REEF RESTORATION & ADAPTATION PROGRAM

In-Situ Experiments to Estimate Fertilisation Success During Coral Spawning – Standard Operating Procedure

May 2024

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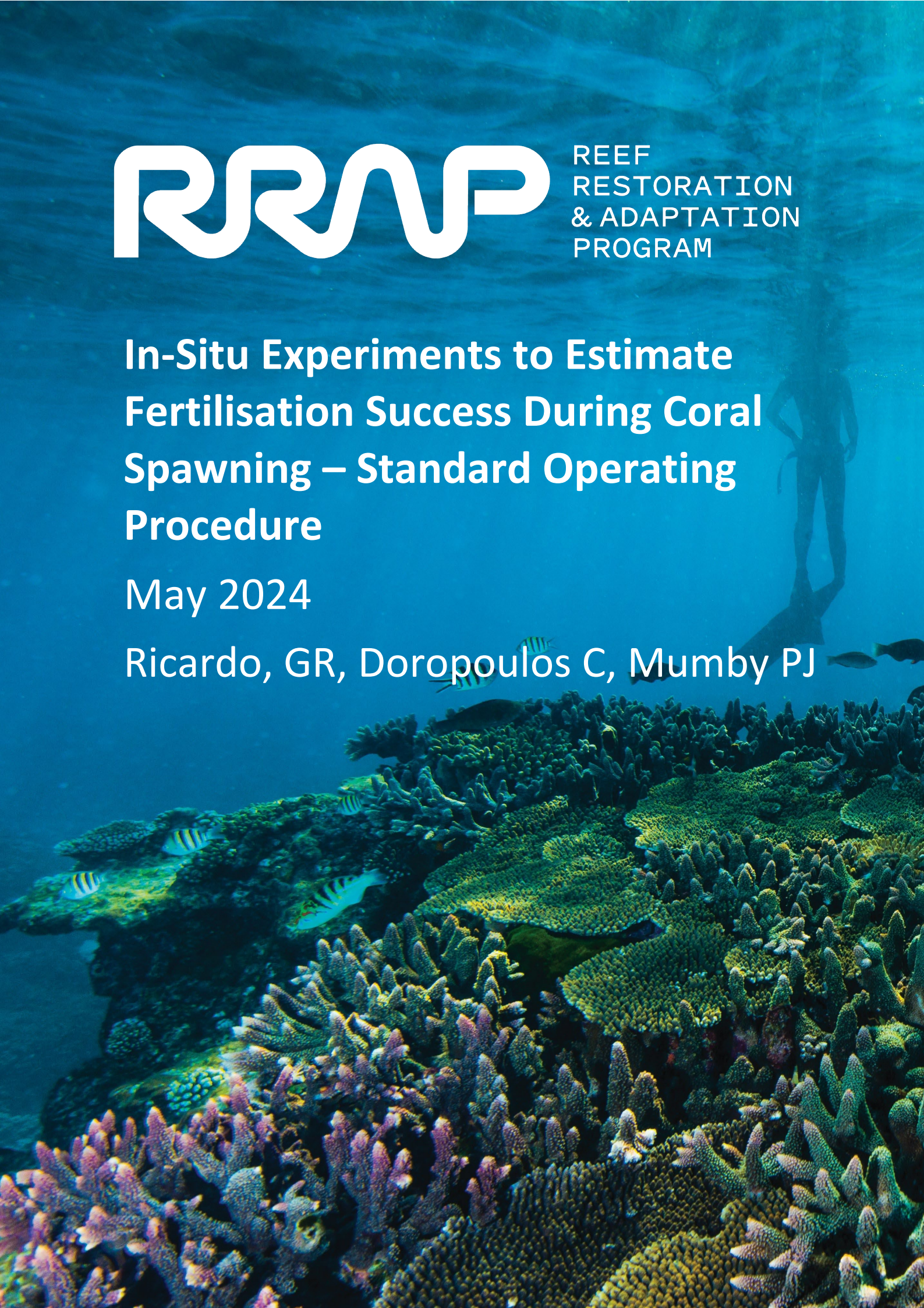

In-Situ Experiments to Estimate Fertilisation Success During Coral Spawning – Standard Operating Procedure

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Cover Images: Coral Reef, Credit: Gary Cranitch, Queensland Museum

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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**
Capricorn Bunker group	Port Curtis Coral Coast TUMRA and inclusive TOs
Palm Island group	Girringun TUMRA and inclusive TOs

We also acknowledge many colleagues and volunteers that have been part of each iteration of the experiments, Dr. James Guest for loaning his spawn catchers, and numerous field stations that have provided support for the project.

Author Contributions

Conceptualization: G.F.R, P.J.M, C.D; Methodology: G.F.R, P.J.M, C.D; Investigation: G.F.R, P.J.M, C.D; Equipment development: G.F.R; Permitting: G.F.R, C.D; Funding Acquisition: C.D, P.M; Reviews: G.F.R.

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1 Executive Summary

The Ecological Intelligence for Reef Restoration and Adaptation Program (EcoRRAP) quantifies natural rates of ecological and genetic reef recovery and adaptation in response to acute and chronic disturbances, as well as key environmental variables related to different coral reef communities. It aims to empirically quantify critical gaps in ecological knowledge to improve the parameterisation of ecological models that support decision making and the design criteria for active, restoration interventions.

This Standard Operational Procedure is produced by the EcoRRAP sub-program *Limits to Early Recovery*. Specifically, it relates to the clustering and density of corals needed to overcome reproduction bottlenecks to ensure larval supply and promote reef function. The information that the procedures described within are used to determine the minimum thresholds of colony size and spacing that are required to guide the densities at which corals need to be deployed so that restored reefs can be self-sustaining and benefit downstream areas through larval supply.

The document describes various approaches to assess coral fertilisation success *in situ* from small coral patch outplants. Tracking a coral slick *in situ* presents numerous challenges owing to i) the uncertainty around a species spawning date and time, ii) spawning occurring at night-time, iii) potential 'contamination' of the slick from the natural spawning population, iv) the logistics of both diving and boating within tight, experimental time constraints, v) species ID and fecundity ID requiring trained staff, and vi) OH&S management. In addition, these occur during unpredictable weather that can influence all field studies. Most of the obstacles can be overcome with careful planning while maintaining sufficient flexibility for unforeseen changes. For example, species and experimental sites need to be assessed once onsite, depending on unpredictable circumstances that can only be known 5 to 7 days prior to spawning events such as wind and swell.

The SOP below describes one common design in detail, and two possible variations. The primary design describes the isolation of the target species from the natural population. Experimental spawning patches are configured on a reef substrate. Coral egg-sperm bundles are transferred into mesh containers from corals spawned on a vessel and placed in the created spawn slick. The design here is used to assess single species fertilisation depression in absence of hydrological convergence zones.

2 Background

The procedure primarily focuses on the execution of manipulative field experiments to assess single-species fertilisation success *in situ*. Thresholds for fertilisation success are used to assess the critical densities of corals that reefs need to maintain reproductive functionality at different levels of reef state – from 'healthy' to 'degraded'. It is critical to understand these thresholds within a restoration context of coral outplanting, as fertilisation failure prevents any program providing benefits beyond a single generation.

The design calls for a team of at least 4 people that includes 3 divers and a vessel operator, and at least one vessel to track the spawn slick. The frequency that the experiment can run is dictated by the number of spawnings at a single location (generally 1–2), whether there is a split-spawning events (two events over two consecutive months), and how many locations the operator can visit. Generally, a 1-week experimental window is needed. In addition, preparation and demobilisation time is needed to locate reproductive adult colonies that are 'ripe' (i.e., ready to spawn), collect, transport and position adult colonies in the experimental spawning location(s), return and attach the corals back to their original locations, and conduct any associated lab work and site characterisations afterwards. Typically, 2 to 4 weeks is needed for the whole field trip.

The method and design of the procedures are mature, having been demonstrated and executed four times in two varying geographic locations (southern Great Barrier Reef and Koror, Palau). Variations in design occur due to i) differences in the objectives of each experiment, ii) species and site conditions, and iii) iterative improvements in the design. While many of the possible confounding variables and experimental artefacts have been addressed through the experimental design, there always remains scope for improvements in this type of experiment that occurs under challenging time and logistical constraints.

3 Objectives and Scope

The purpose of this document is to outline the procedures utilised to conduct *in situ* coral fertilisation experiments with reproductively mature adult colonies using a standardised approach. It applies principles and considerations from laboratory studies on coral fertilisation such as sperm concentration and contact time (e.g., Buccheri et al. 2023) but under the hydrodynamic forces of field settings. This represents the first application of these methods to corals with manipulated spawning patches *in situ*. We adapted principles from field experiments conducted on other spawning invertebrates such as sea cucumbers (Levitan 1991) and abalone (Babcock & Keesing 1999) to suit coral spawning and fertilisation biology.

