



Chapter 3- Spawning

Zebrafish are photoperiodic breeders mating right after sunrise. To maximize this feature the laboratory lights are automated in all fish laboratories to turn on at 9:00 A.M. and off at 11:00 P.M. This photoperiod 14 hours light and 10 hours dark are intended to simulate optimal natural breeding conditions. Since Zebra fish embryos develop rapidly, it is also useful for collecting eggs just after they have been fertilized. Regulating the light and dark cycle allows us collect timed eggs if need be. Fertilized eggs can typically be collected a half-hour to an hour after the lights come on.

Zebra fish can be spawned as early as 2 months old, but are generally most productive between 6-12 months. The fish can be spawned up to every 3 days but once a week if most affective, and reduces the stress involved in spawning. In pair-wise crossings (one male and one female) spawning success of 50% is typical. One can expect to collect up to 100 fertilized eggs, but individual fecundity can be as high 350. Mass spawning, cross of more than one male and female carries a higher success rate and one can expect to collect 100's of fertilized eggs.

There are three different methods for when and how to setup fish. First, fish can be setup late in the afternoon the day before fertilized eggs are needed. This is the easiest and most common method, but you must be aware that the fish may mate the same day before the lights go out, which can be problematic if your expecting to collect timed embryos the next day. Alternatively, fish can be setup late in the afternoon, but with a horizontal tank divider in it separating males from females. The divider must then be removed as soon as the lights come on the following morning. This is useful when you need to know for sure that the eggs were fertilized that morning. Finally, fish crosses can also be setup on the same day just after the lights come on however, this is often less productive, especially for pair-wise (natural) crossings, but also insures recently fertilized eggs.

Fish are breed in spawning traps. There are two different types of traps, 1.5-liter transparent traps that can be used to spawn up to 8 fish per trap and 2-liter opaque traps that can hold 12 fish. In H221, clean traps are located below the workspace in the center of the laboratory. In J083b, they are located on a shelf on the northwest wall. Both types of traps consist of three pieces, a solid outer tank, a screened inset and a lid.

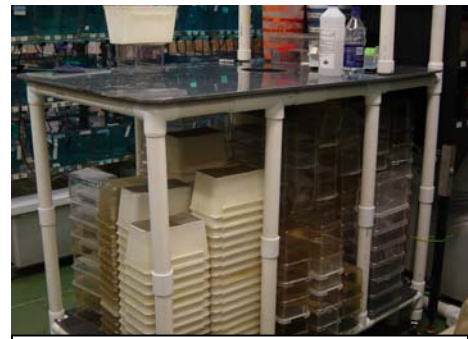


Figure 3.0 H221 Clean spawning trap storage. Located under the central workspace.

Collect clean sets of spawning traps and find an open workspace near the fish tanks you will be working with. Place the screened insets in to the solid tanks. Fill the traps with system water from the either the appropriately labeled hose or faucet. Do not use water from systems labeled R.O. (reverse osmosis). Fill the trap to within a half-inch of the top of the tank. Always leave an air-gap for the fish to gulp air.



3.1 Netting Fish

Obtain a clean net from the blue bin labeled “Nets”. Chose one that is an appropriate size for the fish tank where your fish are located. Net size is also important if you intend to collect a lot of fish or just a choice few. Fish are netted directly out of their tanks. The tanks can be left in place or disconnected from the system and moved to a work area. When doing the latter be sure that you are familiar with how to properly remove and reinstall the tank to and from the system. This varies from system to system see this manuals fish system section for further details or ask zebra fish staff also see section 3.2 below.

Carefully slide back or lift up the fish tank lid making sure that you don’t startle the fish, although small zebra fish can jump out of the tank on to the floor or in the worse case scenario jump in to another fish tank. If the latter situation occurs, notify the laboratory manager or staff as soon as possible. This is also why you don’t want to have adjacent fish tank lids open concurrently.

Be patient when netting. Avoid making sudden movements smashing the net against the inside of the tank in response to fast moving fish. It is easy to crush these small fish between the net frame and the side or bottom of the tank. Avoid netting from back to front handle of the net tilted towards the back of the tank. This generally herds the fish upward and towards the front of the tank where they can jump out.

