Hatchery Protocol for the Blacklip Rock Oyster

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Hatchery Protocol for the Blacklip Rock Oyster *Saccostrea echinata* (Quoy and Gaimard, 1835)

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The Cooperative Research Centre for Developing Northern Australia

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Aboriginal and Torres Strait Island readers should be aware that this publication may contain images or names of deceased persons.

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Contents

1. Introduction

The tropical Blacklip Rock Oyster (BRO), *Saccostrea echinata* (Quoy and Gaimard, 1835), has significant potential for aquaculture in northern Australia and the Indo-Pacific region, because of its large size, local consumption and valued flavour (Fleming, 2015; Nowland et al., 2019b). Blacklip Rock Oysters have been documented to occur throughout the Indo-Pacific, from Japan to New Caledonia, and across northern Australia, from Cone Bay in Western Australia to Bowen in Queensland (Nowland et al., 2019d). Sizeable colonies are considered to be rare, and BROs are generally not very abundant within their range (Glude, 1984). Instead, they occur in patches of isolated individuals towards the low tide level, with a preference for sheltered areas (Thomson, 1953).

The taxonomy of most tropical oyster species remains unresolved, and this is also true for BROs. Currently, the name *Saccostrea echinata*, is used to describe at least two topical rock oyster species; the true BRO and a much smaller, spiny oyster (Codi King et al., 2009; Huber, 2010). However, *S. echinata* is the presently accepted name for BRO on the World Register of Marine Species (WoRMS) (Mollusca Base, 2021). Recent genetic investigation of this species in Australia (McDougall, 2018; Nowland et al., 2019d) has placed the BRO within '*Saccostrea lin. J*' as nominated by Sekino and Yamashita (2016). These studies have highlighted the need for further research to clarify the inconsistencies concerning delineation of tropical oyster species. For the purposes of this manual, and to maintain consistency with the literature and with WoRMS, BRO is referred to as *S. echinata*.

The Northern Territory Government's (NTG) Department of Industry, Tourism and Trade's research and development program for BROs originated from a request from senior Aboriginal Traditional Owners to look at developing farming techniques for this oyster, to reintroduce previous BRO commercial activities undertaken by Aboriginal communities. Blacklip Rock Oyster aquaculture has the potential to provide local economic development and employment opportunities in remote communities that align with the desire to work on country utilising natural resources. It incorporates local knowledge, historical connection, cultural appropriateness and low-tech farming methods.

Initial hatchery trials with BROs began at the government-owned, Darwin Aquaculture Centre (DAC) in 2010. Since that time, dedicated research efforts have developed and refined hatchery protocols, resulting in species-specific, optimised, culture methods and increased spat (juvenile) production (Nowland et al., 2018a; Nowland et al., 2018b; Nowland, 2019; Nowland et al., 2019a; Nowland et al., 2021). These improved hatchery protocols improved larval growth rates, reduced larval mortality, and have significantly increased the proportion of settled larvae (i.e., pediveliger larvae successfully undergoing metamorphosis to spat) from around 0.5% to more than 10%. These improvements have resulted in increased oyster supply to experimental BRO farms, with hundreds of thousands of spat now being grown-out. Further improvements to hatchery production methods are required to increase current settlement rates to at least 30%, which is considered commercially acceptable.

The protocols in this manual reflect the production methods employed at the DAC at the time of publication. Although BRO production at the DAC has come a long way, consistent and routine production of millions of spat has not yet been achieved, and research to improve production is ongoing. The considerable, and growing, interest in hatchery production of this species, throughout Australia and internationally, has prioritised the release of this hatchery production protocol as a benchmark. It is hoped that others will build upon the methods outlined here, so that the commercial potential of this new aquaculture commodity for tropical Australia can be realised as soon as possible.

This protocol describes hatchery rearing methods specific to BROs and is intended for those with an understanding of basic bivalve biology and hatchery rearing techniques. Additional reading is recommended for those interested in a comprehensive description of bivalve biology and hatchery culture. Some references to consider include; Gosling (2003), Helm et al. (2004), O'Connor et al. (2008) and Lucas et al. (2019).

2. Seawater supply

Seawater at the DAC is pumped via submerged pumps, into the hatchery through a primary bank of 12 high-volume, deep-bed sand filters with approximately 98% efficiency for the removal of particles of >10 µm. The seawater is then secondary filtered through a bank of five, low-volume, deep-bed sand filters into a header tank. The seawater from the header tank is either used directly in the BRO nursery or subject to further filtration and 'aged[1]', prior to use in the larval rearing system. Seawater in both the nursery and larval rearing systems may be blended with carbon filtered de-chlorinated freshwater (FW) to adjust salinity as required.

Seawater used in the larval rearing system is drawn from the header tank, filtered through a 1 µm (nominal) filter bag and stored in two 100 kL storage tanks. Freshwater is added to reduce salinity to 25 ppt, and the water is then continually aerated and left to mature for 7–10 days at a water temperature of 28–30°C. As a quality control, the water can be examined by testing for the presence and abundance of *Vibrio* bacteria using Thiosulfate-citrate-bile salts-sucrose (TCBS) plating and counting colonies through serial dilutions, during the maturation process. Once matured, the 25 ppt seawater is pumped into the hatchery, through a pair FSI X100 bag filter housings fitted with 1 µm filter bags placed in series (Figure 1). The two 100 kL storage tanks are filled and used alternately to enable continual supply of microbial matured seawater to the hatchery. While one 100 kL is supplying the hatchery, the other is being filled and matured in preparation for use. After one 100 kL tank of matured culture water has been exhausted, it is drained, rinsed with freshwater and air dried before being refilled for reuse.

Each day the pipework used to transport culture water between the 100 kL maturation tank and the hatchery is disconnected, drained and backwashed with a 200 ppm chlorine solution. The chlorine solution is held in the pipework for 20 minutes before being drained, flushed with FW and left to drain and dry overnight.

Figure 1. Pair of FSI X100 bag filter housings fitted with 1 µm filter bags placed in series. Arrows indicate the direction of water flow. FSW; filtered seawater and FW; freshwater. FW is used to flush the system when cleaning.

[1] Aging or maturing seawater is the practice of leaving it in an aerated holding tank for a period of at least seven days to allow the population of beneficial (non-opportunistic) bacteria in the water to increase and stabilise, and concurrently reduce the numbers of harmful (opportunistic and pathogenic) bacteria, prior to the water being used.

3. Broodstock

To obtain broodstock in good reproductive condition, the DAC hatchery collects a minimum of 40 BRO broodstock from wild populations during the natural spawning period throughout the monsoon season of October through April (Figures 2 and 3). In the Northern Territory, Australia, BROs spawn semi-continuously throughout the monsoon season and have an extended resting phase during the dry season (May–September) (Nowland et al., 2019c). Due to BROs having genetically distinct populations within their distribution, broodstock oysters are collected from within the same management unit[2] as the farm location (Nowland et al., 2019d). On arrival at the DAC, fouling is removed from broodstock before they are cleaned in iodine solution (10 mL of Phoraid in 10 L of FW) and either used for spawning or held within a quarantine area.

Quarantine protocols will be hatchery specific, and those for the DAC are detailed in NTG Fisheries Biosecurity Controls and Policies. Briefly, BRO are held in isolated tanks within a designated quarantine area and effluent seawater is treated with chlorine. Chlorine strength follows AQUAVETPLAN recommendations for the treatment of wastewater (Department of Agriculture Fisheries and Forestry, 2008). After four weeks broodstock may be released from quarantine and added to broodstock holding tanks, subject to satisfactory health examinations and observations. When broodstock are not used for spawning or in quarantine, they are housed in 5,000 L cylindroconical holding tanks with a 120% day^{-1} flow-through water exchange, with ambient temperature and salinity seawater. Methods for feeding broodstock follow those described by O'Connor et al. (2008) for Sydney Rock Oysters.

Broodstock conditioning protocols have not been developed for BROs and existing protocols for temperate oyster species (e.g., Sydney Rock Oysters or Pacific Oysters) have not proven successful for BROs. However, research to develop BRO broodstock conditioning protocols is currently underway and, if successful, will extend the reproductive period of BROs and allow year-round spat supply to farmers and eliminate the reliance and cost of collecting broodstock from the wild.

