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COMMENT

Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease I: Antibiotics in Sperm Extenders

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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. Methods for disinfecting ovarian fluid and unfertilized eggs are available, but methods for disinfecting sperm have not been described. In this study we determined whether sperm extenders containing a mixture of penicillin and streptomycin can be used to eliminate *F. psychrophilum*. In vitro trials demonstrated that when Rainbow Trout *Oncorhynchus mykiss* sperm is mixed with an extender, a 15-min exposure to 0.197 mg penicillin plus 0.313 mg/mL streptomycin is effective at killing the bacteria and has no effect on sperm motility. Small-scale trials showed that egg fertilization rates were not reduced when sperm held in an extender solution containing the same antibiotic mixture for 15 min was used to fertilize eggs. Production-scale trials, however, showed a roughly 18% decrease in egg fertilization rate when sperm stored in an antibiotic containing extender was used. To determine why a reduction in fertilization capacity was observed, a small-scale experiment testing the fertilization of eggs with larger quantities of sperm was performed and showed that increasing the volume of sperm used did not increase fertilization rates. Our results demonstrate that extenders containing penicillin and streptomycin can be used to disinfect sperm, especially when small quantities of eggs are fertilized, but factors negatively affecting egg fertilization and survival on a production scale still need further investigation.

The gram-negative bacterium *Flavobacterium psychrophilum* is the causative agent of bacterial coldwater disease (BCWD). Worldwide, this disease 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). In vertical transmission, one source of the bacterium is the ovarian fluid where *F. psychrophilum* can be found in high concentrations and enters the egg through the micropyle during fertilization (Kumagai and Nawata 2010a). Recently, Kumagai and Nawata (2010a, 2010b) demonstrated that iodophor disinfection of eggs prior to fertilization can help prevent the transmission of BCWD. *Flavobacterium psychrophilum* can also be found in male seminal fluid and also enters the egg during fertilization (Kumagai and Nawata 2011; R. W. Oplinger, personal observation). Few methods are available for disinfecting sperm, which can be attributed to the sensitivity of sperm to chemical treatment and the potential for chemical treatment to cause premature flagellum activation.

Sperm extenders are solutions that are specially designed to store sperm in an unactivated state, and if antibiotics are added to the extenders, these solutions can serve as vehicles for sperm disinfection. Extenders are designed to have ion concentrations and pH that mimic seminal fluid and many sperm extenders contain antibiotics (Stoss 1983). The use of extenders is common in salmonid aquaculture because the sperm of these species is known to rapidly lose motility when stored in a refrigerated state without extension (Stoss 1983). A mixture of penicillin and streptomycin has been added to many sperm extenders to reduce bacterial growth (Stoss 1983; Stoss and Refstie 1983). For example, Brown and Mims (1995) found that the addition of 5,000 IU penicillin plus 5 mg streptomycin/mL to the sperm extender for Paddlefish *Polyodon spathula* increased the length of time that samples could be stored. Similarly, the addition of 10,000 IU penicillin plus 10 mg streptomycin plus 25 μg amphotericin/mL increased the storage time of Channel Catfish *Ictalurus punctatus* semen (Christensen and Tiersch 1996). In Rainbow Trout *Oncorhynchus mykiss*, Stoss et al. (1978) demonstrated that the addition of penicillin and streptomycin up to concentrations of 9,000 IU penicillin plus 9.0 mg streptomycin/mL did not affect the motility of the sperm. In vitro trials have demonstrated

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that 15-min treatments with penicillin concentrations > 10,000 IU/mL or streptomycin concentrations > 5,000 mg/L are effective at killing *F. psychrophilum* (Wagner et al. 2012; Oplinger and Wagner 2013). Also, the combination of penicillin and streptomycin is lethal to *F. psychrophilum* at concentrations > 2.5×10^6 IU/L penicillin plus 2.5 mg/L streptomycin (Oplinger and Wagner 2013). Most work on the addition of antibiotics to sperm extenders has evaluated the effects of antibiotics over long periods of time (days to weeks). Stoss et al. (1978) noted that antibiotic concentrations > 9,000 IU/mL penicillin plus 0.9 mg/mL streptomycin reduced Rainbow Trout sperm fertility and that the lowest antibiotic concentrations tested inhibited bacterial growth (Stoss et al. 1978, lowest antibiotic concentration not specified in manuscript). The preferred antibiotic concentration for most Rainbow Trout sperm extenders is 125 IU/mL penicillin and 0.125 mg/mL streptomycin (Stoss and Refstie 1983; Negus 2008), but the effectiveness of these concentrations at killing *F. psychrophilum* in sperm has not been evaluated.