The best method is to net the fish from back to front with the handle of the net tilted towards the front of the tank, Figure 3.2. In general, keep the top of the net tilted downward in the direction you are moving the net. During netting, move the net slowly, smoothly and follow the fish. Do not use the same net in different fish tanks in order to avoid any potential contamination. Whenever you are done with netting, place the “dirty” net in the net soak disinfectant, Figure 3.1. Be sure to place the fish tank lid securely back in place whenever you’re not using it.



Figure 3.1 Net Soak bucket next to H221 sink.

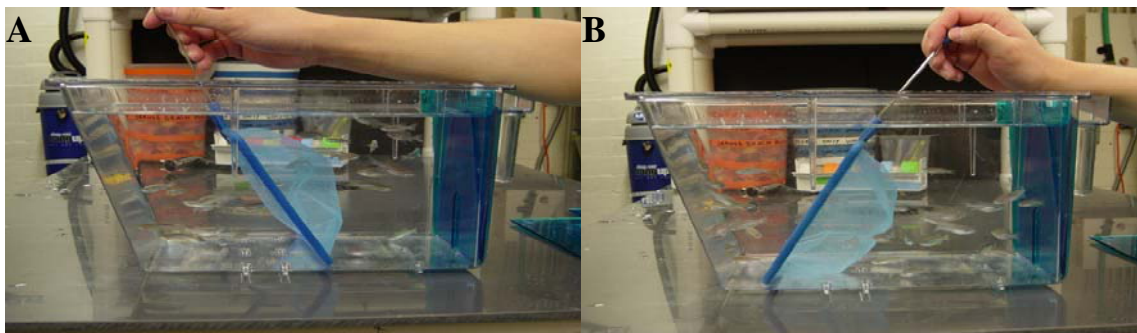


Figure 3.2 A. Proper netting technique, top of the net is tilted down to detour the fish from jumping out of the tank. B. Improper netting, top of the net is tilted upward encouraging fish to jump out of the tank in order to avoid the net. .



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3.2 Transferring Fish During Spawning

Often when setting up crosses it is necessary to temporarily transfer the fish from their tank to a secondary container to transfer them to a work area where your setting up the fish crosses and where often the lighting is better for sexing the fish. Use only clean disinfected vessels that are intended for this action. A clean solid section of a transparent spawning trap or a 2-liter fish tank works best, but other small clear clean tanks work. Be sure to fill the tanks with system water before you begin netting the fish. This is not the preferred method because it requires an additional netting of the fish, which is additionally stressful. The preferred method is collecting the fish out of the tank on or off the fish system and directly placing them in the spawning tank

3.3 Sexing Fish

Accurately sexing fish is crucial not only for spawning success, but also in maintaining individual fish lines in separate tanks. You would not want to accidentally place heterozygous carrier back in to a wild type tank after spawning. It is often useful to have a spawning logbook, which gives a physical description to help distinguish between fish. Some lines of fish are relatively easy to sex while others can be more difficult, especially when young.

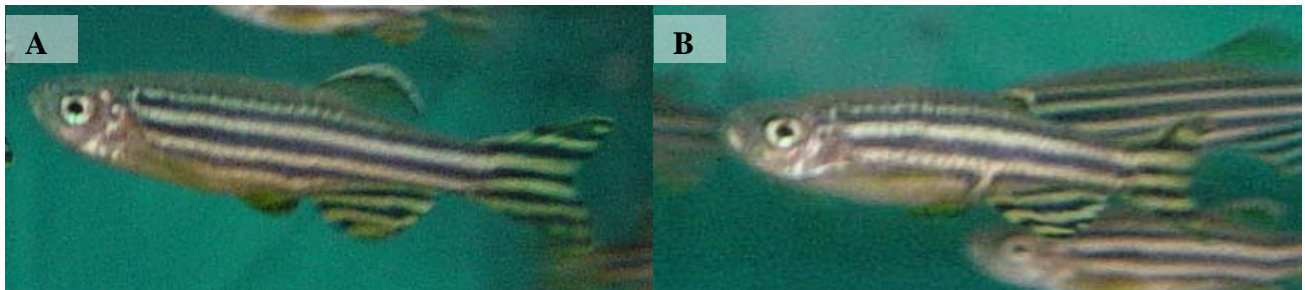


Figure 3.3 A. Male zebra fish (left image) are slender and long with more yellow coloration ventrally, and a overall pinkish hue on the flanks, especially on the caudal peduncle. B. Female zebra fish (right image) have a more robust abdomen, less yellow pigment, and an overall blue and silver appearance as opposed to the pinkish hue of males.