Figure 2. Wild Blacklip Rock Oyster broodstock, collected from Goulburn Island, Australia (11°38'49"S 133°25'23"E), Scale bar = 2 cm.

[2] Within the Northern Territory, there are currently two management units, which relate to the two known distinct populations of BROs occurring either side of the Wessel Islands. New areas interested in farming BROs should undergo genetic stock assessments to determine optimal management practises.

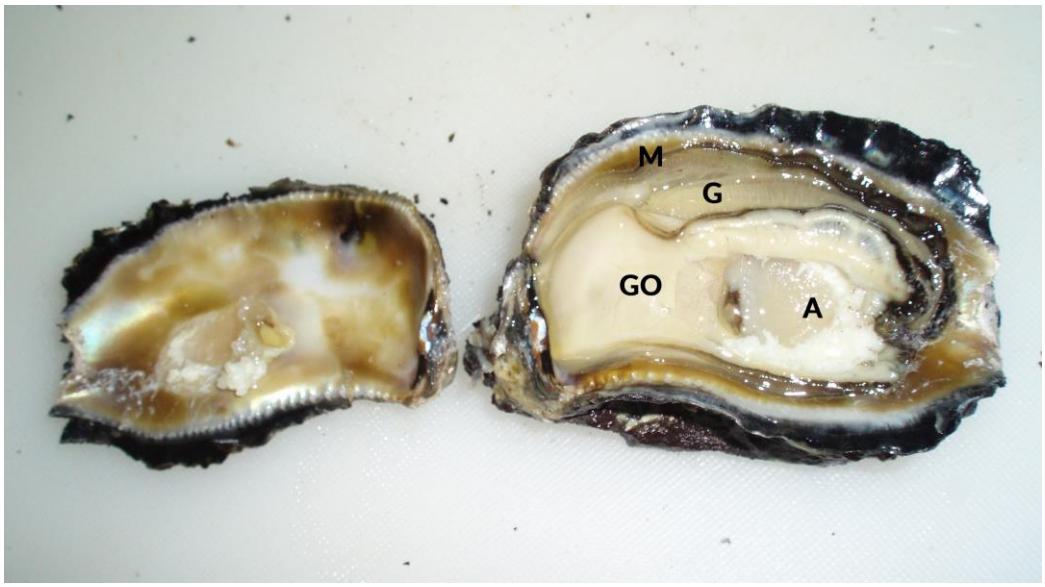

Figure 3. Reproductively mature Blacklip Rock Oyster broodstock, collected from Goulburn Island, Australia (11°38'49"S 133°25'23"E). GO= gonad; M= mantle; G= gills; A= adductor muscle.

4. Spawning

The DAC has evaluated multiple spawning induction techniques for BRO broodstock to determine the most effective method (Nowland et al., 2021). This research has demonstrated a combination of physical and chemical spawning induction, involving reducing salinity and addition of BRO spermatozoa (either naturally released or stripped from a sacrificed male oyster), as the most successful method for BROs.

4.1. Overview and equipment set-up

The day before spawning, one 1,000 L tank is filled with 1 µm (nominal) filtered seawater (FSW) at 36 ppt and one 1,000 L tank and two 5,000 L tanks are filled with matured 1 µm (nominal) FSW from one of the 100 kL storage tanks. All four tanks have gentle aeration (air stone sitting inside central tank outlet), to allow for temperature acclimation to 30±1 °C. A minimum of 40 broodstock are moved to the spawning room and placed (upright) into a 250 L capacity spawn table (empty of water), fitted with a 100 L sump recirculating system (Figure 4). To induce a spawning response, broodstock are left to dry overnight (emersion).

On spawning day, broodstock are subjected to a maximum of three induction cycles so that only fully developed eggs and sperm are used (see section 4.2 below). When spawning occurs, fertilised eggs are rinsed with matured FSW (25 ppt) and stocked into the two 5,000 L larval tanks at maximum density of 10 embryos mL^{-1}. Equipment required for spawning induction includes:

- 1x 250 L spawn table
- 1x 100 L sump
- 1x 24 V submersible pump
- 1x internal standpipe
- 1x small recirculating system that links sump to spawn table
- 2x trestle tables
- 40x 4 L black spawn buckets

- 12x 20 L buckets (with water volume lines marked)
- 12x perforated plungers
- 2x 15 µm nylon mesh nets
- 2x 70 µm nylon mesh debris nets
- 2x 1,000 L tanks of FSW for filling the spawn table and washing (one at 25 ppt and one at 36 ppt)
- 2x 5,000 L tanks of matured FSW for stocking the fertilised eggs (25 ppt and 30±1 °C)
- 2x 12 mm hoses and pumps used for filling spawn buckets and washing larvae
- Refractometer
- Thermometer
- One microscope bench set-up with a compound microscope, 8x Sedgewick-Rafter slides, 1 mL disposable pipettes, 100 µL pipette and tips, counter, paper, pen and 4 small torches

Equation for tank volume;

$$\text{Tank volume} = \pi \times r^2 \times h \qquad \textbf{Eq. 1}$$

Equation for salinity reduction from 36 to 25 ppt;

$$\frac{36}{25}\ \text{ppt} = 0.69\%\ \text{(therefore 69\% of total volume FSW and 31\% FW)} \qquad \textbf{Eq. 2}$$

4.2. Steps for spawning induction

1. Leave clean and dry oysters upright on the spawning table overnight, ~16 h (Figure 4).

Figure 4. Blacklip Rock Oyster broodstock left to dry on spawn table overnight (emersion).

2. Keep the room dark and fill the spawn table sump with FSW from the 1,000 L tank at the required temperature and salinity (30±1 °C and 36 ppt).
3. Turn the recirculating pump on to fill the spawn table with enough FSW to cover the oysters (~15 cm). You will need to top-up the sump with FSW (36 ppt) from the 1,000 L tank.
4. Allow the oysters to acclimate for 1 h and observe to ensure they are open and filtering seawater (Figure 5).

Figure 5. Spawn table filled with filtered seawater and Blacklip Rock Oyster broodstock reaction observed.

5. Freshwater (FW) is added to the sump to reduce the salinity from 36 to 25 ppt. Measure this reduction in salinity using a salinity refractometer. You can also replace 31% of the current table volume with FW (i.e., if the table is currently holding 250 L of FSW, remove 77.5 L of FSW from the sump and replace with FW).
6. Maintain the oysters in these conditions for 1 h.
7. If spawning has not commenced within 1 h of salinity reduction, empty the table and repeat steps 2–6. Note: broodstock are subjected to a maximum of three induction cycles so that only fully developed eggs and sperm are used.
8. If spawning has not commenced after three induction cycles, sacrifice five oysters to get a better indication of broodstock condition. It is possible that broodstock are not in adequate spawning condition, which may affect larval performance.
9. When broodstock begin spawning, males will most likely spawn first. Leave them on the spawn table, because sperm is a chemical stimulus that can trigger mature females to spawn.
10. Once females begin to spawn or if the density of sperm on the table becomes too high to clearly see other broodstock, remove spawning broodstock from the table. Place them into individual labelled 4 L black plastic spawn buckets, filled with FSW from the 1,000 L wash tank (30±1 °C and 25 ppt), where they will continue to spawn.
11. Label the spawn buckets with gender and spawn start time, and record this on a data sheet.
12. Check a sample of sperm under the microscope for percent sperm motility. Then pool sperm from a minimum of five selected males, which can be added to the eggs if needed. Note: check the time of spawning because the activity/efficacy of sperm declines after ~20 minutes.
13. From each spawn bucket containing a female oyster, check a sample of eggs under the microscope to ensure the eggs are water hardening (taking on a round shape) and that enough sperm is present i.e., so that at least one sperm is seen at the periphery of each egg.

