Chapter 4 Nurseries and Cultures

4.0 Fish Nurseries

Successfully developing a fertilized egg to adulthood is of up most importance to the fish facility. From the first days of fertilization to day 20+ are fragile times for young zebra fish. Optimal nutrition, water quality, food and fish densities are critical for success. Refined techniques over the years have lead to 90% survival rate (85% for mutants, 75% transgenic) and continued vigilance is required to maintain it.

Nurseries are held at 28.0 °C this temperature is important because development may not occur properly at other temperatures. The fish are fed 2-3 times a day a diet of paramecium, dry "shaker" food until they are 10 days old at that time they start getting fed artemia nauplii in addition to their other feeds. Each nursery tank has its own water line and valve. Water is dripped in to the nursery tanks at approximately 1-drip/2 seconds. The water supply to the tanks is shutoff via manifolds during the morning feeding and turned back on during the late afternoon feeding. This traps the young fish with their live food.

4. 1 Adding Fish To The Nursery

There is two main nurseries, one in H221 the other in J-083b. The H-lab nursery can hold 36 1.0-L tanks and the J-lab nursery 32. There is a running total of the number of tanks in use on the whiteboard in H225. Once your fish have reached 4 days its time to put them on the nursery. Even if the fish are still in their yolk-sac it's important to adapt them to visual stimuli of motion provided by the nurseries live feed. Find an open nursery space in H or J-laboratories Fill out the nursery production sheet on the clipboard in front of the nursery. It asks for the date to start feeding, family name (genetic id), the approximate number of fish, the number of tanks used, the researchers initials and a question that asks if there is more than one tank can the tanks be combined upon removal from the nursery system usually around day 20 post-fertilization. This question only applies if there is more than one tank and is necessary due to space constraints.

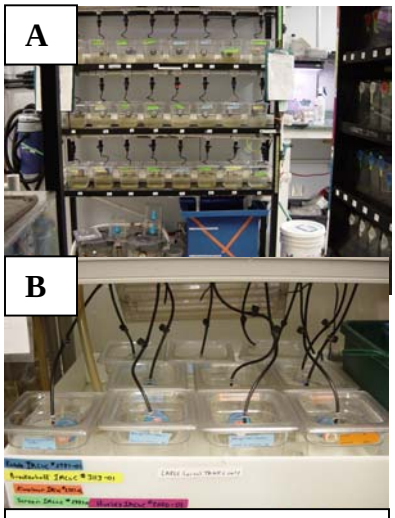

Figure 4.0 A. Nursery system in J-083b. B. Close up of the nursery system in H221

Obtain a clean nursery tank. Make sure a 150 um drain screen is installed in the back of the tank. The screen needs to be examined to make it is not damage, if it is damaged throw it away in the garbage and get a new one out of the nursery screen beaker located on the nursery shelf. Put enough system water in the tank to cover the bottom of it. Take the lid of your petridish and lower it a slight angle down in to the tank. Slowly pour the contents of the dish in to the tank. Get a squirt bottle of system water and gentle rinse out the remaining contents out of the dish in to the tank. Place the label from the petridish on

to the tank and put a clean lid on the tank. Dispose of the petridish in the garbage. Place the tank into a space with a water line. Place the line through the hole in the center of the tank lid. If you're placing a tank on the nursery during the day do not try to turn the water on to the tank. The water is shutoff at the water manifold. If you're placing the tank on the system in the evening or night set the water line valve to 1 drop/2seconds. A special note on H221 nursery use, make sure when connecting, or moving tanks that you do not move any of the water lines out of the drip tray. This may lead to flooding and if the system runs too low on water in can burn up the pump or even worse lead to gas bubble disease in the nursery fish. Most fish will remain on the nursery for 20 days or until their bellies are swollen orange with artemia and they have some of their adult pigmentation (stripes).

4.2 Juvenile Fish

When the time is appropriate the fish facility staff will move the fish off the nursery to a near by zebra fish modular system. Part or all of these systems are used to grow out juvenile fish 20-90 days old until they are big enough to move to adult fish tanks. Fish in these tanks get feed 00 nutra plus (small fish food) and extra artemia, but are no longer fed paramecium. From here individual researchers are responsible for moving their fish from this system to adult tanks. It is crucial to move these fish in a timely fashion or it will cause backups in the nurseries (nowhere to move the fish).

