Controlling the Sex of SALMONIDS

by Grant Feist,
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PREFACE

This manual is designed to be used by aquaculturists who wish to control the sex of salmonids they are producing. The pamphlet provides instructions on how to produce all-female, all-male, and sterile populations of fish using a variety of methods. It also includes methods for the storage of eggs and sperm.

I. INTRODUCTION

There are major economic advantages to controlling sexual development in fish. Modern technologies allow for the production of monosex or sterile stocks that have several advantages. Female fish generally are more valuable than males because of the high value of eggs and the production of offspring. The use of stocks that are either all female or sterile also eliminates the occurrence of sexually precocious males, which are economically undesirable. Sterile fish may also have increased growth potential or adapt better to rearing conditions, such as transfer to seawater. Finally, repeat spawners, such as rainbow trout, can benefit aquaculture operations because manipulated stocks can continue to produce offspring of the desired sex for years.

This manual provides methodologies that you can use to produce all-female, all-male, or sterile (including triploid) stocks of salmonids for aquaculture. Furthermore, the manual describes not only the "recipes," but the theory behind the methods.

To understand how and why the methods described in the booklet can control the sex of salmonids, it is important to understand how sexual development occurs naturally in the fish. Salmonids, like mammals, appear to develop as either males or females, based on the X-Y chromosome-like system of sex determination. Embryos receive one set of chromosomes from the egg and one set from the sperm. Cells or organisms such as sperm that have one set of chromosomes are called *haploid*. Cells or organisms that have two sets are called *diploid*. All eggs carry one X chromosome, whereas half the sperm carry an X and half carry a Y chromosome. Eggs that are fertilized with a sperm carrying an X become XX and are female; those receiving a Y chromosome become XY and are male (figure 1). Fish, however, differ from mammals in that applying hormones at the right stage during early development can change their sex.

There are two general ways to produce 100% female, 100% male, or sterile populations of salmon. The first involves manipulating the chromosomes of eggs and sperm, and the second involves treating fry with sex steroid hormones. You can use male sex steroids (androgens) to produce male or sterile fish and female steroids (estrogens) to produce female fish (figure 2).

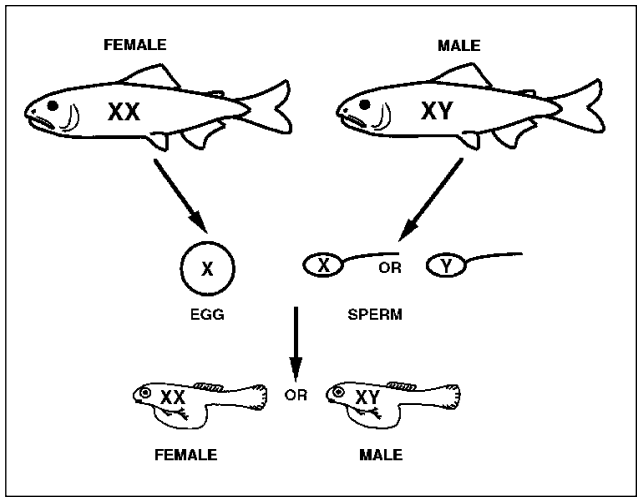


Figure 1. Sex determination in salmon

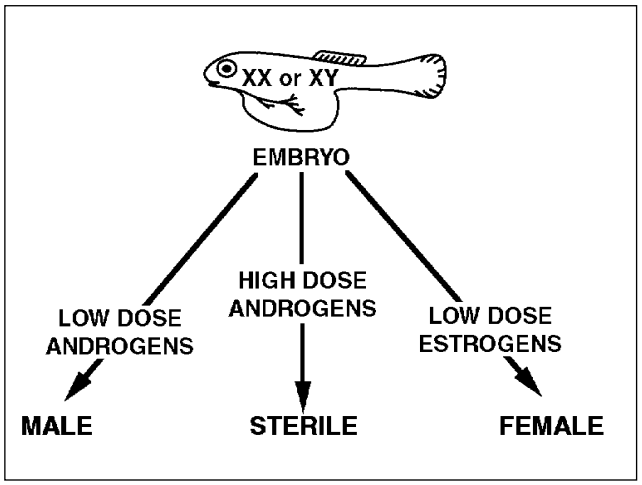


Figure 2. The effects of treating young salmon with sex steroids

Before getting into specific methodologies, we will first introduce some basic concepts of sex control.

A. Production of Females

Producing all females through chromosome manipulation is called *gynogenesis*. It involves inactivating the sperm's DNA so that it contributes no paternal chromosomes to the embryos. The eggs are essentially fooled into believing they have been fertilized. These eggs are then shocked by exposing them to heat or extreme pressure so that a second set of maternal chromosomes (called the *polar body*),

which is usually extruded upon fertilization, is retained. Resulting embryos have two sets of maternal chromosomes and are XX, or all female (figure 3).

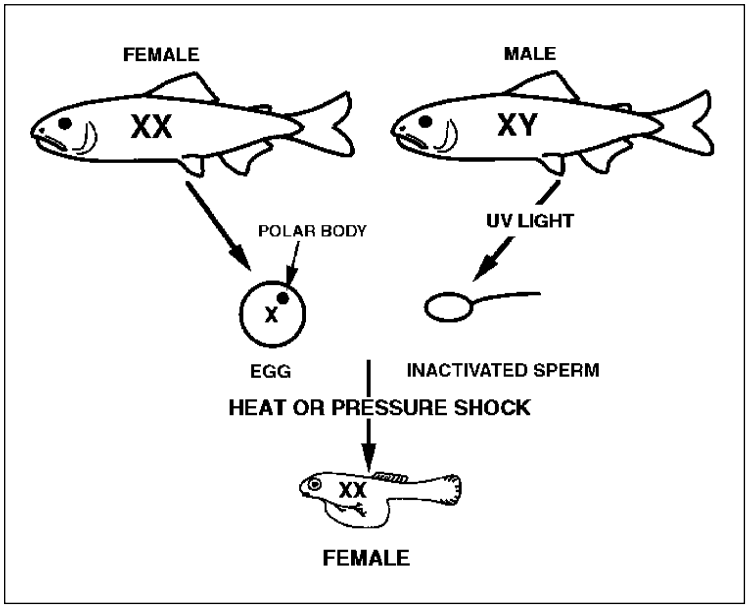


Figure 3. Creating all female populations of salmon by gynogenesis.

Treating young fish with sex steroids to produce all-female stocks usually involves using estradiol, an estrogen. You can apply this steroid by immersing newly hatched fish in it, by feeding fry with a diet treated with estradiol, or by a combination of immersion and feeding.

