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Reducing or Withholding Hydrogen Peroxide Treatment during a Critical Stage of Rainbow Trout Development: Effects on Eyed Eggs, Hatch, Deformities, and Fungal Control

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Abstract.—In two separate trials, eggs of rainbow trout *Oncorhynchus mykiss* were cultured with the use of hydrogen peroxide and formalin treatments to control fungal infections. For the first trial, treatment regimens consisted of no chemical treatment (control) or daily treatments of either hydrogen peroxide at 500 mg/L for 35 min or formalin at 1,667 mg/L for 15 min. Hydrogen peroxide treatment duration was reduced to 5 min daily during 70–140 daily temperature units (DTU°C). In this trial, 27% of control eggs were infected with fungus, compared with 0% for the hydrogen peroxide and formalin treatments. Eyed egg percentages were significantly lower for control eggs compared with the hydrogen peroxide and formalin treatments. Comparing formalin and hydrogen peroxide treatments, percent hatch at 91% and 90% and percent deformities at 1.0% and 1.3%, respectively, were not significantly different. In the second trial, rainbow trout eggs were reared from fertilization to hatch under four treatment regiments: (1) control or no chemical treatment, (2) 500 mg hydrogen peroxide/L for 35 min daily (hydrogen peroxide A), (3) 500 mg hydrogen peroxide/L for 35 min daily with treatment completely withheld during 70–140 DTU°C (hydrogen peroxide B), and (4) 1,667 mg formalin/L for 15 min daily. Within this trial 15% of control eggs were infected with fungus, compared with 1% for hydrogen peroxide B and 0% for both hydrogen peroxide A and formalin. Eyed egg percentages were significantly better for hydrogen peroxide B than for hydrogen peroxide A. Hatch was significantly reduced in the control group compared with the formalin treatment. Incidence of deformities was not significantly altered by treatment type.

Fungal infection of salmonid eggs can greatly reduce hatching success and seriously compromise the success of a particular hatchery stocking or production program. Historically, malachite green was used by culturists to control fungus of eggs. However, its approval was withdrawn by the U.S. Food and Drug Administration (FDA) in 1991 because of its teratogenicity (Meyer and Jorgenson 1983). Currently formalin fills the role of malachite green and is widely used as an egg fungicide. Although formalin is an effective fungicide for eggs, concerns have arisen over user safety related to its odoriferous nature and suspected carcinogenicity and its potential to adversely affect the aquatic environment (Marking et al. 1994). As a result a substantial effort has been invested in the search for an effective alternate fungicidal treatment.

Marking et al. (1994) screened 21 different chemicals for their efficacy in reducing fungus on eggs of rainbow trout *Oncorhynchus mykiss*. Of those tested, hydrogen peroxide, salt, and formalin were found to be the most effective treatments for controlling fungus, and impacts on the environment were less stringent. Hydrogen peroxide can effectively control fungus on the eggs of rainbow trout (Rach et al. 1995; Schreier et al. 1996; Barnes et al. 1998) and chinook salmon *Oncorhynchus tshawytscha* (Waterstrat and Marking 1995). Although salt may be an effective fungicide (Waterstrat and Marking 1995; Schreier et al. 1996), the large quantities required (≥15,000 mg/L) to achieve satisfactory control may make its use on a large scale impractical (Edgell et al. 1993). Hydrogen peroxide has risen to the forefront as an alternate fungicidal treatment because of its apparent effectiveness in the control of fungus and because it degrades to water and oxygen in the environment. Hydrogen peroxide is currently designated as a drug of low regulatory priority (LRP) when administered at 250–500 mg/L, concentrations that do control fungus on fish and fish eggs (Beaulieu et al. 1992).

