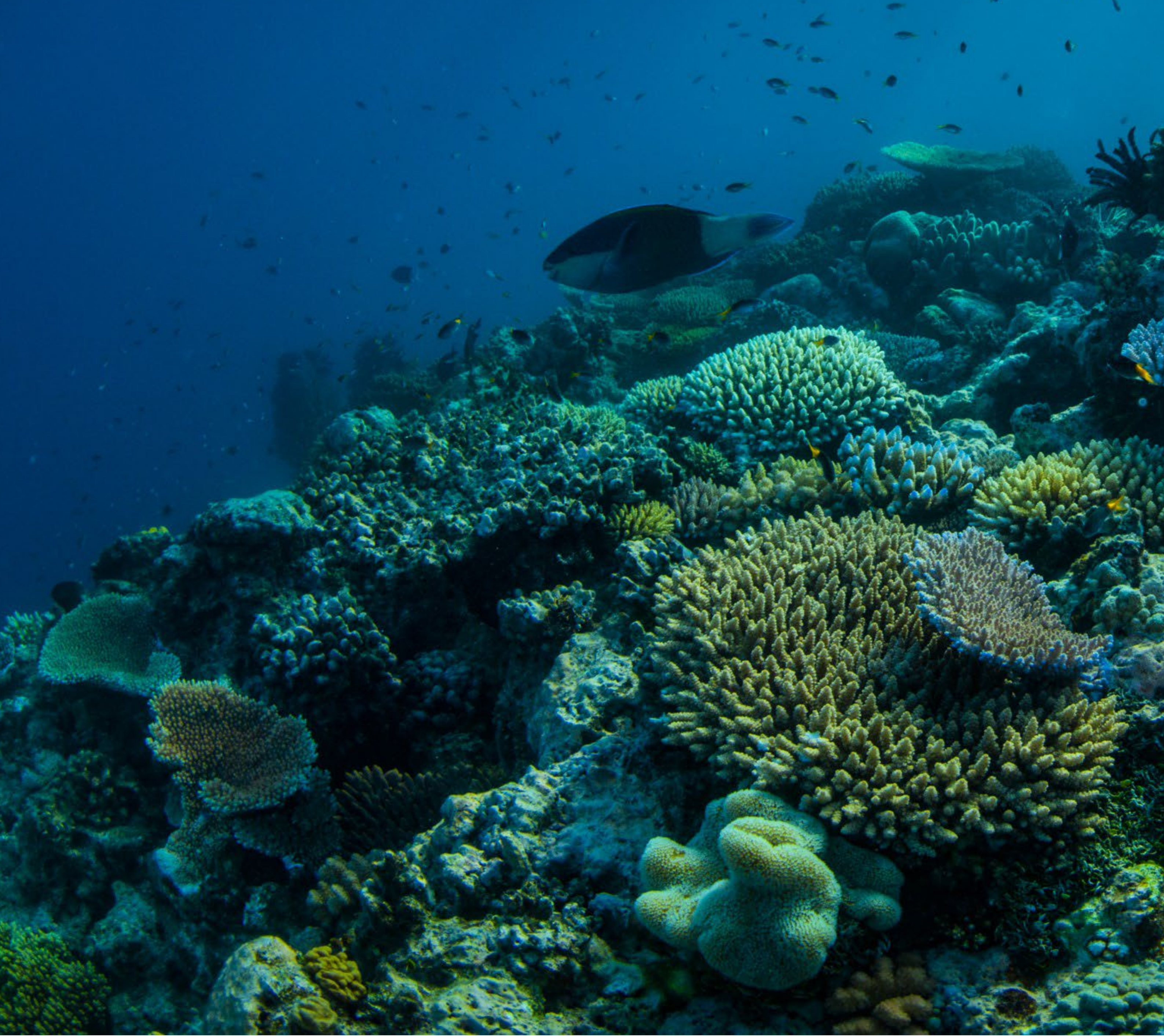
RRAP REEF RESTORATION & ADAPTATION PROGRAM

Assisted Evolution (ECT-02)

Final Report June 2025

van Oppen, M.J.H., Lamb, A., Nitschke, M., Chan, W., Molinaro, G. and Howlett, L.



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This report summarises work undertaken under *Assisted Evolution (ECT-02)* in accordance with the Reef Restoration and Adaptation Program's *Enhanced Corals and Treatments* Project Agreements. It provides a summarised, point-in-time synopsis of activities, methods, findings and outcomes completed in accordance with the approved project scope up to 30 June 2025.

All information reflects project scope and outcomes as of May-June 2025. Subsequent updates, analyses, or scientific developments are not included. This report should be read alongside any associated and publicly available technical reports, datasets, and publications for full detail. This report does not provide scientific inferences, policy guidance or operational instructions beyond the project's defined scope and duration.

