On-farm performance of rainbow trout (Oncorhynchus mykiss) selectively bred for resistance to bacterial cold water disease: Effect of rearing environment on survival phenotype

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A frequent cause of freshwater farmed trout loss is bacterial cold water disease (BCWD) that is also synonymous with rainbow trout fry syndrome (Barnes and Brown, 2011; Nematollahi et al., 2003; Starliper, 2011). The etiological agent of BCWD is a Gram-negative, yellow-pigmented bacterium, Flavobacterium psychrophilum. In Idaho, a primary location of rainbow trout production in the US, most losses due to BCWD occur in fish between 0.2 to 4 g; however, larger fish can also be compromised by BCWD. BCWD can present as an acute outbreak in small fish or as a chronic disease in larger fish, typically causing between 2 and 30% mortality. Production losses from BCWD are due to direct mortality and may also occur due to deformities in fish that survive infection (Madsen et al., 2001; Nematollahi et al., 2003). Current BCWD management frequently involves oral antibiotic therapy, chemical treatment, and/or a reduction in fish density (Barnes and Brown, 2011; Nematollahi et al., 2003; Starliper, 2011).

Selective fish breeding programs for disease resistance comprise an increasingly important role in aquaculture production and offer an additional management tool for reducing bacterial-caused disease losses. Bacterial cold water disease (BCWD) is one of the most frequent causes of elevated mortality in juvenile salmonids, and we have selectively bred three genetic lines of rainbow trout for varying resistance to BCWD. These lines, designated ARS-Fp-R (resistant), ARS-Fp-C (control) and ARS-Fp-S (susceptible), differ in survival following standardized laboratory challenges with the causative agent of BCWD, Flavobacterium psychrophilum. This study evaluated survival of the genetic lines in laboratory challenges and in a production environment. Evaluations of disease resistance demonstrated a reproducible, 30% or greater, survival difference between ARS-Fp-R and ARS-Fp-S lines at body weights ranging from 0.7 to 13 g. Farm trials were performed to evaluate survival over an 80-day growth period starting after the trout began feeding. After a BCWD epizootic, the ARS-Fp-R line displayed significantly greater risk-adjusted survival (95.7%) than the ARS-Fp-S line (91.2%, P < 0.0001) and the ARS-Fp-C line (92.4%, P = 0.0001). Phenotype stability in farm-trial fish was also evaluated using laboratory challenges. The ARS-Fp-R line consistently displayed a higher, but not always statistically significant, survival percentage compared to the other lines and the data suggest that the magnitude of the survival phenotype difference is sensitive to environmental influence. In summary, the overall greater survival of the ARS-Fp-R line provides evidence of genetic improvement under production conditions.

Genetic improvement in disease resistance accomplished through selective breeding offers an additional management tool for controlling disease in finfish aquaculture (Fjølstad et al., 1993; Gjedrem, 2005; Moen, 2011; Henryon et al., 2005). At the National Center for Cool and Cold Water Aquaculture (NCCCWA), we have investigated whether family-based selective breeding of rainbow trout can increase the innate disease resistance of naïve fish against BCWD (Hadidi et al., 2008; Leeds et al., 2010; Silverstein et al., 2009). Since 2005, an odd-year spawning line of pedigreed rainbow trout developed from the intercrossing of four domesticated founder strains has been evaluated and selectively bred for increased BCWD survival based on intraperitoneal injection-challenge evaluation (Silverstein et al., 2009). This closed genetic line has been designated ARS-Fp-R. In addition, we have developed a selection control line, designated ARS-Fp-C, and a susceptible line, designated ARS-Fp-S. All lines were derived from the same resource population.

At present, factors influencing BCWD resistance under field conditions remain poorly understood and optimal trial design for evaluating genetic resistance has not been investigated. Studies reported herein were initiated as part of a multi-year laboratory and field evaluation process to: 1) obtain off-site validation of the resistant and susceptible resistance lines; and 2) evaluate the ARS-Fp-R line under environmental conditions.
phenotypes using standardized laboratory challenges, 2) test the hypothesis that the ARS-Fp-R line will exhibit higher survival than either the ARS-Fp-C or ARS-Fp-S lines, and 3) measure the stability of the disease-resistance phenotype at select time points after farm exposure by removing fish from field evaluation and subjecting them to standardized laboratory challenges.

2. Materials and methods

2.1. Rainbow trout genetic lines, rearing conditions and water quality parameters

Development of the single, 2-year-old, spawning, closed resource population consisting of 71 full-sib families from which the ARS-Fp-R, -C, and -S lines were derived has been previously described (Silverstein et al., 2009). Differentiation of the three lines via selective breeding is shown schematically in Fig. 1. Briefly, the ARS-Fp-R line has been selected each generation for improved survival following laboratory challenge with *F. psychrophilum* as previously described (Leeds et al., 2010). We have utilized survival at 21 days post-challenge as a surrogate measure of disease resistance. The ARS-Fp-S line has undergone 1 generation of selection for poor survival following laboratory challenge with *F. psychrophilum*, and has since been randomly mated as a reference susceptible line. The ARS-Fp-C line represents random mating of the ARS-Fp-R line after only one generation of selection and was developed to directly quantify survival improvement due to effects of continued selection. An average of 76, 19, and 25 full-sib families has been produced and evaluated for BCWD resistance each generation for the ARS-Fp-R, -S, and -C lines, respectively. Details of general animal husbandry practices and rearing conditions are given in Silverstein et al. (2009) and Leeds et al. (2010).