The objectives of our research were to determine whether a mixture of penicillin and streptomycin can kill *F. psychrophilum* when mixed into Rainbow Trout sperm and whether these antibiotics are detrimental to sperm when added to an extender. In our trials, sperm was held in the extenders for 15 min. We selected this duration because it is practical in a hatchery setting where several lots and thousands of fish can be spawned in a single day. Longer storage periods could slow spawning operations since the number of females that are ready to spawn and the percentage of these with good quality eggs is unknown a priori. While extenders allow sperm to be stored for several days (Stoss and Refstie 1983), factors such as temperature and oxygen concentrations lead to the general maxim that the shorter the storage period, the better the quality of the sperm.

METHODS

We conducted several trials that evaluated sperm extenders containing penicillin and streptomycin on Rainbow Trout egg fertilization, sperm motility, and antibiotic effectiveness at killing *F. psychrophilum*. In the applicable trials (see below), a 0.5% NaCl solution was used as the diluent and water hardening occurred in well water (pH = 7.6, hardness and alkalinity = 180 mg/L). The sperm extender used consisted of 6.02 g/L NaCl, 2.98 g/L KCl, and 4.77 g/L HEPES (Stoss et al. 1978; Negus 2008) and was mixed with sperm at a 1:1 (v/v) ratio. Antibiotics were added to the extender as described below. *Flavobacterium psychrophilum* (CSF 259–93 strain: Crump et al. 2001) used in the experiments was cultured in either a maltose-infused tryptone yeast extract broth (MAT; 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, 0.05% $MgSO_4$, 1% maltose, and 0.02% $C_2H_3NaO_2$: Crump et al. 2001) or on a tryptone yeast extract, salt agar medium (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, and 0.05% $MgSO_4$: Holt et al. 1993). All solutions were tempered to ensure that temperatures (9–11°C) were identical to the sperm, eggs, and ovarian fluid used in the trials. Sperm and eggs were collected from Eagle Lake and West Virginia strain Rainbow Trout housed at the Mantua State Fish Hatchery, Box Elder County, Utah, or from Kamloops strain Rainbow Trout housed at the Whiterocks State Fish Hatchery, Uintah County, Utah. Sperm was expressed by hand stripping the fish, filtered through a metallic mesh screen, and collected into a Styrofoam cup. Eggs were also expressed by hand stripping and collected on a fabric mesh screen that separated the eggs from the ovarian fluid. All data were analyzed using the R statistical software package (Hornik 2013). Q–Q plots and Shapiro–Wilks Tests (Kuehl 2000) were used to assess whether the normality and equality of variance assumptions of the analyses were met; transformations were performed as necessary. All results were considered statistically significant at $P < 0.05$. Analyses for individual experiments are described within their respective sections. Antibiotic waste produced during the trials was minimal (<10 L) and was not released into public waterways. No fish that were exposed to antibiotics were stocked, and those individuals who performed the treatments were trained on the safe handling of antibiotics and wore gloves, eye protection, and respirators.

In vitro test of the ability of penicillin and streptomycin to kill F. psychrophilum *when mixed with sperm.*—Two antibiotic concentrations were tested: (1) 0.079 mg penicillin (Russell R-Pen; 1,582 IU/mg) plus 0. 125 mg/mL streptomycin (Sigma-Aldrich S6501) and (2) 2.5 times this concentration (0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin). Growth of *F. psychrophilum* was assessed from five replicate samples taken at 5-min intervals from 0 to 15 min after adding antibiotic. Our lowest antibiotic concentration was identical to what is recommended by Stoss and Refstie (1983) and Negus (2008). The higher concentration was chosen to determine whether antibiotic concentrations approached any toxicity threshold for sperm during a 15-min exposure. The objective of the experiment was to determine whether these mixtures would kill 100% of *F. psychrophilum* when treating sperm in extender solution.

