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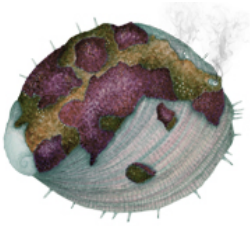
White Abalone Spawning & Culturing Guide



UC DAVIS
BODEGA MARINE LABORATORY

Abalone illustrations above by Carolina Rivera

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Materials list for Bodega Marine Laboratory

1 wild white abalone broodstock (collected in 2000)
15 F1 white abalone broodstock (from 2001 spawning)
?? F2 white abalone broodstock from 2012 & 2013 cohorts
  Will attempt only ripe animals with 2+ GI)
  Might want to select & assess them the day before spawning and keep them in spawning containers in cold room overnight
16 2-gal buckets for spawning F1/wild abalone
16 2-gal bucket liners
30 deli containers for spawning F2 abalone
30 deli container bucket liners for spawning F2 abalone
Spawning protocol
1 L 1M solution of Tris (prepare day before spawning)
  Dissolve 121.1 g Tris (mol. weight = 121.1 g) in 900 mL DI water
  Add DI water to make 1 L solution
1 L 3% hydrogen peroxide solution (prepare on spawning day)
  Add 100 mL 30% H_2O_2 to 900 mL DI water
Extra 100 mL bottle of 30% H_2O_2 (can be older)
Nitrile gloves for mixing chemicals
Spatulas (for removing abalone from tanks)
Metal spatula for examining gonads
Scrub brush for cleaning abs
1-2 balances for weighing abalone (different scales for different cohorts?)
2 sets of calipers (large for F1/wild, small for F2 animals)
Digital camera(s)
6 2-L pitchers
Plunger for egg/embryo mixing
3 25-mL pipette pumps and tips
2 10-mL pipette pump and tips
1 200-µL micropipetteman and blunt tips
2 1000-µL micropipetteman and blunt tips
Jumbo plastic transfer pipettes
3 100-µm mesh sieves (2 2-gal and 1 5-gal bucket size)
50 50-mL Falcon tubes in racks
2 alcohol thermometers
1 digital thermometers
Headlamp(s)
Scalpel blade holder and blades
Permanent markers, pencils, scissors
Label tape
Clip board and Rite-in-the-Rain paper
Compound microscope with phase contrast
1 extension cord
Depression & flat microscope slides
Cover slips
Ice for chilling sperm
Ice bucket for chilling sperm

Materials list for Aquarium of the Pacific

From AoP:
6 F1 white abalone broodstock (from 2001 spawning)
6 buckets for spawning abalone
6 bucket liners
Sieve for rinsing eggs

From BML:
Information folder*
500 mL 1M solution of Tris (prepare before travel)
  Dissolve 60.55 g Tris (mol. weight = 121.1 g) in 400 mL DI water
  Add DI water to make 500 mL solution
500 mL 3% hydrogen peroxide solution (prepare on spawning day)
  Add 50 mL 30% H_2O_2 to 450 mL DI water**
Extra 100 mL bottle of 30% H_2O_2 (can be older)
Nitrile gloves for mixing chemicals
Spatulas (for removing abalone from tanks)
Metal spatula for examining gonads
Scrub brush for cleaning abs
1 balance for weighing abalone
1 set of calipers
Digital camera(s)
4 2-L pitchers
Plunger for egg/embryo mixing
2 25-mL pipette pumps and tips
1 10-mL pipette pump and tips
1 200-µL micropipetteman and blunt tips
1 1000-µL micropipetteman and blunt tips
Jumbo plastic transfer pipettes
50 50-mL Falcon tubes in racks
1 alcohol thermometer
1 digital thermometers
Headlamp
Scalpel blade holder and blades
Permanent markers, pencils, scissors
Label tape
Clip board and Rite-in-the-Rain paper
Compound microscope with phase contrast
1 extension cord
Depression & flat microscope slides
Cover slips
1 125 qt Yeti cooler
9 embryo transport trays with 9 green lids
Ice packs for embryo transport
5 lbs dry ice for keeping ice packs cold
Blankets for insulating embryo transport containers
Duct tape (for attaching digital thermometer during transport)
100 mL concentrated Vortexx, just in case

Materials list for Cabrillo Marine Aquarium

From CMA:
9 F1/F2 white abalone broodstock (from 2003 spawning)
9 buckets for spawning abalone
9 bucket liners
Spatula for removing abalone
Compound microscope

From BML:
Information folder*
2 2-gal buckets
9 bucket liners
500 mL 1M solution of Tris (prepare before travel)
  Dissolve 60.55 g Tris (mol. weight = 121.1 g) in 400 mL DI water
  Add DI water to make 500 mL solution
500 mL 3% hydrogen peroxide solution (prepare on spawning day)
  Add 50 mL 30% H_2O_2 to 450 mL DI water**
Nitrile gloves for mixing chemicals
Metal spatula for examining gonads
Scrub brush for cleaning abs
1 balance for weighing abalone
1 set of calipers
Digital camera(s)
4 2-L pitchers
Plunger for egg/embryo mixing
2 25-mL pipette pumps and tips
1 10-mL pipette pump and tips
1 200-µL micropipetteman and blunt tips
1 1000-µL micropipetteman and blunt tips
Jumbo plastic transfer pipettes
2 100-µm mesh sieves (2-gal bucket size)
50 50-mL Falcon tubes in racks
1 alcohol thermometer
1 digital thermometers
Headlamp
Scalpel blade holder and blades
Permanent markers, pencils, scissors
Label tape
Clip board and Rite-in-the-Rain paper
Depression & flat microscope slides
Cover slips
Small cooler for sperm transport
Ice for chilling sperm (can pick up at AOP)
Small cooler for sperm transport
3 embryo transport trays with 3 green lids
5 embryo transport trays with 8 red lids

* Each information folder will include a contact sheet with phone numbers and email addresses, directions to each facility and the rout between facilities (the “sperm transport route”), a copy of the permit for transporting animals, the spawning protocol, a data sheet for recording information about abalone for this spawning attempt, and extra Rite-in-the-Rain paper.
** Can make 1 L 3% H_2O_2 by adding 100 mL (one bottle) 30% H_2O_2 to 900 mL DI water, then bring half of the solution to each facility. Make sure 100 mL bottle of 30% H_2O_2 is new and kept cool and dark for transport.

