Optimization of Disinfectants for Controlling Fungus Growth on the Eggs of Nilgiri Melon Barb (*Haludaria* fasciata) During Incubation

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Abstract

Fungal infection on fish eggs leading to low hatching rate and production of larvae is a common problem encountered in fish hatcheries. Various disinfectants are applied on eggs for control of infection and improving larval survival of the aquacultured species. In this study, the doseresponse of methylene blue (MB) and hydrogen peroxide (HP) on the fungal infection and hatching success of eggs of Melon barb was studied. Fungal infection on eggs were observed during the incubation. Higher dosages of both MB and HP have resulted in significantly higher egg mortality and hatchling deformity (p<0.05). The MB dosage of 1.5 ppm has prevented the fungal growth on eggs along with improving the hatching rate and hatchling survival rate. HP dosage of 9.5 ppm has prevented fungal growth in the main study. Application of these disinfectants had increased 7-9% hatchling production than without using any disinfectant (control treatment).

Keywords: Egg, incubation, disinfection, Methylene blue, Hydrogen peroxide

Introduction

Bacterial and fungal infections on fish eggs resulting in declined seed production is a common problem encountered in hatcheries (Forneris et al., 2003). Pseudomonas, Aeromonas, Flavobacterium sp., Cytophyta, Chromobacterium and Vibrio sp. are among the harmful bacteria found on fish eggs. Water molds of the class Oomycetes, such as

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Saprolegnia and Achyla, are the most commonly detected fungal diseases of fish eggs (Madsen et al., 2005; Barnes et al., 2009). Saprolegniasis is one of the widely occuring fungal diseases in freshwater fish hatcheries, causing huge mortalities of eggs (Eissa et al., 2013; Yeasmin et al., 2016). It is believed that infestation with fungus usually starts on dead eggs in the spawn and then spreads to nearby live eggs, resulting in mass mortality. Controlling the fungal infection of eggs during incubation in freshwater fish hatcheries is important for hatchery operators.

In fish hatchery, disinfection is one of the most basic methods of preventing infection on eggs (Stuart et al., 2010; Zawada et al., 2014). Dose-response studies of disinfectants on each speciesis required for selecting a disinfectant and determining the proper dosage for their egg incubation. Research findings on egg disinfection methods for Acipenser persicus (Ghomi et al., 2007) Oncorhynchus tshawytscha (Eissa et al., 2007), Oncorhynchus mykiss (Wagner et al., 2008), Pterophyllum scalare (Sanabria et al., 2009), Clarias gariepinus (Yisa et al., 2014), Salmo trutta (Zawada et al., 2014), Gymnocorymbus ternetzi (Chambel et al., 2014), Danio rerio (Chang et al., 2016), Oreochromis niloticus (Jantrakajorn & Wongtavatchai, 2016), Perca flavescens (Abd El-Gawad et al., 2016) are available in the literature. Methylene blue (MB) and hydrogen peroxide (HP) are the two disinfectants often employed in ornamental fish hatcheries (Yanong, 1996; Russo et al., 2007; Chambel et al., 2014; Sipos et al., 2020).

Haludaria fasciata, often known as the Nilgiri Melon Barb, is an indigenous freshwater ornamental fish in India that is collected from the wild for its trade (Sekharan, 2006; Raghavan et al., 2013). Artificial production in hatcheries aid in reducing the burden of wild collection and over exploitation of their natural population and to meet the demand of the market (Ng & Tan, 1997). Its controlled breeding

was demonstrated and the fish is an oviparopus asynchronous spawner which lavs 180 to 415 with a mean 264±86.6 eggs from ten pairs per spawning process (Mercy et al., 2021). Similar to other barbs, eggs of H. fasciata are demersal and adhesive and are therefore manually collected from the bottom of spawning tanks or any substrate for artificial incubation in hatcheries (Yanong, 1996; Mercy et al., 2021). Such eggs may have come into direct contact with detritus and fish feces, both of which potentially harbor pathogens during incubation and viable eggs may be infected with fungus. In the present study we tried to study the dose-response effect of MB and HP in improving the fungal eradication, hatching success and hatchling productivity of H. fasciata eggs during incubation.

