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Investigation of Fertilization, Hatching and Survival Rates of Eggs from Crossbreeding Diploid and Tetraploid in Trout (Oncorhynchus mykiss)

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Abstract

The aim of this study was to compare the sperm size and reproductive performance of tetraploid and diploid rainbow trout (*Oncorhynchus mykiss*) and to investigate the effects of polyploidy on reproductive success. The effects of different ploidy levels on sperm morphology, fertilization rates, embryo development and survival rates were evaluated. Tetraploid and diploid rainbow trout individuals were used in the study. Four different crossover combinations were tested: (i) Diploid male× Diploid female, (ii) Tetraploid male× Tetraploid female, (iii) Diploid male × Tetraploid female, (iv) Diploid female × Tetraploid male. Sperm sizes were measured by microscopic analysis and fertilization rates and survival of offspring were compared. Statistical analyses were performed using ANOVA and correlation tests. Sperm size from tetraploid males was found to be significantly larger than diploid individuals ($p < 0.05$). In terms of fertilization rates, diploid × tetraploid crossovers showed significant fertilization success. When looking at embryo hatching rates, tetraploid individuals were lower; however, when looking at survival rates, there was no significant difference between the groups. In conclusion, tetraploidization may affect sperm size reproductive success. Improving reproductive results by crossing diploid and tetraploid individuals is considered a viable method. However, genetic and environmental factors need to be further investigated to fully understand how this process works.

Keywords: Oncorhynchus mykiss, tetraploid, diploid, sperm, fertilization

1. Introduction

1.1. Purpose and Importance of the Study

Fish farming has become an increasingly important sector in the world, both environmentally and economically. Genetic manipulation methods, especially tetraploidization, play an important role in fish production and sustainability (FAO, 2022). Polyploidy is a genetic process that occurs when an organism's chromosome sets increase, which can affect reproductive capacity or fertilization rates (Havelka et al., 2014).

This study aims to investigate the effects of polyploidy on reproductive success by comparing sperm size and reproductive performance of tetraploid and diploid trout (Oncorhynchus mykiss). Factors such as water temperature, fertilization times, and shock treatments will also be discussed in detail in terms of how they affect reproductive success.

1.2. Study Hypotheses and Research Questions

This study aims to test the following hypotheses:

• Hypothesis 1: Sperm size of tetraploid fish is significantly different from sperm size of diploid fish.

• Hypothesis 2: The reproductive performance (fertilization rate, hatching rate, and offspring survival rate) of tetraploid fish differs from that of diploid fish.

• Hypothesis 3: Different mating combinations of diploid and tetraploid fish (diploid x diploid, tetraploid x tetraploid, diploid x tetraploid) have unique effects on reproductive performance.

In light of these hypotheses, the following research question will be answered:

• Research Question 1: What is the difference in sperm size between tetraploid and diploid fish?

• Research Question 2: How do fertilization, hatching, and offspring survival rates differ among fish with different ploidy levels?

• Research Question 3: What is the effect of different mating combinations of diploid and tetraploid fish on reproductive performance?

Answering these questions will contribute to a better understanding of the reproductive biology of tetraploid fish and will help develop genetic improvement strategies in fish.

1.3. Reproductive Biology in Tetraploid and Diploid Fish

The reproductive biology of fish can vary significantly depending on their chromosomal structure. In natural populations, most fish species have a diploid chromosome set with two copies of each chromosome (Crossman et al., 2022; Hajam et al., 2024). However, individuals with tetraploid or higher chromosome sets can be obtained through polyploidization (Han et al., 2010). Tetraploidization is a technique that uses chemical or physical methods to artificially double the chromosome number (Arai, 2001; Weber et al., 2015). This method is frequently used in aquaculture to improve growth, regulate sex determination, and produce sterile fish (Benfey, 1999).