Previous unpublished research with hydrogen peroxide at the Fisheries Experiment Station (FES), Logan, Utah, found that, given FES water quality, treatment concentrations of 250 and 500 mg/L for 15 min were not successful in controlling fungus. In follow-up studies, treatments of 500 mg/L for 45 min increased deformities and reduced survival, indicating that treatment durations of 45 min were too long. It is possible that deformity and survival problems may be linked to the toxicity of hydrogen peroxide when it is administered during specific egg developmental stages. Gai-

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kowski et al. (1998) identified a developmental stage during which rainbow trout were sensitive to the administration of hydrogen peroxide, even at 500 mg/L for 15-min. They recommended withholding treatment during 70–140 daily temperature units (DTU°C), which coincides with the period between blastopore formation and its closure (Piper et al. 1982). The objective of our trials was to evaluate the effect of hydrogen peroxide treatment of rainbow trout eggs on fungus control, eyed eggs, hatch, and deformities of sac fry when hydrogen peroxide treatment was constant, reduced to 5 min, or was withheld entirely during the sensitive stage of development.

Methods

We conducted two trials to evaluate different hydrogen peroxide treatment regimens. For trial 1, approximately 60,000 rainbow trout eggs (9 eggs/mL) of the Fish Lake-DeSmet strain were fertilized at the Egan State Fish Hatchery, Bicknell, Utah, on February 24, 1999. The eggs were allowed to water harden for 1 h and were then transported to the FES, where they were treated for 10 min in a solution of 100 mg iodophor/L (Argentyne, Argent Chemical Co., Redmond, Washington). Eggs then divided into 10-L upwelling-type egg jars at 6,300 eggs per jar (three jars per treatment) for the formalin, hydrogen peroxide, and control treatments. The jars were similar in construction to those described by Dewey and Wagner (1993) but were constructed from 15.2-cm (inner diameter) polyvinyl chloride. Water flow to the jars was adjusted to the individual jars so that eggs were not moved by the flow. Flow rate to all jars averaged 3.7 L/min (range, 3.0–5.7 L/min).

The chemical treatments were started the following day via peristaltic pumps (Pulsafeeder Dolphin 50, IDEX Corp., Punta Gorda, Florida). The formalin treatment was run daily for 15 min at a nominal concentration of 1,667 mg/L. Hydrogen peroxide treatment was 500 mg/L for 35 min daily for days 1–6. For days 7–11 (70–140 DTU °C at 12.5°C), the duration of treatment was cut to 5 min daily but was returned to 35 min daily from day 12 to the conclusion of the trial. The 5-min treatment was a compromise between no treatment (no treatment effects on eggs) and the need to maintain control of fungal growth on the eggs. The control jars were not chemically treatment. Several days after all eggs reached the eyed stage, they were physically shocked to turn nonviable eggs white by dropping them from approximately 50–60 cm onto a metal screen. Fungal clumps were removed, total number of eggs compromised by fungus were enumerated, and percentages of fungus-infected eggs were calculated. Viable eyed eggs and nonviable white eggs were then separated using a mechanical egg picker (Jensorter model JB, Jensorter, Inc., Bend, Oregon) and the percent eyed eggs was calculated ([number eyed eggs/initial number eggs] × 100). The eyed eggs from each jar were then moved to individual trays of an incubator system (MariSource, Tacoma, Washington). No chemical treatments were administered while the eggs were in the trays. After the eggs had completely hatched, 6 d after being picked, any dead eggs or deformed fry were counted and removed. Fry deformities included joined eggs, curved and bent spines, and deformed yolk sacs. The percent survival to hatching and percent deformed were calculated (100 × number hatched/number eyed eggs; 100 × number deformed/number hatched).

For trial 2, approximately 158,000 rainbow trout eggs (12 eggs/mL) of the Fish Lake-DeSmet strain were fertilized at the Egan State Fish Hatchery on March 6, 2000. The same methodology and materials were used for this trial as mentioned previously, except for the treatment regimens tested. The first hydrogen peroxide treatment (hydrogen peroxide A), consisted of a 500-mg/L concentration for 35 min throughout the study. The second hydrogen peroxide treatment (hydrogen peroxide B) was 500 mg/L for 35 min daily for days 1–6. At days 7–11 (70–140 DTU °C at 12.5°C), hydrogen peroxide was withheld; treatment from day 12 to the conclusion of the trial was 35 min. Formalin treatment was administered daily for 15 min at a nominal concentration of 1,667 mg/L. Control jars were not treated. In trial 2, water flow to all jars was set to the same flow rate of the individual jar that had the least amount of flow with no egg movement. Flow rate to all jars averaged 2.5 L/min (range, 2.0–2.6 L/min).