Materials list for UCSB

From UCSB:
13 F1 white abalone broodstock (from 2001 spawning)
2 folding tables from REEF
Scott's magic abalone removal spatula
Space in room next to the abs for scopes, ice packs
Ice for chilling sperm
Ice bucket or small cooler for chilling sperm

From BML:
Information folder*
15 2-gal buckets
15 plastic bucket liners
4 5-gal buckets (for storing treated seawater)
500 mL 1M solution of Tris (prepare before travel)
  Dissolve 60.55 g Tris (mol. weight = 121.1 g) in 400 mL DI water
  Add DI water to make 500 mL solution
500 mL 3% hydrogen peroxide solution (prepare on spawning day)
  Add 50 mL 30% H_2O_2 to 450 mL DI water**
Extra 100 mL bottle of 30% H_2O_2 (can be older)
Nitrile gloves for mixing chemicals
Filtration/UV treatment unit
Flat-head screwdriver
2 5-µm filter cartridges
4 0.35 µm filter cartridges
Filter canister wrench
Teflon tape
1 extension cord
1 power strip
Spatulas (for removing abalone from tanks)
Metal spatula for examining gonad
Scrub brush for cleaning abs
1 balance for weighing abalone (to 1,200 g)
2 sets of calipers
Digital camera(s)
6 2-L pitchers
Plunger for egg/embryo mixing
2 25-mL pipette pumps and tips
1 10-mL pipette pump and tips
1 200-µL micropipetteman and blunt tips
1 1000-µL micropipetteman and blunt tips
Jumbo plastic transfer pipettes
2 100-µm mesh sieves (2-gal and 5-gal bucket size)
50 50-mL Falcon tubes in racks
1 alcohol thermometer
2 digital thermometers
Headlamp
Scalpel blade holder and blades
Permanent markers, pencils, scissors
Label tape
Clip board(s) and Rite-in-the-Rain paper
Compound microscope with phase contrast
Depression & flat microscope slides
Cover slips
1 125 qt Yeti cooler
12 embryo transport trays with 12 green lids
9 embryo transport trays with 10 red lids
Ice packs for embryo transport
5 lbs dry ice for keeping ice packs cold
Blankets for insulating embryo transport containers
Duct tape (for attaching digital thermometer during transport)

Materials list for the Sea Center

From TWSC:
4 F1/F2 white abalone broodstock (from 2003 spawning)
UV unit for treating seawater
Classroom (or other space) for spawning

From BML:
Information folder*
6 2-gal buckets
5 plastic bucket liners
2 5-gal buckets (for storing treated seawater)
500 mL 1M solution of Tris (prepare before travel)
  Dissolve 60.55 g Tris (mol. weight = 121.1 g) in 400 mL DI water
  Add DI water to make 500 mL solution
500 mL 3% hydrogen peroxide solution (prepare on spawning day)
  Add 50 mL 30% H_2O_2 to 450 mL DI water**
Nitrile gloves for preparing chemicals
Spatulas (for removing abalone from tanks)
Metal spatula for examining gonads
Scrub brush for cleaning abs
1 balance for weighing abalone (to 200 g)
1 balance for weighing abalone + rock (to 2,500 g)
1 set of calipers
Digital camera(s)
3 2-L pitches
Plunger for egg/embryo mixing
1 25-mL pipette pumps and tips
1 10-mL pipette pump and tips
1 200-µL micropipetteman and blunt tips
1 1000-µL micropipetteman and blunt tips
Jumbo plastic transfer pipettes
1 100-µm mesh sieve (2-gal bucket size)
50 50-mL Falcon tubes in racks
1 alcohol thermometer
1 digital thermometers
Headlamp
Scalpel blade holder and blades
Permanent markers, pencils, scissors
Label tape
Clip board and Rite-in-the-Rain paper
Compound microscope with phase contrast
Depression & flat microscope slides
Cover slips
Ice for chilling sperm
Small cooler for sperm transport
1 Igloo chest cooler
9 embryo transport trays with 10 red lids

* Each information folder will include a contact sheet with phone numbers and email addresses, directions to each facility and the rout between facilities (the "sperm transport route"), a copy of the permit for transporting embryos, the spawning protocol, information about the abalone from previous spawning attempts, a data sheet for recording information about abalone for this spawning attempt, and extra Rite-in-the-Rain paper.
** Can make 1 L 3% H_2O_2 by adding 100 mL (one bottle) 30% H_2O_2 to 900 mL DI water, then bring half of the solution to each facility. Make sure 100 mL bottle of 30% H_2O_2 is new and kept cool and dark for transport.

UC Davis Bodega Marine Laboratory White Abalone Spawning and Culturing Protocol

3 months pre-spawning

- Reserve Climate Control Room with ARG/Karl Menard.
- Arrange spawning dates with partner facilities.
- Confirm availability of members of BML spawning team for each attempt.
- Begin seasoning settlement tiles with *Ulvella* sp. and/or encrusting coralline algae.

4-6 weeks pre-spawning

- Inventory all spawning and larval culturing equipment, chemicals, *Navicula*, etc. Make purchases, as necessary.
- For travel: Reserve vehicle(s).
- For travel: Book hotel room(s).
- Confirm space & equipment needs (see materials list above) and spawning day schedule with BML and partner facilities.
- Confirm availability of someone to turn BML system if spawning occurs at partner facility (e.g., Blythe Marshman).
- Set up Climate Control Room system.
- Make sure WAC Lab settlement troughs are ready to receive larvae.

2 weeks pre-spawning

- Begin assembling (for Climate Control Room) and packing (for travel) equipment.
- Determine if visitors from partner facilities will be participating (e.g., NMFS, SWFSC).
- For travel: Arrange for ARG or other abalone personnel (e.g., Blythe Marshman) to do WAC Lab checks during spawning attempts at partner facilities.