Materials and Methods

Fifty numbers of H. faciata having 4-5 cm were collected from a freshwater stream in Eruvessi village in Kerala, India (12°05′19.5_N 75°34′24.3_E). The sampled fish population were identified using the taxonomic key published by Jayaram (2010) and Pethiyagoda (2013). The brood fishes were disinfected by dipping in 4 ppm potassium permanganate and quarantined for one month. They were stocked in glass tanks for getting acclimatizing to lab conditions and feeding regime during the period. The fishes were fed with commercial pellet feed having 32% protein and water exchange was done at 10% daily. For spawning, the brood fishes were sorted and were stocked two days before the experiment in a ratio of 3 female and 1 male fish. The spawning tank was a glass tank having dimension of 90 cm x 30 cm x 30 cm (LxBxH) and filled with water to a depth of 20 cm. Inside the spawning tank a cage having 60 cmx 25 cm x 25 cm (LxBxH) and 4 mm mesh was installed providing 1 cm space between cage and bottom of glass aquaria. Due to asynchronous reproductive pattern of the species, fifteen breeding tanks was arranged to produce the eggs required for this study.

Brood fishes were stocked inside the cage. The mating and spawning occured on second day at the morning hours from 10 am to 1 pm after stocking. The tank was siphoned at 11am and those eggs collected were discarded. Another siphoning was done at 12:30 to collect fresh eggs from the bottom of tank. These eggs were incubated in treatment tubs. Food grade poly polypropylene plastic tubs (EssecTM) with a dimension of 12 cm x 8 cm x 6 cm was used for treatments. Fifteen eggs were incubated in each tub containing 250 ml water. The treatment group and control were kept in triplicates. Mild aeration was provided in the tubs for sufficient water movement.

The research design (Fig. 1) was to conduct the experiments in two steps, the preliminary experiment, and the main experiment. In the preliminary study, the dosage of MB (Emparta®,Merck Life Science, India) was 1, 3, 5, 10 ppm and HP 30% (Emparta®, Merck Life Science, India) was 1, 5, 10, 25, 100 ppm. After understanding the suitable effective dosage range to control fungal growth from the preliminary study, the optimization of doses was done in the main experiment. Along with disinfectant treatment, a triplicate of control was also set. The water quality was maintained as temperature - 27-29°C, pH-6.8, D.O- 5.8 ppm, ammonia-0 ppm, nitrite- 0 ppm, nitrate- 5 ppm, GH-76 ppm. 40% water change was done every 12 h and replaced with the same dose of disinfectant in the renewed water.

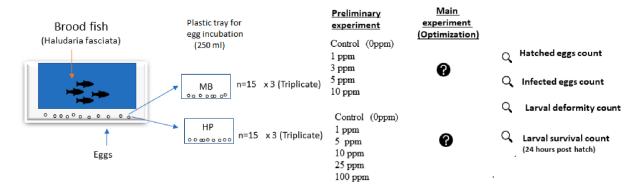


Fig. 1. Schematic presentation of work plan

At 20 h, the eggs were examined for finding the occurrence of any infection. We used a pathological microscope (TM-5.0., Advanced Compound Trinocular Microscope, ESAW, Ambala, India) connected to a camera (CMOS 5MP, 1.0V/LUX-SEC@550n m) for the observation and collecting photomicrograph of fungal infection on eggs. The eggs were wet mounted on a microscope and no stains were applied. The number of hatched eggs, dead eggs, deformed hatchlings (28 h post fertilization or hpf) and larvae survived up to 24 h post hatch (hph) was obtained from the experiment.

The percentage of egg hatching, mortality, infection, deformity, hatchling survival and hatchling productivity was calculated using the following equations.

- Hatching (%) = No. of eggs hatched / No of eggs incubated x 100
- Mortality (%) = No. of dead eggs /
 No. of eggs incubated x 100
- Infected (%) = No. of infected eggs / No. of eggs \times 100
- Deformity (%) = No. of deformed hatchlings / No. of hatched eggs x 100
- Survival rate (%) = No. of survived hatchlings / No. of hatched eggs x 100
- Hatchling productivity (%) = No. of survived larvae / No. of eggs incubated x 100

All statistical analyses were performed with the software Jamovi (Navarro & Foxcroft, 2019). To

determine the differences between the treatments, a One-way Analysis of Variance (ANOVA) with Tukey HSD's multiple comparisons of group means were used. The results of treatments are presented as tables of Mean ± SD of the derived percent rates. For all statistical tests, the significance level was set to p<0.05.