The SOP guides the user through the entire process, with a major focus on the site selection and species selection for the work. These two considerations are the most important, due to the logistical requirements of being able to transport coral colonies into areas where they will not mix with other spawning corals during a spawning event. Thus, spatial and reproductive isolation need strong consideration and planning. In addition, the relatively uncontrollable nature of coral spawning (exact days after the full moon, exact time of release), and the uncontrollable nature of weather, both require careful planning to manage the operational execution of the experiment. The procedural instructions inform users how to plan and manage for these requirements. Further details are outlined in the document in relation to equipment requirements, sampling requirements, and levels of training required for members on the team.

4 Procedure

None required for this operating procedure.

5 Identified Risks and Hazards

Hazards and associated Risks:

- Vessel not compliant with national regulations (including safety equipment)
    - Death - drowning, fire, vessel communication devices not functioning properly, poor or no response to an emergency from base contacts.

- Poor boating navigation and planning
    - vessel collision and associated injuries

- Poor diving navigation and planning
    - disorientation and panic, dive-related injuries.

- Poor communication with Research Station or home base (e.g. mothership)
    - Risks delaying an appropriate or timely response in an emergency.

- Exposure (sun, hot weather, windy conditions).
    - Sunburn, dehydration, hyperthermia, crushing injuries, person overboard.

- Manual handling of equipment/lines.
    - Potential risks include musculoskeletal injuries, strains, or bruises from handling heavy or unwieldy equipment.

- Interaction with wildlife
    - Stings/injury from jellyfish.
    - Bites from marine predators

- Staff non-compliance with sampling procedure.
    - Risks include compromised data integrity, inaccurate research findings, and possible safety hazards.

- Lack of QA/QC of samples
    - Risks include compromised data integrity, inaccurate research findings, and possible safety hazards.

6 Equipment and Materials

6.1 Procedure equipment

Table 1. List of equipment required for procedures.

Equipment	Quantity	Size
Coral collection		
Cutting plier	2	S, M
Club Hammers	1 per diver	~1.5 kg
Cold Chisels	1 per diver	~175mm length
Transport Tubs/baths	5	>40L
Buckets	2	20L
Underwater notepads	1	–
Pencils	1 per diver	–
Catch bag	1 per diver	–
Routine dive equipment	1 set per diver	–
Coral transport		
Handheld GPSs	1	–
Prelabelled tags	100	–
Small cable ties	200	2.5 mm width
Long cable ties	120	300 mm length
Permanent narrow-head markers	2	–
Coral deployment		
Dishracks (or similar)	50	–
Spacing pole	1	Design dependent
Marking line	1	300 m
Marine rope	2	40 m

Transect tape	2	–
Underwater compass	1	–
Aquarium egg crate	Experiment dependent	–
Spawning experimental		
Tupperware holding containers	50	Design dependent
Plankton mesh	1	Length design dependent Mesh size design dependent
Glow/cyalume sticks (marine grade)	1 per mesh container + spare	–
Coral tubs on vessel	4	>40 L
Mesh containers tubs	50	>400 mL
Glue gun	2	–
Cable ties	100	–
Stanley knife	1	–
Metal saw blades	2	–
Dremel (optional)	1	–
Falcon tubes	>100	60 mL
GPS (in water) and waterproof container	2	–
Spawn catchers		
Spawn catchers	1 per colony + spares	
Tent pegs (or similar)	4 per spawn catcher	
PVC tubes	4 per spawn catcher	
Cable ties	100	~150 mm
Night diving		
Waterproof pouches	3	–

Underwater torches	1 per diver	–
Identity dive lights	1 per diver	
AAA Batteries	Instrument dependent	
AA Batteries	Instrument dependent	
Night Boating		
Headtorches	1 per diver	
Sampling pole	1–2	3 m
Anemometer	1	
Lab work		
Counting chambers	1 per user	
Microscopes	1 per user	
Tally Counters	2 per user	
2 mL Eppendorf	>500	2 mL
1 mL Eppendorf	>500	1 mL
Molecular pipettes	1	5 mL
Transfer pipettes	200	~2 mL
Site characterisation		
UAV (drone)	1	–
Rhodamine	>500 g	
Fluorescein	>500 g	
Spoon	2	
Mixing container	1	2 L

6.2 Personal protective equipment (PPE) and other safety equipment

- Gloves
- Hat
- Protective Clothing
- Sunscreen
- Water
- Vinegar
- Wetsuit / stinger suit
- Safety sausage
- Dive torch
- Identity dive light
- Medical O^2 bottle
- O^2 administrative equipment
- Defibrillator on main vessel
- First aid kit
- EPIRB on main vessel

7 Steps for Implementation

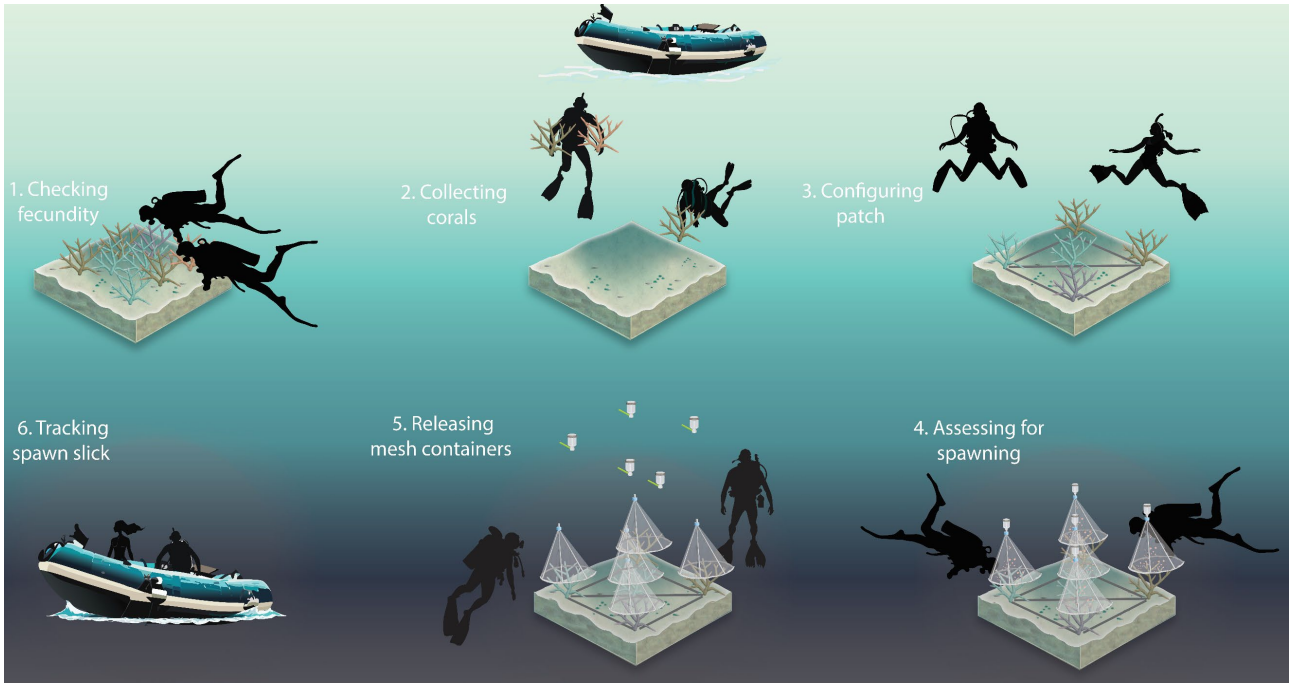

Figure 1. An overview of the experimental process. Step 1: Target species at the location are assessed to see if they contain pigmented eggs. Step 2: When the target species is selected, colonies are collected and transported to the experimental site. Step 3: The collected colonies are configured into a patch of known intercolonial spacing. Step 4: During the potential spawning nights, colonies are assessed for 'setting' and spawning. Step 5: If spawning occurs, mesh containers are released into the water column from either a vessel or using divers. Step 6: Once all containers are released, they are tracked using a vessel.

7.1 Planning

1. The two primary considerations in designing these field experiments are **site selection** and **species choice**.

Location and site selection

- Select a field location with easy access to vessels and a wet laboratory. At least one vessel should be assigned for each spawn slick to be tracked. Fertilisation success (i.e. embryo and egg counts) from experimental slick samples will need to be fixed, so a nearby laboratory (or somewhere with open air flow but covering to protect from potential rain) is often needed.

- A field site must be selected that is isolated from the natural spawning population. The site should leverage various structural and hydrodynamic features to reduce or eliminate cross-contamination of coral spawn between the experimental site and the surrounding 'wild' coral populations of the same species (Figure 2). In addition, isolation from other species within the genus is required to prevent hybridisation, which is common at the embryogenesis stage (see Annex. 8.1). For example, the patch can be set-up behind features such as exposed reef crests on low tide, or within embayments spatially separated from the natural population.