1 week pre-spawning

- Vortexx all spawning and larval culture equipment (allow time for it to dry).
- Clean/sterilize/prepare trough settlement system and install 0.35 µm filter on inflow.
- Finish packing equipment (or preparing it in Climate Control Room for BML attempts).
- Email spawning and culturing protocol and contact list to participants.
- Confirm parties participating.
- For travel: Prepare information packets for each spawning facility and each vehicle. Vehicle information packets should contain copies of the contact list, directions, hotel reservations, white abalone permit, and spawning protocol.
- Order 1-kg *Navicula* sp. from Reed Mariculture to arrive ~2 d prior to predicted settlement. The shipment generally arrives in the afternoon (~4 PM) and can be kept in the refrigerator overnight for inoculation the following morning. Keep in mind that BML Shipping & Receiving is only open Monday through Thursday. If necessary, order can be delivered to Business Services in Bodega Bay on Friday through Sunday.

2 days pre-spawning

- For travel: Pack vehicle(s).

1 day pre-spawning

- For travel: Fill sump in Climate Control Room at a rate of 1 gal/min, if there is time. If sump must be filled more quickly, do not exceed a rate of 3 gal/min. Turn off system once sump is full.
- For travel: Pack ice packs and hydrogen peroxide into vehicle(s).
- For travel: Pick up 5 lbs dry ice for keeping ice packs cold overnight.
- For travel: Drive to spawning location.
- For BML: Arrange/prepare all equipment in WAC Lab, Climate Control Room, and Shellfish Health Lab
- For BML: Consider assessing F2 animals and keeping in treated seawater in individual containers in the Climate Control Room overnight.

Spawning day

5:40-6:00 AM – Arrive at spawning facility. Unpack equipment, and begin spawning protocol.

Spawning protocol

1) Add 3 L treated seawater to each 2-gal bucket.
2) Add 9 mL of 1M Tris solution (3 mL/L, increases the pH of the water) to each bucket, and stir.
3) Wait 15 min.
4) Add 18 mL of 3% hydrogen peroxide solution (6 mL/L) to each bucket.
5) Stir for 10 min.
6) Remove abalone from tanks, scrub with brush and rinse them well in treated seawater to remove debris.
7) Record weight, length, gonad score, shrinkage score, and predicted sex, and take photographs of dorsal side, ventral side, and gonad. Use label tape or other method to ID photos.
8) If spawning at multiple facilities, coordinate with personnel at other facility for timing to begin adding animals to chemicals. Add one abalone to each bucket, labeling each bucket with the tag number of the animal in it.
9) Maintain buckets at ~14°C or ambient seawater temp for 2.5 hrs.*
10) Obtain ice for chilling any sperm that is collected.
11) Add another 9 mL of 3% hydrogen peroxide (3 mL/L) to buckets containing animals not spawning yet.*
12) Wait 1 hr.*
13) Rinse out the chemical solution and replace with treated seawater.
14) Spawning should occur within 1-3 hrs and may continue for many hours.

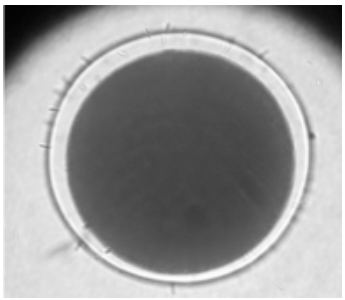
> * If spawning occurs rinse out chemical solution immediately and replace with treated seawater (any eggs/sperm already present will be sacrificed because chemicals damage them). A few mL of water from buckets of spawning animals (or heat-treated sperm) can be added to buckets of non-spawning animals to stimulate spawning.

Gamete Collection:

15) Sperm:
    a. For spawning males, suction highest possible concentrations of sperm from near the respiratory pores and place into Falcon tubes or other sealed containers. Label containers with the tag # of the male and the time of collection (will want to use freshest sperm for fertilization). Immediately place containers on ice.
    b. Check sperm under compound microscope for quality. Sperm motility/quality can be assessed upon dilution of collected sperm (~1/10,000, depending on stock concentration) using darkfield microscopy with phase scope using 10X objective lens.
    c. Consider whether to use some sperm for cryopreservation at CMA. Cryopreservation should occur immediately after collection.

16) Eggs:
    a. Keep eggs cool (8-14°C), especially if holding >15 min.
    b. Check egg shape under compound microscope (should be perfectly spherical).
    c. Rinse/sieve eggs into clean, freshly treated seawater. Be sure not to leave eggs dry on sieve to avoid tearing envelope.

Fertilization:



17) Keeping track of parents:
    a. Eggs: Keep track of female parents to know if eggs are “good” (i.e., fertilize at a high rate) or not.
    b. Sperm: Keeping track of male parentage is also important; however pooling sperm from multiple males could optimize fertilization.
    c. Highest priority should be given to crosses between least related individuals (e.g., between 2001 and 2003 cohort animals, which have different mothers)
18) Getting the correct egg to sperm ratio:
    a. Goal: 5-15 sperm attached to each egg (10 sperm/egg optimal) when looking at embryos under microscope at their widest diameter.

b. For highly concentrated sperm (e.g., collected directly from the respiratory pore upon spawning), place two mL of sperm solution into a 2 L pitcher of seawater with eggs.
c. Gently stir eggs and sperm for 1.5-2 min.
d. Collect a sample of embryos from pitcher to check under the microscope. Focus on the largest diameter of the egg.
    i. <5 sperm/egg at widest diameter: add a little more sperm to bucket and check again.
    ii. >15 sperm/egg at widest diameter: let embryos settle to bottom of bucket, decant water off the top, and add new water to reduce sperm concentrations and reduce the chance of polyspermy.
e. When correct ratio is obtained, let eggs and sperm interact in the bucket for 15-20 minutes minimum. Gently stir the water in pitchers using the pitcher plunger occasionally so that embryos stay suspended and do not clump at the bottom.
f. After 20 or more minutes wash embryos by letting them settle and decanting the water off the top 3 times (or, if decanting not possible, by rinsing *very* carefully through sieve). Check embryos under the scope to make sure sperm is sufficiently rinsed. If not, continue rinsing until sperm:egg ratio is appropriate.

First Cleavage & egg/embryo counts:

19) Watch for first cleavage to occur 1-2 hrs after fertilization. For each cross, assess batches of 15-20 embryos under the microscope to calculate the percent of embryos that are developing and to estimate fertilization rate.
20) Perform counts of eggs/embryos from each cross to estimate total initial number of embryos.

