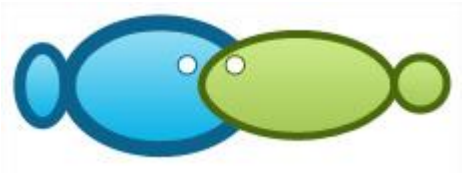

Investigation of the anti-mould property of European alder cone (*Alnus glutinosa*) on fish eggs

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Abstract. One of the biggest challenges encountered during large-scale hatchery operations is the occurrence of water moulds, primarily *Saprolegnia* species belonging to the Oomycetes, which can adversely affect the incubation of fish eggs. The primary objective of the current research was to investigate the effects of alder cone (*Alnus glutinosa*) on the infection and colonization of fish eggs by water mould, while also considering the preservation of fish embryos and larvae under various conditions. Prussian carp (*Carassius gibelio*) and pike (*Esox lucius*) egg hatching tests were conducted using different number of alder cones at different water change methods. In addition, zebrafish (*Danio rerio*) embryotoxicity test was performed with different dilutions of alder cone extract by comparing water of two different fishholding systems. The laboratory tests on *C. gibelio* eggs showed that the effectiveness of alder cones depends on the place of origin and the types of water exchanges in the case of flow-through water treatment. During the experiment with pike, an increasing number of alder cones changed every 24 hours (1-2-3 alder cones per Petri dish) resulted in a significant increase in the hatching rate (Kruskal-Wallis test, $p<0.05$). Alder cone extracts significantly reduced the survival of zebrafish embryos at a relatively high concentration of 47 g of cone L^{-1}.
Key Words: egg treatment, desinfection, antifungal agents, embryotoxicity.

Introduction. Egg disinfection is frequently utilized in aquaculture hatcheries to reduce mortality rates and manage diseases effectively (de Swaef et al 2016). One of the biggest problems in the management of large quantities of hatchery-spawned eggs is caused by water moulds, mainly *Saprolegnia* species of Oomycota. The unfertilised eggs provide a suitable medium for the emerging fungi or fungal pathogens' rapid multiplication. Live embryo containing eggs can also stick to the growing fungal threads, which may be entrapped by the aquatic fungus causing embryo's death or a distorted embryo development (distorted larval development). For this reason, in hatchery practice it is necessary to treat fertilised eggs during the incubation period. Recently, the most commonly used biocidal compound was malachite green oxalate, but the European Commission (37/2010/EU) no longer authorised its use on edible fish and their developmental forms due to its carcinogenic potential (Eszterbauer et al 2018). Since then, research on chemical, physical and biological control of water moulds has been intensified, with several studies investigating possible prevention and elimination options (Marking et al 1994; Eszterbauer et al 2018; Yavuzer 2018; Herrera et al 2021; González-Palacios et al 2019; Verschuere et al 2000; Kucska et al 2024).

In aquaristics, dried alder cones *(Alnus glutinosa)* have long been used as a preventive treatment against various diseases of ornamental fish and shrimps. The recommended dosage used is 4-5 cones 100 L^{-1}. The treatment causes the water to take on a brownish-yellow colour, and the dissolving humic acids reduce the pH of the water, acidifying it if the water to be treated is sufficiently soft (Demény et al 2020). Several pentacyclic triterpenes and β-sitosterols have been identified in supercritical extracts of the bark of the alder. The extracts contained high amounts of lupeol, betulin and betulinic acid, the primary sources of which are the stem, bark, leaves and woody stems (Felföldi-Gáva et al 2012). Research has shown that these chemicals possess antitumor, antiviral, antibacterial, anti-inflammatory, antioxidant and hepatoprotective properties (Felföldi-Gáva et al 2012). Ellagitannins, glutinolines, pedunculagins and praecoxin D have been isolated from the cones by extraction (Ivanov et al 2012). However, little information is available on the composition of solutions prepared from cones soaked in water. Treatment with alder cone has been successfully tested by our research team in the past for egg incubation in several fish species, including crucian carp (*Carassius carassius*) (Demény et al 2020; Szakáli et al 2013) and thermal rudd (*Scardinius racovitzai*) (Müller et al 2018a). Despite the fact that the dosages of the solutions were based on practical observations, no experiments have been conducted to optimise the effect. For instance, there has been no assessment of the dosage required to accurately determine the inhibition of mould infection on egg surface or the impact of the solution on embryogenesis, etc.

