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Hydrogen peroxide, iodine solution and methylene solution highly enhance the hatching rate of freshwater ornamental fish species

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Abstract The aim of this study was to evaluate the effect of hydrogen peroxide, iodine solution (PVP) and methylene blue on eggs disinfection of three ornamental fish species, *Danio rerio*, *Pterophyllum scalare* and *Gymnocorymbus ternetzi*. The main idea was to create conditions to enhance the hatching rates. Eggs of each species were exposed to different concentrations of hydrogen peroxide (5, 10, 15 and 25 mg/L), PVP (0.25, 0.5, 0.75 and 1 mg/L) and methylene blue (0.5 1, 2 and 3 mg/L). The optimal doses ranged between species and chemicals: for *G. ternetzi*, the concentrations that high enhanced the hatching rate were 1 mg/L for the PVP treatment, 25 mg/L for the hydrogen peroxide treatment and 3 mg/L for methylene blue treatment; for *P. scalare*, the best results were achieved with 25 mg/L for hydrogen peroxide treatment and 3 mg/L for methylene blue treatment. By contrast, for all the different chemical did not increased the *D. rerio* hatching rate. Results showed that hydrogen peroxide and methylene blue are the most versatile, effective and safe to use in these species. On the other hand, PVP can be used but with many precautions due to very low safety margin. Results clearly show that the optimal concentration of chemicals for eggs disinfection is fish species dependent and it is completely wrong to extrapolate concentrations between different chemicals and fish species. Our study suggests that *P. scalare* can be used as a model in study of effectiveness of new chemicals with potential to disinfect water and increase hatching rates.

Keywords Ornamental fish · Disinfection fish eggs · Hydrogen peroxide · Iodine solution (PVP) · Methylene blue

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Introduction

The ornamental fish sector is a widespread and global component of international trade, with annual value of the world's wholesale of one billion dollars, most of the aquaculture production of ornamental fish focuses on freshwater species (Chambel et al. in press). Currently, the development of an ornamental aquaculture protocol faces several critical bottlenecks related to production processes and the competition of less expensive specimens collected from the wild (Calado 2006).

The main constraint in the reliable production of most fish species is mortality during the early developmental stages. The quality of eggs and larvae produced in hatcheries is considered an important limiting factor in the larvae production and, consequently, in the development of the aquaculture industry (Kjørsvik et al. 1990; Peck et al. 2004).

The damage by fungi in fish eggs results in an average annual lack of production of about 20 %, with peaks higher than 40 % (Forneris et al. 2003). Due to heavily colonization by pathogens on the external surface of fish eggs, disinfection of eggs has been widely used to reduce egg mortality and to improve rearing success during the yolk sac and first feeding stages and also to reduce mortality of fish eggs incubated in hatchery tanks (Morehead and Hart 2003; Madsen et al. 2005; Stuart et al. 2010).

There are some protocols for fish eggs disinfection, which include the use of malachite green solutions, formalin, hydrogen peroxide, iodine solution (PVP), methylene blue, ozone or sodium hypochlorite (Gaikowski et al. 1999; Arndt et al. 2001; Small and Wolters 2003; Rasowo et al. 2007). However, most published egg disinfection drug efficacy studies were conducted on salmonids or on cool and warm water fish species and concentration of the chemical to be used depends on fish species, the contact time of the chemical and the water temperature (Rach et al. 1998, 2004; Rasowo et al. 2007; Hirazawa et al. 1999; Eissa et al. 2013), To the best of our knowledge, no studies have reported for the chemicals antifungal efficacy on the ornamental fish eggs disinfection.

The goal of this study was to evaluate the effect of hydrogen peroxide, PVP and methylene blue on eggs disinfection of three ornamental fish species, *Danio rerio*, vertebrate fish model in research, *Pterophyllum scalare* and *Gymnocorymbus ternetzi,* two of the most popular ornamental fish species, in order to promote high hatching rates and larval survival to the first feeding.

