STUDIES ON THE DIFFERENT EFFECTS OF SUBSTANCES USED AGAINST ADHESIVENESS OF FISH EGGS IN THE RECIRCULATING AQUACULTURE SYSTEM

L.B. Athanasopoulos[1*], V. Nistor[1], F.M. Dima[1], N. Patriche[1], E. Sîrbu[1], M. Tenciu[1], D.M. Stroe[1], G. Ion[1], C.M. Chivoeanu[1]

[1]Institute of Research and Development for Aquatic Ecology, Fishing and Aquaculture Galați, Romania

Abstract

An important stage in artificial reproduction in fish of the families Acipenseridae and Cyprinidae is the elimination of adhesiveness of egg process. International studies recommended a variety of substances used against adhesiveness of eggs but do not differentiate the major advantages and disadvantages of their use. The present experiment aimed to determine the effects of de-adhesion with mineral silt, talcum and tannin. The final results regarding the effect of the substances used for de-adhesion on embryogenesis were determined by qualitative assessments, and the fungal infestation was quantitatively quantified by the percentages of fish eggs infested with Saprolegnia sp. in relation to the fertilization percentages. The present work emphasizes that the use of tannin for de-adhesion of fish eggs (B3) not only provides medium-good transparency in embryogenesis, but also reduces the risk of fungal infestation by 12.55% compared to de-adhesion with silt (B1), 13,56% with talcum (B2), with comparable fertilization percentages of 87% in B1, 86.9% in B2 and 89.4% in B3, which recommends it for deadhesing of eggs and reducing fungal attacks in aquaculture.

Key words: *substances for fish eggs deadhesion, embryogenesis, saprolegniosis, mineral silt, talcum, tannin*

INTRODUCTION

To meet the food needs of a growing population, solutions must be found to develop new sources of high-quality protein from aquaculture, which has seen an upward trend worldwide in recent years [1]. The variety of fish artificially propagated in modern recirculation systems is large, ranging from commercially valuable fish of the family *Acipenseridae* to fish of the family *Cyprinidae* used for commercial purposes or for breeding and conservation programs [2]. The principle of artificial reproduction in fish is mainly based on the stimulation of gonadal maturation of the breeders by the external supply of gonadotropic hormones [3], the removal of the sexual elements, oocytes and semen, their artificial fertilization and the deadhesion of the eggs for incubation and fish larvae production [4].

The amount of injectable hormone and the prevention of fungal infestation of the fertilized eggs are the most important elements of successful artificial reproduction. Effective antifungal agents (e.g. malachite green) have been banned by international legislation due to their carcinogenic and teratogenic effects [5], and the remaining treatment agents (including gentian violet) are not effective, so fungal infestation in recirculating systems can jeopardize fish production. The aim of the present work is to find substances that

*Corresponding author: lilianablondina@yahoo.com

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achieve a good deadhesion of the eggs and at the same time have antifungal properties. The role of the biochemicals responsible for the adhesiveness of the chorionic membrane of the eggs is their mechanical and biochemical protection, which forms a barrier against external environmental aggressors and pollutants. In some fish species, eggs that come into contact with the ovarian fluid do not exhibit adhesion, but upon contact with water they become sticky by natural mechanisms for attachment to the substrate and protection from predators.

The eggs are treated with various substances that are able to neutralize the action of the glycoproteins represented by mucopolysaccharides in the outer zona radiata membrane of the eggs [6].

Proper egg deadhesion enables an efficient fertilization process, allows stereomicroscopic monitoring of embryogenesis, microscopic assessment of fungal infestation and incubation of eggs under continuous water flow, conditions that ensure the necessary dissolved oxygen for embryonic development and maximum hatching efficiency.

Poor deadhesion impairs the fertilization rate, as the sperm cannot reach the micropyl of the clumped eggs, which dies on hatching and is attacked by fungi.

If the deadhesion was poor but fertilization has taken place, the eggs inside the clump suffer from a lack of oxygen during membrane respiration in the embryogenesis process, which causes their death by asphyxiation.

Fungi of the genus Saprolegnia sp. are grafted onto unfertilized and dead eggs and can reproduce asexually by zoosporangia with zoospores and sexually by the conjugation of oogonia with antheridia, which form motile oospores that can produce a new mycelium under the right conditions. The hyphae system they develop in this way can also rapidly entrap embryonated eggs and cause high losses [7].