The main objective of the study was to investigate the effect of solutions made of alder cone on the infection and colonization of fish eggs by water mould, while also considering the preservation of fish embryos and larvae under various conditions.

Material and Method. Three different experiements were carried out with the following species: Prussian carp (*Carassius gibelio*), pike (*Esox lucius*), zebrafish (*Danio rerio*).

Experiement on Prussian carp egg. For the experiment, we used eggs obtained from induced reproduction of two Prussian carp females. After anaesthesia (Clove oil, *Syzygium aromaticum*) solution (10 drops per 100 L of water), the two females (body weight 240-311 g) were treated with 4.5 mg of pituitary extract (PE) in 0.65% NaCl solution per kilogram of body weight, while the males (body weight 254-261 g) were treated with 2 mg PE kg^{-1} of body weight by intraperitoneal injection. The maturation process was completed after a 12 h latency time in 24°C water, the anaesthetized females were stripped and the two batches of eggs were mixed. The so-called in vitro (also known as dry fertilisation) method was used (Müller et al 2018b). Spermatozoa were activated, and after one minute of agitation, the eggs were swollen with Woynarovich-solution (40 g of salt and 30 g of urea dissolved in 10 L of water). The solution was changed 3 times until the end of the egg swelling (~ 50-60 min) and the eggs just starting to clump were also treated with a tannin solution (5 g of tannic acid 10 L^{-1} of water) for 3 × 15 s. Afterwards, the batches of eggs were selected for treatment. A number of 6314 randomly selected eggs were placed in 84 Petri dishes (Figure 1) (diameter 90 mm, height 15 mm), at a density of 75.2±13.9 eggs by Petri dish (mean±scatter). At the end, 4 eggs from unfertilized batches were also placed in each Petri dish to promote the growth of water mould. No treatment of the batches was carried out in the first 24 hours of incubation. Based on preliminary results, the treatments described in Table 1 were applied, each treatment in 3-3 replicates.

The tannin used in the experiment was ordered from a pharmacy. The alder cone is estimated to contain 15% tannin, so treatments were adjusted to the dose of the alder cone.

Treatment without water exchange (NoWaCh): for treatments without water exchange, we did not exchange the solutions, but we poured the Petri dishes with the corresponding solutions the day after fertilisation, so the egg cells remained in this solution until the end of the experiment.

For the treatments with water exchange (WaCh): the eggs were treated twice daily (9:00 h, 16:00 h). The water was poured off on the top of the eggs, and 60 mL of

the experimental solution was poured over them and left to stand for 20 min. After the time had elapsed, the Petri dishes were filled with 60 ml of clean water from the recirculation system after two washes.

Table 1

Setup of the experiment with *Carassius gibelio* eggs

Water treatment	*Treatment*		*Dose*	*Code*
With water exchange	Control		No treatment	Control - WaCh
	Formalin		1:10 000	Formaline - WaCh
	Tannin		0.06 g L^{-1}	Tannin-0.06 g L^{-1}-WaCh
			0.3 g L^{-1}	Tannin-0.3 g L^{-1}-WaCh
			0.6 g L^{-1}	Tannin-0.6 g L^{-1}-WaCh
	Alder cone solution: place of origin	Gödöllő (Hungary)	0.4 g L^{-1}	0.4 g L^{-1}-WaCh-Gö
			2 g L^{-1}	2 g L^{-1}-WaCh-Gö
			4 g L^{-1}	4 g L^{-1}-WaCh-Gö
		Dunaújváros (Hungary)	0.4 g L^{-1}	0.4 g L^{-1}-WaCh-DuÚj
			2 g L^{-1}	2 g L^{-1}-WaCh- DuÚj
			4 g L^{-1}	4 g L^{-1}-WaCh- DuÚj
		Hannover (Germany)	0.4 g L^{-1}	0.4 g L^{-1}-WaCh-Ha
			2 g L^{-1}	2 g L^{-1}-WaCh- Ha
			4 g L^{-1}	4 g L^{-1}-WaCh- Ha
Without water exchange	Control		No treatment	Control - NoWaCh
	Formalin		1:10 000	Formaline - NoWaCh
	Tannin		0.06 g L^{-1}	Tannin-0.06 g L^{-1}-NoWaCh
			0.3 g L^{-1}	Tannin-0.3 g L^{-1}-NoWaCh
			0.6 g L^{-1}	Tannin-0.6 g L^{-1}-NoWaCh
	Alder cone solution: place of origin	Gödöllő (Hungary)	0.4 g L^{-1}	0.4 g L^{-1}-NoWaCh-Gö
			2 g L^{-1}	2 g L^{-1}-NoWaCh-Gö
			4 g L^{-1}	4 g L^{-1}-NoWaCh-Gö
		Dunaújváros (Hungary)	0.4 g L^{-1}	0.4 g L^{-1}-NoWaCh-DuÚj
			2 g L^{-1}	2 g L^{-1}-NoWaCh- DuÚj
			4 g L^{-1}	4 g L-1-NoWaCh- DuÚj
		Hannover (Germany)	0.4 g L^{-1}	0.4 g L^{-1}-NoWaCh-Ha
			2 g L^{-1}	2 g L^{-1}-NoWaCh- Ha
			4 g L^{-1}	4 g L^{-1}-NoWaCh- Ha