B. Production of Males

Androgenesis is manipulation of chromosomes to produce males. In this method the egg's DNA is inactivated and normal sperm are used for fertilization. The fertilized egg is then shocked with heat or pressure to double the paternal chromosomes. Resulting embryos are either XX (female) or YY (male) (remember that one-half the sperm carry an X chromosome and one-half carry a Y). Offspring from this process must be reared to maturity to determine which are YY and which are XX. Toxic chemicals must be used to inactivate the egg's DNA. Because of these inconveniences, the process of androgenesis is not used extensively. When bred with normal females, however, YY males produce 100% male offspring (figure 4).

A much easier way to produce male populations is to expose young fish to androgens. These male sex steroids, as in all-female production, are applied through immersion or feeding.

C. Production of Sterile Fish

The process by which chromosomes are manipulated to sterilize fish is called *triploidy*. This method uses normal sperm to fertilize the eggs. A heat or pressure shock is then applied, which results in retention of the extra set of maternal chromosomes (polar body). Resulting embryos now have three sets of chromosomes, one of paternal origin and two from the mother. Animals with odd numbers of sets of chromosomes are generally sterile. Fish with three sets of chromosomes are called *triploids* (figure 5).

Sterile fish also can be produced by treating young fish with very high doses of androgens. These doses are much greater than those used for sex reversal. The steroids can be applied through immersion or feeding.

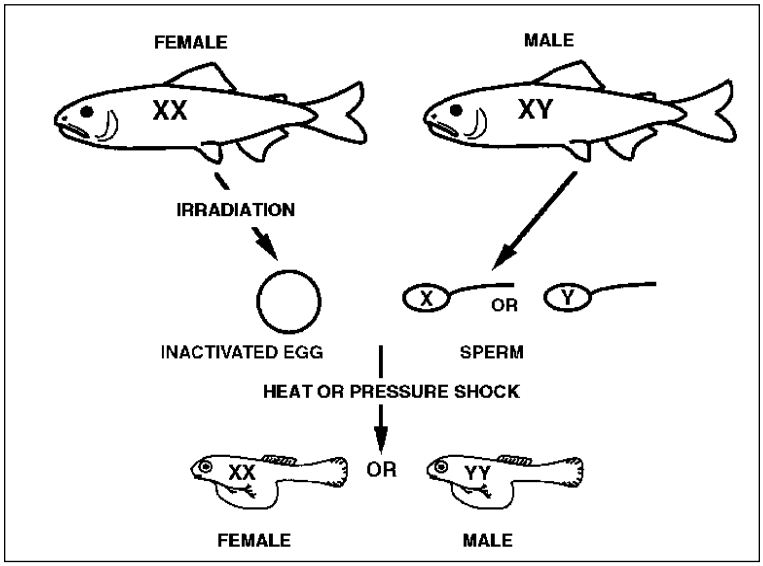


Figure 4. Creating male salmon by androgenesis

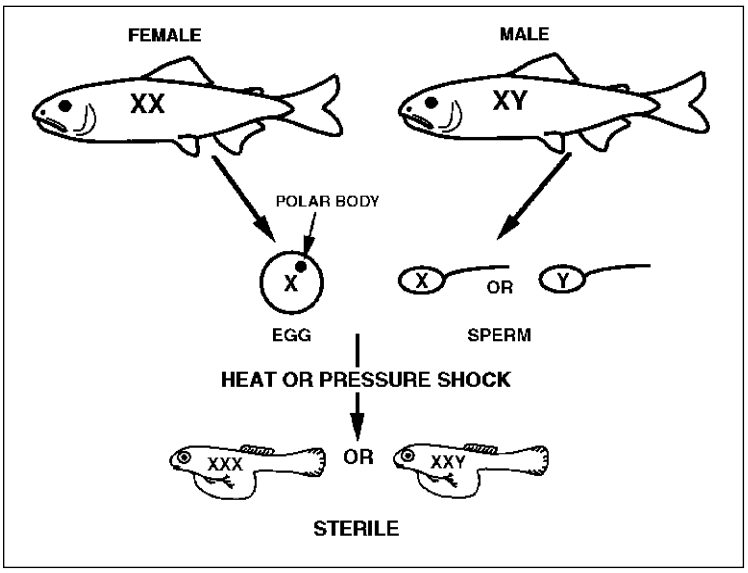


Figure 5. Creating sterile salmon by triploidy

II. CHROMOSOME SET MANIPULATIONS

A. Gynogenesis

The production of all female populations of salmonids by gynogenesis requires that sperm be inactivated before it is used to fertilize eggs. Fertilized eggs are then shocked to induce polar body retention. This technique produces all-female populations for all species of salmonids except coho salmon. Although the procedure seems complicated, it is very simple.

You will need the following items to perform gynogenesis.

1. sperm extender solution
2. UV light source
3. UV light meter
4. UV goggles
5. glass trays
6. hot-water bath

The easiest way to inactivate sperm is to expose them to ultraviolet (UV) light. Other forms of radiation also will inactivate sperm, but these are very hazardous and are not recommended except in laboratory settings. Shortwave (254 nanometer) germicidal lamps, which operate in standard fluorescent fixtures, and UV light meters are available from several sources (see Appendix 1). Direct or reflected UV light is damaging to both skin and eyes. Wear protective clothing and UV goggles when working with UV light sources.

UV light causes links to form in the DNA of the sperm so that it cannot combine with the DNA of the egg following fertilization. Since UV light does not penetrate liquids very far, it is important that all of the sperm be exposed to the radiation because even a small amount of activated sperm can produce many fish that are not female. To ensure that all the sperm are exposed, dilute the sperm with a sperm-extender solution and spread the mixture as a thin layer on glass trays before irradiating. See Appendix 2 for a chart of weights, volumes, abbreviations, and conversions.

Salmonid sperm remain viable for only short periods of time following exposure to water. Extender solutions are composed of high concentrations of salts or sugars that keep the sperm viable until they are diluted with water. There are many kinds of sperm-extender solutions, but we recommend two. The first is a glucose solution. To prepare this solution simply add 54 grams of glucose to 1 liter of distilled water, or 27 grams to 0.5 liters, and so on. The second extender, which works slightly better than the glucose solution, is composed of several salts and glucose. Appendix 3 gives the recipe for this extender.

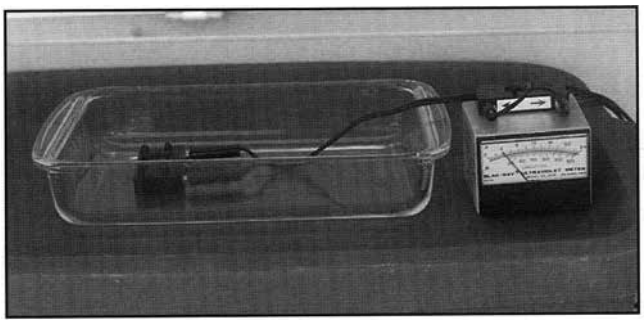
Sperm should receive a total radiation dose of 12,000 to 15,000 ergs/mm^2. Most UV light meters measure UV light as microwatts/cm^2 (1 microwatt = 10,000,000 ergs/second). Irradiating the sperm at 400 microwatts/cm^2 for 2 to 2.5 minutes should inactivate all the sperm. Glass baking trays work well for this procedure.