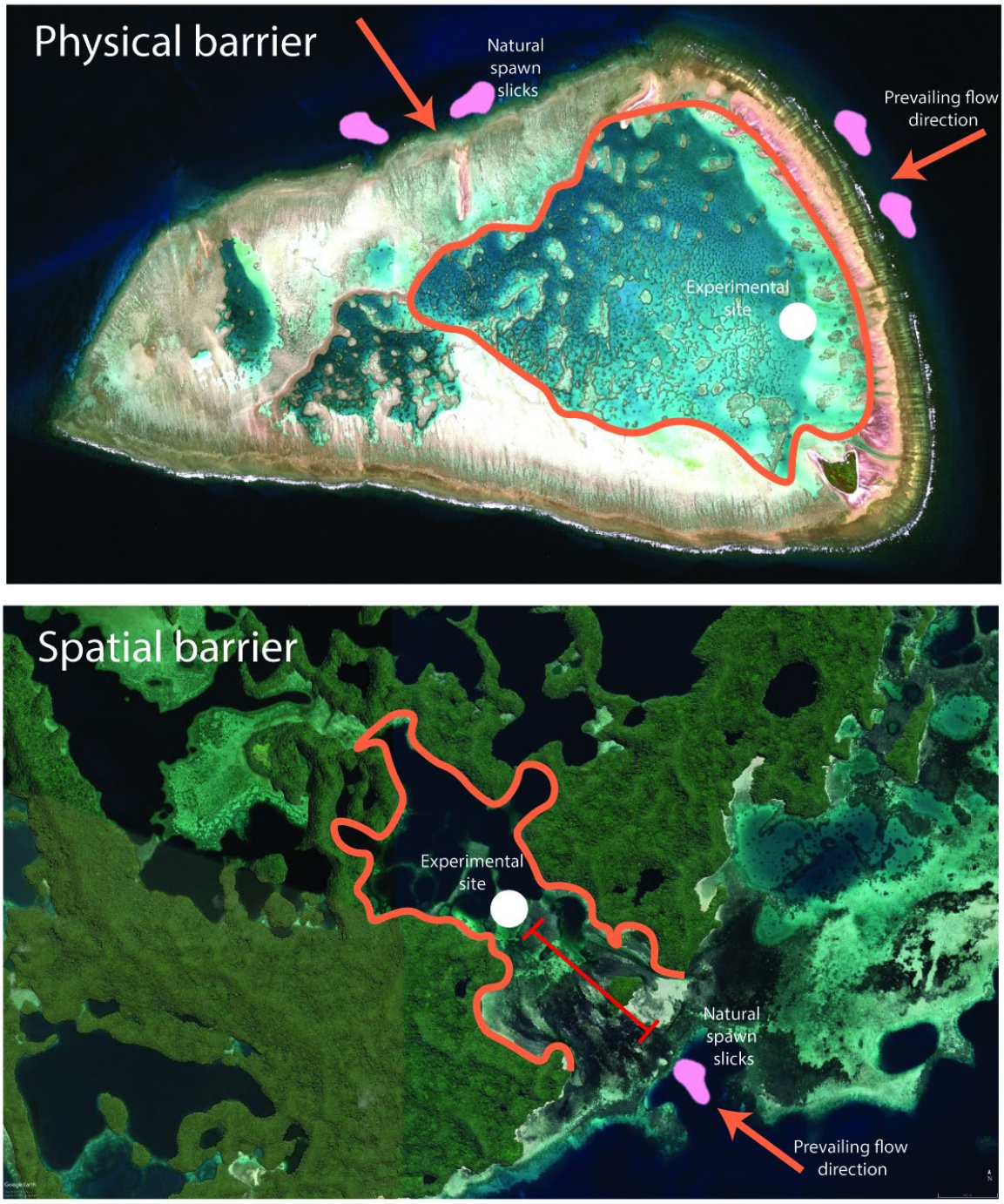

Figure 2. Two examples of site-selection to prevent the risk of contamination from natural spawn slicks. (top) Site: One Tree Island first lagoon. At low tide, the reef flat protrudes from the water's surface creating a physical barrier (orange line). (bottom) Entrance to Nikko Bay, Palau. The rock islands provide a physical barrier (orange line), and selecting a site further within the bay allows for a spatial separation barrier (red line).

- Site assessments often need to consider tidal current predictions, and anticipated wind direction on the night if exposed. Typical flow rates on coral reefs are ~ 0.1 m/s, so it is advisable to allow a minimum of 300 m downstream for the slick to move unencumbered, but ideally, greater than 500 m is preferable. However, the distance available will be limited at many sites because of reef structure. Additionally, although the coral will be transplanted to the site briefly, sites should be selected where adequate water quality can be met so that coral health is not compromised. An optimal site is one that is 2–4 m deep and can accommodate the dimensions of the experimental patch. See *Figure 3* for several possible

experimental issues and artefacts that can influence the outcome of the experiment such as inter and intraspecific contamination, selfing and wave events.

Species selection

- The target species is crucially important. Some species spawn predictably at various locations ±1 day from their mean predicted date, but many species or locations can vary markedly in predictability. An incorrect prediction of spawning times is the most likely cause of experimental failure. Resources such the Coral Spawning Database allow better estimates of local species-specific spawning times (Baird et al. 2021)(Annex. 8.7); however, local variability, sporadic environmental conditions, and unknown factors can influence spawning times. Execution is much easier if some details about the reproductive biology of a target species is well known. Keep in mind the corals will need to be checked each night for bundle setting, requiring considerable resources such as boat time/dive personnel, so utilising a tighter spawning window is preferable. See Table 1 for a framework to assist target species selection.

- Ideally, a scouting trip should assess be conducted for site fecundity (how many gravid colonies are at a potential collection site, Figure 4), and suitability for the experimental site, in the month prior to the coral spawning event. Take a GPS waypoint at each prospective site to accurately ensure a quick find when returning to the site.

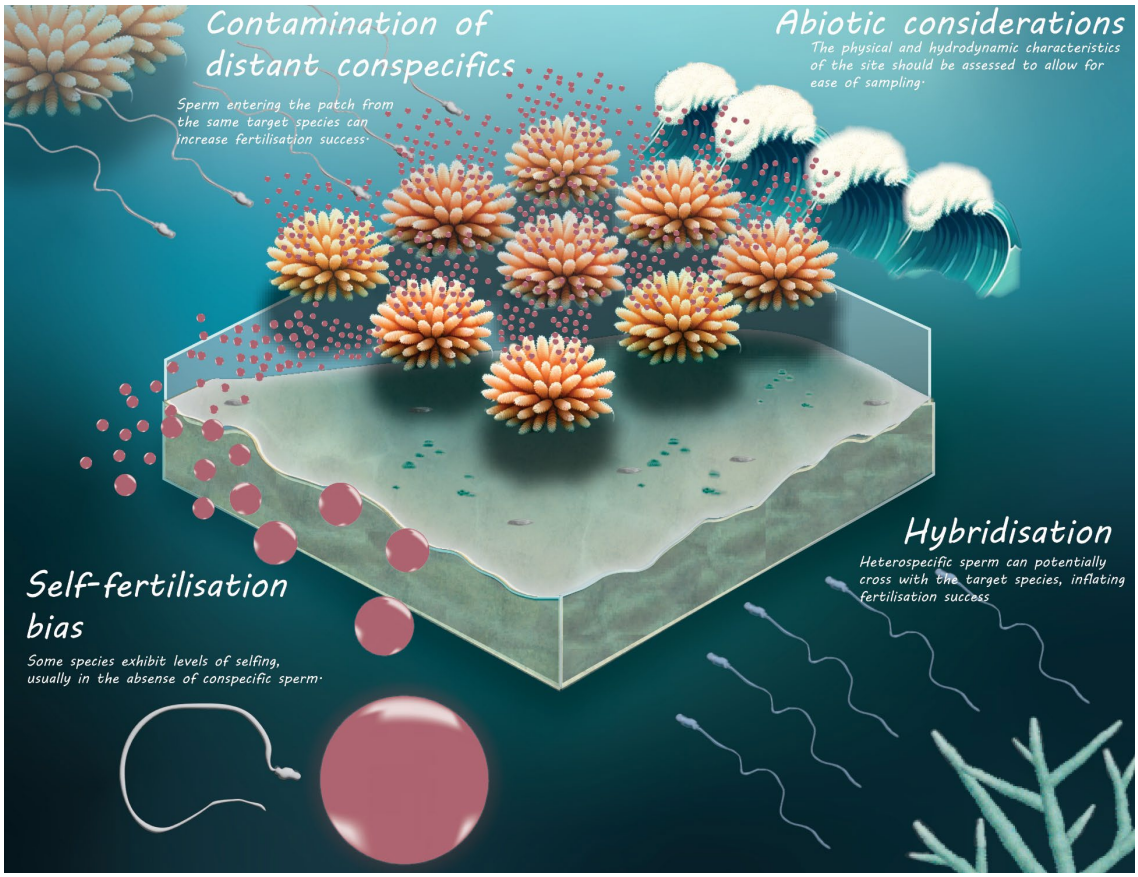

Figure 3. Potential experimental artefacts and issues that can influence the outcome of the experiment including contamination from conspecifics (the same species), interspecific hybridisation, selfing and influences from various hydrodynamic processes.