Water for the two trials was supplied by a gravity-fed well system. Water quality assessment, conducted on one occasion during both trials, produced the following qualities: dissolved oxygen = 7.1 mg/L, temperature = 12.6°C, oxygen saturation = 78 %, and nitrogen saturation = 111%. Oxygen measured in the jar effluent averaged (±SD) 6.9 ± 0.1 mg/L. Oxygen and temperature were measured with a YSI 58 dissolved oxygen meter (Yellow Springs Instruments, Inc., Yellow Springs, Ohio), and gas saturation by a Sweeney Saturometer DS1-A (Sweeney Aquamatic, Stony Creek, Connecticut).

Chemical treatments were conducted using di-

TABLE 1.—Trial 1: mean (±SD) survival to eyed egg stage, eggs with fungus, hatch, and deformities (three replicates) of rainbow trout eggs treated daily with 1,667 mg formalin/L for 15 min, 500 mg hydrogen peroxide/L for 35 min (5 min daily from 70–140 daily temperature units [°C]), or left untreated (control). Within a column, values with a different letter are significantly different from other values ($P \leq 0.05$).

Treatment	Survival to eyed stage (%)	Eggs with fungus (%)	Hatch[a] (%)	Deformities[b] (%)
Control	62.9 ± 6.1 z	27.6 ± 7.1 z	85.0 ± 6.6	0.9 ± 0.2
Formalin	80.1 ± 3.9 y	0.0 ± 0.0 y	90.6 ± 3.3	1.0 ± 0.2
Hydrogen peroxide	81.6 ± 5.7 y	0.0 ± 0.0 y	90.1 ± 1.5	1.3 ± 0.8

[a] Based on number of eyed eggs.
[b] Based on number of hatched eggs.

lutions of 35% hydrogen peroxide (Dyce Chemical, Inc., Ogden, Utah) and formalin (Paracide-F, Argent Chemical Laboratories, Inc., Redmond, Washington). Treatment concentration calculations were based on percent active ingredient and specific gravity of the test chemicals. Assays of the hydrogen peroxide stock solution, treatment dilutions, and jar effluent were made with a commercial test kit (ammonium molybdate/sodium thiosulfate titration method; model HYP-1, accuracy ± 1 mg/L, Hach Co., Loveland, Colorado). Because of dilutions made of hydrogen peroxide concentrations assayed from jar effluent, values may have displayed an inaccuracy of ± 40 mg/L. For trial 1, hydrogen peroxide assays were conducted on one occasion for samples from the jar effluent at 10 and 20 min after treatment initiation. For each hydrogen peroxide treatment in trial 2, hydrogen peroxide was assayed on two occasions for samples removed from the jar effluent at 5, 15, 30, and 40 min after treatment initiation. Formalin concentrations were not assayed.

For both trials 1 and 2, all jars were inoculated with a fungus cultured from waste feed collected in FES raceways. Fungus samples were cultured on petri dishes that contained Bacto corn meal agar (DIFCO 1984); petri dishes were kept in the dark and stored at about 21°C. To inoculate the egg jars, 1 cm^2 of the agar culture was removed and placed into small tissue cassettes (Tissue-Tek III, Uni-cassette, Ames Division, Miles Laboratories, Inc., Elkhart, Indiana), which were then suspended in the egg jar inflow. The tissue cassettes were not removed from the jars during the daily chemical administration of trial 1 but were for trial 2. The cultures were removed during trial 2 because, by leaving the cultures suspended in the jars during chemical administration, we were defeating the purpose of having a seed culture to assist with egg infection. The inoculum was replaced on a weekly basis after the initial inoculation for both trials.

