
Procedural Guide for the Artificial Insemination of the Lyretail Swordtail, *Xiphophorus helleri*



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Introduction

The lyretail trait in the swordtail fish was first identified by Don Adams, a Florida fish farmer, and the lyretail swordtail has since been established as a distinct variety (Gordon and Axlerod, 1968). In this swordtail type the upper and lower rays of the caudal fin are elongated and resemble the Greek musical instrument, giving rise to the term “lyretail” (Fig. 1).

Interestingly, the elongation of fin rays is not restricted to the caudal fin, but all of the fins become extended. The striking appearance of the fins makes this variety higher in value than the common or wild-type swordtail. The estimated farm-gate values of the red common *versus* the red lyretail swordtails at three different body sizes are compared in Fig. 2. Clearly, swordtails that possess the lyretail characteristic are significantly more valuable. As mass production of the lyretail swordtail would have a definite economic impact on the producers, developing methods to reproduce this fish has become one of the objectives of our freshwater ornamental fish activities. The Center for Tropical and Subtropical Aquaculture, University of Hawai‘i Sea Grant College Program and the Hawai‘i Department of Agriculture Aquaculture Development Program have pooled their resources for the publication of a series of “How To” manuals covering the commercial production of a variety of freshwater ornamental fish species. The publication of this manual regarding the lyretail swordtail represents the latest in the series.



Figure 1. Photograph of a male lyretail swordtail. Note the extended gonopodium.

Constraints In Producing Lyretail Swordtails

The elongated fins, characteristic of the lyretail trait, prevent the male lyretail swordtail from mating naturally. While the sperm of the male lyretails are viable, their inability to copulate is a result of the over-development of the gonopodium (Fig. 1). Many breeders of lyretail swordtails have circumvented this problem by mating a lyretail female with a common male. However, a maximum of 50% of the

offspring will possess the lyretail trait, thus limiting the number of progeny that can be produced per generation. Developing a method or methods that can result in the production of a higher percentage of lyretail individuals would obviously have a significant economic impact, as the lyretail is almost three times the value of the common swordtail.

Our investigations began by confirming the breeding constraints of lyretail swordtails in a commercial setting. To begin, 20 adult marigold lyretail females that had already been impregnated by common males were placed inside a cage in a 500-gal tank equipped with constant aeration and water exchange. These females were allowed to bear their offspring over a period of two weeks and were then

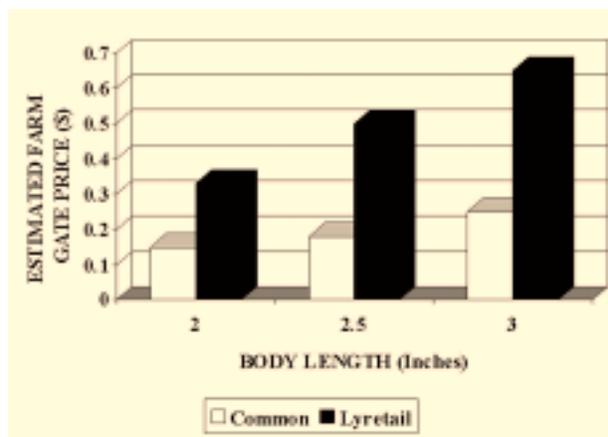


Figure 2. Comparison of estimated farm gate prices for common and lyretail swordtails at different body sizes. Source: Worldwide Aquatics (2000).

returned to their original production tank. The fry were fed a diet of salmon fry starter feed for approximately two months. A total of 286 fry were then sorted into common and lyretail groups resulting in a ratio of 145 common: 141 lyretails. This ratio is consistent with previous reports that about 50% of a particular brood will display the lyretail trait (Norton, 1991; 1992). To increase the production of lyretail individuals, it is necessary to understand how the lyretail trait is inherited.