Table 2. A framework to deciding which species to select for a given experimental location and site.

Target species selection		
Planning	Questions	Action
Initial list	Which species has a well-documented reproductive biology in this region? Use resources such as the Coral Spawning Database and region-specific research papers.	Create a list of potential target species
Refined list	Is there sufficient information to predict spawning within a 5-day window?	Reduce the list to species that spawn more predictably
Target species scoring	Are the species ecologically relevant? Are these species easy to identify? Are these species easy to locate, or is a collection site known? Are these species reproductively isolated? Do these species have known hybrid partners? Are gravid colonies conspicuous? Does the species spawn at night?	The weight given to each question will vary depending on the operator's experience and priorities. Reduce the list to 3 posssible target species.
Species reassessment	Once onsite, is the target species gravid, easy to locate, and easy to identify?	If no, reassess using the decision framework above.

2. **Permits.** Any permits should be applied for well in advance to allow for long processing times. The permit should detail, at a minimum, the number of corals to be collected, any installation of temporary structures during the experiment, and the release of mesh containers. Any auxiliary instruments and equipment such as unmanned aerial vehicles (UAVs), flow meters, drogues etc. should also be detailed.

3. **Equipment and purchasing.** Various specialist coral spawning equipment will need to be purchased and manufactured in advance. See *Equipment and Materials* for the full coral spawning equipment list. Equipment that should be purchased or organised early includes:
    - Plankton mesh
    - Bogorov counting chambers
    - Chemicals such as Sodium β-glycerophosphate
    - Stereomicroscopes
    - Night-time boating and diving equipment.

4. **Prototyping coral spawn catchers and adult colony bases.** The method for capturing the spawn will need to be piloted ahead of the spawning event. A full description of possible designs is detailed in Variations 1 & 2. See Table 2 for considerations associated with each design such as available experimental site characteristics and experimental resources needed to run variations in the designs.

5. **Spawning team and volunteers.** This work requires a reasonably qualified dive team, ideally with coral spawning experience. Many operations within the team will require species-level identification, fecundity assessments, scientific diving, boating experience, as well as basic wet lab and microscopy work. The more roles each team member can fulfill, the easier it is to manage planning, logistics, and OH&S.

6. **Assessing if coral colonies are gravid.** When possible, the method to assess fecundity and an image of gravid colonies should be provided to staff ahead of time. For example, *Acropora* fecundity is assessed by breaking the lower portions of branches, outside of sterile colony zones (Randall et al. 2021). This is typically in the centre of the colony, although for large tabular species, senescence can occur at the centre, and branches are better assessed off-centre. It is usually prudent to check the fecundity of a colony at >1 positions to be confident a coral is not truly non-gravid. A point-and-shoot underwater camera with macro capabilities can quickly aid in confirming fecundity.

   Branching corals (e.g., Acropora) - use pliers or small chisel to snap a fragment at approximately 5-10 cm from the top of the branch. Sample from centre of the colony, and the bases of branches to avoid sterile zones. Typically, eggs are easiest to observe on the colony at the point of the break.

   Submassive corals (e.g., Platygyra) – use hammer and chisel to break open an ~2 x 2 cm fragment. Sample close to the centre of the colony and look for eggs below the tissue layer of the fragment and the colony.

   Observe egg status by eye, magnifying glass, underwater camera, or if very small bundles bring fragments back to microscope. Colonies that are highly likely to spawn at the predicted spawning night will typically have pink/red colour eggs visible.

   *Typically sample a total of 2 to 3 fragments per colony. If eggs are clearly observed in first fragment however, no further sampling is required.

   **Fragments can be placed back where they were sampled from. They will often reattach to the colony by the tissue layer growing over the break point.

7. **Safety risk assessment and field and dive plans.** Most institutions require reasonably detailed field and dive plans. The greatest risks usually involve planned diving operations, especially if night-dives are to be conducted. Night-boating and diving requires considerable planning and pre-departure communication.

8. **Freight and logistics.** Freight of equipment and supplies often needs to be organised weeks to months in advance because of transport times and space availability of stations and vessels. Robust water-resistant boxes such as Nally Bins are very useful for transporting gear.

7.2 Onsite preparation

9. Once on site, allow at least half a day to undertake required inductions, communicate plans with staff, unpack gear and food, and set-up test equipment. Use this time to efficiently handle tasks such as adding batteries to lighting equipment, setting up ropes and lines, attaching gear, and calibrating sensors, etc.

10. It is often prudent to assess for fecundity of the target species at the earliest convenience once onsite. Since it is not uncommon for fecundity to be lower than expected, scouting may be needed to locate suitable target species/sites. Additionally, species morphology varies between location, and local knowledge may be needed to identify corals at species level. If experimental sites have not been confirmed, now is the time to select these.

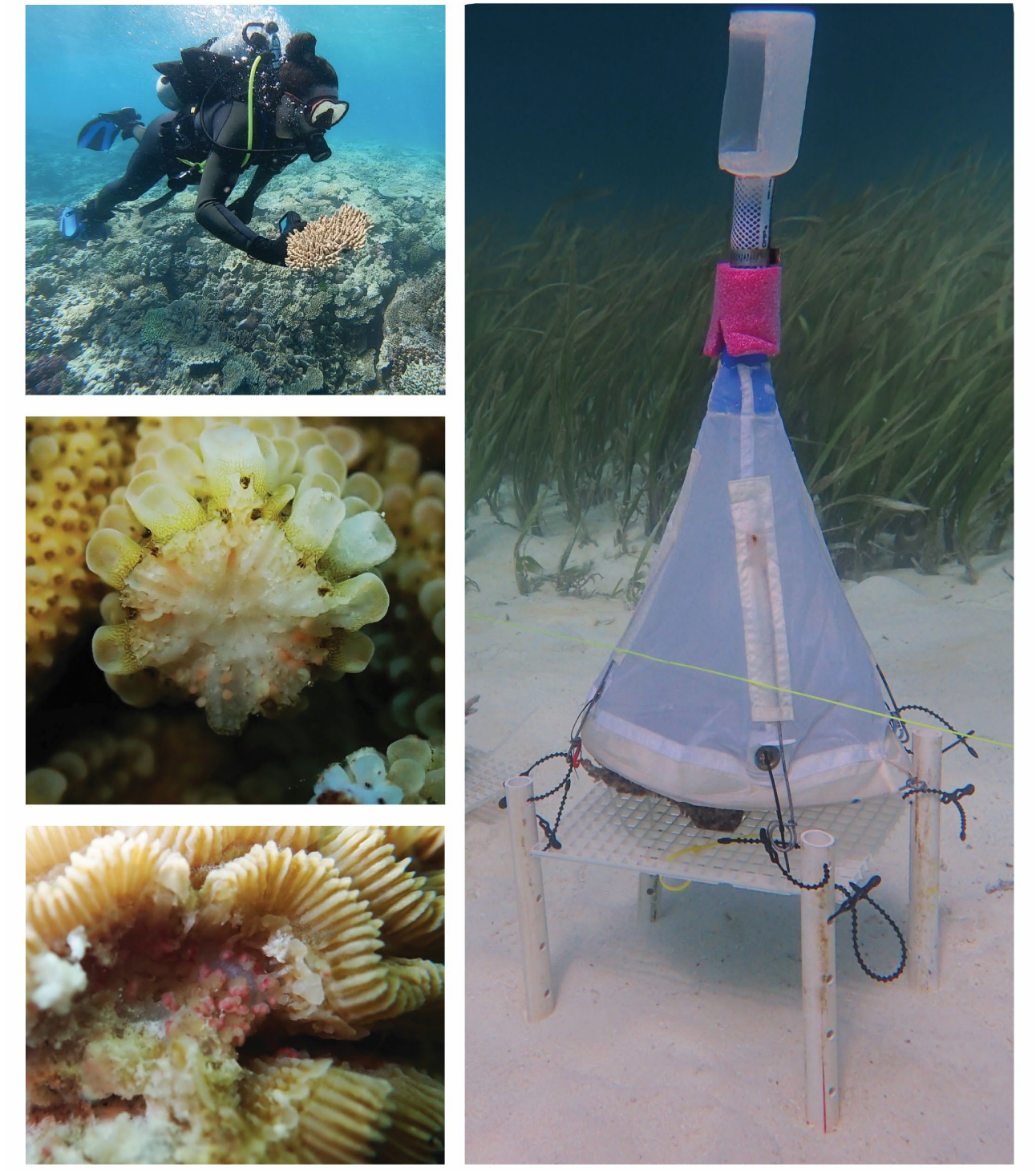

Figure 4. Coral collection and patch set-up. (left-top) A diver transporting a colony following collection. (left-centre) An Acropora *spp. is assessed for fecundity. Eggs can be observed radiating towards the surface of the branch. (left-bottom) Massive and sub-massive colonies often have eggs along a sheath which are more easily missed. (right) A spawn catcher and colony base mounted on a sandy bottom. Photo credit: Gerard Ricardo and Heinrich Breuer.*