14. If needed add sperm to the eggs to ensure fertilisation. Eggs should be fertilised within 60 minutes of release.
15. Take another sample of the eggs to ensure fertilisation has commenced (1st polar body is observed, Figure 6B), visually examine the egg/sperm ratio and percentage fertilisation. Note: BRO eggs are ~50 µm in diameter.
16. Once ~60% of the eggs are fertilised (i.e., the 1st polar body is observed, Figure 6B), very gently wash and retain them on two 15 µm nylon mesh nets (the second net is a backup, in case eggs go through the first net) and rinse them through a 70 µm nylon mesh debris net (to clean off unwanted debris from the spawning table) into a 20 L bucket filled with 10 L of FSW (25 ppt).
17. Take three replicate 100 µL samples to count the number of embryos; fertilised eggs that are undergoing division (Figure 6C–F).
18. Once the majority of eggs are fertilised, cleaned, and starting to form embryos (dividing) stock them into the 5,000 L incubation tanks at a temperature of 30±1 °C and salinity of 25 ppt, with gentle aeration, but no algae. At a maximum stocking density of 10 embryos mL^{-1} (maximum of 50 million embryos per 5,000 L tank). No algae is required until larvae are D-stage veligers at approximately 18 h post fertilisation.

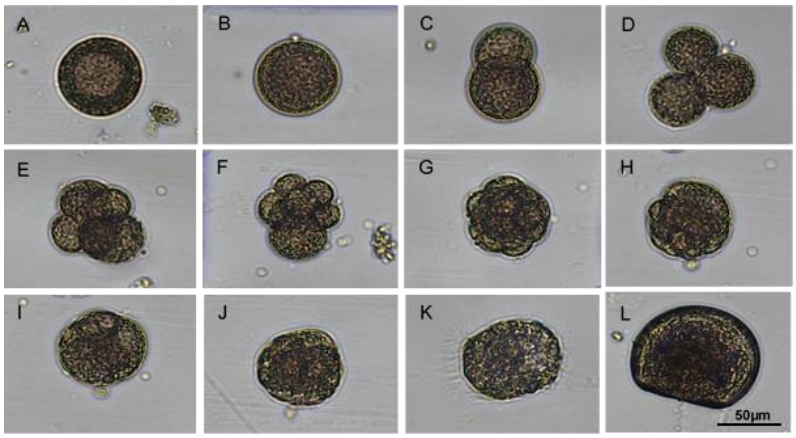

Figure 6. Embryonic development of the Blacklip Rock Oyster, showing: (A) unfertilized egg; (B) fertilized egg with polar body; (C) 2-cell embryo; (D) 3-cell embryo; (E) 4-cell embryo; (F) 8-cell embryo; (G) morula, ~16-cell; (H) morula, ~32-cell; (I) blastula, > 64-cell; (J) stage leading to gastrula; (K) ciliated trochophore; and (L) early D-stage veliger.

5. Larval rearing

Larval rearing begins immediately post fertilisation and lasts for 18–21 days, at which time larvae develop into crawling pediveligers (Figure 7G), before metamorphosing into spat (Figure 7H) and settling to the substrate. For a detailed description of BRO embryonic, larval, and early postlarval development refer to Nowland et al. (2018a).

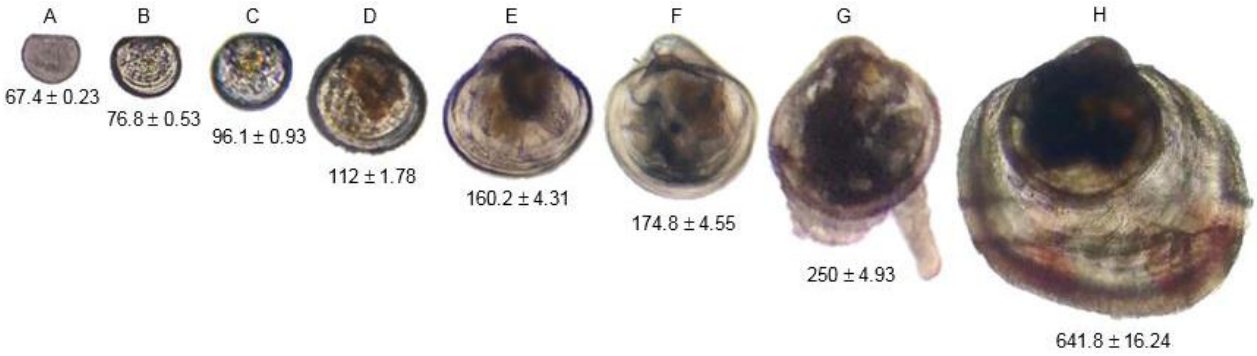

Figure 7. Blacklip Rock Oyster larval development, mean (±SE) dorso-ventral shell measurement (DVM, µm). (A) D-veliger, 1 day post hatch (dph); (B) D-veliger, 3 dph; (C) early umbonate larvae, 7 dph; (D) umbonate larvae, 10 dph; (E) umbonate larvae, 15 dph; (F) eyed umbonate larvae, 16 dph; (G) pediveliger, 18 dph; and (H) 1-day-old spat.

5.1. Overview and equipment set-up

The larval rearing room consists of four 5,000 L cylindroconical larval rearing tanks each with two 50 mm tank outlets (Figure 8). At any one time only two tanks are used, allowing two spare tanks to be maintained, thoroughly cleaned and dried awaiting the next daily water exchange. Ball valves are used on tank outlets that can be removed, totally dismantled and chlorinated after each water exchange to reduce microbial build up in the system (e.g., the Banjo PP ball valve single union). Larval tanks are filled with 1 µm (nominal) matured FSW at a temperature of 30±1 °C and salinity of 25 ppt, and a 0.5 µm filtered air system is used. Photoperiod is 11 h daylight, to match natural conditions, from 0800–1900 low light (20–35 lux) sunrise from 0730–0800 and sunset from 1900–1930, controlled with a light timer.

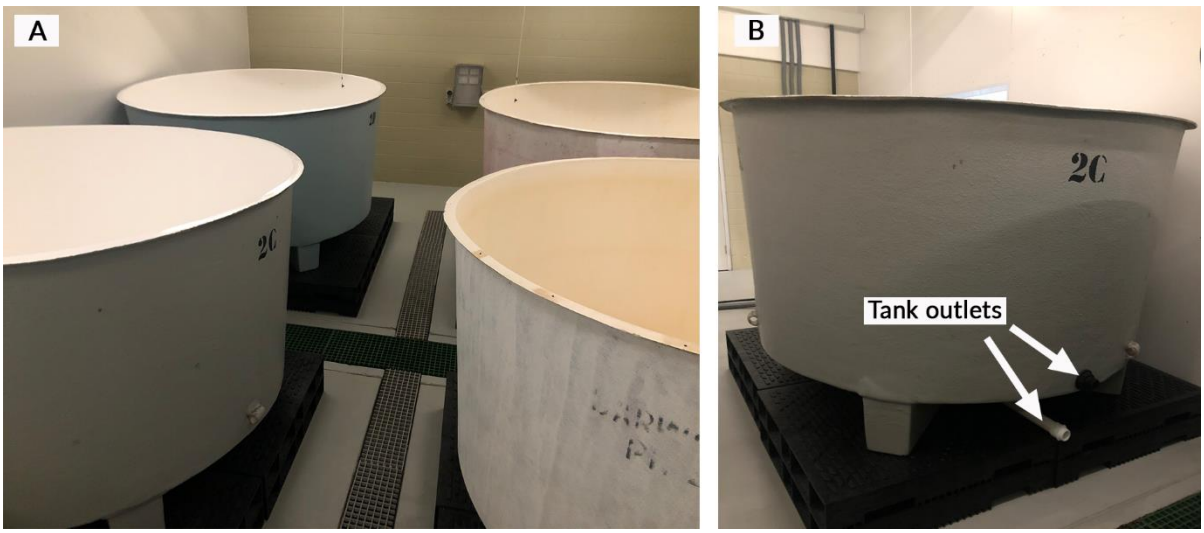

Figure 8. (A) Four 5,000 L tanks in larval rearing room and (B) close up of one 5,000 L larval rearing tank, showing two 50 mm tank outlets.

