Efficacy of Injectable Tulathromycin for Reduction of Vertical Transmission of *Renibacterium salmoninarum* in Spring Chinook Salmon *Oncorhynchus tshawytscha*

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SUMMARY

Bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* (Rs) occurs nearly worldwide where wild or cultured salmonid fishes are present. Control of BKD is confounded by its two modes of transmission, horizontal (fish-to-fish) and vertical (from female parent to progeny via the eggs). A highly successful BKD control strategy employed in Pacific Northwest hatcheries culturing spring Chinook salmon (*Oncorhynchus tshawytscha*) includes: (1) injecting pre-spawning adults with a macrolide antibiotic to improve survival and reduce Rs infection levels, (2) broodstock culling of highly infected females and (3) improved fish husbandry. However, the future availability of the injectable macrolide antibiotic (erythromycin) used for adults is uncertain. This drug shortage has resulted in an urgent need to identify a replacement injectable antibiotic to ensure continued successful control of BKD. The research conducted was intended to provide information for addressing this need via preliminary tests of the safety and efficacy of a new macrolide antibiotic, injectable tulathromycin, which is sold under the trade name DRAXXIN® (Zoetis Animal Health). A long-term goal is to reduce or eliminate the use of antibiotic treatment in spring Chinook salmon hatchery culture. Non-treated females were included in the study to provide empirical data in support of this goal. A subset of pre-spawning spring Chinook salmon at Leavenworth NFH was injected on July 10, 2014 with DRAXXIN at 5 mg per kg body weight (31 fish, left pelvic fin clip). Another subset of females (30 fish, right pelvic fin clip) was left uninjected. The surviving fish (31 DRAXXIN-injected fish and 28 uninjected fish) were spawned between August 18 and September 2, 2014. Although there were apparent trends toward higher pre-spawn survival and lower Rs prevalence and levels for the DRAXXIN-injected females in comparison to the uninjected females, the differences were not statistically significant for any of the Rs assays used ($P > 0.05$). Based on USFWS enzyme-linked immnosorbent assay (ELISA) test results of kidney tissue samples from the spawning females, egg lots from DRAXXIN-injected and uninjected females were assigned to Rs vertical transmission risk groups (low, medium or high). A subset of 220 eyed eggs from each female was transferred to the Western Fisheries Research Center (USGS) on October 8, 2014, hatched and reared until the study was terminated on September 22, 2015. The study results provided no evidence that DRAXXIN injection of adult female Chinook salmon affected their fecundity, egg eye-up, or survival and growth of progeny fry. There was little evidence of Rs infection in progeny of either DRAXXIN-injected or uninjected females, so the effect of DRAXXIN injection on vertical transmission of Rs could not be assessed. To adequately evaluate the efficacy of DRAXXIN injection for reducing Rs vertical transmission to progeny, additional studies should be conducted with larger numbers of DRAXXIN-injected and uninjected Chinook salmon females with a greater range of Rs levels.

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PRODUCTS AND DELIVERABLES TO DATE

1. Summary of research results prepared for Pacific Northwest Fish Health Protection Committee Meeting, February 2015.

2. Oral presentation (webinar) to the Interagency Coordination Meeting on Draxxin Studies by D. Elliott entitled "Efficacy of Injectable Tulathromycin (DRAXXIN®) for Reduction of Vertical Transmission of *Renibacterium salmoninarum* (Rs) in Spring Chinook Salmon." Olympia, WA; May 20, 2015.

3. Oral presentation (webinar) to the USFWS AADAP Aquaculture Drug Approval Coordination Workshop by D. Elliott entitled "Efficacy of Injectable Tulathromycin (DRAXXIN®) for Reduction of Vertical Transmission of *Renibacterium salmoninarum* (Rs) in Spring Chinook Salmon." Bozeman, MT; July 29, 2015.

4. U.S. Geological Survey data release, October 1, 2018 (Elliott 2018).

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INTRODUCTION

Bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* (Rs) occurs nearly worldwide where wild or cultured salmonid fishes are present (Pascho et al. 2002). Rs can cause significant disease and mortality in juvenile salmonids in both fresh water and seawater, and also in pre-spawning adults. A highly successful BKD control strategy has been employed in Pacific Northwest hatcheries culturing spring Chinook salmon (*Oncorhynchus tshawytscha*) for more than two decades, and includes: (1) injecting pre-spawning adults with a macrolide antibiotic, (2) broodstock culling of highly infected females and (3) improved fish husbandry. However, future availability of the injectable macrolide antibiotic (erythromycin) used for adults is uncertain. This drug shortage has resulted in an urgent need to identify a replacement injectable antibiotic to ensure continued successful control of BKD. The research described was intended to provide information for addressing this need via preliminary tests of the safety and efficacy of a new macrolide antibiotic, injectable tulathromycin. In the long-term, the goal is to reduce or eliminate the use of antibiotic treatment in spring Chinook salmon hatchery culture. The inclusion of non-treated females in the study was intended to provide empirical data in support of this long-term goal towards a rational phase-out of antibiotic treatment of adults.