Table 3.0 Gender Key	Male	Female
Body Shape	Long and slender	Shorter, more robust abdomen
Color	Enhanced yellow coloration ventrally, overall pinkish hue on the flanks, especially on the caudal peduncle	Comparatively less yellow color, overall blue and silver appearance.

3.4 Types of Crosses

3.41 Pair-wise or natural crosses-low yield, are comprised of one male and one female, and are used for screening individual fish for mutations, genes, or anytime parents of the progeny need to be known. It is also useful for propagating stocks, and routine low-level embryo production.

3.42 Mass spawning-High yield is used when a large numbers of embryos are needed. Multiple males and females are setup in the same spawning tanks at a ratio 1 male to 2



females. Four males to 8 females is a popular ratio. Make sure to use the appropriate size of spawning trap.

When large numbers of embryos are needed routinely males and females should be separated in to different tanks. These tanks can be rotated from week to week. It's recommended that tanks used in this manner be labeled with the last spawning dates. This is also a good idea for any tank of fish that are spawned often (wild-types, screen).

3.5 Labeling Spawning Traps

Once you have the fish in the spawning trap place the lid over the top. You now need to label the trap. First, because the fish facilities are communal you need to select the appropriate labeling tape color. For those doing work under the protocol of David Raible use blue tape, David Kimelman use orange tape, Susan Brockerhoff use yellow tape, James Hurley use magenta tape and those working with screen fish use green tape. Labeling tape can be found at every workstation. In the upper left-hand corner place the genetic information, and then along side or just below it place the stock number. On the bottom section of the labeling tape place the anticipated date of birth and your initials. For information about genetic information see Chapter 2 Basic Genetics and for stock numbers see Chapter 1 Basic Methods.

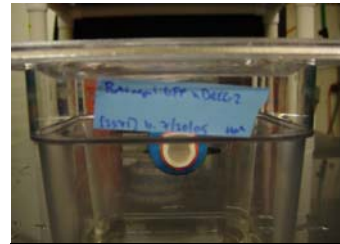


Figure 3.4 Tank label

3.6 Spawning Shelf

Place the crosses on shelves labeled cross or crossings. Do not place crosses outside of the designated area, and do not leave them in work areas. With lids in place it is safe to vertically stack 3-4 sets of traps on top of each other.



Figure 3.5 H221 Spawning Shelf

3.7 Collecting Eggs

An hour after the lights come on spawning traps can be examined for eggs. Those traps that contain eggs can be collected by pulling the inset (inner trap) out of the solid trap (outer trap) and placing it in a spare outer trap that is full of system water. This will separate the fish from the eggs. Next get the egg-collecting screen next to the sink, Figure 3.6.

- 1) Briefly run the egg screen under a stream of system water to remove any oils from previous egg collections.
- 2) Slowly pour the water and eggs from the trap on to the screen.
- 3) Use the system water squirt bottle to run a stream of water around the inside of the trap and gentle swirl the water.
- 4) Pour the water back through the egg screen.



Figure 3.6 Egg screen



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- 5) Briefly rinse off the eggs with the system water to remove any scales and feces.
- 6) Get a petridish and invert the egg screen over the dish no more than an inch above it.
- 7) Gently squirt system water through the backside of the screen washing the eggs in to the petridish.
- 8) Transfer the spawning tank label on to the dish.
- 9) Take the petridish to a microscope and remove any unfertilized eggs, feces, scales and most of the water.
- 10) Then fill the dish 2/3 the way full with embryonic medium.
- 11) Place dish with the lid on in an incubator.
- 12) There is normally a 20-hour die off, so eggs should be inspected the next day and any unviable eggs should be removed.

Be careful to keep adult fish away from any fertilized eggs at all times for they will readily eat them.