The larval rearing area is cleaned by scrubbing and rinsing the area with Virkon Aquatic at 1% (10 gL^{-1}) and chlorine 200 ppm (after FW rinsing, leave to dry-out at least overnight). Both Virkon Aquatic and chlorine are used as cleaning agents, as chlorine is a disinfectant and kills bacteria while Virkon Aquatic has a wider spectrum of activity against viruses, some fungi, and bacteria. The larval rearing room is set up with 4x 5,000

L tanks, 1x 1,000 L washing tank, an airline drying rack, shelving for nylon mesh nets and equipment, footbaths and spray bottles for hands.

Larval nylon mesh nets ranging from 15–500 µm are used to retain larvae when tank water is exchanged (Figure 9A). Before each daily water exchange, nets are checked for holes and small holes are repaired using glue if required. Note: When first increasing the nylon mesh net size, use a back-up net of the previous (smaller) size to ensure no significant losses of larvae.

$$\text{Larval net size to use} = \frac{\text{size of larvae (µm)}}{1.4} \qquad \textbf{Eq. 3}$$

Make sure to have each size nylon mesh net needed and tubs for wet screen harvesting (Figure 9B). Wet screen harvesting is a gentle process of sieving larvae out of the water column to clean and count, before being restocked into new larval rearing tanks. Tanks are drained into the required net size while submerged in seawater to retain larvae (Figure 9B), before being gently washed through a larger debris net, into a 20 L bucket to be counted (see section 5.2 below). Multiple nets can be used to grade larvae, where the larger size grade will be retained, while the smaller size grade will pass through. Grading ensures uniform larval size and allows the assessment of smaller larvae, which can be either kept or discarded (see table 2 for estimated larval harvesting net sizes).

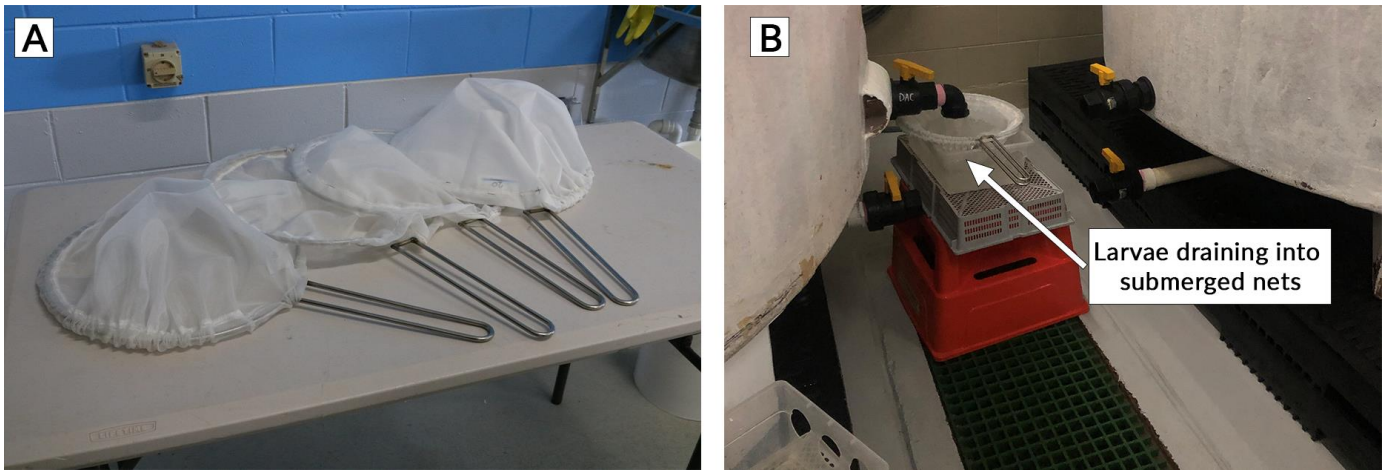

Figure 9. (A) Larval nylon mesh nets (B) Wet screen harvesting of Blacklip Rock Oyster larvae from 5,000 L larval rearing tank.

5.2. Larval sampling and measuring

Equipment to sample, measure and assess larvae includes a compound microscope (with 4x, 10x and 20x magnification), perforated plunger, 10–100 µL automatic pipette, pipette tips, fixative (FSW formalin), counter, calculator and Sedgewick-Rafter slides (Figure 10). The recipe for FSW formalin solution is one volume of concentrated formalin (usually supplied as 37% formaldehyde solution) to nine volumes of clean, FSW.

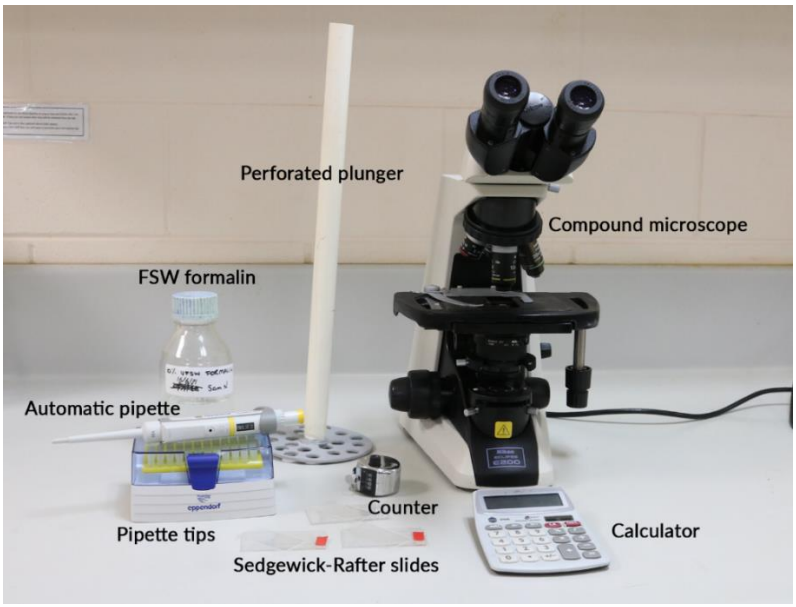

Figure 10. Equipment to sample, measure and assess larvae.

During daily water exchanges, larvae are rinsed from the nylon mesh net into a 20 L bucket containing FSW and gently distributed uniformly in the water column using a perforated plunger. Three replicate 100 µL samples are taken using an automatic pipette and each placed on a Sedgewick-Rafter slide for larval counting. A sample of larvae is examined live to assess their general health before being fixed with a drop of 10% FSW formalin to allow accurate counting and measuring. Healthy larvae are swimming in the water column and on microscopic inspection they should be active, have uniform shell shape, algae in their stomach (i.e. guts dark in colour) and vigorous, unimpeded cilia action (cilia are tiny vibrating hairs that direct food particles towards the mouth as well as providing the motive force for swimming activity). For more information on assessing bivalve larval health refer to Gosling (2003) and Helm et al. (2004). Using a compound microscope, the number of larvae within each sample is counted and the dorso-ventral measurement (DVM) of 10 larvae is measured (Figure 11). This information is used to gauge the growth and survival of the larval batch. These counts and measurements are also used to adjust larval densities and food rate when required (Section 5.3). Larval size (DVM) is compared between runs to assess the health and general condition of the larvae run (Figure 12).

Healthy larvae will typically occupy the water column, therefore on a daily basis the bottom ~300 L of the tank are harvested separately and their health and condition (their gut content, activity and development) are assessed. This information is recorded and a decision is made, based on their observed health and vitality, to either keep or discard these larvae. Regular grades are undertaken during the run to eliminate poor/slow-developing and moribund larvae that are less likely to survive or settle into spat (see larvae culture schedule in Table 2).

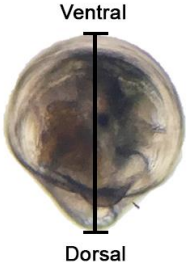

Figure 11. Dorso-ventral measurements (DVM) shown on an eyed umbonate Blacklip Rock Oyster larvae, 16 days post hatch.