A random sub-sample of each generation (n = 60/lot) were tested once or twice per year and determined to be free of viral hemorrhagic septicemia virus, infectious hematopoetic necrosis virus (IHNV), infectious pancreatic necrosis virus, *Oncorhynchus masou* virus, infectious salmon anemia virus, and spring viremia of carp virus. One-year-old fish or older were also tested and confirmed to be free of *Aeromonas salmonicida*, *Yersinia ruckeri*, *Renibacterium salmoninarum*, *Myxobolus cerebralis* and *Ceratomyxa shasta*. While not required for certification, broodstock and one-year-old fish at our facility were swabbed from kidney tissue (n = 60 fish/lot) and cultured on Tryptone yeast glucose agar slants (AFS-FHS, 2010), incubated at 15 °C for 14 days, and were negative for *F. psychrophilum*. Testing was performed either by Kennebeck River Biosciences (Richmond, ME) or by the U.S. Fish and Wildlife Service (Lamar, PA).

Flow-through spring water was used to maintain early life stage growth and pre-spawning broodstock. The average water quality parameters (± 1 s.d.) of the NCCWCA spring-source, as measured from January 2008 through August 2012, were the following: water temperature 12.8 ± 0.4 °C, pH 7.2 ± 0.12, ammonia 0.03 ± 0.06 mg L⁻¹, CO₂ 303 ± 4.3 mg L⁻¹, and water hardness 285 ± 25 mg L⁻¹ CaCO₃. Fish were fed a commercial fishmeal-based diet (Zeigler Bros, Inc., Gardners, PA).

At the Clear Springs Foods (CSF), Inc. farm site (Buhl, ID), where fish were grown for the farm trial, as well as the CSF Research Facility, where animals were transferred for experimental challenge, fish were fed a standard rainbow trout fishmeal-based diet (45% protein; 20% fat; CSF, Inc.). Both the farm site and CSF Research Facility are supplied by spring water from the Snake River Plain Aquifer. At the farm site, water quality parameters were the following: temperature of 14.5 °C, pH 8.2, ammonia <0.01 mg L⁻¹, nitrate-nitrite nitrogen 3.22 mg L⁻¹, and water hardness 255 mg L⁻¹ CaCO₃. At the CSF Research Facility the water quality parameters were the following: temperature of 14 °C, pH 7.9, ammonia <0.01 mg L⁻¹, nitrate-nitrite 2.48 mg L⁻¹, and water hardness 216 mg L⁻¹ CaCO₃.

2.2. Bacterial and viral strains, propagation, enumeration and standardized laboratory challenge

All BCWD challenges used *F. psychrophilum* strain CSF259-93 originally isolated by Dr. S. LaPatra from a clinical case of BCWD in rainbow trout. In 2003, a frozen broth culture was sent to the NCCWCA and a single colony was isolated on Trypton yeast extract salts (TYES) plates (Cain and LaFrentz, 2007), amplified in TYES broth, and a bank of approximately 200 1-ml frozen stocks, containing 10% glycerol, were prepared from this culture and stored at −80 °C to provide a standardized challenge source. Virulence of the NCCWCA subculture was confirmed to be identical to the original stock and the complete genome sequence of this isolate has been determined (G. Wiens, unpublished data). For challenge, a single vial was thawed, dilutions cultured on TYES plates at 15 °C for five days, and the harvested bacteria re-suspended in either Dulbecco’s PBS (Sigma) or sterile saline. Cell concentration was measured by optical density of the suspension at 525 nm and challenge dose adjusted for body weight to maintain a dose above 300,000 CFU per gram average fish body weight, although this value varied between challenge experiments (Table S1). Bacterial cell number was verified at the NCCWCA by direct plate counting. Fish were challenged by i.p. injection as previously described (Hadidi et al., 2008; Overturf et al., 2010). This challenge route results in reproducible mortality when challenging large numbers of fish.

Viral challenge was carried out using IHNV strain 220-90 (LaPatra et al., 1994). Briefly, IHNV was propagated in *Epithelioma populorum cyprini* (EPC) cell line and virus re-isolated as previously described (LaPatra et al., 1994). Fish were waterborne challenged with a concentration of 10⁴ PFU ml⁻¹ tank water (Overturf et al., 2010).

2.3. Phenotype validation studies

Off-site phenotype validation was carried out at the CSF Research Facility using 21 full-sib rainbow trout families from the NCCWCA 2009 year class. Egg hatching was temperature-synchronized as described previously (Leeds et al., 2010) and all families hatched within a 4-day period. At 39 days post-hatching, individual families were transported overnight to the CSF Research Facility using supplemental oxygen. Upon receipt, fish were acclimated for 8 days in ambient water (14.5 °C) prior to BCWD and IHNV challenge. PBS injected groups, or mock waterborne challenge groups, were included.

**Fig. 1.** Schematic of disease resistance breeding and the relative direction of selection applied to three rainbow trout lines. Symbols indicate year class in which BCWD challenges were performed while the steps indicate the generations of selection applied either for increased BCWD resistance (+1) or for increased BCWD susceptibility (−1). Number of full-sib families is given for each line and generation. The ARS-Fp-R was selected for three generations while the ARS-Fp-C and ARS-Fp-S lines were selected for only one generation, and since, randomly bred as reference lines with similar genetic background to the ARS-Fp-R line.
in all experiments to confirm that mortality was due to pathogen exposure. Tanks were checked daily for mortality and at the end of the experiment (21 days) the numbers of remaining live fish counted. Cohorts of the same 21 full-sib families were subsequently BCWD challenged at the NCCWWA at a mean age of 89 days post-hatch as part of a larger resistance phenotyping experiment carried out on all NCCWWA 2009 year class families of rainbow trout (Leeds et al., 2010). Ten of the twenty-one families were subsequently BCWD challenged at a mean age of 123 days post-hatch (Table S1). Animal husbandry procedures and challenge experiments were approved by the NCCWWA institutional animal care and use committee (NCCWWA IACUC protocols 053, 064 and 065). Permits from the State of Idaho Department of Fish and Game and the Department of Agriculture were obtained for fish/egg shipments for farm evaluation.