Results and Discussion

All the live eggs (Fig. 2B) in both preliminary and main treatment hatched between 26 and 28 h. The dead eggs in the effective concentration treatments had no fungal infection. In all other treatments, eggs were infected with the fungus *Saprolegnia* sp. which was observed as white tuft on eggs (Fig. 2A). The fungus was morphologically identified by its long hyphae which is branched and aseptate and presence of clauvate zoosporangium. At 28 h fungus has grown bigger and its hyphae projected radially around the egg (Fig. 3C). The MB and HP treated at an effective dosage prevented the growth of fungus on the dead eggs (Fig. 6 A&B).

The eggs treated at 1 ppm dose of MB had a better hatching rate (91.11±7.69%), lower deformity (2.56±4.44%) and higher hatchling survival rate (97.44±4.44%) (Table.1). Higher and lower concentration dosages than 1 ppm had less hatching rate, and survival rate. Dosage of 1 ppm had reduced the infection and deformity. There was no significant difference in hatching rates between the treatments. The hatchling deformity was significantly higher (p<0.05) in concentrations at 3 ppm and above than control and 1 ppm (Table 1).

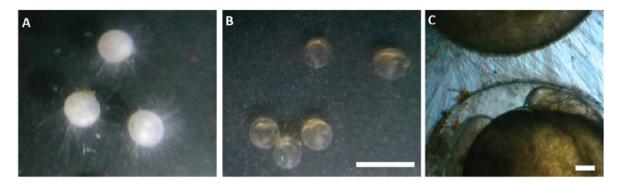


Fig. 2. Eggs of H. fasciata during incubation

A- Dead eggs infected with fungus; B- Live eggs, C- Live eggs being infected by fungus from an infected dead egg (Wet mounted).

Panel A and B scale bar = 2.5 mm, and panel C: scale= 0.1 mm

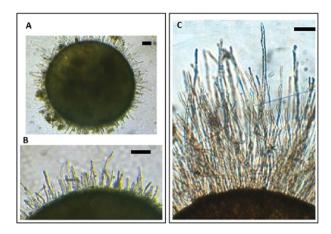


Fig. 3. Photo micrographs of eggs infected with fungus at 12 h (A), 20 h (B) and 28 h (C)

(Wet mounted, Scale= 0.1 mm)

In the HP treatment, the hatching percentage was high in 1 ppm (95.55±3.85%), 5 ppm (95.55±3.85%), and 25 ppm (95.55±3.85%) than in control and 100 ppm treatments (Table 2). But deformity was more observed in 25 ppm. At 100 ppm dosage, the hatching is significantly lower (p<0.05) and is lethal for eggs than all other treatments. In control, 1 ppm and 5 ppm treatments the eggs were observed to have more infection than in higher concentration treatments. The hatchling deformity was significantly higher in 25 ppm than in 1 ppm and 5 ppm treatments (p<0.05) (Table 2).

Based on the fungicidal effect of treatments, we conducted the main experiment/optimization on MB and HP at concentrations between 1.5 ppm and 2.5 ppm and at 5.5 ppm - 9.5 ppm respectively.

Table 1. Preliminary experiment with Methyelene blue

In the main or optimization experiment, the MB treatments at 1.5 ppm dose had the highest hatching rate (95.55±3.85%), hatchling productivity (91.11±3.85%) and low hatchling deformity (4.76±4.12%) among the treatments (Table 3 and Fig. 4). There was no significant difference in the hatching rate between the treatments. There were no infected eggs in disinfectant-treated eggs (Fig. 6A), but the control group had infected eggs.