Rs can cause losses as high as 80% in stocks of Pacific salmon (Evenden et al. 1993), and BKD has been implicated in declines of adult returns of Chinook salmon in the Pacific Northwest (Raymond 1988). Control of BKD is confounded by its two modes of transmission (Evelyn 1993), horizontal (fish-to-fish) and vertical (from female parent to progeny via the eggs). At least some Rs cells are carried inside the eggs, where they cannot be reached by surface disinfectants (Evelyn et al. 1986b). Therefore, culling egg lots from infected females based on results of kidney tissue testing by the enzyme-linked immunosorbent assay (ELISA) and injection of pre-spawning females with antibiotics to reduce Rs levels decreases the probability of Rs vertical transmission at spawning. A multi-year study at Idaho Department of Fish and Game (IDFG) Chinook salmon hatcheries determined that a BKD management strategy utilizing ELISA testing and erythromycin injection of adults resulted in significant reductions in juvenile disease and

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mortality from BKD and secondary infections during hatchery rearing, as well as significant reduction in Rs levels in returning adults (Munson et al. 2010). Erythromycin injection can also significantly decrease adult pre-spawning mortality. In 1980-1981, prior to the medication of adults at Warm Springs National Fish Hatchery (NFH), 48-74% of the wild and hatchery spring Chinook salmon died from BKD before spawning. Injections of erythromycin began in 1982, resulting in an immediate reduction of pre-spawn mortality (Olson and Spateholts 2001). Following concerted efforts with erythromycin injections, ELISA testing and culling of eggs from highly infected females, the ELISA profile of the adult salmon at Warm Springs NFH reversed itself from 86% of the adults having high ELISA values indicative of severe Rs infections in 1984 to only 9% of adults having high ELISA values in 1991 (Leek 1992). Similar results occurred at the other USFWS NFHs with the subsequent reduction of yearly juvenile epizootics in the late 1980's and early 1990's to the relatively rare juvenile outbreak in the 21st century (USFWS Region 1 Fish Health Centers records). The increase in juvenile fish survival has enabled the IDFG and USFWS hatcheries to decrease the number of eggs taken during spawning to meet hatchery production goals, thus requiring fewer adult fish to be trapped and held, and allowing more adults to be left in the river for natural spawning or for tribal and sport fisheries. The success of the BKD control program has also resulted in the elimination of routine medicated feed treatments to spring Chinook salmon juveniles to control mortality due to BKD during the rearing period. This has substantially reduced the amount of antibiotic used in these rearing programs while simultaneously increasing the overall health of the fish released for enhancement and mitigation programs.

Erythromycin, a macrolide, is the principal antibiotic that has been used for pre-spawning injection of adult fish to control Rs. Erythromycin injection can result in therapeutic antibiotic levels in mature eggs of females injected as few as 9 days before spawning (Evelyn et al. 1986a; Armstrong et al. 1989; Lee and Evelyn 1994), and can reduce BKD losses among progeny fish (Moffitt and Kiryu 1999). In recent years the commercial supply of erythromycin injectable solution used in the fish and animal industry has been unreliable. Pacific salmon aquaculture, a minor use industry, has an urgent need to identify a

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replacement antibiotic, one that is safe and effective and will continue to be manufactured, for use in programs for prevention of Rs vertical transmission.

Another macrolide antibiotic, injectable tulathromycin, is sold under the trade name DRAXXIN® (Zoetis Animal Health) for treatment of certain bacterial respiratory diseases in cattle and swine, and has shown efficacy against Rs in preliminary studies. An *in vitro* trial showed significant inhibition of Rs growth at DRAXXIN concentrations as low as 0.11 mg/L (Craig Olson, Northwest Indian Fisheries Commission, personal communication). Pre-spawning injections of spring Chinook salmon with DRAXXIN at a state hatchery resulted in adult survival and Rs levels (based on ELISA testing) comparable to those observed for erythromycin injection the previous year, with fewer apparent toxic side effects observed following DRAXXIN injection (Bryan Quinton, Washington Department of Fish and Wildlife, personal communication). However, the efficacy of DRAXXIN for reducing vertical transmission of Rs has not been determined.

This study with spring Chinook salmon at Leavenworth National Fish Hatchery (NFH) sought to address four questions:

1. Does pre-spawning injection with DRAXXIN result in reduced pre-spawning mortality of DRAXXIN-injected adult Chinook salmon in comparison to uninjected fish?
2. Does pre-spawning injection of adult Chinook salmon with DRAXXIN result in reduced prevalence and levels of Rs in these fish at spawning in comparison to uninjected control fish?
3. Does pre-spawning injection of adult Chinook salmon with DRAXXIN affect success of egg eye-up (all progeny), as well as hatching rates, fry survival and early growth rates (subsample of progeny) in comparison to progeny from uninjected control fish?
4. Does pre-spawning injection of adult Chinook salmon with DRAXXIN result in reduced prevalence and levels of Rs in progeny fish in comparison to progeny of uninjected control fish?

METHODS

Adult Chinook salmon study groups

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The research conducted at Leavenworth NFH was originally designed for comparison of a group of uninjected adult female spring Chinook salmon with groups injected with DRAXXIN and erythromycin, but the erythromycin group (no fin clip) included extra females at spawning such that the injection status of fish in this group could not be verified. Therefore, the erythromycin-injected group was eliminated from the study and will not be discussed.

Injection and fin clipping of adults was done on July 10, 2014, with 5 mg DRAXXIN per kg body weight delivered by intraperitoneal injection to 31 females, which were marked by a left pelvic fin clip. Uninjected females (30 fish) were marked with a right pelvic fin clip.

Adult Chinook salmon samples

Spawning occurred on August 18 and 25 and on September 2, 2014. At spawning, fin-clipped females were identified; 31 females from the DRAXXIN-injected group and 28 females from the uninjected group were spawned. Several samples were taken from each of these fish for Rs detection and quantification; all samples were placed on ice in tubes that had been pre-labeled with the individual fish identification number. After the ventral surface of the fish was swabbed with iodophor before spawning, a 1 mL sample of ovarian fluid was taken via a pipet inserted into the genital papilla; ovarian fluid samples were to be analyzed for Rs cells by solid phase cytometry.

