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PROGRAM

Cryopreservation (CP-01)

Final Report June 2025

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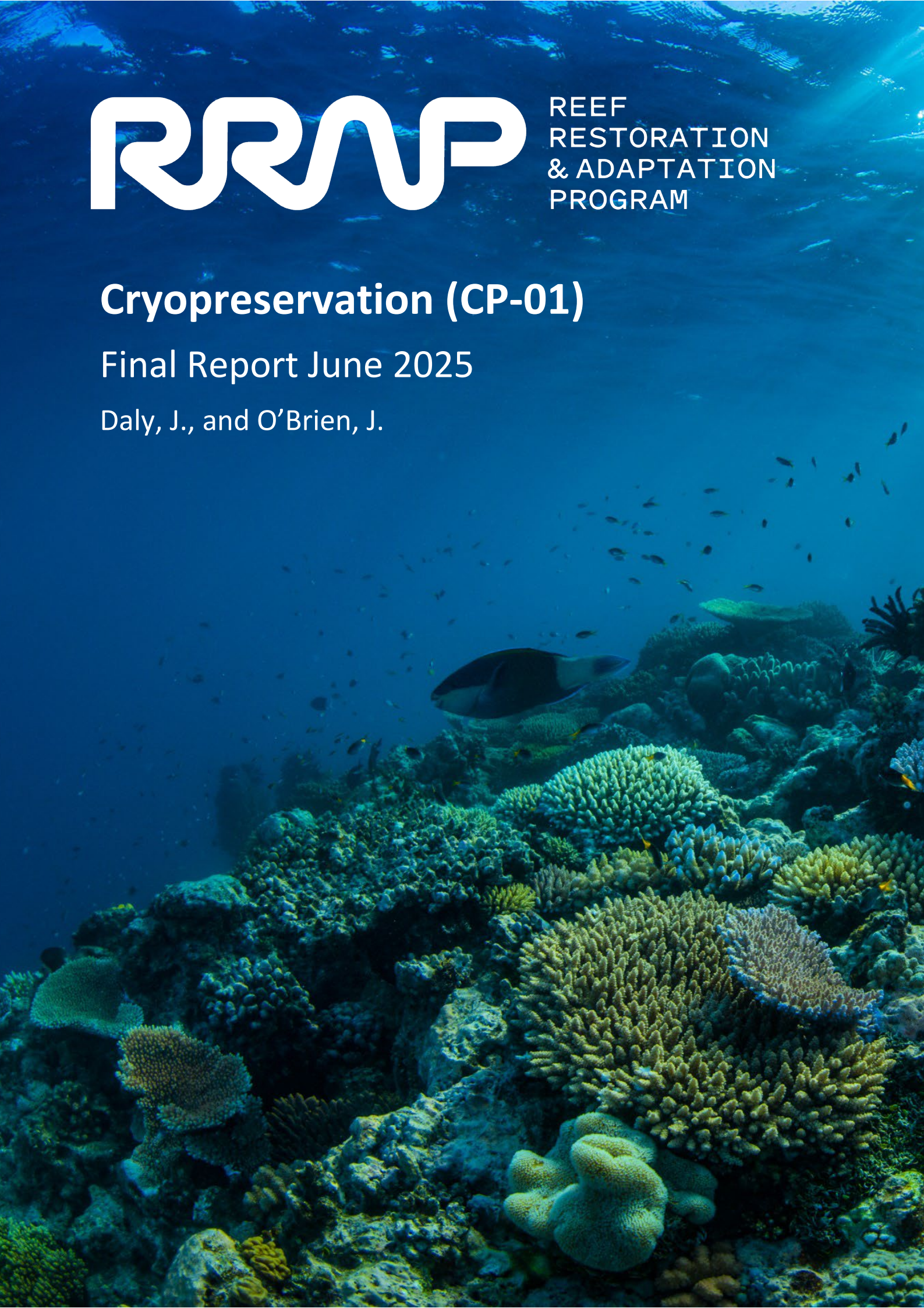

RRAP Cryopreservation (CP-01) Final Report June 2025

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Cover Page: Coral reef, Credit: Gary Cranitch, Queensland Museum

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This report summarises work undertaken under *Cryopreservation (CP-01)* in accordance with the Reef Restoration and Adaptation Program's *Cryopreservation* 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.