Transport:

21) Pack embryos in embryo transport containers (OXO containers with green lids should be filled first, as they are higher quality than the Sterilite containers with red lids):
    a. Test embryo transport container for leakage by filling with treated seawater and inverting.
    b. Empty embryo transport container.
    c. Add ≤300,000 embryos to each tray and top off with treated seawater.
    d. Label each tray with cross and fertilization time.
22) Pack embryo transport containers in cooler:
    a. Line the bottom of the cooler with a single layer of ice packs.
    b. Cover ice packs with blankets.
    c. Add 9 full embryo transport containers (3 wide and 3 high) to the Yeti cooler. If <9 transport containers have embryos, fill the remaining with treated seawater and put those containing embryos in the center of the stack.
    d. Layer blankets over the top layer of transport containers in a manor that an alcohol thermometer and the probe from a digital thermometer can be inserted in the center and can be accessible during the drive.
    e. Add a layer of ice packs, as needed, over the top layer of transport containers.
    f. Use duct tape to attach the display of the digital thermometer to a place where it can be easily read throughout the drive.

23) Maintain the temperature in the coolers at 11-13°C throughout the drive:
    a. Record the temperature every 30-60 minutes.
    b. If the temperature is increasing or decreasing quickly, or if it nears the extremes of the 11-13°C range, adjust ice packs as necessary.
24) Request that someone at BML (e.g., Blythe Marshman) fill the appropriate number of hatching trays. Flow to the sump in the Climate Control Room system should not exceed 1 gal/min.

Embryos:

25) Counts (optional, depending on how accurate counts were prior to transport – if accurate counts were obtained prior to transport, it's best not to disturb embryos by stirring at this point): With embryos in a known volume of water, stir gently so that all embryos are evenly suspended. Take up to 10 aliquots of enough volume that there are around 8-12 embryos per aliquot. Count the number of embryos in each, and scale up to estimate total numbers.
26) Fertilization estimate: For each cross, assess batches of 15-20 embryos under the microscope to calculate the percent of embryos that are developing and to estimate fertilization rate.

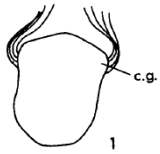
27) Prepare to add 1-2 million embryos in each hatching tray*: According to Hahn 1989, a monolayer of embryos for the hatching trays is about 600-1400/cm2. The bottom area of the hatching trays is about 33 x 55 cm or 1650 cm^2, so **1-2 million embryos per hatching tray** creates a monolayer.
28) Prepare hatching trays: Adjust water level in hatching trays so there is enough room to add the water the embryos are contained in without overflowing. Label each hatching try with the cross, the fertilization time, and the number of embryos going into it.
29) Make sure temperature in transport trays is near 14°C. If not, leave embryos in transport trays until temperature equalizes with the room.
30) Carefully place embryos in hatching trays, pouring them in gently near the bottom of the tray and rinsing transport container and lid to detach any sticking embryos.
31) If hatching tray is not full after adding embryos, top off water level to just below the bulkhead.
32) Keep embryos in stagnant water overnight.
33) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, and the room temperature is 14°C.

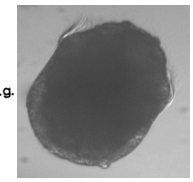
*__Alternatively to hatching trays__ place embryos directly into larval buckets (this is what Doug Bush does at The Cultured Abalone). About 400,000 eggs/embryos will fit as a monolayer on the bottom. Place inflow on top of bucket so that it flows down through the mesh and out the external standpipe overnight.

1 day post-spawning: hatching day

Larvae:

1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, and the room temperature is 14°C.
2) Larvae will begin to hatch 24 hours after fertilization, probably in the late morning or early afternoon.
3) Fill larval buckets and barrel (as needed) with filtered, UV treated seawater.** Be careful to avoid air bubbles getting caught between the bottom of the buckets and the mesh. In the new larval buckets (with white bottoms), you can use the plug at the bottom to help release air under the mesh. Take care not to stretch the fragile mesh with high-pressured water or by pressing it with your hand or other hard object.
4) Appropriately assign a larval bucket or the larval barrel to each hatching tray. Most sources say 5-20 larvae/mL is optimal, but it is probably best not to exceed 10 larvae/mL.
    a. **Buckets:** Gray-bottomed (~16.5 L) and white-bottomed buckets (~14.5 L) each hold an absolute maximum of 300,000 larvae, and an optimum of **150,000 larvae**.
    b. **Barrel:** The larval barrels (~ 171 L) hold a maximum of 3,000,000 larvae and an optimum of **1,500,000 larvae**.
5) Label each bucket or barrel with the crosses going into them.
6) *Very slowly* turn on flow to hatching trays, making sure not to disturb the embryos on the bottom of the tray. Water should flow from the hatching tray down into the larval bucket or barrel below.
7) Once water is flowing from the hatching trays to the larval buckets or barrel, label the water level in each hatching tray. It will be important to regularly check that the water level in the hatching trays does increase because it could be an indication that the flow to the buckets or barrel has been restricted.
8) Check larvae from each cross/container under the compound scope. The ciliated girdle ("c.g." in sketch from Leighton 1974) should be visible. Photograph larvae.
    a. Save photographs in Chinook > Research > Cherr Lab > White Abalone > Photos > "2015_*mm_dd Facility*" ("*mm*" & "*dd*" correspond to the month and day of spawning, and "*Facility*" corresponds to the location(s) of the spawning attempt.
    b. Label photos "2015_*mm_dd_hhmm* F###xM###" ("*hhmm*" corresponds to the hour and minute the photos were taken and "F###xM###" refers to the tag numbers of the mother ["F"] and father ["M"], see photos in folders from previous spawnings for other examples).





9) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, that flows to hatching trays are

** If embryos are already in larval bucket(s), connect sister bucket(s), move the inflow tube to the external standpipe so water flows up through the mesh and over to sister bucket, transporting hatched larvae with it.

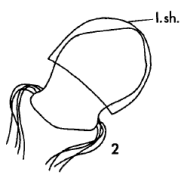
appropriate, and that the larval buckets and barrels are set up appropriately. Also check that the water level in the hatching trays is appropriate and does not exceed the label on the tray.

