

The use of trifluralin to control fungal infection and to increase hatching rate of fertilized eggs of brood ship sturgeon (*Acipenser nudiventris*) in Guilan Province

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Received: July 2017

Accepted: November 2017

Abstract

A brood ship sturgeon with the weight of 10 kg, produced 3.6 kg eggs. After the fertilization process, 300 g of eggs (approximately 8800 ± 120 eggs) were distributed into each box of Youchtchenko incubator (each incubator has 4 boxes). The research was carried out with 4 treatments and 3 replications in each treatment. The research was conducted at the International Sturgeon Research Institute and Beheshti Sturgeon Restoration and Genetic Conservation Center, in 2017. Treatments included control (no added trifluralin herbicide) and 3 different concentrations of trifluralin, treatment 1 (0.1 mg L^{-1}), treatment 2 (0.05 mg L^{-1}), treatment 3 (0.025 mg L^{-1}). Trifluralin was added 24 hours after placing the fertilized eggs in the incubator. 48 hours after incubation, the number of fungal eggs reached the highest level in treatments. However, in the control treatment it was significantly more than other treatments ($p < 0.05$). In treatments 2 and 3 the remaining eggs and the hatching rate, were significantly lower than other treatments ($p < 0.05$). Possibly, the use of trifluralin in two concentrations of 0.025 and 0.05 mg L^{-1} had negative effects, which may be due to low concentrations and use as a temporary bath. After 96 hours, the eggs were completely hatched. Hatching rate in treatment 1, was significantly more than control and treatments 2 and 3 ($p < 0.05$). The results indicated that trifluralin can be successfully used in the proper function of Youchtchenko incubator, at a concentration of 0.1 mg L^{-1} .

Keywords: Trifluralin, Fungal eggs infection, Hatching rate, Youchtchenko incubator, Ship sturgeon.

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Introduction

Fungal contamination of freshwater fish eggs is well known as a problematic disease (Shahbazian *et al.*, 2010). Fungal diseases caused by Saprolegniaceae are of great importance, especially during the incubation of sturgeon eggs (Bauer *et al.*, 2002). Mortality of eggs during this period sometimes reaches 70-90% (Bauer *et al.*, 2002). Saprolegniasis is a kind of fungal disease of fish and their eggs caused by saprolegniacea (Noga, 2000). This fungus lives in fresh water, but some of them can grow in brackish water up to 2.8 ppt (Azari Takami, 1997). Fungal and bacterial growth on fish eggs can seriously compromise egg survival during incubation (Wagner *et al.*, 2012; Zawada, *et al.*, 2014). Sturgeon infection has been reported with the *Saprolegnia* (seven species) at the time of cultivation (Bauer *et al.*, 2002). The most important fungi isolated from sturgeon eggs in Iran (Guilan Province-Sangar) include *Saprolegnia parasitica*, *Fusarium* spp, *Cladosporidium*, *Mucor*, *Aspergillus* spp (Azari Takami, 2009). *Saprolegnia australis* has been reported, observed on the eggs of sturgeon species as *Acipenser guldenstadti persicus*, *A. nudiiventris* and *A. stellatus* (Czeczuga and Muszynska, 1999).

Fungal infection on eggs causes diseases which result in egg mortality, reduce hatching of fertilized eggs and survival of larvae (Yisa *et al.*, 2014). The number of zoosporic fungi growing on the eggs of particular fish species depends not only on the chemical composition of water but also on the eggs itself (Czeczuga and Muszynska, 1999). Dead eggs are a very good medium for developing the