A customary method of summarizing the results of genetic experiments is to use a Punnet square diagram (Norstog and Meyerriecks, 1983). This diagram illustrates the behavior of genes (and their alleles) as they are inherited from one generation to the next. When there are two variants of a particular gene (e.g., lyretail and common swordtail), these two variants are called alleles. Each fish carries two alleles of a particular trait - one that originated from the mother of the offspring and the other coming from the father. It is customary to label the alleles so that they can be followed through the mating process, so in this instance the lyretail allele and the common allele are designated “L” and “l,” respectively. An example of a Punnet square that depicts what will happen to the alleles from a cross between a lyretail female and a common male is presented in Fig. 3. It has been hypothesized that the lyretail trait is a dominant trait, meaning that an individual only needs one lyretail allele for that trait to be expressed (Norton, 1991; 1992). Therefore, the lyretail female possesses a genotype that consists of one allele for the lyretail trait or “L” and one allele for the common swordtail trait or “l.” The genotype of the common male to be used in the mating can be described as consisting of two little l’s. Using the Punnet square we can visualize which allele is being contributed by each parent during the mating process and examine the possible combinations that can result from a cross between this male and female. Only two genotypes can result from the cross depicted in Fig. 3, namely “Ll” and “ll.” That means the next generation that is produced will consist of two kinds of phenotypes (lyretail and common) and they will be produced in equal numbers. The behavior of alleles segregating randomly during the mating process is the foundation of one of biology's most basic principles, The Mendelian Principle of Segregation.

In the case presented earlier, where 286 fry were sorted, the expected ratio of common to lyretail individuals should be 143 common: 143 lyretail, if the expected ratio of common to lyretail individuals is 1 common: 1 lyretail. The actual ratio of 145 common: 141 lyretail is actually a little different from the expected ratio, and this can occur just by chance alone due to the large number of random interactions that take place between eggs and sperm during the fertilization process. A statistical test such as the Chi Square test (Sokal and Rohlf, 1969) is often employed to determine if the deviation from the expected ratio can be explained by chance alone. Using this statistical test, the observed ratio of common to lyretail individuals was found to be consistent with the expected 1:1 ratio (chi square = 0.06, $P>0.05$), deviating from the expected ratio just by chance alone.

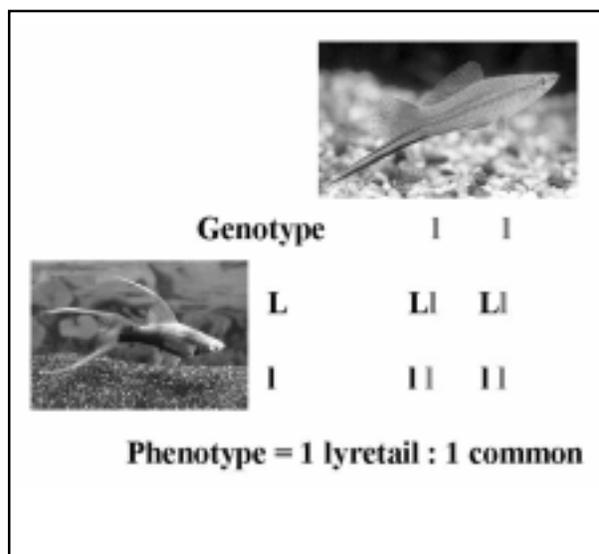


Figure 3. A Punnet Square illustrating the possible genotypic combinations of a cross between a lyretail and common swordtail.

The 141 lyretail swordtails produced from the lyretail-to-common cross were then stocked into a 500-gal tank and allowed to grow to five months of age. During that time, the swordtails were fed *ad libitum* a diet consisting of salmon feed supplemented with live *Moina*. The growout tank had continuous aeration and a water exchange of approximately 10% volume per day. At five months of age these individuals should have already produced fry, as common swordtails normally produce their first fry four months after birth (Tamaru et al., 2001). The lack of fry production in the lyretail group is consistent with previous reports that the lyretail male cannot physically mate with a female individual (Norton, 1991).

Increasing the Percentage of Lyretail Individuals

Following the Mendelian Principle of Segregation of a dominant trait, if a male lyretail and a female lyretail were to be mated one could expect 75% of their offspring to be lyretails and the remaining 25% to be common swordtails. A Punnet square summarizing the alleles inherited from such a mating is presented in Fig. 4. Artificial insemination, a technique that has been described for use with livebearers for over fifty years (Clarke, 1950; Takeshita, 2001), was considered as a means to transfer sperm from a lyretail male into a lyretail female. By circumventing the physical constraints to the natural reproduction of lyretails, the production of lyretail individuals within a single cross could be increased. Therefore, the artificial insemination of lyretail individuals was attempted between November and December 2001 using individuals that were greater than 5 cm in standard length (SL) and 3 g in body weight (BW). Five virgin lyretail females inseminated with the sperm from lyretail males produced offspring from January through March 2002. The clutches of fry were grown to a size when their phenotypes could be scored confidently and the ratios of observed and expected phenotypes could be analyzed statistically (Table 1). The broods at the time when their lyretail and common swordtail phenotypes were determined ranged between 29 and 123 individuals. With the exception of only one cross (female D23), the observed lyretail and common swordtail phenotypes were consistent with the expected 3:1 ratio. Although the common to lyretail ratio for progeny obtained from female D23 was significantly different from the expected ratio, there was still a preponderance of lyretail individuals at a ratio of approximately 2:1. The overall results are consistent with the lyretail trait being a dominant trait and inherited in a Mendelian fashion. Equally important is the demonstration that artificial insemination represents a means by which the number of lyretail individuals can be increased.