2.4. Farm trial evaluation

Farm trials were carried out in 2010 and 2011 at the same CSF farm site. Losses occurring during hatching and prior to feed initiation are common and were excluded from this study due to typical non-disease related causes of mortality including genetic deformity and general frailty. During these trials, fish were grown indoors in cement raceways. In the 2010 farm trial, family pools representing the ARS-Fp-S and ARS-Fp-R lines were sent to the farm site and fish were cultured in separate adjacent raceways (Fig. S1 A). In the 2011 trial, family pools representing the ARS-Fp-S, ARS-Fp-C, and ARS-Fp-R lines were sent to the site and fish were cultured within a single raceway (Fig. S1 B). The rationale for the serial raceway design was to place the ARS-Fp-R line at greatest risk as they were furthest from incoming spring water and also downstream of the ARS-Fp-C and ARS-Fp-S lines known to have higher susceptibility to BCWD in standardized laboratory challenges. In both trials, hatchery personnel were blinded to the genetic identity of the groups of fish during the study period. Eggs were disinfected prior to shipping and also upon receipt using a 10-minute, 100-ppm bath incubation of OVADINE™ (Western Chemical, Ferndale, WA).

To generate eggs for the 2010 field trial, three-year-old females from the 2007 year class were mated with one-year-old neonales from the 2009 year class that had previously received a series of salmon pituitary extract (Argent Laboratories, Redmond, WA) injections to induce early maturation. Egg quantity was estimated separately for each full-sib family using the von Bayer method (von Bayer, 1908). Prior to egg pooling, a total of 300 eggs from each farm trial family were retained at the NCCWWA for hatch evaluation and BCWD phenotype confirmation. Approximately 25,000 ARS-Fp-R line eyed eggs (a mix from 18 families, mean = 1389 ± 177 eggs per family) and 25,000 ARS-Fp-S line eyed eggs (a mix from 9 families, mean = 2778 ± 500 eggs per family) were used in this trial. Percent survival from hatching until the time of transfer into raceways did not differ between the ARS-Fp-R (71.6%) and ARS-Fp-S (71.7%) lines, thus each line had similar numbers of fry at the time of transfer.

To generate eggs for the 2011 farm trial, two-year-old females were mated with two-year-old males, both from the NCCWWA 2009 year-class. Approximately 20,000 ARS-Fp-R line eyed-eggs (a mix from 17 families, mean = 1,176 ± 699 eggs per family), 4000 ARS-Fp-C line eyed eggs (a mix from 3 families, mean = 1360 ± 620 eggs per family), and 20,000 ARS-Fp-S line eyed-eggs (a mix from 9 families, mean = 2222 ± 1055 eggs per family) were used in this trial. In contrast to the 2010 farm trial, percent survival from hatching until the time of yolk-sac fry transfer into raceways was lower and differed between lines: ARS-Fp-R (50% hatch), ARS-Fp-C (55% hatch), and ARS-Fp-S (36% hatch). The lower survival was not specific to the farm site as similarly-low hatch percentages were observed in the cohorts of eggs retained at the NCCWWA. Furthermore, egg eying rates were generally lower in 2011 for all genetic lines of fish at the NCCWWA broodstock facility compared to previous years, thus suggesting an unknown environmental factor adversely affecting egg quality. A total of 1000 eggs from each pool were retained at the NCCWWA for hatch evaluation and experimental challenge.

Once eggs hatched, fry were moved into shortened raceways of approximately 2.8 m³ with flow-through water. Fry numbers were estimated at raceway transfer by weighing five groups of 100 fish to obtain an average individual fish weight in grams. This value was then used to divide the total weight of the group of fish to determine the population number. In the 2010 trial, fry feeding started approximately 15 days after transfer from the upwelling incubators into the raceways, while in the 2011 trial, fry were fed starting approximately 9 days after transfer from the upwelling incubators into the raceways. At the end of the indoor rearing period, subsamples of counted fish were weighed and total weight of the remaining raceway biomass determined to estimate final fish numbers present in each raceway at the end of the trial.

2.5. Microbial and F. psychrophilum culture from fish grown at the farm trial site

After first feeding, expected baseline mortality rate is approximately 0.02 to 0.05% fish day⁻¹, and above this threshold clinical investigation was initiated. Clinical investigation involves microscopic examination, bacteriological culture on Trypticase soy agar (TSA) and TYES plates using swabs inoculated from the fish external skin surface as well as spleen and kidney tissue, and virus isolation using standard methods (AFS-FHS, 2010). Yellow pigmented bacteria, cultured on TYES plates, were confirmed to be F. psychrophilum by PCR (described in Section 2.6). To identify the other non-F. psychrophilum species, single bacterial colonies were subcultured on TYES or TSA media, cells harvested from plates and whole cell extracts were normalized to an OD₅₆₀ of 1.0 and 1 ml pelleted cells were dissolved in 100 μl of SDS-PAGE loading buffer. A total of 10 to 20 μl lysate was subjected to 12.5% SDS-PAGE (30:0.8 acrylamide–bisacrylamide) followed by standard Coomassie Blue staining. Isolates that differed in whole cell profiles were subsequently subjected to 16S rRNA gene amplification and direct sequencing of the PCR product as previously described (Marancik and Wiens, 2013). Bacterial isolates were assigned genus using the Ribosomal Database Project (rdp.cme.msu.edu) and sequence similarity was compared using BLAST analyses of the NR database. 16S rRNA gene sequences have been deposited in GenBank as accession nos. (KCs330341–KCs330369).