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The RRAP partners acknowledge Aboriginal and Torres Strait Islander Peoples as the first marine scientists and carers of Country. We acknowledge the Traditional Owners of the places where RRAP works, both on land and in sea Country. We pay our respects to elders; past, present, and future; and their continuing culture, knowledge, beliefs, and spiritual connections to land and sea Country.

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

Location	**Traditional Owner Group**
Palm Islands	Manbarra
Davies Reef	Bindal

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1 Executive Summary

The Assisted Evolution (ECT-02) Project within the Reef Restoration and Adaptation Program (RRAP) aimed to enhance coral thermal tolerance through two complementary approaches and sub-projects:

- ECT-02.1: selective breeding of coral hosts
- ECT-02.2: manipulation of algal symbionts

Sub-project ECT-02.1, under the Enhanced Corals and Treatments Sub-program, developed breeding interventions, including selective breeding, artificial selection, and intraspecific hybridisation, to produce corals with improved thermal tolerance, and tested their performance and potential trade-offs under ecologically relevant conditions. Sub-project ECT-02.2 focused on the laboratory evolution and introduction of heat-tolerant algal symbionts (Symbiodiniaceae) into coral hosts, assessing their effects across life stages and species while evaluating ecological risks and benefits. Together, these sub-projects aimed to generate validated methods to accelerate coral adaptation, identify potential trade-offs for heat tolerance, and inform restoration strategies capable of supporting reef survival under climate change.

Both sub-projects delivered key scientific findings aligned with their core research objectives. In ECT-02.1, significant variation in phenotypic traits, including survival and growth, was observed among purebred and hybrid offspring, with clear evidence that parental lineage influenced performance under heat stress. This highlighted the critical role of parental effects and the need for strategic broodstock selection in future large-scale deployments, including the Pilot Deployments Program (PDP). Genomic analyses further reinforced this, showing that not all inter-regional crosses led to adaptive outcomes, underscoring the necessity of incorporating genetic data to guide breeding programs and avoid maladaptive combinations. In ECT-02.2, heat-evolved Symbiodiniaceae strains demonstrated phenotypic traits including enhanced photosynthetic efficiency when exposed to elevated temperatures, indicating improved thermal tolerance in culture. Further, some heat-evolved strains imparted their enhanced thermal tolerance to their coral host when in symbiosis, both in lab and in field experiments. This enhanced thermal bleaching tolerance did not come with a trade-off against coral growth. Additionally, the use of chemical bleaching to manipulate and repopulate symbiont communities in adult corals was successfully validated, providing a flexible and scalable method for introducing beneficial algal strains independent of coral spawning events. Together, these findings contribute to a growing toolkit of interventions that can be used to support coral adaptation and inform future reef restoration strategies.

To build on the foundational work of the RRAP Project Assisted Evolution (ECT-02) and accelerate the transition from research to large-scale implementation, several key recommendations for future research have been identified. For selective breeding, future efforts should focus on quantitative genetic analyses to identify reliable phenotypic and genotypic predictors of offspring performance, alongside rigorous field trials across ecologically diverse reef environments. Longitudinal studies tracking enhanced coral throughout their life span will be critical to validate sustained benefits. Novel strategies such as “in-field selective breeding”, where thermally tolerant broodstock from genetically distinct populations are co-deployed in close proximity to each other, warrant further investigation. Furthermore, cross-breeding experiments coupled with comprehensive offspring phenotyping under stress and in field conditions will enhance selection precision for thermally tolerant parents. In parallel, algal symbiont research should prioritise the upscaling of field deployments of corals inoculated with heat-evolved symbionts across multiple sites, species, and symbiont types, including the development of techniques for culturing and enhancing vertically transmitted symbionts. Continued development of new heat-evolved strains using multiple stressors (e.g. temperature and light) is needed to broaden thermal tolerance. Infrastructure such as mobile, containerised facilities will support region-wide symbiont distribution. Finally, developing high-throughput phenotyping and genotyping workflows will be essential for identifying candidate corals with enhanced thermal tolerance *in situ*.

2 Background and Justification for the Research

ECT-02.1: Selective Breeding for Enhanced Thermal Tolerance

Coral reefs are degrading due to rising sea surface temperatures and the increased frequency of summer heat waves. Coral species harbour natural variation in their heat tolerance within and between populations. Natural selection can act on this variation to increase the frequency of alleles (genetic variants) within populations that confer heat tolerance and support species adaptation and survival. However, the rate of reef degradation occurring globally is indicative that natural adaptation may occur too slowly to ensure the future of reefs.

The goal of the first sub-project was to develop managed breeding interventions that enhance coral bleaching tolerance: selective breeding, artificial selection, and intraspecific hybridisation. If successful, these interventions would generate coral stock that can be used to supplement populations with heat tolerance conferring alleles and thereby speed up their rate of adaptation and increase their chance of survival.