Saprolegnia fungi. A *Saprolegnia zoospore* can grow on dead egg and produce a mass of mycelium. These mycelia surround live eggs and suffocate them so another mass of eggs die and are invaded directly by the fungi. This cycle continues until all the eggs die. Malachite green is used to control this disease, but due to its harmful effects and slow decomposition in nature, using it was prohibited by the Food and Drug Administration (FDA) (Marking *et al.*, 1994). It is reported that, malachite green can cause carcinogenesis, mutagenesis, chromosomal fractures, teratogenicity and respiratory toxicity. Most effects occurred in biochemical factors of blood in the fish exposed to this substance (Srivastava *et al.*, 2004). Toxicity created in some mammals, covering organ injury, mutagenic, carcinogenic and anomalies of development (Srivastava *et al.*, 2004). The banning of malachite green increased the demand to find an effective alternative material to control the disease (Banavreh *et al.*, 2004). Also, Brock and Bullis (2001) reported that saprophytes will rapidly colonize dead eggs, and if unattended, the infection can quickly spread to adjacent, healthy eggs. Disinfected water can be applied by using widely available water treatment approaches such as ultraviolet light, ozone or chlorination-dechlorination and water filtration (down to 1 μ m) to remove fungal zoospores. Precautionary treatment of embryos, nauplii and early larvae of marine shrimp, by continuous bath at 0.01 to 0.2 mg L⁻¹ of the herbicide trifluralin, betadine (20-50 ppm for 30 s), formalin (200-300 ppm for 30 s) has been reported (Brock and Bullis, 2001). Lio-Po *et al.*

(1982) investigated in vitro effects of trifluralin on hyphal growth and saprogenesis of *Lagenidium* spp. isolated from *Penaeus monodon* larvae and *Scylla serrate* eggs. Knen and Cava (2008) evaluated the genotoxic effects of trifluralin on Nile tilapia. Also the effects of trifluralin on biochemical and histological parameters of fish was investigated (Poleksis and Karan, 1999). The purpose of this study was to use the trifluralin to reduce fungi and increase the hatching rate of eggs of ship sturgeon, in Youchtchenko incubators.

Materials and methods

A brood ship sturgeon weighing 10 kg, produced 3.6 kg eggs. After fertilization 300 g of eggs (approximately 8800 ± 120 eggs) were distributed into each box of Youchtchenko incubator (each incubator has 4 boxes). The research was conducted at the International sturgeon research institute and Beheshti Sturgeon Restoration and Genetic Conservation Center in 2017. The research was carried out with 4 treatments and 3 replicates in each treatment. Treatments included control (No added trifluralin) and 3 different concentrations of trifluralin, treatment 1 (0.1 mg L^{-1}), treatment 2 (0.05 mg L^{-1}), treatment 3 (0.025 mg L^{-1}). After 24 hours trifluralin was added as a 15 min bath once daily for up to 48 hours and twice daily until the end of incubation time (96 hours). Fungal eggs were syphoned and weighed daily. The total number of fungal eggs was determined by counting the number of fungal eggs per g of sample. Water temperature, dissolved oxygen and pH were measured daily. Hatchability percentage, fertilization percentage,

fecundity (Yisa *et al.*, 2014) and the number of fungal infected eggs (Sharifpour *et al.*, 2015) were determined according to the following formulae

Hatchability percentage = $\text{No. of fry} \times 100 / \text{No. of fertilized eggs}$

Fertilization percentage = $\text{No. of fertilized eggs} \times 100 / \text{No. of eggs stripped}$

Fecundity = $\text{Total weight of stripped eggs} \times \text{Total No. of eggs in sub-sample} / \text{weight of eggs in sub-sample}$

Fungally infected eggs = $[\text{No. of infected eggs} / \text{total eggs}] \times 100$

This experiment was conducted based on a completely randomized design. All statistical analyses were carried out using the SPSS statistical package version 16.0. One-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to identify significant variations at 0.95 confidence limits ($\alpha=0.05$).

Results

24 hours after incubation, fungal eggs were observed and the eggs were not hatched (Table 1). 48 hours after incubation, the number of fungal eggs significantly increased, and the hatching rate was low ($p<0.05$). In this period, the number of fungal eggs in treatments 1, 2 and 3 were significantly lower than in the control treatment ($p<0.05$). 72 hours after incubation, the number of fungal eggs decreased, but hatching rate increased significantly ($p<0.05$). After 96 hours, the eggs were completely hatched. The number of larvae in treatment 1 was significantly more than treatments 2, 3 and control ($p<0.05$, Table 1). The weight of larvae decreased from beginning to the complete hatching of eggs (Table 1). The mean number of larvae to the number of

eggs in treatment 1 was significantly more than in treatments 2, 3 and the control ($p<0.05$, Table 2). A number of eggs, fertilized eggs, and fertilization rate (%) in each box of the incubator, and practical fecundity are presented in Table 2. Dissolved oxygen, water temperature and pH were measured every day (Table 3).