All eggs were counted in each Petri dish at time 0 of the experiement (75.2±13.98 eggs by Petri dish) and the hatched larvae on day 4 (fish were kept in the Petri dish until two days old, when they were not feeding). During the experiment, the water temperature was 26-27°C.

The following parameters have been counted:

Hatching rate (%) = (number of hatched larvae / number of eggs) × 100

Mould growth rate (%) = (number of *Saprolegnia hyphae* infected eggs / total number of eggs) × 100

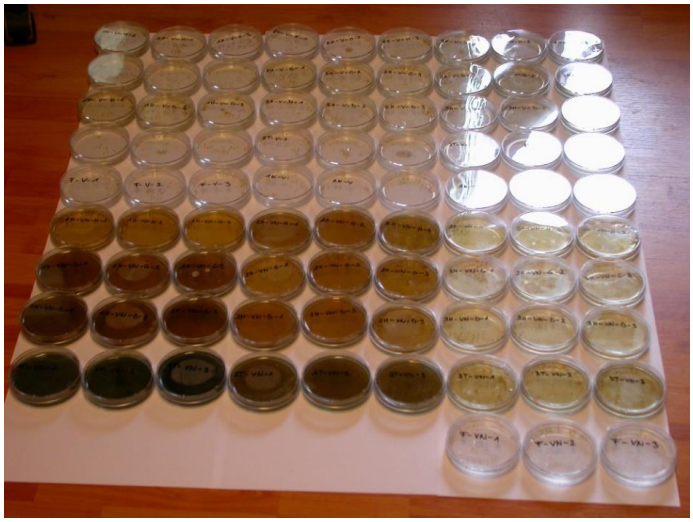

Figure 1. Setup of the experiment with *Carassius gibelio* eggs.

Experiement 2. Pike (E. lucius) egg hatching test. The fertilised 4 day-old pike eggs were obtained from the hatchery of the Ráckeve Danube Branch Angling Association (Kossuth str. 94. 2300 Ráckeve, Hungary). A pike egg batch with a relatively lower proportion of fertile eggs was selected and specifically used a sample with unfertilised eggs from the Zuger jar. The sample was transported to the fish laboratory of the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, and kept for one day spread in aerated and oxygenated net cages. Afterwards (5 days after fertilization), the fish were transferred to a fish egg incubation system (average water flow rate 55.3 L min^{-1}, water temperature 14.6-14.8°C) in Petri dishes (diameter 90 mm, height 15 mm, height of mosquito net 25 mm from the edge of the Petri dish, in order to prevent egg hatching and larvae drift). Randomly selected eggs (average 216 eggs per Petri dish) were subjected to 4 treatments with solutions prepared using commercially available dried alder cones (Green Aqua alder cones, Green Aqua Kft, Budapest):

- Control: without treatment, n=4 repetitions
- Alder 1: 1 alder cone (0.7-0.8 g) by Petri dish, changed every 24 hours 3 times, n=4 replicates
- Alder 2: 2 alder cones (1.4-1.6 g) by Petri dish, rotated every 24 hours 3 times, n=4 replicates
- Alder 3: 3 alder cones (2.1-2.4 g) by Petri dish, rotated every 24 hours 3 times, n=4 replicates

The pike larvae started to hatch on day 4 from the start of the experiment. The experiment was finished on day 5, when the larvae were counted and the hatching rate calculated: Hatching % = (number of larvae / number of eggs) × 100.

