

COMMENT

Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease II: Efficacy of Using Antibiotics in Diluents and During Water Hardening

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Abstract

Bacterial coldwater disease, caused by *Flavobacterium psychrophilum*, has led to the loss of significant numbers of hatchery-reared salmonids. The bacteria can be spread from parent to progeny within contaminated sperm and ovarian fluid and can enter the egg during fertilization. The addition of antibiotics to diluents and water-hardening solutions could prevent the spread of the disease. In separate trials, a mixture of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin was added to both a 0.5% sodium chloride fertilization diluent and hatchery well water during hardening. Tests showed that the addition of the antibiotics to the diluent and during up to 60 min of water hardening had no effect on the eye-up, hatch and deformity rates of Rainbow Trout *Oncorhynchus mykiss* eggs compared with the nonantibiotic-treated controls. Also, significant reductions in the prevalence of *F. psychrophilum* on the surface and inside eggs were observed when compared with controls. These results indicate that the addition of penicillin and streptomycin to diluents and during water hardening can prevent the vertical transmission of bacterial coldwater disease.

Worldwide, bacterial coldwater disease, caused by *Flavobacterium psychrophilum*, has been implicated in the loss of significant numbers of cultured salmonids (Nematollahi et al. 2003). *Flavobacterium psychrophilum* can be transmitted both horizontally and vertically (Brown et al. 1997; Kumagai and Nawata 2010a). During vertical transmission, bacteria from the ovarian or seminal fluid enter the micropyle during fertilization (Kumagai and Nawata 2010a). The bacterium incubates within the egg and *F. psychrophilum* outbreaks can occur after the eggs hatch (Kumagai and Nawata 2010a). Three possible methods for preventing the vertical transmission of *F. psychrophilum* are vaccination, iodine disinfection prefertilization, and antibiotic treatment. Vaccines against the bacteria are currently under development leaving the iodine (Kumagai and Nawata 2010a) and

antibiotic treatments as the best available methods for limiting the spread of the disease from parent to progeny. Antibiotic treatment is a particularly appealing method for preventing the vertical spread of *F. psychrophilum* because it allows for the treatment of sperm, eggs, and ovarian fluid, whereas iodine treatment is only intended for eggs and ovarian fluid (Kumagai and Nawata 2010a). The bacterium is not susceptible to every antibiotic (e.g., tobramycin: Kumagai et al. 2004) but is susceptible to several including florfenicol (Bruun et al. 2000), oxytetracycline (Bruun et al. 2003), erythromycin (Brown et al. 1997; Hesami et al. 2010), penicillin (Wagner et al. 2012), doxycycline, sarafloxacin, and enrofloxacin (Rangdale et al. 1997).

Penicillin and streptomycin are inexpensive, readily available antibiotics that show promise for controlling *F. psychrophilum*. Previous in vitro trials have demonstrated at 15°C, that 15-min treatments with penicillin (>6.3 mg/mL: Wagner et al. 2012), streptomycin (>5,000 mg/L: Oplinger and Wagner 2013), and a mixture of these two antibiotics (1.6 mg/mL penicillin plus 2.5 mg/mL streptomycin: Oplinger and Wagner 2013) are effective at killing 100% of the bacterium. Toxicity trials have shown that Rainbow Trout *Oncorhynchus mykiss* eggs can tolerate 1-h exposures to high penicillin concentrations (up to 63.2 mg/mL: Wagner et al. 2012) and eggs can tolerate a mixture of penicillin and streptomycin at concentrations of 5.7 mg/mL penicillin plus 9.0 mg/mL streptomycin (Stoss et al. 1978). In contrast, most Rainbow Trout sperm extenders target antibiotic concentrations of 0.08 mg/mL penicillin mixed with 0.125 mg/mL streptomycin (Stoss and Refstie 1983; Negus 2008). In vivo trials in which *F. psychrophilum* had been mixed into sperm and eggs showed that a concentration of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin is required to kill the bacterium (Oplinger and Wagner 2015, this issue).

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One possible method for preventing the vertical transmission of *F. psychrophilum* is the application of antibiotics during fertilization and water hardening. Disinfection of salmonid eggs using Betadine or erythromycin during water hardening has been tested for multiple fish species and pathogens (e.g., Evelyn et al. 1986; Leary and Peterson 1990; Kumagai and Nawata 2010b). Research with erythromycin has shown that the antibiotic is incorporated into the perivitelline fluid during the hardening of eggs from Coho Salmon *O. kisutch* and Chinook Salmon *O. tshawytscha* (Evelyn et al. 1986). In theory, although not thoroughly tested, the vertical transmission of *F. psychrophilum* could be prevented by applying antibiotics during egg fertilization. One benefit of the application of antibiotics during fertilization is that it allows for the simultaneous disinfection of sperm, eggs, and ovarian fluid. Also, antibiotics could enter the egg through the micropyle, which would allow the treatment to continue after water hardening.