Figure 4.1 Zebra fish modular system used for juvenile fish rearing

4.3 Shaker Food

Is comprised of 1-part Argent artificial plankton, 1-part spirulina powder, and 1-part O.S.I artificial rotifers. Food is dispensed from a 50 ml Falcon tube with 3 holes in the cap. Food is shaken from the tube, one shake per nursery tank. The food is used not only for its nutrients but also to adapt them to eating non-living fed. Food is stored in the refrigerators in H and J-laboratories. Make sure each shaker container has an expiration date on it.

4.4 Artemia Culture

Artemia franciscana has 15 life stages but it is the first one nauplii (instar 1) that is important to the fish. These crustaceans are full of protein, and omega III acid. They are also easy to store and culture and given their size 400-500 um their perfect for young fish 10 days or older. The process of culturing artemia involves hydration of cysts, semi-decapsulation (the removal of the outer shell) of the cyst by means of oxidation, 24 hours exposure to salty water and harvesting. Semi-decapsulation is important because it reserves energy the emerging nauplii would have needed to break through the shell, it lowers the bacterial count in the hatching medium and it helps prevent the introduction of non-digestible cyst shells in to the fish tank. These shells might obstruct the digestive tract of fish.

Setting up a new Hatchery

1) Get Artemia cysts out of the refrigerator in H225-lab. For the 10 L hatcheries #1,2, and 3 weigh out 12 grams of cysts in to a clean 250 ml beaker using the scale in the H225. Then add enough system water to cover the cysts in the beaker. Hydrate the cysts for 20 minutes.
2) Open the valve on the bottom of the hatchery and hose it out with RO water (labeled green hose) in to a waste bucket below it. Close the valve. Then use this water to fill the hatchery to the 5-liter mark. Dumb the waste bucket down the sink.
3) Go to the carboy containing the super salt solution in H221. Stir the contents of the jug with the PVC pipe at the top. Decant 750 ml salt solution from the carboy in to a graduated cylinder and pour it into the hatchery.
4) Add 5 ml ammo-lock to the hatchery. Organic ammonia is a natural byproduct of artemia development.
5) Pour the hydrated cysts into an artemia net (labeled white mesh nets located above the sink). Fill the glass bowl near the faucet with 5-6.8% sodium hypochlorite (bleach) located below the sink. Swirl the net containing the cysts in the bleach for 90 seconds. Make sure the cysts are immersed in the bleach. Lift the cysts out of the bleach and place them under the system faucet. Turn on the water and slowly swirl rinse the cysts for an additional 90 seconds. Be very careful when using bleach its toxic to humans and fish.
6) Take the net and cysts to the newly set up hatchery. Place the net in the salty solution and invert the net.
7) Attach the airline hose to the bottom of the hatchery, and open the valve. There should be a strong flow of air bubbles tank.
8) Place the hatchery lid on to the tank.
9) Record the date on the tape on the lid include AM or PM.

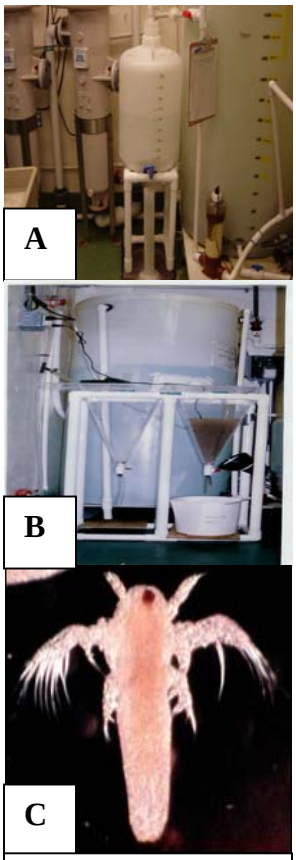

Figure 4.2 A. Carboy of salt mix B. Artemia hatchery. C. Artemia nauplii

Harvesting

1) Find the oldest hatchery. It should be more than 24 hours old, but no older than 36 hours. Shut off the air supply to the hatchery by closing the valve at the bottom. Let the stagnate hatchery settle for at least 10 minutes before harvesting. The cysts should settle down into the bottom most layer, the mesa layer should be artemia, and the upper most layer will be artemia along with hatched cyst shells.
2) Place a waste vessel under the spout, open the valve and drain out the unhatched cysts usually the first 500 ml. Drain the mesa layer of the hatchery into a clean 5.0 L beaker(s) until the very top layer is left in the bottom of the hatchery. Pour the rest of the hatchery in to the waste bucket. Pour the waste bucket down the sink.
3) Take the beaker to the sink and place the acrylic artemia screens labeled top and bottom in to the sink. Place the top screen onto of the bottom screen and decant ~1.0 liter of artemia from the beaker in to the top of the screen. Grab the white system hose and gently spray the artemia through the top screen into the bottom screen. Remove the top screen and decant the contents of the bottom screen into a clean 2.0 L beaker using the system hose. Continue this process until all of the artemia have been put through the screen and decanted into the beaker. Occasionally, flip over the screen and spray them with the hose to remove debris wedged into the screens, a small brush can also be used for this.
4) If the artemia is not going to be used right away they can be stored in the refrigerator on the bottom shelf. Place the green airline hose in the artemia vessel.

