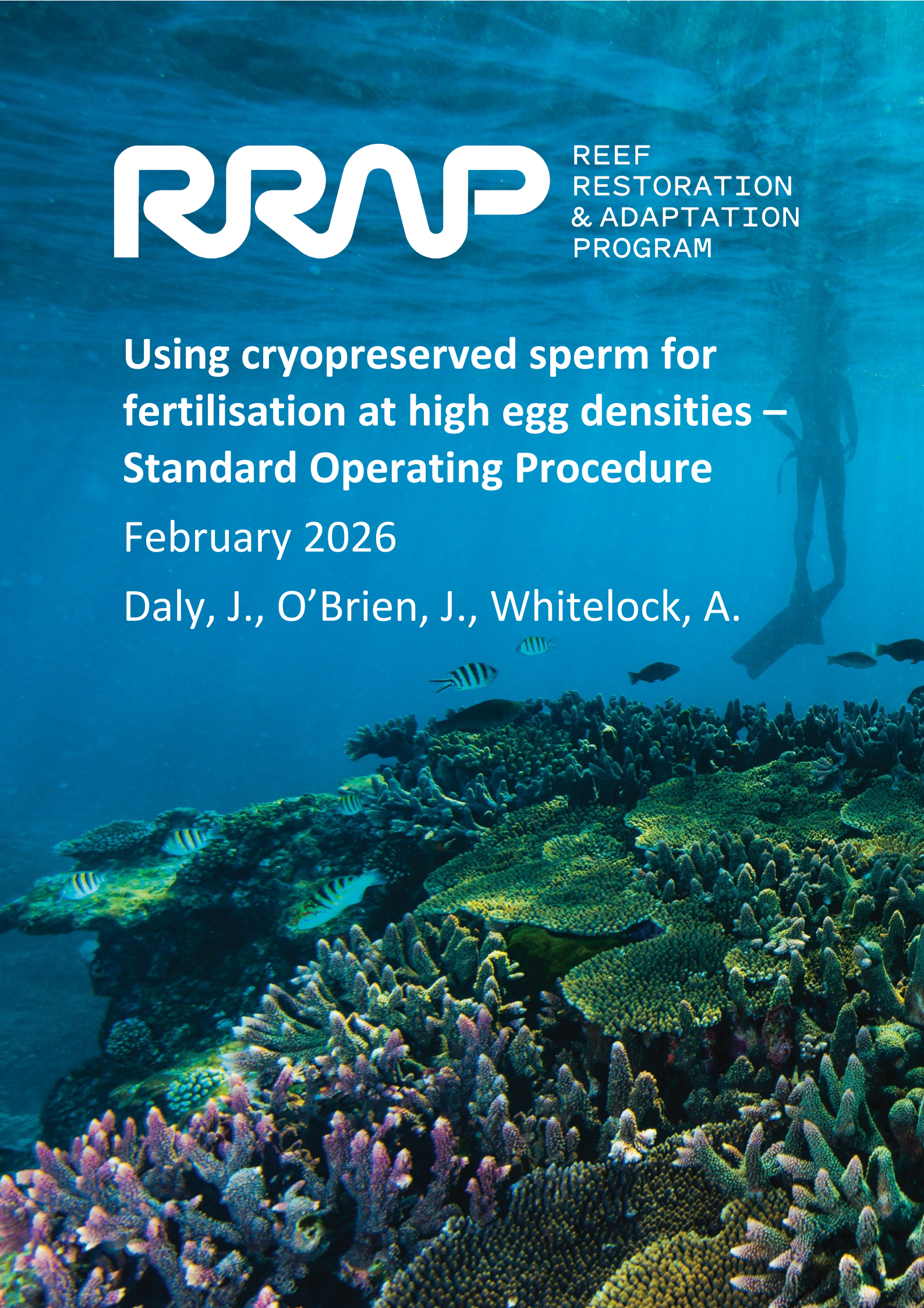
REEF RESTORATION & ADAPTATION PROGRAM

Using cryopreserved sperm for fertilisation at high egg densities – Standard Operating Procedure

February 2026

Daly, J., O'Brien, J., Whitelock, A.