This study was undertaken to determine whether the vertical spread of *F. psychrophilum* can be prevented by the application of antibiotics during egg fertilization and water hardening. We tested the addition of a penicillin-streptomycin mixture (0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin (Oplinger and Wagner 2015). The antibiotics were added to both a 0.5% sodium chloride fertilization diluent and a water-hardening solution (antibiotic mixed with well water) to determine whether the antibiotics (1) had an effect on Rainbow Trout egg fertilization, hatch, and deformity rates, and (2) significantly reduced the occurrence of *F. psychrophilum* on both the surface and interior of eggs.

METHODS

Egg safety trial.—Six treatments were tested in this trial: antibiotics were added (1) to the diluent only, (2) after the post-fertilization egg rinse and during water hardening for 15 min after fertilization, (3) after the postfertilization egg rinse and during water hardening for 60 min after fertilization, (4) to both the diluent and during water hardening for 15 min after fertilization, and (5) to both the diluent and during water hardening for 60 min after fertilization, and (6) as a control (no antibiotics added) the normal diluent and water-hardening process was followed. Four replicate lots of eggs, each consisting of eggs from three female Rainbow Trout and sperm from two males were produced. Each lot was split into six equal-sized portions (prior to fertilization), each of which contained approximately 1,250–1,500 eggs and was subjected to a different treatment. The diluent solutions consisted of 0.5% NaCl with or without 0.197 mg/mL penicillin powder (Russell R-Pen, 1,582 IU/mg) and 0.313 mg/mL streptomycin powder (Sigma-Aldrich S6501). To fertilize the eggs, sperm was added and flagella were activated by adding 300 mL of the appropriate (with or without antibiotic) diluent solution. Three minutes after fertilization, the eggs were rinsed three times with hatchery water (pH = 7.6,

total hardness and alkalinity = 180 mg/L). Then they were water hardened in hatchery well water for 60 min after fertilization. When applicable, antibiotics were added to the water-hardening solution at the same concentration as in the diluent. Eggs that were subjected to antibiotics for the first 15 min were also rinsed 15 min after fertilization with hatchery water and then left to sit in hatchery water (without antibiotics) for the remainder of the water-hardening process. In accordance with Utah Division of Wildlife Resources (UDWR) policy (eggs collected and reared in UDWR's Mantua Hatchery, Box Elder County), 60 min after fertilization all eggs were disinfected for 15 min in a 100-mg/L iodine solution (Amend 1974). Eggs were incubated in Heath trays and percent eye-up, hatch, and the deformity rate among the eggs was determined by visually examining every egg in each tray. Percent eye-up was determined 5 d after eyes first appeared within the eggs, and percent hatch and the deformity rates were determined 7 d after the first eggs began to hatch. Data were analyzed as a randomized complete block design using Program R (Hornik 2013) and were considered statistically significant at $P < 0.05$. For this study, replicate groups of eggs were considered blocks.

Egg disinfection trial.—Gametes from six male and six female fish were collected. Approximately 20 min after the gametes were stripped, *F. psychrophilum* (CSF 259-93 strain; Crump et al. 2001) from a 96-h-old culture of a maltose-infused tryptone yeast extract (MAT) broth (incubated at 15°C; Crump et al. 2001) was added to both the sperm and eggs to reach a target concentration of 10,000 bacteria cells/mL. To reach this target concentration, the bacteria density in the 96-h culture was determined using a spectrophotometer (Thermo Electron Corporation Genesys 10 UV, wavelength set at 525 μm) and converted to an estimate of CFU/mL using the formula, $\text{cells/mL} = -5,155,052.107 + 137,140,282.280 \times \text{absorbance}$ (Oplinger and Wagner 2012). The estimated density in the 96-h culture was 6.68×10^7 *F. psychrophilum* cells/mL. Then a 100-fold dilution of this 96-h culture was made by adding 1 mL of this culture to 99 mL of uninoculated MAT broth. Then to reach the target concentration of 10,000 bacteria cells/mL in the sperm and eggs, the volume of sperm and eggs (with ovarian fluid) was determined and 14.97 μL of the 100-fold dilution of the 96-h-old culture per milliliter of sperm or eggs was added. No active outbreaks of bacterial coldwater disease were observed among the broodfish and as a result the density of *F. psychrophilum* naturally occurring in the sperm and eggs was assumed to be negligible (Kumagai and Nawata 2010a).