The recirculating aquaculture system, in which fish are artificially propagated, utilizes technological water treatment modules with minimal additional water supply (maximum 20% per day) intended to supplement the water level lost mainly through filtration of solid wastes and evaporation [8, 9, 10, 11].

Although recirculation systems contain UV lamps to sterilize the technological water, they do not succeed in preventing the spread of *Saprolegnia sp.* in the system and increasing fungal contamination [11]. The effectiveness in eliminating egg stickiness was evaluated qualitatively and quantitatively and the results on the degree of fungal infestation were correlated with the percentage of fertilized eggs. The present work aims to investigate the different effects of deadhesion agents on *Acipenseridae* and *Cyprinidae* eggs used in artificial propagation, in recirculating aquaculture systems. The experiment was carried out using three substances for egg stickness removal: mineral slime, talcum and tannin. The effectiveness of the deadhesion was evaluated qualitatively and quantitatively and the results on the degree of fungal infestation were correlated with the percentage of fertilized eggs.

MATERIAL AND METHOD

The experiment was carried out in 2024 at the I.C.D.E.E.A.P.A. Galati recirculation aquaculture system for the artificial propagation of Russian sturgeon and carp, and the results obtained were correlated with the results of the experiments from previous years. Three substances were evaluated for eggs deadhesion: mineral silt in the form of a creamy black suspension, talcum, an inorganic compound of magnesium silicate (3MgO4SiO2H2O) in the form of a white, odorless powder, and tannin, a plant compound in the form of a brownish powder with a complex chemical (polyphenolic) structure that has the ability

to precipitate proteins, has bactericidal and fungicidal properties and is used to extend the shelf life of food and in oenology [12]. Organic tannin called Gran Tannin-C, extracted from the native chestnut (*Castanea sativa*) was used.

The mineral silt was processed by passing it through a fine sieve and sterilized at high temperatures to destroy resistant forms of some parasites or pests remaining in the sludge of the fish ponds, which pose a risk of infesting the eggs during the incubation period.

The experiment was started for the Russian surgeon on 12.05.2024. after artificial fertilization, the destickiness treatment for sturgeon eggs was carried out for 250 g portions in two variants with mineral silt and talc and then incubated at 16^{00} hours. The destickiness treatment for carp eggs was carried out with tannin on 13.05.2024 and was transferred to the incubators at 14^{30} hours. The doses used to prepare the deadhesion solutions and the times required to remove the stickiness of the sturgeon eggs were: 1 L of mineral silt/5 L of water, with a mixing time of 35-45 minutes and 100 g of talcum/5 L of water, with a mixing time of 45-60 minutes [13]. The treatment to remove stickiness from carp eggs was carried out with 0.9 $^{0}/_{00}$ tannin solution (instead of $0.5^{0}/_{00}$ mentioned in the literature) for 20 seconds, followed by 2-3 consecutive rinses (after Rouabah Abdelkader et al. modified method).

In order to standardize the experimental conditions, only Brateș incubators were used, although these are not suitable for harvesting carp larvae. The Brateș incubator is a parallelepipedal enclosure with separation by a sieve to prevent the eggs from escaping. The usable capacity of the incubator can be adjusted to a volume of 7-9 liters via a water outlet. Incubation is ensured by generating vortex-like water flows that keep the egg in suspension. This is ensured by the periodic movement of a drawer and the regular supply of water from the drawer with counterweight, which is turned upside down at regular intervals after filling. This system ensures the oxygen supply to the water during the incubation period of the eggs. Fertilized eggs, free of adhesions, were placed in the incubators in portions of 250 g. The water temperature during incubation was between 19 and 24°C and the dissolved oxygen in the water was between 6.9and 8.7 mg/l. The flow rate in the incubators was between 3.5 and 8 l/min, depending on the water temperature and developmental stage of the embryos.

During incubation three antifungal treatments were carried out at an interval of 24 hours, with 1% gentian violet at a dose of 3.5 ml/l administered for 20 minutes [14]. Gentian violet is a synthetic dye with antifungal properties and a complex chemical formula 4-[4,4-bis(dimethylamino benzhydrylidene]cyclohexa-2,5-dien-1-yl-idenedimethylammonium chloride, which is used in aquaculture for the treatment of fish eggs [15]. Fertilized Russian sturgeon eggs freed from adhesions were transferred to the incubator Brateș B1 (treated with mineral silt), Brateș B2 (treated with talcum) and Brateș B3 carp eggs (treated with tannin). The technological water in the recirculation system showed a uniform fungal infestation potential at all the incubators, and at the time of the massive infestation of the sturgeon eggs with *Saprolegnia sp*., the carp eggs were not infested.