2.6. F. psychrophilum-specific PCR

A single-step PCR was used for species-specific amplification of a 1089 bp F. psychrophilum 16S rRNA gene fragment using described primers PSY1 and PSY2 (Wiklund et al., 2000). A Taqman qPCR assay was used to quantify F. psychrophilum load in spleen tissue from field trial fish as previously described (Marancik and Wiens, 2013).

2.7. Standardized laboratory challenge of farm trial fish

To experimentally assess the phenotypes of the farm fish, fish were removed from each raceway or section of a raceway and transported to the CSF Research Facility for standardized laboratory challenge. In the 2010 farm trial, 300 fish were removed from each raceway on trial days 32 and 55, transported to the CSF Research Facility, and subjected to standardized BCWD challenge (Table S1, 2010 #1 and #2 CSF Res. challenges). In the 2011 farm trial, either 200 or 300 fish from each line were removed on trial days 16, 52 and 81, transported to the CSF Research Facility, and subjected to standardized BCWD challenge (Table S1, 2011 #1-3 CSF Res. challenges). Cohorts of the field trial fish were also retained at the NCCWWA and challenged at similar developmental times [degree days (°C×days)] as field trial fish (Table S1, 2011 #1-3 NCCWWA challenges).
2.8. Statistical analyses

Average family differences between genetic lines were tested for significant difference by comparing, using a Student’s t-test, family cumulative percent survival transformed by the arcsin square-root to normalize variance. Differences in mortality kinetics were determined by averaging family mean days to death (MDD). Mean days to death is equal to the sum of numerical day of death for all fish that died, divided by the total number of fish that died within the challenge period. For farm trials or experimental challenges of pooled-family lines, difference in survival was determined using the product limit method of Kaplan and Meier and calculations were performed using GraphPad v4.0 software. Log-rank (Mantel–Cox) test was used to compare survival curves. In farm trials, fish numbers were measured prior to raceway transfer and also at the termination of the experiment. Because final counts are considered more accurate than initial counts, the initial counts were adjusted if there was a discrepancy between input and output after taking into account mortality and sampling. Final percent mortality per line was calculated based on the numbers of fish at risk and was adjusted due to live fish sampling. While statistical significance for all experiments was P<0.05, before initiating field trials, a second set of criteria were established for judging the economic relevance of line improvement that included a ≥5% difference in risk-adjusted survival.

3. Results

3.1. Rainbow trout ARS-Fp-R and ARS-Fp-S lines exhibit a reproducible and stable BCWD resistance phenotype when grown under laboratory conditions

Prior to farm trial testing, a blinded study was conducted to independently BCWD resistance phenotype 11 ARS-Fp-R families and 10 ARS-Fp-S families. Percent survival of the ARS-Fp-R line families, challenged at CSF Research Facility at 48 day post-hatch (631 degree days), averaged 75±18% (s.d.) and was significantly greater than the survival of the ARS-Fp-S line families that averaged 35±11% (P<0.0001, two-tailed t-test, Fig. 2A and B). The kinetics of mortality did not significantly differ between the rainbow trout lines (P=0.17, two-tailed t-test). The MDD of the ARS-Fp-R line families was 10.9±1.6 days while MDD of the ARS-Fp-S line families averaged 10.1±1.1 days. Body weights measured before challenge did not significantly differ between lines (data not shown). Both lines were equally susceptible to IHNV challenge with mean survival of 8±5% for ARS-Fp-R families and 6±5% for ARS-Fp-S families (P=0.34, two-tailed t-test). There was no correlation between BCWD and IHNV family post-challenge survival (Pearson r=0.05, P=0.82, n=21). To confirm and compare the BCWD resistance phenotype across locations and ages, full-sibs from the same 21 families, that had been retained at the NCCCWA, were phenotyped at 89 days post-hatch (1133 degree days). Mean family body weight at the start of the challenge was 4.6±0.5 g (~6-fold larger). The mean survival of the 11 ARS-Fp-R families was 84±10% and was significantly greater (P<0.0001, two-tailed t-test) compared to ARS-Fp-S families (34±17%). There was a significant positive phenotypic correlation in percent family survival between the two challenge experiments initiated at 48 days and 89 days post-hatch (Pearson r=0.59, one-way ANOVA). The MDD of ARS-Fp-R line families challenged at day 89 post-hatch was 9.9±2.5 days and the MDD of ARS-Fp-S line families was 10.0±2.1 days. Additional BCWD resistance phenotyping using five families each from the ARS-Fp-R and ARS-Fp-S lines was carried out at the NCCCWA at 123 days post-hatch (mean body weight 13±2 g). Survival measured at 123 days post-hatch was significantly higher for ARS-Fp-R families (87±2% vs. 35±13%) (P<0.0001, two-tailed t-test).