The process of sperm inactivation is shown in figure 6. Place the light meter in the glass tray and raise or lower the light source or the tray until the meter reads 400 microwatts/cm^2. Move the meter to all areas of the tray to make sure that the light is evenly distributed.

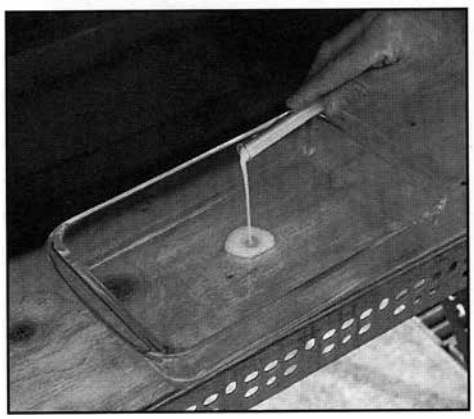
Dilute sperm in the extender to 10%, or 1 part sperm to 9 parts extender. Mix the diluted sperm well, pour it into the glass container, and shake it from side to side so that the sperm is distributed evenly. It is very important that the diluted sperm in the tray be about 0.5 mm deep or less so that all

of it is exposed to UV light. For example, 10 ml of diluted sperm in a 16- x 25-cm tray will have a depth of about 0.25 mm. Next place the tray under the lamp for 2 to 2.5 minutes. Be sure the tray is even so the sperm does not form pools that will result in liquid depths greater than 0.5 mm. After 2 to 2.5 minutes place the eggs directly in the tray containing inactivated sperm and cover them with water. We have found that 10 ml of diluted sperm fertilizes about 1500 salmon eggs or about 3000 trout eggs. After 2 minutes rinse the eggs with water to remove excess sperm and cover them with water for an additional 8 minutes. It is important to apply the heat shock to the eggs around 10 minutes after fertilization.

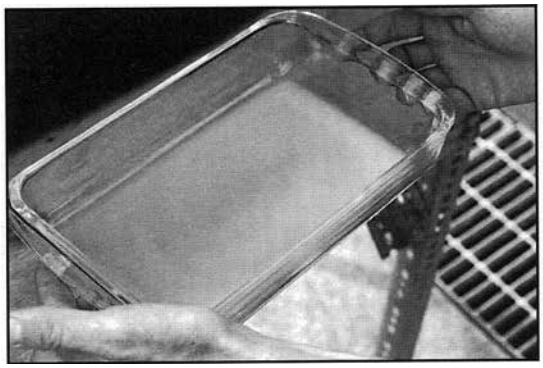
Heat shock eggs at either 29 °C for 10 minutes or 26 °C for 20 minutes. Both temperatures will give



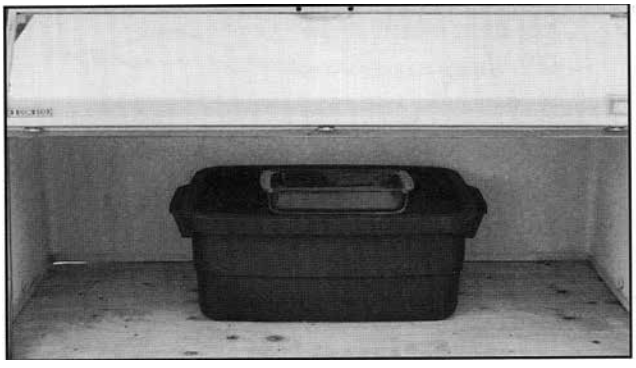
1. Place light meter in glass tray.



2. Dilute sperm in the extender and pour into tray.



3. Shake tray to distribute sperm.

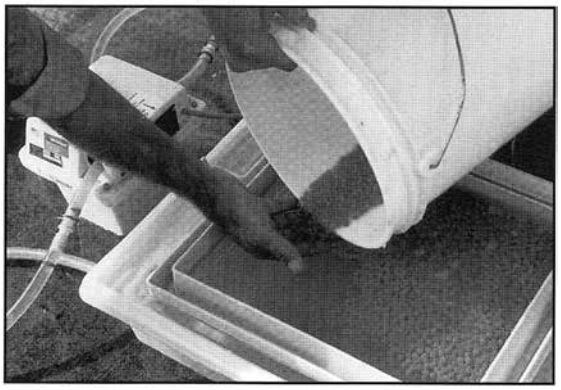


4. Irradiate sperm.

Figure 6. The process of sperm inactivation

similar results, but the heat shock must be at least 15 degrees higher than the ambient water temperature. Temperatures greater then 29 °C will kill most salmonid eggs. Eggs can be heat shocked in a variety of containers, including PVC rings, floating mesh baskets, or the bottom tray of a Heath tray (figure 7). Aerate the water in the bath and use a circulating bath to ensure even distribution of heat shock to the eggs.

You can place eggs directly in Heath trays for water hardening following the heat shock. Gynogenesis typically results in egg mortality



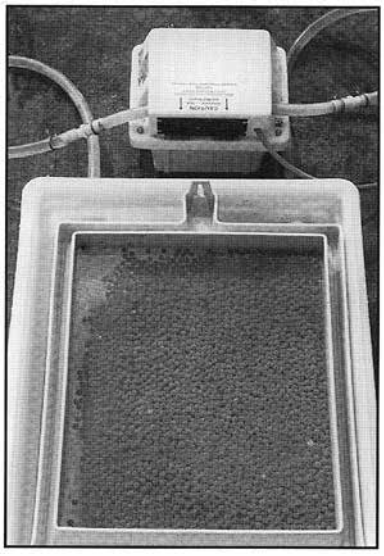


Figure 7. Heat shocking eggs

of 30 to 90%. Keep fungus down with a formalin drip during incubation, and pick the eggs carefully during the eyed stage.

Gynogenesis can be accomplished only if none of the sperm's DNA is incorporated into the egg. There are many ways to verify that gynogenetic techniques have been successful. The first is to ensure that 100% female populations have been produced.

You can determine the sex of salmonids visually at age 6 months or greater by dissection. The gonad lies underneath the kidney and air bladder (figure 8). To dissect the fish, cut from the vent up to the gills and gently pull away the gut (figure 9).