3.8 Collecting Embryos for Microinjections

To collect embryos for microinjections set the fish up as normal then on the morning the embryos are to be collected fill a clean solid spawning trap with system water. Once the fish in the trap have given enough embryos for you to start injections lift the insert of the spawning trap out along with the fish and quickly place them (inset and fish) in to the clean solid tank so they can continue breeding. Collect the embryos from the first solid spawning trap, rinse it out, and refill it with clean system water to repeat the process.

3.9 Holding Fish Off-System

If your phenotyping embryos to establish the genotype of the parents or other screen work you can return the fish back to their spawning tank for a maximum of 5 days during this procedure. Make sure to place a tank divider in to the spawning trap to separate males and females. This is very important because the fish will likely continue trying to spawn after the eggs have been collected if they are not separated. This is can be very stressful, and even fatal at times.

Fish that do not produce eggs on the first morning can be held over an additional day to see if they will give eggs the next day. If they do not give eggs on the second day return the fish back to their tank because it is unlikely any further attempts will be successful and the fish will be stressed.

Do not hold fish off-system for more than 5 days. The water quality in the tank declines rapidly (mainly pH), and the fish are not feed off-system. They can become more susceptible to disease either in their off-system tank or when they are eventually put back on to a fish system where they would experience drastic change in water quality parameters.



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3.10 Dirty Spawning Traps

After you have taken down your crosses take them to the sink. Separate the traps, and thoroughly wash them off with R.O water. Additionally, take the solid section of the trap and scrub it with brush located at sink, and rinse with R.O. Neatly stack the dirty traps on the cart designated for dirty dishes. They will be autoclaved and returned clean spawning trap area as soon as possible.



Figure 3.7 Dirty Spawning traps placed on a cart.

3.11 Incubator

After you have collected and cleaned your eggs they need to be placed in a 28.5°C until day four when the swim bladder develops and their moving around. At this time the young fish can be moved to a nursery system in H221 or J083B. Incubators are located in the individual P.I.'s laboratories. They are not incubated in the zebra fish facility.

3.12 Spawning Trouble

The fish facility tries to maintain the best environment possible for fish breeding. Typically, pair-wise crosses are ~50% successful and mass crosses are ~75%. Spawning success depends on numerous factors. Some lines are very productive, some are not and some are both from generation to generation. Here are some tips that might help.

- The most common mistake that we see is the homosexual crosses. Solution, ask and fish facility manager and others who are familiar with the fish or fish line to help you with sexing. Take your time setting up crosses.
- Obese females are often the worst fish to try to spawn. They become egg-bound and are not able to reabsorb eggs efficiently. This can be due to overfeeding, or simply have not been breed enough. Do not use these females in crosses. Try squeezing them see section 3.13.
- If the fish do not give eggs in the smaller 1.5-liter spawning traps try switching them to the 2.0-liter traps.
- If the fish don't give the 1st day try placing them in fresh system water and check again the following day.
- Talk to fish facility manager by different strategies.

3.13 *In Vitro* Fertilization (Walker and Streisinger, 1993)

Simultaneously maturing embryos in large numbers can be produced via *in vitro* fertilization. This is also a handy technique for fish that do not or cannot reproduce naturally. It generally entails, anesthetizing males, collecting sperm, and temporarily suspending it while you anesthetize females and collect their eggs. The two are then brought together with water. The procedure needs to be performed just the lights come on in the laboratory this is when the fish are prime for reproduction.

Precautions

- ✓ Always collect sperm before collecting eggs. It is much more difficult to collect suitable amounts of sperm than eggs. If you cannot collect sperm there is no point in collecting eggs.



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- ✓ Watch your water. Water activates fish sperm and chorions on eggs begin to swell with water after contact making fertilization more difficult.
- ✓ Do not leave fish in tricaine too long you have only a few minutes after the gill movement has slowed. Some fish may die due to prolong exposure to tricaine. It is possible to resuscitate some fish by gently running system water across the gills.
- ✓ When using spoons and spatulas during the procedure slide it under the fish starting from the head down to the tail.
- ✓ After squeezing females label their tank with the date of the procedure and do not squeeze them again for 4 weeks, for males 3 weeks. They can be spawned naturally after 1 week.

- ✓ Timing is crucial be fast and efficient.