11. **Adult coral collection**. Fecund corals should be collected several days before the experimental window starts. The method for removal is species specific, but generally from the base of the coral. For corals attached to the reef, it is typically better to use a chisel on the adjacent reef rather than directly at the coral's base. The size of the colonies should reflect the goal of the experimental design, but generally ~20 cm diameter corals are sufficient. It is often better to aim to collect a whole coral, rather than a fragment. This reduces the stress on the corals, and fragments can create further confusion distinguishing between genetically distinct colonies. Divers should resist the temptation to collect two fragments from the same colony – these can easily be mixed up during the transfer to the site, and two fragments of the same individual often have low self-fertilisation rates and therefore could compromise the experiment (see Annex. 8.2). Rather, more emphasis should be placed on proper collection technique. To prevent multiple dive ascents, colonies are typically left near a float line or anchor chain until the end of the dive.

    Rather than transporting immediately to the site and hoping for acclimation, it is generally better to move the corals on the day of the first spawning checks and return the colonies soon after the experiment is completed.

12. Corals are generally fine to be transported by vessel submerged in seawater for 30 min without requiring water changes. If the transport is anticipated to be longer or the air temperature is high, a refresh of the water should be conducted while onboard the vessel.

13. If possible, adult corals are best transported to a protected natural reef in natural crevice or grooves. Corals can temporarily tolerate low light conditions, and this should be selected over higher light conditions. Corals do not tolerate sand and silty benthos well and should not be placed here unless they are placed on deployed bases, for example on structures or dishracks (see Figure 4). Anecdotally, stressed adults may increase spawning asynchrony, and possibly cause reabsorption of the gametes, although this appears to occur inconsistently.

14. Any further manufacturing of the field set-up should be completed in this pre-spawn period. Such tasks include manufacture of the egg-mesh containers, manufacture of the adult colony bases, if spawning nets are to be used, these and methods for attachment.

15. The patch can now be placed on the benthos at the experimental site in a desired design. Logistically and for modelling exercises, a lattice grid is the easiest method for placement of corals, but this will depend on the research question to be answered. For designs where spawning on the vessel is required, extra colonies should be placed adjacent to the patch. All colonies should be labelled in a way to reflect the design. For example, using a lattice grid, the first number could signify the row, and the second number the column of the grid e.g., 1_2, 1_3 etc.

16. **Vessel navigation**. Manoeuvring vessels at night near reefs contain various risks. Floating hazard lights such as SOLAS lifebuoys should be used to mark the vessel's location and other potential obstacles. Temporary night lights on marker buoys set up in advance can assist in safely navigating the site during low visibility and low tide conditions. The divers will need to be within relatively

proximity to the vessel, and adequate anchoring to allow the vessel to be 5 to 10 m of the experimental site is needed. An extra navigational hazard because of low tides should also be considered. Setting up temporary night lights on marker buoys in the days before the nighttime work can assist with nighttime navigation of the site.

17. **Diver navigation**. Night-time diving can be disorientating; therefore, clear reference points underwater will greatly assist the divers moving through the site. Diver identity lights or glow sticks, are useful for marking the perimeter and specific areas of the coral patch as well as the anchor line, enhancing underwater visibility and orientation. Marking lines can help direct divers between various colonies of the same row. Flagging tape and labels are also useful to mark out specific rows.

18. **Diver preparation**. Every diver should be provided with an underwater notepad, pencil, dive torch with red filter, identity light (ideally a specific colour), sinkers to prevent gear from floating, and bag (Ziplock or catch bag). If using a Ziplock bag, puncture holes in it to allow air to escape and prevent the bag from unintentionally opening due to trapped air. Divers should submerge the bag at the surface to ensure it fills with water and sinks before they descend, reducing the risk of losing equipment.

7.3 Experimental window

19. The experimental window to commence checking corals for setting should begin at least three days before the predicted night of spawning when spawning times at the location are well known, and possibly earlier if spawning times are poorly known. The first night should be conducted as a 'dry-run', which allows the divers and crew to familiarise themselves with the experimental set-up under less time-constrained conditions and to troubleshoot initial experimental issues. Allow adequate time for a safety briefing, and pre-departure experiment briefing. Clearly define everyone's roles and establish communication protocols for the duration of the experiment, including signals for divers' entry and exit from the water.

Figure 5. Experimental window and spawning. (left-top) A diver waits for tagged colonies to spawn. (left-centre) An Acropora *spp. is assessed for setting. Bundles can be observed at the base of the branch. (left-bottom) Bundles of* Platygyra *spp. observed in the valleys. Note that for some species setting can be difficult to observe. (top-right) A mesh container following the breaking of the egg-sperm bundle leaving only eggs remaining. (bottom right) Fertilised and unfertilised eggs. Note cell cleavage at the 2 and 4 cell stage which is the optimum time to fix. Photo credit: Gerard Ricardo and Natalia Robledo Amaya.*

20. The crew should move to the site with at least 1 hour before the expected time of spawning. This time can be used to take initial notes, finalise lighting, and collect seawater for holding containers

(see below). Although coral setting can be assessed by free diving, it is not recommended unless highly experienced because of disorientation and the time needed to check all individuals. Two SCUBA divers should descend and methodically check for setting (Figure 5). The percent set of the colony should be recorded, which can then be used to estimate gamete output. Two colonies that are adequately set should be removed from the patch and placed on the vessel. If setting occurs during daylight hours, these colonies can be placed in black covered tubs which can expediate spawning times.

21. When corals commence spawning on the vessel, operators on the vessel should move quickly to add greater than 50 bundles (about the size of a 20-cent coin) to each mesh container. Any additional bundles added should be proportional to the mesh container's surface area, to prevent clogging of the mesh. See Variation 1 below for diver release of the mesh containers.

22. Operators on the vessel should promptly place the mesh containers randomly within the patch, specifically in the area where bundles from the coral colonies in the experimental patch are observed ascending. See Variation 1 below for diver release of the mesh containers.

23. **Controls**: Set up several controls to account for potential extraneous factors affecting fertilisation success:
    - **Contamination control**: Place two containers upstream of the patch to test for external contamination; ideally, these should show no fertilisation.
    - **Self-fertilisation control**: Bundles from each colony within the vessel should be added to a closed container with seawater. The level of self-fertilisation represents the maximum self-fertilisation rate that may occur in the experimental containers. This value should be deducted from any recorded fertilisation success observed in the containers. For example, if the fertilisation success in the experiment was 5% and the self-fertilisation control was 2%, the actual fertilisation success should be 3%.
    - **Development rate control**. Each colony on the vessel should be crossed in closed containers immediately during spawning. The sperm concentration should appear lightly cloudy. The embryo development in this control can serve as a guide to when to fix all the containers later (see below). It can also give insights into gamete compatibility, although because of the limited number of crosses, this should be interpreted with caution.
    - **Sperm penetration control**. Bundles from one individual can be added to a mesh container and placed in sperm from another individual. This confirms that sperm can move freely through the mesh used.

24. **Notes**: Notes should be taken at any convenience. Important notes to consider are:
    - Time of spawning
    - Time of first container deployment
    - Time of last container deployment
    - Sea state, wind speed, wind direction, tidal height, tidal direction, flow rate, flow direction.

25. Once all containers have been deployed, divers should immediately enter the vessel.

26. The vessel should track the mesh containers while being careful to avoid creating turbulence near them, as this might influence their trajectory or increase dispersion. If possible, take numerous waypoints during the mesh container tracking, which may identify topographical fronts.

27. Generally, 90 min is considered sufficient to assess fertilisation success. Topographical barriers may prevent extended timelines and additional fertilisation success is unlikely without the presence of convergence factors.

28. When mesh containers are collected, they need to go into individualised closed and labelled holding containers. These holding containers should have seawater collected prior to spawning to prevent contamination. Mesh containers should be removed slowly from the water, as rapid water pressure differences can damage eggs and embryos against the mesh.

29. Fixing the samples is best conducted with a formaldehyde-based fixative e.g. Bettie's fixative (see Annex. 8.3). Fixing the sample allows a standardisation of contact times between sperm and eggs and prevents embryo fragmentation. Samples may be counted for the proportion of embryos anytime thereafter. For most *Acropora* cleavage occurs within 90 to 120 minutes to the two-cell stage. However, samples are preferably fixed at the 4-cell stage, which allows adequate time for later inseminated eggs reach initial cleavage (Figure 5). Embryo fragmentation is common after the 8-cell stage, so fixation should ideally occur before this stage because it can be difficult to distinguish a 2-cell embryo from a 2-cell fragment. The operator can use the one-to-one cross performed at the start of spawning (development rate control) for a guide of when to fix the samples.