Pools of sperm were formed, each containing the sperm from five fish; each pool was divided into two 10-mL aliquots, and one aliquot was extended at each antibiotic concentration. Since the prevalence of *F. psychrophilum* in infected sperm can vary widely (Kumagai and Nawata 2011) and the prevalence in the stocks used was unknown, 100 µL of a 96-h-old MAT broth culture containing *F. psychrophilum* was added to each aliquot approximately 1 min before the addition of the extender to ensure the presence of *F. psychrophilum* (~20% of similar five-fish pools lack the bacterium: R.W.O., unpublished data). The extender was sterilized by autoclaving prior to use. Antibiotics were added to the extender solutions at the start of the experiment. Tenfold serial dilutions on TYES agar indicated that the concentration of *F. psychrophilum* in the sperm solution (after MAT broth addition) was $\sim 1 \times 10^5$ cells/mL. This bacteria concentration is greater than naturally found in salmonids in Japan (10^1 to $10^{4.5}$ CFU/mL: Kumagai and Nawata 2011), but is similar to concentrations of bacteria challenges used in other in

vitro studies (10^4–10^5: Bullock and Stuckey 1977; 10^5: Darwish et al. 2008). Developing target treatment concentrations using higher bacteria numbers also helps to reduce the probability of developing antibiotic-resistant strains of bacteria. Furthermore, concentrations needed for in vivo treatment can be much higher than for in vitro treatment of the same bacterium (Gee and Sarles 1942).

Verification of bacterial growth or its inhibition was the only endpoint monitored in this experiment. Samples were taken 0, 5, 10, and 15 min after the addition of extender to the sperm. The 0-min sample was taken about 10 s after mixing the sperm and served as a control. This control was viewed to be preferable to a separate "untreated control" because it allowed us to assess the potential effects of transfer of antibiotic to the media plate with the inoculum as well as have a tighter relationship between control and antibiotic treatment; the number of bacteria in each particular replicate and sample was the same. To take the samples, the bacteria–antibiotic extended sperm solution was diluted by taking 100 μL of extended sperm and mixing it with 10 mL of a sterile phosphate-buffered saline (PBS) solution. Then, 100 μL of the sperm–PBS mixture was withdrawn and plated onto a TYES agar and incubated at 15°C for 10 d. After incubation, the presence or absence of *F. psychrophilum* growth was noted by assessing colony color, morphometry, Gram stains, oxidase tests, and catalase tests, and treatments with no *F. psychrophilum* growth were considered successful at controlling the bacterium. Based on the growth of bacteria at the lower concentration and an interest in testing additional replicates to verify the lethality of the higher concentration, additional replicates ($N = 4$) of the higher antibiotic concentration were tested on a second date.

Tests of antibiotic effect on sperm motility.—To test the effect of the antibiotics on sperm motility, antibiotics were added to the sperm extender at five different concentrations: (1) 2.05 mg/mL penicillin plus 3.25 mg/mL streptomycin, (2) 3.95 mg/mL penicillin plus 6.25 mg/mL streptomycin, (3) 7.90 mg/mL penicillin plus 12.5 mg/mL streptomycin, (4) 15.8 mg/mL penicillin plus 25.0 mg/mL streptomycin, and (5) control (no antibiotics added). Five pools (replicates) of sperm were formed and each pool contained sperm from three males. Each pool was divided into five aliquots containing 2 mL of sperm and each aliquot was extended at a different antibiotic concentration. Sperm motility checks were performed 15 min after extender addition by mixing 100 μL of sperm with 500 μL of a 0.5% NaCl diluent. From this mixture, 50 μL was pipetted onto a microscope slide and cover-slipped, and sperm was viewed at 400 × magnification. A visual estimate of the percentage of motile sperm was made (~10 s after the activation of the sperm) and the time until motility ceased (measured from when sperm was activated) was determined. Sperm motility was defined as moving more than one body length in any given direction; sperm that were just vibrating in place were not considered motile. A one-way, randomized, complete block design ANOVA (Kuehl 2000) was performed to assess differences in mean percent motility and activity time among antibiotic concentrations (blocked by replicate sperm pool).

