

**STUDY OF THE EFFECT OF TRIPLOID STATE ON THE DEVELOPMENT
OF THE GENITAL ORGANS AT RAINBOW TROUT (*Oncorhynchus mykiss*)**

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Abstract

The aim of the paper was to study the influence of triploid state at rainbow trout on the development of the sexual glands and to study the degree of sterility of the gonads. Triploid state was induced by thermic shock at 5-10 minutes from wet fecundation. The eggs were introduced in a warm water bath at 28-30 °C, for 5-10 minutes. Triploid state was assessed by measuring erythrocyte size and karyotype. The degree of sterility of the individuals was established by histologic study of the genital organs at females and males. The sterility degree is manifested different from total absence of the gonads to the presence of asymmetric developed gonads and even gonads apparently normal. Triploid individuals that have presented testicles, they have a lysed cytoplasm in the cells, seminal epithelia is present, is noticed the absence of sperm from membrane and different dystrophic processes.

Key words: rainbow trout, triploid, thermic sock, sterility, erythrocyte dimension, karyotype

1. INTRODUCTION

Polyploidy in fish is characterized by the modification of the normal diploid chromosomes set (2n) in triploid (3n) tetraploid (4n) etc. Polyploidy can be lethal at some species (mammals and birds). Triploid state can be induced by forcing the retention of the second polar body, which results after normal fertilization. Inducing triploid state can be performed by a shock immediately after fertilization in order to result in the retention of the second polar body, and can be accomplished with temperature (high or low), hydrostatic shock, anesthetic or chemical substances, but also can occur spontaneous (Bohl 1999, Dunham 2004, Sellars et. al. 2006, Quillet et.al.,1991).

The first experiments at fish for inducing polyploidy were realized by Swarup in 1956. When polyploidy is odd the resulted individuals are sterile, because of the unequal chromosome in sexual cells. Generally diploid individuals have a higher rate of growth until sexual maturity is reached, compared with triploid individuals, after which the role is inversed. Triploid individuals are lacking or have under developed sexual organs, in a high percent, which allows for the energy that would be used for their development to be directed towards somatic development (Beaumont & Hoare 2003, Bohl 1999, Dunham 2004, Kalbassi & Johari 2008).

Because of the fact that polyploidy comes with a multiple set of chromosomes the cells will have a larger nucleus and the cell size will be adjusted accordingly, resulting in bigger cells and organs respectively.

Determining polyploidy can be accomplished by karyotyping (staining and counting the cromosomes), Flowcytometry, measuring cell size Coulter Counter Channelizer or blood smear, silver staining of the nucleolus regions (RON), izoenzymes analysis. Karyotyping is the most sure technique but is labor intensive and slow. Coulter Counter method is fast, the equipment is inexpensive. Measuring the

erythrocyte from blood smears is a relatively precise method (Benfey, Sutterlin & Thompson 1984, Garcia-Abiado et.al. 1999). RON analysis is the most inexpensive of all and can be applied without sacrificing the animal. The principle of this method is counting the nucleolus (1RON is expected/haploid genome)(Kapuscinski & Miller 2007, Phillips et.al. 1986).

2. MATERIALS AND METHODS

The experiments were performed in a fish farm, specialized in rainbow trout breeding. The experiments were conducted in natural breeding season of the animals, on a total number of 80,000 eggs. For eggs fecundation we used wet method and for the eggs from one female we used sperm from two males.

2.1. Inducing triploid at rainbow trout

Eggs harvesting and milt was performed in natural breeding season of rainbow trout. At females high maturation degree of eggs is indicated by soft abdomen turgescence genital pore. For harvesting, females were dried with a cotton cloth and the eggs were harvested by applying a gentle pressure on the abdomen from head to caudal fin (fig.1. A, B.).



Figure 1. A,B Aspects from eggs harvesting

At males, the maturation of the milt is assessed by gentle pressing the abdomen, if there is a sperm discharge, than the male can be harvested. The milt recovery is performed similar with the eggs, by gentle pressure of the male abdomen. When wet fecundation is desired the milt are recovered directly over the eggs. Homogenizing the milt with the eggs is performed with a feather for avoiding damages to the eggs (fig. 2).

Eggs fecundation was performed by adding water to the gametes mixture. After 5-10 minutes from sperm activation and eggs fertilization a thermic shock was applied, in order to induce the second polar body retention. The thermic shock was achieved by introducing the fecundation pot in a water bath at 28-30 °C for 5-10 minutes (fig. 3).



Figure 2. Milt recovery over the eggs



Figure 4. Inducing the thermic shock

After water shock the fertilized eggs were introduced in classic incubators for hatching.