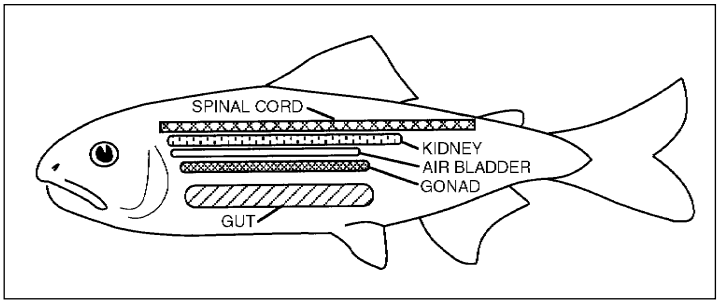


Figure 8. Diagram of location of gonads, which are paired and lie to the side of the kidney

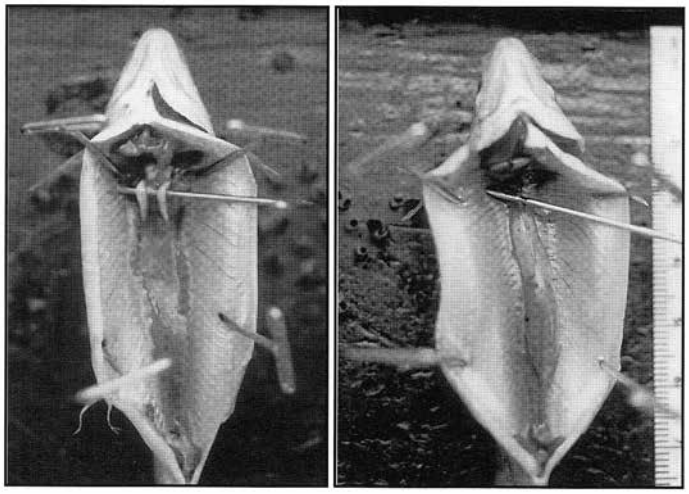


Figure 9. Opened fish showing female (left) and male (right) gonads, which are lifted by pins

Ovaries appear as enlarged tissue toward the head of the fish, whereas testes are much thinner. Closer examination of ovaries should reveal the presence of many tiny spheres, which are oocytes, or the future eggs.

Another way to verify successful gynogenesis is to use irradiated sperm from a closely related species. You can recognize any paternal contribution to the egg by either inviable hybrids or morphologically distinct hybrids (see section IV D). You also can employ color or morphological markers to provide proof of gynogenesis when using sperm from the same species to fertilize eggs. If the sperm carries dominant color or morphological genes, then any offspring exhibiting these characters contain at least part of the sperm's DNA and gynogenesis has not occurred.

B. Triploidy

You can produce sterile or triploid animals by fertilizing eggs with normal sperm and then heat shocking the egg to induce polar body retention. Triploid fish contain three sets of chromosomes. One-half the fish will be XXY, or triploid males, and one-half will be XXX, or triploid females. Both males and females should be sterile. To produce triploid fish simply fertilize the eggs with normal sperm, wait 10 minutes, and then heat shock the eggs the same way as for gynogenesis, either at 29 °C for 10 minutes or at 26 °C for 20 minutes.

Triploid males will probably show more gonadal development than triploid females. Gonads from a mature triploid male may appear similar to those of a normal fish except they will be smaller and will not produce sperm. Gonadal development should be inhibited in triploid females. Triploid females should have stringlike gonads. Gonads of both triploid males and females should look like those of normal males if they are sexed at around six months of age (see figure 9).

Triploid individuals should have one-third more DNA in their cells because they have three sets of chromosomes, compared with two sets in normal diploid animals. You can buy expensive equipment that can measure the amount of DNA in an individual's blood cells, but the easiest way to verify that triploid animals have been produced is to sex them by dissection after they're six months old and see whether all the fish have similar malelike gonads. Hybrid triploids also can be produced by fertilizing eggs with sperm from closely related species (see section IV D).

C. Androgenesis and Tetraploidy

Androgenesis is the method used to produce YY males. With this technique the DNA of the egg is inactivated before being fertilized with normal sperm. The fertilized egg is then shocked by temperature, pressure, or chemical treatment to suppress the first cell division. Resulting animals are either XX females or YY males. *Tetraploidy* is the production of animals with four sets of chromosomes. Normally fertilized eggs are shocked the same way as in androgenesis. Resulting animals are either XXXX females or XXYY males. Crossing tetraploid fish with normal diploid fish results in sterile triploid offspring, which contain three sets of chromosomes.

Both androgenesis and tetraploidy are very difficult techniques to perform. It is hard to inactivate the DNA of the egg with UV light, and you must use toxic chemicals to accomplish this. Suppression of the first cell division of the egg is also very difficult. For these reasons androgenesis and tetraploidy are recommended for laboratory settings only. For an easier way to produce YY males, see section IV B.

III. HORMONES

Aquaculturists can change the sex of a fish by exposing the fish to sex steroid hormones at the right time during early development. Exposure to androgens can produce males; exposure to estrogens can produce females. Using hormones to control sex has several advantages over manipulating chromosomes, including higher survival rates and less expensive or less cumbersome methodologies. The disadvantage in using hormones to control the sex of salmon is that some compounds used to reverse the sex of fish cannot be used in animals produced for human consumption. Check the rules and regulations of the United States Department of Agriculture, the Federal Drug Administration, and the Drug Enforcement Agency before using these agents. Also, exercise care when handling steroids, for example, when preparing diet formulations.

Exposure to steroids does not always result in complete sex reversal. It is possible to obtain 100% female or male populations, but a small percentage of the animals may become hermaphrodites (intersex) or sterile. Exposure to low doses of androgens will generally increase normal growth rates, whereas exposure to low doses of estrogens or high doses of androgens will generally decrease growth rates.

A. Production of Females

The steroid most commonly used to sex-reverse salmon into females is 17-beta-estradiol (17b-estradiol), a naturally occurring hormone. You may also use synthetic steroids such as estradiol benzoate or diethylstilbestrol. See Appendix 1 for a list of sources of steroids. Very small doses of estradiol will sex-reverse most salmonids, except for rainbow trout, which require slightly higher doses. See Appendix 2 for a chart of weights, volumes, abbreviations, and conversions.

The easiest way to apply the steroid is to incorporate it into the fish food. You can do this by dissolving the steroid in pure ethanol and then spraying it on the food. Feed estradiol at 5 parts per million (ppm) for 8 to 12 weeks (approximately 670 to 1000 ˚C days) beginning at the onset of feeding (see table 1). Feed the fish at least three or four times daily. This dose will result in nearly 100% female populations. Rainbow trout, unlike coho, chinook, chum, and so on, require 20 ppm for 8 to 12 weeks. Parts per million is the same as milligrams (mg) of steroid/kilograms (kg) of diet.

Table 1. Sex-reversing salmon to females by exposure to estradiol

METHOD	DOSE	DURATION	WHEN
feeding	5 or 20 ppm	8-12 weeks	onset of feeding
immersion	400 µg/l	2 hours	at 50% hatch

You will need a sensitive scale to measure out the estradiol, or you can buy the steroid in premeasured quantities. Wear latex gloves and a filter mask when handling the steroid.