Small-scale egg fertilization trials.—In the paired design employed in this trial, one-half of each sperm pool was treated with the antibiotic and the other half was not. The experiment used 12 replicate pools of sperm, and each pool consisted of sperm from three fish. Each pool was divided in half, and an equal volume of extender containing 0.197 mg penicillin plus 0.313 mg/mL streptomycin was added to one-half of the sperm pool and no extender was added to the other half. Twenty-four groups of eggs were formed by transferring 50 mL of eggs (later determined to be 800–1,500 eggs per replicate) from a bucket containing eggs pooled from 20 females. After the sperm was exposed to the antibiotics for 15 min, 1.0 mL was withdrawn and used to fertilize half of the egg groups. At the same time the remaining egg groups were fertilized using 0.5 mL (equal sperm count to 1:1 diluted extender group) of nonextended sperm (nonextended sperm also used 15 min after collection). After fertilization the eggs were incubated and percent survival to the eyed egg stage (eye-up) and to hatch, and deformity rates were determined for each replicate by hand counting. For analysis, each performance metric (percent eye-up, hatch, and deformity) from eggs fertilized using extended sperm was compared with the eggs fertilized using nonextended sperm from the same sperm pool using paired *t*-tests.

Production-scale egg fertilization trials.—To determine whether eggs could be successfully fertilized at a production scale using antibiotic-treated sperm, three replicate trials were performed on separate dates. Eggs from 20 female Rainbow Trout were used in the first trial, 60 females were used for the second trial, and 30 females were used for the third trial. Eggs were fertilized in a male : female ratio of 1:2. Pools of eggs and sperm were formed and divided in half. One-half of the sperm was placed on extender containing 0.197 mg penicillin plus 0.313 mg/mL streptomycin and used to fertilize one-half of the eggs. The other half of the eggs were fertilized using nonextended sperm (control). Eggs were fertilized with extended sperm 15–34 min (depending on date; variation due to differences in time required to strip eggs from females) after the sperm was extended, whereas eggs in the control group were fertilized immediately after collection. The eggs collected on a date were pooled into a single eyeing jar for each treatment (i.e., antibiotic sperm versus control sperm). Each spawn date represented a separate replicate in the analysis. Paired *t*-tests were used to compare the eye-up rates of the eggs that were fertilized using antibiotic-treated sperm with their respective paired controls.

Effect of increasing volume of extended sperm relative to egg volume.—The purpose of this trial was to determine whether reductions in sperm fertility caused by extender use on a production scale (see Results) can be offset by using more sperm. Treatments were (1) sperm placed in antibiotic extender (0.197 mg penicillin plus 0.313 mg/mL streptomycin) and eggs fertilized

15 min after extender addition, (2) same as treatment 1, but double the volume of extended sperm used to fertilize the eggs, (3) sperm mixed into extender without antibiotic and eggs fertilized after 15 min with same sperm concentration as treatment 1 (extender control), and (4) control (no extender, no antibiotic, fertilized at the same sperm concentration as treatment 1, and used immediately after collection).

For each of four replicates, gametes were pooled from three males and two females. Each replicate pool of eggs was then subdivided into four beakers so that each contained 50 mL of eggs. The sperm was divided into four beakers; three of those beakers received one-fifth of the volume of pooled sperm within the replicate (used for treatments 1, 3, and 4 above) and the fourth beaker received two-fifths of the sperm volume (used for treatment 2). Extender with or without antibiotics was added to the appropriate treatments. After 15 min, the sperm–extender mixtures were added to the eggs to initiate fertilization. After fertilization, eggs were rinsed with well water and treatment replicates were incubated in separate trays, which were randomly allocated with a seven-tray stack. Survival to the eyed egg stage, percent hatch, and counts of deformed fry were performed after hatch and one-way ANOVA was used to compare these metrics among treatments.

RESULTS

In Vitro Test of the Ability of Penicillin and Streptomycin to Kill *F. psychrophilum* when Mixed in Sperm

We found that the ability of the penicillin–streptomycin mixture to kill *F. psychrophilum* varied with concentration. Controls exposed to antibiotic for just a few seconds all had bacterial growth. The lowest antibiotic concentration (0.079 mg penicillin plus 0.125 mg/mL streptomycin) did not kill the bacteria in any of the 5- or 10-min exposure replicates. After 15 min, growth was found in three of five replicates. In contrast, growth of the bacteria at the higher antibiotic concentration (0.197 mg penicillin plus 0.313 mg/mL streptomycin) was observed in five of nine of the replicates after 5 min, three of nine of the replicates after 10 min, and none of nine replicates after 15 min.