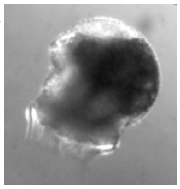
2 days post-spawning

1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, that flows to hatching trays are appropriate, and that the larval buckets and barrels are set up appropriately.
2) Check hatching trays for hatched larvae that have not made it down to the larval buckets or barrel. If many healthy larvae remain swimming normally in the hatching try, carefully transport them to their corresponding buckets or barrel using a blunt-tipped pipette.
3) Once all healthy larvae are in the larval buckets and barrel, disconnect inflow from the hatching trays, and stick it down the external standpipe of the bucket(s) or sink it to the bottom of the larval barrel. Keep the flow low to avoid the impingement of larvae on the mesh bottom of the sister bucket during transfers (or banjo filters of the barrel). If not all larvae have transferred from the hatching tray(s) to the bucket(s) or barrel, transfer healthy larvae by hand using a transfer pipette.
4) Larval transfer:
    a. Larval buckets, transfer from “old” to “new” bucket:
        i. Attach the clean, empty sister bucket.
        ii. Fill the new sister bucket with seawater taking care not to create any bubbles in between the bottom of the buckets and the mesh. Fill to just below the fitting between the bucket pair, adjusting the external standpipe as necessary.
        iii. Adjust the standpipe in the old sister bucket containing larvae so that the water level rises above the fitting conjoining the bucket pair. You should be able to see water and larvae flowing from the old bucket with larvae to the new, clean sister bucket without larvae.
        iv. Let the transfer run for ~24 hours.
        v. Once the transfer is complete, begin to slowly lower the water level in the old bucket, which should no longer contain many larvae.
        vi. In the bucket that now has larvae, slightly lower the water level so that the water line is enough below the attachment to the sister bucket that slight sloshing of the bucket will not result in larvae spilling out. As you lower the level, larvae are likely to become suck on the dry sides of the bucket. Use the inflow hose to rinse them down into the water, and attach the inflow hose using a PVC clip to the top of bucket with larvae (outflow from this bucket should continue out the external standpipe while cleaning the sister bucket in the following steps).
        vii. Continue to lower the water level in the old bucket that no longer has larvae to about 1-2 inches above the mesh.
        viii. Detach the old bucket.
        ix. Look at larvae remaining in the bucket under the microscope. These larvae are likely dead or unhealthy and may contain ciliates. Unless these larvae look particularly healthy, discard larvae in the old bucket down the freshwater drain. (If they do look healthy, put them in a labeled, 2-gal bucket and perform manual water changes daily until settlement.)
        x. Carefully rinse the old bucket with hot freshwater and DI water. Be sure not to use too high pressure on the mesh so that it doesn’t stretch out.
        xi. Begin a new transfer (i.e., repeat, beginning with step “a.i.” above).
    b. Larval barrel, transfer from “old” to “new” barrel:
        i. Fill the clean barrel with seawater to just below the level of the bulkhead.

ii. On the effluent of the old barrel, remove the 2” threaded pipe nipple with the 90° fitting and hose, and attach it to the effluent bulkhead of the new barrel.
iii. Place the threaded cap over the effluent of the old barrel.
iv. Carefully remove the banjo filter assemblage from the effluent of the old barrel and transfer it to the new barrel. PLEASE NOTE that the some of the attachments on the banjo filters are soft plumbed and can come apart! If there are larvae attached to the banjo filters, make sure to gently swish the filters around in the new barrel.
v. Remove the 1” 90° fitting ad hose from the inside of the old barrel, and transfer it to the inside of the new barrel. (This attachment might change, or we might not use it at all – we are still investigating the best way for larvae to travel gently from one barrel to the other.)
vi. Let transfer run for ~24 hours.
vii. Once the transfer is complete, slowly slower the water level in the old barrel by getting creative with a siphon and a sieve.
viii. Rinse old bucket with freshwater.
ix. Begin new transfer (i.e., repeat, beginning with step “b.i.” above).

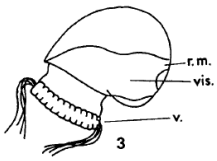
5) Check larvae from each cross/container under the compound scope. The larvae should be developing their shells (“l.sh.” in sketch from Leighton 1974). Photograph larvae.
6) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.

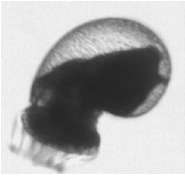




3 days post-spawning

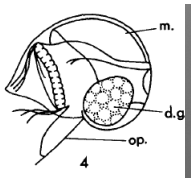
1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.
2) Perform larval transfers (see step 4, 2 days post-spawning).
3) Check larvae from each cross/container under the compound scope. Larval shells should be growing and yolk should be shrinking. In the sketch, “v.” is velum, “vis.” is viscera, and “r.m.” is retractor muscle (Leighton 1974). Photograph larvae.
4) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.

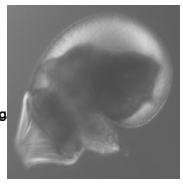




4 days post-spawning

1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.
2) Perform larval transfers (see step 4, 2 days post-spawning).
3) Confirm readiness of WAC Lab settlement troughs and make adjustments as necessary.
4) Check larvae from each cross/container under the compound scope. You should be able to see the operculum developing. In the sketch, “op.” is operculum, “d.g.” is digestive gland, and “m.” is mantle (Leighton 1974). Photograph larvae.
5) Perform larval counts:
    a. Larval buckets: Set bucket to a known volume (measure height of water above mesh and calculate volume). If larvae are dense in a bucket, you can probably just sample directly out of the bucket with it full. If larvae are sparse, you might need to lower the height of the water to condense the larvae for a more accurate count. Perform counts by distributing larvae in the bucket evenly using the bucket plunger and taking ten aliquots of ~10-µL to 1-mL in volume





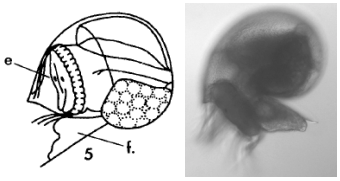
from the bucket using a blunt pipette tip. Make sure the larvae in the buckets are evenly mixed prior to taking an aliquot. Ideally, you want about 10 larvae/aliquot. Average the larvae you get per aliquot, and scale up to determine the number of larvae in the bucket.

b. Larval barrel: You will probably need to subsample the larvae in the barrel. Evenly distribute larvae in the barrel using the barrel plunger, use a 2 L pitcher to take a sample of larvae in the barrel. In the pitcher, distribute the larvae evenly using the pitcher plunger (embryo mixer). If larvae are not very dense in the barrel, you might need to take an aliquot greater than 1 mL to achieve ~10 larvae/aliquot.

6) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.

5 days post-spawning

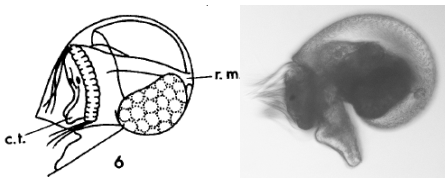
1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.
2) Perform larval transfers (see step 4, 2 days post-spawning).
3) Check larvae from each cross/container under the compound scope. Eyespots should be starting to become visible. In the sketch, “f.” is foot and “e.” is eyespot (Leighton 1947). Photograph larvae.



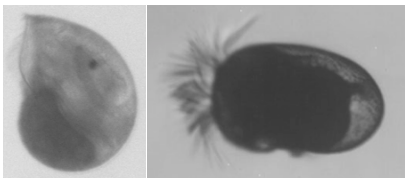
4) Check algal growth and readiness of WAC Lab settlement troughs. Adjust lighting as needed.
5) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.

6 days post-spawning

1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.
2) Perform larval transfers (see step 4, 2 days post-spawning).
3) Check larvae from each cross/container under the compound scope. Eyespots should become more visible. The buds of the cephalic tentacles may be just barely showing. In the sketch, “c.t.” is cephalic tentacle and “r.m.” is retractor muscle. Photograph larvae.



4) Inoculate WAC Lab troughs with *Navicula* when the shipment arrives. The number of troughs inoculated will depend on settlement space needs – see settlement instructions on “8 days post-spawning” to estimate surface area requirements. See “White Abalone Feeding Protocol” for details on trough inoculation with *Navicula*). Consider starting a *Navicula* culture in the Greenhouse on extra tiles or other surfaces.
5) Consider whether to start settlement competency tests. Under the compound scope, look for well-developed cephalic tentacles (see sketch and photo for tomorrow, “7 days post-spawning”), animals that are not swimming (see photo to the right of larva pulled into its shell), and possibly crawling on the bottom of the microscope slide to test for settlement (see photo on far right). Larvae will begin to spend more time near the bottom of the larval buckets and barrel rather than swimming near the surface when they are ready for settlement. Competency tests can be conducted as follows:

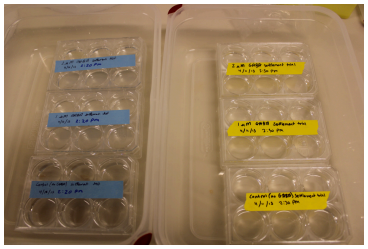


a. **Make 1 L of 1000 µM GABA solution:** Find newest GABA on general chemical shelf A in Room 305. Mix 0.103 g GABA (mol. weight = 103.12 g) with 1 L treated seawater.
b. **Make 1 L of 10 µM GABA solution:** Add 10 mL 1000 µM GABA to 990

mL treated sweater.

c. Dedicate a 6-well plate to each of three settlement competency treatments: No GABA, 1 μM GABA, and 2 μM GABA – and dedicate at least two wells per plate for each cross or larval container. If there are more than 3 crosses or larval containers, you will need more than one plate per treatment. A hypothetical plate set up for one of the three settlement treatments might look like this:

Well 1: Bucket 1 larvae	Well 2: Bucket 2 larvae	Well 3: Bucket 3 larvae
Well 4: Bucket 1 larvae	Well 5: Bucket 2 larvae	Well 6: Bucket 3 larvae



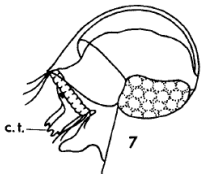
d. Prepare settlement competency trails as follows:
   i. No GABA treatment: In a 6-well plate, add ~10-15 larvae/well from the appropriate bucket, using as little water as possible. Make sure larvae in each bucket or barrel are well-mixed prior to collection so that they animals both having a tendency to swim near the surface and the bottom are tested. Add 5 mL filtered, UV treated seawater to each well.
   ii. 1 μM GABA treatment: In a 6-well plate, add ~10-15 larvae/well from the appropriate bucket, using as little water as possible. Make sure larvae in each bucket or barrel are well-mixed prior to collection so that they animals both having a tendency to swim near the surface and the bottom are tested. Add 4.5 mL filtered, UV treated seawater to each well. Add 0.5 mL 10 μM GABA to each well, and mix gently.
   iii. 2 μM GABA treatment: In a 6-well plate, add ~10-15 larvae/well from the appropriate bucket, using as little water as possible. Make sure larvae in each bucket or barrel are well-mixed prior to collection so that they animals both having a tendency to swim near the surface and the bottom are tested. Add 4 mL filtered, UV treated seawater to each well. Add 1 mL 10 μM GABA to each well, and mix gently.

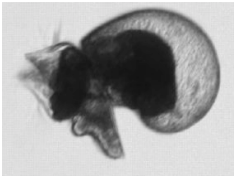
e. Cover each plate, and label the plate covers with the date, time, GABA treatment, and larval crosses. Three plates fit nicely into one embryo transport tray. Leave in Climate Control Room overnight.

6) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.

7 days post-spawning

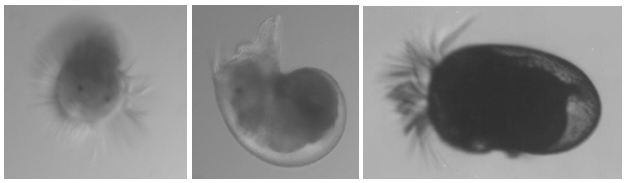
1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.
2) Perform larval transfers (see step 4, 2 days post-spawning). As larvae develop a greater number of healthy larvae may stay toward the bottom of the larval buckets and not make the transfer. Before discarding the "dregs" at the bottom of the old larval barrels, check these larvae under the scope. If they look healthy, consider saving them in an extra 2-gal bucket. If they have ciliates or look otherwise unhealthy, discard them in the freshwater drain.
3) Check larvae from each cross/container under the compound scope. You should be able to see the cephalic tentacles ("c.t." in the sketch from Leighton 1974). Photograph larvae.