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We specifically acknowledge and thank the following Traditional Owners of Country on which this report was prepared:

Location	**Traditional Owner Group**
Mosman, NSW	Cammeraygal
Kensington, NSW	Bidjigal

Table of Contents

List of Figures

List of Tables

1 Executive Summary

This SOP provides a practical, scalable method for using cryopreserved sperm to produce coral larvae at a restoration-relevant scale. The SOP was developed in *Acropora* species commonly spawned for aquaculture and resilience research in Australia (e.g., *A. kenti* and *A. millepora*), but is broadly applicable to all bundle spawning hermaphroditic coral species. The high-density fertilisation approach described in this SOP utilises a standard fertilisation unit based on cryopreservation sampling methods (5 mL gamete bundles over 5 mL seawater), which yields sperm at approximately 2×10^9/mL and ~25,000 eggs. To streamline the workflow, a packed cell volume approach is used to estimate egg numbers for fertilisation in a volume of 100 mL seawater in 1 L Imhoff cones and a target sperm concentration of 1×10^6 motile cells/mL. The full process, from gamete collection through to fertilisation assessment, takes approximately four to five hours and is aimed at coral aquaculture and restoration practitioners who may use this SOP to facilitate specific crosses for genetic management as part of coral conservation breeding programs.

2 Background

Cryopreservation and biobanking can play a crucial role in large-scale reef restoration programs by facilitating genetic management of populations through selective breeding, reducing the dependence on synchronous spawning, supporting research, and preventing the extinction of coral species. In vitro fertilisation of coral eggs is usually based on a fertilising medium containing approximately 10 eggs/mL and a sperm concentration of 1×10^6/mL. To date, fertilisations using cryopreserved sperm have been done in relatively small volumes (i.e., <500 mL) in comparison to fertilisations using fresh sperm, which often utilise volumes in the range of litres to hundreds of litres. Although the small fertilisation volume minimises the number of cryopreserved samples that need to be thawed, it also limits the number of eggs that can be fertilised if the 10 eggs/mL conventional density is maintained. Previous uses of cryopreserved sperm for larval production have generally aimed to produce hundreds to thousands of larvae for experimental purposes. The production of coral recruits using cryopreserved sperm for reef restoration at scale will therefore require upscaling by several orders of magnitude to permit the production of hundreds of thousands to millions of larvae for brood stock management or deployment purposes.

This SOP applies to hermaphroditic coral species that produce gamete bundles containing both eggs and sperm during spawning. While it has been developed specifically in *Acropora* species commonly spawned for aquaculture and resilience research in Australia (e.g., *A. kenti* and *A. millepora*), the process is broadly applicable to all bundle spawning coral species. This SOP utilises a standard fertilisation unit based on the sampling method used to generate high concentration sperm samples for cryopreservation. Samples are collected at a ratio of 5 mL of gamete bundles over 5 mL of seawater, which equates to a sperm concentration of approximately 2×10^9/mL and approximately 25,000 eggs after bundle break up. The isolated sperm are assessed to estimate sperm concentration and motility, and can be used for fertilisation either fresh or after cryopreservation and thawing. Eggs for fertilisation must be cleaned thoroughly to remove excess autologous sperm, after which the cleaned eggs can remain separated by colony or combined into a pool immediately prior to fertilisation. After fertilisation the eggs are cleaned again then transferred into rearing systems for larval development. The procedure described in this SOP takes approximately 4–5 hours, from gamete bundle collection through to assessment of fertilisation outcomes. The procedures described in this SOP have been demonstrated over several years, with fertilisation rates ranging from 20% to 95%. Additional refinement is still required to understand this variability and to optimise fertilisation outcomes.

3 Objectives and Scope

The purpose of this SOP is to provide guidance on using cryopreserved sperm to generate coral larvae at a restoration-relevant scale. The SOP is aimed at coral aquaculture and restoration practitioners who may use this SOP to facilitate specific crosses for genetic management as part of coral breeding programs. The SOP provides a list of required equipment and steps for all stages of the fertilisation processes, along with additional information on best practices for consumables and equipment. In addition to fertilisation of eggs from individual coral colonies, the SOP also provides procedures for pooling eggs from multiple colonies where larger egg numbers are required.