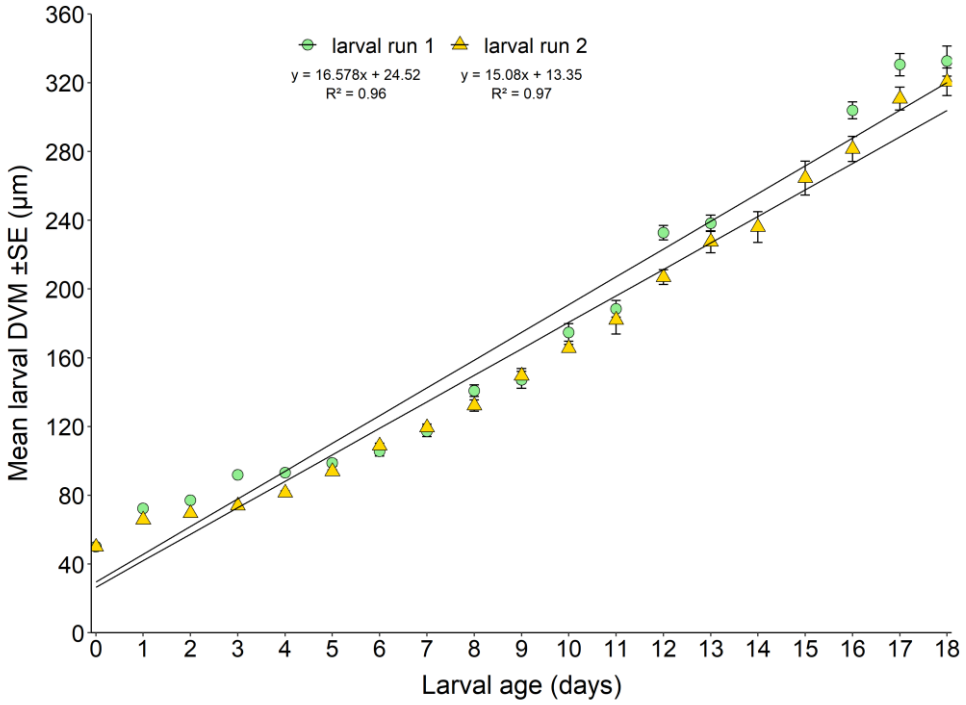

Figure 12. Changes in mean (±SE) dorso-ventral measurements (DVM, µm) of Blacklip Rock Oyster larvae over two hatchery runs (Nowland, 2019).

5.3. Larval food

Live microalgae starter cultures are obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian National Algae Supply Service (Table 1). Microalgae is cultured using DAC standard operating procedures, modified from those described by Andersen (2004). Microalgae is introduced the day after spawning, and after the first water exchange. This first water exchange occurs as soon as practically possible, at ~18 h post fertilisation, to ensure food is available to larvae. Algae food rates are measured as cells of algae mL^{-1} day^{-1}, food rates and species composition change as the larvae develop (see Table 2). If excess food in the culture water is observed the food rate may be reduced, conversely if larvae have a light gut colouration or are growing very well (days ahead in growth) the food rate may be increased. Larvae are batch fed twice a day, half in the morning and half in the afternoon (see section 5.5). Food rates are calculated daily, based on algae cell counts and stocking density (Table 2). Residual algae is determined daily with a handheld fluorometer (Turner Designs, USA) and food rates should be adjusted, based on consumption, before the afternoon (remaining) algae is fed.

Table 1. Live microalgae species used to feed Blacklip Rock Oyster larvae, their dry weights (pg) and reference, *Tisochrysis lutea* (T-iso) dry weight equivalent and CSIRO accession number (CS Number).

	Chaetoceros muelleri	***Pavlova sp.***	***Cheatoceros calcitrans***	***Tisochrysis lutea***
Dry weight (pg)	20.00	23.00	15.00	19.00
Source of dry weight data	(Nell and O'Connor, 1991)			
T-iso dry weight equivalent	0.95	0.83	1.27	1.00
CSIRO CS Number	CS-176	CS-50	CS-178	CS-177

5.4. *Vibrio* testing

Testing for *Vibrio* spp. bacteria is conducted daily using TCBS agar plates that are held in an incubator at 29°C for 48 h. TCBS agar plates are made in advance, from T.C.B.S. (Old Formulation) (CM1025, Oxoid Limited, England) according to manufacturer instructions. Samples taken and tested daily include; (1) the current larval tank FSW (excluding larvae), (2) new larval tank FSW and (3) algae food mix. This testing is not preventative, the information obtained is used for monitoring purposes and better understanding the tank environment over time. As *Vibrio* spp. are associated with larval mortality (i.e. run crashes), which are a current issue under investigation. However, routine testing for *Vibrio* spp. in the algae culture room, using TCBS plates, is a quality control measure and is undertaken daily to ensure no *Vibrio* spp. contaminated food is used for the larvae (Figure 13).

Steps for *Vibrio* testing include;

1. Rinse sample container and lid 3 times with sample water (note: take larval tank sample from water after it has passed through a 20 µm screen to prevent collecting larvae).
2. Seal containers and take to the laminar flow cupboard.
3. Label TCBS plates with the date and sample location.
4. Use automatic pipette set at 50 µL and sterile pipette tips (note: use a new pipette tip for each sample).
5. Take sample from jar, making sure not to touch any edges with the pipette.
6. Inoculate TCBS plate with the 50 µL of sample water.
7. Seal TCBS plate with parafilm.
8. Once liquid has been absorbed into the plates sit them upside down in the incubator.
9. Check plate at 24 and 48 h for bacteria colonies and record data; presence/absence and colour of the colony. At the DAC isolation and DNA testing has shown that yellow colonies are *Vibrio harveyi* and green colonies are *Pseudomonas* sp.

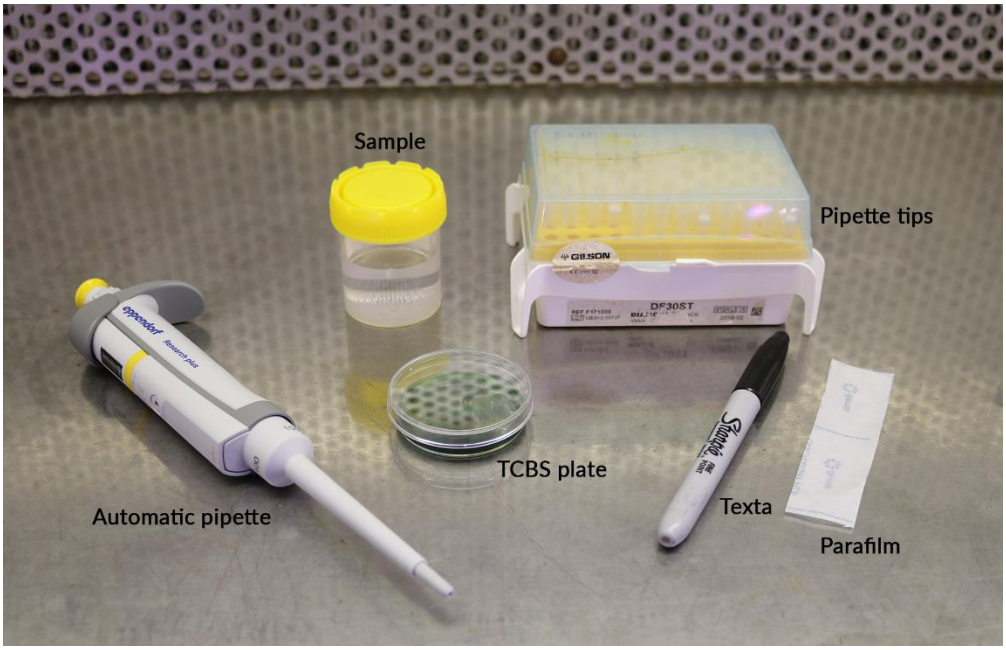

Figure 13. Equipment for *Vibrio* testing inside laminar flow cupboard.