To induce polyploidy, developing embryos are subjected to heat or pressure treatments. This results in differences in gamete size, sperm structure, fertilization success, and embryonic development between tetraploid and diploid fish (Arai, 2001; Comber and Smith, 2004). Studies on the reproductive biology of polyploid fish show that tetraploid males have larger sperm-producing cells, but reduced sperm motility. This may affect their ability to fertilize eggs (Lu et al., 2022; Nynca et al., 2023). Some previous studies have provided some findings on the sperm characteristics and fertilization efficiency of tetraploid fish. The findings reported that tetraploid individuals produce larger sperm cells than diploid individuals, but tetraploid fish have larger sperm cells and lower motility, which may directly affect reproductive success (Manor, 2009; Yavuz, 2013). The increase in sperm cell size may directly affect fertilization outcomes by changing their interaction with eggs (Nagler, 2018). Some studies suggest that tetraploid sperm have a poorer ability to cross the egg membrane than diploid sperm (Benfey, 1999; Benfey, 2011; Scott et al., 2015). Studies have also shown that eggs from tetraploid females have a larger diameter than those from diploid individuals and that their composition may change during embryonic development (Han et al., 2010; Liu et al., 2020). Studies on egg production by tetraploid females indicate that fertilization success and hatching rates are affected by factors such as water temperature, genetic composition, and environmental conditions (Hershberger and Hostuttler, 2007; Havelka et al., 2014). Although polyploid manipulations offer advantages such as genetic stability, faster growth, and changes in reproductive capacity, the reproductive success of tetraploid individuals may sometimes be lower than diploid fish (Arai, 2001; Weber et al., 2015). Especially in crossbreeding between different species, ploidy incompatibility can negatively affect embryonic development. For example, In crossbreeding experiments between American spoonbills (Polyodon spathula) and sterlet fish (Acipenser ruthenus), only viable offspring were obtained from crosses of female sterlet and male spoonbill; in the reverse combination, embryos stopped developing before 120 h (Káldy et al., 2024). Similarly, in the naturally tetraploid Siberian sturgeon (Acipenser baerii), the presence of spontaneously arising hexaploid males is striking. These individuals are fertile and produce pentaploid offspring when mated with normal tetraploid females (Havelka et al., 2014). This demonstrates the important influence of ploidy levels on reproductive success and offspring survival. On the other hand, assuming the extinction of the Chinese spoonbill (Psephurus gladius), alternative preservation methods for polyploid fish, such as germ cell transplantation, have been investigated (Ye et al., 2020). In one study, spermatogonia cells from American spoonbills were successfully transplanted into the gonads of Yangtze sturgeon, and these cells remained viable for at least seven months (Ye et al., 2020). These findings demonstrate the potential of polyploidy-based conservation strategies and interspecies genetic material transfer. Furthermore, studies on triploid Atlantic cod (Gadus morhua) by Feindel, Benfey, and Trippel (2009) revealed that triploid males continued to produce sperm, but their offspring had lower survival rates than diploid individuals. This suggests that the reproductive success of polyploid individuals may be reduced and that genetic factors play a critical role in embryonic development. In summary, studies on the reproductive biology of tetraploid and diploid fish indicate that polyploid manipulation has a significant effect on reproductive performance, gamete characteristics, and embryonic development. Therefore, the advantages and limitations of polyploid techniques in aquaculture should be carefully evaluated and their application in fish farms should be optimized to the best extent.

2. Materials and Methods

2.1. Study Area and Experimental Design

This study was conducted at Abalıoğlu Balık ve Gıda Ürünleri A.Ş. Kayseri Karagözler Trout Production Facility. It aimed to compare the reproductive performance of tetraploid and diploid trout (Oncorhynchus mykiss). Experiments were conducted under controlled laboratory conditions, considering variables such as water temperature, shock duration and fertilization duration.

2.2. Fish to be Used for the Experiment

In the experiment, diploid eggs were produced by crossing functional male trout that had previously been transformed from female to male with diploid females. In this study, tetraploid fish obtained as a result of heat shock application of eggs obtained from complete female fish on 10.12.2020 and diploid fish routinely produced in the facility were used.