Figure 4. Eggs incubators

2.2. Determining the triploid rate by colchicine treatment

After three weeks of incubation the eggs the triploid rate was assed. 100 eggs were analyzed from the heat shock treated lot and 30 eggs were analyzed from control lot. The eggs were treated with a colchicine solution of 0.01% for 6 hours, and then were transferred in a 0.4% KCl solution for 20-30 minutes. Eggs were fixed in 3:1 ethanol: acetic acid fixator. Fixed eggs were kept in fixator solution at 4°C until the chromosome analysis was performed. The triploid assessment was performed by removing the embryos with a fine scalpel. Pieces of the embryos were stained with carmine acetic and examined at microscope for triploid determination.

2.3. Determining the triploid state at rainbow trout by erythrocyte aria and perimeter measure

The preparation of the blood smears for the two years fish is described: blood was collected and smeared on a microscope blade, the sample is fixed in air, for avoid the erythrocyte deformation, than it was colored by panoptic Pappenhein method. Briefly two substances are used May-Grünwald and Giemsa (4-10%). From the blood smears at least 10 microscope fields were analyzed. The erythrocyte aria and perimeter was measured for establishing the diploid and triploid state. For the triploid the erythrocyte diameter is bigger compared with the diploids. The evaluation was performed with immersion objective using Olympus CX41 microscope and Quick-Photo software.

2.4. Anatomic appreciation of the genital organs by histologic analysis

After scarification of the animals and blood collection, the genital organs were analyzed. The genital organs were analyzed from the individuals that were identified from blood smear as triploid. Anatomic descriptions of the organs were performed and tissue samples were collected. The tissue fragments were fixed in formalin 10%, and were dehydrated, clarified, embedded in histologic paraffin, after which the samples were cut at 4 μ m (with manual rotary microtome Leica) and stained for evidencing the tissue structures (Mallory trichromic method). The evaluation was performed with immersion objective using Olympus CX41 microscope.

3. RESULTS AND DISCUSSIONS

3.1. Triploid determination with colchicine treatment

Microscopic samples from embryos fragment were examined and the chromosome number was counted, in several fields, from the same embryos. In normal diploid individuals the expected number of chromosomes is 60 and in triploid individuals we expected 90 chromosomes. For accurate determination of diploid or triploid state we counted at least 10 metaphase fields from each individual.

We examined 62 sampled from which 4 were eliminated since the accurate count of the chromosomes was not possible. After analyzing the good samples we concluded that 48 of the individuals were triploid and 10 were diploid. The rate of triploid obtaining was 82.76%, which is a satisfactory rate and comparable with other reports from specialty literature.

3.2. Triploid determination from blood smears

In order to determine the triploid inducing rate at rainbow trout we started at the idea that thermic sock applied after 15-20 minutes from fecundation has as effect the blocking of the elimination of the second polar body ($2n+n=3n$). From the experimental lot we chose 30 individuals that were sacrificed. Blood samples were collected.

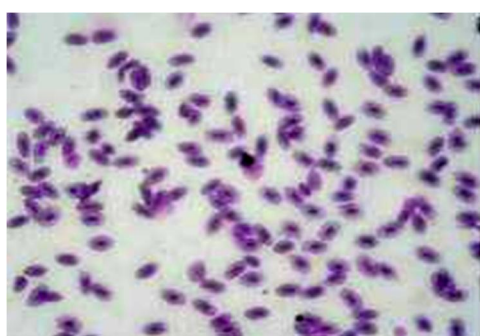


Figure 5. Triploid erythrocyte (MGG; 1000x)

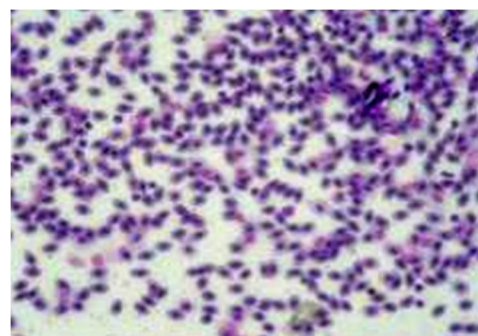


Figure 6. Diploid erythrocyte (MGG; 1000x)

From the blood smear at least 10 microscopic fields were analyzed. The erythrocyte area and perimeter was measured for establishing the diploid and triploid state. From the data obtained we noticed that erythrocyte area has a mean value of 42.44 μm^2 with minimal values between 32.7 and 58.2 μm^2 . For erythrocyte perimeter we obtained a mean value of 25.40 μm with a minimum of 21.9 μm and a maximum of 29.6 μm .

From the 30 samples of blood analyzed for determining triploid state 28 samples were identified as triploid, one as diploid and for one the determination of diploid or triploid state was inconclusive. The data from table 1 are presenting the statistical analysis of the area (μm^2) and perimeter (μm) for the erythrocyte at triploids.