Fig. 2. Evaluation of full-sib fish families for survival following F. psychrophilum challenge. (A) Kinetics of survival of individual families over 21 days post-challenge (n=50 fish per family, avg BW=0.7±0.1 g). (B) Cumulative percent family survival by genetic line. (C) Correlation between day 48 and day 89 BCWD post-challenge survival (Pearson r=0.82). (D) Correlation between day 48 and day 123 BCWD post-challenge survival (Pearson r=0.88). For all panels, blue squares denote the ARS-Fp-R line and red circles ARS-Fp-S line families.
correlated with survival results from 48 to 89 days post-hatch challenges (Fig. 2D, day 48 compared to day 123 challenge, Pearson r = 0.88, P = 0.0008; day 89 compared with day 123 challenge, Pearson r = 0.80, P = 0.0052, not shown). In summary, these results indicate that the phenotypes of the ARS-Fp-R and ARS-Fp-S lines were reproducible after fish were transported and challenged and consistent over a 75-day growth period ranging from an average body weight of 0.7 to 13 g. Furthermore, selective breeding did not alter susceptibility to IHNV infection. Thus, these results validated BCWD selective breeding efforts and provided rationale to conduct small-scale farm trials.

3.2. Farm trial survival of ARS-Fp-R, ARS-Fp-C and ARS-Fp-S lines of rainbow trout

Evaluation was carried out during early life stage rearing (feed initiation to 80 days or 1200 degree days), when BCWD epizootics can be most severe. In the 2010 trial, the initial counts of ARS-Fp-R and ARS-Fp-S line fish at first feeding were estimated at 17,554 and 17,272 respectively. Over the 80 day trial period, 350 mortalities were recorded in the ARS-Fp-R line raceway (97.9% risk-adjusted survival) and 515 mortalities were recorded in the ARS-Fp-S line raceway (96.9% risk-adjusted survival) and typical disease signs associated with BCWD or IHNV outbreaks were not observed in either of the raceways. The survival of both lines was generally considered to be good by hatchery personnel. A transient elevated mortality event occurred on trial day 35 in the ARS-Fp-R fish (0.18% daily mortality, n = 30) that was not caused by F. psychrophilum. A mixed population of bacterial species was identified from both ARS-Fp-R and ARS-Fp-S line fish on trial day 35 that is described in further detail in Section 3.5. Final fish growth and feed utilization of both lines were within variations observed for standard production lots at the farm site.

A second trial was carried out in 2011 at the same farm site using a modified experimental design. In this trial, fish were cultured in one raceway that had been divided into three sections, and ARS-Fp-C line fish were included in the study as these fish were intermediate in phenotype between ARS-Fp-R and ARS-Fp-S line fish. In this trial, the initial count numbers of ARS-Fp-R, ARS-Fp-C, ARS-Fp-S, and line fish at first feeding were estimated at 10979, 1799, and 6215 respectively. During this trial, a BCWD outbreak was observed within the ARS-Fp-S line that was first diagnosed on day 33 of the hatch house evaluation period (Fig. 3). Elevated mortality in the ARS-Fp-S line occurred for approximately three weeks following diagnosis and averaging 0.20±0.13% per day during this time, and by day 55 of the study period, returned to baseline. Over the 80-day hatch-house evaluation period, a total of 492 mortalities were recorded in the ARS-Fp-S line (91.2% risk-adjusted survival), 111 mortalities were recorded in the ARS-Fp-C (92.4% risk-adjusted survival), and 451 mortalities were recorded in the ARS-Fp-R line (95.7% risk-adjusted survival, Fig. 3). Survival of the ARS-Fp-R line was 4.9% greater than the ARS-Fp-S line (P<0.0001, hazard ratio = 0.48, with a 95% confidence interval ranging from 0.42 to 0.55) and 3.6% greater than the ARS-Fp-C line (P<0.0001, hazard ratio = 0.49, with a 95% confidence interval ranging from 0.38 to 0.64). There was not a significant survival difference between the ARS-Fp-C and ARS-Fp-S lines (P=0.40).

3.3. Isolation and detection of F. psychrophilum from farm trial fish

In the 2010 farm trial, F. psychrophilum was not detected in either the ARS-Fp-R or -S lines. In the 2011 farm trial, all three lines were infected within the serial raceway experiment. From day 33 through day 54 of the study, F. psychrophilum was cultured either from spleen or kidney tissue from 17 of 27 (63%) ARS-Fp-S, 1 of 8 (12%) ARS-Fp-C, and 2 of 11 (18%) ARS-Fp-R fish sampled. From each fish, 1–2 colonies were subcultured and a total of 53 isolates were confirmed by 16S rRNA gene PCR as well as real-time qPCR. At the end of the trial period (day 81), 30 fish from each of the ARS-Fp-R, -C and -S lines were randomly sampled, weighed, and spleen tissue cultured on TYES plates or processed for qPCR detection of F. psychrophilum loads. There was no difference in mean body weights between the three lines (data not shown) and F. psychrophilum was not cultured from any spleen sample (n = 90). Quantitative PCR analyses detected only very low-levels of F. psychrophilum DNA in two ARS-Fp-S line fish and one ARS-Fp-C line fish (Cq values of >37) which are below the limit of quantitation. No ARS-Fp-R line fish were positive by qPCR. In summary, F. psychrophilum presence was confirmed within all three genetic lines; however, the infection appeared limited in scope and had resolved by the end of the hatch house rearing period.