4 Pre-requisites

4.1 Sperm quality assessment

Assessment of sperm sample fertility potential (also referred to as 'sample quality' assessment) is an essential step in the cryopreservation pathway and for using sperm for fertilisation. Sperm sample quality is typically determined by estimating the number of cells in the sample (concentration), the proportion of cells that are actively moving (motility). These assessments can be made either manually or with the aid of computer-assisted sperm analysis (CASA). Manual assessment involves the visualisation of a sample aliquot using a phase microscope to subjectively estimate the percentage of motile cells and vigour of movement, and manual cell counting using a haemocytometer to determine concentration. CASA provides an automated and standardised method to simultaneously estimate sperm concentration and motility and is much less time consuming than manual methods, but relies on expensive proprietary software. Detailed steps for assessment of sperm concentration and motility in fresh and cryopreserved samples using CASA are provided in Daly et al. (2024).

Regardless of the analysis method used, the use of a sperm activation solution containing caffeine and bovine serum albumin (BSA) is recommended for motility assessment. This removes the need to wait for sperm activation to occur naturally (which can take up to 30 min) and ensures that all samples are exhibiting maximal motility at the time of sample assessment. Instructions for the preparation of sperm activation solution are provided in Daly et al. (2025). For samples collected as described in **step 7.1** (i.e., approximately 2×10^9/mL) an aliquot of sperm should be diluted at a ratio of 1:99 (sperm sample:sperm activation solution, v/v). Sperm motility can then be assessed by placing a droplet of activated sperm on a plain glass slide with a coverslip and estimating the proportion of cells actively moving, to the nearest 10%. For manual assessment of concentration, sperm should first be inactivated by exposure to 10% (v/v) ethanol in filtered seawater (FSW) to ensure accurate counting. For samples collected as described in **step 7.1** (i.e., approximately 2×10^9/mL) an aliquot of sperm should be diluted at a ratio of 1:999 for counting (sperm sample: 10% ethanol in FSW, v/v). This can be done in two steps by first diluting the sample aliquot 1:99 with FSW (sperm sample: FSW, v/v), followed by 1:9 with 10% (v/v) ethanol in FSW. Sperm concentration can then be estimated using an improved Neubauer counting chamber (haemocytometer) according to the manufacturer's instructions.

4.2 Sperm cryopreservation

Coral sperm are cryopreserved at a concentration of approximately 1×10^9/mL in FSW with 10% (v/v) dimethyl sulfoxide (DMSO). A custom made, 3D-printed, cryopreservation device is used to cool 1 mL aliquots in 2 mL cryovials at a rate of –20 °C/min using liquid nitrogen, as described in Zuchowicz et al. (2021). A detailed description of the sperm processing pathway for coral sperm cryopreservation, including sample thawing and post-thaw quality assessment, can be found in Daly et al. (2024).

4.3 Calculating egg densities

To maximise the chances of successful fertilisation, gametes need to be combined as soon as possible after spawning, ideally within two hours of sample collection. Manually counting eggs can be time consuming and is often not feasible given the time required to isolate and prepare coral gametes for fertilisation, especially when there are multiple colonies spawning. To reduce the amount of resources required and minimise the time between spawning and fertilisation, this SOP uses a packed cell volume (PCV) approach to estimate the number of eggs used for fertilisation.

Most *Acropora* species commonly spawned for restoration in Australia (e.g., *A. kenti* and *A. millepora*) have egg diameters in the range of 500–600 µm (Randall et al. 2024). Packed cell volume calculations in this SOP are based on the upper end of this range to provide a more conservative estimate of egg numbers. For an egg diameter of 600 µm, volume can be calculated as 0.113 µL, which means that 1000 eggs would occupy a volume of approximately 113 µL:

$$V_{egg} = \frac{4}{3}\pi r^3 \approx 4.18879 \times 0.3\ mm^3 \approx 0.113\ \mu L$$

$$0.113\ \mu L \times\ 1000 = 113\ \mu L$$

In practice, this volume is rounded up to 120 µL per 1000 eggs for ease of measurement and to allow for a small amount of water space between eggs.

For *Acropora*, the number of eggs in a sample can therefore be conservatively estimated by dividing the PCV of eggs in microlitres by 0.12.

