

Artificial Insemination in *Xiphophorus*

Frequently, researchers using *Xiphophorus* must perform artificial insemination (AI), because divergent species sometimes have behavioral barriers to mating. The technique originally was published by Eugenie Clark in 1950 (Science; 112: 722-723); it remains essentially the same, with only a few changes based on new technologies, such as the production of quality plastic pipette tips. One should keep in mind the very basics of AI in *Xiphophorus*:

- Fertilization in *Xiphophorus* is internal.
- *Xiphophorus* can store sperm for several months. Our records indicate that storage can be up to 9 months and possibly longer.
- Males do not always produce sperm, regardless of the external physical appearance or age.
- Females may NOT be physiologically able to reproduce; in particular, older females may be sterile.

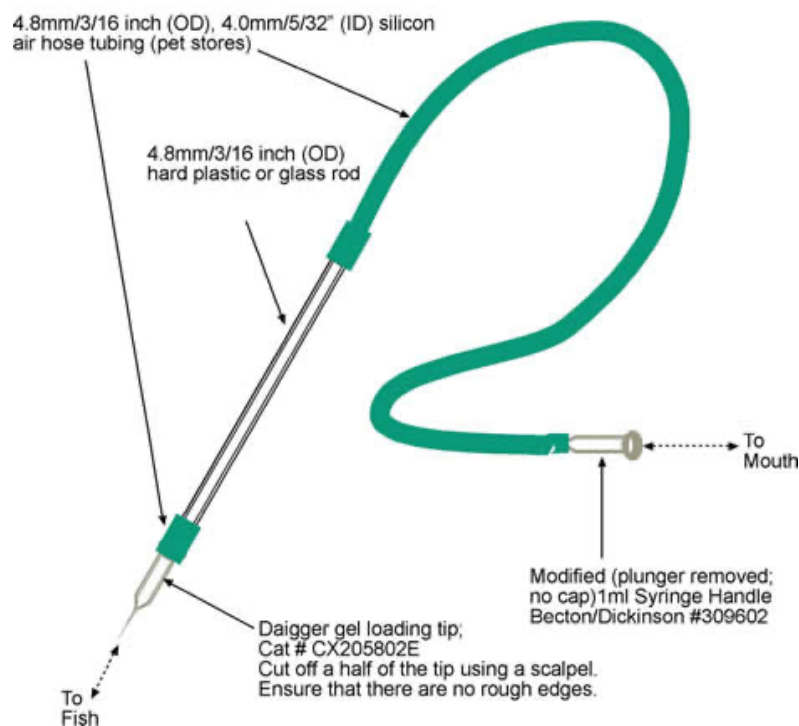
AI procedures should be undertaken only when absolutely necessary. It is a time-consuming technique which requires skill, and several things can go wrong with the procedure (see below). It should NOT become a routine practice for all matings. AI can be used in non-hybrid matings when the situation dictates, such as when a mating pair has not produced offspring after several months of co-habitation. When making hybrid fish, one needs to develop experience with the species/strains employed. Some species pairs will readily breed, while others will show little or no interest in a tank mate. For example, a *X. helleri* male typically will not inseminate a *X. variatus* female. However, we know from experience that a male *X. variatus* will mate with *X. helleri* females.

Materials

- 100 ml of anesthetic such as MS-222, (Tricane methane sulfite) diluted to 0.06% concentration using fish tank water (see anesthesia section).
- 0.7 % NaCl solution ("saline"; preferably autoclaved/sterilized).
- Minimally, 3 specimen containers, one for the female, one for the male and one dedicated container for the anesthetic. Specimen containers from Lee are suitable.
- An microcentrifuge tube (1.5-2.0 ml) or equivalent.