3.4. Standardized challenge demonstrates an effect of rearing environment on BCWD phenotype in fish grown in either parallel or serial raceways

The effect of rearing environment on BCWD resistance phenotype was examined in both the 2010 and 2011 field trials by removing fish from the raceways, transportation to laboratory challenge facility and subjecting to standard experimental injection challenge. In the 2010 trial, fish were removed on trial days 32 (841 degree days post-hatch) and 55 (1131 degree days post-hatch). Surprisingly, BCWD post-challenge survival of the ARS-Fp-R lines was only modestly higher, 11.2% and 10.9% respectively at the two time points (Table 1), and was not statistically different from the ARS-Fp-S line (P=0.15 and P=0.23 respectively). In contrast, when the ARS-Fp-R and ARS-Fp-S families were individually challenged at the NCCWFA at 845 degree days, a significant survival difference was observed between lines (P=0.0002, two-tailed t-test). The ARS-Fp-R line averaged 89±9% survival, which was 42 percentage points greater than the ARS-Fp-S line, 47±21%. We next examined whether pooling ARS-Fp-R or ARS-Fp-S families would alter challenge results as compared with individual family challenge. Approximately equal numbers of fish from each of the tested ARS-Fp-R and ARS-Fp-S families were pooled, and subsequently, a subsample of 75 fish was challenged at 1270 degree days post-hatch. Post-challenge survival of the ARS-Fp-R line, (87.9±6.8%, n = 3 tanks, 74 fish total) was significantly higher than the ARS-Fp-S line (52.7±4.3%, n = 3 tanks, 72 fish total, P<0.001).

To more fully compare phenotypes of laboratory and farm reared fish, in 2011 farm trial fish removed from the raceway on trial days 16, 52 and 81 corresponding to 450, 1015, and 1407 degree days...
Although not statistically significant was intermediate between the ARS-Fp-R and ARS-Fp-S lines; furthermore, phenotypic changes appear to be greatest within the ARS-Fp-S line as compared to the laboratory-reared cohort.

3.5. Culture of yellow pigmented bacteria and other eubacteria from farm trial fish

In order to better characterize the microbial flora of farm trial fish, both non- F. psychrophilum yellow pigmented bacteria, as well as other eubacteria, were cultured and identified by 16S rRNA gene sequencing. Initially, yolk-sac fry were removed 7 days prior to the first feeding and external swabs from 20 fry (10 per raceway, 5 live and 5 dead) were inoculated onto TYES plates. 16S rRNA gene sequencing identified that 10 of the 14 isolates belonged to the genus Chryseobacterium.

Four isolates belonged to the genus Flavobacterium (GenBank accession nos. KC330341, KC330345, KC330349 and KC330350). In the 2011 field trial, two isolates were cultured from ARS-Fp-C and ARS-Fp-R line fish that belonged to the genus Flavobacterium (GenBank nos. JX827613 and JX827614, respectively). These results demonstrate the presence of yellow pigmented bacteria species associated with fish following hatching and during field trial evaluation.

During the 2010 field trial, a minor elevated mortality event started on farm trial day 35 and moribund fish were examined (6 ARS-Fp-R, 6 ARS-Fp-S) and cultured on TYES and TSA. While no yellow pigmented bacteria were isolated from TYES plates, microbial cultures grew on

![Table 1](image)

Comparison of BCWD post-challenge survival of 2010 farm- and laboratory-reared ARS-Fp-R and ARS-Fp-S line fish.

<table>
<thead>
<tr>
<th>Challenge #, location</th>
<th>TDD° (Date)</th>
<th>Genetic line</th>
<th>Fish n</th>
<th>Tanks n</th>
<th>BW, (± s.d.), g</th>
<th>Survival (± s.d.), %</th>
<th>Delta b</th>
<th>MDD c d</th>
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<tr>
<td>#1 CSF Res.</td>
<td>841 (16-Aprile-10)</td>
<td>ARS-Fp-R</td>
<td>103</td>
<td>2</td>
<td>1.01</td>
<td>61.2</td>
<td>11.2</td>
<td>12.5</td>
</tr>
<tr>
<td>#2 CSF Res.</td>
<td>1131 (6-May-10)</td>
<td>ARS-Fp-R</td>
<td>98</td>
<td>2</td>
<td>1.69</td>
<td>74.5</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>#1 NCCCWA</td>
<td>845 (6-May-10)</td>
<td>ARS-Fp-R</td>
<td>728</td>
<td>66</td>
<td>1.3 ± 0.2</td>
<td>89.1 ± 8.6</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>#2 NCCCWA</td>
<td>1270 (10-June-10)</td>
<td>ARS-Fp-R</td>
<td>74</td>
<td>3</td>
<td>4.06</td>
<td>87.9 ± 6.8</td>
<td>6.8</td>
<td></td>
</tr>
</tbody>
</table>

a. Temperature degree days at challenge.
b. Percentage point survival difference between ARS-Fp-R and ARS-Fp-S lines.
c. MDD = mean days to death.