E.g., for a PCV of 1 mL:

$$\frac{1000\ \mu L\ PCV\ eggs}{0.12} \approx\ 8{,}333\ eggs$$

This approach can also be used to calculate the PCV of eggs required to achieve a target egg density, for example 1000 eggs/mL in a 100 mL fertilisation volume:

$$1000\ eggs/mL \times 100\ mL\ = 100{,}000\ eggs$$

Assuming 120 µL per 1000 eggs,

$$100{,}000\ eggs = 100\ \times 120\ \mu L = 12{,}000\ \mu L = 12\ mL\ PCV\ eggs$$

This SOP utilises a standard fertilisation unit based on the number of eggs present in a sample collected for cryopreservation at a ratio of 5 mL of gamete bundles over 5 mL of seawater. In our experience with Australian *Acropora* species, 5 mL of bundles equates to approximately 3 mL of eggs once those bundles have broken down, i.e., approximately 0.6 times the volume of bundles. Using the packed cell volume calculations outlined above, this would mean that the standard fertilisation unit contains approximately 25,000 eggs, which equates to a density of 250 eggs/mL in a 100 mL fertilisation volume:

$$\frac{3000\ \mu L\ PCV\ eggs}{0.12} = 25{,}000\ eggs$$

$$\frac{25{,}000\ eggs}{100\ mL} = 250\ eggs/mL$$

5 Identified Risks and Hazards

Table 1. Generalised hazard and risk table for handling and use of chemicals and liquid nitrogen

Task or Process	Hazards	Potential Consequence	Control Measures	Risk Category	Risk Rating		
					Consequence (of hazard)	Frequency (or likelihood)	Risk
Chemical use	Chemical spills, personal safety	Poisoning or incapacitation; contamination of environment	• Store chemicals in original packaging in the appropriate environment as specified in the SDS • Use appropriate PPE as defined in the SDS (gloves, safety glasses, lab coat, enclosed shoes). • Label aliquots and prepared solutions with contents, date, and initials. • Refer to SDS and local laws for disposal.	Safety	Minor	Possible	Low
Working with liquid nitrogen	Exposure to low oxygen environment	Asphyxia	• Ensure adequate ventilation of working area • Only trained personnel allowed to work with liquid nitrogen • Limit the volume of liquid nitrogen used to 50L to prevent asphyxiation in case of spillage	Safety	Major	Unlikely	Medium
	Exposure to sub-zero temperatures	Burns and frostbite	• Use cryo gloves, forceps and safety glasses when handling liquid nitrogen and cryopreserved samples • Only trained personnel allowed to work with liquid nitrogen	Safety	Minor	Possible	Low

NOTE: Always consult the product SDS provided by the manufacturer prior to use and follow institutional guidelines for chemical use and disposal.

Always follow institutional guidelines for storage and handling of liquid nitrogen.

6 Equipment and Materials

6.1 Procedure equipment and consumables

Table 2. Equipment and consumables needed for fertilisation of coral eggs at high-densities .

Item	Supplier	Product number
Sample collection and preparation		
Plastic cups	Generic	–
50mL tubes – racked	Corning	CLS430829
Transfer pipettes	Labco	650.050.503
100µm cell strainers	Corning	352360
100mm diameter filter with 106µm mesh	Generic	–
Squirt bottle	Generic	–
2L plastic bowl	Generic	–
1L graduated measuring cylinder	Generic	–
Fertilisation		
Air pump	Generic	–
Rigid airline tubing	Generic	–
1L Imhoff polycarbonate cone	Hach	206700
Plastic 2-way, ball valve, push fitting (tap)	John Guest	PPSV040808W
P1000 100–1000 µL pipettor	Eppendorf Research Plus	3123000063
100–1000 µL pipette tips	Interpath	24800
Electronic pipette controller	VistaLab ali-Q VS	2100-1005
10mL serological pipettes	Corning	CLS4100
6-well plates	TPP	Z707767

NOTE: Supplier information and product numbers are for reference only and users may choose their own equivalent product and supplier.

*Small volumes of seawater can be hand-filtered through a 0.22µm syringe filter using a rubber- and lubricant-free syringe.

6.2 Personal protective equipment (PPE) and other safety equipment

Table 3. PPE required for fertilisation of coral eggs at high-densities.

Item	**Manufacturer**	**Product number**
Safety glasses	Generic	–
Lab coat	Long sleeve, full length	–
Nitrile gloves	Generic	–
Cryogloves (pair)	Tempshield	Mid-arm

7 Steps for Implementation

7.1 Gamete preparation

NOTE: Ensure that gamete handling and fertilisation are undertaken using clean, sanitized equipment, in an area that is not being concurrently used for activities that generate chemical vapours or aerosols (e.g., from fixatives or cleaning products) that might present potential toxicity to gametes and embryos. Disposable gloves are not necessary for the collection and handling of gamete samples but may be worn if preferred. Hands should be washed thoroughly with fresh water, or gloves changed, between each gamete sample to avoid cross-contamination. Ambient temperature in the laboratory should be set to 24–28°C when possible to avoid cold- or heat-shocking sperm.