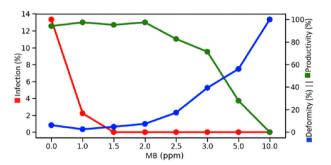


Fig. 4. Effect of MB on egg incubation success factors (mean values)

In the case of treatment with HP, a higher hatching rate was observed in 6 ppm (93.33±6.67%), 6.5 ppm (97.78±3.85%) and 9.5 ppm dosages (93.33±6.67%), (Table 4 and Fig. 5). The egg mortality was higher in 8.5 ppm, 8 ppm, 7 ppm and in control. The egg infection was considerably reduced at 9 ppm and 9.5 ppm (Fig. 6B) when compared with the infection on dead eggs. The hatchling deformity was lower than control in all other treatments and there was no deformity at the 5.5 ppm dose. A higher hatchling survival rate was observed at 5.5 ppm (91.11±3.85%) and 6 ppm (91.11±3.85%). The progressive reduction in fungal infection on dead eggs was observed only

Parameters	Dosage of MB						
	0 ppm (Control)	1.1		5 ppm	10 ppm		
Hatched %	84.44±16.77 ^a	91.11±7.69a	84.44±3.84a	80±6.67a	20±3.85a		
Mortality %	15.55±16.77 ^a	8.89±7.69 ^a	11.11±7.7 ^a	20±6.67a	17.78±3.85a		
Infected %	15.55±16.77 ^a	2.22±3.85 ^a	0^a	0^{a}	0^a		
Hatchling deformity %	4.78±4.17 ^a	2.56±4.44 ^a	39.31±6.45 ^b	56.04±8.96 ^b	100 ^c		
Hatchling survival %	96.67±5.77a	97.44±4.44 ^a	71.37±11.05 ^a	27.89±5.16 ^b	$0_{\rm p}$		
Hatchling productivity %	82.22±20.36 ^a	88.89±10.18 ^a	60±6.67a	22.22±3.84b	$0_{\rm p}$		

abc Within a row, means without a common superscript differ (p<0.05)

Parameters	Dosage of HP							
	0 ppm (Control)	1ppm	5 ppm	10 ppm	25 ppm	100 ppm		
Hatched %	86.66±13.33a	95.55±3.85a	95.55±3.85a	93.33 ^a	95.55±3.85a	$0_{\rm p}$		
Mortality %	13.33±11.54 ^a	4.44±3.85a	4.44±3.85a	6.66 ^a	4.44±3.85a	100 ^b		
Infected %	13.33±11.54 ^a	4.44±3.85a	4.44±3.85a	0^a	0^a	0^a		
Hatchling deformity %	2.22±3.85a	2.38±4.12a	2.38±4.12a	4.76±4.12a	16.35±4.42ab	0 ^{ac}		
Hatchling survival %	97.78±3.85 ^a	97.62±4.12a	97.62±4.12a	88.1±4.12 ^a	83.65±4.42a	0_{p}		
Hatchling productivity %	84.44±7.69a	93.33±6.67a	93.33±6.67a	82.22±3.84a	80±6.66a	0_{p}		

Table 2. Preliminary experiment with Hydrogen peroxide

Table 3. Main experiment with Methylene blue (Optimization)

Parameters	Dosage of MB						
	0 ppm (Control)	1.5 ppm	2 ppm	2.5 ppm			
Hatched %	88.88±10.18 ^a	95.55±3.85 ^a	91.11±3.85a	91.11±7.7 ^a			
Mortality %	11.11±10.18a	4.44±3.85a	8.88±3.85 ^a	8.88±7.7a			
Infected %	11.11±10.18a	0^{a}	0^a	O ^a			
Hatchling deformity %	7.56±0.84 ^a	4.76±4.12a	7.32±7.14 ^a	17.26±5.14a			
Hatchling survival %	91.67±14.43a	95.24±4.12a	97.44±4.44 ^a	82.74±5.14a			
Hatchling productivity %	82.22±20.36 ^a	91.11±7.69 ^a	88.89±7.69a	75.56±10.18 ^a			

from increasing concentration from 8.5 ppm to 9.5 ppm. At these concentration, there was no significant difference in hatching rate, hatchling survival and hatchling productivity with other treatments. There was no significant difference (p>0.05) between the treatments on the parameters in main study for both MB and HP.