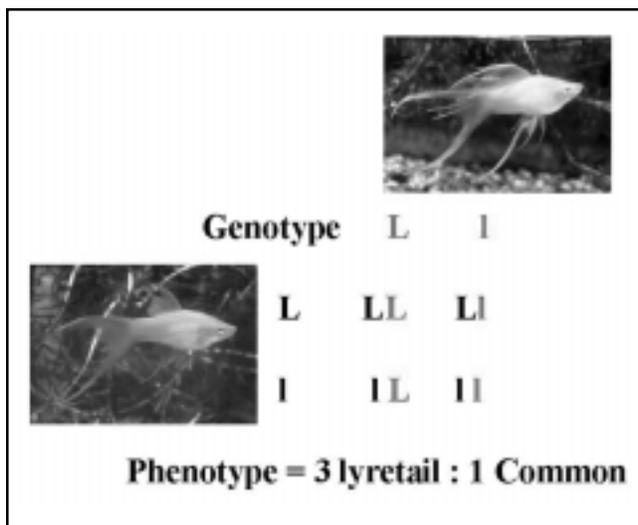


Figure 4. A Punnet Square illustrating the possible genotypic combinations of a cross between a male lyretail and a female lyretail swordtail.

Table 1. Summary of expected and observed ratios of lyretail and commons swordtails resulting from the insemination of virgin lyretail females using sperm from lyretail males.

Clutch Number	Lyretails	Common
D9 Born January 15, 2002		
Observed	22	7
Expected	22	7
Chi Square = 0.00, $P > 0.05$		
D23 Born January 22, 2002		
Observed	79	44
Expected	92	31
Chi Square = 5.69 $P < 0.05$ **		
D6 Born February 4, 2002		
Observed	53	19
Expected	54	18
Chi Square = 0.07, $P > 0.05$		
D10 Born February 18, 2002		
Observed	43	22
Expected	49	16
Chi Square = 2.06, $P > 0.05$		
D7 Born March 28, 2002		
Observed	32	13
Expected	34	11
Chi Square = 0.30, $P > 0.05$		

Homozygous Lyretail Genotype

A consequence of the lyretail-to-lyretail cross is that 25% of the offspring should be a homozygous genotype for the lyretail trait (Fig. 4). The homozygous genotype has two “L” alleles of the lyretail trait and all progeny produced by a homozygous lyretail individual should be lyretail. The challenge is to identify which individual is homozygous for the lyretail trait as morphologically, a homozygous individual cannot be distinguished from a heterozygous individual. This, however, can be accomplished using a technique called progeny testing which is summarized using the Punnet Square presented in Fig. 5. In this case, a homozygous lyretail individual can be identified by the progeny it produces, 100% lyretail as opposed to 50% or the 1:1 ratio expected from a heterozygous individual (see Fig. 3). F-1 lyretail females from a lyretail-to-lyretail cross were placed with common male swordtails at a ratio of 3F: 1M in June 2002. They were allowed to mate randomly for one month, after which individual females were placed into separate aquaria. Clutches of fry from each female were isolated and grown to a size when the phenotypes could be scored confidently. An example of a homozygous lyretail female identified by progeny testing is presented in Fig. 6. Of the 13 females that have been producing fry since late July 2002, three females have produced clutches that are 100% lyretail. This is consistent with the expected ratio of 3 out of 13 females (about 25%) if the lyretail gene is being inherited in