Acknowledgement

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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**
Davies Reef	Bindal
The Palms	Manbarra
Konomie	Woppaburra
Moore Reef	Gunggandji
Mosman, NSW	Cammeraygal
Kensington, NSW	Bidjigal

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1 Executive Summary

Over the past four years the RRAP Cryopreservation Sub-program has advanced the development, upscaling, and use of cryopreservation and biobanking technologies to support coral reef restoration and resilience on the Great Barrier Reef (GBR). By enabling the long-term storage of coral germplasm, tissues, and symbionts, cryopreservation can assist the maintenance of coral broodstock for aquaculture, support the development of enhanced corals and Symbiodiniaceae for research, and secure genetics to help mitigate the loss of genetic and species diversity on the reef. The program had three objectives: (1) establishing cryobiology research infrastructure and expanding biorepository capacity; (2) developing scalable cryopreservation technologies; and (3) securing coral biodiversity through targeted biobanking.

Key outcomes from the Sub-program include the expansion of the Taronga CryoDiversity Bank to accommodate thousands of samples using a modular biosecure storage system, the development of a semi-automated workflow for sperm cryopreservation, and development of a high-density fertilisation method to enable the use of cryopreserved coral sperm to produce larvae at scale. Coral recruits produced from cryopreserved sperm using this fertilisation approach have shown comparable *in situ* survival rates to recruits produced from fresh sperm in field deployments. Progress was also made in larval cryopreservation, and cryopreservation protocols for cultured Symbiodiniaceae strains were developed, supporting the establishment of laboratory protocols for routine cryopreservation of symbiont cultures for research. Additionally, from 2021 to 2023, 1,035 samples were cryopreserved from 152 coral colonies across 13 species, collected from four sea Countries. A cross-cultural approach to biobanking was developed in partnership with Traditional Custodians, ensuring that samples maintain links to Country in perpetuity through the incorporation of sea Country information in the CryoDiversity Bank database and including the requirement for free, prior and informed consent (FPIC) in the Taronga material transfer agreement.

Research outputs included eleven peer-reviewed publications, three technical reports, and two standard operating procedures. Future research should focus on upscaling of germplasm conservation to support the development of thermally tolerant corals, ongoing research to develop larval cryopreservation, development of biorepository systems and processes to support the management of thermally tolerant genotypes and phenotypes, and the continued refinement of culturally safe practices for collection and use of germplasm (sperm, larvae, symbionts) in partnership with Traditional Custodians.

2 Background and Justification for the Research

Cryopreservation and biobanking can enable the long-term frozen storage of living genetic material in liquid nitrogen (-196ºC). Through the development and application of innovative cryopreservation techniques for coral germplasm, tissues, and symbionts, the RRAP Cryopreservation Sub-program aims to integrate with, and provide support for, several potential RRAP interventions. This includes cryopreserving reproductive products to assist the development and maintenance of coral broodstock for aquaculture and to support coral deployment and securing enhanced coral and Symbiodiniaceae for research and development of thermally tolerant strains. Additionally, biobanking is one of the most effective methods to secure biodiversity, and cryopreservation of living coral samples from healthy reefs could help to mitigate the loss of genetic and species diversity caused by predation, natural disasters, and bleaching events on the Great Barrier Reef (GBR). Cryopreservation and biobanking of sperm from Great Barrier Reef corals at the Australian Institute of Marine Science (AIMS) began in 2011 as a collaboration between Taronga Conservation Society Australia and the Smithsonian Institute in the United States of America (Hagedorn et al. 2012). From 2011 to 2020, cryopreservation of gametes was based on opportunistic sampling during spawning at AIMS, with the goal of banking as many cryopreserved coral sperm samples as possible to secure genetic diversity from the Great Barrier Reef. While improvements have been made to the cryopreservation process over this time, most notably with the addition of Computer Assisted Sperm Analysis (CASA) for gamete quality assessment (Zuchowicz et al. 2021), the biobanking of coral sperm in Australia and around the world has generally been undertaken on a relatively small scale. Similarly, the use of cryopreserved sperm to generate larvae has been limited (Hagedorn et al. 2021, Daly et al. 2022), so the knowledge required to scale the use of cryopreserved sperm for aquaculture production is limited.