Tests of Antibiotic Effect on Sperm Motility

We found that the four antibiotic treatments, which had concentrations up to 15.8 mg/mL penicillin (= 24,996 IU/mL) plus 25 mg/mL streptomycin, had no pronounced effect on the motility of the sperm compared with controls. Across treatments, average sperm motility ranged between 32% and 68%, and no differences in the percentage of sperm that were motile were observed among antibiotic concentrations ($F_{4,12} = 0.57$, $P = 0.69$; Table 1). The average motility time of the sperm ranged between 68 and 82 s and did not vary among treatments ($F_{4,12} = 0.70$, $P = 0.60$).

TABLE 1. Average percentage of Rainbow Trout sperm that were motile and the time required between sperm activation and the cessation of motion (s) after 15 min of storage in a sperm extender containing various concentrations of penicillin and streptomycin. The SD for each mean is presented in parentheses.

Treatment	% Motile	Time motility ceases (s)
2.05 mg/mL penicillin plus 3.25 mg/mL streptomycin	43 (43)	68 (17)
3.95 mg/mL penicillin plus 6.25 mg/mL streptomycin	46 (32)	70 (16)
7.90 mg/mL penicillin plus 12.5 mg/mL streptomycin	68 (62)	82 (12)
15.8 mg/mL penicillin plus 25.0 mg/mL streptomycin	52 (33)	81 (12)
Control (no antibiotics added)	32 (35)	72 (15)

Small-Scale Egg Fertilization Trials

Eye-up rates among eggs fertilized using extended sperm in the small-scale trials did not differ significantly from their respective paired controls ($t_{1,11} = 0.13$, $P = 0.90$; Figure 1; Table 2). Similarly, hatch and deformity rates were similar and did not vary between treatments (both $P > 0.72$)

Production-Scale Egg Fertilization Trials

Between 39,000 and 140,000 eggs were fertilized on each date. On average, the eye-up rate of the eggs that were fertilized using the antibiotic-treated sperm was 18.0 ± 6.2% (mean ± SD)

TABLE 2. Eye-up rates of Rainbow Trout eggs fertilized using either antibiotic-treated or nonantibiotic-treated sperm (control) in small scale trials (800–1,500 eggs per trial). The mean and SD of the replicates are shown for each treatment.

Replicate	Antibiotic-treated eye-up rate (%)	Control eye-up rate (%)
1	99.6	98.1
2	99.7	99.8
3	64.8	97.3
4	90.3	97.7
5	97.9	99.8
6	96.4	90.5
7	91.0	98.4
8	99.7	91.7
9	79.3	23.5
10	60.2	70.9
11	93.3	91.9
12	68.3	71.7
Mean	86.7	85.9
SD	14.7	22.1