After spawning, kidney tissue samples were taken for Rs analysis by several assays. Samples for ELISA testing for Rs antigen were taken by USFWS personnel from the cranial, mid, and caudal kidney (AFS-FHS 2014) of each fish (total tissue weight about 1 g) with clean forceps for each individual fish according to USFWS protocol. Forceps for ELISA sampling were cleaned by removal of large debris with a clean paper towel, then cleaned in Tergazyme™ detergent (Alconox), followed by rinses in two different beakers of 70% ethanol. Forceps used to sample suspected BKD lesions were subjected to additional scrubbing in detergent before reuse, to prevent Rs antigen carryover.

A separate sample (≤ 25 mg) was taken from a different area of the mid kidney of each fish by use of separate RNAse-free forceps and placed in RNA stabilization solution (RNALater, Qiagen); this sample

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was to be divided later for extraction of DNA and RNA for PCR testing. The extracted DNA was to be assayed for Rs DNA by real-time quantitative polymerase chain reaction (qPCR) to detect a specific DNA sequence of the Rs major soluble antigen (*msa*) gene, as a proxy for total Rs bacterial load (Elliott et al. 2015). Extracted RNA was to be used for performance of reverse-transcription qPCR (RT-qPCR) to detect expression of messenger RNA (mRNA) of the Rs *msa* gene. Since mRNA has a short half-life usually measured in minutes, it is considered an indicator of the presence of viable bacteria, and RT-qPCR for Rs *msa* mRNA expression has been used as a proxy for viable Rs bacterial load (Metzger et al. 2010). Forceps used for sampling kidney tissue for Rs DNA and RNA testing were disinfected in iodophor solution, washed in detergent, soaked for 15 minutes in 10% chlorine bleach diluted with tap water, rinsed thoroughly with deionized water, dried and autoclaved before reuse.

Samples were transported to the USFWS Olympia Fish Health Center on ice and then frozen at -20°C until testing by USFWS or transfer to the USGS Western Fisheries Research Center (WFRC). The kidney tissue samples in RNALater were held at 4°C for 24 h before freezing.

Adult Chinook salmon Rs testing

Adult fish kidney samples were tested for Rs antigen by polyclonal ELISA (AFS-FHS 2014) at the USFWS Olympia Fish Health Center. The kidney tissue samples that had been stored in RNALater and the ovarian fluid samples were transferred to the WFRC for Rs testing. For kidney tissue samples, DNA was extracted by the method of Chase et al. (2006) and RNA was extracted by the procedure of Purcell et al. (2004); total DNA and RNA concentrations in extracted samples were also determined (Badil et al. 2011). The qPCR for detection of Rs DNA was performed by the AFS-FHS Blue Book method (AFS-FHS 2014), and RT-qPCR for Rs RNA expression was done as described by Metzger et al. (2010). A sample was considered positive by qPCR or RT-qPCR if two replicates showed amplification, and the mean cycle threshold (C_T) value was less than 38 (Elliott et al. 2015).

A procedure modified from the water sample testing method of Elliott et al. (2015) was used for detection of Rs cells in ovarian fluid by solid phase cytometry (SPC), an indirect membrane filtration-

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fluorescent antibody technique (MF-FAT) that uses automated laser scanning of filters (SPC-FAT), with microscopic verification of results. The SPC-FAT is more sensitive than the standard MF-FAT in which filters are scanned manually by an observer using a fluorescence microscope (Elliott et al. 2015). Modifications to the SPC-FAT procedure of Elliott et al. (2015) were as follows: 0.5 mL of ovarian fluid was combined with 0.5 mL of 0.01 M phosphate-buffered saline (PBS, pH.7.1) containing 0.05% (v/v) Tween 20 (PBS-T20), followed by vortex mixing. The sample was then centrifuged at 15,000 *g* for 10 min, the supernatant was poured off, and the pellet was resuspended in 1 mL PBS-T20. Then, 0.5 mL filtered trypsin solution, prepared as described by Elliott and McKibben (1997), was added, and the mixture was heated at 50°C for 10 min. The prepared sample was then triturated through a syringe fitted with a 22-gauge needle prior to membrane filtration. The remaining steps in the SPC procedure were performed as described by Elliott et al. (2015). Samples in which Rs counts were ≥ 10 bacteria per mL were considered true positives.

Chinook salmon egg transport, hatching and rearing

Eyed eggs from DRAXXIN-injected and uninjected groups (220 eggs per female) were transported from Leavenworth NFH to the WFRC on October 8, 2014 and placed into standard egg incubator trays supplied with sand-filtered, UV-treated Lake Washington water. Eggs were separated into trays according to adult fish treatment group (DRAXXIN-injected or uninjected) and Rs vertical transmission risk group (low, medium or high) based on kidney tissue ELISA optical density (OD) values from the female parent of each egg lot. Because there were about twice as many eggs in the uninjected and DRAXXIN-injected medium ELISA risk groups as in the other groups, eggs from the medium ELISA uninjected and DRAXXIN-injected groups were divided into two trays each, and eggs from the low and high ELISA uninjected and DRAXXIN-injected groups were incubated in one tray per group. After hatching and swim-up (completed by November 28, 2014), fish were transferred to 278-L tanks (Two tanks each for the medium ELISA uninjected and DRAXXIN-injected progeny groups and one tank each for the other groups) and fed a commercial diet (Bio-Oregon) until the study was terminated. The fish were hatched

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and reared in single-pass, sand-filtered and UV-treated ambient temperature Lake Washington water. Water temperature (mean ± SD) was 12.6 ± 1.2 °C during egg incubation and 11.5 ± 6.5°C during fish rearing. Remaining fish were humanely euthanized with an overdose of buffered tricaine methanesulfonate (MS-222) on September 22, 2015.