Materials and methods

Eggs and experimental facilities

The study was conducted at the Ornamental Aquaculture Laboratory of Polytechnic Institute of Leiria. All eggs were hatched in the laboratory. Males and females of *D. rerio* and *G. ternetzi* were maintained separately in glass aquaria (60L) and *P. scalare* broodstocks were maintained in glass aquaria (100 l), both with external filtration, constant aeration, photoperiod 14:10 light–dark cycle, temperature of 27 ± 1 °C, pH 6.5–7.5 and fishes were fed ad libitum three times *per* day with commercial granulate food. Tanks were cleaned daily, and water quality parameters were measured twice a week.

One day before reproduction males and females of each species *D. rerio* and *G. ternetzi* were joined and in next morning fish spawned. After spawning, eggs were gently siphoned and equally divided among experimental design. In the case of *P. scalare* 2–3 days before spawning, the pair selects and begins cleaning the spawning site, using their mouths to bite

and scrub the surface of the slate. After spawning, all eggs were gently siphoned from the hatching slates and equally divided among experimental design.

Preliminary study

To determine the concentrations of each chemical used in this study, a preliminary test was performed with the species *G. ternetzi*. To evaluate the effect of hydrogen peroxide, PVP and methylene blue treatment dose on the hatching rate, 20 eggs were placed in aerated 400 mL beakers with the concentrations of 25, 500, 100 and 200 mg/L of hydrogen peroxide (PANREAC QUIMICA, Spain), 1, 3, 5 and 10 mg/L of PVP (MEDA Pharma, Portugal) and 1, 5, 15 and 25 mg/L of methylene blue (Merck SA, Germany). All concentrations were tested separately and defined on the active ingredient substance.

Each treatment was tested on three replicates, the disinfectants were applied at the beginning of each experiment, and a complete water change was performed after 24 h (time to eggs hatching).

Main study

The main study was realized testing the chemicals concentrations of 5, 10, 15 and 25 mg/L of hydrogen peroxide, 0.25, 0.5, 0.75 and 1 mg/L of PVP and 0.5, 1, 2 and 3 mg/L of methylene blue on *D. rerio*, *G. ternetzi* and *P. scalare* in same conditions as the preliminary study. Complete water change was performed after 24 h to *D. rerio* and *G. ternetzi* and 48 h for *P. sclare* (time to eggs hatching).

Determination of hatching rate

The hatching rate was expressed by the percentage of number of larvae hatched, 24 h (*D. rerio* and *G. ternetzi*) or 48 h (*P. sclare*) after each treatment.

Statistical analysis

All data were checked for normality and homoscedasticity. One-way analysis of variance (ANOVA) with Tukey HSD's multiple comparison of group means was employed to determine significant differences between the different treatments (Zar 2009). When normality and homoscedasticity were violated, Kruskal–Wallis nonparametric test was used with Games–Howell multiple comparison test (Zar 2009). Additionally, linear regression analysis was used to measure the relationship between chemicals concentrations and hatching rates.

Where applicable, results are presented as mean ± SEM. For all statistical tests, the significance level was set at $p \leq 0.05$. All calculations were performed with IBM SPSS Statistics 20.

Results

During the experimental period, the water quality of broodstock was maintained in appropriate values for maintaining these species, OD > 8.0, pH between 7.1 ± 0.4 temperature 27 ± 1 °C, total ammonia and nitrite below 0.5 mg/L and nitrate <10 mg/L.

Preliminary study showed that hatch rate obtained in control group and treatment groups varied between 0 and 100 % (Fig. 1). The results showed that the average hatching rates in all chemical treatments increased when compared with the control group ($p < 0.05$). The only exception was obtained for the treatment with PVP at concentration of 15 mg/L, where no differences to control group were found ($p > 0.05$). On the other hand, at 25 mg/L, the hatch rate was lower compared with all treatments including the control group ($p < 0.05$). Furthermore, this study showed that the lowest concentration tested of PVP and hydrogen peroxide promoted a hatching rate higher than the control group and no better hatching rates were obtained for the higher concentrations.

Hatching rates observed in main study for each species and treatments are shown in Figs. 2A–C, and Table 1. The control groups showed lowest hatching rates, namely, 53 ± 7.7 % to *D. rerio*, 50 ± 2.8 % to *G. ternetzi* and especially 1.86 ± 1.86 % to *P. scalare*.