For example, to make 1 kg of feed containing 5 ppm estradiol, dissolve 1 gram of estradiol in 1 liter of ethanol. This produces a concentration of 1 mg/ml. Take 5 ml of this solution, add it to 50 ml of ethanol, and place the mixture in a spray bottle. Spread 1 kg of food into a thin layer on aluminum foil, cookie sheets, or something similar. Next spray the food until it is moist. It should take only a small part of the solution to do this. Mix the diet thoroughly with a spatula and repeat the process until all of the solution has been used. This will ensure that the steroid is evenly distributed throughout the food. Next add 20 ml more of ethanol to the bottle to rinse out any leftover steroid, and apply it to the food in a similar manner. Let the food sit out overnight until the smell of ethanol is gone.

Another way to apply the steroid is to immerse the fish in a very weak solution of estradiol. The timing of this immersion is critical. Immerse fish on the day when one-half of the fish from an egg take (50% of the eggs in an incubation tray) have hatched (a "50% hatch"). Immersing animals later or earlier will result in a decrease in females. The advantage of this technique is that it requires only one treatment with a very small amount of steroid. A single immersion can result in more than 80% of the animals developing as females. Note that this technique works for chinook, coho, chum, and so on, but it does not appear to work with rainbow trout.

Immerse animals in 400 mg of estradiol/l of water for a period of 2 hours (see table 1). Make sure the water in which you are immersing the animals is well aerated (use an air pump or an oxygen cylinder). There will be some variation in the effects of immersion depending on the particular hatchery system. You may have to make minor adjustments to the dose or the duration of the dose for more efficient sex reversal. Check some fish for gonadal development (see figure 9) to determine the amount of sex reversal.

For example, to immerse animals in 10 liters of water, take 4 ml from a solution of 1 mg of estradiol/ml of ethanol and add it to 10 l of hatchery water. This will make 400 mg/l. Try to keep the concentration of ethanol in the water at about 0.04%. Next place the eyed eggs and hatchlings in the solution for 2 hours. After immersion, rinse the fish with water to remove excess steroid.

We suggest placing the fish in baskets or containers before immersing them. This makes it easier to carry the fish from the incubation trays to the immersion water. It is also a good way to keep treated animals separate from untreated ones. We have used Tupperware containers with the tops and bottoms removed and screens inserted. This works well because the containers float. You can also use plexiglas or other plastic containers. Make sure there is adequate circulation through the container.

Fish can also be immersed while in an incubation tray (the tray in which the eggs are placed after fertilization). Determine the volume of water in the tray, pull the tray out of the water flow, and add the amount of estradiol in ethanol to make 400 mg/l. Make sure the water is well aerated and the steroid is evenly distributed throughout it. After 2 hours simply return the tray to the water flow. Remember that any other trays beneath the treatment tray will be exposed to the steroid. You can reinsert trays from the top down in the stack to help flush out any remaining steroid.

You also can employ a combination of immersion and feeding. This should increase the percentage of females produced.

B. Production of Males

There are a number of synthetic androgens that will masculinize salmon. We suggest using either the synthetic steroid 17-alpha-methyltestosterone, or MT (17a-methyltestosterone), or the naturally occurring steroid 11b-hydroxyandrostenedione (OHA). As with estradiol, these two steroids can be applied through immersion or feeding. Unlike estradiol, the use of MT or OHA will also effectively sex-reverse rainbow trout and not just salmon.

Feed fish to be exposed to these steroids at 0.5–3 ppm (0.5–3 mg steroid/kg of diet) for MT and at 3 ppm for OHA for 8 weeks (table 2). Prepare the steroid solutions and diet in the same manner as for estradiol.

Although treatment with androgen should produce nearly 100% males, some of the fish fed MT will be functionally sterile. This is because they do not develop sperm ducts (the duct that carries sperm from the gonad to the vent). If sperm is required from these sex-reversed animals, it will have to be removed surgically. Fewer fish will become male if treated with 0.5 ppm MT but more will have intact sperm ducts. Similarly, more fish will become male if given 3 ppm MT but fewer will have intact sperm ducts. The majority of fish fed OHA, however, will have intact sperm ducts. If functional males are required, we suggest feeding OHA at 3 ppm or MT at 0.5–1 ppm.

Table 2. Sex-reversing salmon to males by exposure to MT or OHA

STEROID	METHOD	DOSE	DURATION	WHEN
MT	feeding	0.5-3 ppm	8 weeks	onset of feeding
MT	immersion	400 µg/l	2 hours	1 week after 50% hatch
OHA	feeding	3 ppm	8 weeks	onset of feeding

For immersion, fish should be exposed once to 400 mg/l for 2 hours (table 2). As with females, the timing of this exposure is critical. Immerse fish at 1 week after 50% hatch. The immersion technique is the same as that for producing females. This technique works only with MT and not with OHA. A combination of immersion and feeding for OHA should produce higher percentages of males than feeding alone. An advantage to immersing fish in MT is that a large percentage of the males produced will have intact sperm ducts.

In summary, if functional males are required, we suggest feeding the fish OHA or immersing them in MT. If masculinization only is required, we suggest feeding MT.

C. Production of Sterile Fish

High doses of androgens will sterilize fish but may result in decreased growth rates and increased mortality. Lower doses of androgens also can produce functionally sterile fish. Remember that exposure to MT at 3 ppm for 8 weeks produces males that usually do not develop a sperm duct.

To sterilize fish with MT, feed them at 25 to 100 ppm for 15 to 17 weeks. Higher doses (50 to 100 ppm) may result in a feminizing effect in some of the fish. This is called "paradoxical feminization," since treatment with androgen results in the formation of females. Immersing fish in MT to sterilize them is impractical since they must be immersed for long periods of time or with very high doses.

IV. BREEDING

Exposing fish to hormones or manipulating sets of chromosomes will result in fish that, when bred, produce all-female, all-male, or sterile populations. You also can employ a combination of administering hormones and manipulating chromosomes. These techniques are particularly useful for animals that can be bred more than once, such as rainbow trout. Established broodstock can produce monosex populations for years. Furthermore, gametes (sperm or eggs) from such broodstock may be stored for long periods of time (see section V).

A. Production of Females

Treating all-female populations obtained by gynogenesis with MT produces males whose sperm carry only X chromosomes ("XX males"). Breeding

these sex-reversed males with normal females then produces 100% female populations (figure 10).