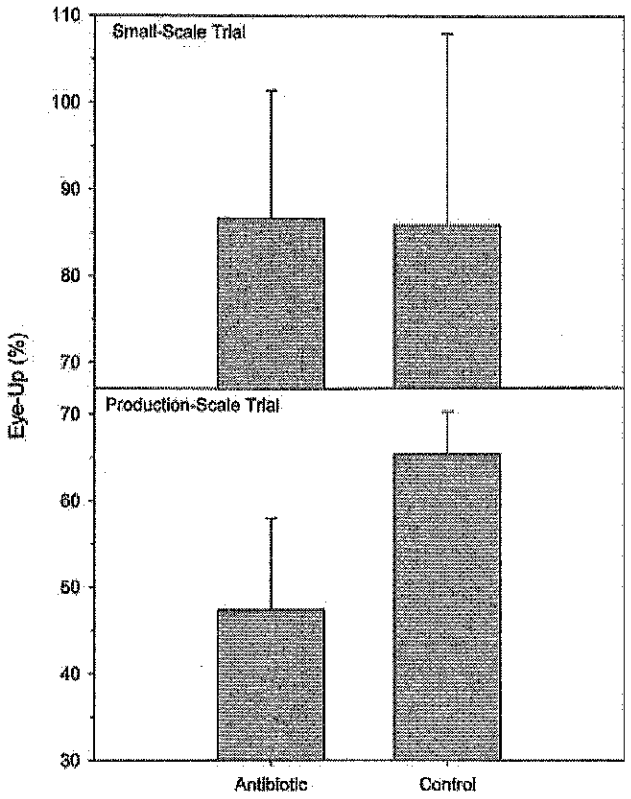

FIGURE 1. Percent survival to the eyed egg stage (eye-up) of Rainbow Trout eggs fertilized using sperm in an extender containing a penicillin–streptomycin mixture compared with eggs fertilized using nonextended sperm immediately after sperm collection (control). Data in the top panel are from small-scale experiments ($n = 12$), whereas data in the bottom panel are from production-scale trials ($n = 3$). Error bars represent + 1 SD of the mean.

lower than their respective paired control ($t_{1,2} = 5.03$, $P = 0.04$; Figure 1).

Effect of Increasing Volume of Extended Sperm Relative to Egg Volume

Eye-up of the eggs ranged between 95.0% (double sperm volume treatment) and 97.2% (control, no extender) and there were no significant differences among treatments ($F_{3,15} = 0.35$, $P = 0.79$; Table 3). Hatch rates ranged between 84.9% (double sperm volume treatment) to 91.1% (control, no extender) and also did not vary among treatments ($F_{3,15} = 0.34$, $P = 0.80$). The rate of deformities was less than 2% and did not significantly differ among treatments ($F_{3,15} = 0.28$, $P = 0.84$).

DISCUSSION

These results show that a mixture of penicillin and streptomycin, when added to a sperm extender, can potentially prevent the vertical transmission of *F. psychrophilum* via sperm. Our tests determined that penicillin concentrations of 0.197 mg/mL mixed with streptomycin concentrations of 0.313 mg/mL were adequate to kill *F. psychrophilum* with 15 min of exposure. Penicillin and streptomycin are often mixed together because these two antibiotics act synergistically, i.e., kill bacteria at lower concentrations of the two antibiotics than if used separately (Jawetz et al. 1950). Moellering and Weinberg (1971) showed in enterococci that this synergism can be attributed to an increased uptake of streptomycin when bacteria are incubated in the presence of penicillin.

Despite this synergism, there is some evidence that streptomycin is more toxic to Rainbow Trout sperm than is penicillin and that motility is reduced at antibiotic concentrations greater than 9,000 IU penicillin mixed with 9,000 μg streptomycin/mL (Stoss et al. 1978). Antibiotic concentrations lower than these thresholds were tested in our study. Reduced sperm motility can be a problem when extenders are used because nonmotile sperm can clog egg micropyles and limit the ability of motile sperm to fertilize eggs (Levanduski and Cloud 1988). Poor sperm motility would be a concern in a hatchery because reduced fertility could reduce the percentage of egg hatch, which in turn would decrease the number of fish produced. In our study, simple motility tests did not detect any antibiotic-caused decrease in sperm motility. Still, reductions in fertilization were observed in production-scale tests. It is not evident why reduced fertility was observed in these tests and not detected in the small-scale trials. The longer duration of antibiotic treatment in some production-scale replicates is one possible factor, but the lack of negative effects of in vitro tests where higher antibiotic concentrations were tested suggests that the antibiotic toxicity to sperm is low. The small-scale tests suggest that extender use per se is not a factor since egg survival was not significantly reduced by extender use in these tests. Instead, it is possible that the use of the extender led to a small reduction in sperm motility and this reduction was not detected using our crude sperm motility assessment (estimated precision within ± 5–10%). Small reductions in sperm motility may have been offset in our small-scale trials by the use of an excess quantity of sperm, whereas in production-scale tests, proportionally less sperm was used. Also, better mixing of sperm and eggs in the beakers used for the small-scale trials may have occurred compared with the larger buckets in which sperm and eggs were mixed for the production-scale tests. Another possible factor in the large-scale trials was air temperature, which was well below freezing during some of the trials. While efforts were made to keep sperm and extender solutions protected in coolers from the air temperatures and no freezing of solutions was noted, the cold temperature may have had some indirect effect on either eggs or sperm. Further research should isolate and evaluate the factors related to larger-scale production, such as the effect of increased volume of sperm, effects of environmental variables (air and water temperature, light, oxygen), effects of egg batch size, and diluent-to-egg ratios.