The hatching rate for *D. rerio* (Fig. 2A) oscillated from 37.8 ± 5.8 % (1 mg/L of PVP treatment) to 66.7 ± 10.1 % (25 mg/L of hydrogen peroxide treatment). The hatching rate obtained for each chemical was 46.67 ± 10.2–56.67 ± 9.7 % with methylene blue treatment, 37.8 ± 5.8–66.63 ± 6.7 % with PVP treatment and 42.2 ± 5.9–66.6 ± 10.1 % with hydrogen peroxide treatment. However, no statistically significant differences were found when compared between treatments ($p > 0.05$).

The *G. ternetzi* hatching rate was significantly enhanced by all the chemical treatments ($p < 0.05$) when compared with the control, with the exception of 0.5 and 1 mg/L for the methylene blue treatment, 5 mg/L for the hydrogen peroxide treatment and 0.25 mg/L for the PVP treatment (Fig. 2B). Furthermore, the highest *G. ternetzi* hatching rate was obtained for the highest concentration tested, which was 90 ± 2.8, 86.6 ± 1.6 and

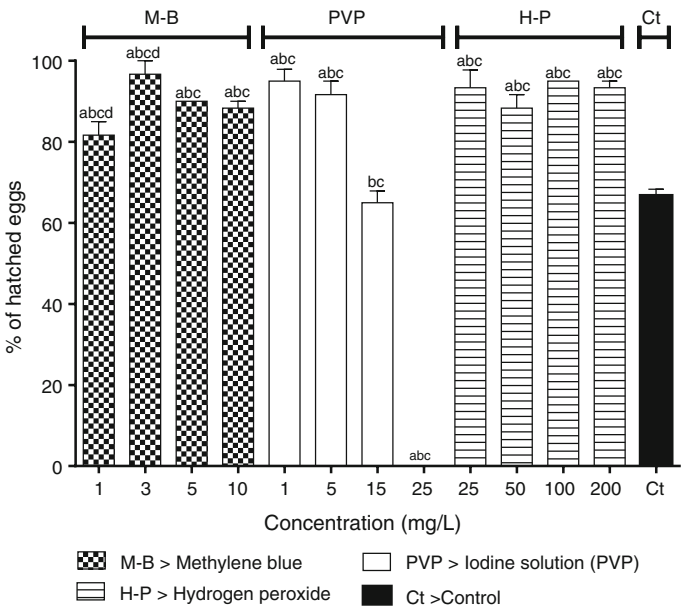

Fig. 1 Effect of methylene blue, PVP and hydrogen peroxide in percentage of hatched eggs of *G. ternetzi* (preliminary study). Values are presented as mean ± SEM ($n = 3$). Lowercase represents significant statistical differences at level $p < 0.05$: **a** between treatments with control group; **b** between treatments with 15 mg/LPVP **c** between treatments with 25 mg/L PVP; **d** between 1 and 3 mg/L methylene blue