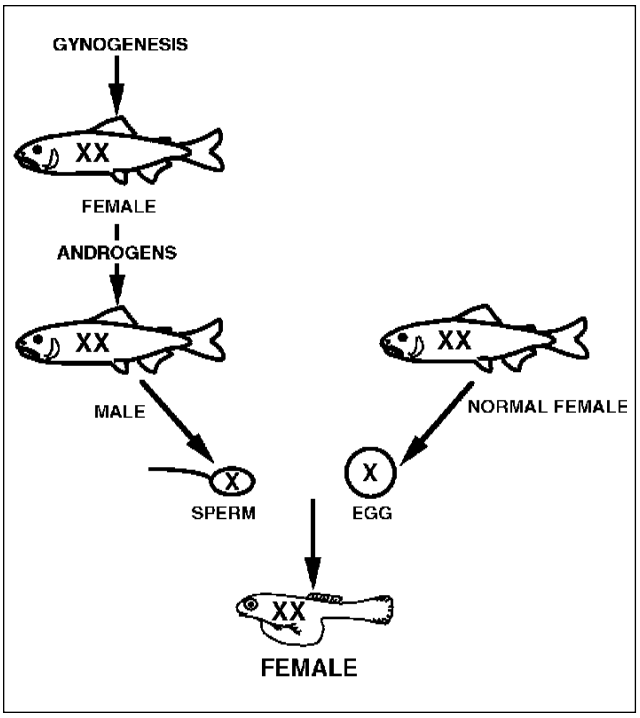


Figure 10. Creating females by combining gynogenesis and hormone treatment

An easier way to obtain "XX males" is to treat normal bisexual groups of fish with MT, which will produce a population that contains 50% genetic males and 50% sex-reversed males. Crossing all of these males with normal females will produce 75% females and 25% males (figure 11). This technique is a quick way to obtain a larger proportion of females without having to perform gynogenesis. To obtain "XX males," simply keep track (by tagging, isolation, and so on) of which males produce all-female offspring. You can then breed these animals in the future or store their sperm to produce additional all-female groups.

B. Production of Males

Producing males by treating androgenetic populations of fish is not as easy as producing females. This is because half of the fish produced by androgenesis will be female (XX) and half will be male (YY), and it is not possible to readily tell which are which until maturity. Androgenetic populations can, however, be treated with estradiol to produce populations that are 75% male (figure 12).

YY males also will be produced, and they can be kept track of and bred to produce all-male populations, as for "XX males." An easier way to produce males and obtain YY males is to treat normal bisexual groups of fish with estradiol, which will result in a population of 62.5% males, 12.5% of which will also be YY males (figure 13).

C. Production of Sterile Fish

You can produce sterile fish by breeding tetraploid animals with normal diploids (see section II C). Resulting offspring will have three sets of chromosomes (triploid) and thus will be sterile.

D. Hybrids

Another way to produce sterile fish for culture is to cross males and females from two different species. This method is called interspecies hybridization. Interspecies hybrids may have the added benefit of possessing the advantageous attributes of their parental species. Of course, they may also exhibit the less favorable features, much as a "rabbage" has the roots of a cabbage and the leaves of a radish.

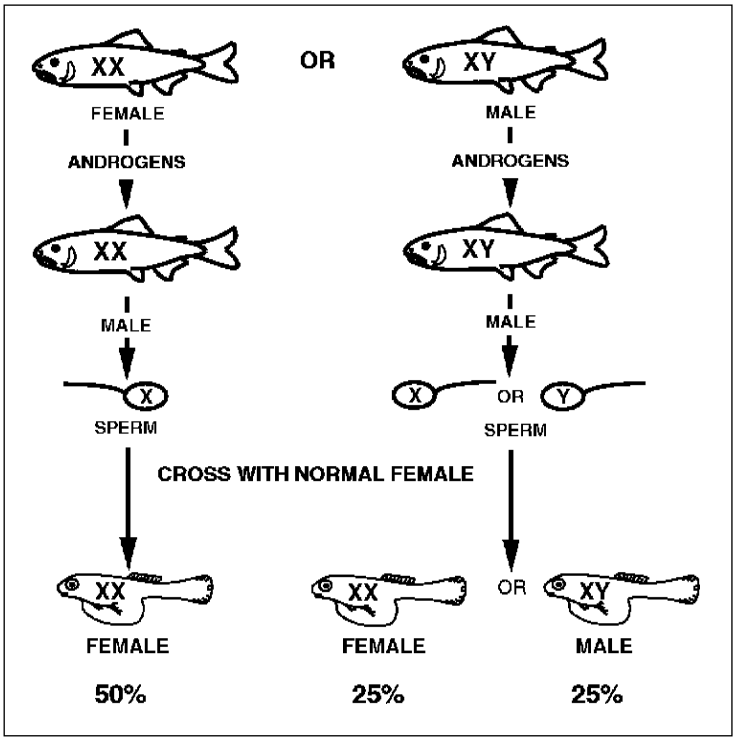


Figure 11. Creating females by breeding hormone-treated fish

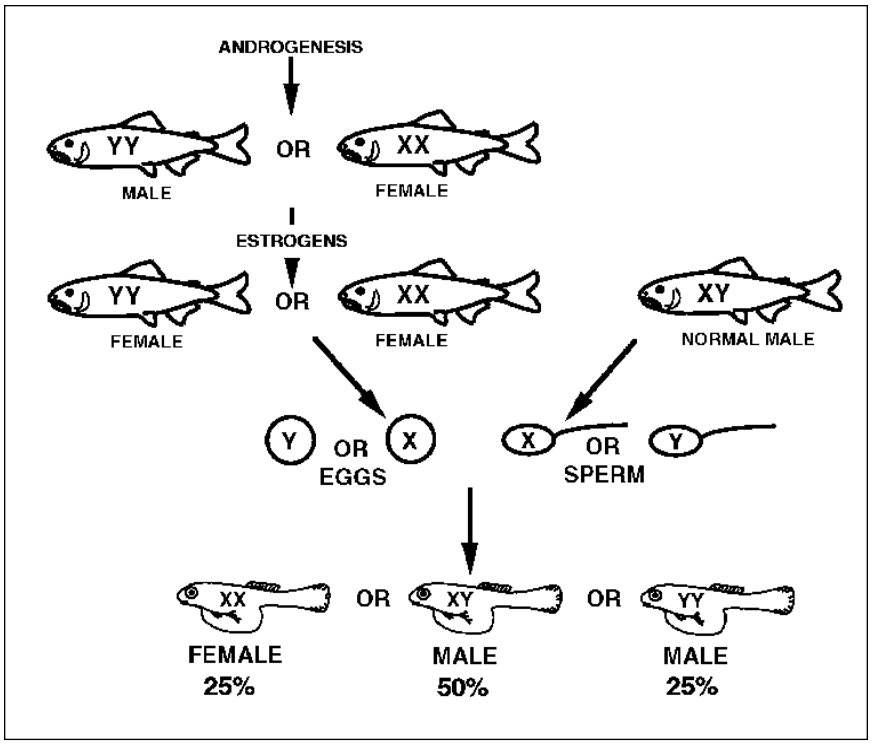


Figure 12. Creating males by combining androgenesis and hormone treatments

You can make diploid hybrids simply by fertilizing the eggs of one species with the sperm of a second. Not all hybrids between species are viable; moreover, the direction of the cross (that is, which species is male and which is female) may be important. For example, the chum female by chinook male cross is viable, but the chinook female by chum male is not. Table 3 summarizes the information about the viability and sexual maturation of salmonid interspecies hybrids.

Viable diploid hybrids among Pacific salmon species are often sterile or nearly sterile. Because each species has different chromosomes, there is no way for the chromosomes to pair up to produce eggs or sperm. Sterility has two potential advantages in aquaculture: sterile fish tend to grow more than normal fish because they are not putting energy into forming eggs or sperm, and sterile fish are unable to breed with wild populations.