d. Table 2. At approximately similar developmental stages, fish from the farm site and cohorts maintained at the NCCCWA were challenged with F. psychrophilum (Fig. 4). In each of the three challenge experiments carried out at the NCCCWA, the average survival of the ARS-Fp-R line was significantly greater than the ARS-Fp-S line (P < 0.0001) with survival differences between the lines for the three respective experiments of 33.9, 38.1 and 39.1 percentage points (Table 2). In all three experiments, the survival of the ARS-Fp-C line was intermediate between the ARS-Fp-R and ARS-Fp-S lines; although not statistically significant from the ARS-Fp-R line in the first and second challenges (Fig. 4B and D). In the first evaluation of the farm trial fish, the relative difference between lines was equivalent to that observed between lines at the NCCCWA (Fig. 4A compared to B, Table 2). Also, it should be noted that the fish used in this challenge study were removed from the raceway 18 days before the diagnosis of BCWD (Fig. 3). At the second evaluation time point, which was 19 days after the diagnosis of BCWD, the ARS-Fp-C line did not significantly differ in survival from the ARS-Fp-S line (Fig. 4C). Most striking was the phenotype difference that was measured in fish removed after the trial ended on day 81. Within this challenge, the ARS-Fp-R and ARS-Fp-S lines did not significantly differ from one another but both were greater than the ARS-Fp-C line (Fig. 4E). In summary, these results suggest that farm raceway environment can alter BCWD resistance phenotype during early rearing;
TSA plates from spleen and kidney tissue of five fish (2 ARS-Fp-R, 3 ARS-Fp-S) and a total of 24 colonies were subcultured and whole-cell protein profiles compared using SDS-PAGE. For isolates with different protein patterns, the 16S rRNA gene was amplified and directly sequenced. Within ARS-Fp-R fish, 4 of 7 isolates belonged to the genus *Hafnia* (GenBank nos. KC330356 to KC330360), while the remaining isolates belonged to the genus *Aeromonas* (GenBank no. KC330368), *Citrobacter* (GenBank no. KC330369), and *Kluyvera* (GenBank no. KC330363). From the ARS-Fp-S fish, 5 of 8 isolates belonged to the genus *Hafnia* (GenBank nos. KC330356 to KC330360), 2 belonged to *Citrobacter* (GenBank nos. KC330355, KC330361), and one could only be identified to the family level Enterobacteriaceae (GenBank no. KC330362). Thus, mixed populations of bacterial species were identified and associated with the mortalities examined during the farm trial. Whether the isolated bacteria are pathogenic is unknown, but most isolates were resistant to serum killing suggesting they may be adapted to survive within fish (data not shown).

4. Discussion

Herein, we have selected for and evaluated the survival of three genetic lines of rainbow trout grown under farm production conditions and assessed the stability of the BCWD resistance phenotype as measured by standardized laboratory challenge. Inclusion of the control and susceptible lines, having similar founder genetic background to each other and to the resistant line, allows the separation of genetic effects due to selective breeding from environmental effects. Resistance phenotyping, carried out both off-site and at the NCCCWA, and maintained after the 80-day evaluation period, the ARS-Fp-R line displayed significantly greater risk-adjusted survival than either the ARS-Fp-S line or the ARS-Fp-C line. However, laboratory challenge results carried out on farm-exposed fish demonstrate that unknown environmental factors can compromise the stability of the resistance phenotype. To our knowledge, this is the first report of improved farm survival of rainbow trout selectively bred for BCWD resistance. In addition, this is the first demonstration that rearing environment can have an important influence on the expression of genetic resistance.

4.1. The BCWD resistance phenotype is stable in fish grown under laboratory conditions

Previously, we have observed, using a limited number of families (n = 6), that the relative BCWD resistance/susceptibility phenotypes, measured using either survival or bacterial clearance as endpoints, were correlated as fish grew from 2.4 g to over 800 g in size suggesting that the relative resistance mechanisms are not expressed in a transitory manner during development (Hadidi et al., 2008). This study has confirmed and extended these findings using both a larger number of individual families (n = 21) and by evaluating pooled families tested as three separate genetic lines in three year classes of fish. Interestingly, the relative phenotypes were apparent as early as 30 days post-hatch (mean body weight = 0.2 g). This body weight is approximately 5-fold smaller than the reported minimum size at which protective immunity can be first induced by immersion vaccination with killed or *Vibrio anguillarum*, 1.0 to 2.5 g (Johnson et al., 1982), or by immersion with attenuated *Flavobacterium psychrophilum* (Lorenzen et al., 2010), and is considerably smaller than when salmonid fish grown under commercial settings are vaccinated by immersion (2–5 g) or by injection vaccination (>20 g) (Bravo and Midtlyng, 2007). Importantly, the relative resistance was independently validated and maintained after fish transport. These findings are consistent with other studies that have examined resistance of rainbow trout to IHNV challenge (Pearce et al., 2010; Quillet et al., 2007) and have found that genetic resistance is a stable trait during growth, at least for fish reared and tested under laboratory conditions.

4.2. Increased survival of the ARS-Fp-R line during early life-stage farm rearing

Two years of farm trials were carried out at the same Idaho farm site using different trial designs. The farm site was chosen due to...