Table 2. Blacklip Rock Oyster larvae culture schedule showing; larval age in days post hatch, stage of development, estimated microalgae food rate, microalgae species composition of daily food, larval tank stocking density, estimated larval harvesting net size and estimated debris net size. Note: CAL= *Cheatoceros calcitrans*, PAV= *Pavlova sp.*, ISO= *Tisochrysis lutea* and MUEL= *Chaetoceros muelleri.*

Blacklip Rock Oyster larvae schedule									
day	**stage**	**est. food rate (cells mL^{-1})**	**CAL**	**PAV**	**ISO**	**MUEL**	**stocking density (larvae mL^{-1})**	**est. larval net size**	**est. debris net size**
0	Embryo	0	0	0	0	0	10	15	70
1	D-veliger	15,000	50%	25%	25%	0%	8	40	80
2		15,000	50%	25%	25%	0%	8	40	80
3		15,000	50%	25%	25%	0%	8	40	100
4		15,000	50%	25%	25%	0%	8	40	100
5	Early umbonate	15,000	50%	25%	25%	0%	8	40/63	120
6		20,000	50%	25%	25%	0%	7	63	150
7	Umbonate	25,000	50%	25%	25%	0%	7	63/80	200
8		25,000	40%	20%	20%	20%	6	80	220
9		25,000	40%	20%	20%	20%	6	80	220
10		30,000	40%	20%	20%	20%	5	80/100	250
11		30,000	40%	20%	20%	20%	5	100/120	250
12	Eyed umbonate	30,000	30%	20%	20%	30%	4	120	280
13		30,000	30%	20%	20%	30%	4	120/150	280
14		30,000	30%	20%	20%	30%	3	150	none
15		30,000	30%	20%	20%	30%	3	150	none
16		30,000	20%	20%	20%	40%	3	150	none
17	Eyed pediveliger	30,000	20%	20%	20%	40%	3	150/180	none
18		30,000	20%	20%	20%	40%	2	180	none
19		30,000	20%	20%	20%	40%	2	180/220	none
20		30,000	20%	20%	20%	40%	2	180/220	none
21		30,000	20%	20%	20%	40%	2	180/220	none

5.5. Daily steps for larval rearing

1. At the start of the day (~0800) record temperature, salinity and pH of the old and new larval tanks and wash tank and residual algae concentration of the current larval tank (see Appendix 1 for data sheet template).
2. Take a 1 mL sample of the larvae and examine larval size, gut content, motility and general health, record this data.
3. Collect samples and undertake *Vibrio* testing (see section 5.4).
4. Collect algae count and calculate daily algae amount (Table 2).
5. Check quality of harvested algae on a haemocytometer slide by visually assessing; cell condition and shape, clumping, bacteria load and other contaminants (e.g. opportunistic ciliate organisms).
6. Add the morning food (½ total algae) to new larval tanks before the tank is stocked with larvae.
7. Slowly drain the old larvae tanks through a larval net of appropriate size (wet screen harvest; Figure 14). Stagger harvesting to allow time between tanks. Note: When first increasing the larval net size use a back-up net of the previous size to ensure no significant losses of larvae.
8. Gently rinse the larvae on the larval net to remove any excess food or small debris (Figure 15).
9. Wash the larvae retained on the larval net through a debris net and into a 20 L bucket, and take 3 x 100 µL samples.
10. Check larval motility before fixing the samples with a drop of FSW formalin.
11. Determine the total number of larvae present (count) and stock the new larval rearing tanks at the recommended density (Table 2).
12. Keep the slides to measure the DVM of 10 individual larvae and take photos (if your microscope has a photographing function).
13. Wash the empty tanks out with FW and scrub using Virkon Aquatic at 1% (10g L^{-1}), leave for 5 min then rinse **thoroughly**.
14. Rinse the tanks at least twice with FW and fill the new larval rearing tanks with 25 ppt matured FSW ready for the next day.
15. Chlorinate airlines and all harvesting equipment, rinse well with FW and hang up to dry until the next day.
16. Record all data on the data sheet and enter into the computer spreadsheet.
17. At the end of the day (~1600), add the afternoon food (remaining algae) to larval tanks. Note: check stocking density and food rates and adjust accordingly.

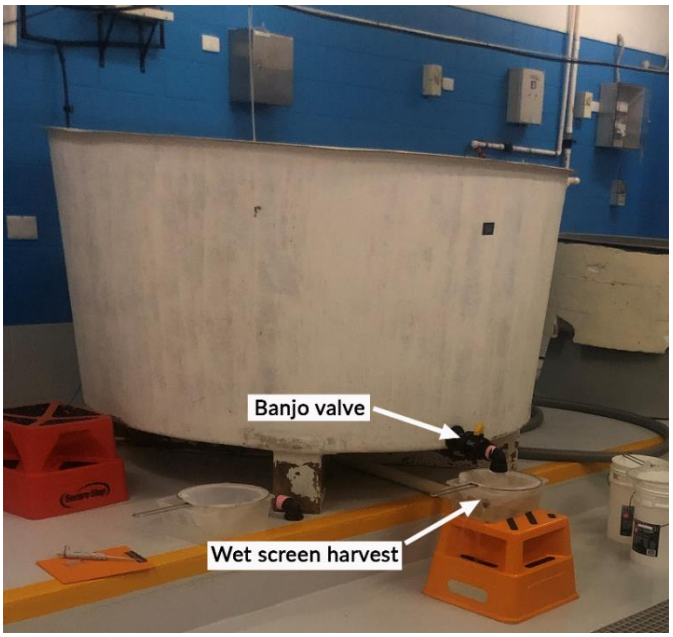

Figure 14. Wet screen harvesting showing larvae passing though 50 mm valve outlet into a nylon mesh larval net.

Figure 15. Larvae being gently rinsed in the larval net using the wet screen harvest method.

6. Settlement

During daily microscopic assessment, from ~17 dph eyed pediveliger larvae motility should be closely observed, as when more than 35% of larvae are crawling pediveligers it is a strong indicator that larvae are ready to metamorphose into spat and it is time to set the larvae (Figure 7G). Larvae retained on a 220 µm larval net are put to set with the settlement-inducing chemical, epinephrine (see section 6.1). Upon removal from the larval rearing tanks, larvae are rinsed into a known volume of FSW and total numbers determined. Larval epinephrine treatment is typically conducted in a 25 cm diameter 150 µm nylon mesh screen, within a 10 L tub of FSW (30±1 °C and 25 ppt). Immediately following treatment with epinephrine, larvae are stocked into a downweller settlement system at a max density of 150,000 larvae per 25 cm diameter 150 µm nylon mesh screen. Epinephrine is made up freshly as required.

6.1. Epinephrine treatment protocol

Procedures for the use of epinephrine are modified from those described by O'Connor et al. (2008) for use with the Sydney Rock Oyster (*Saccostrea glomerata*).

Chemical

Epinephrine Bitartrate Salt
Sigma E-4375
C9H13NO3-C4H6O6
FW=333.3

General use of epinephrine

1.2 g of epinephrine per 20 L of FSW with a maximum of 50 g of larvae for 60 minutes treatment. Note: When *S. glomerata* are retained on a 212 µm screen 1 million larvae are assumed to weigh 18 g.

At the DAC we typically treat up to 500,000 BRO larvae with 0.6 g of epinephrine in 10 L of FSW for 60 minutes of treatment.

Epinephrine deteriorates in light and is **HIGHLY TOXIC**: Technicians must use appropriate personal safety equipment when using epinephrine and read the associated safety data sheet before using.

Equipment

- 1 x 10 L black/dark container (epinephrine degrades in light)
- 150 µm screen to retain larvae being treated
- Epinephrine (Highly toxic to heart, lungs and respiratory organs) **MUST USE: GLOVES, FUME HOOD OR RESPIRATIOR**. Epinephrine should be mixed with a small concentration of FW
- Alfoil to cover jar containing epinephrine
- Black plastic to cover the screen and 10 L container during treatment

Method

1. Determine the number of oyster larvae that are ready to settle (pediveligers) retained on a 220 µm nylon mesh larval net.
2. Weigh out epinephrine, typically 0.6 g per 10 L of FSW and dissolve in a small volume of FW (**use gloves, respirator or fume hood and safety glasses**).
3. In a dark container, add epinephrine solution to 10 L of FSW and mix well. Make sure the seawater used is at the same temperature and salinity as the larval culture water.
4. Add the 150 µm screen to the container holding a maximum of 500,000 larvae and gently mix well.
5. Cover container with black plastic. Epinephrine denatures in light, therefore the container needs to be covered with black plastic so that no light can penetrate onto the larvae (if possible carry out this procedure in a dark room).
6. Leave larvae in the container for 60 minutes.
7. After treatment is completed wash the larvae thoroughly with FSW and gently brush off any adherent larvae from the walls of the screen with a soft-tipped brush and gentle flow of FSW (Figure 16).
8. Add treated larvae to 150 µm settlement screens in the set system (~150,000 larvae per 25 cm diameter 150 µm nylon mesh screen).