Feeding Artemia To Nursery Tanks.

Artemia nauplii are feed to nursery fish tanks 10 days or older. From a clean transfer pipette fed 3 drops per tank.

4.5 Disinfecting Artemia Hatcheries

Once a month the artemia hatcheries need to be disinfected. The records of disinfection are kept on labeling tape on the individual hatcheries. Hatcheries should be disinfected after they have been harvested and before they are set back up again. Before bleaching rinse out the hatchery thoroughly with RO water. Fill the hatchery with RO water to the 7.5 liters mark on the side of the tank. Restore the airflow back to the hatchery and add 150 ml bleach. Let it stand for 1 hour. Drain the hatchery in to a waste bucket and thoroughly rinse out the hatchery with RO again and refill to the 7.5 liter mark with RO and then add 15 grams sodium thiosulfate, restore the air flow and let it run for 15 minutes. Drain and rinse with RO. Record disinfection date on the hatchery.

4.6 Paramecium Culture

Paramecium are a commonly observed protozoan. *Paramecium multimicronucleatum* such as the ones used in the fish facility is the largest species at 150-350 um. It has one macronucleus and 3 or 4 micronuclei. These foot-shaped ciliates are easily maintained and cultured. Paramecium make the perfect first food for developing fish due to their size and nutritional value. The food chain involved in culturing these protists starts with the decomposition of seeds by bacteria, small flagellates called *Chilomonas* feast upon the bacteria and in turn are feed upon by Paramecium. When you culture para you are culturing this food chain.

Figure 4.3 *Paramecium multimicronucleatum*

Pellet stock and stock culture are the reagents used for making bottle and tub cultures. If there is very little demand for paramecium bottle cultures can be used for feeding and propagating. The tub cultures are used for feeding the fish while the bottle cultures are mainly used to maintain the paramecium line. Bottle cultures need to be propagated every 8-12 days after vitamins are added to them. Tub cultures need to be used when they are around 8-12 days old. After vitamins are added to these cultures the paramecium population slowly increases till about 8-12 days, depending on temperature, and then sharply drop off (food chain fails) and become unusable. At anytime in using the reagents or cultures you to detect hydrogen sulfide smell (swampy, rotten egg smell) it means the substance has gone anaerobic and needs to be disposed of. Reagents and cultures should have a fruity smell that usually does not smell terrific, but does not smell as bad as rotten eggs. To avoid anaerobic conditions always keep a large surface-area at the top of the bottle. Do not fill bottles up in to the tapered neck, and leave the caps loose on inoculated bottle cultures. Always disinfect the work-area countertop with NPD or isopropyl before beginning any work. Keep a logbook of paramecium culturing and how often tubs are being used. Timing is very important making bottle and tub cultures. Estimating future use should be based upon the number of tanks in the nursery and past usage based on the paramecium logbook. Always keep sterilized RO in the 5 gallons carboys ready for tub culture.

4.7 Pellet Stock

1. Obtain 10 clean 1-liter bottles with caps from the Hurley laboratory J631.
2. Obtain a clean 5-liter beaker or flask and add 2.5 liters of reverse osmosis (RO) water or D.I.
3. Add 5 grams of salt to the RO water
4. Place beaker on hot plate/stirrer.
5. Place a large stir bar into the beaker and turn on the hot plate and stirrer.
6. Just before the solution starts to boil add protozoan pellets (Carolina Biological), 3 or 4 at time for a total of 50 protozoan pellets. Warning adding pellets will encourage the water to boil.
7. Allow of the pellets to dissolve completely, and with autoclave gloves remove the beaker from the burner.

8. Using a funnel add 500ml of boiled stock to five bottles and slowly top off each bottle to 1000ml mark with RO water.
9. Repeat steps 2-8 to get ten bottles of stock.
10. Label each bottle with the title pellet stock include the date, your initials and the total number of bottles made and number each bottle example 1/10, 2/10, …10/10 for ten bottles.
11. Tightly screw lids on to the bottles and place autoclave tape across the lids.
12. Take the bottles to the autoclave. **LOOSEN** the caps from the bottles. Obtain an autoclavable tub and fill the tub with ~1" of water and place the bottles in the water. Autoclave for 40 minutes on the liquid setting.