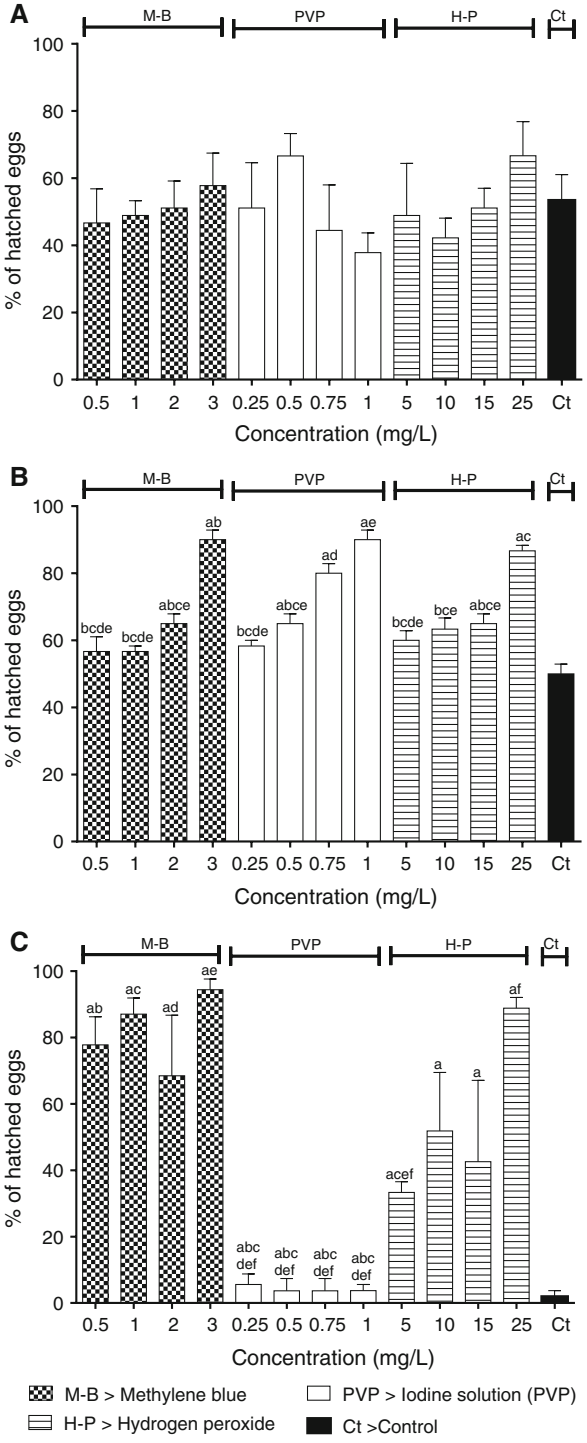

Fig. 2 Effect of methylene blue, PVP and hydrogen peroxide in percentage of hatched eggs on three species under study (main study). Values are presented as mean ± SEM ($n = 3$). **A** *D. Rerio*; **B** *G. Ternetzi*; lowercase represents significant statistical differences at level $p < 0.05$: (*a*) between treatments with control group; (*b*) between treatments with 3 mg/L methylene blue (*c*) between treatments with 25 mg/L hydrogen peroxide; (*d*) between treatments with 0.75 mg/L PVP; (*e*) between treatments with 1 mg/L PVP; **C** *P. scalare*; lowercase represents significant statistical differences at level $p < 0.05$: (*a*) between treatments with control group; (*b*) between treatments with 0.5 mg/L methylene blue (*c*) between treatments with 1 mg/L methylene blue; (*d*) between treatments with 2 mg/L methylene blue; (*e*) between treatments with 3 mg/L methylene blue; (*f*) between treatments with 25 mg/L hydrogen peroxide

Table 1 Values of variation in hatching rate obtained as a function of the concentration of methylene blue, hydrogen peroxide and PVP for the three species under study

Specie	Chemical	R^2	p value*	Variation of hatching rate (%)/mg L^{-1} chemical
D. rerio	Methylene blue	0.1041	0.3065	4.220 ± 3.916
	Hydrogen peroxide	0.2067	0.1375	1.042 ± 0.6458
	Iodine solution (PVP)	0.1431	0.2253	−24.83 ± 19.21
G. ternetzi	Methylene blue	0.7886	0.0001	13.28 ± 2.174
	Hydrogen peroxide	0.7857	0.0001	1.343 ± 0.2218
	Iodine solution (PVP)	0.9	<0.0001	44.00 ± 4.638
P. scalare	Methylene blue	0.04017	0.5322	3.702 ± 5.722
	Hydrogen peroxide	0.4035	0.0265	2.592 ± 0.9966
	Iodine solution (PVP)	0.01783	0.6791	−2.200 ± 5.164

* Significance level was set at p level ≤ 0.05. Values are presented as mean ± SEM ($n = 100$ eggs/chemical/species)

90 ± 2.8 % for 1 mg/L of PVP treatment, 25 mg/L of hydrogen peroxide treatment and 3 mg/L of methylene blue treatment, respectively.