Experiement 3. Zebrafish (D. rerio) embryotoxicity test. For experiments with zebrafish, the broodstock was obtained from our own breeding facility (MATE Szent István Campus, Institute of Aquaculture and Environmental Safety). Laboratory-strain AB zebrafish were bred in experimental spawning tanks using programmed (light-rate controlled) spawning (Gazsi et al 2021). Embryos showing normal cleavage were selected under a light microscope and transferred to a 24-wells plate, with 5 embryos $well^{-1}$ (in 2 mL wells) in 3 replicates. Embryo survival and hatching were monitored daily from 8-16 cells post fertilization until 5 days of age. The test was carried out at 25.5 ± 1°C under an illumination altenating 10 h of light with 14 h of dark. The plots for mortality and survival results represent mean and standard deviation. Two straining solutions were used for testing:

1). 3 alder cones were soaked in 50 mL zebrafish embryo medium (E3: 503 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$-$2H_2O$, 0.33 mM $MgSO_4$-$7H_2O$) for 1 h. Total weight of the cones: 2.35 g. Concentration of the stock solution: 47 g L^{-1}. Initial pH of the embryo medium used for the extract: 7.1. After soaking, pH of the extract: 4.0. Treatment

groups: 100% (V/V)-E3: stock solution (alder cone extract) undiluted; 50% (V/V)-E3: 50% stock solution + 50% E3 solution; 25% (V/V)-E3: 25% stock solution + 75% E3 solution;
2). 3 alder cones were soaked in 50 mL system water, which was used in Experiment 2, for 1 hour. Total weight of the cones: 2.35 g. Concentration of the stock solution: 47 g L^{-1}. The initial pH of the system water used for the extract is 8.1. After soaking, the pH of the extract is 5.41. Treatment groups: 100 % (v/v) R: 100% stock solution (alder cone extract) undiluted; 50% (v/v) R: 50% stock solution + 50% system water, 25% (v/v) R: 25% stock solution + 75% system water.

Statistical analysis. Statistical analysis was performed in two experiments (prussian carp and pike experiments), where the data were compared using a non-parametric Chi2 test (Npar Kruskal-Wallis test). Significance values are presented in the figures and explanations below the experimental results.

Bioethics resolution. According to the Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes (European Parliament and the Council 2010), experiments on fish that are not yet in the autonomous feeding stage do not require authorisation. In the research work, fish in the self-feeding stage for experiments were not used.

Results

Experiement 1 - on prussian carp. Significantly higher hatching rates were observed for the 0.4 g L^{-1} alder cone treatment with water exchange and the formalin treatment with water exchange from cones collected at different geographical locations (Figure 2) compared to the control group.