Sperm motility and fertilization ability are likely correlated with physiological condition (Levanduski and Cloud 1988), and cells that remain motile in samples that have overall low

TABLE 3. Comparison of the combined effects of doubling Rainbow Trout sperm quantity (1.5:1 male : female ratio) and a 15-min antibiotic (penicillin–streptomycin) treatment of sperm with controls for each factor. Performance metrics were mean (± SD) egg survival to the eyed stage (% eye-up), percent hatch, and the deformity rate (percentage of crippled fry).

Treatment	Male : female ratio	Eye-up rate (%)	Hatch rate (%)	Deformity rate (%)
Antibiotic extender	1.5:1	95.0 (4.9)	84.9 (13.8)	8.5 (9.3)
Antibiotic extender	0.75:1	96.1 (2.9)	88.2 (8.2)	6.4 (6.3)
Extender without antibiotic	0.75:1	96.8 (2.0)	90.2 (6.8)	4.1 (3.3)
Control: no extender, no antibiotic	0.75:1	97.2 (2.7)	91.1 (7.0)	6.9 (7.6)

percentage motility may have reduced fertilization capacity compared with sperm in samples in which a higher percentage of sperm are motile. This reduced physiological condition may exacerbate the effect that small changes in percent motility may have on egg fertilization. One option to combat reduced sperm motility associated with extender use could be to simply increase the volume of sperm used. We tested this and found that doubling the quantity of sperm used did not increase the percentage of eggs fertilized. In retrospect, these trials should be repeated at a production scale as it is likely that sperm were present in excess, even in the treatment with low sperm concentration. Regardless, there is evidence that increasing the volume of extended sperm may be counterproductive because this also increases the number of nonmotile sperm that could clog the micropyle and prevent fertilization by motile sperm (Stoss and Holtz 1981).

The results from this research demonstrate that the use of a sperm extender containing penicillin and streptomycin can be used to disinfect sperm and prevent the vertical transmission of BCWD in salmonids. Given the increase in antibiotic-resistant bacteria and concerns for both human and fish health related to aquaculture (Davies 1994; Wiklund and Dalsgaard 1998; Wang et al. 2012), antibiotics should be used responsibly. That is, antibiotic solutions should be properly disposed of (e.g., chemical or thermal degradation, not dumped down the drain) and applied in concentrations and durations that preclude development of antibiotic resistance. Antibiotics should not be used prophylactically (i.e., they should be used only to correct a particular disease problem at a given time). In addition, humans who are handling these antibiotics should be trained in the proper handling of these chemicals and wear appropriate personal protection equipment.

It appears that small numbers of eggs can be fertilized using extended sperm without compromising hatch. However, eye-up and hatch rates decreased when sperm extenders were used at a production scale. This reduced fertility could be offset by dividing large groups of eggs into smaller aliquots for fertilization and afterwards pooling the eggs for incubation. Future research should target the optimization of the use of sperm extenders at a production scale and should determine whether a reduction in *F. psychrophilum* density in the sperm leads to a reduced occurrence of BCWD after hatch.

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REFERENCES

Brown, L. L., W. T. Cox, and R. P. Levine. 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Diseases of Aquatic Organisms 29:213–218.

Brown, G. G., and S. D. Mims, 1995. Storage, transportation, and fertility of undiluted and diluted paddlefish milt. Progressive Fish-Culturist 57: 64–69.

Bullock, G. L., and H. M. Stuckey. 1977. Ultraviolet treatment of water for destruction of five gram-negative bacteria pathogenic to fishes. Journal of the Fisheries Research Board of Canada 34:1244–1249.

Christensen, J. M., and T. R. Tiersch. 1996. Refrigerated storage of Channel Catfish sperm. Journal of the World Aquaculture Society 27:340–346.

Crump, E. M., M. B. Perry, S. C. Clouthier, and W. W. Kay. 2001. Antigenic characterization of the fish pathogen *Flavobacterium psychrophilum*. Applied and Environmental Microbiology 67:750–759.