Progeny fish sampling and testing

Mortality of juvenile fish was monitored daily, and kidney tissues of fresh mortalities removed from tanks between April 2 and July 27, 2015 (when fish were large enough for kidney tissue culture) were cultured on modified selective KDM2 agar medium (Elliott et al. 2013). Bacterial colonies were tested by the direct fluorescent antibody test (Elliott et al. 2013) for identification of Rs. Weights (g) and fork lengths (mm) were obtained from a subsample of 30 individual live fish per group from each of the progeny groups on June 11, 2015 and August 24, 2015.

Progeny fish were sampled twice by repeated dip-netting from each group for ELISA testing. The first sampling occurred May 26 – June 10, 2015, with kidney and spleen tissues sampled from 160 fish per progeny group and processed as twenty 8-fish pools. The second sampling was accomplished September 2 – 10, 2015, with kidney and spleen tissues sampled from 100 fish per progeny group and processed as twenty 5-fish pools. The pooled tissue samples were diluted 1:8 (w/v) in PBS-T20 (pH 7.4), and were processed and tested by polyclonal ELISA according to standard procedures (AFS-FHS 2014). For each sampling, the five pooled samples from each progeny group that showed the highest ELISA OD values were tested for Rs DNA by qPCR (AFS-FHS 2014). Because of the small amount of kidney tissue available in each sample, the DNA for qPCR testing was extracted from the tissue pellet remaining after ELISA testing, as described by Nance et al. (2010).

Statistical analysis

Statistical analyses were performed using InStat v3 (GraphPad Software) and IBM PASW v18 (IBM). The Fisher exact test was used to evaluate differences in total mortality, Rs prevalence or infection

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level category between two groups, and the chi-square test was used to assess differences among three or more groups. For comparison of Rs levels or fish lengths and weights between or among groups data were first tested for normality by the Kolmogorov-Smirnov method. For data sets that passed the normality test (without or with log transformation), a parametric *t* test (two groups) or analysis of variance (ANOVA; three or more groups) was used for comparisons. Tukey's HSD multiple comparison procedure was applied for post-hoc analysis if a significant result was obtained by ANOVA. If at least one data set in each comparison failed the normality test ($P < 0.05$) after data transformation was performed, the nonparametric Mann-Whitney *U* test (two groups) or Kruskal-Wallis test (three or more groups) was used for these comparisons. Dunn's multiple comparison procedure was applied when a significant result ($P < 0.05$) was obtained using the Kruskal-Wallis test. Concordance (observed agreement) between assays was calculated as described by (Smith 2006). Concordance was not evaluated by means of the kappa statistic due to the bias of this estimate when prevalence is either very high or very low (Feinstein and Cicchetti 1990), as occurred in this study. Survival curves were estimated by the Kaplan-Meier method and compared by log-rank tests.

RESULTS

Pre-spawning mortality

All 31 adult female Chinook salmon that had been injected with DRAXXIN (left pelvic fin clip) survived until spawning. Among the 30 uninjected females (right pelvic fin clip), 28 were spawned. Although no fish with right pelvic fin clips were noted among adult fish mortalities, it was presumed that the two missing fish died before spawning. There was no significant difference in overall survival ($P = 0.24$) between the DRAXXIN-injected and uninjected females.

Rs prevalence and levels in spawning females

Based on results from ELISA testing of kidney tissue samples from spawning females, the fish were divided by USFWS fish health personnel into three risk groups for Rs vertical transmission: low, medium

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and high (Table 1). Among the DRAXXIN-injected females, 36% were assigned to the low-risk group, 45% were included in the medium-risk group, and 19% were assigned to the high-risk group (Table 1). Among the uninjected females, 21% were included in the low-risk group, 54% were assigned to the medium-risk group, and 25% were included in the high-risk group (Table 1). The proportion of females occurring in each ELISA OD risk group was independent of whether or not the fish had been injected with DRAXXIN ($P = 0.47$). There was a trend toward higher ELISA OD values in the uninjected females (median OD 0.127; 95% confidence interval 0.084, 0.469) compared with DRAXXIN-injected females (median OD 0.105; 95% confidence interval 0.095, 0.143), but the difference was not significant (Mann-Whitney *U* test; $P = 0.18$).

Results of qPCR testing of kidney tissues indicated that samples from 10% (3 of 31 fish) in the DRAXXIN-injected group and 21% (6 of 28 fish) in the uninjected group were positive for Rs DNA (Table 2). The difference in Rs DNA prevalence between the two groups was not significant ($P = 0.29$). Testing of the kidney samples by RT-qPCR revealed the presence of Rs RNA in 6% (2 of 31 fish) in the DRAXXIN-injected group and 21% (6 of 28 fish) in the uninjected group (Table 3); the differences in prevalence were not significant ($P = 0.13$). Both Rs DNA and Rs RNA were detected in 7 of the 59 total females tested, Rs DNA but no Rs RNA was detected in 2 fish, and Rs RNA but no Rs DNA was detected in a single fish (Table 4). Observed agreement between the DNA and RNA PCR assay results (both positive and negative) was 95%. The highest quantity estimates of Rs DNA (Table 2) and Rs RNA (Table 3) were obtained from uninjected fish. Among fish positive for Rs DNA, the geometric mean quantity estimate was 0.7 copies/µg total DNA in DRAXXIN injected fish and 35.3 copies/µg total DNA in uninjected fish. Among fish positive for Rs RNA, the geometric mean quantity estimate was 0.6 copies/µg total RNA in DRAXXIN-injected fish and 23.9 copies/µg total RNA in uninjected fish.