The results of the efficiency of eggs disinfection of *P. saclare* are showed in Fig. 2C. All treatments and concentrations of methylene blue and hydrogen peroxide enhanced the hatching rate comparatively to the group control ($p < 0.05$). On the other hand, no differences were found between hatching rate in PVP treatment and control group ($p < 0.05$) and with this chemical, hatching rates are lowest comparatively with other chemicals independently of the concentrations ($p < 0{,}05$). The hatching rate ranged from 1.86 ± 1.86 % (control group) to 94.4 ± 3.2 % (3 mg/L of L methylene blue). The higher hatching rates obtained were 3.7 ± 3.7 % at 0.5 and 0.75 mg/L of PVP, 88.87 ± 3.2 % at 25 mg/L of hydrogen peroxide and 94.4 ± 3.2 at 3 mg/L of methylene blue.

The results achieved by means of linear regression analysis (Table 1) showed statistical significant dependences to hydrogen peroxide, iodine active and methylene blue for *G. ternetzi* and hydrogen peroxide for *P. scalare* ($p < 0.05$). The statical linear trends are presented in Figs. 3a–d. The hatching rate increases when increases the chemical concentration used on the treatment, which means there is a direct positive correlation between these two attributes. This relation was more prominent for PVP effect on *G. ternetzi* when compared with other species. For this species, an increment of 1 mg/L of PVP leads to an increase in hatching rate of 40 ± 4.6 %.

Discussion

This study was intended to assess the effect of three of the most commonly used chemicals on the hatching success of *Danio rerio*, *Pterophyllum scalare* and *Gymnocorymbus ternetzi* eggs.

All of chemicals used in study showed capacity in increasing hatching rate at least in one specie, and the optimal doses obtained varied between *G. ternetzi* and *P. scalare* species and PVP, methylene blue and hydrogen peroxide. By contrast, for all the different chemical did not increased the *D. rerio* hatching rate.