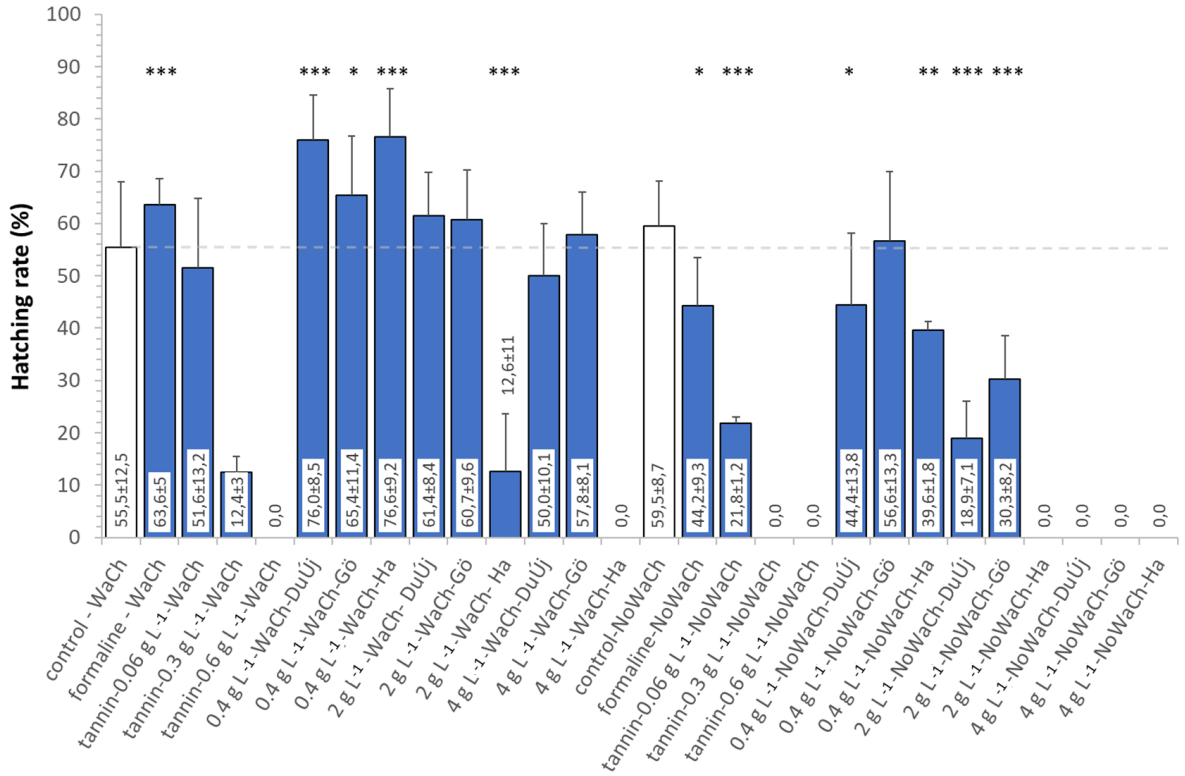

Figure 2. Summary diagram showing the effects of treatments on hatching rate. Asterisks represent a comparative statistically significant result comparing to 'control-WaCh (*p<0.05; **p<0.01; ***p<0.001). WaCh=water changes, NoWaCh=no water changes, DuÚj=Dunaújváros, Gö=Gödöllő, Ha=Hannover.

In the treatments without water exchange, no treatment had a significantly higher hatching rate compared to the control. Treatments with larger dilution rates resulted in 100% mortality (tannin-0.3 g L^{-1}-NoWaCh, tannin-0.6 g L^{-1}-NoWaCh, 2 g L^{-1}-NoWaCh-Ha, 4 g L^{-1}-NoWaCh-DuÚj 4 g L^{-1}-NoWaCh-Gö 4 g L^{-1}-NoWaCh-Ha, where DuÚj= Dunaújváros, Gö=Gödöllő, Ha=Hannover stand for the type of solution, according to the cone origin). In both water treatment procedures, solutions extracted from ginger alder pupae from Hannover had a significantly less favourable effect on embryo survival than solutions from Hungarian cone (Dunaújváros, Paks). The 4 g L^{-1} alder solutions without water exchange hardened the eggshell to such an extent that the larvae were unable to hatch, irrespective of their origin. During the experiement, mould growth was only observed in 5 treatment groups (mean mould growth rate = control - WaCh: 9.8%, control - NoWaCh: 14.4%, tannin-0.06 g L^{-1}-WaCh (5.1%), 4 g L^{-1}-NoWaCh- DuÚj (3.6%), 0.4 g L^{-1}-WaCh-Gö (1.1%).

Experiement 2 - with pike eggs. Summary hatching results of the pike experiment are shown in Figure 3.