The number of infected eggs 24, 48 and 72 hours after the beginning of incubation are presented in Figs. 1, 2 and 3, respectively. Also the number of larvae 96 hours after the beginning of incubation in treatments is presented in Fig. 3.

Table 1: The number of fungal infected eggs and larvae in different treatments during incubation.

Hours after beginning incubation	Treatment	Number of infected eggs	Number of larvae	Mean weight of larvae (g)
24	Control	35±7.07 ^a	The eggs were not hatched	The eggs were not hatched
	1	14±5.65 ^b		
	2	35.5±4.94 ^a		
	3	39±5.65 ^a		
48	Control	2650±212.13 ^a	88±11.31 ^a	0.056±0.001 ^a
	1	1575±106.06 ^b	95±7.07 ^a	
	2	123±32.52 ^d	58±11.31 ^b	
	3	175±33.94 ^c	55±7.07 ^b	
72	Control	126±36.76 ^a	1432±141.42 ^b	0.031±0.001 ^b
	1	70±14.14 ^b	2535±162.63 ^a	
	2	129±19.79 ^a	1201±62.22 ^c	
	3	132±11.31 ^a	995±84.85 ^d	
96	Control	The eggs were completely hatched	1657±145.66 ^b	0.015±0.001 ^c
	1		2836±132.93 ^a	
	2		1470±84.82 ^c	
	3		1429±79.19 ^c	

Each value is mean ± SD of 3 individual observations. Different letters in each row mean significant difference (Duncan's multiple comparison tests, $p<0.05$).

Table 2: Effects of different concentrations of trifluralin on the number of larvae and number of larvae to the number of eggs of ship sturgeon (mean±SD).

Treatments	Mean number of larvae to number of eggs	Mean number of larvae	No of eggs in each box of incubator	No of fertilized eggs in each box of incubator	Fertilized rate (%)	Practical fecundity
Control	0.313±0.027 ^a	1657±145.66 ^a	8800±120	5280±65	60±2	106000±1500
1	0.537±0.024 ^b	2836±132.93 ^b				
2	0.278±0.015 ^{ac}	1470±84.82 ^c				
3	0.270±0.014 ^c	1429±79.19 ^c				

Each value is mean ± SD of 3 individual observations. Different letters in each column mean significant difference (Duncan's multiple comparison tests, $p<0.05$).

Table 3: Average of some water parameters during incubation (mean±SD)

Parameters	Day after eggs hatching			
	1	2	3	4
Dissolved oxygen (mg L ⁻¹)	8.92±0.2	8.82±0.1	8.81±0.2	8.80±0.2
Water temperature (°C)	18.16±0.57	19.16±0.76	20±0.5	20.25±0.25
pH	7.40±0.18	7.38±0.23	7.45±0.12	7.32±0.38

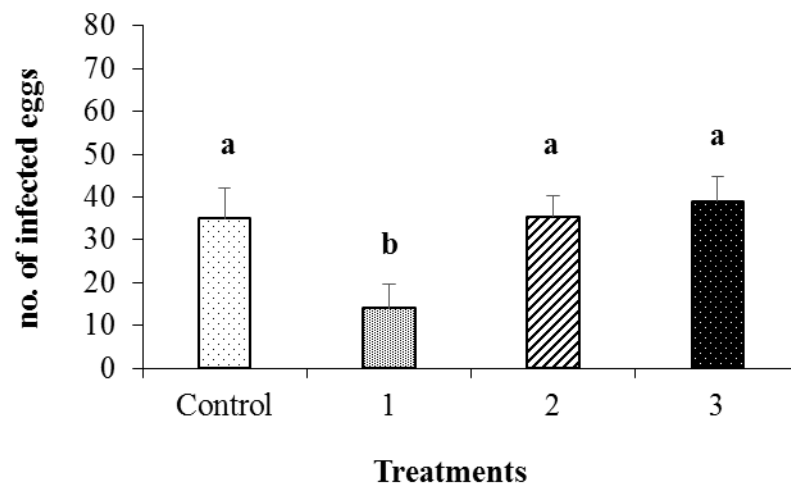


Figure 1: No. of infected eggs 24 hours after beginning of incubation in treatments (mean±SD).