By SPC-FAT testing, ovarian fluid samples from some fish in both the DRAXXIN-injected and uninjected groups at each of the ELISA risk levels tested Rs-positive (Table 5). Overall, ovarian fluid samples from 39% of the DRAXXIN-injected females and 25% of the uninjected females tested Rs-positive, and geometric mean Rs levels in positive fish were 62 and 792 Rs cells/mL of ovarian fluid in

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DRAXXIN-injected and uninjected females, respectively. The overall difference in Rs levels between DRAXXIN-injected and uninjected fish were not significant (unpaired *t*-test; $P = 0.13$). The highest geometric mean Rs concentrations were observed in the high ELISA risk groups of DRAXXIN-injected and uninjected fish (Table 5).

Progeny Chinook salmon survival and growth

Leavenworth NFH personnel determined fecundity (total number of eggs per female) and egg survival to eye-up for 21 females in the DRAXXIN-injected group and 15 females in the uninjected group from which eggs were retained at the hatchery for hatching and rearing (Table 6). The eggs retained were from females in the low ELISA risk group (all females retained), and from females in the medium ELISA risk group with mean ELISA OD values ≤ 0.123. Among the females with egg lots retained by the hatchery, there was no significant difference in fecundity (Mann-Whitney *U* test; $P = 0.19$) or egg survival to the eyed stage (Mann-Whitney *U* test; $P = 0.87$) between DRAXXIN-injected and uninjected fish.

For the sub-groups of eyed eggs from all females transported to the WFRC, survival to the swim-up stage ranged from 98.6% in the DRAXXIN-injected low ELISA group to 99.6% in the DRAXXIN-injected high ELISA group (Table 7). There was no significant difference in survival among the groups to the swim-up stage ($P = 0.12$). Analysis of post-ponding survival of progeny fish between November 28, 2014 and the termination of the study on September 22, 2015 indicated that most mortality occurred between 40 and 80 days after ponding (Figure 1A). Only three mortalities were recorded after 180 days post-ponding, two in the DRAXXIN low ELISA group and one in the uninjected medium ELISA group (data not shown). Total survival at the termination of the experiment ranged from 29% in the DRAXXIN low ELISA risk group to 72% in the uninjected low ELISA risk group (Figure 1B). A high prevalence of fry with ‘corkscrew’ deformities was noted in the DRAXXIN low ELISA group (data not shown). Comparison of survival curves by log-rank tests indicated significant differences among the six progeny groups ($P < 0.0001$), and significantly higher survival ($P < 0.0001$) in the combined progeny groups from

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uninjected females in comparison to combined progeny groups from DRAXXIN-injected females. However, there was no consistent trend in survival in relation to DRAXXIN injection or ELISA risk group (Figure 1).

Comparison of fork lengths and weights among the progeny groups at two time points after ponding revealed significantly greater mean length ($P \leq 0.009$) and mean weight ($P \leq 0.001$) of progeny in the uninjected low ELISA risk group than mean lengths and weights of fish in all other progeny groups in the first sample taken 195 days after ponding. In the second sample taken 268 days after ponding, mean length and mean weight in the uninjected low ELISA risk group were significantly greater than mean lengths and weights ($P \leq 0.002$) for all other groups except the DRAXXIN-injected low ELISA risk group ($P > 0.05$). There were no significant differences in mean length or weight among any of the other progeny groups ($P > 0.05$). The uninjected low ELISA risk progeny group contained the lowest number of fish per tank immediately after ponding, and the number of fish in the DRAXXIN-injected low ELISA risk group was greatly reduced by high mortality (71%) after ponding.

Rs testing of progeny fish

No Rs was isolated in culture from kidney tissue of post-ponding mortalities. The ELISA testing of kidney and spleen tissues from juvenile fish sampled on May 26 – June 10, 2015 (8-fish pools) showed very low ELISA OD values (< 0.080) for most of the pooled samples (Table 8). The highest ELISA OD values obtained were from two tissue pools in the DRAXXIN-injected low ELISA risk group (ELISA OD values 0.081 and 0.086), one pool in the DRAXXIN-injected medium ELISA risk group (ELISA OD 0.135), and one pool in the uninjected high ELISA risk group (ELISA OD 0.097). All kidney-spleen pools from fish sampled on September 2 – 10, 2015 (5-fish pools) showed ELISA OD values < 0.080 (Table 9). None of the tissue pools tested by qPCR from the first or second sampling period tested positive for Rs DNA (data not shown).

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DISCUSSION

Inclusion of higher numbers of adult female spring Chinook salmon in the DRAXXIN-injected and uninjected fish groups spawned at Leavenworth NFH in 2014 would have increased the power of statistical comparisons in this study. Although there were apparent trends toward higher pre-spawn survival and lower Rs prevalence and levels for the DRAXXIN-injected females in comparison to the uninjected females, the differences were not statistically significant for any of the Rs assays used ($P > 0.05$). Positive results from ELISA, qPCR for Rs DNA and SPC-FAT assays did not confirm the presence of viable Rs in spawning females in either group (see review in Elliott et al. 2013), but RT-qPCR results for Rs RNA suggested that DRAXXIN injection reduced, but did not completely eliminate, viable Rs from injected fish.