Corals inhabiting reefs with histories of high temperatures may be adapted to high temperatures. It had been demonstrated that warm-adapted corals could be bred with corals from reefs with cooler thermal histories (i.e. intraspecific hybridisation) to generate heat-tolerant offspring. Heat-tolerant hybrid offspring could theoretically be deployed onto reefs with cooler thermal histories to guard them against climate change. However, the evidence supporting the use of intraspecific hybridisation in coral conservation was limited to several combinations of species, life stages, and environments. We aimed to test the performance of intraspecific hybrid stock from multiple species across key life stages and ecologically relevant environments.

Selective breeding involves using broodstock phenotypes and/or genotypes to inform breeding and produce enhanced offspring. Selective breeding had successfully been applied to improve agricultural stocks, and it had more recently been suggested as a conservation tool. However, there was very limited research on selective breeding in coral prior to the beginning of RRAP and an accurate and rapid assay for identifying heat tolerant broodstock had not been developed. We aimed to determine whether rapid heat stress assays could be used to inform the production of enhanced coral stock for reef restoration initiatives.

Artificial selection involves thermally selecting coral larvae to generate enhanced stock. Thermal stress can be applied to a bulk pool of larvae that is fatal to sensitive larvae and generate a relatively resilient pool of surviving larvae. If thermal resilience in larvae is conferred via similar mechanisms as it is in later life stages, then this intervention might be used to generate enhanced coral stock for reef restoration. However, there was no evidence prior to RRAP that artificial selection of coral larvae could be used to produce enhanced stock for reef restoration initiatives; we aimed to test this.

Across interventions, the objectives of the first sub-project were:

1) Assess whether breeding interventions can generate heat-tolerant coral stock for key coral species.
2) Test the performance of offspring bred for enhanced heat tolerance under ecologically relevant conditions in the laboratory and field.
3) Measure trade-offs in phenotypic traits (e.g. growth and survival) in offspring bred for enhanced heat tolerance.
4) Contribute to our understanding of coral heat tolerance and how it might be harnessed for reef conservation.

ECT-02.2: Manipulation of Algal Symbionts for Enhanced Thermal Tolerance

The goal of the second sub-project was to enhance coral heat tolerance by manipulating algal symbionts (Symbiodiniaceae). Heat-evolved algae were developed via thermal selection in the laboratory and introduced into corals to assess their impact on thermal tolerance. Proof-of-concept for this approach was demonstrated prior and during the first two years of the project, where heat-evolved symbionts enhanced thermal bleaching tolerance in *Acropora* larvae, recruits, and adults. However, further investigation was needed to determine if these benefits were sustained under field conditions and across a broader range of coral species and life stages and algal symbiont species. The project also explored the potential trade-offs and risks of heat-evolved symbionts to ensure a thorough risk-benefit analysis. We developed an adult system based on chemical bleaching which allowed for the uncoupling of experiments from coral spawning events, enabling year-round testing of new coral-symbiont pairings. This work used phenotyping, transcriptomics, genome resequencing, and metabolomics to understand the genetic basis of enhanced thermal tolerance and optimise selection strategies for experimental evolution. These findings were expected to aid in the prediction of which heat-evolved algal strains would confer increased thermal bleaching tolerance on the coral holobiont.

The objectives of this sub-project were two-pronged: (1) to develop new heat-evolved algal symbiont strains, and (2) to test their effect in multiple life stages of the coral holobiont in the laboratory and field. This sub-project aimed to provide insight into whether the use of heat-evolved symbionts can be implemented at an ecologically relevant scale by expanding the number of coral host and algal symbiont species tested. It also developed important knowledge for assessing risks and benefits of implementation – a key goal of the RRAP Enhanced Corals and Treatments (ECT) Sub-program.

Whole RRAP Assisted Evolution (ECT-02) project

The two sub-projects worked in synergy, combining coral host breeding and algal symbiont manipulation to enhance coral resilience against rising ocean temperatures, ultimately helping to prepare coral populations for future climate change challenges. The combined objectives (Section 3) included:

1. Measure phenotypic variation in thermal bleaching tolerance for a core set of coral species across environmental and latitudinal gradients on the Reef using rapid performance experiments and natural bleaching events and assays that span host and symbiont physiology.
2. Test phenotype assays to rapidly identify candidate coral colonies for selective breeding and asexual propagation within coral populations.
3. Perform genome-wide single-nucleotide polymorphism (SNP) and transcriptomic analyses to identify adaptive mechanisms and coral host and symbiont genetic markers linked to heat and bleaching tolerance.
4. Conduct phenotypic analyses to assess the existence of potential trade-offs with growth and reproduction.
5. Develop heat-tolerant algal symbionts via experimental evolution and assess whether they impart enhanced thermal tolerance on early and adult coral life stages.

3 Research Objectives and Key Findings

A current list of project outputs are listed on the RRAP website: gbrrestoration.org. Key research objectives and findings are detailed below.