Darwish, A. M., B. D. Farmer, and J. P. Hawke. 2008. Improved method for determining antibiotic susceptibility of *Flavobacterium columnare* isolates by broth microdilution. Journal of Aquatic Animal Health 20:185–191.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science 264:375–382.

Gee, L. L., and W. B. Sarles. 1942. The disinfection of trout eggs contaminated with *Bacterium salmonicida*. Journal of Bacteriology 44:111–126.

Holt, R. A., J. S. Rohovec, and J. L. Fryer. 1993. Bacterial cold-water disease. Pages 3–22 *in* V. Inglis, R. R. Roberts, and N. R. Bromage, editors. Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, UK.

Hornik, K. 2013. The R FAQ. Available: http://CRAN.R-project.org/doc/FAQ/R-FAQ.html. (March 2014).

Jawetz, E., J. B. Gunnison, and V. R. Coleman. 1950. The combined action of penicillin with streptomycin or chloromycetin on enterococci in vitro. Science 111:254–256.

Kuehl, R. O. 2000. Design of experiments: statistical principles of research design and analysis, 2nd edition. Brooks/Cole Publishing, Pacific Grove, California.

Kumagai, A., and A. Nawata. 2010a. Mode of the intra-ovum infection of *Flavobacterium psychrophilum* in salmonid eggs. Fish Pathology 45:31–36.

Kumagai, A., and A. Nawata. 2010b. Prevention of *Flavobacterium psychrophilum* vertical transmission by iodophor treatment of unfertilized eggs in salmonids. Fish Pathology 45:164–168.

Kumagai, A., and A. Nawata. 2011. Concentration of *Flavobacterium psychrophilum* in the ovarian fluid and milt of cultured salmonids. Fish Pathology 46:116–119.

Levanduski, M. J., and J. G. Cloud. 1988. Rainbow Trout (*Salmo gairdneri*) semen: effect of non-motile sperm on fertility. Aquaculture 75:171–179.

Moellering, R. C., and A. N. Weinberg. 1971. Studies on antibiotic synergism against enterococci II: effect of various antibiotics on the uptake of ^{14}C labeled streptomycin by enterococci. Journal of Clinical Investigation 50:2580–2584.

Negus, M. T. 2008. Salmonid sperm cryopreservation techniques. Minnesota Department of Natural Resources Special Publication 167.

Nematollahi, A., A. Decostere, F. Pasmans, and F. Haesebrouck. 2003. *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases 26:563–574.

Oplinger, R. W., and E. J. Wagner. 2013. Control of *Flavobacterium psychrophilum*: tests of erythromycin, streptomycin, osmotic and thermal shocks, and rapid pH change. Journal of Aquatic Animal Health 25:1–8.

Stoss, J. 1983. Fish gamete preservation and spermatozoan physiology. Pages 305–350 *in* W. S. Hoar, D. J. Randall, and E. M. Donaldson, editors. Fish physiology volume IX, part B. Academic Press, New York.

Stoss, J., S. Buyukhatipoglu, and W. Holtz. 1978. Short-term and cryopreservation of Rainbow Trout (*Salmo gairdneri* Richardson) sperm. Annales de Biologie Animale Biochime Biophysique 18:1077–1082.

Stoss, J., and W. Holtz. 1981. Cryopreservation of Rainbow Trout (*Salmo gairdneri*) sperm: I. Effect of thawing solution, sperm density and interval between thawing and insemination. Aquaculture 22:97–104.

Stoss, J., and T. Refstie. 1983. Short-term storage and cryopreservation of milt from Atlantic Salmon and sea trout. Aquaculture 30:229–236.

Wagner, E. J., R. W. Oplinger, and M. Bartley. 2012. Penicillin-G: efficacy against *Flavobacterium psychrophilum* and evaluation of lethal dose limits for Rainbow Trout. Open Journal of Animal Sciences [online serial] 2: 150–158.

Wang, H., J. C. McEntire, L. Zhang, X. Li, and M. Doyle. 2012. The transfer of antibiotic resistance from food to humans: facts, implications and future directions. Revue Scientifique et Technique (International Office of Epizootics) 31:249–260.

Wiklund, T., and I. Dalsgaard. 1998. Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. Diseases of Aquatic Organisms 32:49–69.