The eggs were fertilized 60 min after the bacteria were added. Eggs mixed with *F. psychrophilum* were fertilized with sperm containing *F. psychrophilum*. Four separate treatments were tested: (1) eggs fertilized using diluent containing antibiotics and antibiotics applied during the first 20 min of water hardening, (2) eggs fertilized using diluent containing antibiotics and antibiotics applied during the first 60 min of water hardening, (3) a positive control (*F. psychrophilum* added to sperm and eggs

and no antibiotics added to the diluent or during water hardening), and (4) a negative control (*F. psychrophilum* not added to sperm and eggs and no antibiotics added to the diluent or during water hardening). The antibiotic concentration for all applicable treatments was 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin. Four replicates of each treatment were tested with ~200 eggs in each treatment. The eggs were thoroughly rinsed with sterile well water 60 min after fertilization to remove the antibiotics but no external egg disinfection occurred. Twenty-five eggs from each replicate were then individually transferred using sterile forceps to sterilized 1.5-mL microcentrifuge tubes containing 0.5 mL of sterile MAT broth. Each egg was macerated with a sterile pestle and incubated at 15°C. After 24 h, the mixture was agitated briefly on a vortex mixer, 100 µL of solution was distributed with a sterile spreader on tryptone yeast extract salt agar with the antibiotic tobramycin added (TYES + T; 4.0 µg/mL tobramycin; Kumagai and Nawata 2010a). The dishes were incubated at 15°C for 10 d and counts of *F. psychrophilum* colonies were made at the end of this incubation period. Isolates were made from all putative *F. psychrophilum* colonies from each treatment replicate on TYES + T media and a PCR assay was performed to confirm that the bacteria were *F. psychrophilum* (Wiklund et al. 2000). Data were normalized using arcsine-square root transformations and analyzed as a one-way ANOVA using Program R (Hornik 2013). Data were considered statistically significant at $P < 0.05$.

RESULTS

Egg Safety Trial

Eye-up, hatch, and deformity rates among eggs treated with antibiotics during various stages of fertilization and water hardening did not significantly differ from eggs that were not treated with antibiotics (all $P \geq 0.12$). Mean survival to the eyed egg stage (eye-up) was similar among treatments, ranging from 67.9% to 79.6% (Table 1). The percent hatch averaged from 60.2% to 71.7% among treatments. Deformity rates were low in all treatments (<1%). The results showed that the addition of antibiotics to both the diluent and water-hardening solution were safe for Rainbow Trout eggs.

Egg Disinfection Trial

Significantly fewer eggs were infected with *F. psychrophilum* ($F_{3,12} = 17.88$, $P < 0.01$) and the average number of CFU on the eggs were lower ($F_{3,12} = 21.14$, $P < 0.01$) on eggs treated with antibiotics than on the untreated control eggs. On average, *F. psychrophilum* was recovered from $29 \pm 24\%$ (mean \pm SD) of the eggs in the negative control treatment and $32 \pm 31\%$ of the eggs in the positive control ($t_{1,6} = 0.36$, $P = 0.73$; Figure 1). In contrast, no *F. psychrophilum* was found among the antibiotic-treated eggs and the number of colonies other than those of *F. psychrophilum* did not differ between the two antibiotic treatment regimes (Tukey's honestly significantly different [HSD] test: $P = 0.88$).

DISCUSSION

The results from our trials showed that antibiotics can be added to both sperm diluents and during water hardening. The results from our egg safety trials demonstrate that the addition of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin to 0.5% sodium chloride sperm diluents and to well water during the water-hardening process does not decrease the number of eggs reaching eye-up and the percent hatch or increase the deformity rate among Rainbow Trout eggs. Also, antibiotic treatment during fertilization and water hardening appears to kill 100% of the bacterium *F. psychrophilum*. Thus, the use of antibiotics during these egg production steps appears to prevent the vertical transmission of bacterial coldwater disease. Interestingly, the percentage of eggs infected with *F. psychrophilum* did not vary between the positive and negative control treatments (positive control was "spiked" with *F. psychrophilum*) indicating that the addition of extra bacteria did not increase the rate of detection of bacterial coldwater disease.