Table 1: Key findings of the Project aligned to the overarching and specific research questions for each sub-project.

Objective		Key Findings and/or Outcomes
1. Selective breeding among coral populations		
1 (a)	Measure phenotypic trait variation across purebred and hybrid coral offspring including in heat tolerance and growth. Offspring will be produced from parent corals from the same or different populations stretching across a wide temperature gradient.	Corals were spawned and cross-bred from several reefs along a thermal gradient to compare the fitness of offspring bred from the same region (intra-region) and different regions (inter-region) of the Great Barrier Reef (GBR) (Macadam et al. 2025, Biological Conservation). A larval heat stress assay was carried out on three species (*Acropora kenti, A. hyacinthus, and Goniastrea retiformis*) and a juvenile heat stress assay on two species (*Acropora kenti* and *A. hyacinthus*). Results showed higher survival in some of the inter-region larvae and juvenile crosses when compared to intra-region crosses, however, other phenotypic traits of the juvenile offspring (growth, colour, and effective quantum yield of photosystem II) were not affected by inter-region hybridisation, and intervention-linked enhancement of offspring varied by species. Furthermore, parents from reefs modelled to have heat-tolerant genotypes did not always produce heat-tolerant offspring. These findings suggest some potential for thermal enhancement using intraspecific hybridisation between regions of the GBR for some species, but also that outcomes are complex and likely influenced by the population genetic structure of species and local adaptation within populations.
1 (b)	Assess genetic traits underpinning phenotypic trait variation in purebred and hybrid offspring.	• Analyses of reduced representation sequencing data revealed that inter-region crosses between reefs do not always generate genetically distinct coral stock with enhanced adaptive capacity (Edmunds et al. 2025). This demonstrates the value of using genetic data to inform broodstock selection and stock production for restoration initiatives. • We have developed a genetic management framework that aims to preserve the functional integrity of ecological communities and presents the Great Barrier Reef as a case study (Pavlova et al. in prep [70% complete]). The framework represents a data-driven workflow that recommends management practices based on species traits, genetic data, and data availability. The framework further identifies data-deficient species and proposes a proactive approach for their management that is rooted in evolutionary theory. • Ribonucleic acid (RNA) sequencing (RNAseq) data: Analyses of RNA sequencing data from samples collected during heat stress experiments have advanced our understanding of the

Objective		Key Findings and/or Outcomes
		molecular mechanisms underlying coral heat tolerance. We identified conserved and unique differentially expressed genes (DEGs) and enriched Gene Ontology terms under elevated temperatures in offspring from between-reef and within-reef crosses. These DEGs represent candidate loci that may play key roles in coral heat tolerance and could serve as potential markers for broodstock selection in future managed breeding efforts.
1 (c)	Measure trade-offs between phenotypic traits (i.e. growth and survival) in offspring bred for enhanced thermal tolerance under different temperatures in the lab and field.	• A trade-off was observed in a laboratory experiment between enhanced survival and growth under elevated conditions and bleaching susceptibility in selectively-bred *A. spathulata* offspring of broodstock from Davies Reef (Lamb et al. submitted to Science Advances). • A trade-off was observed across environments in selectively-bred *A. spathulata* offspring of broodstock from Davies Reef which displayed enhanced heat tolerance in the laboratory (Lamb et al. submitted to Science Advances) but reduced performance in the field (Morgans et al. 50% complete). • A trade-off was observed in a chronic heat stress experiment between growth under ambient and survival and bleaching resilience under elevated conditions in recruits inoculated with heat-evolved Symbiodiniaceae (Lamb et al. 2025, in review). • Notably, no trade-off was observed between heat tolerance (based on rapid heat stress assays) and fecundity in coral broodstock tolerance (Lamb et al. submitted to Conservation Science and Practice) that would impede selectively-bred stock production.
1 (d)	Estimate the scope of trait enhancement via selective breeding in corals.	Rapid heat stress assays were used to inform selective breeding that successfully enhanced coral recruit but not larval heat tolerance (Lamb et al. submitted to Conservation Science and Practice; Lamb et al. submitted to Science Advances). The effect of selective breeding on recruit performance was also dependent on the batch of broodstock that was spawned and crossed (Lamb et al. submitted to Science Advances). Furthermore, field deployments have demonstrated that selective breeding can be used to generate stock that is viable under ecologically relevant conditions (Lamb et al. in prep (20% complete)) but can underperform in some environments (Morgans et al, in prep (50% complete)). The results from this body of work simultaneously caution against generalisations about the effect of selective breeding on the performance of coral stock across life stages, populations, and environments. Finally, using two interventions – selective breeding and inoculation with heat-evolved symbionts (see second sub-project) – in tandem rather than either in isolation was beneficial in some cases but redundant or counterproductive in others (Lamb et al. submitted to Science Advances).