Table 1. Statistical analysis for the area (μm^2) and perimeter (μm) for the erythrocyte at triploids rainbow trout

No. crt.	Specification	$\bar{X} \pm S_x$	Standard deviation	Variance (s^2)	C.V.
1	Area (μm^2)	42.44 $\pm$ 1.26	6.8	46.27	16.02
2	Perimeter (μm)	25.40 $\pm$ 0.36	1.9	3.2	7.4

From the data in table 1 it can be noticed that area has the mean has a value of $42.44 \pm 1.26 \mu\text{m}^2$, standard deviation has a value of 6.8, and the variability coefficient is 16.02, these data are indicating that our measures have a mean variability. For erythrocyte perimeter the mean value was $25.40 \pm 0.36 \mu\text{m}$, standard deviation was 1.9 and variability coefficient 7.4, which represents a low variability for this character.

The data for erythrocyte area and perimeter at rainbow trout at diploid from the control lot are presented in table 2.

Table 2. Statistical analysis for the area (μm^2) and perimeter (μm) for the erythrocyte at diploid rainbow trout

No. crt.	Specification	$\bar{X} \pm S_x$	Standard deviation	Variance (s^2)	C.V.
1	Area (μm^2)	24.10 $\pm$ 2.09	5.53	30.61	22.94
2	Perimeter (μm)	19.30 $\pm$ 0.94	2.50	6.29	12.95

From the data in table 2 it can be noticed that area has the mean has a value of $24.10 \pm 2.09 \mu\text{m}^2$, standard deviation has a value of 5.53, and the variability coefficient is 22.94, these data are indicating that our measures have a high variability. For erythrocyte perimeter the mean value was $19.30 \pm 0.94 \mu\text{m}$, standard deviation was 2.50 and variability coefficient 12.95.

From the analysis of the data presented in table 1 and 2, we observe that the difference from the area of the diploid and triploid erythrocyte is $18.34 \mu\text{m}^2$ which shows that the erythrocytes from triploid individuals have an area with $1.76 \mu\text{m}^2$ higher compared with diploid individuals.

For the erythrocyte perimeter from diploid and triploid individuals the difference is $6.1 \mu\text{m}$, which shows that for triploid the perimeter is higher with $1.31 \mu\text{m}$ compared with diploid individuals.

3.3. The appreciation of anatomic aspect for the genital organs and the results obtained by histologic methods and for determination of integrity of the genital organs

In order to achieve our aim 30 individuals from triploid lot were sacrificed in order to analyze the genital organs and to establish the sterility degree. In figure 7 there are presented comparatively the normal genital organs (from diploids) and modified (from triploids).

	The shape of the individuals	Internal organs	Observations
Diploid			350g Female, normal sexual organs
Diploid			300g Male, normal sexual organs
Triploid			750g Asymmetric sexual organs
Triploid			850g Sterile
Triploid			850 g Sterile
Triploid			700 Sterile, enlarged liver
Triploid			400g Asymmetric sexual organs
Triploid			700 g Asymmetric sexual organs
Triploid			700 g Asymmetric sexual organs
Triploid			800g Sterile

Figure 7. Comparative presentation of the aspect of sexual organs at diploid and triploid individuals

The comparative presentation of the genital organs at diploids and triploids individuals we can notice that from 30 individuals sacrificed, 16 are phenotypical sterile and manifest an atrophy until the total lack of genital organs, 9 males are with asymmetric testicles and 5 individuals are manifesting apparent normal sexual organs.

Individuals from the experimental lot (triploids) and from control lot (diploids) were weighed before scarification. The mean weight of the individuals from experimental lot was 703.33 ± 36.01 g. The mean weight of the individuals from control lot was 330 ± 25.5 g. The weight of the triploids was 2.13 times higher than the diploids.

Table 3. Statistical analysis of the weight at diploid and triploid rainbow trout

No.	N	Specification	$\bar{X} \pm S_x$	Standard deviation	C.V.
1	30	Triploid	703.33 ± 36.01	139.47	19.83
2	5	Diploid	330 ± 25.5	57	17.27

From the variability coefficient of the diploid and triploid we notice that at the experimental lot the variability coefficient was 17.27, these means that the two lots have a mean variability, and the individuals are unevenly.

From the sexual organs of the triploid fish, histologic prepares were made and are presented in figure 8.