To determine the percentage of fertilization of the Russian sturgeon, homogeneous egg samples were collected nine hours after fertilization and counted in triplicate on 10 cm diameter Petri dishes - according to the method of T.A. Dettlaff, et al [16]. The samples were preserved in a solution of 1 part 40% formalin and 9 parts distilled water. Eggs were counted under an Olympus SZ 61 stereomicroscope equipped with a 3MP Olympus SC 59 camera at the stage of second segmentation at the animal pole with the formation of the four visible blastomeres, a stage that allows the

detection of polyspermic eggs that cannot be detected at earlier stages and is contraindicated beyond this stage because unfertilized eggs continue their division until gastrulation, when they rapidly degenerate. The number of polyspermic, activated, unfertilized and normal oocytes was evaluated. Samples were taken in triplicate 3 hours after fertilization to evaluate the fertilization percentage of carp eggs. To qualitatively determine the effects of the applied deadhesion treatments on the transparency and brightness of the eggs examined under the microscope, the comparative method was used in the blastula stages (Photo 1-ABC) and in the last stages of larval development before hatching (Photo 2-ABC).

During embryogenesis there was a massive infestation of *Saprolegnia sp*. (despite antifungal treatments), which was recirculated with the water in the system until the larvae hatched. From the point of view of fungal infestation, it was found that in the incubators where eggs adhesiviness was treated with mineral silt -B1 and with talcum- B2, the percentages of infested eggs were 28.34% and 26.23% respectively, while in B3 incubator, where the carp eggs adhesiviness was treated with tannin, the percentage of fungal infestation was 2.09%. The results were statistically processed in Excel program using ANOVA and post hoc analysis performed by using Tukey test.

RESULTS AND DISCUSSIONS

The results on the substances used were obtained from the qualitative assessment of the deadhesion efficiency and transparency of the chorionic membrane and the quantitative statistical calculation of the mean values to determine the percentage of fertilisation and fungal infestation of the fish eggs.

Qualitative assessments were obtained by optical analyses performed in triplicate on samples of embryonated eggs in the triplicate. The eggs samples were taken at random, incubated under the same conditions of temperature and flow rate of technological water, counted under the stereomicroscop and evaluated in terms of transparency, mineral load of the membrane, and viability.

When viewed under the stereomicroscope, the characteristics of the eggs differ morphologically in the three variants: rough appearance, maximum adhesion of silica crystals and mineral impurities, and high opacity for the eggs treated with mineral silt (photo 1-2 A), smooth appearance and good transparency for the eggs treated with talcum (photo 1-2 B) and smooth appearance and medium to good transparency for the eggs teeated with tannin (photo 1-2 C).

For the evaluation of transparency, a rating scale was used based on three main categories of transparency: poor, average and good, with intermediate values also included in the evaluation. In B1 the eggs showed a poor degree of transparency due to mineral adhesions and poor silt deadhesion capacity, in B2 the talcum deadhesion was total leading to good transparency of the eggs and in B3 deadhesion was effective, but transparency was assessed as medium in the early stages of embryo segmentation, becoming good in the pre-hatching stages (Table 1).

Table 1 Qualitative determination by optical assessment of embryogenesis in Russian sturgeon and carp

Stages of embryogenes is analyzed	Chorionic membrane transparency		
	Silt	Talc	Tannin
The segmentation stage of blastula formation	poor	good	medium
Stage the motile larva before hatching	poor	good	good

Embryogenesis in sturgeon and carp in different variants of eggs deadhesion treatments is shown in the blastula stage (photo 1- A, B, C) and in the pre-hatching motile larval stage (photo 2-A, B, C).