Objective		Key Findings and/or Outcomes
1 (e)	Develop high-throughput tools for the assessment of heat tolerance and growth. This includes efforts to develop single larval and juvenile protocols for shallow whole genome sequencing as well as the operationalisation of Machine Learning and 3D scanning pipelines for trait measurements in selectively-bred corals.	• A machine learning pipeline was developed to rapidly and accurately measure coral juvenile fitness (Macadam et al. 2021) that was then applied to increase the efficiency of data processing across projects (e.g. Macadam et al. 2025, Biological Conservation and Lamb et al. submitted to Science Advances). • Laboratory protocols were developed that optimised the quantity and quality of RNA extracted from individual coral larvae and recruits (Edmunds et al. 2025). • High-performance computing using Nextflow to run the *nf-core/rnaseq* and *nf-core/taxprofiler* pipelines was used for the first time at AIMS for efficient and high-quality pre-processing and mapping of RNA sequence data to a reference genome (Edmunds et al. 2025). • Custom R scripts were developed for subsequent differential gene expression analysis that utilised three independent approaches (*edgeR*, *limma voom*, and *DeSeq2*) as well as Gene Ontology based gene set enrichment analysis that utilised *clusterProfiler* (Edmunds et al. 2025).
2. Treatment with algal symbionts (Symbiodiniaceae)		
2 (a)	Select and screen new Symbiodiniaceae strains with established and new methods.	• Strains of *Durusdinium trenchii*, *Fugacium kawagutii* and *Symbiodinium pilosum* were exposed to ethyl methanesulfonate to induce random mutagenesis, and then underwent thermal selection at high temperature (31/33°C). After 4.6– 5 years of experimental evolution, the *in vitro* thermal tolerance of these strains was assessed. Heat-evolved *D. trenchii*, *F. kawagutii* and *S. pilosum* strains all exhibited increased photosynthetic efficiency under thermal stress. However, trade-offs in growth rates were observed for the heat evolved *D. trenchii* lineage at both ambient and elevated temperatures. (Scharfenstein et al. 2023 DOI: 10.1111/eva.13586). • We exposed a *D. trenchii* strain to stable and fluctuating temperature profiles, which varied in oscillation frequency. After 2.1 years (54–73 generations), we characterised the adaptive responses under the various experimental evolution treatments by constructing thermal performance curves of growth from 21 to 31°C for the heat-evolved and wild-type lineages. Additionally, the accumulation of extracellular reactive oxygen species, photophysiology, photosynthesis and respiration rates were assessed under increasing temperatures. Of the fluctuating temperature profiles investigated, selection under the most frequent oscillations (diurnal) induced the greatest widening of *D. trenchii*'s thermal niche. Continuous selection under elevated temperatures induced the only increase in

Objective		Key Findings and/or Outcomes
		thermal optimum and a degree of generalism. (Scharfenstein et al. 2024 doi: 10.1111/nph.19996).
2 (b)	Test whether chemical bleaching of adults and re-inoculation with heat-evolved algal symbionts is feasible for a range of coral species and measure a range of phenotypic traits to assess performance and trait trade-offs.	• To test whether acquisition of symbionts from the environment occurs, we subjected adult fragments of corals (six species in four families) to a chemical bleaching treatment (menthol and Dichorophenyl-dimethylurea (DCMU)). The treatment reduced the native microalgal symbiont abundance to below 2% of their starting densities. The bleached corals were then inoculated with a cultured *Cladocopium proliferum* strain. Genotyping of the Symbiodiniaceae communities before bleaching and after reinoculation showed that fragments of all six coral species acquired the strain used for inoculation. Our results provide strong evidence for the uptake of Symbiodiniaceae from the environment by adult corals. We also demonstrate the feasibility of chemical bleaching followed by reinoculation to manipulate the Symbiodiniaceae communities of adult corals, providing an innovative approach to establish new symbioses between adult corals and heat-evolved microalgal symbionts. (Scharfenstein et al. 2022 https://doi.org/10.1038/s41396-022-01203-0). • To inoculate adult corals with heat-evolved Symbiodiniaceae, we typically use chemical bleaching to disrupt the native symbiont community and create an available niche for the introduced symbionts. We investigated how the extent of chemical coral bleaching impacts the uptake of heat-evolved symbionts and host health during the recovery phase. Results showed that while mild bleaching (low and medium treatments) leads to significantly better survival and recovery, uptake of heat-evolved symbionts was low, occurring in ~5% (low treatment) and ~25% (medium treatment) of inoculated fragments. Conversely, severe chemical bleaching maximises the chance of uptake (uptake in ~69% of inoculated fragments) but leads to more partial and total mortality due to the longer duration of being in a starved state. Coral genotype also played a role in all treatments, suggesting that some genotypes are more sensitive to the impacts of chemical bleaching than others. This knowledge contributes to the optimisation of the bleaching and inoculation process, which is essential when considering the scalability and implementation of this approach into reef restoration practices. (Allen et al. in prep. [80% complete]).
2 (c)	Inoculate aposymbiotic larvae and juveniles of a range of coral species (incl. *Isopora, Acropora* and *Platygyra*) with cultures of wild type or heat-evolved Symbiodiniaceae and measure a range of phenotypic traits to assess performance and trait trade-offs.	• Replicate cultures of the generalist species, *Cladocopium proliferum*, were exposed to elevated temperature (31°C) for >10 years, and one heat-evolved strain (SS8) as well as a wildtype strain 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 five months of the experiment and