The high concordance of positive and negative results from qPCR and RT-qPCR testing of the same samples suggested that the presence of Rs DNA in adult Chinook salmon kidney tissue samples could be used as an indicator of recent Rs exposure or active infection, although testing of larger sample sets with a higher prevalence of PCR-positive fish is needed. Good concordance of results between qPCR and RT-qPCR assays for Rs DNA and Rs mRNA, respectively, has been previously reported under experimental conditions, with the DNA assay showing higher analytical sensitivity (Suzuki and Sakai 2007), as appeared to be the case in the current study. It is likely that Rs DNA only persists in fish tissues for a few days to weeks after the bacterium is killed (reviewed in (Pascho et al. 2002). Conversely, the ability of Rs antigens to persist and be detectable by ELISA testing for several months in the absence of live bacteria (Pascho et al. 1997) may have been reflected in the relatively low percentage of females in the medium to high ELISA OD groups from which kidney samples tested positive for Rs DNA by qPCR; kidney samples from only 5% of DRAXXIN-injected females and 27% of uninjected females in these ELISA OD groups tested positive for Rs DNA.

The ability of ELISA to detect *R. salmoninarum* antigens that circulate into the kidney from infection sites in non-sampled tissues may also have contributed to the higher number of females positive by

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ELISA than by PCR (see reviews in Elliott et al. 2013; 2015). The only non-kidney sample tested for Rs from the spawning females in this study was ovarian fluid, which is derived from secretory epithelia in the ovaries as well as blood plasma (Lahnsteiner et al. 1995) and surrounds post-ovulated eggs while they are held in the body cavity until they are released through the genital papilla at spawning (Rime et al. 2004). Sampling of ovarian fluid via a pipet inserted into the genital papilla in this study helped to avoid contamination by urine and feces (Smith and Bell 1975). The relatively high percentage of DRAXXIN-injected females and uninjected females positive for Rs cells in ovarian fluid by SPC-FAT testing (39% and 25%, respectively) and Rs concentrations >1 x 10^3 cells/mL in some females from each group indicated the presence of the bacterium in a location other than the kidney in these fish.

The study results provided no evidence that DRAXXIN injection of adult female Chinook salmon affected their fecundity, egg eye-up, or survival and growth of progeny fry. Deformed fry were prevalent in the DRAXXIN low ELISA transmission risk group, but not in the DRAXXIN medium and high ELISA risk groups. The presence of 'corkscrew' deformities in a single fry group suggested a genetic cause of the abnormalities that was likely limited to one or a few mating pairs (Bruno et al. 2013). An increase in the number of DRAXXIN-injected and uninjected adult female Chinook salmon in the study would have enabled the use of replicate tanks for each treatment and for each ELISA risk group within a treatment, to reduce potential impacts of anomalies associated with individual mating pairs and provide a better assessment of effects of DRAXXIN injection on the survival and growth parameters monitored in progeny.

Results of ELISA testing of progeny fish sampled at two points during rearing did not demonstrate progression of Rs infections in progeny of either the DRAXXIN-injected or uninjected females. Only four 8-fish kidney-spleen tissue pools—three from progeny of DRAXXIN-injected females and one from progeny of uninjected females—yielded ELISA OD values > 0.080 in the first sample, and none of the 5-fish tissue pools in the second sample showed ELISA OD values > 0.080. One factor that may have influenced the apparent lack of Rs vertical transmission is the relatively low rate of intra-ovum infection reported (about 6% – 15%), even in egg lots from highly infected females (Evelyn et al. 1984a; 1984b;

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1986b). Although the Rs levels in ovarian fluid and kidney tissues of a one uninjected female exceeded 1.8×10^7 cells/mL and 2.8×10^4 DNA copies/ug, respectively, a subsample of only 220 eggs from each female was transferred to WFRC. Thus, there may not have been sufficient numbers of infected eggs transferred for successful vertical Rs transmission, amplification of Rs numbers in infected individuals, and subsequent horizontal transmission. Because Rs infections develop slowly, Rs levels may have increased if progeny had been held longer (Pascho et al. 1991). Additionally, the pooling of tissue samples may have diluted Rs antigens to levels below detection limits in most samples.

The finding that none of the progeny samples tested by qPCR were positive for Rs DNA was not surprising. The diagnostic sensitivity of polyclonal ELISA is often higher than that of qPCR (Elliott et al. 2013; Elliott et al. 2015), and concordance between ELISA and qPCR results is relatively poor when low ELISA OD values are obtained from sample testing (Elliott et al. 2013).

In conclusion, whereas the results of this study suggested that DRAXXIN injection was associated with increased pre-spawn survival and decreased Rs prevalence and levels in female spring Chinook salmon spawned at Leavenworth NFH, the numbers of DRAXXIN-injected and uninjected fish included in the study were too low to yield statistically significant differences between the groups. There was no evidence that DRAXXIN injection at 5 mg DRAXXIN per kg body weight was toxic to the adult females or their progeny. The Rs antigen prevalence and levels in progeny of both DRAXXIN-injected and uninjected females was extremely low, and the study was thus not able to determine if DRAXXIN injection of spawning females affected vertical transmission of Rs to progeny. To adequately evaluate the efficacy of DRAXXIN injection for reducing Rs vertical transmission, additional studies should be conducted with larger numbers of DRAXXIN-injected and uninjected females in Chinook salmon populations with a greater range of Rs infection levels.