7.1.1 Gamete collection

1. Collect gamete bundles from the surface of the water using a clean, sanitised, plastic cup.
2. Using a 3 mL transfer pipette, transfer bundles into a labeled 50 mL tube at a ratio of 5 mL of gamete bundles over 5 mL of seawater (10 mL total volume) (**Fig. 1**).
3. Agitate the sperm–egg bundles by gently swirling the sample by hand until the bundles have broken apart.

7.1.2 Separate sperm from eggs

1. After bundle break up, let the sample sit for two minutes in a tube rack to allow eggs to float to the top of the sperm sample below. Eggs will form a layer at the surface and individual eggs will be visible.
2. Gently aspirate the sperm sample from the bottom of the tube using a clean 3 mL transfer pipette.
3. Transfer the sperm sample to a 100 µm cell strainer sitting atop a new sterile 50 mL tube.
4. If necessary, gently tap the filter to encourage flow or use a clean transfer pipette to collect any residual sample from the underside of the filter.
5. Remove the filter, then cap the tube loosely to allow air exchange and label with the colony ID and relevant metadata from the original sample tube.
6. Add approximately 40 mL FSW to the tube containing eggs then cap the tube loosely to allow air exchange.
7. Transfer the eggs for cleaning (**step 7.3**).
8. Transfer the sperm sample for cryopreservation (see **section 4.2**)

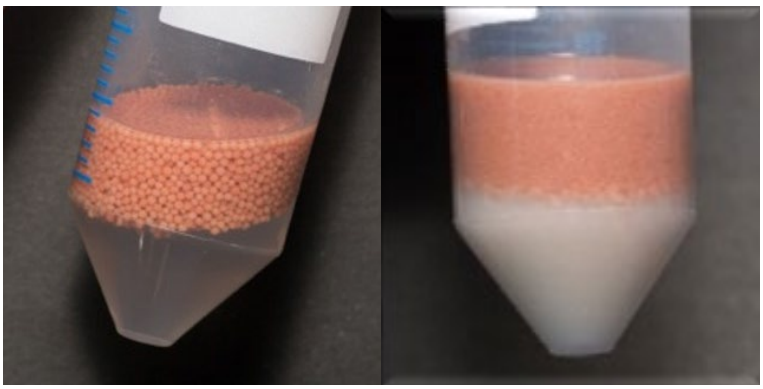

Figure 1. Coral gamete sample before (left) and after (right) bundle break up.

7.2 Calculation of sperm addition

1. Determine sperm concentration and motility.

NOTE: See **section 4.1** for information on assessing sperm quality using CASA and manual methods.

2. Calculate the motile concentration of the sample by multiplying the sperm concentration by the proportion of motile sperm.
    - E.g., a sperm sample that has a concentration of 1×10^9/mL and 50% motility, would have a motile concentration of 5×10^8 sperm/mL (i.e., $0.5 \times (1 \times 10^9$/mL)).
3. Calculate the volume of sperm sample required to achieve a motile concentration of 1×10^6/mL in the fertilisation volume.
    - E.g., a motile concentration of 1×10^6/mL in a fertilisation volume of 100 mL gives a total of 1×10^8 motile sperm (i.e., $100 \times (1 \times 10^6$/mL)). If the sample has a motile concentration of 5×10^8 sperm/mL, then the volume of sperm needed would be 0.2 mL (i.e., $1 \times 10^8 / 5 \times 10^8$).

NOTE: The suggested concentration of motile sperm in the fertilisation volume (1×10^6/mL) aims to approximate that of standard artificial fertilisation methods using fresh sperm. If sample quality post-thaw is poor (i.e., requiring the addition of a large volume of cryopreserved sperm to achieve a motile concentration of 1×10^6/mL), then it may be necessary to target a lower motile concentration as there is evidence in some species that fertilisation may be impacted if the total sperm concentration in the fertilisation volume exceeds approximately 1×10^7/mL (Oliver and Babcock 1992). Addition of a lower sperm volume will also minimise the amount of DMSO added to the fertilisation volume, which may also impact on fertilisation in some species (Howells et al. 2022).