Objective	Key Findings and/or Outcomes
	enhanced adult coral thermotolerance relative to 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. (Chan et al. 2023 DOI: 10.1111/gcb.16987). • Four-month old *Platygyra daedalea* recruits were inoculated with eight different symbiont treatments (one heat-evolved and one wildtype strain of *Durusdinium trenchii* and *Cladocopium proliferum*, as well as all possible combinations including one *Durusdinium* and one *Cladocopium* strain) through a simulated thermal stress event. Overall, the corals accumulated a total of 30+ Degree Heating Weeks (DHWs). Survival, size, reflectivity, photosynthetic efficiency, and community composition were monitored at ambient (29℃) and elevated (32℃) temperatures for the duration of the experiment. Preliminary results indicate that hosting multiple heat-evolved symbionts greatly enhances (+6 DHWs) a coral's bleaching resilience. Future analyses are expected to shed more light on the costs and benefits of hosting multiple symbiont strains and how community composition can change when a thermal stress event is experienced. (Ivory et al.in prep. [70% complete]). • We chemically bleached *Platygyra daedalea* colonies to eliminate homologous symbionts, following which we inoculated the corals with cultured (both wild-type and heat-evolved *Cladocopium proliferum*) and freshly isolated Symbiodiniaceae. After four months of recovery, we subjected the new symbioses to a simulated heatwave, delivering 6.1 experimental Degree Heating Weeks of heat stress. Genotyping of the Symbiodiniaceae communities before bleaching, after reinoculation and heat stress showed *P. daedalea* acquired and retained *C. proliferum* in mixed communities with homologous *Cladocopium* and *Durusdinium* strains. We characterised the bleaching extent (coral pigmentation, Symbiodiniaceae density), photophysiology (maximum quantum yield of PSII, photosynthesis and respiration rates) and metabolic content of corals according to their Symbiodiniaceae community composition. Both the heat-evolved and wildtype *C. proliferum* enhanced tolerance relative to native *Cladocopium*. Bleaching was less severe in corals inoculated with heat-evolved rather than wild-type *C. proliferum*, though only if present as the dominant strain (i.e. over 50% relative abundance) in the Symbiodiniaceae community. (Nitschke et al. in prep. for Science Advances [80% complete]).

Objective	Key Findings and/or Outcomes
	• We inoculated the offspring of heat-tolerant broodstock (*Acropora spathulata*) and control recruits with heat-evolved (SS8) and wildtype (WT10) Symbiodiniaceae (*Cladocopium proliferum)* and assessed their performance over two months under ambient and elevated temperature conditions in the laboratory. Inoculation with heat-evolved symbionts enhanced recruit survival and bleaching resilience (colour and photochemical efficiency) under elevated, but reduced growth under ambient temperatures. (Lamb et al. submitted to Science Advances). • We investigated the uptake and establishment of experimentally evolved *Cladocopium proliferum* and *Durusdinium trenchii* in mixed inocula and examined the effects hosting multiple symbiont strains has on the growth and survival of juvenile corals (*Platygyra daedalea*). Our results revealed that while both strains in the mixed culture inocula were taken up, most recruits were dominated by *D. trenchii*. Corals hosting *D. trenchii* over *C. proliferum* displayed faster growth despite previous studies showing the opposite. We show that the costs and benefits of hosting mixed communities are highly dependent on the identities of the individual strains in the communities. (Ivory et al. 2025 https://doi.org/10.1007/s00338-025-02632). • We field-deployed bio-engineered corals over the course of one annual thermal cycle which included a summer heatwave to understand a) the phenotypic performance (i.e. survival, growth, and bleaching tolerance) of corals inoculated with heat-evolved symbionts, and b) the stability and spread of heat-evolved symbionts in experimental corals and into the environment (water, sediments, and nearby wild corals). Results showed that heat-evolved symbionts were able to persist in >60% of corals deployed with them through summer (five months post-deployment) and that corals harbouring heat-evolved symbionts (heat-evolved *Cladocopium* or *Durusdinium*) or naturally heat-tolerant symbionts (native *Durusdinium*) performed better than those hosting native *Cladocopium* communities. There was also an effect of previous chemical bleaching treatment, with corals that had been severely chemically bleached performing better than more mild chemical bleaching during summer. Throughout winter, the performance of all fragments homogenised, with no difference between any of the chemical bleaching treatments. While persistence of heat-evolved symbionts decreased after summer, they were still able to be detected at the thermal minima (22°C) ten months post-deployment. (Allen et al. in prep. [65% complete]).

