Sport Fish Research Studies

Federal Aid Project F-394-R13

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Project Title: Sport Fish Research Studies

Period Covered: July 1, 2013 – June 30, 2014

Project Objective: Investigate methods to improve spawning, rearing, and survival of sport fish species in hatcheries and in the wild.

Job No. 1 Breeding and Maintenance of Whirling Disease Resistant Rainbow Trout Stocks

Job Objective: Rear and maintain stocks of whirling disease resistant rainbow trout.

Hatchery Production

The whirling disease resistant rainbow trout brood stocks reared at the Bellvue Fish Research Hatchery (BFRH; Bellvue, Colorado) are unique, and each requires physical isolation to avoid unintentional mixing of stocks. Extreme caution is used throughout the rearing process and during on-site spawning operations to ensure complete separation of these different brood stocks. All lots of fish are uniquely fin-clipped and most unique stocks are individually marked with Passive Integrated Transponder (PIT) and/or Visible Implant Elastomer (VIE) tags before leaving the main hatchery. This allows for definitive identification before the fish are subsequently used for spawning.

Starting in the middle of October 2013, BFRH personnel checked all of the Hofer[1] (GR), Harrison Lake (HL), Hofer × Harrison Lake (GR×HL) brood fish (2, 3, and 4 year-olds) weekly for ripeness. Maturation is indicated by eggs or milt flowing freely when slight pressure is applied to the abdomen of the fish. The first females usually maturate two to four weeks after the first group of males. As males are identified, they are moved into a separate section of the raceway to reduce handling and fighting injuries. On November 27, 2013, the first group of GR females were ripe and ready to spawn.

Before each fish was spawned, it was examined for the proper identification (fin-clip, PIT, or VIE tag), a procedure that was repeated for each fish throughout the winter. Fish were spawned using the wet spawning method, where eggs from the female were stripped into a bowl along with the ovarian fluid. After collecting the eggs, milt from several males was added to the bowl. Water was poured into the bowl to activate the milt, and the bowl of eggs and milt was covered and left undisturbed for several minutes while the fertilization process took place. Next, the eggs were rinsed with fresh water to expel old sperm, feces, egg shells, and dead eggs. Eggs were poured into an insulated cooler to water-harden for approximately one hour.

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[1] Hofer (H) is used interchangeably with GR throughout this document to describe the resistant strain of rainbow trout obtained in 2003 from facilities in Germany.

Water-hardened fertilized (green) eggs from different crosses of the GR, HL, and GR×HL were moved to the BFRH main hatchery building. Extreme caution was used to keep each individual cross separate from all others. Upon reaching the hatchery, green eggs were tempered and disinfected (PVP Iodine, Western Chemical Inc., Ferndale, Washington; 100 ppm for 10 min at a pH of 7). Eggs were then put into vertical incubators (Heath Tray, Mari Source, Tacoma, Washington) with 5 gallons per minute (gpm) of 11.1ºC (52ºF) of flow-through well water. The total number of eggs was calculated using number of eggs per ounce (Von Bayer trough count minus 10%) multiplied by the total ounces of eggs. Subsequent daily egg-takes and specific individual crosses were put into separate trays and recorded. To control fungus, eggs received a prophylactic flow-through treatment of formalin (1,667 ppm for 15 min) every other day until eye-up.

Eggs reached the eyed stage of development after 14 days in the incubator. The eyed eggs were removed from the trays and physically shocked to detect dead eggs, which turn white when disturbed. Dead eggs were removed (both by hand and with a Van Galen fish egg sorter, VMG Industries, Grand Junction, Colorado) for two days following physical shock. The total number of good eyed eggs was calculated using the number of eggs per ounce multiplied by total ounces. Eyed eggs were shipped via insulated coolers to other state and federal hatcheries three days following physical shock. Select groups of eggs were kept for brood stock purposes at the BFRH.

Table 1.1. Bellvue Fish Research Hatchery on-site spawning information for the Hofer (GR), Harrison Lake (HL), and Hofer × Harrison Lake (GR×HL) rainbow trout strains during the winter 2012-2013 spawning season**.**

Strain	Date Spawned	No. Spawned Females	No. Green Eggs	No. Eyed Eggs	Shipped To
100% HL	12/23/13-1/20/14	204	6,365	5,728	Fish Research Hatchery
100% GR	11/19/13-12/10/14	185	196,126	176,513	Fish Research Hatchery/CPW Hatcheries
GR×HL	12/10/13	173	294,733	123,007	Fish Research Hatchery/Utah Hatchery Triploids
Total	11/19/13-1/20/14	562	497,224	305,248	

The FRH 2013/2014 on-site rainbow trout production spawn started on November 19, 2013, with the last groups of HL females spawned on January 20, 2014. The initial goal was to produce 253,000 eyed eggs; egg take exceeded the production needs with 305,248 eyed eggs produced (Table 1.1). With the availability of both ripe males and females from several year classes and combinations of previous years crosses of GR, HL, and GR×HL, BFRH personnel produced seven different lots during the spawn. BFRH personnel were able to fill all GR, HL, and

GR×HL production and research directed project egg requests for Colorado in 2013/2014. The GR×CRR brood stock are not mentioned in this report because they have been fully transitioned into production at the CPW Glenwood Springs Hatchery and Poudre Rearing Unit.

Research Projects

Eggs produced specifically for research projects and brood stock management comprises a large proportion of the total production from the BFRH. Specific details of those individual crosses and families created for laboratory and field experiments are described in their respective sections of this report. The bulk of these family group descriptions appear in Job No. 2: Improved Methods for Hatchery and Wild Spawning and Rearing of Sport Fish Species.

Job No. 2 Improved Methods for Hatchery and Wild Spawning and Rearing of Sport Fish Species

Job Objective: Provide experimental support for both hatchery and wild spawning and rearing of sport fish species as they arise.

Formalin Sensitivity in Rainbow Trout - Overview

Formalin is one of the most effective and widely used compounds in fish culture for therapeutic and prophylactic treatment of fungal infections and external parasites of fish and fish eggs (Bills et al. 1977). Formalin has been shown to effectively prevent fungal infections on rainbow trout eggs at concentrations as low as 250 ppm; however, at 1,000 ppm, formalin not only prevented infection, but also decreased existing infection and increased hatching rates at exposure times ranging from 15 to 60 minutes (Marking et al. 1994). In addition to being a fungicide, formalin has been shown to be an egg disinfectant, reducing bacteria abundance on the surface of the egg at concentrations of up to 2,000 ppm (Wagner et al. 2008).