- A petri dish (or equivalent) with wet cotton where the fish will be placed on its "back".
- A good stereo microscope with adequate lighting such as that provided by fiber optic lighting units. The magnification used is typically between 10 and 30X.
- An artificial inseminator tool (or equivalent device), as depicted in the figure below:

Artificial insemination tool



Procedure

- 1) First of all, prepare everything! The microscope, the positioning of your seat, the microfuge tube, the saline, the inseminator, and so on. The success of the procedure and the lives of the fish rely on the preparedness of the experimenter.
- 2) Pipette 30 microliters of saline solution into the lid of the microcentrifuge tube (or a microtiter plate). This will serve as a mixing platform for the sperm packets.
- 3) Anesthetize the male by placing him into the dedicated anesthetic container with 100 ml of 0.06% anesthetic (MS-222; in water). The swimming movements of the fish will slow down gradually. It will lose its ability to stay upright in the water and keel over on its side or totally lie on the bottom upside down. The

mouth and gill arches will still be moving albeit very slowly. It will take ~2 minutes for the fish to become anesthetized, at which point it should be removed with a wet gloved hand and placed on the wet cotton under the microscope. The fish is extremely vulnerable to injury, particularly around the ocular area, so take care not to scrape the fish against the sides of the container.

4) Gently hold the male on his back with one hand to expose his underside, and with the other hand, simultaneously squeeze his body in a direction from behind the operculum to the gonopodial area. This movement, which can be repeated 3-4 times, should lead to an eruption of sperm at the base of the gonopodium. This aqueous secretion is usually replete with sperm packet, otherwise known as "spermatozeugmata". The liquid should be pipetted into the lid of the microcentrifuge tube which already contains 30 microliters of saline solution. Then, put the male back into an observation container and monitor his recovery; continue to do this as you go on to the next step - you cannot leave the sperm too long. Occasionally, fish may have a hard time recovering from anesthesia: expedite their recovery by extremely gently moving the fish back and forth through the water, thereby clearing the anesthetic from the gills and restoring the regular breathing rhythm.

5) Next, anesthetize the female by placing her in the same dedicated anesthetic container. The procedure is identical to that described above. Once again, remember that the fish is extremely vulnerable to injury, particularly in the ocular region, so take care not to rub off the fish's scales against the sides of the container. Then, lie the sedated female on the cotton pad, positioned so that you can clearly see the urogenital opening.

6) Pipette the sperm solution into the tip of the micropipette tip and hold the solution in place. Try to avoid air bubbles as they can be injected into the female and cause her to lose her balance. The urogenital opening is directly anterior to the anal fin. (NOTE: The anal opening is a bit more anterior). Hold the female in place with one hand, and hold the artificial inseminator with the other hand. The inseminator should be held like one holds a pencil. Insert the plastic tip into the urogenital opening and gently push in, angling it towards the anterior region (this leads internally to the genital tract). You may feel some resistance, particularly in virgin females, since they can develop genital "plugs". When the tip of the pipette has penetrated a few mm, begin to gently blow the sperm solution into the female. Sometimes, the female's visceral cavity inflates as this is done. Avoid blowing air bubbles into the body.

7) Put the female should be put back in an observation tank and monitor her recovery. Expedite recovery as described for the male fish.

8) Carefully monitor both males and females for several subsequent days. Males are usually less affected by this technique. Females, on the other hand, can become infected at the site of injection and may have to be treated with

antibiotics. Sometimes, they swim in an abnormal fashion; this effect usually subsides within a few days.

9) Finally, make sure all records are updated, noting which fish were used, when the insemination was performed, and how many fry were produced (common sense but worth mentioning).

Success with artificial insemination does not come instantaneously. You must be gentle, so not to harm the fish, yet you must be fast, so that the fish does not revive while you are carrying out the procedures. Therefore, you may wish to familiarize yourself first with the anatomy of the female livebearing fish. A valuable source of information is a chapter by George B. Constanz in the book "Ecology and Evolution of Livebearing Fishes" G.K. Meffe and F.F.Snelson, 1989. Prentice Hall, Englewood Cliffs, NJ

We also found it was useful to practice beforehand to gain a feel for the technique and for the time it takes to accomplish the various steps. For example, by using females that had just been killed for other assays (to remove tissues and tumors), we learned how to carefully insert the pipette into the urogenital opening without lacerating the female. Similarly, with males, we learned the best way to massage the body to obtain sperm. We also timed the stages and depths of anesthesia and the pattern of recovery. Best of all, we were able to observe an expert carrying out the procedure.

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