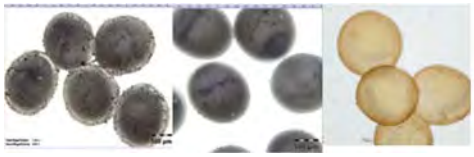


Photo 1A Image with poor transparency of eggs, possibly in the late blastula stage

Photo 1B Good transparency image for eggs treated with talcum, stage 28A

Photo 1C Medium transparency for eggs treated with tannin, early blastula stage

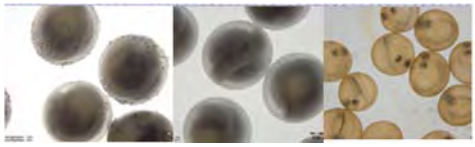


Photo 2A- Low transparency, due to deadhesion treatment with split, eggs at stage 30 A

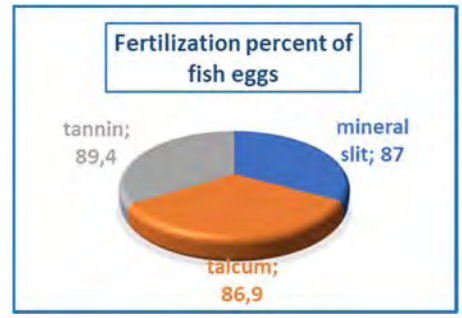
Photo 2B- Good transparency of eggs treated with talcum for deadhesion at stage 34 A

Photo 2C - Image of eggs treated with tannin for deadhesion, good transparency

The developmental stages of embryogenesis in sturgeons are presented according to T.A. Dettlaff, A.S. Ginsburg, O.I. Schmaihansen.

Quantitative assessments were made by stereomicroscopic counting of fertilized eggs at the segmentation stage with four blastomeres to determine the percentage of fertilization [17] in the eggs of Russian sturgeon and carp (Figure 1).

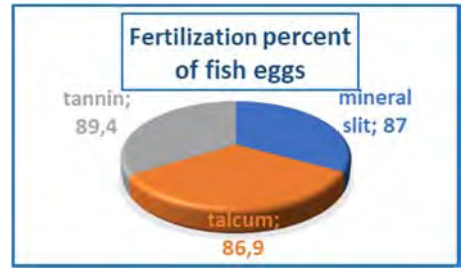
Graph no 1



To determine the degree of eggs infestation with *Saprolegnia sp.* in the differentiated deadhesion treatments, counts were made in triplicate under a stereomicroscope, the mean values were calculated and expressed in graph 2. The results of the experimental variants B1, B2, B3 differed statistically significantly at p-values < 0.05.

Foto no 3 Samples with sturgeon (left) and carp eggs with *Saprolegnia sp*.

Graph no 2



The ratio value between the eggs of Russian sturgeon and carp with saprolegniosis was 13.56 (Table 2).

Table 2 The results of eggs deadeshion in the experimental varians B1, B2, B3.

Deadhesion substances	Silt (B1)	Talc (B2)	Tannin (B3)
Deadhesion percentage (%)	100	98	76
Percentage of fertilization (%)	86,9	87	89,4
Percentage of eggs infested with *Saprolegnia sp*. (%)	26,2	28,34	2,09
Unfertilized eggs/ eggs with Saprolegniosis (%)	0,50	0,46	5,08
Hatching percentage (%)	76	72	97

Analysis of the data presented in Table 2 shows that the use of tannin for deadhesion of eggs is less effective than deadhesion with other substances [18]. Although some studies claim that tannin at high doses can cause abnormalities in embryonic and postembryonic development [19], there are also studies that conclude that there are no major changes in terms of abnormalities in larval development and hatching percentage [20, 21], which is consistent with the results of the present experiment, since the magnitude of these abnormalities is incomparably small compared to the losses caused by the fungal attack. According to the data presented in Table 2, the results of this experiment confirm the beneficial properties of tannin for deadhesion, prevention and treatment against fungal attack, which is in concordance with other previously conducted studies [12, 22, 23].

CONCLUSIONS

In recirculating aquaculture system, fungal infestation during the incubation period causes the greatest loss of biological material, as the recirculating water carries fungal spores that are evenly distributed in all incubators. During the same period, when the same water was heavily contaminated with fungi, the Russian sturgeon eggs suffered a massive infestation with *Saprolegnia sp*. while the carp eggs showed a low infestation at comparable fertilization percentages. This was due to the deadhesion treatment of the eggs with tannin, a non-toxic and environmentally friendly plant extract with a strong antifungal effect. Due to the medium to good visibility and the exceptional antifungal effect at the mentioned dosages, deadhesion of carp eggs with tannin is recommended for use in recirculating aquaculture systems to drastically reduce saprolegniosis and increase the production of hatched larvae.

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