Formalin is effective against most ectoparasites, including *Trichodina*, *Costia*, *Ichthyophthirius*, and monogenetic trematodes (Piper et al. 1982). Typical formalin exposure concentrations range from 125 – 250 ppm for up to one hour (Piper et al. 1982), however, concentrations of up to 400 ppm have been used experimentally in toxicity tests (Wedemeyer 1971; Howe et al. 1995). A poll of Colorado Parks and Wildlife hatchery managers found that a range of concentrations from 130 – 250 ppm were used, with the most common treatment being 167 ppm for 30 minutes.

Differential formalin sensitivity has been demonstrated for various strains of rainbow trout when exposed post-hatch (Piper and Smith 1973); however, there has been little to no research on differential strain sensitivity to formalin exposure during egg incubation. In addition, the formalin sensitivity of fingerling rainbow trout exposed to varying levels of formalin during egg incubation is unknown. Therefore, whirling disease resistant strains of rainbow trout were exposed to various formalin concentrations at multiple life stages and under various hatchery conditions to examine if and under what conditions sensitivity (measured by mortality after exposure) to formalin occurs.

Four whirling disease resistant rainbow trout strains and crosses were used to determine formalin sensitivity: 1) Hofer (GR), 2) Harrison Lake (HL), 3) Hofer × Harrison Lake 50:50 (GR×HL 50:50), and 4) Hofer × Harrison Lake (GR×HL 75:25). All four of these strains and crosses are maintained as brood stock at the BFRH. Three experiments were designed to examine the sensitivity of these four strains to formalin. The first experiment, conducted in 2012, was designed to examine formalin sensitivity when eggs were exposed to three different concentrations (1,667, 2,000, and 5,000 ppm) of formalin for fungal control. The results of this experiment were presented in previous reporting cycles. The second experiment, described below and conducted in 2013, was designed to determine if there is differential sensitivity to varying formalin concentrations used to control external parasite infections as fingerlings following exposure to varying levels of formalin used to treat fungal infections during egg incubation. The third experiment, being conducted in 2014 with the methods described below, is designed to determine if certain hatchery conditions, such as size-at-exposure, crowding, reduced flow, and day-of-feeding, can affect sensitivity to formalin in rainbow trout fingerlings. The results of the third experiment will be presented in the next reporting cycle.

Experiment 2: Rainbow Trout Egg and Fingerling Formalin Sensitivity

METHODS

Spawning

Spawning occurred in December 2012. GR egg groups were created by pooling the eggs from 18 pairs of two-year-old GR females spawned with three-year-old GR males. The eggs from three pairs of two-year-old HL males spawned with three-year-old HL females, and 18 pairs of three-year-old HL males spawned with two-year-old HL females, were pooled together to create the HL strain egg groups for the experiment. The GR×HL 50:50 cross egg groups were created by pooling the eggs from 20 pairs of two-year-old GR males spawned with two-year-old HL females. The eggs from 37 pairs of two-year-old GR×HL 50:50 females spawned with two-year-old GR males were pooled together to create the GR×HL 75:25 egg groups for the experiment. Following spawning, eggs were disinfected with iodine and water hardened for one hour before being distributed in the egg tray towers for incubation and formalin exposure.

Egg Formalin Sensitivity

Two, five gpm flow-through egg tray towers were utilized for the egg formalin exposure experiment, with one formalin treatment per tower. Six egg trays within the seven tray towers were used for the experiment. Two, three inch diameter, screen-bottomed PVC inserts were placed in each of the six trays, a total of 12 PVC inserts per treatment (Figure 2.1). Each PVC insert contained 500 eggs from a given strain or cross, providing three 500 egg replicates per strain or cross, per treatment. Strains and crosses were assigned to PVC inserts within a treatment using a random number generator (Table 2.1). Eggs from each strain or cross were initially counted out by hand to determine the number of ounces containing 500 eggs. This measurement was then used to distribute approximately 500 eggs to each of the PVC inserts.

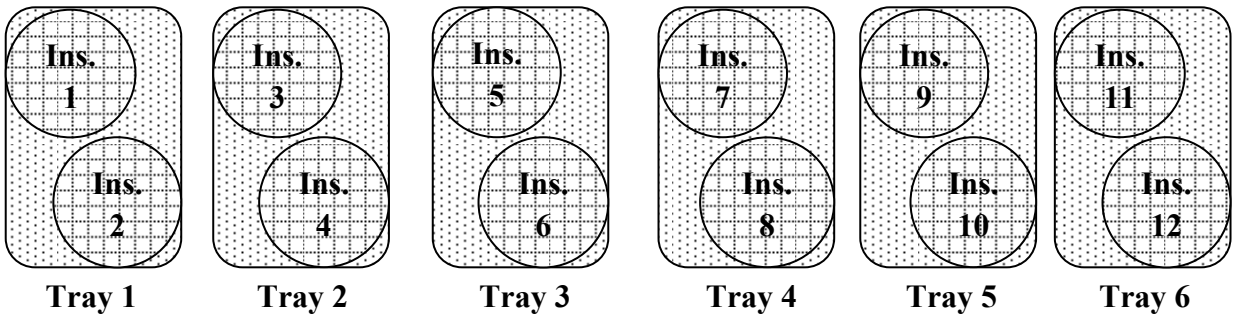

Figure 2.1. Arrangement of 12 screen-bottomed PVC inserts in the six trays (1-6, from top of tower down) used in each formalin treatment group. Strains and crosses were randomly assigned to an insert, within a treatment, using a random number generator (see Table 2.1).

Two formalin treatment levels were used to determine rainbow trout egg formalin sensitivity. The control formalin concentration was the same as that traditionally used to treat eggs at the BFRH. Eggs in the control treatment were exposed to 1,667 parts per million (ppm) of formalin, equating to 16 oz of formalin in a one gallon chicken feeder for an exposure period of 15 minutes with a flow of five gpm. A traditional control, consisting of no formalin treatment, was not included in this experiment because experience had shown that pre-hatch mortality would be high due to fungal infection if the eggs were not treated.

The second formalin treatment, the high formalin concentration, was five times the effective treatment level (1,000 ppm) for control of fungus (Marking et al. 1994). Eggs in the high formalin concentration treatment were exposed to 5,000 ppm of formalin, equating to 48 oz of formalin in a one gallon chicken feeder for an exposure period of 15 minutes with a flow of five gpm. This concentration was thought to be a toxic concentration of formalin to rainbow trout eggs (Marking et al. 1994); however, in a similar experiment, toxicity to eggs (defined as a 10% or more decline in hatching rate) was not apparent at a concentration of 5,000 ppm for exposures of 15 or 30 minutes (Marking et al. 1994). In a similar experiment conducted in 2012, the 5,000 ppm egg treatment was the only one of three treatments (1,667, 2,000 and 5,000 ppm) in which one strain, the GR×HL 50:50, showed increased mortality relative to the other two formalin concentrations.