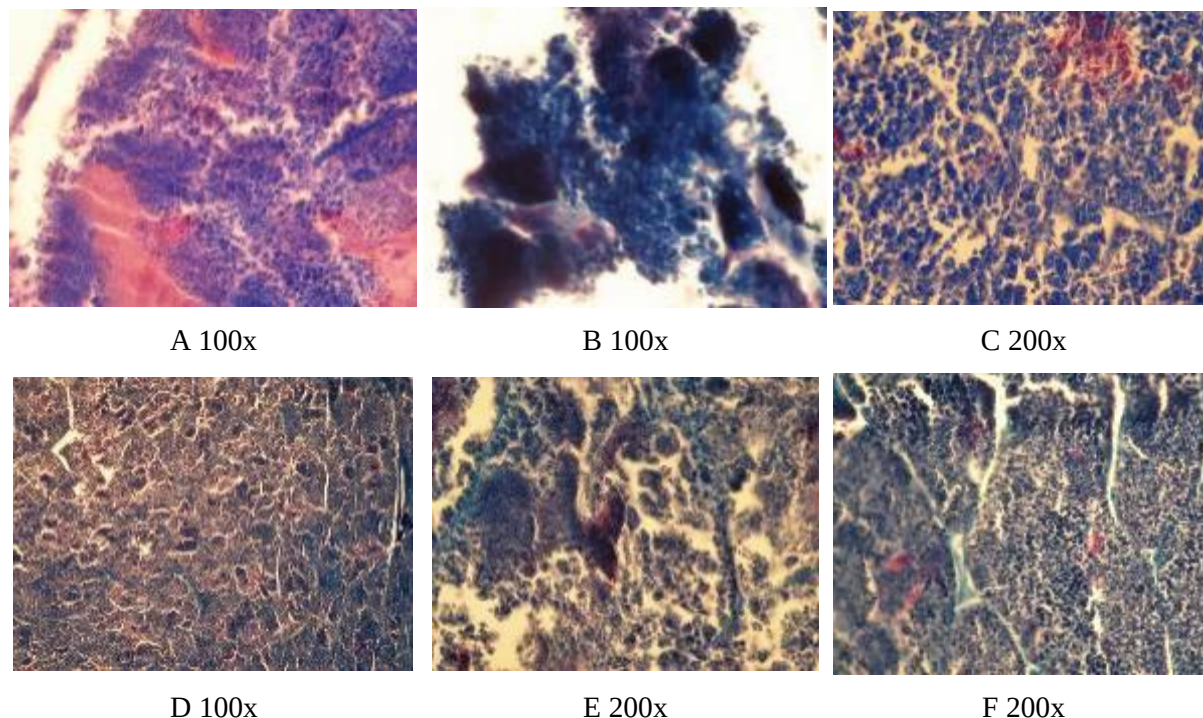


Figure 8. Histologic prepares at rainbow trout

A. Cytolysis aspect; B Cytolysis aspect in lumen, atrophy of the seminal epithelia, absence of sperm, the epithelia is lysed; C. Cytolysis aspect, at 200x magnification we noticed lyse of the seminal

epithelia and the absence of sperm; D. Cell lyses aspect, the detachment of the membrane epithelia; E. Dystrophic process, cell lyse; F. Normal aspect epithelia

From the data presented in figure 8, it can be noticed that we had some individuals with cytolysis epithelia, atrophy of the seminal epithelia, cytolysis of the lumen, also the absence of the sperm. At 200x magnification lyse of the seminal epithelia and the lack of sperm was observed. At 100x magnification we noticed the detachment of the membranes, cell lyse, dystrophic processes, cell lyse aspect and lack of sperm. Figure 8 F. is presenting a normal histologic aspect of the gonads.

4. CONCLUSIONS

In order to evidence the chromosomes using carmine acetic is allowing a good visualization of the chromosomes.

Water shock of the fertilized eggs can be used successfully for triploids obtaining. From the total of the treated eggs a triploid percent of 82.76%.

The erythrocyte area can be an effective indicator of the polyploidy state. The area of the erythrocytes of the diploids was lower compared with triploid individuals. The area of the erythrocytes at triploids is 1.76 times higher than diploids individuals.

The perimeter of the erythrocyte can be an effective indicator of the polyploidy state. The perimeter of the erythrocytes of the diploids was lower compared with triploid individuals. The perimeter of the erythrocytes at triploids is 1.31 times higher than diploids individuals.

The variability coefficient for area of erythrocyte has a middle value fact that indicates a precision of the data acceptable for determining the triploid state at rainbow trout.

The variability coefficient for perimeter of erythrocyte has a low value fact that indicates a precision of the data acceptable for determining the triploid state at rainbow trout.

The sterility degree of the triploid individuals is manifested differently from the total absence of the gonads, to asymmetric development of the gonads but also individuals with apparent normal developed gonads. From total 30 individuals sacrificed, 16 were phenotypically sterile, with atrophy of the sexual organs, 9 males with asymmetric developed gonads and 5 individuals had apparently normal sexual organs.

The triploid individuals at which the testicles are present have cytolysis aspect, the lyse of the seminal epithelia, the absence of sperm, the detachment of the epithelia from the membranes and dystrophic processes are observed.

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