

# Salmonid Triploiding Experiment

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## Introduction Purpose of Triploiding

As the fish approaches spawning, triploiding/sterilization when initiated early in the fertilized egg's cell division process causes the sexual maturation of the resulting fish to not occur, whereby, the fishes' energy, normally used for spawning, is diverted to fish growth, with the anticipated end result culminating in the following item(s):

1. Increase fish growth.
2. Fish live longer as fish are not stressed out with the riggers of spawning.
3. Control fish numbers by reducing/eliminating natural reproduction, thus eliminating the undesirable stunted fish populations.
4. Reduces the chance of planted fish from spawning with endemic species resulting in compromising the native fishes' genetics.

## Triploid Methods

Egg sterilization can be accomplished by interrupting early cell development divisions by the following four methods:

1. Increased temperature treatment-sudden hot water temperature rises for a short period of time. Usually total sterilization is not practical in remote field operations with cold air temperatures because of the difficulty of maintaining the constant, elevated temperatures for the time period necessary in these adverse environmental conditions. Also bulky equipment is needed and it is difficult getting that equipment to stream-side fish spawning locations.
2. Chemical treatment-addition of reactionary chemicals does not consistently produce the desired results, and it conflicts with most health standards. This method is more favorable in laboratory or hatchery applications than in field situations.
3. Electric shock treatment-similar problems as #2.
4. Hydrostatic pressure-intense pressure up to a little more than 10,000 pounds per square inch (psi), or five minutes, with light mobile equipment, at least with initial pilot project equipment, for stream application. In most recent years, most researchers favor this application, to best achieve most consistent results with the highest percentage of successful triploiding.

Most recent researchers have determined that the hydrostatic pressure system is the best method to triploid fish. A hydrostatic pressure machine is capable of producing up to 10,000 pounds per square inch (psi) on as many as 24 ounces of fertilized fish eggs per cylinder. The stainless steel chamber, pressure gauge, bleeding valve and extra replacement rubber seal kit from Aqua Ecosystems, Inc., and weighs approximately 40 pounds and it costs approximately \$1200 delivered. A 12-ton capacity bench press and hydraulic pump (an automobile wheel bearing press) are needed to apply the necessary pressure to the cylinder. It alone weighs 171 pounds, and costs \$800 delivered. The equipment was purchased by Kokanee Power, a non-profit organization interested in making future kokanee fishing better in California, and they offered the equipment to DFG for that purpose. This unit was ideal to conduct triploid tests, but if test results are favorable, a larger unit is available from this same company. The larger cylinder is capable of

treating up to 2.4 liters (81 ounces), almost four times the egg capacity of the smaller cylinder. The \$13,000 price tag for this tool includes a gas-powered hydraulic system which produces cylinder pressures up to 10,000 psi.

The importance of producing 100% triploid fish depends on the needs of that particular water and introduced fish. If there are no tributary streams for fish spawning, and there are no resident fish populations to protect, there is not any need to sterilize the entire introduced populations, unless triploid fish are intended to grow larger fish. If, in the case of inland salmon introduction, triploiding may interrupt, or delay the salmon's normal, spawn-and-die process. The applications have to be determined by a case-by-case situation.

### Historical Triploiding Research

According to PhD. Tollman Benfey, Department of Biology at University of New Brunswick, triploid fish have three sets of chromosomes rather than the normal (diploid) two sets. Although both triploid sexes are sterile, the males develop on-reproductive gonads and take on secondary sexual characteristics, even though they do not have viable sperm. Since the mid-80's, triploidy has been conducted on a variety of animals including carp and other fishes in the minnow family, catfish, salmon (all American species EXCEPT sockeye and kokanee), trout (browns, brooks, and rainbows), bluegills, largemouth bass, yellow perch, talapia, siamese fighting fish, mussels, abalone, oysters, shrimp, crabs, scallops and clams from 1984-1997, the year of the bibliography.

The thermal-triploiding method was tested by DFG's trout hatcheries in the 1980's with varying degrees of success in a variety of lakes. These "English Ladies" rainbow trout did produce some 5-7 pounders in Lake McClure, Stanislaus County (anglers' report), but the results in other tested lakes were not as favorable.