Table 2.1. Assignment of strain to PVC insert within a given treatment via a random number generator. Each treatment contains two 500 egg replicates per strain or cross.

PVC Insert	Control	High Formalin
1	GR×HL 50:50	GR×HL 75:25
2	GR×HL 75:25	HL
3	GR×HL 50:50	GR×HL 50:50
4	GR	HL
5	GR	GR
6	HL	GR×HL 50:50
7	GR×HL 75:25	GR×HL 75:25
8	GR	GR×HL 50:50
9	HL	GR×HL 75:25
10	GR×HL 75:25	GR
11	HL	HL
12	GR×HL 50:50	GR

The experiment started with the distribution of eggs to the PVC inserts within each treatment. Formalin treatment began on the second day of the experiment, with treatment occurring every other day until the eggs were eyed. Once the eggs eyed, treatments ceased. Eyed eggs were physically shocked by pouring the eggs into a second tray where the dead and unfertilized eggs were identified, counted, and removed. Pre-hatch mortality was calculated using the equation $\%\, prehatch\ mortality = 100 \times \frac{mortality\ before\ hatch}{initial\ number\ of\ eggs}$ (Barnes et al. 2000). Mortality before hatch was calculated by summing the number of eggs that were picked-off (those eggs that turned white prior to eyeing), dead eggs that were removed following physical shock, and eggs that remained unhatched once hatching had occurred.

Upon hatching, each replicate was transferred to a labeled, two gallon tank and held until the fish swam up. Post-hatch mortality was calculated using the equation $\%\, posthatch\ mortality = 100 \times \frac{mortality\ after\ hatch}{initial\ number\ of\ eggs}$ (Barnes et al. 2000). Mortality after hatch was calculated by summing the number of crippled fish that did not survive to swim-up, and the number of deformed fish that were not counted as "healthy" upon completion of the experiment. These deformed fish were removed and counted as mortalities while a final count of swum-up fish was obtained. The initial number of eggs, used in both of the equations presented above, was back-calculated upon conclusion of the experiment by counting the number of fish that were remaining at the end of the experiment, and adding the number of pre- and post-hatch mortalities that occurred. Percent total mortality, including both pre-hatch and post-hatch mortality was calculated using the equation $\%\, total\ mortality = 100 \times \frac{prehatch+posthatch\ mortality}{initial\ number\ of\ eggs}$.

Statistical analyses were conducted using the GLM procedure in SAS (SAS Institute 2011). Differences in percent pre-hatch, post-hatch, and total mortality were analyzed using a two-factor analysis of variance (ANOVA), with strain/cross and treatment as the factors ($N = 24$). Percentages were arcsine-square root transformed prior to analysis. Values for all analyses were reported from the type III sum of squares. If significant effects were identified ($P < 0.05$), the

least-squares means method with a Bonferroni adjustment was used to determine which treatments caused significant differences in mortality within a strain or cross.

Fingerling Formalin Sensitivity

Figure 2.2. Visual implant elastomer (VIE) tags behind the eye of the (clockwise from the top) HL, GR×HL 50:50, GR, and GR×HL 75:25 fish, as seen fluorescing under a black light.

Upon conclusion of egg formalin sensitivity experiment, strain replicates within a formalin treatment were combined into a single rearing trough, for a total of eight troughs (1,667 GR, 1,667 HL, 1,667 GR×HL 50:50, 1,667 GR×HL 75:25, 5,000 GR, 5,000 HL, 5,000 GR×HL 50:50, and 5,000 GR×HL 75:25). All groups were fed a similar ration of 2.5% of their body weight day^{-1} in the interim between experiments, and were reared under similar environmental conditions (i.e., flows, temperatures, etc.), until they reached 3" in length (fingerlings).

Two weeks prior to initiation of the first fingerling formalin sensitivity experiment, fish were marked with a visual implant elastomer (VIE) tag in the adipose tissue behind both the left and right eyes, preventing misidentification if a tag was lost from one of the sides during experimentation. One VIE color was used for each of the four strains, regardless of egg treatment level (GR: red, HL: green, GR×HL 50:50: orange, GR×HL 75:25: pink; Figure 2.2).

Twelve tanks (74.8 L) were used in each formalin trial (Figure 2.3), providing three replicates of each of four treatment levels: 0 ppm, 167 ppm, 250 ppm, and 500 ppm. Treatment was randomly assigned to tank using a random number generator (Table 2.2). Five days prior to a trial, 20 fish of each strain were randomly distributed to each of the twelve tanks, resulting in a total of 80 fish per tank. The five day pre-experiment monitoring period was used to account for any mortality that occurred as a result of moving fish from inside the hatchery to FR1. Feeding of the fish in FR1 was ceased the day prior to conducting a formalin trial.

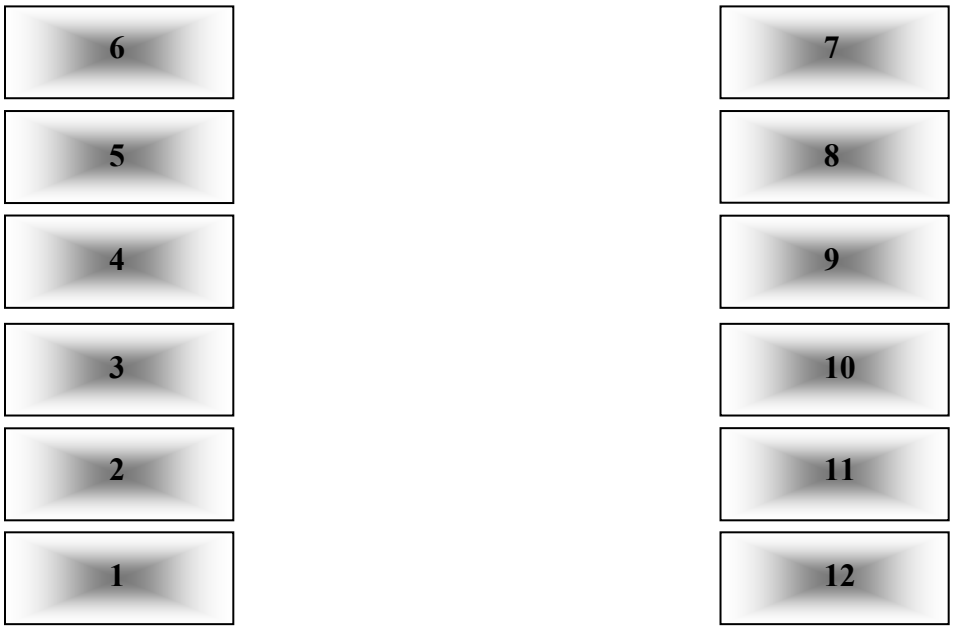

Figure 2.3. Arrangement and numbering of the twelve experimental tanks used in the fingerling formalin sensitivity experiments, housed in FR1 of the BFRH.