The use of antibiotics for control of vertical transmission of bacterial pathogens has been attempted previously by injecting broodstock with antibiotics (Brown et al. 1990; Haukenes and Moffitt 2002), but studies that have focused on egg treatment are few. Among those, Arenzon et al. (2002) used penicillin during egg incubation and Jensen et al. (1981) used a 0.002-mg/mL erythromycin phosphate solution for rinsing eggs. Erythromycin (0.002 mg/L for 1 h) was also used during water hardening

TABLE 1. Eye-up, hatch, and deformity rates (%; SD in parentheses) among Rainbow Trout eggs that were treated or not treated with a mixture of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin during fertilization or during either the first 15 or 60 min of water hardening. Eggs in the control treatment were not subjected to antibiotics during either fertilization or water hardening. A. represents antibiotic treatment and N.A. represents the absence of antibiotics.

Treatment	Eye-up rate (%)	Hatch rate (%)	Deformity rate (%)
A. Diluent only	70.6 (19.5)	63.7 (22.7)	0.18 (0.07)
A. Diluent + 15 min hardening	78.0 (14.6)	70.3 (19.7)	0.71 (0.51)
A. Diluent + 60 min hardening	67.9 (24.5)	60.2 (27.3)	0.84 (0.48)
N.A. Diluent + 15 min hardening	75.6 (16.6)	66.5 (22.2)	0.32 (0.37)
N.A. Diluent + 60 min hardening	79.6 (13.8)	71.7 (19.2)	0.29 (0.29)
Control	69.8 (21.9)	60.4 (31.4)	0.42 (0.21)

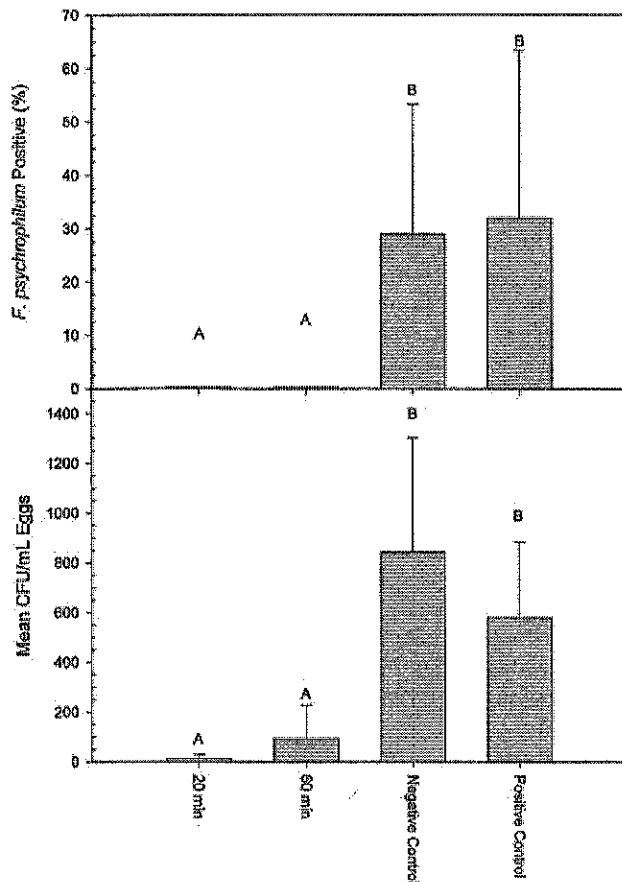


FIGURE 1. Percentage of eggs infected with *F. psychrophilum* (top panel) and average number of *F. psychrophilum* as CFU/mL (bottom panel) observed on eggs that were fertilized using diluent containing a penicillin and streptomycin mixture and water hardened with the same antibiotic mixture for either the first 20 or 60 min after fertilization. The effect of the antibiotics is compared against two control treatments that were not exposed to antibiotics. To ensure infection, *F. psychrophilum* was added to the eggs in the positive control treatment and was not added to the negative control. Letters above the bars represent the results of a Tukey's mean separation test with treatments that have statistically significant differences, which are denoted with different letters. Error bars represent +1 SD of the mean.

of eggs from Coho Salmon and steelhead (anadromous Rainbow Trout), but abnormal development resulted (Jensen et al. 1981). To our knowledge, the trials we conducted are the first to use penicillin–streptomycin during fertilization and during the water-hardening process. The results indicate that a mixture of penicillin and streptomycin can be safely added to fertilization diluents and water-hardening solutions and that the addition of these antibiotics could potentially reduce the vertical transmission of *F. psychrophilum*.