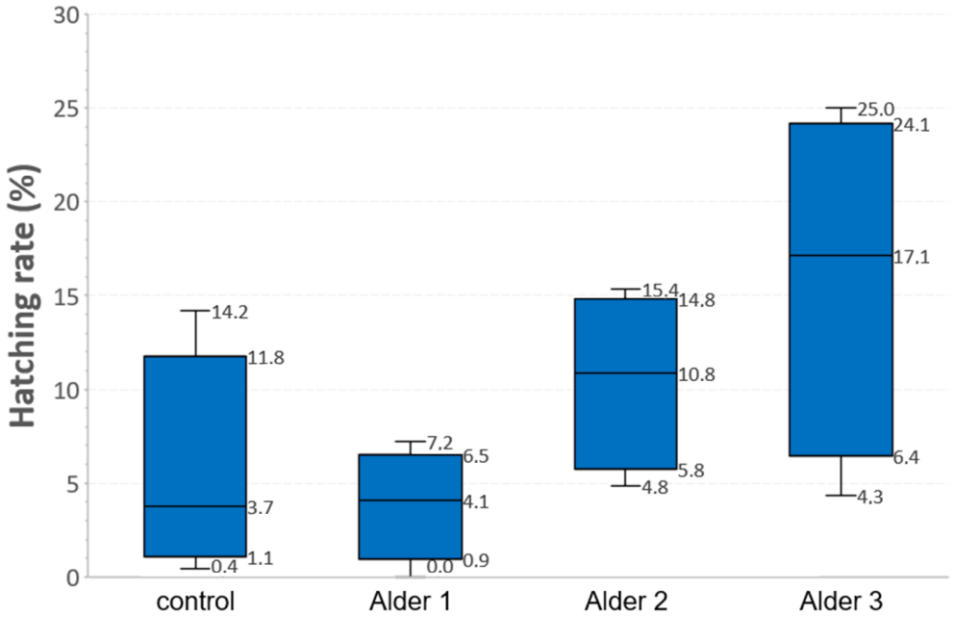

Figure 3. Combined Box plot diagram of the treatments (The bottom of the box represents the first quartile (Q1), the column in the middle is the median or second quartile (Q2), the top of the box is the third quartile (Q3), the interquartile range is the height of the box, i.e., the difference between Q3 and Q1, the upper whisker is the maximum, the lower whisker is the minimum. Asterisks representstatistically significant differences compared to control (***p<0.001).

As the number of alder cones in the Petri dish increased, the hatching rate also increased. The 1 alder cone by Petri dish treatment did not show any difference in the hatching rate compared to the untreated control treatment. In the untreated control samples, the water mould proliferated and clumped in the egg grains. The tannin released from the alder cone solution "stained" the eggshells, giving a brownish-yellowish colouration to the eggs of all three species (Figure 4, for pike). Compared to the control, the size of the mould colonies was smaller in the alder cone treated samples (all replicates of each treatment had mould colonies) and fewer egg grains were interwoven (aggregated).

Figure 4. Within the treatment group, the samples are as follows: a) Control (without treatment), b) 1 alder cone by Petri dish, c) 2 alder cones by Petri dish, d) 3 alder cones by Petri dish.

Experiement 3 - embryotoxicity test on zebrafish. The effects of the different alder cone extracts on embryo survival and hatching are summarised in Figures 5 and 6. No mortality was observed in any of the two types of the media used for the test (zebrafish embryo medium and pike hatchery system water) under control conditions (100% survival). However, the effects of the alder cone extracts showed striking differences depending on the water used. In the case of extracts prepared with zebrafish medium, all embryo died by the end of embryonic development in the stock solution (100%-E3) and in the 50% dilution stock solution (50%-E3), whereas this phenomenon was only observed in the stock solution (100%-R) when pike system water was used. The rate of decline in survival also showed a striking difference between the two different water treatments (Figure 5). All individuals within each group that survived hatched by the end of embryonic development. It is important to highlight that the alder cones significantly modified the pH of the water used for the extracts (E3: 7.1 → 4; R: 8.1 → 5.1).

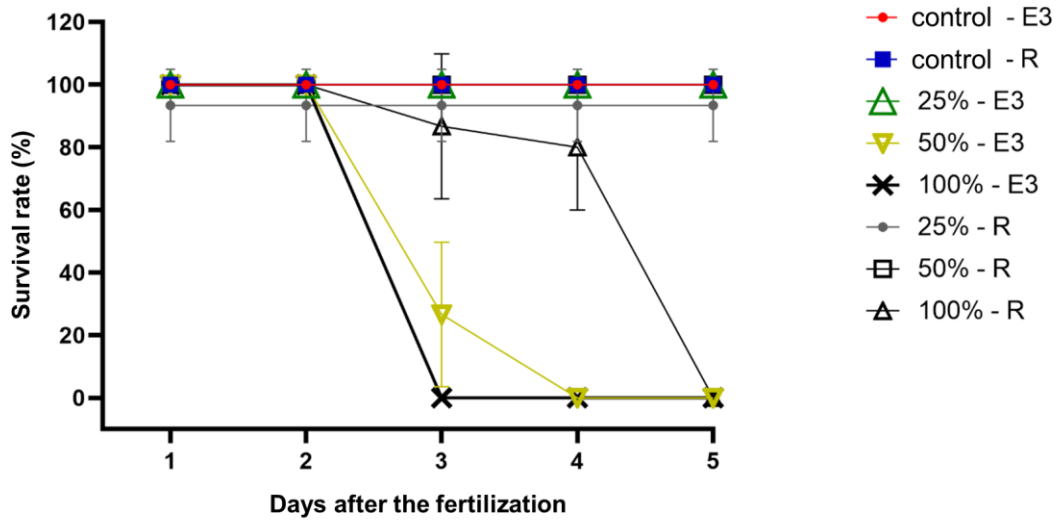

Figure 5. Summarized results of survival at different water qualities and different dilutions of alder cone extract. E3: embryo medium; R: fish-holding system water.