Peristaltic meter pumps were used to deliver the formalin at the correct rate to produce the desired formalin concentration in each tank. Formalin was delivered at a rate of 1.26 ml minute^{-1} for the 167 ppm treatment, 1.89 ml minute^{-1} for the 250 ppm treatment, and 3.78 ml minute^{-1} for the 500 ppm treatment. Because formalin is known to remove oxygen from the water (1 ppm oxygen removed for every 5 ppm formalin within 30-36 hours; Piper et al. 1982), oxygen levels were monitored during treatment. Treatments occurred for either 30 or 60 minutes, and treatment time was the same across all tanks within a trial. As a result, four trials were conducted: fish treated at 1,667 ppm as eggs treated for 30 minutes as fingerlings, fish treated at 1,667 ppm as eggs treated for 60 minutes as fingerlings, fish treated at 5,000 ppm as eggs treated for 30 minutes as fingerlings, and fish treated at 5,000 ppm as eggs treated for 60 minutes as fingerlings. Mortalities that occurred during and after a trial were identified using the VIE tags, and the length (mm), weight (g), and time and date found were recorder for each mortality.

It is known that fish treated with excessive concentrations of formalin may suffer delayed mortality, with the onset of death occurring within 1 to 24 hours of treatment, but potentially occurring up to 48 to 72 hours later depending on size and condition of fish, and water temperatures (Piper et al. 1982). Therefore, fish were retained within the experimental tanks for five days following formalin exposure so that residual mortality could be recorded. Fish were checked in the morning and afternoon during this post-exposure monitoring period. The time at which mortalities were found, as well as the strain, length, and weight of each fish, was recorded. Fish remaining at the conclusion of the post-exposure monitoring period were euthanized using an overdose of MS-222, counted, measured and weighed. Following removal of fish, tanks were cleaned and prepared for the next formalin trial.

Table 2.2. Assignment of treatment to tank and order in which the experimental treatments were applied in the four formalin trials (fish treated at 1,667 ppm as eggs treated for 30 minutes as fingerlings [1,667 for 30 Min], fish treated at 1,667 ppm as eggs treated for 60 minutes as fingerlings [1,667 for 60 Min], fish treated at 5,000 ppm as eggs treated for 30 minutes as fingerlings [5,000 for 30 Min], and fish treated at 5,000 ppm as eggs treated for 60 minutes as fingerlings [5,000 for 60 Min]).

Treatment	1,667 for 30 Min		1,667 for 60 Min		5,000 for 30 Min		5,000 for 60 Min	
(ppm)	Tank	Order	Tank	Order	Tank	Order	Tank	Order
0	9	10	5	10	2	3	8	4
0	10	2	1	8	4	7	10	1
0	5	8	12	5	6	10	5	7
167	7	5	8	12	9	4	2	11
167	12	11	6	9	7	2	7	5
167	2	1	11	6	3	9	9	10
250	1	12	10	4	11	5	1	9
250	3	4	4	1	1	6	3	3
250	6	7	7	2	5	11	12	8
500	4	9	9	3	8	1	4	6
500	11	6	3	7	10	8	11	2
500	8	3	2	11	12	12	6	12

To evaluate the effects of egg formalin concentration (EGG; 1,667 or 5,000 ppm), fingerling formalin concentration (FINGER; 0, 167, 250, or 500 ppm), exposure duration (DUR; 30 or 60 minutes), and strain or cross (STRAIN; GR, HL, GRxHL 50:50, GRxHL 75:25), a general linear model was implemented in SAS Proc GLM (SAS Institute 2011). An intercept-only model was included in the model set, as were all singular and additive combinations of the four treatment factors. In addition, models in which there was an interaction between egg formalin concentration and formalin concentration (testing if exposure to various formalin concentrations as eggs increased mortality with an increase in concentration as fingerlings) and models with interactions between egg formalin concentration, fingerling formalin concentration, and duration of exposure were also included in the model set. Model weights and delta Akaike Information Criterion corrected for small sample size (ΔAICc) rankings were used to determine support for each of the models included in the model set, and parameter estimates were reported from the candidate model with the lowest AICc value (Burnham and Anderson 2002).

RESULTS

Egg Formalin Sensitivity

As mentioned in the methods, 500 eggs from each strain or cross were counted by hand and measured to determine how many ounces of eggs constituted 500 eggs. After the initial count, eggs were measured out, not counted out, using this known measurement. Using this procedure to distribute the eggs resulted in an average (± SD) of 506 (± 29) eggs per PVC insert. Average number of eggs did not differ among strains/crosses or treatments ($F = 0.86$, $P = 0.560$).

Average pre-hatch mortality differed both between the treatments ($F = 9.37$, $P = 0.008$), and among the strains/crosses ($F = 29.54$, $P < 0.001$); the interaction was also significant ($F = 7.73$, $P = 0.002$). Eggs within the 5,000 ppm treatment experienced significantly higher average (± SD) percent pre-hatch mortality (32.4 ± 13.3%) than did the control treatment (26.8 ± 6.5%). The GR×HL 75:25 exhibited significantly higher percent pre-hatch mortality (43.0 ± 13.0%) than all of the other strains and crosses ($P < 0.001$). The HL strain exhibited significantly higher average percent pre-hatch mortality (30.0 ± 2.0%) than the GR strain ($P = 0.012$), but did not differ from the GR×HL 50:50 cross. The GR×HL 50:50 cross and the GR strain did not differ from each other in average percent pre-hatch mortality (GR×HL 50:50: 23.9 ± 2.7%, GR: 21.5 ± 3.0%; $P = 1.000$).

On average, the greatest mortality was observed in the form of eggs that turned white and were picked off prior to eyeing up (14.6 ± 7.8%), and eggs that did not survive to eye-up and were removed following bumping of the eyed eggs (11.4 ± 4.3%). On average, only 3.6% (± 1.6%) of the eggs not removed during the physical shock removal did not survive to hatching; these were removed following hatching of all of the eggs within a PVC insert.

In addition to exhibiting a higher average percent pre-hatch mortality than the other strains and crosses, the GR×HL 75:25 cross was the only strain or cross to exhibit sensitivity to formalin, pre-hatch (Figure 2.4). GR×HL 75:25 eggs in the high formalin treatment exhibited significantly higher mortality (53.4 ± 2.4%) than did those in the control treatment (32.6 ± 5.3%; $P = 0.001$). None of the other strains or crosses exhibited a significant increase in mortality with an increase in formalin treatment concentration, pre-hatch ($P = 1.000$; Figure 2.4).