4) If settlement competency tests were started yesterday, assess the results 24 hours after they began. Assess tests as follows:
   a. There are data sheets for analyzing settlement competency trials on Chinook > Research > Cherr Lab > White Abalone > Abalone Spawning > "GABA settlement competency trial data sheet.xlsx". The spreadsheet can be amended for the number of crosses in the culture, and a few copies can be printed so they are ready for subsequent trials.
   b. On the data sheet, record the date and time the test started, the date and time sampled, and the hours elapsed in between.

c. For each well, record the number of larvae that are swimming, on their side, crawling, or dead (no shell, empty shell, otherwise not living). See examples below:



Swimming *On Side* *Crawling*

d. Calculate the percentage of living larvae that are crawling in each well.
e. Calculate the average percent of living larvae that are crawling in each treatment.
f. After settlement competency test is assessed, perform water changes in each well. *Change out water in this manor for all settlement competency tests daily until larvae are transferred to settlement areas.*
   i. Decant water out of each well using a transfer pipette, making sure not to suck up any larvae.
   ii. Add new treated sweater to each well.

5) Based on the results of the settlement competency test, assess whether to settle larvae soon, or to set up another settlement competency test to run of the next 24 hours. Follow step 5 under "6 days post-spawning" to set up another settlement competency test.
6) Check diatom growth in WAC Lab settlement areas. Adjust lighting as needed.
7) At the end of the day, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.

8 days post-spawning

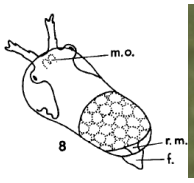
1) First thing in the morning, check that the UV unit is on, the inflow to the system is about 1 gal/min, the seawater temperature is 14°C, the room temperature is 14°C, and flows to larval bucket(s) and barrel are appropriate.
2) Check larvae from each cross/container under the compound scope. Reference photos and sketch from yesterday to look for developmental characteristics. Photograph larvae.
3) If you have decided not to settle larvae today, perform larval transfers (see step 4, 2 days post-spawning), follow procedures 3-7 from yesterday, and omit settlement steps below. If you have decided to settle larvae today, do NOT perform larval transfers, and proceed as follows.
4) Perform larval counts (see step 4, 2 days post-spawning).
5) Determine the number of settlement surfaces required, which settlement surfaces will be used, and how many larvae will be added to each settlement surface. Any questionable or unhealthy larvae should be placed in troughs at the bottom of the rack because they are they last to receive seawater. A **2 larvae/cm^2** settlement density is recommended; however, this can be increased slightly, depending on space needs.
   a. Trough settlement area:
      i. Each trough without tiles has ~7,000 cm^2 surface area.
      ii. Each trough has 18 15x15 cm half-tiles, or another ~4,000 cm^2 surface area.
      iii. Each trough has a total surface area of **11,000 cm^2**, or space for **22,000 larvae**.
      iv. In one entire trough rack (12 troughs), there is room for ~**300,000 larvae**.
   b. **Make 1000 µM GABA solution.**
      i. Find newest GABA on general chemical shelf A in Room 305. Mix 0.103 g GABA (mol. weight = 103.12 g) with 1 L treated seawater. Repeat, as necessary to create enough GABA for settlement.
      ii. When filled to the top of the standpipe, **each trough holds ~30 L seawater**. To create 1 µM GABA solution in each trough, prepare **30 mL of 1000 µM GABA solution** for each trough (****These measurements should be confirmed for new trough rack****).
   c. Settlement in troughs:
      i. Drain troughs, siphon out debris, and prepare settlement tiles.
      ii. Add 0.35 µm filter cartridge(s) to appropriate inflowing seawater line(s).

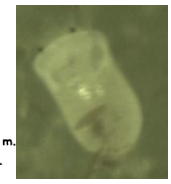
iii. Fill seawater troughs with treated seawater, and then siphon enough off the top that there is plenty of space left to add the larvae in the volume of water they are suspended in without the trough water level exceeding the level of the standpipe. By lowering water level 2 cm below the standpipe, there is about 6-8 L of space remaining for adding larvae (the top 1 cm holds about 4 L, and each lower cm holds a little less).
iv. **Add 30 mL of 1000 µM GABA to each trough**. Can us bubble air through to stir.
v. Slowly adjust larvae from Climate Control Room temperature to the temperature in WAC Lab troughs.
vi. Gently pour ≤ **22,000** larvae into each trough, making sure that water level does not exceed that of standpipe.
    1. If transferring larvae from a larval bucket:
        a. Lower water level in bucket so that it is just under half-way full.
        b. Use a 2-L pitcher to transport larvae from the larval bucket into a 5-gal bucket until the larval bucket is empty enough to lift and pour into the 5-gal bucket.
        c. Using a 2-L pitcher, transfer larvae from the 5-gal bucket into the troughs.
        d. Using 0.35-µm filtered, treated sweater, carefully rinse inside and outside of all pitchers/buckets to make sure no larvae are left behind.
    2. If transferring larvae from the larval barrel:
        a. Carefully siphon water out of the barrel using a sieve as a barrier between the larvae and the siphon hose or slowly drain water out the bottom of the barrel and catch larvae in a wet sieve below.
        b. Figure out how to consolidate larvae into a 5-gal bucket (or some other way to consolidate them).
        c. Using a 2-L pitcher, transfer larvae to troughs.
        d. Using 0.35-µm filtered, treated sweater, carefully rinse any surfaces the larvae have touched to make sure none are left behind.
vii. Top off seawater level to just below level of standpipe.
viii. Label each trough with the cross present in it.
ix. Let sit stagnant for 6-24 hours.
x. Once larvae are settled, turn flow on very low for 24 hours. **Leave air off.**
xi. Turn up flow slightly 2 d post-settlement.

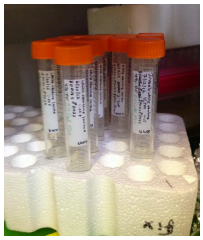
6) General WAC Lab checks should cover afternoon checks of the trough system. Check and record temperature in the troughs. Make sure that the air and water flow in the troughs is off.