7.3 Cleaning eggs

7.3.1 Using a mesh filter

1. Place a 100 mm diameter filter with 106 µm mesh in a 2 L bowl and top up with FSW so that the water level is approximately 2 cm below the top of the filter.
2. Fill a second 2 L bowl with FSW to the same level.
3. Carefully pour coral eggs from **step 7.1.2** into the filter and gently swirl the filter in the FSW to rinse sperm off the eggs.
4. Slowly raise the filter out of the water, continuing to swirl the eggs so that they do not get sucked onto the mesh, and transfer the filter with eggs into the second bowl containing FSW.
5. Empty the contents of the first bowl then rinse the bowl thoroughly with fresh water followed by FSW, then refill the bowl with FSW.
6. Gently swirl the filter containing eggs in the FSW in the second bowl, then transfer back to the bowl with clean FSW.
7. Repeat this process at least five times, or until the water is completely clear and the eggs move freely when swirled.
8. Use a squirt bottle to gently rinse the eggs from the filter into a clean bowl containing FSW and label with the colony ID.
9. Place an airline in the bowl and provide gentle aeration at approximately one bubble/s.
10. Eggs can be maintained under these conditions for up to one hour prior to fertilisation.

7.3.2 Using a 1 L Imhoff cone

1. Fill a clean 1 L Imhoff cone with a 2-way tap the bottom to approximately 800 mL with FSW.
2. Carefully pour coral eggs from **step 7.1.2** into the cone and allow the eggs to float to the surface.
3. Open the tap at the bottom of the cone and slowly drain the water until the level reaches approximately 100 mL (or as low as possible without losing eggs), keeping a close eye on the eggs to ensure that they remain at the surface.

NOTE: If the eggs become dispersed in the water column while the water is draining, close the tap and allow the eggs to float back to the surface before resuming.

4. Refill the cone to approximately 800 mL with FSW and allow the eggs to float back to the surface.
5. Repeat this process at least five times, or until the water is completely clear and the eggs move freely.
6. Refill the cone to 800 mL with FSW and label the cone with the colony ID.

7. Position a rigid airline at the bottom of the cone and provide aeration at approximately one bubble/s.
8. Eggs can be maintained under these conditions for up to one hour prior to fertilisation.

7.3.3 Pooling eggs

NOTE: The egg cleaning process is unlikely to remove one hundred percent of sperm so pooling of eggs should be done immediately prior to fertilisation to minimise the risk of background fertilisation.

1. Remove aeration and allow the eggs to settle at the water surface.
2. If eggs were cleaned using a filter and bowls, concentrate the eggs by siphoning from the bottom of the bowl to reduce the water volume, or by pouring the eggs gently into a 100 mm diameter filter with 106 µm mesh in a 2 L bowl.
3. If eggs were cleaned using 1 L Imhoff cones, remove the rigid airline from the cone, allow the eggs to float to the water surface, then open the tap at the bottom of the cone and slowly drain the water until the level reaches 100 mL.
4. Combine the eggs by gently transferring the contents of each bowl or cone into a 1 L graduated measuring cylinder.
5. Using a 3 mL transfer pipette, remove triplicate aliquots of at least 100 eggs each and transfer them to individual wells containing 5 mL FSW in a 6-well plate, to use as no-sperm controls.

NOTE: The rate of background fertilisation can be determined approximately two hours after the eggs are combined by calculating the proportion of dividing embryos in the no-sperm control samples.

6. Immediately proceed to fertilisation, as described in **step 7.4.2**.

7.4 Fertilisation

7.4.1 Individual colonies

1. If eggs were cleaned using a filter and bowls, concentrate the eggs and transfer them to 1 L Imhoff cones containing approximately 800 mL of FSW, as described in **step 7.3.3**.
2. Remove the rigid airline from the 1 L Imhoff cones, allow the eggs to float to the water surface, then open the tap at the bottom of the cone and slowly drain the water until the level reaches 100 mL (**Fig. 2**).
3. Add the required amount of sperm (calculated in **step 7.3**) using a micropipette and start a timer for 60 minutes.

NOTE: Cryopreserved sperm should be added to the fertilisation volume as soon as possible after thawing. It is recommended to do a test thaw from each cryopreservation batch to assess motility and concentration so that the number of samples required for thawing can be anticipated (see **step 7.2**). If multiple vials are required, it is recommended to thaw them all concurrently and to pool the samples for post-thaw assessment and use to minimize variation and time required for sample preparation.