9. 2 h after being placed onto screens wash the larvae/spat again with FSW and gently brush any recently settled spat off the screen sides and mesh. This process stops spat from attaching to the screen material.
10. Grade out spat on a 300 µm screen every second day and place in a separate downweller system (Figure 17). This removes healthy spat from any larvae that do not metamorphose and might die in the set system.
11. Repeat the epinephrine treatment process for non-settled larvae 2–3 days post epinephrine treatment.

Figure 16. Small specs on the 150 µm screen are Blacklip Rock Oyster larvae adhering to the walls and mesh after epinephrine treatment.

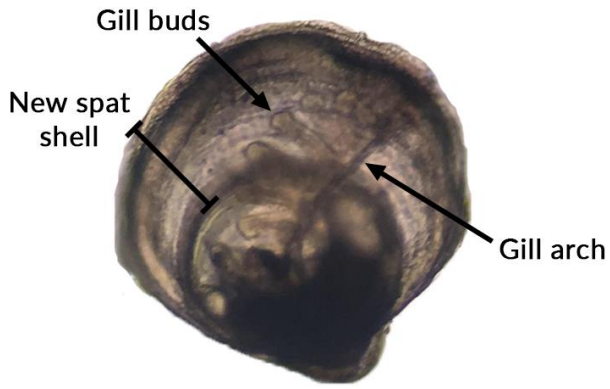

Figure 17. Recently settled Blacklip Rock Oyster spat showing new shell growth, gill buds and gill arch.

6.2. Downweller settlement system

A recirculating downweller system is used for larval settlement. The downweller system consists of a 250 L fibreglass table fitted with a 100 L sump recirculating system and spray bars that can hold up to ten 25 cm diameter 150 µm screens (Figure 18). Temperature and salinity are the same as larval rearing; 30±1 °C and 25 ppt. Ready to set larvae that have been treated with epinephrine are stocked into the 150 µm screens at a maximum density of 150,000 larvae per screen. The spray bar provides flow and food to the larvae from the sump and up to four systems are maintained. At any given time only two systems are used, allowing the spare systems to be cleaned and dried awaiting the next water exchange. Daily cleaning involves pumps, hoses, airlines and spray bars cleaned with chlorine and scrubbing the tank and sump with Virkon Aquatic at 1% (10g L^{-1}; leave for 5 min then rinse **thoroughly**). The tanks are then filled with FSW ready for the next day. Every day the larvae and spat are viewed and measured to determine and record growth and development. When spat are large enough to be retained on a 300 µm larval net they are transferred to a separate downweller system, to separate them from settling larvae (Figure 19). Spat can be cleaned (gently rinsed) with FW from approximately one week post set.

Max number screens = 20 x 150,000 larvae per screen = 3 million larvae/spat **Eq. 4**

Food rations will continue according to the larval culture schedule (Table 2; 30,000 cells mL^{-1}), as batches of larvae/spat undergoing metamorphosis may temporarily stabilise or reduce feeding. Tanks must be visually inspected to determine if the food rate is excessive or needs to be increased. Spat are fed in the morning and afternoon each day.

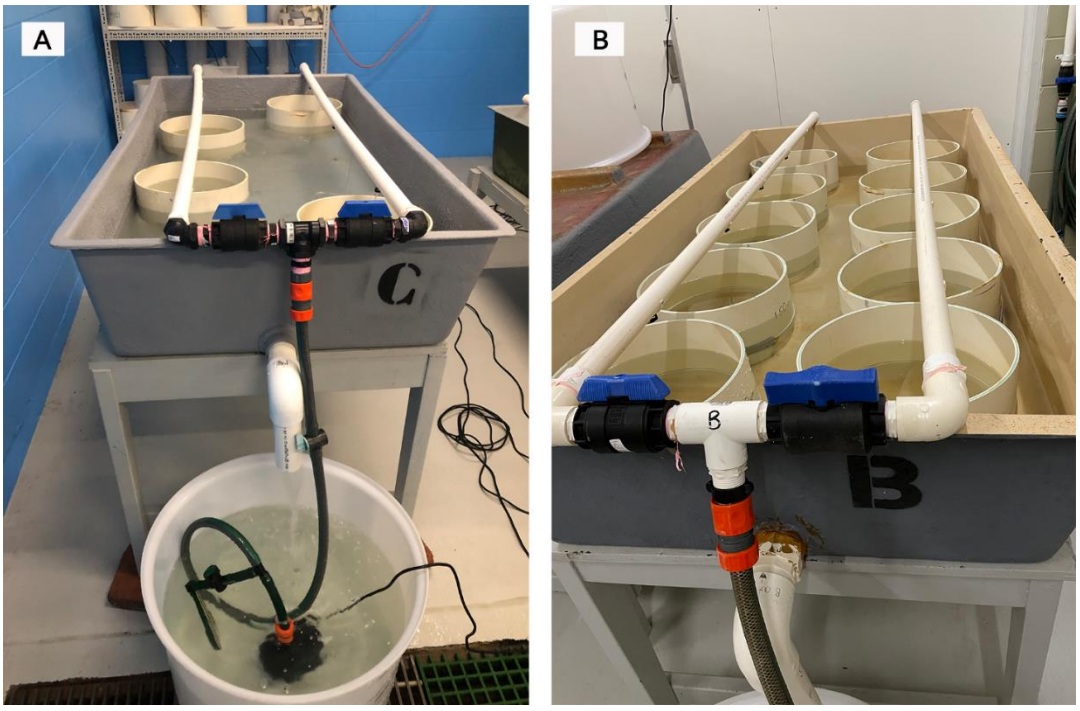

Figure 18. (A) Recirculating downweller system for settlement and (B) close up of spray bars and holding screens in the downweller system.

Figure 19. Recently settled Blacklip Rock Oyster spat that have been separated from setting larvae.

7. Nursery

Spat are transferred to either a downweller or upweller nursery system when they are large enough to be retained on a 500 µm larval net. The downweller nursery system is similar to the downweller settlement system (section 6.2) as it provides a downwards flow of FSW and food for spat (Figure 20). While the upweller nursery system provides an upwards flow of FSW and food for spat, and aids the removal of debris (e.g., faeces) that can get trapped on the nylon mesh screens (Figure 20). A series of 50 cm diameter screens are maintained, fitted with either 150 or 500 µm nylon mesh. With an initial stocking density of approximately 100,000 spat per screen. Spat are viewed daily and measured to determine growth and development and the system is cleaned with FW; screens, pumps, hoses, airlines and spray bars are rinsed. Three times a week the screens of spat are removed and the systems are cleaned with 200 ppm chlorine. At this stage, spat are large enough to be left out of water during cleaning (typically 1–2 hours).

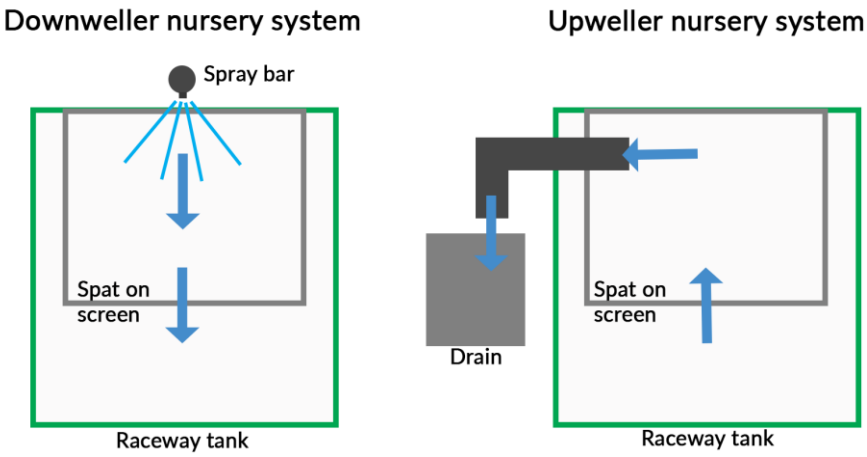

Figure 20. Downweller and upweller nursery system diagram. Arrows indicate the flow of seawater thorough the systems.