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Table 1. Numbers of adult DRAXXIN-injected and uninjected female Chinook salmon (Leavenworth NFH 2014 spawn) assigned by USFWS fish health personnel to low, medium and high Rs vertical transmission risk groups based on ELISA OD values obtained from testing kidney tissue.

	Treatment Number of fish (range of values for ELISA OD-blank)	
ELISA OD risk group	DRAXXIN	No injection
Low	11 (0.037 – 0.083)	6 (0.035 – 0.083)
Medium	14 (0.085 – 0.163)	15 (0.085 – 0.168)
High	6 (0.175 – 0.305)	7 (0.176 – 2.294)

Table 2. Prevalence and quantity estimates of Rs DNA detected by qPCR in kidney tissues sampled from DRAXXIN-injected and uninjected female Chinook salmon at spawning (Leavenworth NFH 2014 spawn.) Samples yielding mean cycle threshold (C_T) values < 38 in two technical replicates were considered positive for Rs DNA.

ELISA OD risk group (Number of females)	Number of Rs-positive fish (% of group)	C_T range positive fish	Range of Rs DNA copies/µg total DNA
	DRAXXIN –injected (31 females total)		
Low (11)	2 (18%)	36.6 – 34.8	1.4 – 2.1
Medium (14)	0	---	---
High (6)	1 (17%)	37.0	0.1
	Uninjected (28 females total)		
Low (6)	0	---	---
Medium (15)	1 (7%)	35.5	0.9
High (7)	5 (71%)	37.4 – 21.1	0.6 – 2.8 x 10^4

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Table 3. Prevalence and quantity estimates of Rs messenger RNA expression detected by RT-qPCR in kidney tissues sampled from DRAXXIN-injected and uninjected female Chinook salmon at spawning (Leavenworth NFH 2014 spawn.) Samples yielding mean cycle threshold (C_T) values < 38 in two technical replicates were considered positive for Rs RNA.

ELISA OD risk group (Number of females)	Number of Rs-positive fish (% of group)	C_T range positive fish	Range of Rs RNA copies/µg total RNA
	DRAXXIN –injected (31 females total)		
Low (11)	1 (9%)	37.1	1.3
Medium (14)	0	---	---
High (6)	1 (17%)	37.2	0.3
	Uninjected (28 females total)		
Low (6)	0	---	---
Medium (15)	2 (13%)	36.8 – 32.3	1.3 – 27.5
High (7)	4 (57%)	34.9 – 27.6	3.1 – 800

Table 4. Presence (positive) or absence (negative) of Rs DNA and Rs RNA in kidney tissue samples from spawning female Leavenworth NFH Chinook salmon, preserved in RNALater and subsequently tested by qPCR (DNA) and RT-qPCR (RNA).

	Rs RNA	
Rs DNA	Positive	Negative
Positive	7 (11.9%)	2 (3.4%)
Negative	1 (1.7%)	49 (83%)

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Table 5. Prevalence and quantity estimates of Rs in ovarian fluid of spawning female Leavenworth NFH Chinook salmon by the solid phase cytometry-fluorescent antibody test (SPC-FAT). Ovarian fluid samples in which Rs counts were ≥ 10 bacteria/mL were considered to be true positives.

ELISA OD risk group (Number of females)	Number of Rs-positive fish (% of group)	Range of Rs cells/mL ovarian fluid (geometric mean)
	DRAXXIN –injected (31 females total)	
Low (11)	3 (27%)	18 – 308 (70)
Medium (14)	7 (50%)	14 – 1,480 (51)
High (6)	2 (33%)	44 – 232 (101)
	Uninjected (28 females total)	
Low (6)	1 (17%)	1,026
Medium (15)	3 (20%)	40 – 1,060 (175)
High (7)	3 (43%)	10 – 1.8 x 10^7 (3,287)

Table 6. Fecundity and egg survival to eye-up for females and progeny in the ELISA OD risk groups (low and medium) for which egg lots were retained by Leavenworth NFH from the 2014 spawning.

	Treatment	
	DRAXXIN (21 females)	No injection (15 females)
Mean eyed egg survival (± SD)	0.913 (± 0.041)	0.927 (± 0.016)
Eyed Eggs on hand	78,903	58,869
Dead eggs	2,953	1,645
Mean fecundity (± SD)	4,116 (± 523)	4,234 (± 585)

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Table 7. Initial survival of Leavenworth NFH progeny groups from DRAXXIN-injected and uninjected female Chinook salmon after transportation of eyed eggs to the WFRC (October 8, 2014) until swim-up (November 28, 2014). A total of 220 eggs from each female were transported to the WFRC.

	Treatment Number of progeny surviving to swim-up/Total (%)	
ELISA OD risk group	DRAXXIN	No injection
Low	2363/2397 (98.6%)	1278/1295 (99.1%)
Medium	3009/3044 (98.9%)	3248/3276 (99.4%)
High	1304/1309 (99.6%)	1518/1527 (99.0%)
All ELISA risk groups	6676/6750 (98.9%)	6044/6098 (99.1%)

Table 8. Results of ELISA testing of tissue samples taken from progeny fish on May 26 – June 10, 2015 (first sample). Kidney and spleen tissues were sampled from a total of 160 fish from each ELISA OD risk group within a treatment group (DRAXXIN-injected or uninjected), and samples were processed as twenty 8-fish pools.