For trial 1 statistical comparisons were made using SigmaStat 2.03 Statistical Software (SPSS, Inc., Chicago, Illinois). Because all data were expressed on a percentage basis, they were square-root- arcsine transformed and analyzed by a one-way analysis of variance (ANOVA). Duncan's multiple-range test was used for multiple comparison testing. When nonnormally distributed data were encountered by the Kolmogorov–Smirnov test, a Kruskal–Wallis ANOVA on ranks was used. All tests were conducted at $\alpha = 0.05$. For trial 2 the percentages of eggs that hatched or became infected with fungus were not normally distributed, so after running a Kruskal–Wallis test, multiple comparisons were run by making pairwise comparisons between all treatments using the Mann–Whitney test.

Results and Discussion

In trial 1 the control group had significantly more eggs infected with fungus (28%) than either the formalin or the hydrogen peroxide treatments; no fungus was observed in either treatment group (Table 1). Survival to the eyed stage was not significantly different between the hydrogen peroxide (82%) and formalin treatments (80%) but was significantly lower for the control group (63%; $P = 0.026$). The percent hatch was not influenced by treatment type and ranged from 85% for the control to 91% for the formalin treatment. The number of deformities in embryos among the treatments was not significantly different ($P = 0.138$). In trial 1, where hydrogen peroxide treatment duration was cut to 5 min during the critical stage, fungus control, survival to the eyed stage, percent hatch, and the percentage of deformities were not significantly different than those in the formalin-treated eggs. Other researchers have found favorable results when using hydrogen peroxide at 500 mg/L but for shorter treatments of 15 min (Marking

TABLE 2.—Trial 2: mean (±SD) survival to eyed egg stage, eggs with fungus, hatch, and deformities (three replicates) of rainbow trout eggs treated daily with 1,667 mg formalin/L for 15 min, 500 mg hydrogen peroxide/L for 35 min, 500 mg hydrogen peroxide/L (no treatment from 70–140 daily temperature units [°C]), or left untreated (control). Within a column, values without a letter in common are significantly different from other values ($P \leq 0.05$).

Treatment	Survival to eyed stage (%)	Eggs with fungus (%)	Hatch[a] (%)	Deformities[b] (%)
Control	59.6 ± 18.5 yz	14.6 ± 12.1 z	87.7 ± 6.7 z	0.3 ± 0.1
Formalin	77.9 ± 2.9 y	0.0 ± 0.0 y	95.4 ± 0.2 y	0.3 ± 0.1
Hydrogen peroxide A	51.9 ± 5.8 z	0.0 ± 0.0 y	95.1 ± 0.5 yz	0.5 ± 0.1
Hydrogen peroxide B	75.9 ± 6.0 y	0.9 ± 1.5 yz	94.5 ± 0.5 yz	0.3 ± 0.1

[a] Based on number of eyed eggs.
[b] Based on number of hatched eggs.

et al. 1994; Schreier et al. 1996; Barnes et al. 1998).

Treatment effects were also found in trial 2. By the end of the trial significantly more eggs were infected with fungus in the control jars (15% infected; $P = 0.040$) than in the hydrogen peroxide A or formalin treatments. Within the control group, 19% of the eggs in the first jar were infected, 1% in the second, and 24% in the third. Only one other jar among the remaining three treatments exhibited any fungus on the eggs: a hydrogen peroxide-B jar that had 3% of eggs infected. Survival to the eyed stage was significantly lower ($P = 0.041$) for eggs in the hydrogen peroxide-A treatment than in the hydrogen peroxide-B or formalin treatments (Table 2) but, for the controls (60%), was not different from the treatments. Percent hatch for the control group was lower than in the formalin treatment ($P = 0.029$), but it was not different than the two hydrogen peroxide treatments. The percentage of deformities ($P = 0.072$) was not influenced by treatment type. Hydrogen peroxide concentrations assayed from A and B treatment series were not significantly different, although they tended to be slightly higher for series B (Table 3).

In trial 2, withholding hydrogen peroxide treatment during the critical stage of egg development as described by Gaikowski et al. (1998) led to differences in survival to the eyed egg stage and fungus control compared with the constant hydrogen peroxide treatment. With daily chemical administration throughout the study, egg infection was completely controlled, whereas in the hydrogen peroxide-B treatment (hydrogen peroxide withheld during days 7–11), 3% of eggs were infected in one jar. Survival to the eyed stage was superior for eggs withheld treatment (76%) compared with the constant treatment (52%), which may indicate greater toxic effects of hydrogen peroxide during sensitive periods in rainbow trout egg development. Deformities were also slightly higher for the constant treatment (0.5%) compared with those in the withheld treatment (0.3%), but these differences were not significant.