2.2.a Tetraploid Procedure

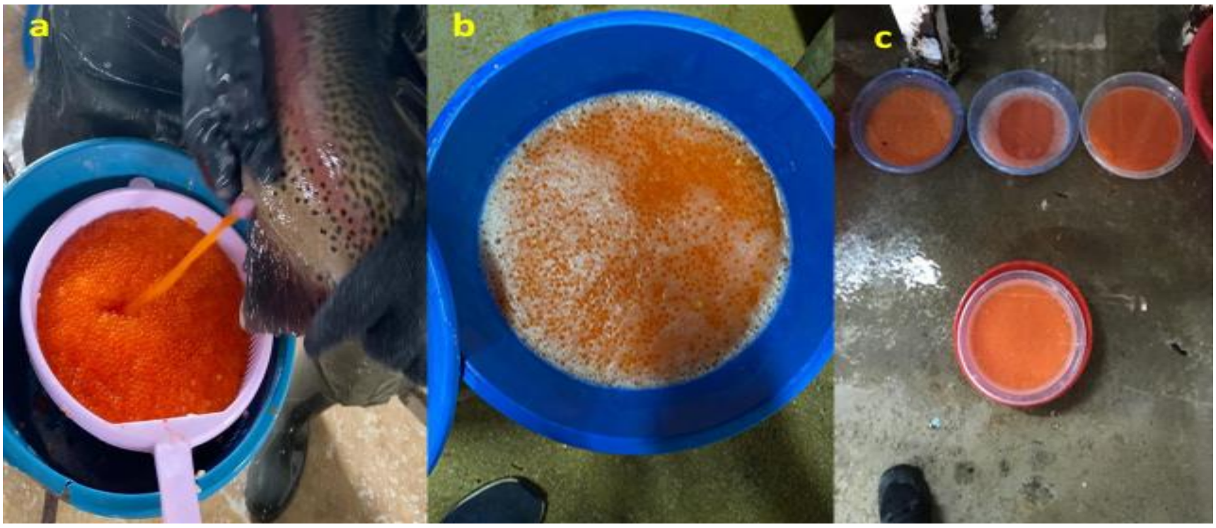

Figure 1. Spawm the eggs a; fertilization b; and waiting after fertilization before thermal shock c. (Original, 2019)

After the eggs were taken from the fish by milking, the fertilization process was performed. The eggs were kept for 3.5 hours after fertilization, as seen in Figure 1.

Figure 2. Shocking apparatus a,b and incubation cabinet c (Original, 2019).

The eggs that were kept were then taken into a cabin with a water temperature of 28 degrees and subjected to thermal shock for 10 minutes, as seen in Figure 2. Then, they were placed in incubation cabinets and waited for observation and opening.

The health and physiological conditions of the fish were continuously monitored in a controlled manner until they became adults after the experiment. At the same time, 1000 juvenile fish from tetraploid female individuals were masculinized by giving 17α-Methyltestosterone (2 mg/kg-MT/feed) hormone feed for

650 degrees/day from the time they started to withdraw their food sac and take feed from outside. Before milking, 5 fish from each group were taken and their length, weight, egg and sperm amounts (Table 1) were determined and the eggs and sperm of each group were mixed and homogenized. At the end of two years, on 25.11.2022, eggs taken from diploid and tetraploid female individuals by milking and sperm taken from diploid and tetraploid functional males by dissection were matured in Cortland fluid and crossed.

GROUPS	Diploid Female	Diploid Functional Male	Tetraploid Female	Tetraploid Functional Male
Average Weight gr	2650±73	1870±46	2730±98	1910±62
Average Height cm	67±4	56±3	71±6	58±4
Egg Quantity Number	3960±110		3520±91	
Sperm Quantity ml		30±2		32±3

Table 1. Height, weight, sperm and egg data of the groups

2.3. Fertilization Procedure

The sperm and eggs obtained from the fish used in the experiment were subjected to routine fertilization procedures in the farm. After milking, the eggs that were cleaned from the ovarian fluid were placed in a clean and dry container. They were grouped as in Table 2. After adding 0.4% Trizma (NH2C(CH2OH)3) and sodium hydrogen bicarbonate (NaHCO3) solution to be 1 mm above the grouped eggs, the eggs were left to be fertilized by mixing the sperm at a rate of 2 ml sperm per 1000 eggs.

All groups were kept under standard farm conditions throughout the experiment, the water temperature was 10±2°C. The oxygen level was measured as 8.5±1 ppm on average. After fertilization, the eggs completed their development processes in the hatching tanks.

2.4. Statistical Analysis

The collected data were analyzed using SPSS 26.0 software. The data are presented as mean ± standard error, and one-way ANOVA was used to determine the differences among the groups. Results with a P value less than 0.05 were considered statistically significant.

Used Statistical Tests:

• One-way ANOVA: To analyze the variance among different crossing groups.

• Correlation Analysis: To examine the relationship between fertilization success and sperm morphology.

All analyses were evaluated at a 95% confidence interval.

3. Findings

3.1. Fertilization Success and Hatching Rates

Fertilization rates and hatching rates were compared between experimental groups. It was observed that fertilization rates were lower in tetraploid individuals than in diploid individuals, but hatching rates remained constant in tetraploid x tetraploid crosses. Significant differences were found between diploid x tetraploid and reciprocal crosses (Diploid Female x Tetraploid Male, Diploid Male x Tetraploid Female).

GROUPS	Control DPD ♀ x DPD* ♂	Control TTPLD ♀ x TTPLD ♂	DPD ♂ x TTPLD ♀ 1. Group	2. Group	3. Group	DPD ♀ x TTPLD ♂ 1. Group	2. Group	3. Group
Fertilization Rate %	93±3	84±5	87±3	91±3	86±4	83±2	86±4	85±3
Opening Rate %	96±2	86±2	84±2	86±4	88±2	84±2	79±6	83±2
Life Rate %	86±5	76±1	72±5	73±5	76±5	73±6	71±5	74±3

Table 2. Fertilization, Hatching and Survival Rates of Diploid and Tetraploid Reproductive Cells