In this study, we discovered that up to 33% of eggs incubated in the control treatment was infected by

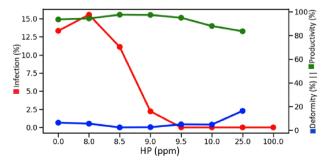


Fig. 5 Effect of HP on egg incubation success factors (mean values)

fungus. It is an opportunistic pathogen that can be found in all bodies of water and poses a damage to the aquaculture industry. Infected eggs usually have a cotton fluff covering the surface of the egg. A living embryo can be killed by the fungus infection, which can spread from egg to egg (Thoen et al., 2011). Previous research has shown that hyphae of fungus entered the egg envelope and fungal spores were found in the cytoplasm, allowing for egg expansion and mortality (Paxton & Willoughby, 2000; Hanjavanit, 2008). The *fungus* species that infected the eggs was not identified in the study. This is owing to its intricacy, which necessitates molecular analysis (Sandoval-Sierra et al., 2014).

The eggs were disinfected in the study's trials by applying HP and MB in various dosages in a prolonged bath treatment (28 h). Higher concentrations of MB and HP had a substantial impact on the hatching rate and survival of the larvae in the preliminary experiment. According to the previous studies most disinfectants have hazardous effects at higher concentrations (Bentivegna & Piatkowski,

Table 4. Main experiment with Hydrogen peroxide (Optimization)

Parameters	Dosage of HP									
	0 ppm (Control)	5.5 ppm	6 ppm	6.5 ppm	7 ppm	7.5 ppm	8 ppm	8.5 ppm	9 ppm	9.5 ppm
Hatched %	86.66± 11.54 ^a	91.11± 3.85 ^a	93.33± 6.67 ^a	97.78± 3.85 ^a	88.89± 3.85 ^a	88.89± 7.7a	84.44± 16.78 ^a	80± 13.33a	88.89± 3.85 ^a	93.33± 6.66 ^a
Mortality %	13.33± 11.54 ^a	8.89± 3.85	6.67± 6.67 ^a	2.22± 3.85 ^a	4.44 ± 3.85^{a}	11.11± 7.7 ^a	15.56± 16.78 ^a	20± 13.33 ^a	11.11± 3.85 ^a	6.67± 6.67 ^a
Infected %	13.33± 11.54 ^a	8.89± 3.85 ^a	6.67± 6.67 ^a	2.22± 3.85 ^a	4.44 ± 3.85^{a}	11.11± 7.7 ^a	15.56± 16.78 ^a	11.11± 13.88 ^a	2.22± 3.85 ^a	0 ^a
Hatchling deformity %	10.56± 5.35 ^a	0 ^a	2.22± 3.85 ^a	8.89± 10.18 ^a	2.56 ± 4.44^{a}	2.78± 4.81 ^a	5.55± 5.09 ^a	2.38± 4.12 ^a	2.56 ± 4.44^{a}	4.95± 4.29 ^a
Hatchling survival %	89.44± 5.35 ^a	100ª	97.43± 4.44 ^a	91.11± 10.18 ^a	97.43± 4.44 ^a	97.22± 4.81 ^a	94.44± 5.09 ^a	97.62± 4.12a	97.44± 4.44 ^a	95.05± 4.29 ^a
Hatchling productivity %	77.78± 13.88 ^a	91.11± 3.84 ^a	91.11± 10.18ª	88.89± 7.69 ^a	86.67± 6.67 ^a	86.67± 11.54 ^a	80± 17.64 ^a	77.78± 10.18 ^a	86.67± 6.67 ^a	88.89± 10.18 ^a

^aWithin a row, means without a common superscript differ (p< 0.05)

1998; Chang et al., 2015). The greater concentrations of both disinfectant treatments on *H. fasciata* eggs resulted in a significant embryo mortality rate, malformed hatchlings and a reduced larvae survival rate. The entire malformed and fragile hatchlings under the treatment died within 24 h. Nutritional variables, defective swim bladders, infections, genetic factors, water current and water quality may all have contribute to the malformation of embryo hatchlings and larvae in hatcheries (Chanu et al., 2010; Berillis, 2015).