In addition to coral sperm, there is a need to cryopreserve coral larvae and symbionts (Symbiodiniaceae) to support reef restoration activities and effort to increase thermal resilience. While cryopreserved sperm represent a valuable genetic resource, the use of this sample type to generate new corals is limited to a few days or weeks each year during annual spawning events when fresh oocytes are available for fertilisation. Cryopreservation of coral oocytes would circumvent this issue but, as with the oocytes of most animal species, is not currently possible due to the low surface to volume ratio and high lipid content that increases the risk of damaging ice formation during cooling. An alternative approach is to cryopreserve coral larvae, which is currently possible for some species (Daly et al. 2018) but has not been widely tested to understand differences that may occur among species and larval sizes. The ability to cryopreserve coral larva during spawning would potentially go some way to increasing the scale of deployment of coral recruits by providing a source of larvae year-round. Cryopreservation of Symbiodiniaceae has been reported for symbionts isolated from coral tissues but has not been applied to single species strains held in living culture. Development of protocols to cryopreserve cultured Symbiodiniaceae strains would provide an important tool to support the generation of thermally-tolerant symbionts to support reef resilience by providing a back-up of different stages of the selection process and reducing the need to maintain living cultures.

The overarching goal of the RRAP Cryopreservation Sub-program is to build on this previous work to establish an ongoing biobanking program for the GBR that can operate at the scale required to support the activities being developed by RRAP partners.

To achieve this, the RRAP Cryopreservation Sub-program had three initial objectives:

1. Establish cryobiology research facilities and expand biorepository capacity to support the transfer of cryopreservation technologies for coral to Australia.
2. Develop new cryopreservation technologies and flexible freezing systems to enable large-scale cryopreservation of living coral genetic samples (in the thousands) to support the activities of RRAP partners.
3. Secure coral biodiversity on the GBR through targeted biobanking of living coral germplasm, tissues, and symbionts from vulnerable and prioritised species, populations, and reefs.

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.	Establish cryobiology research facilities and expand biorepository capacity to support the transfer of cryopreservation technologies for coral to Australia.	• Expansion of biorepository systems at the Taronga Institute of Science and Learning (Cammeraygal Country, Sydney) to accommodate thousands of samples. Assessment of available storage options determined that a modular approach utilising a series of 6000-sample storage systems would be the most flexible and cost-effective approach to biorepository expansion, as this would allow for increasing capacity while reducing the amount of unused space and permitting samples to be separated or spread across systems as needed. • An audit of historic sample collections made between 2011–2020 determined that the Taronga CryoDiversity Bank held 2614 cryovials of cryopreserved sperm sourced from 381 coral colonies, with 230 colonies (60%) represented by single genotypes (i.e. sampled as individuals) and the remaining 151 colonies represented in pooled sperm samples comprising two or more colonies. These samples were collected from 30 identified species of hard coral, originating from 10 Traditional Custodian sea Countries across the northern, central, and southern regions of the GBR Marine Park.
2.	Develop new cryopreservation technologies and flexible freezing systems to enable large-scale cryopreservation of living coral genetic samples (in the thousands) to support the activities of RRAP partners.	• Development of a semi-automated approach to streamline the assessment, handling, and cryopreservation of coral sperm during spawning. The process builds on existing technologies and combines computer-assisted sperm analysis, barcoded cryovials, and a series of linked auto-datasheets for simultaneous editing by multiple users to improve the efficiency of both sample processing and metadata management in the field. • Fertilisation using a standard sperm concentration (1 x 10^6/ml) with higher egg densities found no impact on fertilisation rate even at 1000 eggs/mL (i.e. 100,000 eggs in 100 mL of filtered sea water (FSW)). This high-density fertilisation method did not impact on the number of plugs with settled recruits using either fresh or cryopreserved sperm, and there was no difference in the survival of those recruits after eight weeks post-settlement. • Coral recruits produced using cryopreserved sperm and deployed on Manbarra sea Country as part of RRAP Large Field Trials (LFT) showed survival on 44% of devices and on 20% of the settlement tabs after three months, with assessment ongoing. These findings compare