Average post-hatch mortality differed only among the strains ($F = 4.18$, $P = 0.023$); post-hatch mortality did not differ among formalin treatments ($F = 0.69$, $P = 0.419$), and the interaction between treatment and strain was not significant ($F = 0.49$, $P = 0.695$). The GR×HL 50:50 cross exhibited significantly higher average percent post-hatch mortality (9.9 ± 2.6%) than the GR strain (4.6 ± 3.2%; $P = 0.038$), but did not differ significantly from the GR×HL 75:25 cross (6.3 ± 2.9%) or HL strain (4.7 ± 3.0%; $P > 0.053$). The GR, GR×HL 75:25, and HL did not differ from each other in average percent post-hatch mortality ($P = 1.000$; Figure 2.5).

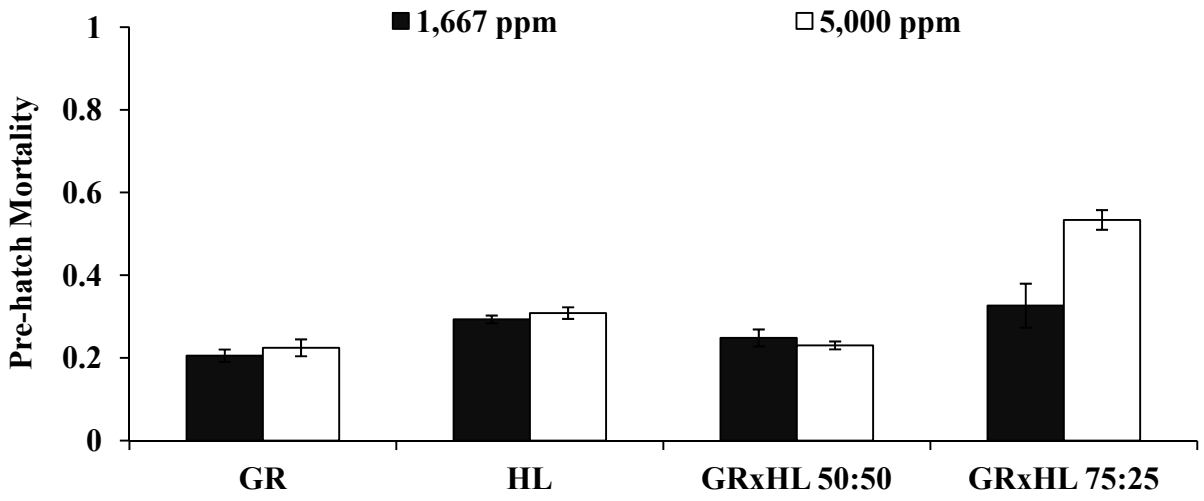

Figure 2.4. Average proportion pre-hatch mortality (SE bars) by strain and treatment.

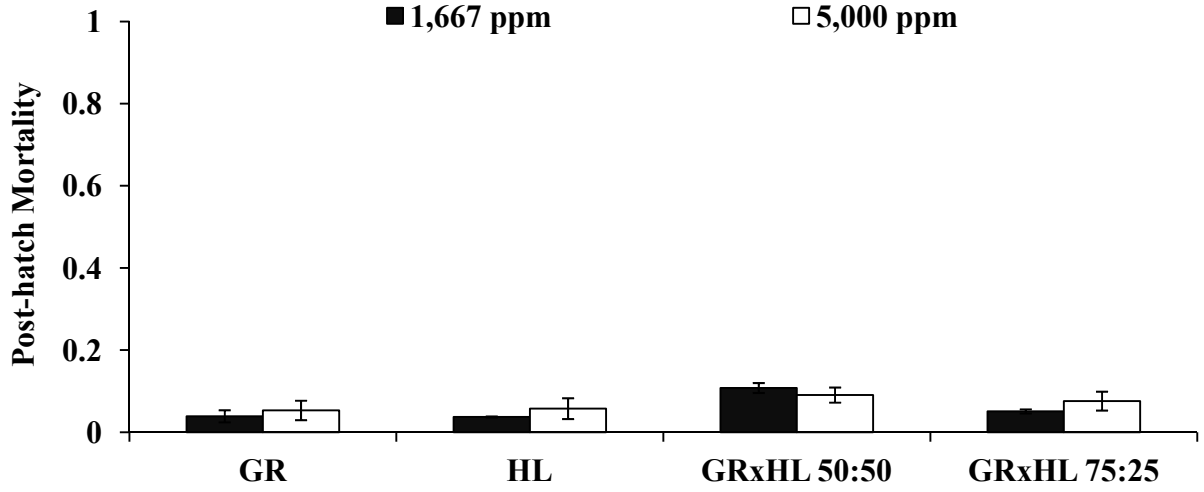

Figure 2.5. Average proportion post-hatch mortality (SE bars) by strain and treatment.

On average, the greatest post-hatch mortality (4.2 ± 2.9%) was observed in the form of crippled fish that were removed either post-mortem, or pre-mortem if it was obvious that the fish was unable to swim up due to deformities. Only a small percentage of post-hatch mortality (1.7 ± 1.1%) occurred in the form of deformed, unhealthy fish that were removed while counting fish at the end of the experiment.

Average percent total mortality differed both between the treatments ($F = 8.70$, $P = 0.009$), and among the strains/crosses ($F = 18.70$, $P < 0.001$); the interaction was also significant ($F = 6.33$, $P = 0.005$). Fish within the high formalin treatment exhibited significantly higher average percent total mortality (39.3 ± 13.9%) than the control treatment (32.7 ±7.5%; $P = 0.009$). The GR×HL 75:25 cross exhibited significantly higher average percent total mortality (49.3 ± 14.8%) than any of the other strains or crosses ($P < 0.002$). The other three strains did not differ

significantly from each other in average percent total mortality (GR: 26.1 ± 4.4%, GR×HL 50:50: 33.8 ± 4.5%, HL: 34.8 ± 2.7%; $P > 0.062$; Figure 2.6).