1-d post-settlement

1) General WAC Lab checks should cover morning checks of the the trough system. Check and record temperature in the troughs. Make sure that the air and water flow in the troughs is off.
1) Test for settlement by gently waiving a credit card over settlers to see if they are attached and/or looking at tiles under the stereo (dissecting) microscope.
2) If animals have settled, turn water flow on very low 6-24 hours post-settlement. **Leave air off.**
3) Check animal development and settlement success on sample tile(s).
    a. Fill an embryo transport tray with treated seawater and place tile in tray.
    b. Examine and photograph under stereo (dissecting) microscope. In the sketch, “f.” = foot, “r.m.” = retractor muscle, and “m.o.” = mouth (Leighton 1974)
    c. Estimate settlement success if you can (may be very difficult since larvae prefer sides of trough to tiles) by counting the number of live/settled vs. dead/unsettled animals.



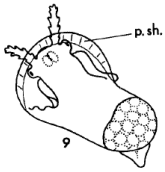


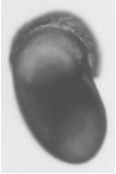


4) If possible and desired, fix 20 new settlers in 4% paraformaldehyde.
    a. Collect 20 new settlers and put in a 15 mL Falcon tube with 3 mL treated seawater.
    b. Label Falcon tube with “White Abalone, date, time, cross, and 4% PF”.
    c. Add 1 mL 16% paraformaldehyde (in fume hood in Room 314).

   d. Put samples in Cherr Lab chemical refrigerator directly behind Rom 314 to the east (use gloves when in this refrigerator!).
   e. In 24 hours, aspirate off solution leaving animals at the bottom, and add 4 mL 0.44 µm filtered seawater from Milli-Q in South Wing Wet Lab. Aspirate solution off again, and add again add 4 mL 0.44 µm filtered seawater. Change label on tube to reflect new solution: cross out 4% PF and add 0.44 µm FSW. Return sample to refrigerator.
5) Check diatom growth in WAC Lab settlement areas. Adjust lighting as needed. Consider whether it might be necessary to order more *Navicula* from Reed Mariculture for post-settlement seeding.
6) Think about starting to clean up Climate Control Room. Some priorities:
   a. Make sure not to leave any plastic or mesh to dry with seawater. Freshwater rinse hatching trays, larval buckets, larval barrels, banjo filters and sieve.
   b. Remove filter cartridges and leave filter canisters disassembled.
   c. Empty sump.
7) Now that animals are all in the WAC Lab, system checks will be part of daily WAC Lab checks.
8) Pat yourself on the back!

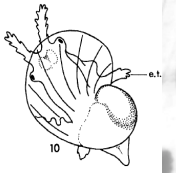
First week post-settlement

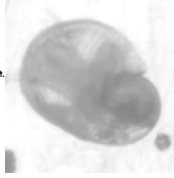




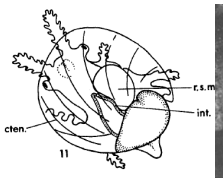
- Daily: Check animal development and settlement success on sample tiles.
  - Fill an embryo transport tray with treated seawater and place tile in tray.
  - Examine and photograph under stereo (dissecting) microscope. In the sketch, “p.sh.” = peristomial shell (Leighton 1974).
  - Estimate settlement success if you can (may be very difficult since larvae prefer sides of trough to tiles) by counting the number of live/settled vs. dead/unsettled animals.
- Daily: Assess diatom cover and change lighting as necessary. Consider whether to supplement with Shellfish Diet, add extra *Navicula* from Greenhouse culture, or order more *Navicula* from Reed Mariculture.
- Every two days: If possible and desired, preserve a sample of 20 new settlers for preservation in paraformaldehyde.
  - Collect 20 new settlers and put in a 15 mL Falcon tube with 3 mL treated seawater.
  - Label Falcon tube with “White Abalone, date, time, cross, and 4% PF”.
  - Add 1 mL 16% paraformaldehyde (in fume hood in Room 314).
  - Put samples in Cherr Lab chemical refrigerator directly behind Rom 314 to the east (use gloves when in this refrigerator!).
  - In 24 hours, aspirate off solution leaving animals at the bottom, and add 4 mL 0.44 µm filtered seawater from Milli-Q in South Wing Wet Lab. Aspirate solution off again, and add again add 4 mL 0.44 µm filtered seawater. Change label on tube to reflect new solution: cross out 4% PF and add 0.44 µm FSW. Return sample to refrigerator.
- **2-3 days post-settlement, turn air on in troughs.**

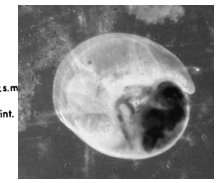
First 3 mo. post-settlement





- Weekly: Check animal development and settlement success on sample tiles.
  - Fill an embryo transport tray with treated seawater and place tile in tray.
  - Examine and photograph under stereo (dissecting) microscope. In the sketch, “e.t. = epipodial tentacle, “cten.” = ctenidium, “int.” = intestine, and “r.s.m.” = right shell muscle (Leighton 1974).
  - Estimate settlement success if you can by counting the number of live/settled vs. dead/unsettled animals.
- Weekly: Assess diatom cover and change lighting as necessary. Consider supplementation as necessary.



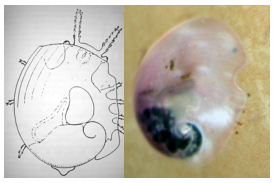


3 mo. post settlement

- Rinse and siphon out troughs to get rid of feces, dead animals, etc.
- Perform a thorough survey of the population to get an accurate abundance estimate.
- Remove 0.35 µm filtertation.

3 – 5 mo. post-settlement

- Bi-weekly: Check animal development and settlement success on sample tiles.
  - Fill an embryo transport tray with treated seawater and place tile in tray.
  - Examine and photograph under stereo (dissecting) microscope. The first respiratory pore should begin to form around 3 months, at which point the abalone become juveniles. The sketch from Leighton 2000 shows the notch of the first respiratory pore forming just to the right of the head.



5 mo. post-settlement

- Begin feeding juveniles dulse.
- Continue to supply diatoms as necessary.
- Perform count of all animals.
- Consider when to move juveniles to the Grow-Out and Experimental Area of the WAC Lab. When they are moved, photograph and take vitals (length and weight) of each animal. Note any shrinkage or gonad development.