Objective		Key Findings and/or Outcomes
		favourably with those of recruits produced using fresh sperm (coral recruits observed on 26% of devices and 10% of settlement tabs), deployed on the same site. • Advances in understanding the cryobiology of coral larvae from a range of species. During preliminary experiments at AIMS in 2022, larvae from the mushroom coral (*Fungia fungites*) were cryopreserved using cryomesh technology and recovered with a 70% survival rate. Working with our international partners we have been able to make progress in larval cryobiology and applying this technology to coral species that have larger larvae (i.e. >300 µm diameter). We have also discovered that there are species differences in how larvae respond to cryoprotectants, and larval quality is a key factor affecting outcomes of experiments. Research is ongoing in partnership with the Smithsonian Institution and the University of Minnesota. • Development of cryopreservation protocols for cultured symbionts at the AIMS Symbiont Culture Facility. Using a slow-cooling protocol with cryo straws we have seen successful cryopreservation and recovery of a broad range of symbiont cultures from across all the major Symbiodiniaceae Genera that associate with coral, including some heat-evolved strains of Cladicopium. Research is ongoing to optimise recovery of the selected strains and to standardise workflows.
3.	Secure coral biodiversity on the GBR through targeted biobanking of living coral germplasm, tissues, and symbionts from vulnerable and prioritised species, populations, and reefs.	• During annual spawning in 2021, 2022, and 2023, a total of 1035 samples were cryopreserved and banked from 152 colonies across 13 species of coral. These samples were collected from corals that originated from the Bindal, Manbarra, Gunggandji, and Woppaburra sea Countries. • Creation of a cross-cultural approach to coral biobanking that helps to ensure that samples at Taronga maintain links to Country in perpetuity, in partnership with Woppaburra Traditional Custodians and the AIMS Indigenous Partnerships team. Key elements identified include designation of the Gamay Rangers as Cultural Custodians of the samples, inclusion of Country and contact information in the sample database, inclusion of FPIC requirements in the Materials Transfer Agreement for any sample requests, and regular review of sample information and permissions to hold the samples at the CryoDiversity Bank. This approach will provide a framework for culturally safe biobanking practices for Great Barrier Reef corals more broadly, in consultation with Traditional Custodian representatives from all sea Countries from which Taronga collects coral samples, and with Traditional Custodian representatives from Cammeraygal and Wiradjuri Country, where the Taronga CryoDiversity Banks are located.

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
Traditional Custodian collaboration	The development of a cross-cultural approach to biobanking was not part of the initial research objectives for the subprogram. The inclusion of this component was due to our participation in an on-Country spawning event on Konomie (North Keppel) Island with Woppaburra Traditional Custodians and AIMS scientists in 2022. The on-Country event enabled informal discussions with Woppaburra TUMRA members about the cryopreservation and biobanking process and cultural concerns related to the transfer of samples to the Taronga CryoDiversity Bank at the end of spawning. These initial discussions resulted in a Biobanking Cultural Safety workshop held at Taronga in September 2023 that included Woppaburra Traditional Custodians, members of the AIMS Indigenous Partnerships team, the Gamay Rangers, and the Taronga Cultural Programs and Science teams. Outcomes from these discussions are outlined in Table 1.

4 Future Research Recommendations

Future research in this area should focus on continued cryopreservation technology development, scaling and integration of cryopreservation technologies into aquaculture production pathways, and the technical and bio-cultural requirements for coral biorepository development.

Upscaling of germplasm conservation to support the development of thermally tolerant corals

Collection and processing pathways for coral germplasm preservation will need to operate at a scale suitable to support the large-scale production and deployment of coral recruits onto reefs. Activities to achieve this should include the refinement and standardisation of protocols for the cryopreservation and use of sperm and symbionts at required scales, and the testing and integration of high-throughput processing systems for cryopreservation of coral germplasm at a commercial scale to support aquaculture production.

Development of larval cryopreservation

Cryopreservation of coral larvae at scale during spawning would permit deployment of coral recruits throughout the year. This will require ongoing research to understand species differences in the response to the cryopreservation process and the biological, physical, and technological limitations of current cryopreservation technologies to permit upscaling. Testing of cryopreservation processes will also require field trial deployment of coral recruits derived from cryopreserved larvae to examine the potential for year-round deployment.

Development of biorepository systems and processes to support the management of thermally tolerant genotypes and phenotypes

Effective biorepository development will require the translation of cryopreservation and biobanking technologies from research-level activities to commercial-scale pathways suitable for industry application. Activities to achieve this could include simulation modelling and process mapping to understand relationships and improve the efficiency of collection and use of coral germplasm at scale, and the continued development and maintenance of biorepository and database systems to support the storage of coral genetic resources.

Refinement of culturally safe practices for collection and use of germplasm (sperm, larvae, symbionts) in partnership with Traditional Custodians

Protocols are required to ensure that samples held off-sea Country maintain their links to sea Country in perpetuity, and that future use of samples can occur in a culturally safe manner, providing new tools for Traditional Custodians to protect the biodiversity of their sea Countries. Achieving this will require consultation with Traditional Custodians to guide the development of protocols for culturally safe and equitable biobanking, and the refinement of Materials Transfer Agreements to incorporate Traditional Custodian requirements and FPIC for future sample access.

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