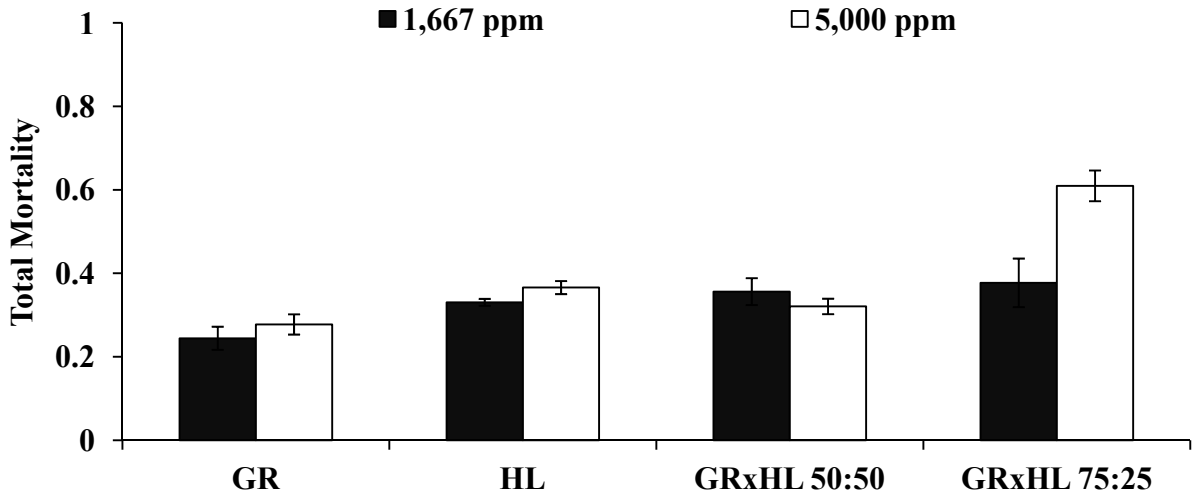

Figure 2.6. Average proportion total mortality (SE bars) by strain and treatment.

In addition to exhibiting the highest average percent mortality, the GR×HL 75:25 cross was the only strain or cross to exhibit sensitivity to formalin, as measured by percent total mortality differences among the treatments. GR×HL 75:25 fish in the high formalin treatment exhibited significantly higher mortality (61.0 ± 3.7%) than did those in the control treatment (37.7 ± 5.8%; $P = 0.003$). None of the other strains or crosses exhibited a significant increase in total mortality with an increase in formalin treatment concentration ($P = 1.000$; Figure 2.6).

Fingerling Formalin Sensitivity

Model selection results for the fingerling formalin sensitivity experiment indicated that the model that included interactions between egg fingerling concentration, fingerling formalin concentration, and fingerling exposure duration, and the additive component of strain was the top supported model of the set (AICc weight = 1.0; Table 2.3). Mortality increased as fingerling formalin concentration increased, and mortality within a concentration was higher with longer treatment durations (30 versus 60 minute; Figure 2.7). Overall, previous exposure to low or high formalin concentrations as eggs did not appear to have an effect on mortality within a treatment concentration and duration combination. For example, fish treated at 1,667 ppm formalin as eggs exhibited 0.27 (SE = 0.05) percent mortality when exposed to 500 ppm formalin for 60 minutes. Similarly, fish treated at 5,000 ppm formalin as eggs exhibited 0.25 (SE = 0.04) percent mortality when exposed to 500 ppm for 60 minutes.

Strains showed differential mortality following exposure to formalin as fingerlings, averaged across egg and fingerling formalin concentration, and duration of exposure (Figure 2.8). Mortality was higher in the pure GR versus the pure HL strains, and among the crosses, the GRxHL 50:50 exhibited higher mortality than the GRxHL 75:25. The GR and GRxHL 50:50 both exhibited similarly higher mortality rates than the HL or GRxHL 75:25, which also

exhibited similar mortality rates despite the fact that the GRxHL 75:25 more closely resembles the pure GR versus pure HL genetically.

Table 2.3. Model selection results for factors influencing formalin sensitivity in *Myxobolus cerebralis* resistant rainbow trout strains. Models are ranked by their AICc difference (Δ_i) relative to the best model in the set and Akaike weights (w_i) quantify the probability that a particular model is the best model in the set given the data and the model set.

Model	R^2	log(L)	K	AICc	Δ_i	w_i
EGG*FINGER*DUR+STRAIN	0.64	491.25	20	-937.58	0.00	1.00
FINGER+DUR+STRAIN	0.55	470.44	10	-919.67	17.91	0.00
EGG+FINGER+DUR+STRAIN	0.55	470.57	12	-915.40	22.18	0.00
EGG*FINGER+DUR+STRAIN	0.56	471.97	14	-913.57	24.01	0.00
EGG*FINGER*DUR	0.54	467.42	16	-899.73	37.85	0.00
FINGER+DUR	0.45	450.82	6	-889.18	48.40	0.00
EGG+FINGER+DUR	0.45	450.92	8	-885.06	52.52	0.00
EGG*FINGER+DUR	0.45	452.06	10	-882.91	54.67	0.00
FINGER+STRAIN	0.41	444.48	8	-872.16	65.41	0.00
EGG+FINGER+STRAIN	0.41	444.57	10	-867.93	69.65	0.00
EGG*FINGER+STRAIN	0.42	445.64	12	-865.54	72.04	0.00
FINGER	0.31	429.15	4	-850.09	87.49	0.00
EGG+FINGER	0.31	429.24	6	-846.02	91.56	0.00
EGG*FINGER	0.31	430.14	8	-843.50	94.07	0.00
DUR+STRAIN	0.24	420.50	6	-828.55	109.03	0.00
EGG+DUR+STRAIN	0.24	420.58	8	-824.37	113.21	0.00
DUR	0.14	408.36	2	-812.66	124.92	0.00
EGG+DUR	0.14	408.43	4	-808.64	128.93	0.00
STRAIN	0.10	404.24	4	-800.26	137.32	0.00
EGG+STRAIN	0.10	404.30	6	-796.15	141.43	0.00
Intercept-only	0.00	393.89	1	-785.76	151.82	0.00
EGG	0.00	393.95	2	-783.83	153.74	0.00

Supplemental Analyses

To supplement the analyses conducted above, the data for the egg and fingerling formalin experiment was given to a statistics consulting class at Colorado State University for analysis. Two different analyses were run, a logistic regression analysis, and a Cox proportional hazard model, both of which aid in additional interpretation of the data. The summary report produced by the statistics department at CSU can be found in Job No. 2 Appendix 1.