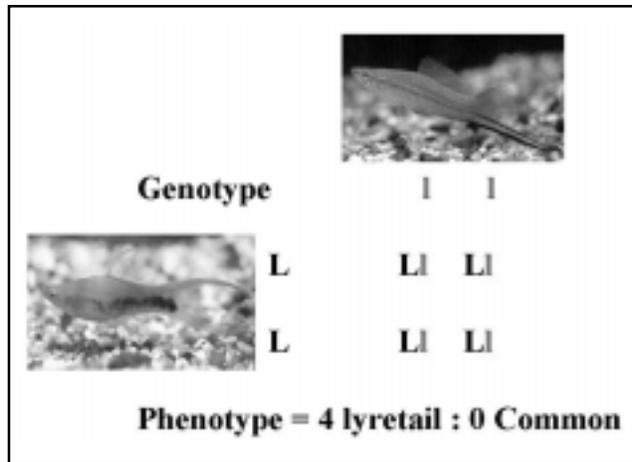


Figure 5. A Punnet Square illustrating the possible genotypic combinations of a cross between a female lyretail and a common male swordtail.

Mendelian fashion. Using females resulting from a completely separate lyretail-to-lyretail cross, the mating and progeny testing was repeated. In this instance, a total of 22 females underwent progeny testing and 5 homozygous lyretail females were identified. The expected number (25% of 22 = 6) of homozygous females is 6, which is consistent with the observed ratio. These results validate the mode of inheritance of the lyretail trait in swordtails and demonstrate that the use of the artificial insemination technique is a means to generate a higher percentage of lyretail offspring by producing individuals that are homozygous for the lyretail genotype.

Artificial Insemination

Artificial insemination is often done to increase the number of offspring of a prized individual animal and for the breeding of endangered species. The technique of artificial insemination involves the collection of sperm from a male and artificially injecting the sperm into a female to result in pregnancy. Artificial insemination is usually conducted in situations where the male of the species cannot or should not be involved in the natural mating process. The technique is also recognized as a means by which widespread improvement in farm animals could be achieved and currently the practice is common in the cattle and sheep industries.

Researchers working with livebearing fishes of the genus *Xiphophorus* use the artificial insemination technique to crossbreed different species that normally would not mate under natural conditions. The barrier may be physical, as with the lyretail swordtails, or it may be based on a behavioral characteristic. The technique used for livebearing fishes was first described by Clark (1950) and has remained essentially unchanged with only minor modifications over the years (Takeshita, 2001). Points to keep in mind, according to Kazianis et al. (2002), when conducting artificial insemination with fishes belonging to the genus *Xiphophorus* are:

- The fertilization of fishes belonging to the genus *Xiphophorus* is internal;
- Sperm can remain viable in the female for several months;
- Males do not always produce sperm; and
- Females may not be physiologically able to reproduce, particularly older females.

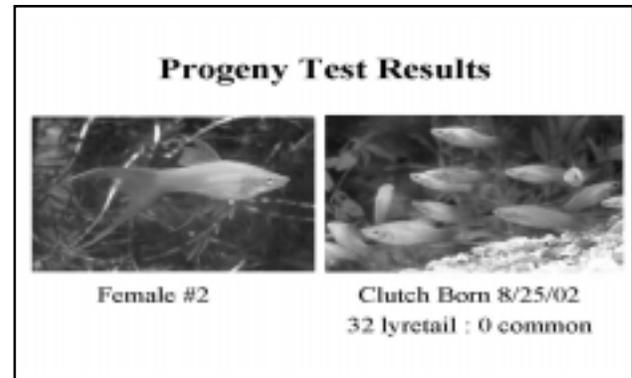


Figure 6. Result of a progeny test to identify homozygous lyretail female.

Materials and Methods

Supplies

Pasteur pipettes (glass)
tuberculin syringe (1 cc)
22-gauge hypodermic needle (1.5 in)
fine tip forceps
safety pin
scissors
alcohol lamp
needle nose pliers
fine gauge file or fine grit sandpaper
petri dish
gauze
microscope slide
anesthesia (clove oil, MS22 or 2-phenoxyethanol)
dissecting microscope (optional)
jewelers glasses (optional)
saline solution (0.9% NaCl)

Getting Started

Several tools are necessary to carry out the artificial insemination of lyretails, the first of which is a device to transfer sperm from the male into the female oviduct. Two devices have been found to work. The first (described by Takeshita, 2001) is a glass pasteur pipette with a tip that has been stretched to a very narrow point. The tip of the glass pipette is first heated using an alcohol lamp and, using needle nose pliers, the glass tip is then stretched to form a very narrow capillary (Fig. 7). The excess portion of the tip is broken off and the rough edges are smoothed with fine grit sandpaper. It is best to make several of these and keep extras on hand. A second device is a 1-cc tuberculin syringe with a 22-gauge needle that is 1-1/2 inches long. A fine grit sandpaper, file or a honing stone can be used to grind the sharp, pointed tip off the needle.