recurrent BCWD loss during early life stage rearing although the occurrence and severity of outbreaks are generally unpredictable across raceways and years. In the first trial (2010), there was no BCWD outbreak in either the ARS-Fp-R and ARS-Fp-S line during the 80-day trial evaluation period. However, it seems likely that fish may have been exposed to the pathogen, as there was an outbreak in an adjacent raceway that was not part of this trial and these raceways receive water from a common source. In the 2011 farm trial, in which the three genetic lines were situated serially within a single raceway, there was a diagnosed outbreak of BCWD within the ARS-Fp-S line resulting in elevated mortality that resolved approximately three weeks after the first diagnosis. Although this outbreak was considered limited in duration, there was a 4.9% greater survival of the ARS-Fp-R line above the ARS-Fp-S line, and 3.6% greater survival compared to the ARS-Fp-C line. The magnitude of the survival difference approached the a priori 5% survival difference criteria for economic relevance of line improvement, and it is possible that the differences between lines would have increased given a more severe outbreak. Extrapolation from this trial to future performance should be made with caution as there were low absolute numbers of fish within the raceway compared to a typical production lot at this location (~220,000 fish). Furthermore, there were unequal numbers of starting fish per line and an unexplained poor hatching rate that was observed in this entire year class of fish. The impact of placing the ARS-Fp-S upstream of the ARS-Fp-C and ARS-Fp-R lines is unknown. It is assumed that the ARS-Fp-C and ARS-Fp-R line fish were in a disadvantaged location and exposed to greater numbers of \( F. \) psychrophilum as infected rainbow trout are reported to shed \( 10^4 \) to \( 10^8 \) bacterial cells fish \(^{-1} \text{ h}^{-1} \) into surrounding water (Madetoja et al., 2003), although we did not measure \( F. \) psychrophilum levels in the water during the outbreak. At present, the correlation between bacterial numbers in water and infection rate is uncertain, as we and other scientists have found that laboratory waterborne challenge elicits no or low mortality suggesting other cofactors or events may contribute to transmission (Decostere et al., 2000; Lorenzen et al., 2010; Madsen and Dalsgaard, 1999). Precise quantification of bacterial loads in the water and on raceway surfaces combined with a better understanding of transmission dynamics within and between the genetic lines warrants further study. A total of 53 \( F. \) psychrophilum isolates were obtained from the 2011 farm trial establishing that there was an active infection within all three groups. At present it is unknown if these isolates are clonal in origin or represent different strain variants. Frequently, multiple strains are isolated from BCWD outbreaks in rainbow trout, salmon and ayu (Arai et al., 2007; Avendaño-Herrera et al., 2009; Chen et al., 2008; Dalsgaard and Madsen, 2000; Del Cerro et al., 2010; Hesami et al., 2008; Lorenzen et al., 1997; Madetoja et al., 2001, 2002). Studies are underway to genotype the isolates from this farm trial, determine virulence attributes, and examine whether there is broad-based resistance within the ARS-Fp-R line against divergent \( F. \) psychrophilum strains or other gram negative bacterial pathogens.

**4.3. Why is there a difference between laboratory phenotype and farm phenotype?**

A surprising result from both field trials was the lack of difference in the post-challenge survival phenotype between the ARS-Fp-R and ARS-Fp-S line fish after rearing in production raceways. In the 2010 trial this was observed when fish were removed from the farm site on either days 32 and 55, transported to the CSF Research Facility, and subjected to standardized laboratory BCWD challenge (Table 1). This experiment compared 100 fish per line to allow sufficient power (0.99) to detect the 42% survival difference we detected between these two lines at the NCCCWA when cohorts of these same fish were challenged with the same strain, same route, at similar age and with a similar challenge dose. In the second trial, the lack of difference between the ARS-Fp-R and ARS-Fp-S was observed at a later point, day 81, in the trial (Fig. 4E). There are likely multiple environmental variables between the laboratory and farm site that include water quality and flow parameters, differences in tank composition and feed rates, exposure to different environmental microbes, and potential differences in generation of adaptive immunity due to on-farm exposure. Microbiological plate culture of fish-associated microbes, either prior to the first feeding or during elevated mortality events, identified a number of different microbial genera. Of note, we isolated yellow-pigmented bacteria belonging to the genera *Chryseobacterium* and *Flavobacterium* (non-*F. psychrophilum*) that are becoming increasingly recognized either externally or internally within rainbow trout and whose presence has been linked to elevated mortality events (Bernardet et al., 2005; Zamora et al., 2011). Future research to determine the virulence of these isolates and whether waterborne exposure to these isolates differentially alters resistance of the three genetic lines to BCWD is of interest.

**4.4. Does raceway exposure differentially affect the susceptible line?**

In the second farm trial, the ARS-Fp-C line fish were included in the study as these fish were intermediate in phenotype between ARS-Fp-R and ARS-Fp-S line fish, and we hypothesized that they would aid resolution of whether the ARS-Fp-R or ARS-Fp-S lines were uniquely affected by farm exposure. Following the BCWD outbreak, there was a gradual increase in the percent survival of the ARS-Fp-S line in the second experimental challenge (Fig. 4A vs. E), and as discussed above, in the third CSF challenge there was only a 1.5% difference in survival from the ARS-Fp-R line (Table 2). These across-challenge comparisons suggest that the ARS-Fp-S line was most affected by the environmental exposure while the ARS-Fp-R and ARS-Fp-C lines remained comparatively stable. The reason for this shift is unknown and may be due to factors including: 1) greater BCWD-related mortality in the ARS-Fp-S line causing biased post-outbreak samples (i.e. only the most resistant ARS-Fp-S fish were alive at the time of sampling), or 2) induction of a greater adaptive immune response due to higher infection or a greater capacity to mount a protective antibody response. The data clearly do not indicate an increase in susceptibility for any of the three lines after farm exposure. Humoral immunity elicited by exposure to a \( F. \) psychrophilum high molecular weight fraction or live attenuated cells provides partial protection against experimental challenge (LaFrentz et al., 2002, 2003, 2004). Further investigation of the genetic basis of resistance is underway and may clarify whether environmental conditions are modulating the innate vs. the adaptive arms of the immune response.

**5. Conclusions**

In summary, family-based selective breeding is an effective means of improving BCWD resistance. Our resistance phenotyping and subsequent selection efforts have solely focused on increasing family survival after standardized laboratory challenges with strain CSF259-93. The overall greater survival of the ARS-Fp-R line compared to the survival of the ARS-Fp-S and ARS-Fp-C lines in the farm trials indicates that laboratory-based selection using standardized challenges can be an effective means of reducing fish mortality in a production environment. The farm trials also illustrate the importance of the environment on the expression of the BCWD resistance phenotype. In these field trials, observations of feeding behavior and growth rate have been favorable for the ARS-Fp-R line, but long-term growth trials (i.e., to a standard market size) have yet to be conducted under production conditions. These initial farm-trial results support the release of germplasm from the ARS-Fp-R line and its continued evaluation in large-scale production trials.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2013.01.018.
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