1) Separate males and females in to different tanks the day before in vitro fertilization is going to take place. Spawning tanks with dividers work well.

Male Gamete Collection (just after the lights come on)

- 2) Anesthetize a male fish in tricaine (see 3.14 Anesthetizing Fish).
 - a. After gill movement has slowed lift fish out of the anesthetic using a plastic spoon.
 - b. Momentarily rinse the anesthetized fish in system water and place them belly up in a slit on a damp sponge.
 - c. Gently plot the genital region with a kimwipe to remove any water.
 - d. With your fingers or forceps gently stroke the sides of the fish starting at the head and moving slowly down to the genital pore, repeat.
 - e. Sperm will come out the pore just above the base of the anal fin. If no sperm is ejected place the fish back in to system water.
 - f. Collect the sperm using microcapillary tube with gentle suction. Viable sperm should have a milky white appearance. Place the male in system water to recover.
 - g. Collect sperm from several males and place it in ice cooled full-strength Hank's saline (see recipes). Sperm from 5-10 males will fertilize 100's of eggs for up to 90 minutes after collection.
 - h. Gently add the sperm from the microcapillary in to a microcentrifuge tube and add enough Hanks saline to make a cloudy suspension.

Female Gamete Collection

- 3) Anesthetize a female in tricaine
 - a. After gill movement has slowed lift fish out of the anesthetic using a plastic spoon.
 - b. Momentarily rinse the anesthetized fish in system water and dry by rolling the fish on a paper towel with a spoon.
 - c. Place the female in a petridish and with moist fingers press gently along the abdomen. If she is ripe with eggs they will come out easily.
 - d. Concentrate the eggs with a spatula in center of the dish.

Fertilizing Eggs

- 4) Add 30-50 μ l of sperm suspension to the eggs. Mix gently. Add 0.5 ml system water.



Add another 1.0 ml after a minute. Fertilization will take place upon the addition of water.

3.14 Squeezing Fish

Squeezing fish has many purposes such as *in vitro* fertilization, haploid embryos for identification, alleviating swollen females, embryos for heat shock and early pressure experiments. The steps described in *in vitro* fertilization can be used to collect gametes. Be certain to label tanks that have been used in squeezing procedures with the date of squeezing. Wait 4 weeks before squeezing the same females again and 3 weeks for males. The fish can be used for normal breeding activity after a week. Heat shock and early pressure procedures are not performed in the fish facility, so there is no protocol for such procedures in this protocol.

3.15 Anesthetizing Fish

Tricaine (MS-222 or 3-amino benzoic acid ethylester) powder is used to create the following stock reagent for anesthetizing fish.

400 mg tricaine powder
100 ml reverse osmosis or deionized water
2.0 ml 1 M Tris (pH 9).
Adjust pH to 7.0 using 1 M sodium bicarbonate
Mix in bottle with screw cap and store in the freezer.

To use tricaine as an anesthetic add 4.2 ml reagent to 100 ml system water. Do not leave fish in the anesthetic for more than 90 seconds. Place fish in system water to recover them.

3.16 Chorion Removal

Remove as much of the water as possible from the eggs. Place them in 5.0 ml of 0.5-mg/ml pronase for 3.5 minutes. Then dilute the eggs with 200 ml system water and then drain. Repeat 3 more times. Pronase may not remove the entire chorion from all of the eggs; depending on the degree of removal required manually removing chorions with forceps might be necessary.

3.17 Fin Clipping (source S. Johnson)

Zebra fish fins may be amputated for various reasons including screening fish for molecular, genetic, or biochemical markers, or fin regeneration experiments. Fins will regenerate within a few weeks if properly clipped. The caudal fin is easiest to clip. Anesthetize the fish in tricaine see section 3.14. Pick up the fish with a plastic spoon and place it parallel on your index finger. Slide a pair of scissors between the fin and your finger and cut. Place the fish immediately in to system water and the fin in to an appropriate tube. Fins will regenerate up to the point where the scales extend on to the body, but for most experiments clipping the fin halfway between this point and the end of the fin is sufficient. Anal and dorsal fins are also easy to clip but regeneration is less reliable. The pectoral and pelvic fins are harder to clip but are more reliable in fin regeneration.