30. Colonies should be photographed with a size reference, marked with a GPS waypoint, and if genetic testing is required, a small sample of tissue taken from each colony. This can often be done after the experiment has been conducted.

31. Colonies should be moved back and attached to their natal reef as soon as possible in the days following spawning.

7.4 Variation 1 – Bundles captured by nets and released by divers.

Background: A variation to the design is to have spawn capture nets over the colonies. This has some advantages such as a more representative gamete compatibility within the population, and ensures that released bundles are closer to their spawning adult. This is particularly useful for assessing individual fertilisation success, rather than patch levels spawning success. The negatives are that this requires more divers, and greater communication challenges. Wave activity or high flow can also damage the nets or the adult colonies, so nets are best removed at the end of each day, which creates additional work.

1. Design coral structures if needed. See Annex. 8.4 for an example.

2. Construct spawn catchers and mesh containers. See Annex. 8.5 and 8.6 for examples. Often only a portion of the bundles are desired to be collected per individual colony, as the non-caught bundle

will contribute to the in-situ sperm cloud. This can be achieved by covering only part of the colony or releasing the mesh container quickly after initial spawning.

3. As mesh containers need to be released while the adult colony is spawning, the experiment is time-constrained, and each diver's responsibility should be discussed. An allocated diver responsibility should be to have extra mesh containers to carry out some of the controls needed on the vessel. It should be made clear to other divers not to release these containers.

4. All divers should have a method of communication and time to come to the surface. The vessel skipper can act as an intermediary, relaying communications from surfacing divers, especially if any require more time.

7.5 Variation 2 – Natural reef patches

Background: The experiment can also be conducted in natural reef settings; however, this removes some levels of control while adding more environmental realism (Figure 6). The greatest concern conducting the research on a natural reef setting is the possibility of contamination, both from conspecific upstream, and from heterospecific species (via hybridisation). Selection of species that are temporally isolated (spawn at different times) is imperative, as hybridisation during fertilisation is not well understood in corals but can be >50% for closely related species. Additional considerations should be discussed on approaches to track the slick if it moves into shallow unchartable areas. This design lends better to a combination of Variation 1, because placement of mesh containers from a vessel is challenging, both logically and accurately.

1. Individual fecund corals are surveyed across a stretch of a reef. Typically for a reef-slope, the patch will be long and thin. The length survey should consider that greater than 30 m is difficult for an individual diver to manage. A depth range of 4–6 m is generally optimal. A lagoonal patch is also possible, which may take the shape of a squarer grid. Reef crests are not suitable for these experiments because of accessibility of divers and vessels during low tide.

2. More exposed areas, such as reef slopes, require greater consideration due to their increased exposure to waves.

3. Closely related species should also be assessed for fecundity due to the risk to hybridisation. If there are only a few individual colonies, these may be moved outside of the experimental plot. Divers should note any other proximal species that are spawning at the same time as the target species.

4. Waypoints for each individual colony need to be collected.

5. Ideally, samples should be taken from larvae, potential parents, and possible hybrid partners to confirm that contamination and has not occurred. Genetic tools can then be used to assess the likelihood of this (not explained here).

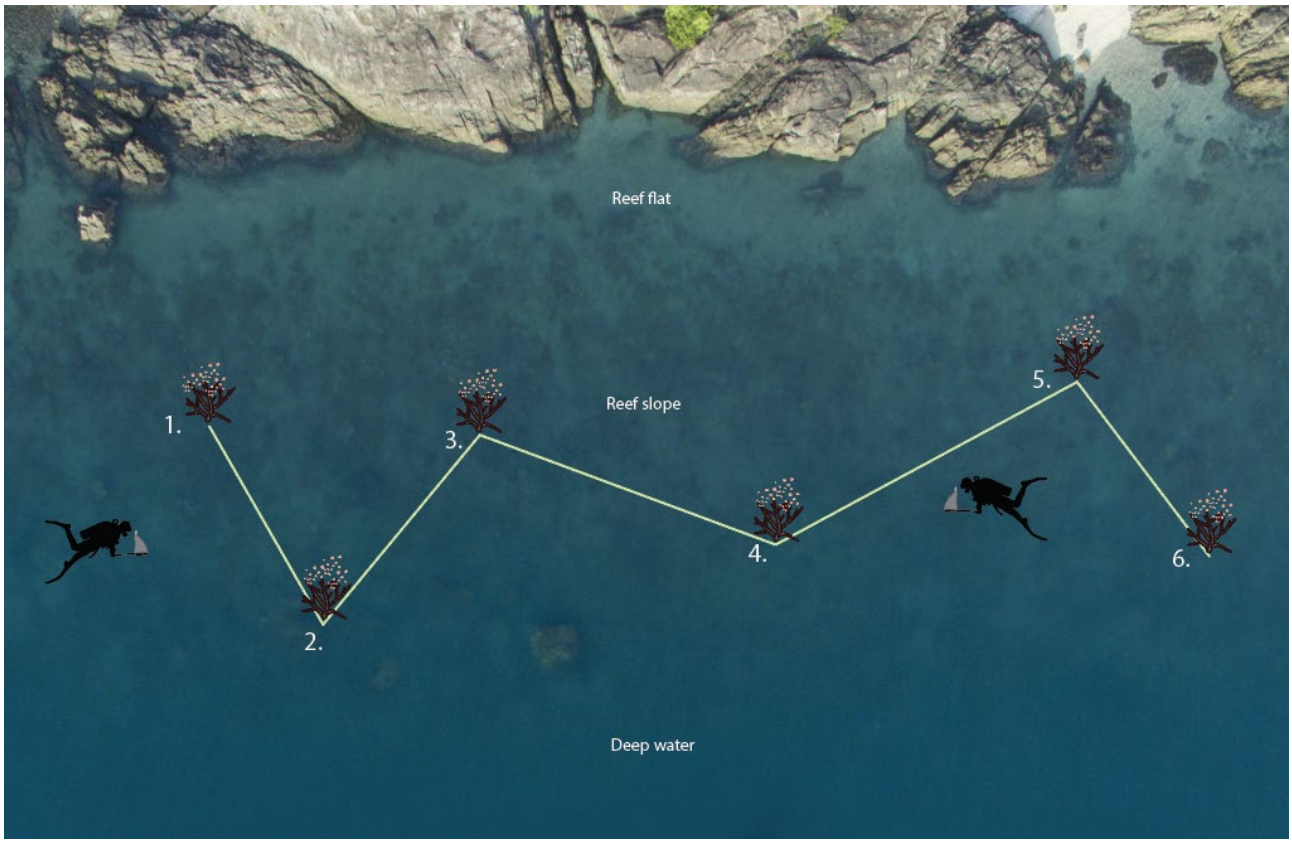

Figure 6. A natural reef design utilising a dive-team. Here divers move between spawning colonies via a line and release mesh containers containing their bundles into the water column.

Table 3. Pros and Cons of various experimental designs and site characteristics to assess in situ *fertilisation success.*

Decision	Pros/Cons	Considerations
Isolated patch	• Higher level of control • Lower risk of contamination • Better to assess restoration effectiveness • Reduced environmental realism	• The patch must be isolated (physically, spatially) from conspecifics and possible hybrid patrners. • Allow time for coral collection and transportation.
OR		
Natural Reef	• Uses natural reef configurations and sizes • Better to assess natural reef degradation • Often in wave exposed enviornments • Findings more site-specific	• Selection of reproductively (temporally) isolated corals is critical • Select species that are unlikely to hybridise (see Annex 9.1) • Ideally genetic work should be done to confirm no hybridisation took place.
Sandy bottom	• Greater control • Easier to find flat space for the patch • Risks of sediment scour and altered biogeochemistry near the benthos layer • Allow extra time to create and install colony bases	• Adult colony bases are needed. Allow extra time to manufacture these.
OR		
Reef substrate	• Generally easier to setup • Typicaly more suitable for coral health. • Greater potential for hybridisation • Often varying in depths	• If wave exposed, care must be taken to prevent spawn catchers (if used) are not damaged
Vessel release	• Simpler communication and execution • Less reliant on the expereince of personnel • Reliant on onboard corals spawning at suitable times • Reliant on the onboard corals being compatiable with others	• Collect extra adult corals for the vessel. If the corals have been removed from the reef, transferring the first spawning individuals to the boat ensures bundles can be partitioned into mesh containers in time.
OR		
Diver release	• Individual contribution can be assesed • Better timing of release • Requires greater resources • More opportunities of issues	• Clear plans need to be discussed of when to exit the water and commence tracking. • An extra vessel is often needed: one to attend to divers, and another to commence tracking of the slick.