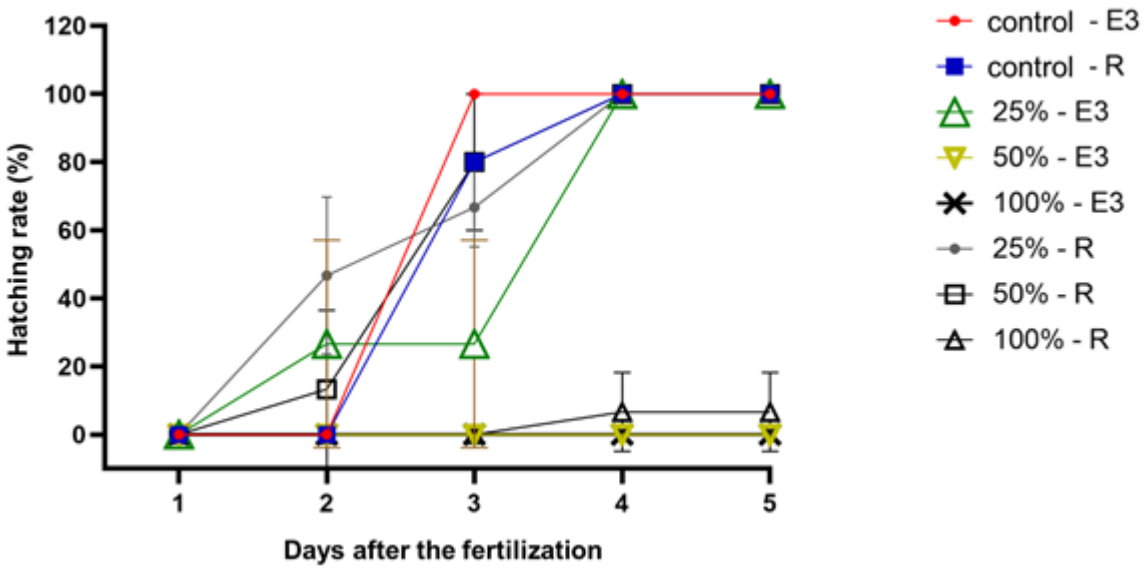

Figure 6. Summarized results of hatching at different water qualities and different dilutions of alder cone extract. E3: embryo medium; R: fish-holding system water.

Discussion. Treatment against mould is essential during incubation. In the prussian carp model fish species, a 0.4 g L^{-1} alder cone solution with water exchange treatment was found to have a statistically proven improvement of the hatching results, even compared to the formalin and tannin treatments used in practice. The alkylating treatments without water exchange caused hardening of the eggshell at higher concentrations, which inhibited the embryo hatching rates. Significant differences in the effects were observed for the solutions made from alder cones collected from different geographical locations. Only at a relatively high concentration of 47 g L^{-1} did the alder cone extracts cause a drastic mortality in zebrafish embryos. The survival of zebrafish embryos below pH 4 (and above pH 10) showed a sharp decrease (Andrade et al 2017), so the pH reducing property of alder cone extracts may have contributed significantly to the mortality observed. However, further experimental work is needed to understand the potential harmfull effects of the different compounds present in the alder cone extracts.

Conclusions. The experiments demonstrate that a solution of alder fruit is effective in protecting against saprolegnia. However, standardizing the solution is challenging due to several factors: (i) The origin of the alder cone significantly affects efficacy, as alder cones collected from different geographical locations exhibit varying effectiveness. (ii) The optimal dose varies depending on the method of application; once-daily treatment proves more effective than egg incubation in the solution and is also enhanced in a flow-through water system. (iii) The quality of the water used for the solution largely determines its toxicity. Given these considerations, conducting a test trial before larger-scale application of alder cone solution is recommended.

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Conflict of interest. The authors declare no conflict of interest.

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