*: Diploid (DPD) ♂: male
**:Tetraploid (TTPLD) ♀: female

As a result of the crossing of diploid and tetraploid fish reproductive cells; when examined in terms of control and experimental groups, it was determined that the fertilization rate in the control group was higher in the diploid female x diploid male fish cross than in the tetraploid female x tetraploid male cross. In the experimental group, the highest fertilization rate was observed in the diploid male x tetraploid female cross.
When the egg hatching rate was examined, it was determined that the diploid male x diploid female fish cross was significantly higher in the control group than in the tetraploid male x tetraploid female cross. In the experimental group, the highest egg hatching (hatching) rate was again observed in the 2nd and 3rd groups of the diploid male x tetraploid female cross.
When the control groups were compared in terms of survival rate, it was seen that the diploid female x diploid male cross was higher than the tetraploid female x tetraploid male cross, and when the other groups were compared, it was seen that the lowest survival rate was in the diploid female x tetraploid male group. Generally, when Table 2 is examined, the highest
It was observed that the fertilization, survival and hatching rate were in the diploid female x diploid male group, while the lowest rate was seen in the diploid female x tertarploid male group.

3.2. Sperm Morphology and Motility Analyses

Sperm samples collected from tetraploid and diploid individuals were examined under a microscope. While the spermatozoa of tetraploid individuals were 35-40 µm, the spermatozoa of diploid individuals were 25-30 µm in length and were significantly longer ($p < 0.05$); however, when their motility was compared, it was observed that the spermatozoa taken from tetraploid individuals were lower.

4. Discussion and Conclusion

4.1. Discussion

The main discussion definition in the study is the comparison of fertilization, hatching and survival rates of sperm and eggs of tetraploid and diploid fish in the reproductive efficiency of rainbow trout.

As a result of the studies and statistical evaluations, it was concluded that there are differences in sperm structure in diploid and tetraploid fish and that this causes significant differences in the fertilization and development of eggs.

According to the results of the study, diploid female x diploid male showed higher fertilization success than tetraploidy female x tetraploidy male cross. This significant difference may be due to sperm size, and egg quality is also thought to be clearly effective (Arai, 2001; Piferrer et al., 2009).

In some previous studies, it was found that there are some genetic changes in the gametes of polyploid fish that will directly affect reproduction (Leggatt and Iwama, 2003). When the results of the two studies are examined, there is a consistent agreement between the findings of the two studies. It is seen that the accuracy and effects of the examined effects on reproduction are the same.

It was determined that spermatozoa obtained from tetraploids were significantly larger than spermatozoa obtained from diploids, but their mobility was lower. Peruzzi et al. (2009) and Bartley et al. (2001) reported similar results, which showed that although sperm size is larger in tetraploids, this negatively affects fertilization. This study supports the hypotheses in the studies conducted. When hatching and survival rates were examined, diploid fish were more successful than the other.

It was concluded that genetic stress in tetraploidization embryos causes developmental loss (Piferrer et al., 2009).

In order to increase the performance in the reproductive behaviors of these fish, stability in their genes and environmental conditions is important.

Statistical analyses (ANOVA and correlation) revealed a significant relationship between sperm morphology and fertilization rates ($p < 0.05$). The importance of sperm size for complete fertilization has been proven by studies. Although observations and findings show that the reproduction of tetraploid individuals is lower than others, more detailed studies are needed.

4.2. Conclusion

The general purpose of the study is to examine the ploidy levels of tetraploid and diploid trout reproduction. Detailed research and statistical studies were conducted and important results were obtained. As a result of the studies, it was determined that fertilization and embryo development were higher in diploid fish compared to tetraploids.

Another finding obtained from the study is that the morphological structure and different behavioral patterns in sperm motility in tetraploid trout positively affect fertilization. The results and findings provide several conclusions that will contribute to future studies on the reproduction of tetraploid trout:

Studies to increase egg and sperm quality in tetraploid trout will have a positive effect. External factors are effective in maintaining embryo viability in the same fish, so these conditions need to be improved. Methods that activate sperm or methods that support hormones should be investigated and implemented. This type of fish farming should definitely be investigated with its positive and negative aspects before making an investment. Input and output expectations are important.

It would be more effective to conduct these studies by genetically investigating them in larger populations. This study will be an important source to show the advantages and disadvantages of tetraploid fish farming. More detailed studies on the effects of reproductive factors on tetraploid trout in particular will be beneficial for the future of aquaculture in the future.

This study will lead to further research on the effects of genetic manipulations in trout farming. In addition, developing new strains using tetraploidy may be an important step towards increasing the productivity of fish. In the future, conducting such studies on a larger scale will contribute to the establishment of sustainable strategies for trout farming.

In conclusion, crossing diploid and tetraploid trout is not only a scientifically important research topic, but also has the potential to increase productivity in fish farming. The progress of such studies may help us understand the genetic diversity of trout and develop new strategies.

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