Objective		Key Findings and/or Outcomes
2 (d)	Assess long-term stability of symbioses with selected Symbiodiniaceae strains in the adult system (if it performs) and in juveniles.	Symbioses with selected Symbiodiniaceae strains persisted for the duration of various lab and field experiments. • **Laboratory**: two years in adult *Galaxea fascicularis* (Chan et al. 2023); 4.2 months and throughout 6.1 eDHWs of heat stress in adult *Platygyra daedalea* (Nitschke et al. in prep.); two months in *Acropora spathulata* recruits (Lamb et al. submitted; Johnston et al. in prep.); 10 months in *Acropora kenti* recruits (Quigley et al. 2023 https://doi.org/10.1007/s00338-023-02426-z). • **Field**: 10 months in adult *P. daedalea* (Allen et al. in prep.); 1 yr in juvenile *P. daedalea* (Ivory et al. in prep.).
2 (e)	Determine the adaptations that have led to increased thermal bleaching tolerance using genomic, transcriptomic and metabolomic analyses in a number of heat-evolved coral holobionts. This knowledge will assist in the identification of adaptive genetic variation, and the optimisation of selection regimes for experimental evolution.	• Selection of *Durusdinium trenchii* under (a) diurnal fluctuation between ambient and elevated temperatures and (b) continuous exposure to elevated temperatures (ratchet design) led to greater increases in tolerance at temperature extrema relative to selection under (c) symmetrical fluctuations between ambient and elevated temperatures every third week, corresponding to two to three generations at each temperature, and (d) symmetrical fluctuations between temperatures with ambient maintained for three weeks and elevated for six weeks, which corresponds to four to five generations. Regime (a) induced thermal generalism (increased thermal breadth); regime (b) led to a higher thermal optimum. (Scharfenstein et al. 2024 doi: 10.1111/nph.19996). • We compared the allele frequencies of single nucleotide polymorphisms (SNPs) in previously generated transcriptomes of four coral-symbiont pairings, using one heat-evolved strain that improved holobiont thermal tolerance (SS8), two heat-evolved strains that did not (SS3, SS5), and one unselected progenitor strain (WT10). By mapping transcripts back to the reference genome and using stringent filtering criteria, we identified 15,602 SNPs across 4,739 scaffolds, covering 36% of the genome. Most SNP loci were polymorphic and shared across strains, with some observed allele fixation, but no fixed differences. Observed genomic characteristics suggest that strain differentiation was driven by recombination of standing genetic variation, rather than novel mutations in coding regions. Since significant SNPs impacted genes involved in thermal tolerance and photosynthesis, the recombination of beneficial alleles into novel haplotypes likely enhanced the strains' thermal resistance in response to the laboratory evolution. These findings show the importance of existing genetic variation for the adaptation of Symbiodiniaceae and offer new insights into the genetic basis of thermal tolerance. (Buerger et al. in prep. [90% complete]). We are in the process of generating chromosomal

Objective	Key Findings and/or Outcomes
	scale assemblies of the heat-evolved strains that will allow a detailed genomic comparison among them. • *Acropora spathulata* recruits were inoculated with either heat-evolved (SS8) or wild-type (WT10) *Cladocopium proliferum* and reared for eight months. Recruits were then exposed to either ambient (27 °C) or elevated temperatures, which ramped up gradually by 0.625 °C per day until reaching 32 °C, where they were held for an additional 15 days. Physiological responses—including growth rate, colour reflectance (as a proxy for bleaching), and mortality—were monitored throughout. There were no significant differences in coral growth rates or mortality (<2%) between SS8 and WT10 recruits under either temperature condition. However, under heat stress, SS8 recruits bleached less severely than WT10, as indicated by lower reflectance values, suggesting increased thermal tolerance conferred by SS8 symbionts. *In hospite* Symbiodiniaceae cells were then collected from all treatments and analysed using Fourier transform infrared (FTIR) single-cell microspectroscopy at the Australian Nuclear Science and Technology Organisation (ANSTO), Clayton, Victoria. FTIR analysis revealed strong, conserved biomolecular responses to heat stress across both strains, including reductions in total protein and increases in free amino acids, carbohydrates, carboxylates, and ester carbonyls. Importantly, FTIR distinguished between SS8 and WT10 under heat stress: although both strains followed similar response patterns, WT10 exhibited more extreme shifts across multiple biomolecular categories, indicating a more severe physiological response. The milder reduction in protein content in SS8 suggests reduced protein degradation or sustained *de novo* protein synthesis, potentially preserving key components such as the D1 protein and antenna pigments essential for photosynthetic function. The stronger accumulation of carboxylates and carbohydrates in WT10 may indicate impaired lipid biosynthesis or translocation under energy-limited conditions, such as diminished adenosine triphosphate (ATP) production from heat-compromised photosynthesis. In contrast, SS8's moderated accumulation of these metabolites suggests better maintenance of cellular energy balance and metabolic function, further supporting its enhanced thermal resilience. (Johnston et al. in prep. [80% complete]). • Mass spectrometry imaging suggests that carotenoid algal pigments involved in photobiology and oxidative stress were the greatest contributors to the thermotolerance differences between *Galaxea fascicularis* corals hosting heat-evolved (SS8) versus wild-type (WT10) *Cladocopium proliferum*. These pigments may have increased photoprotection in the heat-evolved symbionts. Of the 13 metabolites that were significantly different between SS8- and WT10-corals under elevated temperature, the