One would expect triploidy in interspecies hybrids to further disrupt sperm and egg formation and repress gonadal development. You can produce triploid hybrids by using polar body retention techniques described in section II A. For these hybrids, the female parent

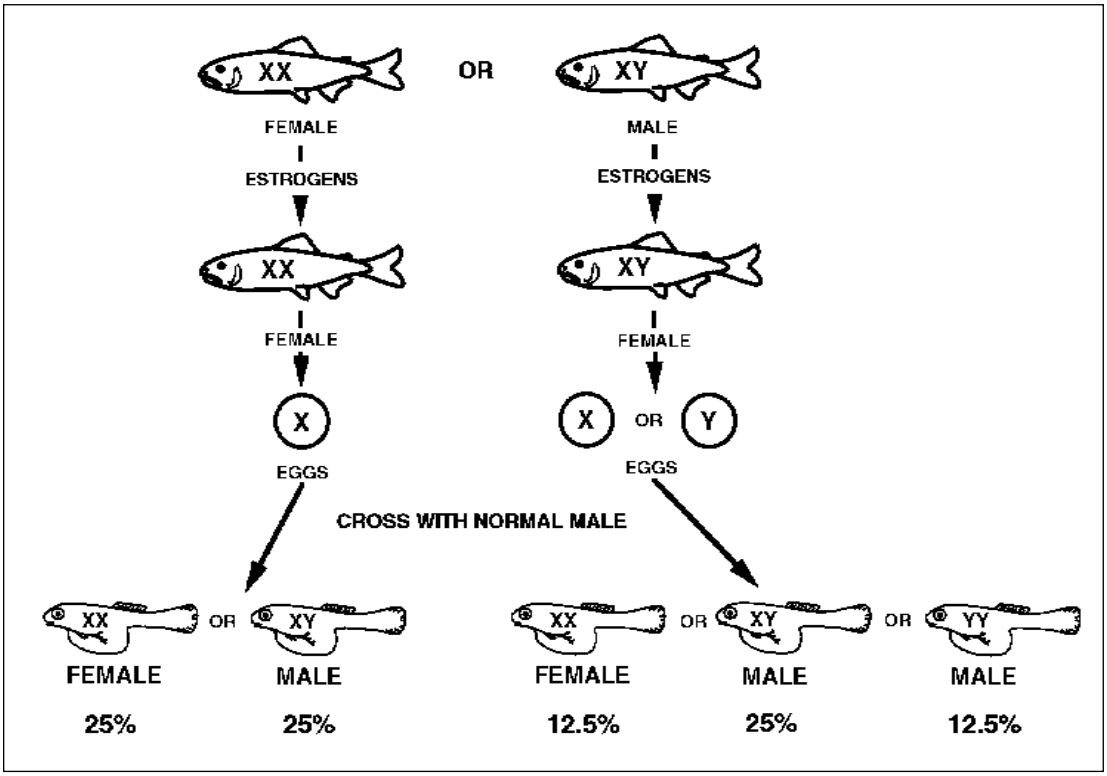


Figure 13. Creating males by breeding hormone-treated fish

Table 3. Viability and maturation characteristics for diploid (2n) and triploid (3N) hybrids between selected salmonid species. The three entries in each block are

(1) viability: G = good; P = poor; 0 = none
(2) gonad development: Y = yes; N = little or none; ? = lack of information
(3) fertile in some back crosses: F = fertile; I = infertile; ? = lack of information

In addition, (m) means that the observation was made for males and (f) for females.

Female Species		Male Species: pink	chum	sockeye	chinook	coho	masu	rainbow	Atlantic
pink	2n	G; Y; F	G; Y; I	P; ?; ?	G; Y; ?;*	G; ?; ?	G; ?; ?		0
	3N	G; Y; ?	G; Y (m); ?		G; Y; ?;*				
chum	2n	G; Y; I	G; Y; F	G; Y; I	G; Y (m); ?;*	P; Y; F (f)	P; ?; ?	0	0
	3N	G; ?; ?	G; ?; ?		G; Y (m); ?;*	P; ?; ?			
sockeye	2n	0	G; Y; F	G; Y; F	G; Y (m); ?	0	0	0	
	3N			G; ?; ?					
chinook	2n	G; Y; ?;*	0	0	G; Y; F	P; Y (m); F (m)			
	3N	G; Y (m); ?;*	0		G; Y; ?	P; ?; ?			
coho	2n	P; ?; ?	0	P; ?; ?	G; ?; ?	G; Y; F		P-0; ?; ?	0
	3N		0			P; ?; ?		0	0
masu	2n	P; ?; ?	0	0			G; Y; F	0	
	3N						G; ?; ?		
rainbow	2n		0	0		G-P; ?; ?;**	P; ?; ?	G; Y; F	0
	3N		P; ?; ?			0-P; ?; ?		G; Y; I	P; ?; ?
Atlantic	2n		0			0		0	G; Y; F
	3N		0			0		0	G; ?; ?

* These fish exhibit early seawater tolerance and high growth rates.
** These fish exhibit increased resistance to IHN.

contributes two chromosome sets and the male parent one set. As with diploid hybrids, the direction of the cross can be important for viability. Because the female parent provides more genetic material, the triploid hybrid is more likely to resemble the female than the male parent.

As with single-species triploids, the growth and survival of triploid hybrids may be inferior to those of comparable diploid hybrids. However, in some cases it appears that triploidy actually increases the viability of interspecies hybrids. The benefit of the triploid hybrid should be realized when normal conspecific diploid fish begin to mature sexually. At this time, the triploid fish, particularly females, should continue growing rather than developing gonads. The conditions required to induce polar body retention depend on the temperature of the water in which the fish are being held (ambient temperature—see section II A). The degree of temperature change appears to be more important in providing the shock than the actual temperature. You can obtain induction of triploidy, which averaged 90% for chum, pink, and chinook salmon and their hybrids, by elevating the water temperature 10 minutes after fertilization from ambient 10 to 26 °C for 20 minutes. Induction of triploidy, which averaged 95% for chum, pink, and chinook salmon and their hybrids, was obtained by elevating the temperature 10 minutes after fertilization from ambient 14.5 to 28 °C for 20 minutes. The higher level of triploid induction was accompanied by decreased survival from egg to hatching. If you require higher efficiency, you may want to run several tests to determine the best temperature shock treatment.

V. SPERM AND EGG STORAGE

Although it is recommended that you use sperm or eggs as soon as they are taken out of the fish, it is possible to store salmonid gametes. Short-term storage (up to 20 days) is possible for both sperm and eggs, whereas long-term storage (up to years) is possible only for sperm. You also can use short-term storage when transporting gametes. Long-term storage of sperm is called cryopreservation.