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9 Acronyms and glossary

Bundle setting – The movement and protrusion of the egg-sperm bundle to the moth of the polyp just prior to spawning.

Benthos – The seabed, which is typically sediment or reef.

Conspecific – Belonging to the same species

Convergence zones – Hydrological zones where two or more water bodies meet.

Egg-sperm bundles – a mode of packaging gametes for release.

Embryogenesis – The development of the fertilised egg following insemination.

Drogue – A sea anchor often attached to a drifter to allow measurements of depth-dependent flow rates.

Fecundity - a measure of the reproductive capacity of an individual or population

Gametes – eggs and sperm

Hybridisation – Progeny produced from sexual fertilisation of two distinct species.

Interspecific cross – Cross-fertilisation between two distinct species.

Isolation – Factors (such as physical, spatial, or reproductive) that allow a particular group of corals to spawn separately from nearby corals.

Site fecundity – The percentage of colonies of a target species that are found within a site.

Spawning – the release of gametes

Spawn slick – An often-visible congregation of eggs, embryos and decaying gametes following mass spawning.

Sterile colony zones – Areas of the colony that are either immature or aged and do not produce gametes.

Annexure

9.1 Hybridisation pairwise likelihoods

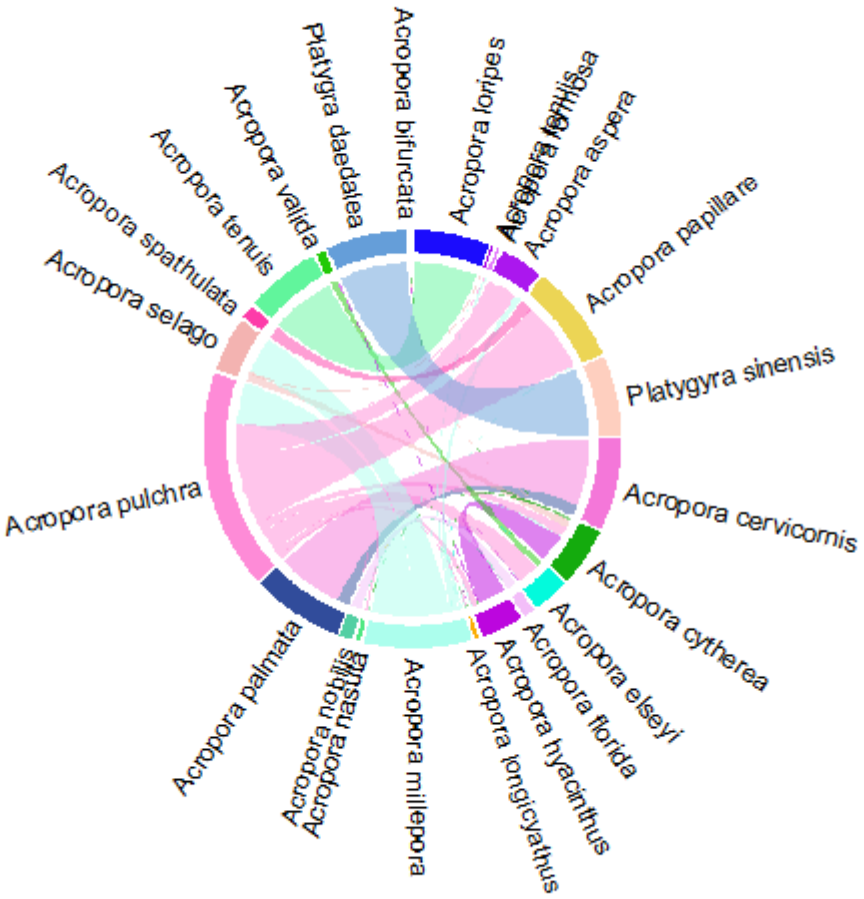

Figure 7. A chord diagram representing fertilisation success between various species. Wider bands indicate higher levels of fertilisation success between interspecific crosses (Willis et al. 1997, Van Oppen et al. 2002). Note that this plot is not exhaustive of all broadcast spawning species reported and known and should only be used as a guide.

9.2 Self-fertilisation probabilities

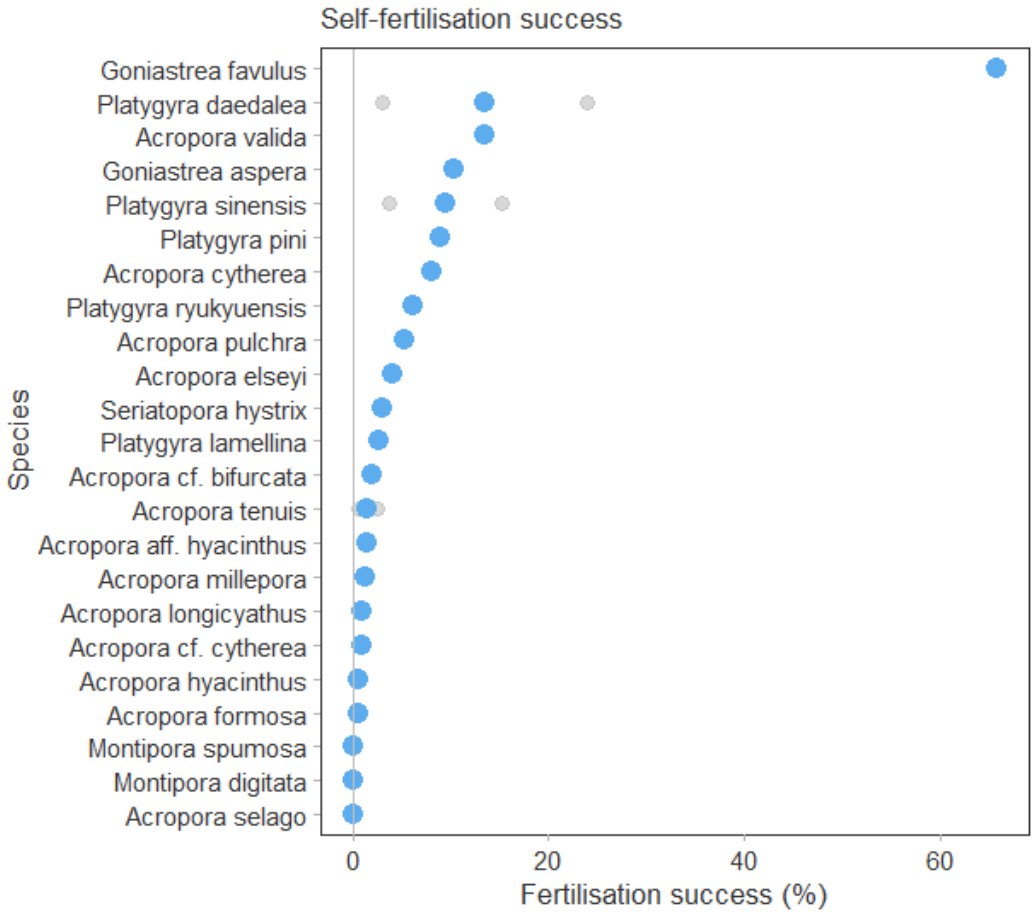

Figure 8. Self-fertilisation success reported in several Acroporidae *and* Favidae (Miller & Babcock 1997, Willis et al. 1997)*. Note that this plot is not exhaustive of all broadcast spawning species reported and known and should only be used as a guide.*

9.3 Bettie's fixative

Bettie's fixative requires dissolving 20 g of Sodium ß-glycerophosphate in 100 mL of 37-40% formaldehyde (equivalent to 100% formalin). This mixture is then combined with 900 mL of filtered seawater, resulting in a solution that is 10% formalin. It is typically used at a ratio of 1:4 fixative to sample volume (5 mL of fixative in 20 mL of sample water), which dilutes the final concentration to 2% formalin.

9.4 Adult colony structures

Background: For a sandy bottom, corals are best raised to prevent sediment scouring and partial smothering. Avoid anything nonporous as wave pressure can easily knock these over.

1. Stilts. Cut four 50 mm PVC tube into 50 cm long sections. Multiply by the number of structures needed.
2. From one end, drill a 10 mm hole 200 mm all the way through for cable-ties to pass.
3. Stand. Depending on the maximum diameter of the adult colonies, cut a square of egg crate. For tabular species with a stem, a small hole may need to be cut into the centre to allow the colony to sit upright.
4. If spawn catchers are to be attached, or other sensors, an additional hole 30 mm from the end will be useful.

*See Figure 9 below.