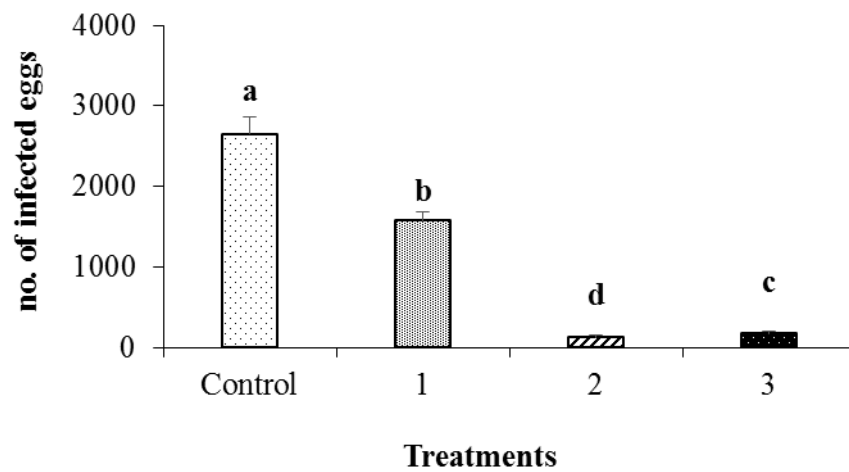


Figure 2: No. of infected eggs 48 hours after beginning of incubation in treatments (mean±SD).

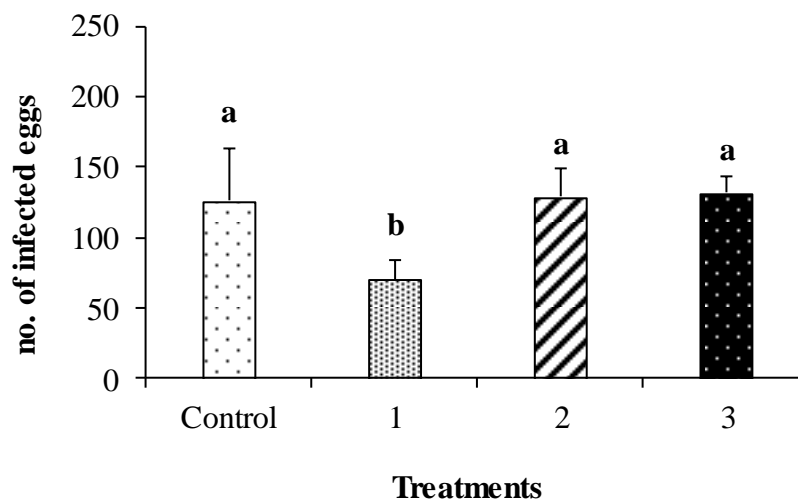


Figure 3: No. of infected eggs 72 hours after beginning of incubation in treatments (mean±SD).