Artificial insemination requires much close-up work during the collection of sperm and the actual insemination of the female. For these activities, using a dissecting microscope or a pair of jewelers glasses (loupe) during the insemination process is recommended (see the bottom left and right photos of front cover). Take time to acquaint yourself with the tools and practice manipulating them under high magnification before actually doing any insemination. You will be surprised at the degree of coordination it takes.



Figure 7. Pulling a glass pipette over an alcohol lamp.

Anesthesia

The insemination process is a very stressful activity for the fishes. To alleviate the trauma and stress, it is recommended that the fishes be anesthetized prior to the procedure. Anesthetics such as MS-222, 2-

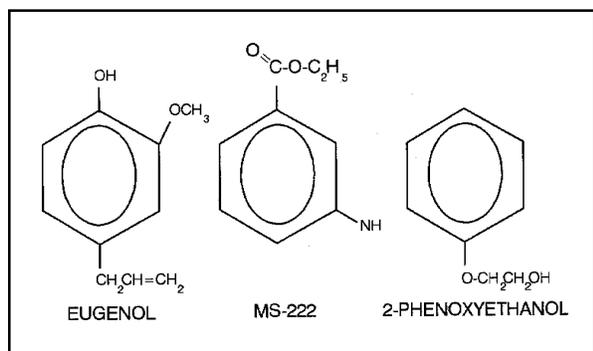


Figure 8. Chemical formulae for various kinds of fish anesthetics. Source: Merck Index.

phenoxyethanol, and clove oil are currently used in fish culture. As seen from the chemical formulas (Fig. 8) these three fish anesthetics are closely related. Because of the importance of this particular step in the insemination process, additional investigations were carried out.

Efficacy, availability, cost and adverse effects on fish, humans, and the environment should be considered when choosing an anesthetic for use in fish culture practices. One of the reasons for carrying out the investigations was to find an anesthetic that was easy to obtain and relatively low in cost. One that is becoming popular is clove oil, a natural oil derived

from the clove plant, *Eugenia aromatica* (Tamaru et al., 1995). Clove oil is available at local drug store and comes in a small vial that contains about 4 ml which costs about \$3.20. Clove oil can be used directly from the bottle to make up a working solution of 25, 50, 100, 300 or 600 ppm. When using clove oil a 1-ml tuberculin syringe can be used to measure out the small amounts needed. Another way to measure out the clove oil is to first make up a 10% stock solution (1 ml clove oil + 9 ml of tap water). If you want to make a 100 ppm working solution, take 1 ml of the stock solution and mix it with one liter of tap water. A 50 ppm solution is 0.5 ml stock solution/liter and a 25 ppm stock solution is 0.25 ml stock/liter and so on. If you do not have a device to measure out the small quantities of clove oil, one drop of clove oil in 400 ml of tap water results in a good working solution. Even though the bottle of clove oil may be small, it will make quite a lot of working solution.

To demonstrate the effectiveness of clove oil, swordtails averaging 14.0 ± 2.0 g BW and 8.0 ± 0.6 cm TL were exposed to various dosages of clove oil and the time to loss of equilibrium was recorded. Five individuals were tested at each dosage. A summary of the average time to loss of equilibrium at each of the dosages tested is presented in Fig. 9. Response time at dosages less than 100 ppm were observed to be quite variable, ranging from 50 sec to over 150 sec. However, at dosages of 100 ppm or greater, response time was rapid - taking place in less than a minute after exposure to the clove oil. All fish used in the experiment were successfully revived and no mortalities had occurred by 72 h post-treatment.

MS-222 or Tricane Methanesulfonate is considered to be one of the most effective anesthetics for fish. MS-222 is usually sold as a water-soluble white powder. To make a stock solution, simply add 5 g of MS-222 to one liter of freshwater and mix until the powder is completely dissolved. The stock solution can be stored for at least six months in a refrigerator at 5 C. Use 10 ml of stock solution for every liter of water in which the fish is to be held. Increase the amount of MS-222 slowly to attain the same effect as described with clove oil.