4. Position a rigid airline at the bottom of the cone and provide aeration at approximately one bubble/s to keep eggs suspended in the water.

NOTE: Even with aeration, it may still be necessary to gently agitate the eggs periodically (e.g., by slowly and carefully swirling the rigid airline) to prevent the formation of a thick surface layer that could lead to anoxia. Care should be taken at this step to avoid excess agitation that may cause damage or deformation of the eggs.

5. At the end of the fertilisation period, remove the rigid airline and fill the cone to approximately 800 mL with FSW.
6. Clean the eggs by draining and refilling the cone five times, or until the water is completely clear and the eggs move freely, as per **step 7.4.2**.

7. Allow the eggs to float to the water surface then use a 3 mL transfer pipette to remove triplicate aliquots of at least 100 eggs each and transfer them to individual wells containing 5 mL FSW in a 6-well plate, to assess fertilisation rate.

NOTE: The fertilisation rate can be determined approximately three hours after sperm addition (i.e., two hours after cleaning) by calculating the proportion of dividing embryos in the triplicate aliquots.

8. Carefully reduce the water volume to approximately 100 mL then gently empty the fertilised eggs into a 2 L sanitised plastic jug and transfer to a larval rearing system for embryo and larval development.

7.4.2 Pooled samples

1. Allow the eggs to float to the water surface in the measuring cylinder and record the total volume of the combined eggs.
2. Calculate the packed cell volume of eggs required for the target fertilisation density by multiplying the number of eggs needed in thousands by 0.12 mL (see **section 4.3** for an explanation of this calculation).
    - E.g., for a fertilisation volume of 100 mL and a target egg density of 1000 eggs/mL, the number of eggs needed is 100,000, and the required packed cell volume of eggs would be 12 mL (100 × 0.12).
3. Measure the required packed cell volume of eggs using a serological pipette (volumes >1mL), or using a P1000 micropipettor with a pipette tip cut cleanly with a razor blade or similar to increase the diameter of the opening (volumes <1 mL).
4. Transfer the eggs to 1 L Imhoff cones containing approximately 800 mL of FSW
5. Allow the eggs to float to the water surface, then open the tap at the bottom of the cone and slowly drain the water until the level reaches 100 mL (**Fig. 2**).
6. Add the required amount of sperm (calculated in **step 7.3**) using a micropipette and start a timer for 60 minutes.
7. Position a rigid airline at the bottom of the cone and provide aeration at approximately one bubble/s to keep eggs suspended in the water.

NOTE: Even with aeration, it may still be necessary to gently agitate the eggs periodically (e.g., by slowly and carefully swirling the rigid airline) to prevent the formation of a thick surface layer that could lead to anoxia. Care should be taken at this step to avoid excess agitation that may cause damage or deformation of the eggs.

1. At the end of the fertilisation period, remove the rigid airline and fill the cone to approximately 800 mL with FSW.
2. Clean the eggs by draining and refilling the cone five times, or until the water is completely clear and the eggs move freely, as per **step 7.4.2**.
3. Allow the eggs to float to the water surface then use a 3 mL transfer pipette to remove triplicate aliquots of at least 100 eggs each and transfer them to individual wells containing 5 mL FSW in a 6-well plate, to assess fertilisation rate.

NOTE: The fertilisation rate can be determined approximately three hours after sperm addition (i.e., two hours after cleaning) by calculating the proportion of dividing embryos in the triplicate aliquots.

4. Carefully reduce the water volume to approximately 100 mL then gently empty the fertilised eggs into a 2 L sanitised plastic jug and transfer to a larval rearing system for embryo and larval development.

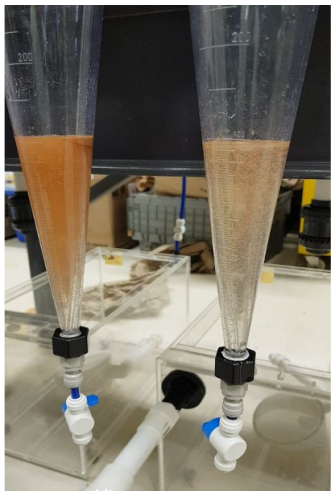

Figure 2. Imhoff cones for high-density fertilisation

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