Objective	Key Findings and/or Outcomes
	majority (~40%) were algal pigments localised within the algal symbionts. WT10- and SS8-corals displayed contrasting pigment profiles under elevated temperature. One of these pigments (m/z 524.17) was completely absent in WT10-corals under elevated temperature, but was highly abundant in SS8. (Chan et al. 2023 DOI: 10.1111/gcb.16987). • Mass spectrometry imaging identified potential biomarkers that separated *Platygyra daedalea* coral with WT10 or SS8 under temperature stress, and we have ongoing research to annotate these makers (they are most likely lipids) to reveal the mechanisms underpinning thermal adaptations. (Nitschke et al. in prep. For Science Advances [80% complete]). • *Acropora kenti* larvae with a heat-evolved (HE) symbiont that conferred enhanced tolerance (SS8) exhibited lower constitutive expression of algal photosynthesis genes, possibly compensated for by higher expression of carbon fixation genes. It also showed upregulation of coral heat tolerance genes, which was induced by the symbiosis with strain SS8. (Buerger et al. 2020 DOI: 10.1126/sciadv.aba2498). • HE symbiont strains that confer enhanced tolerance on coral larvae showed trait values under ambient conditions *in vitro* that differed from trait values of non-conferring and wildtype strains. There were low levels of intracellular reactive oxygen species (ROS), low levels of mitochondrial activity and high levels of reduced glutathione. Further work is required whether this pattern holds for other conferring HE strains and whether these trait values can be used to predict the *in hospite* effect of a HE strain. (Buerger et al. 2023 DOI 10.3389/fmars.2023.1094792).

Adjustments to key research objectives

Table 2: Variation in the Project over time.

Initial Research Question	Explain when, how and why the research question changed
Develop phenotype assays, deoxyribonucleic acid (DNA) markers or combined polygenic predictor scores to rapidly identify candidate coral colonies for selective breeding and asexual propagation within coral populations.	Some components of this objective turned out to be too overlapping with RRAP Genetic basis of key traits (ECT-01) Project goals. What we did do was to test phenotype assays to rapidly identify candidate coral colonies for selective breeding and asexual propagation within coral populations.
Can intraspecific hybridisation be used to generate heat tolerant coral stock for reef restoration?	In response to the large degree of variation in heat tolerance observed within coral populations (e.g. see RRAP Project Genetic basis of key traits (ECT-01) data), we broadened our approaches to managed breeding to harness within and between population variation in coral heat tolerance by investigating selective breeding and artificial selection, in addition to intraspecific hybridisation.

4 Future Research Recommendations

ECT-02.1: Selective breeding among coral populations

- Quantitative genetic analyses to identify phenotypic or genotypic predictors of offspring performance.
- Field testing of enhanced coral across ecologically relevant environments.
- Testing of enhanced coral throughout their lifespan.
- 'In-field selective breeding' by deploying parental broodstock from genetically distinct populations in warm regions close together on cooler (e.g. Central GBR) reefs.
- Evaluating strategies for choosing thermally tolerant parents via crossing experiments where offspring are phenotyped across life stages, under stress and in the field.

ECT-02.2: *Treatment with algal symbionts (Symbiodiniaceae)*

- Upscaling of field deployment of corals with heat-evolved (photo)symbionts: multiple sites in the central GBR, coral and symbiont species, including the development of strategies to culture vertically transmitted symbionts.
- Establishment of new heat-evolved symbiont cultures and explore multiple selective agents to further enhance tolerance breadth (e.g. temperature and light).
- Delivery of symbiont cultures to the northern and southern GBR via a containerised facility.
- Managed breeding combined with symbiont and bacterial treatments; sourcing of biological material from extreme environments; and transplanting over larger spatial and evolutionary distances.
- Amplifying essential workflows in coral phenotyping and genotyping to predict thermally tolerant corals in the field.
- Respecting, engaging and empowering Australian Indigenous and international communities.

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