A. Short-Term Storage

Short-term storage of sperm and eggs may be required when you transport gametes or when males and females mature at different times. Store sperm or eggs at cool temperatures and keep them under oxygen. We do not recommend that you leave gametes inside fish carcasses. If you must do this, keep the carcasses cool. After three hours the fertilization rate of gametes held inside carcasses begins to decrease dramatically.

To transport eggs or sperm, simply fill a cooler with crushed ice and place newspapers on top of the ice. Place gametes in plastic bags on top of the newspapers. Adding oxygen to the bags (especially with sperm) greatly increases the viability of the gametes. Treat sperm in this manner if you hold it for short periods during gynogenesis or triploidy.

Optimum conditions for short-term storage of sperm are temperatures of 1 to 5 °C and the presence of oxygen and antibiotics. Add antibiotics as 500 international units (IU) penicillin and 6,000 IU streptomycin per ml of semen. You can hold sperm under these conditions for up to 20 days with only about a 20% decrease in viability. Store salmon eggs at about 3 °C under oxygen. Eggs lose their viability more rapidly than sperm. The easiest way to store gametes is to place them in sealable plastic bags, fill the bags with oxygen, and place them in a refrigerator at about 3 to 5 °C.

B. Long-Term Storage (Cryopreservation)

Cryopreservation of sperm involves freezing the sperm at very low temperatures and then storing it in liquid nitrogen. Although liquid nitrogen storage facilities are expensive, valuable sperm, such as that from XX or YY males, can be stored for years and used to produce thousands of offspring. Before freezing sperm, dilute it in a solution containing cryoprotectants so that ice crystals do not form and cause cell injury. Dilute sperm at 1 part semen to 3 or 4 parts extender. There are many kinds of extenders but we recommend a solution composed of 5.4% glucose, 9% DMSO (dimethyl sulfoxide), and 10% fresh hen's yolk. Mix these constituents well and cool them to 4 °C before adding the sperm at 1 part semen to 3 parts extender. Then mix the sperm and extender well also.

Exercise care when handling liquid nitrogen or

dry ice (frozen carbon dioxide). Both are extremely cold (-196 ºC for liquid nitrogen and -57 ºC for dry ice). You can obtain containers, gloves, and cryopreservation tubes from the vendors listed in Appendix 1.

Place the diluted sperm in a cryopreservation tube with a screw cap, and leave an airspace of about 20% of the volume of sperm to be frozen. For example, 1 ml of sperm would be 0.2 ml of air space, 5 ml would be 1 ml of air space. It is important that the tubes have a screw cap, or the sperm will explode out of the tube when it is removed from the liquid nitrogen. You also can use plastic straws for this purpose. Use a hot pair of thin needle-nosed pliers or a hemostat to crimp the end of a plastic straw. Fill the straw with diluted sperm, leave an airspace, and then crimp the other end.

Freeze diluted sperm in straws or tubes gradually before placing them in liquid nitrogen. Placing the sperm directly in liquid nitrogen is very stressful to the cells. Place the filled tubes or straws directly on crushed dry ice in a cooler for at least 5 minutes before placing them in liquid nitrogen for at least 60 minutes. Once the sperm is in liquid nitrogen you can store it for years as long as you keep it frozen.

To fertilize eggs with cryopreserved sperm, simply remove them from the liquid nitrogen, thaw them at 5 ºC, add the thawed sperm to the eggs, and activate with water. We have also found that you can simply crack the tubes open, add the frozen pellet to the eggs, activate the mixture with water, and mix. You can expect a fertilization success rate of around 50% when using cryopreserved sperm.

VI. CLOSING REMARKS

Most of the techniques described in this brochure may seem complicated but they are in fact relatively easy to carry out. If you take time to learn the basis of how sexual development is controlled by the fish itself, described in the introduction, you will have a better understanding of how and why these methods control sex. Some experimentation is required for these methods to work correctly. Remember to be patient and keep detailed notes of the steps you take when performing these techniques. If the methods are not working or are only partially working, try making small changes in how you perform the chromosome set manipulations or hormone treatments. These changes should alter the outcome and, we hope, result in an efficient method of obtaining the result you want.

APPENDICES

Appendix 1. Sources of Products

(This is a partial list of many possible suppliers and is not intended as an endorsement of these vendors.)

Steroids

ICN Biomedicals, Inc.
3300 Hyland Avenue
Costa Mesa, CA 92626 USA
Telephone 800-854-0530

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178-9916 USA
Telephone 800-325-3010

Steraloids Inc.
P.O. Box 310
Wilton, NH 03086 USA
Telephone 603-654-9509

UV lamps, meters, protective eye wear, water baths, and cryopreservation

Baxter Diagnostics, Inc.
Scientific Products Division
1430 Waukegan Road
McGaw Park, IL 60085-6787 USA
Telephone 800-234-5227

VWR Scientific
Many U.S. locations
Telephone 800-932-5000

Whatman LabSales
P.O. Box 1359
Hillsboro, OR 97123-9981
Telephone 800-942-8626

Appendix 2. Weights, Volumes, and Conversions

WEIGHT	UNIT	ABBREVIATION	# OF GRAMS
	gram	g	—
	kilogram	kg	1000
	milligram	mg	1/1000
	microgram	µg	1/1,000,000
	pound	lb	453.6
VOLUME			# OF LITERS
	liter	l	—
	milliliter	ml	1/1000
	gallon	gal	3.8

Appendix 3. Sperm Extender Solution

Solution I

KCl	9.0 g/l
NaCl	2.35 g/l
NaH_2PO_4	0.51 g/l
$MgSO_4.7H_2O$	0.29 g/l
$CaCl.2H_2O$	0.29 g/l

Solution II

$NaHCO_3$	5.0 g/l
Glucose	5.0 g/l
Streptomycin sulfate*	6.0 g/l
Benzylpenicillin, potassium salt*	5,000,000 IU/l

Solutions may be stored frozen separately.

Mix 4 parts solution I to 1 part solution II.

For storing extended sperm, use 1 part mixture to 1 part fresh sperm. For immediate use of extended sperm, use 9 parts mixture to 1 part fresh sperm.

*Not necessary if solutions are not to be used for storage (for example, gynogenesis).

Glossary

androgen	Male sex steroid
androgenesis	The method used to produce males with two Y chromosomes
diploid	Cells or organisms that have two sets of chromosomes
estrogen	Female sex steroid
haploid	Cells or organisms that have one set of chromosomes, such as sperm
polar body	The extra set of maternal chromosomes in an egg
triploid	Having three sets of chromosomes. A triploid animal is sterile.
triploidy	The process by which chromosomes are manipulated to sterilize fish
gynogenesis	Producing all females through chromosome manipulation
paradoxical feminization	The feminizing effect achieved when fish are treated with androgens
gamete	Sperm or eggs
tetraploid	Having four sets of chromosomes
interspecies hybridization	Crossing males and females from two different species. The resulting offspring are often sterile.
cryopreservation	Long-term storage of sperm

NOTES