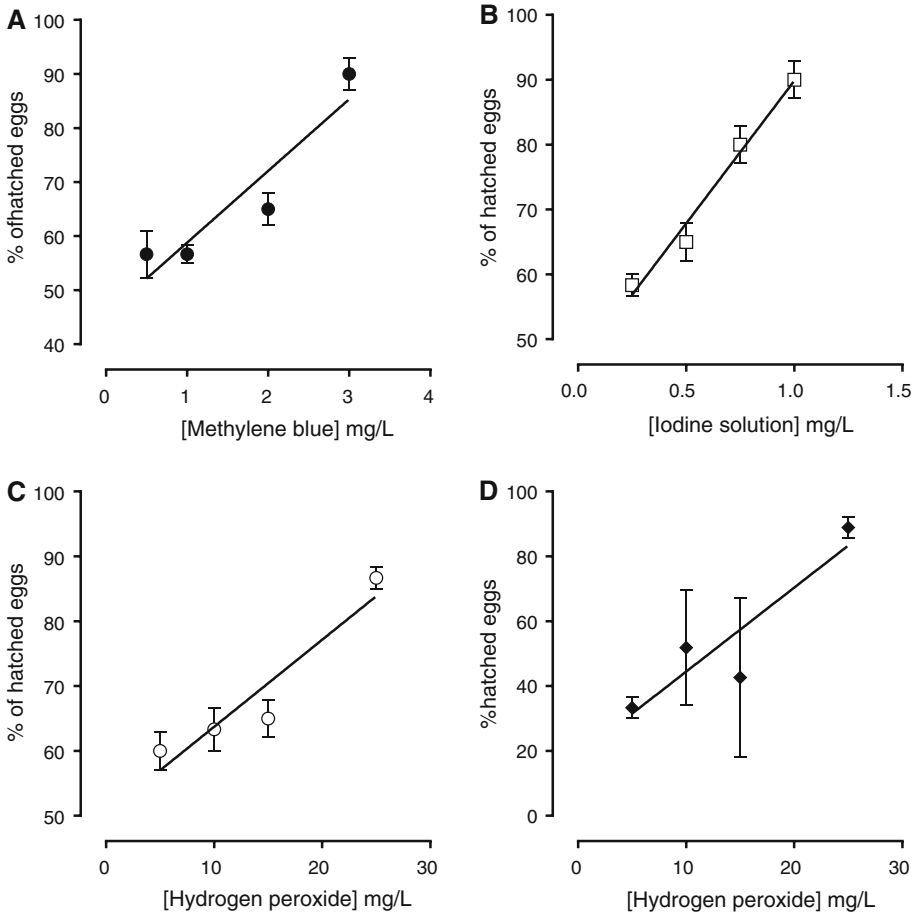

Fig. 3 Linear regression analysis for hatching rate (%) as function of methylene blue (**a**), PVP (**b**) and hydrogen peroxide (**c**) concentration for *G. ternetzi* and hydrogen peroxide (**d**) concentration for *P. scalare*. Values are presented as mean ± SEM ($n = 3$)

The hatching rate obtained to *P. scalare* was very low in control group, however, in the natural environment or in captivity without removing eggs from the parents, usually hatching rate is higher (Farahi et al. 2011). This low percentage of hatching relates to the fact that eggs are taken from the parents, to be subjected to treatment, since in normal conditions, the breeders have parenting functions, removing most of unfertilized and nonviable eggs by constantly agitating the water to hinder the attachment and proliferation of fungi (Degani and Yehuda 1996), In Egypt, Saprolegniosis is considered one of the most important causes of mortality among angelfish (Ahmed et al. 1990). In the absence of parents, eggs are more vulnerable, leading to near zero hatching rates.

The methylene blue showed capacity to increase hatching rate for *G. ternetzi*, and hatching rate shows high dependence of concentration, by contrast for *P. scalare* all concentrations increasing hatching rate but without dependency of concentration. The safety of this chemical in disinfecting eggs is reported by Sanabria et al. (2009) who tested

the effect of methylene blue in eggs and larvae of *P. scalare* showing that 24-h bath post fertilization have no effects on survival and swim bladder.

Hydrogen peroxide shows high capacity in increase hatching rate in a concentration dependent manner for *P. scalare* and *G. ternetzi*. In both species, the highest concentration used (25 mg/L) can reach the maximum hatching rates. This chemical is currently used in aquaculture, but some authors suggests use of hydrogen peroxide at a concentration of 1,000 mg/L in some species of fish and also that the use of 500 mg/L can be lethal for *P. scalare* (Schreier et al. 1996; Barnes and Gaikowski 2004; Sanabria et al. 2009).

Iodine solution only proved to be effective for the *G. ternetzi* specie, although many authors consider it a good chemical for fish eggs disinfection of several species. Stuart et al. (2010) suggested the use of 50 mg/L PVP with a 5 min bath. On the other hand, Eissa et al. (2013) obtained very efficient results against the mold infection on egg stocks of *P. scalare* with the use of 60 mg/L of PVP as immersion solution during 30 min. However, in our preliminary study, the use of 1 or 5 mg/L PVP of and increased the *G. ternetzi* hatching rate. By contrast, the treatment with 15 mg/L of PVP reduced the *G. ternetzi* hatching rate. Moreover, the treatment with 25 mg/L of PVP was toxic for all the eggs. The toxicity was previously referred in baths of fish eggs with 75 and 100 mg/L during 30 min for Chinook salmon and rainbow trout, respectively (Alderman 1984; Fowler and Banks 1990). In line with the preliminary results obtained in this study, Aydın et al. (2011) also found the hatching rates reductions and a significantly increased abnormalities in turbot eggs after iodine treatments.

Our results showed that hydrogen peroxide and methylene blue are the most versatile, effective and safe to use on the three tested species. PVP can be also used but with many precautions due to the very low safety margin.

The results presented here confirm the importance of determining the optimal dose of each chemical disinfectant for each species of fish in order to ensure the health and welfare of the specimens used in aquaculture and laboratory work. One of the key points of our study is related with the view that the optimal chemical doses used on each specie cannot be extrapolated from species to species, independently of the similarity they have. Taken together, both the low hatching rate observed on *P. scalare* control group and the high increases of the *P. scalare* hatching rate induced by the disinfectants, this species could be used as an experimental model in the study of effectiveness of new chemicals for water disinfection and increase the hatching rates.

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