ELISA OD risk group	Mean ELISA OD (SD)	Range of ELISA OD values
	DRAXXIN –injected	
Low	0.067 (0.007)	0.061 – 0.086
Medium	0.069 (0.016)	0.063 – 0.135
High	0.066 (0.002)	0.064 – 0.070
	Uninjected	
Low	0.067 (0.003)	0.063 – 0.074
Medium	0.063 (0.002)	0.061 – 0.068
High	0.063 (0.008)	0.061 – 0.097

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Table 9. Results of ELISA testing of tissue samples taken from progeny fish on September 2 – 10, 2015 (second sample). Kidney and spleen tissues were sampled from a total of 100 fish from each ELISA OD risk group within a treatment group (DRAXXIN-injected or uninjected), and samples were processed as twenty 5-fish pools.

ELISA OD risk group	Mean ELISA OD (SD)	Range of ELISA OD values
	DRAXXIN –injected	
Low	0.061 (0.006)	0.056 – 0.077
Medium	0.061 (0.004)	0.057 – 0.069
High	0.063 (0.004)	0.058 – 0.070
	Uninjected	
Low	0.062 (0.005)	0.057 – 0.075
Medium	0.059 (0.002)	0.057 – 0.065
High	0.059 (0.001)	0.056 – 0.063

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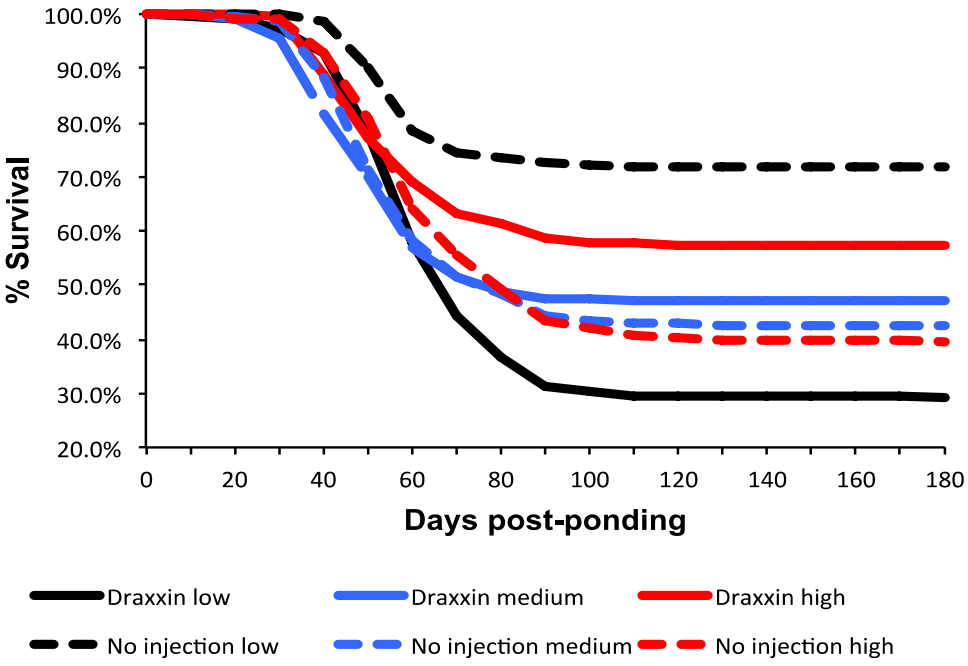
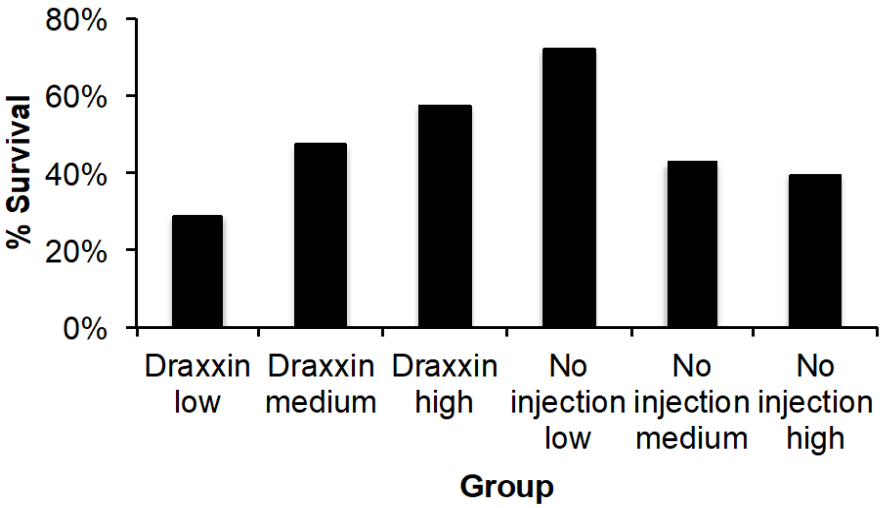
Figure 1. Survival of progeny Chinook salmon from November 28, 2014 (ponding) through study termination on September 22, 2015. (A) Survival curves for Chinook salmon ELISA *R. salmoninarum* transmission risk progeny groups (low, medium and high) from DRAXXIN-injected females (solid lines) and uninjected females (dashed lines) through 180 days post-ponding (scale 20% to 100% for better visualization). (B) Total survival of progeny groups at the termination of the study 298 days after ponding (scale 0% to 80% for better visualization).

(A)



B.



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