4.8 Stock Culture

1. Obtain 10 clean bottles.
2. Add 100ml of pellet stock to each bottle.
3. Top off each bottle to 900ml mark with RO water.
4. Label each bottle with the title-pellet stock include the date, your initials and the total number of bottles made and number each bottle, example 1/10, 2/10, …10/10 for ten bottles.
5. Tightly screw lids on to the bottles and place autoclave tape across the lids.
6. Take the bottles to the autoclave. LOOSEN the caps from the bottles. Obtain an autoclavable tub and fill the tub with ~1" of water and place in the water. Autoclave for 40 minutes on liquid setting. Can be autoclaved concurrently with carboy RO, and pellet stock.

4.9 Bottle Culture

Need to be propagated every 8-10 days.

1) Obtain 4 bottles of stock culture.
2) Clean the countertop with NPD and a large Kemwipe.
3) Assess health, density and purity of a paramecium (see section 4.11) bottle for propagation (these are located on the shelves in the office H225). Do not use cultures older than 12 days.
4) Each bottle culture to be inoculated should be filled to the 900 ml mark on each bottle.
5) Thoroughly mix paramecium culture that is to be the innoculum by inverting the bottle several times. Pour 100 ml of innoculum in to the 4 stock culture bottles.
6) Add syringe-filtered (Luer-Lok 0.45 micron Millipore) vitamins 5 ml/bottle see adding vitamins 4.10 below.
7) Boil 20 wheat berries (Carolina Biological) for 10 minutes. Add 5 wheat berries to each bottle.
8) Place culture on the shelf in H225 with the <u>cap loose.</u>

4.10 Adding Vitamins To The Cultures

1) Fill a 1.0 ml microcentrifuge tube to the 1.0 ml mark with Nekton bird vitamins. Fill to the 1.0 ml mark for 4 tupperware cultures repeat for 8 tubs.

2) Transfer vitamins to a 50cc conical tube and add 25 ml autoclaved RO water to solubilize the vitamins (25 ml for 4 para tubs). This should be enough for 5 ml/bottle.
3) Secure Millipore filter to Luer-Lok of syringe and add vitamin solution to the syringe barrel.
4) Uncap bottle, depress plunger while holding syringe over the culture. Dispense 5ml/bottle.
5) The syringe filter will plug up with insoluble ingredients and you will need to exchange for a new one every couple of bottles (10-15 ml).
6) Label bottle with the date, initials and "V+".

4.11 Assay Paramecium Density, Purity and Health

Density is determined by thoroughly mixing the bottle or tub. Spray the cap of bottle culture with NPD or simply lift the lid on tubs and extract 1.0 ml sample. Place the sample in a cell plate in H225 that is divided in to 4 quadrants. Under the dissecting scope on low-magnification count the number of paramecium in each quadrant. Then sum the quadrants. Paramecium can be sedated with 1-2 drops of proto-slow (MS-222 can also be used) to ease counting. This will drastically slow the paramecium movement. There is also a hand-counter to aid in counting.

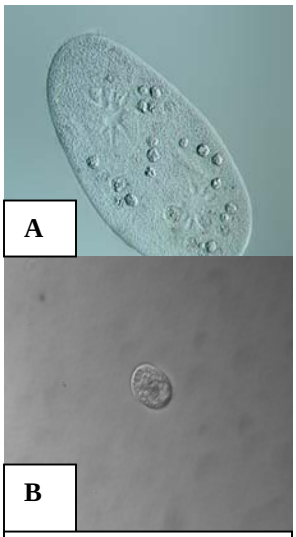

Figure 4.4
A. Paramecium multimicronucleatum approx. 150 um. B. Colep approx. 10 um.

Zooming in to the cell plate and looking top to bottom for Coleps and any other microorganism assess *purity*. If anything other than paramecium are found in the culture do not use it, and alert the laboratory manager. Pellet stock, stock culture, bottle and tub culture should be briefly smelt. If a hydrogen sulfide smell (rotten egg, swamp gas) scent is detected the container has gone anaerobic and is not to be used.

The shape of the paramecium cell and its mobility assesses *health*. If there is a lot of skinny or immobile paramecium do not use the culture. Note mobility needs to judged with out the addition of proto-slow.