From both of these trials it does seem evident that reducing the treatment duration to 5 min or withholding treatment completely during the critical stage may be an effective way of controlling egg fungus when using hydrogen peroxide. It could be debated, however, that the presence of any fungus-infected eggs within a jar or incubator is less than desirable. Withholding hydrogen peroxide treatment appeared to allow fungal infection to become established in one of the hydrogen peroxide-B jars. Even after chemical treatment was reinitiated the fungus was not eliminated. Allowing the treatment to run for 5 min as we did in trial 1 may have created the difference in total

TABLE 3.—Trial 2: mean (±SD) hydrogen peroxide concentrations (mg/L) from hydrogen peroxide-A (500 mg/L for 35 min daily) and hydrogen peroxide-B (500 mg/L for 35 min daily) treatments withheld from 70–140 daily temperature units (°C). Concentrations were assayed in the egg jar effluent at 5, 15, 30, and 40 min after the initiation of the 35-min treatment.

Treatment and replicates	Hydrogen peroxide concentration (mg/L) at			
	5 min	15 min	30 min	40 min
Hydrogen peroxide A	440 ± 57	515 ± 81	505 ± 44	95 ± 50
Hydrogen peroxide B	480 ± 0	535 ± 19	510 ± 38	90 ± 60
Replicates	2	5	4	4

fungus control. It is also possible that the slight difference in fungus control experienced between the two trials with respect to the withheld treatments was caused by the size and quantity of eggs used, differences in flow regimens, and handling of inoculum. In trial 1, fewer (6,300 eggs/jar) but larger (9 eggs/mL) eggs than in trial 2 (13,200 eggs/jar; 12 eggs/mL), resulted in a total depth of eggs in the eyeing jars of 3.9 cm, compared with 5.7 cm in trial 2. Less egg depth and increased interstitial space between eggs in trial 1 presumably should have reduced the likelihood of fungal growth. Flows were also higher for trial 1 (3.0 L/min) compared with trial 2 (2.5 L/min). In trial 1 the tissue cassettes containing the fungus culture were left in place during chemical treatment, whereas in trial 2 they were removed. This did not appear to have contributed to a higher infection among treatments in trial 2, as might be expected. In trial 1, 28% of control eggs were infected with fungus compared with 15% in trial 2, but this difference was not significant.

The type of egg jars used for this trial was generally effective in maintaining treatment concentrations. During trial 1, after 10 min into chemical administration the assayed concentration was only 387 mg/L, but it reached 567 mg/L after 20 min. Desired concentrations were more quickly obtained during treatments in trial 2. After 5 min, hydrogen peroxide concentration was 460 mg/L (mean of both series A and B) and between minutes 15 and 30 of the 35-min treatment the concentration averaged 516 mg/L. Five minutes after the end of chemical delivery the hydrogen peroxide concentration had dropped to 93 mg/L. In Utah state hatcheries, production eggs are generally placed into large (34 cm diameter) upwelling-type egg jars immediately after water hardening until the eyed stage. So for conditions in Utah, the use of the smaller jars we used for the trials was a realistic evaluation of hydrogen peroxide and concentration ranges associated with such a set up. Care must be taken when working with different egg incubator designs to achieve the anticipated treatment concentration. Past studies have found that calculated target concentrations may actually be less when run through Heath-type or Clark–Williamson incubators, compared with McDonald egg jars (Waterstrat and Marking 1995; Rach et al. 1997).

The use of hydrogen peroxide is also a cost-effective alternative to formalin. Based on the cost of the actual amount of chemical used during trial 1, the total cost of formalin was US $26.85 compared with $7.01 for the hydrogen peroxide (1998 prices). The results from these trials also suggest no significant difference in survival between either hydrogen peroxide or formalin treatments for rainbow trout eggs. Further tests on a larger production scale with different water quality parameters may still be necessary.

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