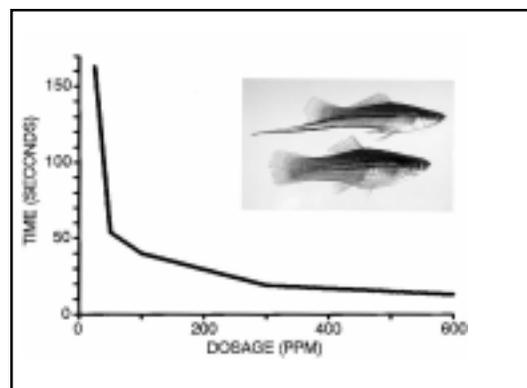


Figure 9. Time to loss of equilibrium for swordtails exposed to varying dosages of clove oil.

Sources for obtaining MS-222 are provided in the appendices.

Another very similar acting anesthetic is 2-phenoxyethanol. It is purchased as a clear liquid that is diluted to a concentration between 200-300 ppm (0.2ml - 0.3ml per liter). At this concentration, fish lose their equilibrium within 1-2 min and will recover within 2-5 min. Sources for 2-phenoxyethanol are provided in the appendices.

Insemination Procedure

Step 1: Chemical Solutions:

Place a drop of physiological saline solution on a glass slide or in a depression slide. Prepare the anesthesia solution.

Step 2a: Removing Sperm from Males:

There are two ways to obtain sperm from a male swordtail. If you desire to keep the male alive, select a male individual and place it in the anesthesia until the fish loses equilibrium and does not struggle when caught. Using a damp piece of gauze or cotton balls, hold the fish upside down between your thumb and index finger. Using your other hand hold the tip of the gonopodium and rotate it back and forth in an anterior to posterior direction (or “crank” it) about 10 times. Starting from the gills, begin to apply gentle pressure using your thumb and index finger and work backward towards the gonopodium. Watch for sperm oozing from the gonadopore just anterior to the base of the gonopodium. If the male is ripe, a pool of white to cream-colored sperm packets will be extruded just anterior to the gonopodium. This should be collected with a pipette as soon as it can be seen. Expel the sperm into the drop of saline that is on the glass slide. After the sperm has been collected, place the individual into a recovery tank containing clean, fresh water and aeration. If sperm is not extruded with the use of gentle pressure after several tries, this male will not be productive by this method and can be used by the next method or placed into a recovery tank for future use. The male will recover in approximately 3-5 minutes.

Step 2b: Removing Sperm from Males:

In the event that no sperm could be obtained from a male, the individual can be sacrificed by first overdosing it with anesthesia (> 600 ppm clove oil) and then decapitating the individual using a scissors. Then, using the scissors, make an incision starting from the anus and work anterior towards the gills. Pull apart the rib cage exposing the coelomic cavity where the testis can be seen. The testis is a whitish paired organ just in front of the gonopodium and usually adhering to the coelomic wall. The entire testis is removed from the individual and placed into the drop of saline on the glass slide. The testis is then teased apart using a fine tip forceps to release the sperm packets which can then be used in the insemination process. It should be noted that the sperm packets obtained by teasing the testis apart (Fig. 10a) do not

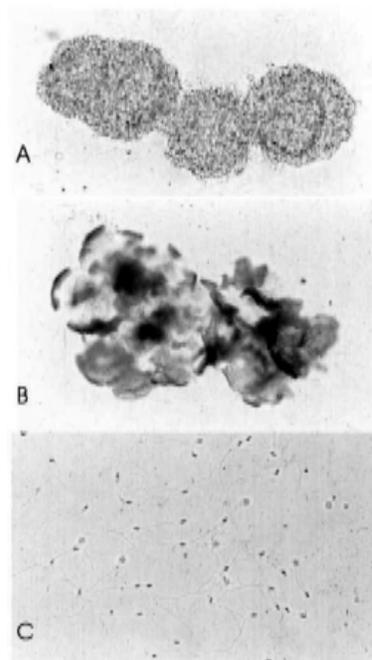


Figure 10. Sperm packets teased from the testis (A) or extruded (B) from a male swordtail. Individual sperm cells of a male swordtail (C).

have the same appearance as those that have been extruded using the first method, (Fig. 10b). If a coverslip is placed on top of the extruded sperm packets they readily break apart and individual sperm cells can be identified (Fig. 10c). The differences in appearance of the sperm packets may reflect varying stages of maturation, but sperm packets obtained by either method has produced successful results in the artificial insemination of the females.