Figure 4.5 A. Autoclaved R.O. water in carboys. B. Paramecium bottle culture. C. Tub culture.

4.12 Tub Cultures

1) Start by boiling a minimum of 120 wheat berries (or 15/tub) in a 250 ml glass beaker filled with sterilized RO from the carboys.
2) Clean the countertop with NPD and Kemwipe.
3) Disinfect 8 tupperware containers and lids by spraying them with NPD and wiping the solution evenly across all surfaces with a Kimwipe. Place the lid on the container as soon as you're done sanitizing it.
4) Obtain 2 bottles of pellet stock. Spray the caps with isopropyl. Add 250 ml pellet stock directly out of the bottle in to the tub.
5) Spray the spigot on the RO carboy with isopropyl. Add ~2.0L of sterile RO water from the carboys to the tub until it is filled to with in an inch of the top of the container. Fill all 8 tubs.
6) Add 15 of the boiled wheat berries to each tub using sterile tweezers.
7) Assess the density, purity and health of the most recent bottle cultures of paramecium (innoculum) see section.
8) Label the culture bottle with the density, date, initials, and "clean" meaning to be free of Coleps.
9) Add 250 ml of bottle culture to the tubs. Quickly reseal the lid after the addition.
10) Label the tub with the date, tub number, innoculum volume and density, V- if no vitamins were added or V+ if vitamins were added, and initials of preparer.

4.13 Paramecium Processing

Before nursery fish can be fed we need to count and inspect the paramecium tub culture. Paramecium are stored in H221 on a shelf next to the sink. Take down the paramecium tub furthest left. This should be the oldest tub on the shelf. Get a pipette pump and a clean 5 ml pipette. Remove the pipette from its package and insert in to the pump. Lift up the tub lid and extract 1 ml of paramecium culture. Place the lid back over the tub. Take the pipette to the dissecting scope and dispense the liquid in to the 6-cell plate that is divided in to 4 quadrants. Shake the plate until the liquid completely covers the bottom of the plate. Place the plate under the dissecting scope and under low-magnification count the number of paramecium in each quadrant. Paramecium generally move around along the wall of the cell plate so look for them there. If you find the paramecium are moving too fast for you to count use 1 drop of proto-slow located next to the scope. There is also a hand-counter to assist in the counting, see also section 4.11 Assay Paramecium Density, Purity and Health. After counting increase the magnification and look for any other organisms, particularly coleps, Figure 4.4 B. If coleps or any other organisms are found in the culture dispose of the tub and inspect a new one. Coleps are benthic and will feed on zebra fish before their swim bladders develop (while they are unable to move freely). After counting and inspecting the tub create a label with the date, number of paramecium per milliliter, initials of the person who counted paramecium, and the word -clean to show that the culture has been inspected for coleps and other organisms. Place the label on the lid of the paramecium tub.

4.14 Feeding Paramecium

Each tank is fed 4,000 paramecium. The nursery tanks actually hold about 500-750 ml water per tank so this would be 5-8 para/ml. Get a tub of paramecium if the tub has not been previously inspected see section 4.13. On the label of the tub there should be a paramecium count per milliliter. Divide this number in to 4,000 and round to the nearest 5.0 ml. This is the amount to feed to each tank. For example, if the tub has 150 para/ml take 4,000/150= 26.6 feed 30 ml per tank. Consult the white board in H225 for the total number of nursery tanks in use in G, H, J-laboratories. Update this list every time you feed. Multiply the total number of tanks by the feed per tank to estimate the volume of paramecium needed to feed each lab.

Before being fed to fish paramecium must be passed through a fine mesh net to remove larger organic debris. Place a clean appropriately sized beaker in to the sink. Obtain the paramecium net (labeled) hanging above the sink in H221. Set the net frame across the top of the beaker and lay the handle on the sink counter top. Obtain a spatula from the shelf above the sink and disinfect it with a spray of isopropyl. Take the lid of the paramecium tub and stir it with the spatula. Decant the desired amount of paramecium from the tub through the net in to the beaker. Replace the lid back on the top of the tub and place it back on to the shelf in the same place from where you got it from. Use a clean small beaker 50 or 100 cc to feed each tank. To transport paramecium from H225 to J083b pour them in to a clean 2.5-liter pitcher and insert the lid in to it. To transport paramecium to G-617 quarantine-lab use a paper cup with an aluminum foil lid. There is no free-exchange of dishware between the main colonies of H and J laboratories and the quarantine room. Do not bring non-disposable dishware to the quarantine laboratory.