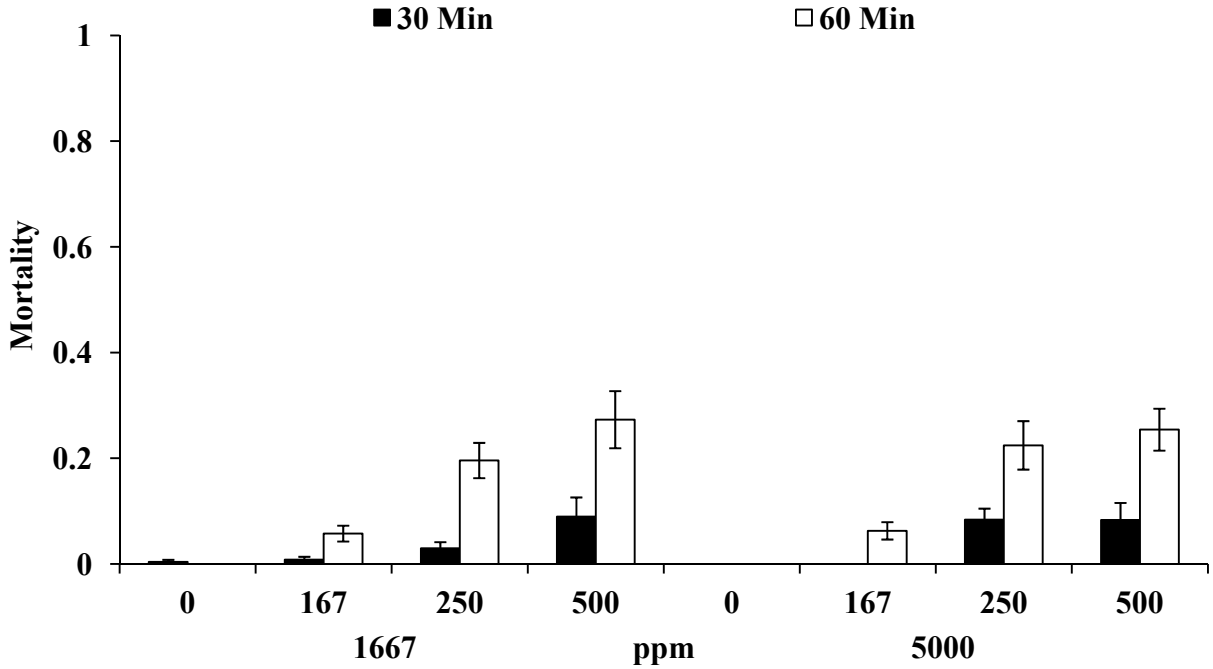

Figure 2.7. Average proportion mortality (SE bars) of fish exposed to either 1,667 or 5,000 ppm formalin as eggs, and re-exposed to formalin as fingerlings at concentrations of 0, 167, 250, or 500 ppm for 30 or 60 minutes.

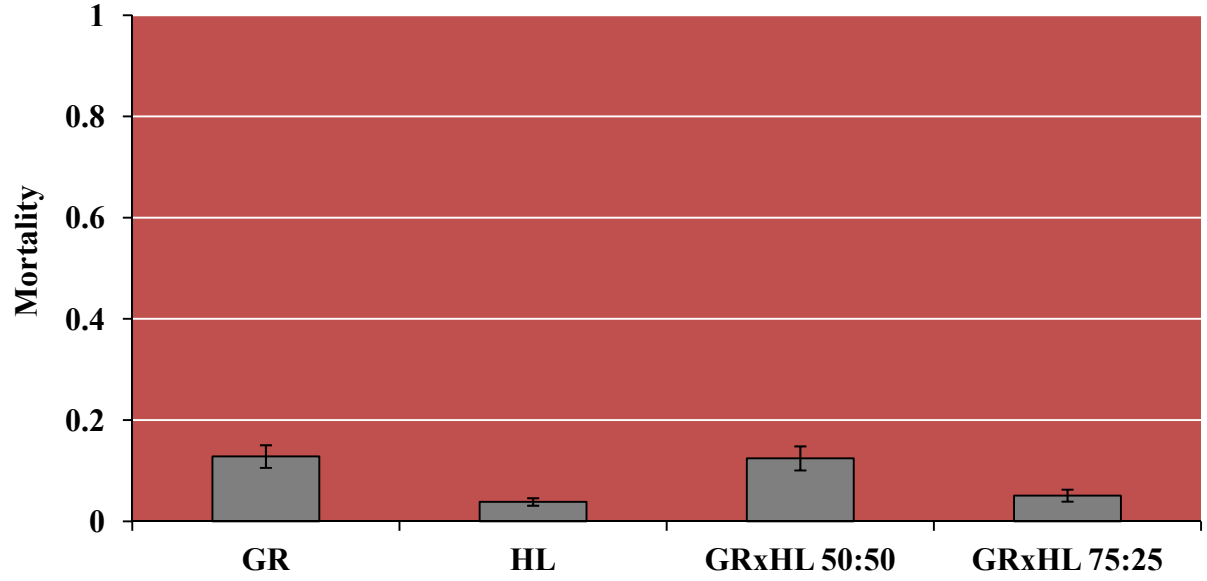

Figure 2.8. Average proportion mortality (SE bars) by strain, averaged across egg and fingerling formalin concentration and duration of exposure.

CONCLUSIONS

At the onset of this experiment, it was believed that the GR strain had a higher sensitivity to formalin treatment because large die-offs of GR strain fingerling fish had occurred in Colorado hatcheries following treatment of with formalin. However, it was unknown whether this sensitivity was exhibited in the egg stage of the life cycle as well. The results of the egg formalin sensitivity experiment suggest that neither the pure GR nor HL strains are sensitive to formalin treatment during the egg life stage, as no increase in total mortality was observed with an increase in formalin treatment concentration. The same was not true, however for the GR×HL 75:25 cross, which did show an increase in egg total mortality with an increase in formalin treatment concentration, and therefore, sensitivity to formalin at higher concentrations. The majority of the mortality experienced in this strain occurred pre- versus post-hatch. In a similar experiment conducted in 2011, the GR×HL 50:50 cross also showed an increase in mortality with an increase in formalin concentration. Taken together, these results suggest that crosses between the two pure strains are more likely to exhibit formalin sensitivity during egg treatments than either of the pure strains, and caution should be used when using higher concentrations of formalin to treat GR-cross eggs.

The results of the fingerling formalin exposure experiment suggest that the GR strain does exhibit sensitivity to formalin when treated as fingerlings. In addition, the GR×HL 50:50 strain also exhibited a sensitivity to formalin. Currently, it is difficult to determine what may have caused the sensitivity in the GR×HL 50:50 cross. If sensitivity were genetically determined, and the GR strain was more sensitive than the HL strain, we would have expected to see higher mortality in the GR×HL 75:25 as it contains a higher proportion of GR genes than the GR×HL 50:50 cross. Further investigation is needed. The results do suggest, however, that caution should be used when using increased formalin concentrations to treat heavy infestations by external parasites on GR and GR-cross fish. The results suggest that if formalin treatments are necessary, a concentration of 167 ppm for 30 minutes should result in little to no mortality; however, mortality may increase even at this concentration if environmental or health stressors are elevated at the time of treatment or if more than one treatment is needed.