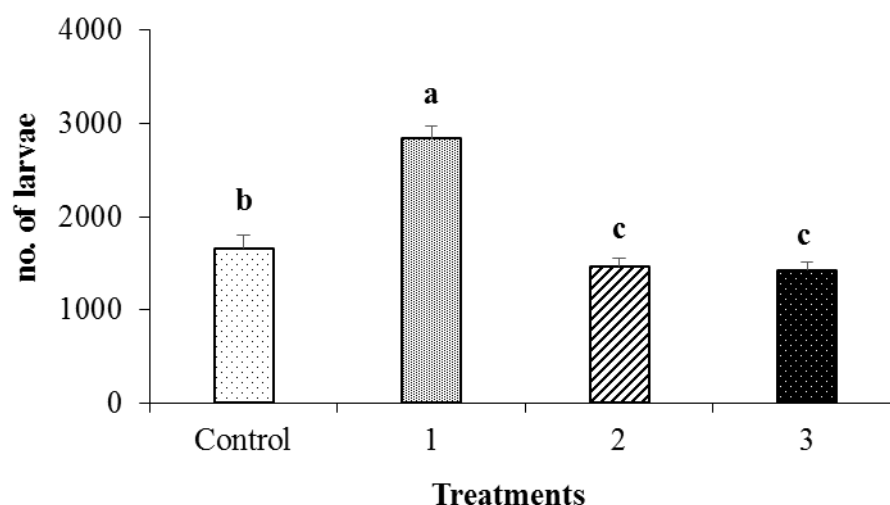


Figure 4: No. of larvae 96 hours after beginning of incubation in treatments (mean±SD).

Discussion

The use of trifluralin, a widely used herbicide for treating eggs of fish, has not been reported. Generally herbicides can have negative effects on aquatic organisms. Determining the effect of this herbicide (trifluralin) on the genome and fish growth performance (especially sturgeon fish) are important issues that need to be examined further. Knen and Cava (2008) studied the genotoxic effect of trifluralin (1, 5 and 10 $\mu\text{g L}^{-1}$ for 3, 6, 9 days) in the species, *Oreochromis niloticus* (Nile tilapia), and found that it significantly increased the micronucleus frequencies in peripheral erythrocytes. So in total it may be concluded that, trifluralin has a genotoxic potential in fish. Also the effects of trifluralin (0.02 mg L^{-1} for 14 days) on the biochemical and histological parameters of carp were investigated (Poleksis and Karan, 1999). They concluded that, trifluralin increased functional enzyme activity in blood serum and had a negative effect on the relative growth rate, and some biological parameters (gills, liver, and kidney) of

fish. As previously mentioned, precautionary treatment of embryos, nauplii and early larvae of marine shrimp, was carried out by a continuous bath of 0.01 to 0.2 mg L^{-1} of trifluralin (Brock and Bullis, 2001). In the present study, as shown in table 1, in treatments of 2 and 3, the remaining eggs and the hatching rate, were significantly lower ($p < 0.05$) than in other treatments (after 48 hours). Possibly, the negative effects observed from the use of trifluralin in two concentrations of 0.025 and 0.05 mg L^{-1} may be due to its low concentrations and use as a temporary bath (15 min). This requires additional research. The effect of trifluralin in higher concentrations (0.1 mg L^{-1}) led to an increase in hatching rate and a decrease of fungal eggs that indicates the positive effects of trifluralin at this concentration. In this connection, Lio-Po *et al.* (1982) reported that, *in vitro*, zoospores of the fungus, *Lagenidium* spp. in the black tiger shrimp (*P. monodon*) larvae and mud crab (*S. serrata*) eggs, were inhibited by $< 1 \text{ mg L}^{-1}$ of trifluralin. This issue shows the effective role of high concentrations of

trifluralin in countering saprognosis of fungi.

In the present study, as seen in table 1, the weight of larvae decreased from the beginning until the complete hatching of eggs. In line with the results of this study, Gisbert and Dorshov (2006) reported that three different phases were detected in allometric growth in weight. From hatch to 6 dph (days post-hatch) growth was negatively allometric, reflecting utilization of yolk for morphogenesis, growth, and metabolic energy. The growth coefficient increased in late lecithotrophic phase (6-14 dph) and the growth was positively allometric during the exogenous feeding phase (15-50 dph).

The sensitivity of eggs to fungal disease depends on something, such as water quality, the flow rate of water and density of eggs in incubators (Banavreh *et al.*, 2004). The capacity of each box of Yushchenko incubator varies from one species to another, and the average capacity of each box is 1.5 kg of fertilized eggs with a flow rate of water of 2-2.5 liter per second (Azari Takami, 2009). In this study, in order to ease the count of larvae and fungal eggs, 300 g of fertilized eggs, were distributed into each incubator box.