Washington Department of Fish and Wildlife (1995) thermally shocked Lake Watcom kokanee salmon resulting in 87% triploid (sterilized) fish with high mortality—50% mortality of the non-treated fish, with 85% mortality of the treated fish. The surviving, treated fish were slightly smaller than the controls, and appear thinner.

British Columbia utilized hydrostatic pressure to repeatedly produce 100% triploids. They were able to shock large numbers of eggs in a short period of time and equipment was compatible to the remote fish spawning sites. They used 8,500 psi for five minutes and obtained 90% egg survival to the eyed stage and 87% through the fish fry stage, when fish feeding begins and mortality usually becomes insignificant.

Research projects in the Province of Alberta applied 10,000 psi hydrostatic pressure just before the second polar body starts to be released resulting in a 100% sterilization rate.

Starting several years ago, some of the more progressive hatcheries started using hydrostatic pressure to produce triploid fish to grow larger fish in a short period of time. These 10-20 pound rainbow trout are now being released by private aquaculturalists into Southern and Eastern California where the demands are high. This illustrates the demand of the California anglers for larger fish and the practicality of its use. So with this need for larger fish and the science to produce this event, it is time for DFG to conduct triploid tests in attempts to optimize California's

fisheries.

### Current Methods

On April 11, 2000, at Mt. Whitney Hatchery (Independence, CA), Whitney rainbow trout (RTW) were experimentally triploided under the controlled environmental of the hatchery building. Enough eggs (291 eggs/ounce) were obtained from 5-7 female, and enough sperm from 5-7 two-year-old males, fish to provide approximately 1,500 fertilized eggs for each of the seven experiments.

After the individual gametes were extracted from the fish, precautions were taken to keep all foreign materials (blood, fish slime, and especially any moisture) from contaminating the eggs and sperm, which were held separately from one another in zip-lock bags and allowed to sit on ice, until they were needed to start the next experiment. This dry spawning method was used because it provided on source of both eggs and sperm through the entire testing period, so if differences in fish growth occurred it would be caused by the effects of triploiding rather than different parents' genetics.

Based on previous research, the best triploid tests with rainbow trout, the hydrostatic pressure needs to be applied to the fertilized eggs, between the first and second meiotic cell division which is when the fertilized eggs reach 300 temperature/time units (TTU). The 300 TTUs is determined by measuring the water temperatures surrounding the fertilized eggs until the hydrostatic pressure is applied. Since the hatchery water supply stayed consistent throughout the testing, at 50 degrees F (10 degrees C), then the time element was determined to be 30 minutes, as computed by the formulas-300 Tus divided by the 10-degree C water temperature. The colder the water, the longer the period between fertilization and initial pressure application; therefore, at 1 degree C, it would take 300 minutes, or five hours between the time when the eggs were fertilized and the hydrostatic pressure applied for each individual test.

After approximately 1,500 eggs were fertilized for the first experimental test, they were placed in a cheese cloth bag, immersed in an anti-bacteria, iodine solution for 10 minutes and then 20 minutes in the water/egg holding container, until the 30 minute waiting time was over, and the next experiment was initiated. All fertilized eggs passed through the appropriate TTU period, they were placed in the pressure chamber and the pressure applied evenly over the next 30 seconds until the desired psi level was reached. That constant pressure was maintained for five minutes, and the pressure was released evenly over the next 30 s, thus completing one individual experiment. The control experiments (other than #1 and #7, but include #3 and #6) were conducted in the same manner as the hydrostatic pressure tests, only without the pressure being applied. The pressure tests (#2, 4, and 5) were conducted at 10,400 psi, 9,700 psi, and 9,150 psi, respectively (Table1).

After each experiment was completed, the string-tied, cheese cloth bags of eggs were suspended in the milk can container which was  $\frac{3}{4}$  full of 50 degree F water and transported to the San Joaquin Hatchery (SJH), Friant, CA. Before these eggs went into the hatchery, they were soaked in iodine for 10 minutes and then moved into the egg hatching building, where they were placed into the egg hatching baskets within five hours after the final test was completed. Daily mortality data was kept on the different experiments. After the fish developed to a total length of 2-3 inches, blood was easily taken from the fish to determine triploid success. The Fish Health

Laboratory personnel extracted blood from individual fish which was then spread onto a glass slide and later evaluated to determine triploid success rate. The stained cells on the glass slide were carefully examined, noting the erythrocytes (red blood cells) irregular deformed and smaller-than-normal erythrocyte (red blood) cells characteristic of triploid fish.