The downweller system consists of a 1,400 L fibreglass raceway recirculating system and spray bar that can hold up to eight 50 cm diameter screens (Figure 21A). A low voltage submerged pump (24v, 4,000 L h^{-1}; Ascento Reefe RP4000LV), is used to circulate water through the system. Flow rate into each screen is governed by the spray nozzle size, currently 1x 110 L h^{-1} nozzles are used per screen. While the upweller system consists of a 1,400 L fibreglass raceway recirculating system with a gutter, that can hold up to eight 50 cm diameter screens (Figure 21B). Water is directed up through the table by two submerged pumps (230v, 6,300 L h^{-1}; Pentair Jung Pumpen U3KS Special), and exits the screens via PVC pipe into the drain. A flow rate through each screen of 25 L $minute^{-1}$ is targeted, which is high enough to encourage screen cleaning but slow enough not to lift spat off the screen. A backup screen is also placed over the PVC pipe outlet to prevent escapes. In both systems, the temperature is ambient and salinity is maintained the same as larval rearing (25 ppt).

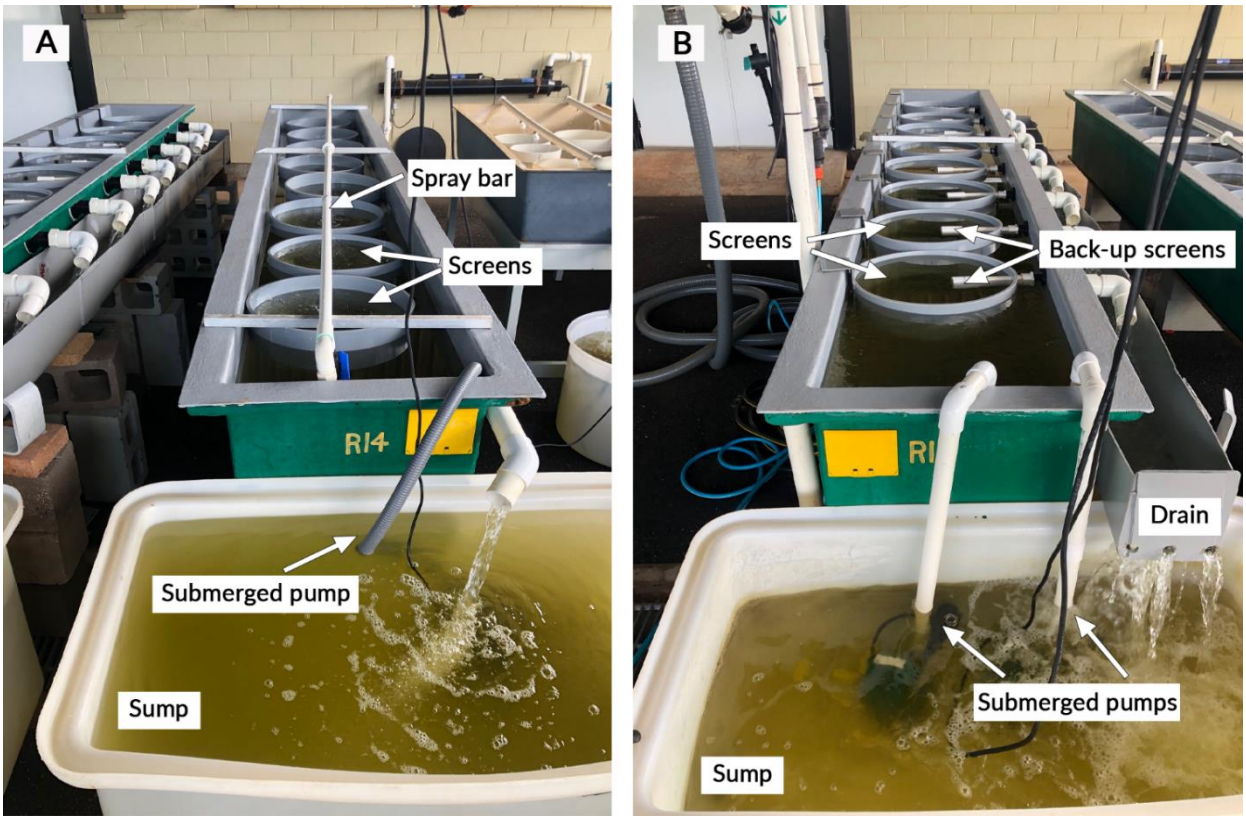

Figure 21. (A) Downweller nursery system and (B) upweller nursery system.

Feeding rates are calculated by tank volume and start at approximately 100,000 cells mL^{-1} day^{-1}. This is increased or decreased depending on the number of spat stocked into the system and according to the rate of algal consumption (e.g., food rates for spat 50 dph are approximately 600,000 cells mL^{-1} day^{-1}).

Grading is conducted using a wet screen method with FSW. Spat are placed onto the desired screen size, rinsed with FSW and sieved gently (Figure 22). The larger spat are retained on the screen, whilst the smaller size grade passes through and is retained on a secondary back-up screen or in the tub. Once in the nursery system grading is carried out weekly and spat are placed on screens specific to their size grade.

Figure 22. Grading Blacklip Rock Oyster spat using the wet screen method.

8. Transportation

When the spat are ≥5 mm they are transferred to ocean farms for grow-out (Figure 23). For transport, spat are removed from the water, rinsed and retained on pieces of cloth (e.g., Chux® cloth or other suitable moisture retaining material). The cloth is then bundled up and the top fixed with an elastic band before being placed on a layer of moist (not wet) cloth in an insulated and plastic-lined foam box (Figure 24). Transportation times are kept to a minimum and always less than 24 h. Once on-site, spat are deployed to the farming system into 3 mm baskets (Hexcly or SEAPA baskets have been used in the NT and both are suitable). Smaller spat can be deployed in 20 mm baskets fitted with a fine mesh sock or into field nursery system. If smaller spat are deployed, they should be closely monitored as fine mesh can easily clog, which will cause mortalities.

Figure 23. Blacklip Rock Oyster spat.

Figure 24. (A) Yagbani Aboriginal Corporation oyster team preparing Blacklip Rock Oyster spat for transport (B) Blacklip Rock Oyster spat on pieces of cloth before being fixed with an elastic band for transport.

8.1. Health assessment

In the Northern Territory an application is required for the movement of fish or aquatic life, under Section 11 of the Fisheries Act, and no activity is permitted until advised of the decision of your application. A subset of the cohort of spat to be deployed/ transported from the hatchery are submitted for a histopathology health check, before deployment. This helps to satisfy that no significant disease processes or histological evidence of reportable diseases are present within the cohort.

9. Photo credits

All photos have been used with the permission of those depicted and were taken by Samantha Nowland, Cameron Hartley, Layla Hadden, Evan Needham and Paul Armstrong.

10. Acknowledgements

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11. Appendix 1

Larval daily data template.

Blacklip Run XXX				
OLD TANKS	**TANK 1**		**TANK 2**	
Day				
Name				
Date				
Current tank temp (°C)				
New tank temp (°C)				
Current tank pH				
New tank tank pH				
Current tank sal (ppt)				
New tank sal (ppt)				
Algae residual AM (k cells)				
Wash tank (temp, pH and sal)				
Larval net size (µm)				
Debris net size (µm)				
Algae added AM (k cells)				
Algae added PM (k cells)				
Algae spp and ratio				
Development stage				
	Counts/100µL		**Counts/100µL**	
Litres				
1				
2				
3				
Average				
Number larvae (x10^6)				
10 larvae measured (tick)				
NEW TANKS	**TANK 1**		**TANK 2**	
Tank A, B, C or D				
Oysters Stocked (x10^6)				
Average larvae DVM				
Min larvae DVM				
Max larvae DVM				
***Vibrio* test (tick)**				
Other comments				

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