9.5 Spawn catchers

Background: To collect bundles released from the adult colony, nets are widely used and have been described elsewhere (Edwards et al. 2010, Omori & Iwao 2014, Roff et al. 2023). Spawn catchers made from flexible and porous material such as plankton nets are preferred over solid materials such as plastic cones. Briefly, a net is made of a cone like mesh that directs the bundles to a central point at the tip of the cone. The circular base of the cone either needs to be weighted with a material such as a chain or attached to structures via cable ties. The cone often requires struts to maintain shape. The top of the cone feeds to a reducer made buoyant by float such as plastic foam. Care needs to be given so that there is minimal edge between the mesh and reducer, as bundles can collect at these edges. The attachment point to the mesh containers can be done in various ways depending on the container used. A reducer such as silicon hose has proved adequate to connect with mesh containers made of bottle. Metal fasteners can be used to temporarily secure the containers to the hose, and these can be removed by the diver.

*See Figure 9 below.

9.6 Mesh containers

Background: Mesh containers are a means to contain eggs from individuals to assess fertilisation success. They allow sufficient samples to be collected from low density slicks and prevent contamination from non-target species.

1. Use a mesh size suitable for the eggs of the target species, such as 125–250 µm mesh. Larger mesh sizes have the benefit of allowing sperm to freely move in and out of the containers.
2. The containers are typically best created from plastic. Although validation of the containers is difficult because of the limited opportunity to prototype the containers against coral spawn, coral eggs are barely buoyant and often move subsurface because of water surface turbulence. In this case, above-water processes such as windage are considered relatively low, with wave movement and surface water wind drag being more dominant processes. Therefore, containers should primarily be subsurface.
3. A larger surface area of mesh increases sperm movement through the mesh; however, larger containers are more difficult to manage and take up considerable space on the vessel once placed within a holding container. We have found 200 mL plastic containers to be a decent trade-off that work well between various designs.
4. To maintain a consistent orientation of the containers, floats can be placed on the wider side of the containers. A sinker can be glued to the lid of the container as a counterweight.

*See Figure 9 below.

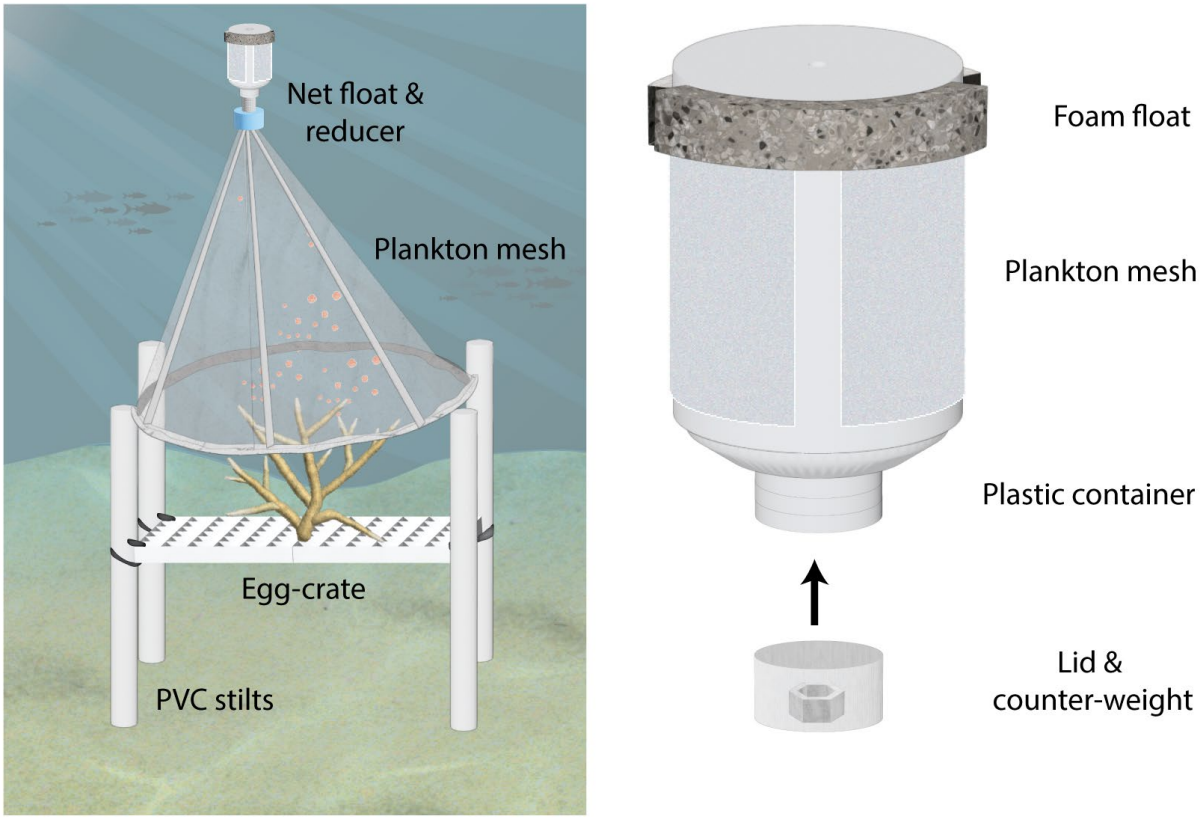

Figure 9. Design drawings of adult colony structures, spawn catchers, and mesh containers.

9.7 Spawning time relative to the full moon

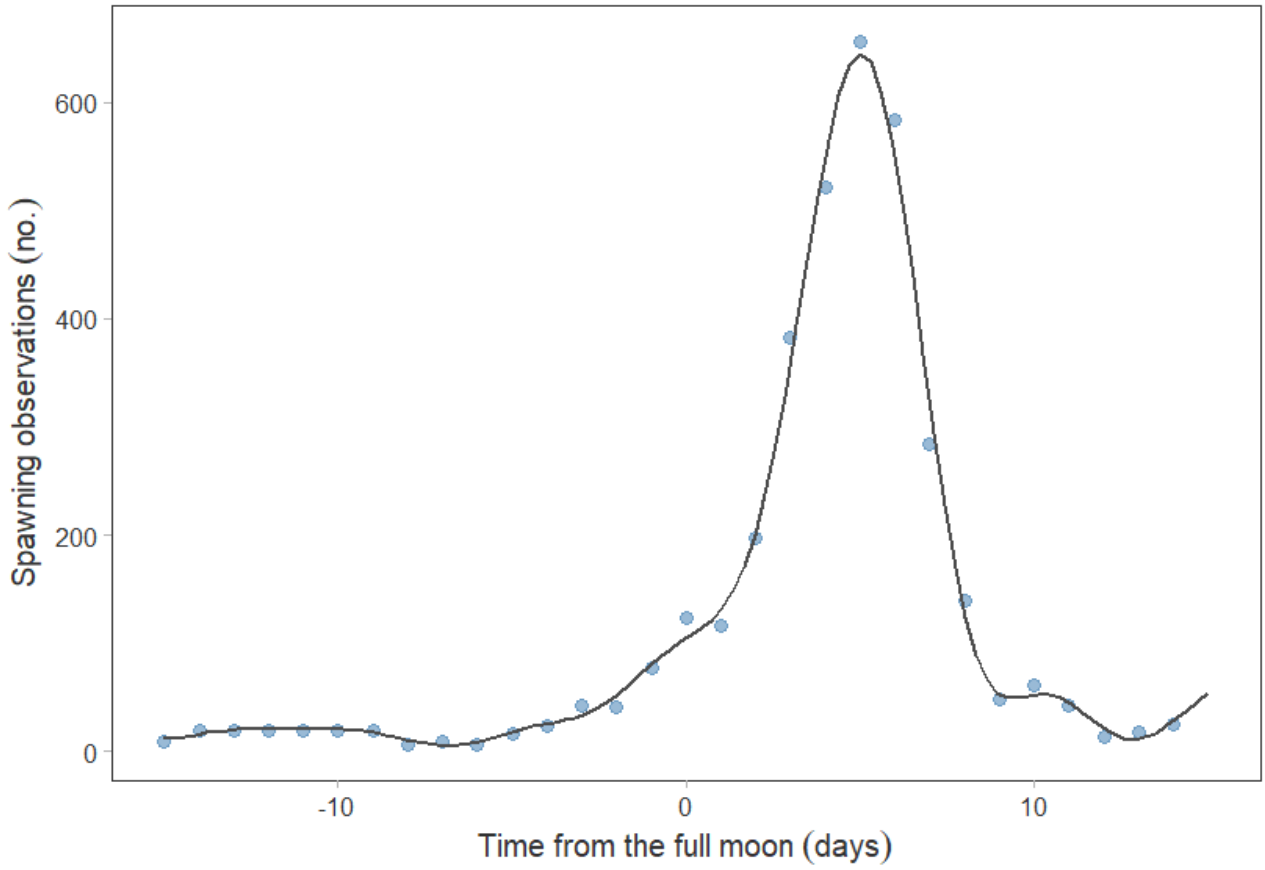

Figure 10. The number of observations relative to the full moon (n = 244 species). Sourced from coral spawning database (Baird et al. 2021).

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