One primary advantage to the use of antibiotics during these production stages is that this treatment limits *F. psychrophilum* that is transmitted in both the female and male reproductive fluids, thus contrasting with other early fertilization disinfection (<1 h after fertilization) methods. For example, the iodine

disinfection method of Kumagai and Nawata (2010b) focuses on transmission through eggs and ovarian fluid and the antibiotic method of Oplinger and Wagner (2015) focuses on sperm. Another advantage is that much less antibiotic is needed to treat a small volume of eggs compared with later developmental stages, which would require more antibiotic. Regardless, the National Pollutant Discharge Elimination System developed by the U.S. Environmental Protection Agency requires the reporting of antibiotic use in hatcheries within the United States (USEPA 2014), and the use of these antibiotics with fish is subject to indexing under the Minor Use and Minor Species Animal Health Act (USFDA 2014).

While not evaluated, it is possible that lower antibiotic concentrations are required if eggs are treated for 60 min after fertilization compared with shorter treatments. The results demonstrate that similar reductions in bacteria numbers are observed regardless of whether antibiotics are applied for 20 or 60 min during water hardening. This indicates that antibiotic use can be discontinued at the hatchery's discretion anytime during this period without reducing the effectiveness of the treatment. Most salmonid hatcheries disinfect eggs shortly after fertilization and iodine is a common disinfectant. Many hatcheries perform this disinfection 60 min after fertilization but some hatcheries disinfect during water hardening (5–60 min after fertilization). It is not known whether the mixing of iodine with antibiotics reduces egg eye-up or hatch rates or whether iodine reduces the effectiveness of the antibiotics. Our results indicate that iodine treatments performed >60 min after egg fertilization are safe to the eggs and do not inhibit the effect of the antibiotics. The development of antibiotic resistance is a growing problem in aquaculture and the rate of development of antibiotic-resistant bacteria strains may be accelerated when suboptimal antibiotic concentrations are used (Schmidt et al. 2000). Thus, the antibiotic concentration used should be adequate to kill all bacteria within the desired exposure period. Many Rainbow Trout produced in western North America are sterilized (Kozfkay et al. 2006; Budy et al. 2012) and triploid induction may be a logical time to discontinue antibiotic treatment during water hardening. In the egg safety trial we had treatments in which we tested antibiotic application for 15 min after fertilization, and we tested a 20-min application in the egg disinfection trial. This shift in time corresponds with when the UDWR induces triploidy and was made for convenience and to minimize egg handling.

We observed that the use of antibiotics did not increase the incidence of larval deformities. This deformity assessment was made 7 d after hatch and was based on the number of fry that had obvious deformities (e.g., in the spine or occurrence of two heads) or swam in circles. Research on the use of the antibiotic erythromycin during water hardening has shown that this antibiotic increases the incidence of meristic count asymmetry between the left and right side of the body (Leary and Peterson 1990); those investigators suggested that the antibiotics interrupted larval development. It is not known whether similar effects occur when a penicillin–streptomycin mixture is

used during water hardening, but nothing abnormal has been observed in a production group of Rainbow Trout eggs we treated with these antibiotics (R. W. Oplinger, unpublished data). In 2013, we applied the antibiotic treatment (diluent plus 20 min water hardening) on a production scale at the Mammoth Creek State Fish Hatchery (UDWR). The survival to the eyed stage was 74% for eggs from 3-year-old female Rainbow Trout (694,532 eggs) and 80% for 4-year olds (328,176 eggs). These data affirm the safety of the antibiotic treatment on a production scale. Survival of these eggs was comparable with what was observed in previous years (65–75%) at this same hatchery.

Our results have demonstrated that a penicillin–streptomycin mixture could interrupt the vertical transmission of *F. psychrophilum* in salmonids. Based on these results, we are optimistic that this antibiotic mixture, applied during fertilization and water hardening, could be a useful tool in the control of bacterial coldwater disease. We recommend that antibiotics be used sparingly, with the knowledge that bacterial resistance is inevitable. There is ample literature noting that antibiotic resistance is a growing concern for human health (Davies 1994; Waters et al. 2011; Wang et al. 2012). Antibiotic resistance in aquaculture also has been a concern, not only for fish health (Schmidt et al. 2000), but also for resistant bacteria moving up the food chain into aquaculture products (Khan et al. 2009; Wang et al. 2012). If antibiotics are added to diluents or water-hardening solutions, antibiotic susceptibility tests should be performed to ensure that *F. psychrophilum* is not developing resistance to penicillin and streptomycin. Periodic drying of eyeing jars, incubation trays, and rearing troughs and treatment with other chemicals such as bleach could slow the development of antibiotic-resistant strains of the bacterium. Future developments in vaccine research, which have shown promise (e.g., LaFrentz et al. 2008; Madetoja et al. 2008; Long et al. 2013), should help provide a more sustainable method of disease control. Until these vaccines are widely available, antibiotic treatments, coupled with rigorous disinfection efforts at both brood and production hatcheries, could help maintain healthy fish production.

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