Step 3: Preparing the Female:

Select a female to be inseminated and anesthetize as described previously. Using a damp piece of gauze or cotton balls, hold the female upside down between your thumb and index finger. Hold an open safety pin so that the pin section is parallel to the body of the female and gently insert the tip of the safety pin into the oviduct. (See bottom right photograph on cover.) Gently push the pin into the oviduct up to a depth of about one-half inch. This ensures that the oviduct is open, allowing for the placement of the sperm into the ovary.

Step 4: Insemination:

Using the glass pipette with the modified tip or the tuberculin syringe, remove an aliquot of sperm solution from the glass slide (approximately 10 packets or two drops). Insert the tip of the glass pipette or syringe containing the sperm into the oviduct (Fig. 11) following the path determined by insertion of the safety pin. The pipette or syringe tip should travel approximately one-half inch to one inch into the oviduct, depending on the size of the female. The fluid is then expelled to inseminate the female. One insemination should be sufficient for several clutches of fry.



Figure 11. Female swordtail being artificially inseminated.

Step 5: Recovery:

After removing sperm from the male or inseminating the female, place the treated fish in a container of clean, fresh water with aeration while they recover from the anesthesia. After the fish are swimming freely, return them to their respective aquaria. The females need a steady diet of a commercial feed high in protein (40-50% crude protein) and with a crude fat content of 10-15% (Tamaru et al., 2001). If available, a steady supply of a live food (e.g., mosquito larvae, *Moina*, or *Daphnia*) is also highly recommended (Tamaru et al., 1997). After three weeks, a dark spot (gravid spot) should appear on the females abdomen which is an indication that the insemination procedure was a success. At this time the females should be placed into spawning baskets which will allow the newborn fry to avoid being cannibalized. Males have been found to regenerate sufficient sperm to be used again in about two weeks.

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Appendix 1: Glossary

Allele: any of several forms of a gene usually arising through mutation.

Chi Square Test: a statistical test that examines the mathematical fit of a frequency curve to an observed frequency distribution.

F1: First filial generation (e.g., sons and daughters).

Gene: the basic unit of heredity, a linear sequence of nucleotides along a segment of DNA that provides the coded instructions for synthesis of RNA, which when translated into protein, leads to the expression of hereditary character.

Genotype: the genetic makeup of an organism with reference to a single trait, or sets of traits.

Gonopodium: a modified anal fin of a poeciliid fish serving as an organ to copulate.

Heterozygous: having dissimilar pair of genes for any given pair of hereditary characteristics.

Homozygous: having identical pair of genes for any given pair of hereditary characteristics.

Insemination: to inject semen into a female reproductive tract; to impregnate.

Lyretail: the caudal fin of a fish that has elongated top and bottom fin rays that resembles a "lyre" an ancient Greek musical instrument.

Mendelian Principle of Segregation: the principle originated by Gregor Mendel stating that during the production of gametes the two copies of each hereditary factor segregate so that offspring acquire one factor from each parent.

Phenotype: the observable constitution of an organism.

Progeny: a descendant or offspring

Punnet Square: a schematic device that aids in visualizing the behavior of genes (and their alleles) as they are inherited from one generation to the next.

Appendix 2: List of Distributors

Listing in this appendix does not constitute an endorsement of products or services. For a more comprehensive listing, consult your local extension agent or Aquaculture Magazine Buyer's Guide and Industry Directory.

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Fax: (407)-886-6787

E-mail: aes@aquaticceco.com

<http://www.aquaticceco.com>

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Argent Chemical Laboratories

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Redmond, WA 98052

Phone: (425)-885-3777/(800) 426-6258

Fax: (425)-885-2112

E-mail: email@argent-labs.com

<http://www.argent-labs.com/argent.htm>

(MS-222, 2-phenoxyethanol, laboratory equipment and supplies)

Penn Tool Co.

1776 Springfield Avenue

Maplewood, NJ 07040-2931

Phone: (800)-526-4956

Fax: (973)761-1494

E-mail: info@pentoolco.com

<http://www.penntoolco.com>

(binocular magnifier)

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