In the preliminary study on MB dosages, the concentrations of 3 ppm and above were effective in controlling fungal infection, but in contrast less viable for egg and hatchling survival than its lower concentrations. Similar effect was reported for the cyprinid Cyprinus carpio with 3 ppm and 5ppm, resulting in a poorer hatching and survival rate than the lower dose of 1 ppm (Yeasmin et al., 2016). The hatchling malformation rate of H. fasciata eggs treated at 3 ppm, 5 ppm and 10 ppm increased from 39.61 percent to 100 percent, proportionally to the increasing concentration. Previous studies recommends dosages of MB for eggs incubation at 3 ppm for a cichlid P. scalare (Chambel et al., 2014) and 1 ppm for cyprinids C. carpio (Yeasmin et al., 2016) and Labeobata (Rahman et al., 2017) to achieve better egg viability. In the main experiment of the study the fungal infection was diminished and the hatchling productivity was increased at 1.5 ppm for *H. fasciata*.

In the preliminary experiment with HP, the hatching rate was better in the 1, 5, 10 and 25 ppm treatments. The egg infection was reduced at 10, 25, and 100 ppm, while all eggs were dead at 100 ppm. At 10 ppm and 25 ppm, hatchling deformity increased with increasing concentration, indicating that HP had a negative effect in prolonged baths at these concentrations. At 1 ppm and 5 ppm, hatchling survival and production were higher than the other values. HP at these concentrations had favorable effects on embryo survival and there by reducing the number of dead eggs available for harbouring infection. On the other hand, the fungicidal impact of HP was lower at doses below 5 ppm and higher at 10 ppm. In the main study, the fungal infection was reduced at doses from 8.5 ppm to 9.5 ppm, and the fungus was effectively removed. HP was largely used as a dip and short-term bath therapy for egg

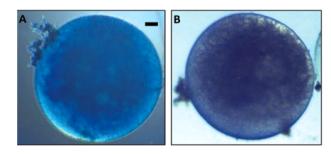


Fig. 6. Dead eggs in the treatments which effectively disinfected with methylene blue (A) and hydrogen peroxide (B) (Wet mounted, Scale= 0.1mm)

disinfection. For trout species Oncorhynchus mykiss eggs, HP at 1000-2000 ppm treatment in a short term bath of 60 min was effective (Kitancharoen et al., 1997; Wagner et al., 2008) and for catfish species Ictalurus punctatus at a concentration of 250-500 ppm in both bath and flush treatments was effective (Small & Wolters, 2003; Mitchell et al., 2009). The hatching and survival rates were improved for a cichlid P. scalare in the prolonged bath treatment of eggs with HP at 25 ppm (Chambel et al., 2014). The 6 ppm, 6.5 ppm, and 9.5 ppm treatments showed a greater hatching rate in *H. fasciata* in prolonged bath treatment. Hatching and survival rates have a direct impact on hatchling productivity. Because of increased hatchling deformities, which lowered hatchling productivity, the control and 6.5 ppm treatments had poorer survival rates. The rates of malformation and hatching were not proportional to the HP treatment doses below 10 ppm. The deformities and survival rate of larvae fluctuated with a concentration equivalent to the hatching rate, which could be a result of a combination of other favourable factors of the disinfectant that were not assessed in this study.

Aside from the fungal infection, both may have affected the hatching rate and survival rate. In aquaculture, HP is utilized as a dissolved oxygen supplement and for ammonia control (Pedersen & Pedersen, 2012; Bögner et al., 2021). The fluctuating hatching rates reported in the HP treatment could be due to the drug's effect on bacterial load and water quality, which affects egg survival. The MB has an oxidizing impact, which may help the embryos survive the experiment. The optimum concentration of HP for fungal irradication, on the other hand, was comparatively less productive than MB. More biological studies are needed to determine which factors of the disinfectant favoured embryo development and thereby increased hatching and hatchling survival rates. Both MB and HP were shown to be effective in disinfecting the fungus on H. fasciata eggs in this investigation.

The *H. fasciata* is a commercially important ornamental fish and its egg disinfection method should be developed for their hatcheries. We found that the optimum concentration for limiting fungal growth on *H. fasciata* eggs was 1.5 ppm for MB and 9.5 ppm for HP. Apart from reduction of fungal growth lower dosage than optimized dosage of HP had improved the egg and hatchling viability. More biological studies are required for finding the

beneficial effect of HP and MB on water quality and embryonic development during egg incubation. We recommend fish hatcheries can use MBand HP at prescribed concentrations for controlling fungal infection on eggs in hatcheries of *H. fasciata* with environmental conditions described in this work.

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