The following day, approximately 200 trout from each of the five triploid/control test groups (#2 through 6) were fin clipped and each different group had a different clip or combination of clips to identify which indicated what test they represented when they were examined later in the experiment (Table 1). After all 1,000 fish were marked, the trout were placed in the same hatchery trough/raceway, so they will all undergo the same environmental fluctuations and competition for food. They will be examined throughout their hatchery life, especially as they approach, and reach sexual maturity, to monitor their physical parameters (mortality, growth, and deformities), and to see if triploid fish can be beneficial to future California fisheries.

Table 1. Hydrostatic Pressure Testing

Test Number	Test Description
1	Control – the first test to measure the effects of dry spawning during the three houses of testing. The fertilized eggs enclosed in a cheese cloth bag were submersed in water for 30 minutes, while resting on the bottom of an egg hatching basket suspended off the bottom of the water trough. During the first 10 minutes of this half hour, the eggs were subjected to an anti-bacteria bath (this procedure is used on all of the following tests).
2	10,400 psi pressure test – after the 30-minute waiting period following egg fertilization, a pressure of 10,400 psi was applied for five minutes on the eggs following the normal protocol of taking 30 seconds to reach the desired pressure level, and another 30 seconds to release the pressure, after the five minute duration of 10,400 psi pressure test.
3	Control for the Group #2-10, 400 psi pressure test with the same handling methods (ie. triploid methodology) of pressurizing the eggs as Group #2, without any pressure applied.
4	9,700 psi pressure is applied following the methods used in Test #2.
5	9,150 psi pressure was applied following the same methods as in Test #2 and #4.
6	Control for Group #5-9, 150 psi, same as #3, again, without any pressure applied.
7	Control for Group #1, with the same handling methods as performed in Test #1 to determine if the three hours that passed between Test #1 and #6, to see if dry spawning had any triploid impacts on mortality or fertilization rates.

### Results and Conslusions

The seven different experimental egg batches were monitored daily for 123 days, and the mortalities recorded by the individual groups during the egg-to-fingerling portion of the study (Table 2).

Table 2. Hydrostatic Tested RTW Total Mortality (April 11, 2000 – August 8, 2000, 123 days)

## Experimental Testing Groups and Results

Test Number	1	2	3	4	5	6	7	
Group Description (see Table 1)	Start Control	10,400 psi	Control for 2	9,700 psi	9,150 psi	Control for 5	Finish Control	Total/Fish Development Stage
Fertilized-Eyed Egg Mortalities 4/4-5/7 (32 days)	42	42	56	25	59	85	123	432
Egg Hatching 5/8-5/19 (11 days)	333	370	784	259	558	693	928	3925
Total Egg Mortality	375	412	840	284	617	778	1051	4357
Sac Fry-Fingerlings Total Mortality* 5/20-8/3 (80 days)	63	63	123	69	77	32	40	467
Grand Total Mortality	438	475	963	353	694	810	1091	4824
Fin Clip Mark (No. of fish marked)	None	Adipose/Right Ventral (189)	Adipose/Left Ventral (197)	Adipose (210)	Right Ventral (207)	Left Ventral (198)	None	-1001

\*Insignificant number of fish mortalities were observed after this time and no group of fish had any significant higher mortalities than any of the other groups. Most of these mortalities were as a result of fish jumping out of the water, therefore not due to experiments or natural mortality.

Analysis of the Table 2 are as follows:

1. As expected, even in normal egg hatching operations, most egg development mortality occurred during the very sensitive maturation of eggs as they are transformed into fry. With the triploiding, 81% (3,925 of 4,824) of the mortality was compiled within the 11 days it took for the eyed eggs to develop into fish, which was only 9% of the 123 days when the experiment terminated. Only 10% of the total mortalities (467 of 4824) occurred after the eggs developed into fish and the fingerlings were fin clipped; although this was 65% of the total time of the entire experiment time (80 of the 123 days), and most of those mortalities were caused by fish jumping out of the water troughs. Since this latter group of mortalities was not a direct result of the triploiding process, the following mortality figures will be based upon egg development to sac fry development period (Total Egg Mortality column), which is more germane to the triploid causing mortality issue.
2. There was inconsistency in the results between the two control groups (#1 & 7) – the time control experiment used to evaluate the effects of time on the egg mortality, and the other five groups (#2 through 6) which underwent triploid methodology, triploided Groups #2, 4, & 5 with pressure applied, and Control Groups #3 & 6 that were handled the same way as pressurized tests without the pressures being applied. There was a 180% increase in egg mortality between the time control groups (Group #1 & 7, 375 compared to 1,052, respectively) that was not comparable to the earliest pressure/control test (Group #2 & 3) which produced the highest pressure/control difference (108%); whereas the last pressure/control test (Group #5 & 6) produced the only 26% increased mortality. Also there appeared to be little, if any relationship between time and the three pressure tests' mortalities (Group #2 with 412 mortalities, Group #4 with 284, and Group #5 with 617). Group #4 (9,700 psi) was located in the middle of the time control test, and it had the lowest mortality of all pressurized tests (284) even lower than Group #1 (375) which being first should have been the

lowest, if time from egg gathering was the only factor. Also the control test #6 for the last pressure test (Group #5), had the lowest increased mortality rate comparison with its' pressure counterpart (24%) of any of the controls even the first pressure/control rate test (112%). While the controls for the pressure tests, though time during the testing had decreased mortalities—Group #3 (control for #2 had 840; while the last test, Group #6 (control for Group #5) had 778 mortalities, an 8% decrease compared to the time-control test of a 180% increase (Table 3).

3. The fish sampled to determine the success of the triploid experiment were easily separated into triploid vs. control (non-triploided eggs) groups from all of the seven test groups by the easily triploid-determined erythrocyte irregularities (deformities and larger than normal cell size) is indicative of triploid fish. According to Mr. John Modin, DFG Fish Health Laboratory pathologist, who examined the fish, these abnormalities are very apparent when stained slides are magnified and the cells can be compared to the control fishes' red blood cells. The few samples taken from triploided groups all tested positive for triploid. If a 95% confidence level was desired for ensuring all trout sampling were sterile would have required sacrificing 60 fish from each group for blood samples. It was felt that it was more important to keep alive at least 200 fish from five different tests than it was to sacrifice any more fish. The blood samples were collected from sacrificed small fish (2-3" fork length) because of the urgency of sampling the fish as soon as possible throughout the group sizes to see if the triploiding was successful. Future testing of the 200 fish samples of control and triploided fish to see what the long-term effects of that hydrostatic pressure has on the tested trout groups in regards to fish health, growth and longevity. At this time, approximately four months after egg fertilization, as expected, there is basically little difference in size between the five groups (three pressure and two control levels). The five fish groups were marked differently with different fin clips which illustrated there was an average size of all fish of 3.151" fork apparent as they approach maturity.

### Conclusions

Hydrostatic pressure has produced 100% triploid trout within the three pressure ranges between 9,150 and 10,400 psi. The best egg to fry survival rate of the pressurized tests were those eggs with 9,700 psi pressure applied. Therefore, future pressure applications should be applied at the 10,000 psi level, also with similar research findings, which is the closest to the 9,700 psi level. The dry spawning method should not be used in the future, as the mortality rates seem excessively high, and the need for one set of fertilized to have same genetic groups will not be necessary in future testing. The process is easy, inexpensive and very successful during the short-term evaluations. As these hatchery raised triploid trout approach their sexual maturity, it is expected, based upon some research results that this gene altering process may cause the fish to grow larger than the control fish as their energy will continue to be diverted from developing sexually, thus producing larger fish. Triploid mortality at this stage of testing amounted to 61% survival factor (2931 of 7,500 initial eggs used in the triploid testing). In the year 2000, several DFG inland triploid testing on inland salmon eggs, have resulted in initial egg-to-fish survival rates of 40-51%. Hopefully, the long-term triploid tests will illustrate those desired effects will take place, and in the future, DFG can use this fisheries research tool to improve California fisheries for our constituents. If triploiding is to become the tool of the future, there are financial offerings from non-profit groups which can be utilized to produce the larger cylinder to drastically reduce the triploiding time by as much as four fold over the present pressurizing tool.

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