One way to control Saprolegniasis is using chemical materials. However it should be noted that, chemical materials always cannot be used in the same way, because their efficacy and toxicity varies with the existing organic matter and quality of physicochemical parameters of waters (Rach *et al.*, 1997). It has been shown that, lake sturgeon eggs (*A. fulvescens*) are the most sensitive species with a mean hatching rate of 54% in 1500 μ L of formalin (used for treating fungal

infections) than other species, including non-eyed eggs of walleye (*Stizostedion vitreum*), common carp (*Cyprinus carpio*), white sucker (*Catostomus commersoni*), channel catfish (*Ictalurus punctatus*) (Rach *et al.*, 1997). Also, the use of 330 ml of 4% formalin for 10 min (in each box of Youchtchenko incubator), and methylene blue at 10 g for 15 min is recommended (Azari Takami, 2009). In the present study, it was found that the eggs of ship sturgeon are not sensitive to short term baths in trifluralin. Determining the effects of trifluralin in a longer bath or continuous bath, and higher concentrations requires further research.

The effect of a chemical disinfectant (formalin) on hatching of eggs of African catfish (*Clarias gariepinus*), as well as survival and growth performance of fry, showed that treatment with formalin at 1 ml concentration for 60 seconds is appropriate to disinfect eggs. The treatment is hence recommended for disinfecting African catfish eggs before incubation (Yisa *et al.*, 2014). Immersion treatment of fertilized eggs of Nile tilapia with 250 mg L⁻¹ bronopol for 10–30 min exposure proved effective against bacteria cultured from fertilized eggs by significantly reducing bacterial load and improving larval survival (Jantrakajorn and Wongtavatchai, 2015). The effect of different concentrations of anolyte on Saprolegniasis in comparison with green malachite in rainbow trout hatcheries, in the northern part of Iran, was determined. It is concluded that constant use of 0.25 ppm of neutral electrolyzed oxidized water (NEOW) is a more effective anti-fungal solution with the least side effects in comparison with 2 ppm of green malachite

(Sharifpour *et al.*, 2015). The mentioned research indicates that it has always tried to examine the effect of different materials on reducing egg losses and increasing hatching rate, but so far there has been no consensus on the introduction of one or a limited number of substances for the control of fungal disease (except for green malachite, which is prohibited). Malachite green was used to control this disease.

In other studies on this subject, the effects of three types of antifungal agents (Nanosil, Chloramine-T and hydrogen peroxide) were compared against fungal infection of Persian sturgeon larvae (Ghazvini *et al.*, 2012). Antifungal treatments were conducted for a period of 18 days as 15 min baths per day. They concluded that 80 mg L⁻¹ Nanosil, had a better effect in decreasing fungal infection than the other treatments, followed by 40 mg L⁻¹ hydrogen peroxide (Ghazvini *et al.*, 2012). Also, the effects of potassium permanganate concentrations in the prevention of infection in Persian sturgeon eggs (*A. persicus*) showed that at 5.37 mg L⁻¹, this material can prevent fungal disease (Banavreh *et al.*, 2004). Treatment of lake sturgeon eggs with 50 mg L⁻¹ iodine for 30 min resulted in an average of 57.8 % reduction in bacterial CFU g⁻¹. While this is a significant reduction, bacteria were not completely eliminated (Chalupnic *et al.*, 1999). Treatment of common carp (*C. carpio*) eggs with sodium chloride at 1.5 g L⁻¹ for 60 min, daily for 4 days showed significantly higher hatching and survival rates (Yahya *et al.*, 2013). A solution made from rock salt is used on sturgeon as a prophylactic treatment to prevent disease and as a treatment for bacterial and fungal

infections, for sac fry, fingerlings, adults and broodstock (Conte *et al.*, 1988).

Despite the wide range of materials, used to treat eggs, for the selection of the substance, their effect on the reduction of egg fungi and the increase of hatching ratio of eggs, should be considered. It can be concluded that trifluralin can be successfully used in Youchtchenko incubators, with proper and non-defective function at a concentration of 0.1 mg L⁻¹.

At the time of egg incubation, dissolved oxygen level should be maintained in excess of 7.5 mg L⁻¹, and water temperature for ship sturgeon is better kept at 14-18 °C (Chebanov and Galich, 2013), and pH at 6.8-7.2 (Azari Takami, 2009). In the present study, most of the demanding physico-chemicals parameters of water were in the proper range although the water temperature was slightly higher than the maximum normal range (Table 3).

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