Spawning Protocol for *Mytilus galloprovincialis*

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Spawning takes approximately 2-3 hours if the mussels are gravid. We have outlined seven steps to ensure successful spawning:

1. COLLECTING GRAVID ADULTS
2. SELECTING INDIVIDUALS FOR SPAWNING
3. CLEANING MUSSELS
4. APPLYING TEMPERATURE SHOCK
5. CREATING WATER MOVEMENT
6. SPAWNING
7. GAMETE COLLECTION AND QUALITY CHECK

During this activity, there are 3 things to always remember:

1. *ALWAYS CHANGE OR WASH WITH VERY HOT WATER ANYTHING THAT TOUCHED OR HAS BEEN NEAR SPERM, MOST OF ALL HANDS!*
2. *KEEP SPAWNING MALES SEPARATE FROM FEMALES*
3. *CHECK UNFERTILIZED EGGS TO MAKE SURE THERE HASN'T BEEN SPERM CONTAMINATION*

1. COLLECTING GRAVID ADULTS

Mussels should be collected during their gravid season (e.g. October for Sète population, February for Villefranche populations). Gravid mussels have full gonads (Fig. 1): ovaries are orange/pink, testis are white/yellow but colors can vary among individuals. Mussels should be cut open to assess the state of gonads. In our experience, if gonads are full (i.e., ripe) the mussel will likely spawn with induction. Gravid adults can be kept at constant temperature (15 ˚C) in flow through seawater tanks and fed Shellfish Diet to maintain gravid adults for weeks to months.

Materials:

- mussels
- pasteur pipettes
- seawater
- scalpel
- slides
- microscope

Method:

1. Cut mussel open
2. Rinse inside of mussel with some seawater to get rid of feces or other material
3. Cut into gonads
4. With a Pasteur pipette suck some of the liquid coming out of the gonad
5. Check under the microscope (might need to dilute the droplet with seawater)

- *Males:* sperm is activated by seawater (few minutes), good males have active sperm
- *Females:* you'll see eggs (60-70 µm in diameter. Fig. 2). Unfertilized eggs can be very misshapen and not perfectly round. Irregular eggs still fertilize.

Fig. 1: Example of a gravid female cut open during fieldwork at the marine station in Sète

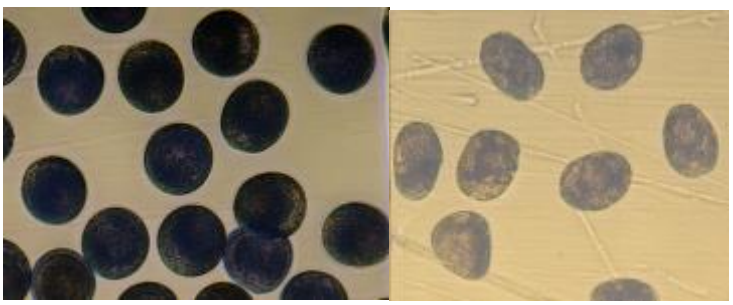

Fig. 2: Example of round and irregular mature Mytilus galloprovincialis *eggs*

2. SELECTING INDIVIDUALS

Since *M. galloprovincialis* does not have sexual dimorphism, randomly choose mussels for spawning = at least twice the number of mussels needed for the experiment usually =

3. CLEANING MUSSELS

Mussels need to be cleaned prior to spawning:

1. This ensures clean collection of gametes
2. Cleaning provides mechanic stimulation necessary for spawning

Materials:

- ample amount of filtered seawater for rinsing mussels
- scissors
- brush
- screwdriver (for scraping off epibionts)
- gloves (optional)

Method:

1. Take a mussel
2. Cut off byssal threads
3. Brush and clean the shell for at least 1 minute
4. Wash the mussel in clean seawater
5. Move the mussel to a small container with filtered seawater (Fig. 3)

*each mussel should be in its own individual container to avoid cross-fertilization among males and females.

Fig. 3: Example of a mussel after cleaning (spawning female)

4. APPLYING TEMPERATURE SHOCK*

After cleaning, move mussels into a container with filtered seawater that is ~10°C warmer than their holding temperature. We do this by putting mussels in small containers (Fig. 3) in a larger tray to which we can add warm water. Check temperature inside the small containers with the mussels every now and then to make sure it's not too hot. The maximum temperature we use is 28 °C.

5. CREATING WATER MOVEMENT

In addition to temperature shock, it is important to gently move the containers with mussels so the water inside will shake them and stimulate spawning. Keep on moving mussels every now and then, even when once they start spawning. Gently scratching their shell with the tip of a pipette also seems to help induce spawning.

6. SPAWNING

Before the actual spawning there will be a pre-spawning activity that consists in the release of some fecal material, froth, and/or small amounts of gametes. Once the mantle is extruded (Fig. 4), the mussels is close to/actively spawning. Once spawning, males and females are easy to recognize: males spawn a white cloud that dissolves in the volume of water, females spawn pink/orange masses that are groups of hundreds of eggs stuck together.

Fig. 4: Female mussel spawning with mantle tissue visible

7. GAMETE COLLECTION AND QUALITY CHECK

Spawning can last 3 hours, so we try to keep gametes at cool temperatures.

1. Eggs can be transferred into a beaker with seawater at maintained at ~15°C
2. Sperm spawned into seawater can be transferred into a tube and put on ice until fertilization

*We have not tested how long gametes last, but fertilization has never failed if performed on the same day as spawning.

*It's a good idea to check gamete quality under the microscope. If sperm contamination occurred, fertilized eggs will have polar bodies or already have undergone division (Fig. 5). Again, good sperm is very active.

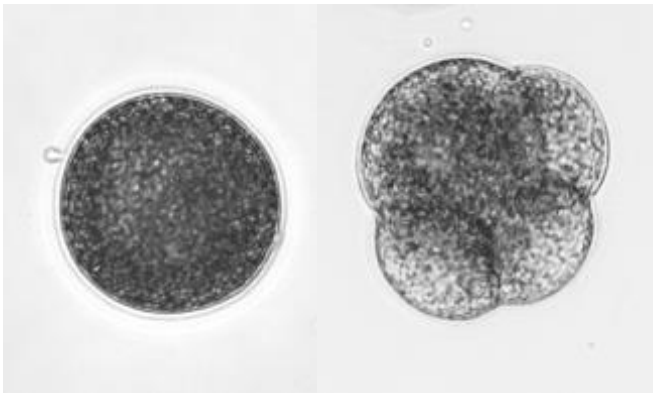

Fig. 5: Fertilized egg showing polar body and a divided embryo