Experiment 3: Hatchery Practice Effects on Formalin Sensitivity

METHODS

Egg Incubation and Formalin Exposure

The same four strains of whirling disease resistant rainbow trout fingerlings used in Experiments 1 and 2 were used to determine strain sensitivity at different ages, and under various flow, density, crowding, and feeding conditions. Seven trays within one tray tower were used for egg incubation, and each tray contained three screen-bottomed PVC inserts (with the exception of tray 7, which only had two inserts). Inserts within each of the treatments were numbered 1-20 (Figure 2.9), strains were randomly assigned to an insert using a random number generator (Table 2.4). Each insert contained 500 eggs, providing five, 500 egg replicates per strain.

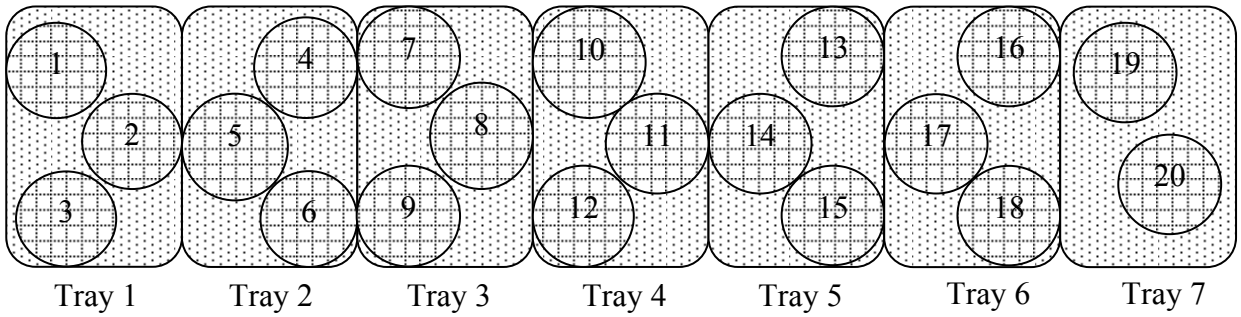

Figure 2.9. Arrangement of the 20 screen-bottomed PVC inserts in the seven trays (from top of tower down) used during egg incubation. Strains were randomly assigned to an insert using a random number generator (Table 2.4).

The formalin concentration to which the eggs were exposed during incubation is that which is traditionally used to treat eggs at the BFRH. All eggs were exposed to 1,667 parts per million (ppm) of formalin (16 oz of formalin in a one gallon chicken feeder) for an exposure period of fifteen minutes at a flow of five gpm. Eggs were treated every other day until eye-up. A traditional control, consisting of no formalin treatment, was not included in this experiment because experience has shown that pre-hatch mortality is high due to fungal infection if the eggs are not treated.

Table 2.4. Assignment of strain to PVC insert within the tray tower via a random number generator. The tray tower contained five, 500 egg replicates per strain.

PVC Insert	Strain
1	Hofer x Harrison 50:50
2	Hofer x Harrison 50:50
3	Hofer x Harrison 75:25
4	Hofer x Harrison 75:25
5	Hofer x Harrison 50:50
6	Harrison
7	Hofer x Harrison 50:50
8	Harrison
9	Hofer x Harrison 75:25
10	Hofer
11	Hofer
12	Hofer
13	Hofer
14	Hofer x Harrison 50:50
15	Harrison
16	Harrison
17	Harrison
18	Hofer
19	Hofer x Harrison 75:25
20	Hofer x Harrison 75:25

Similar to Experiments 1 and 2, two measures of mortality were calculated during egg incubation and swim-up, pre-hatch and post-hatch mortality (similar to Barnes et al. 2000). Pre-hatch mortality was determined by counting the number of dead eggs picked out of each of the inserts as they were removed. Upon hatching, sac fry were transferred to rearing tanks (separated by strain and replicate) to determine post-hatch mortality (to swim-up). Separation was maintained at all levels (strain and replicate) so that replication within a strain was maintained throughout the entirety of the experiment. Equations used to calculate pre-hatch, post-hatch, and total mortality (Barnes et al. 2000) were the same as those described above for Experiment 2.

Statistical analyses were conducted using the GLM procedure in SAS (SAS Institute 2011). Differences in percent pre-hatch, post-hatch, and total mortality were analyzed using a single-factor analysis of variance (ANOVA), with strain/cross as the factor ($N = 20$). Percentages were arcsine-square root transformed prior to analysis. Values for all analyses were reported from the type III sum of squares. If significant effects were identified ($P < 0.05$), the least-squares means method with a Bonferroni adjustment was used to determine which treatments caused significant differences in mortality within a strain or cross.

Rearing of Rainbow Trout Fingerlings

Upon completion of the egg incubation portion of the experiment, strain replicates were combined into four troughs, one per strain, and fish were reared to fingerling size for use in Experiment 3. All four strains were fed a similar ration of food (i.e., 2% body weight per day) in the interim between experiments, and were reared under similar environmental conditions (i.e., flows, temperatures, etc.), until they reached 3" in length. A subset of fish (180 per strain) was evaluated for formalin sensitivity at 1.5" in length. An additional subset of 180 fish will be retained through September to be evaluated at 5" in length (see below).

Two weeks prior to initiation of the first formalin sensitivity experiment, all fish were marked on both sides with a VIE tag in the adipose tissue behind the eye, preventing misidentification if a tag was lost from one side during experimentation. VIE tags were used for individual mortality identification as fish from each of the four strains were combined in each replicate of the experiment. One VIE color was used for each of the four strains (e.g., GR: red, HL: green, HxH 50:50: orange, HxH 75:25: purple; see figure 2.2 for example of identification using VIE tags).

Experimental Design

Twelve 20 gallon tanks were used in each trial (Figure 2.3), achieving three full turnovers during the 30 minute treatment, allowing us to produce the desired formalin concentration during the treatments. Treatments were randomly assigned to tanks using a random number generator. Five days prior to the experiment, 20 (normal density) or 40 (increased density) fish of each strain were randomly distributed to each of the experimental tanks. The five day pre-experiment monitoring period was used to account for any mortality that occurred as a result of moving fish from inside the hatchery to FR1. Mortalities, and their lengths and weights, were recorded daily in each tank, and were identified to strain using the VIE tags. The final pre-experiment feeding occurred the day prior to conducting an experiment, with the exception of the feeding trial (described below).

On the day of an experiment, peristaltic metering pumps were used to deliver the formalin at the correct rate to produce the required concentration of formalin in the tank (1.26 ml per minute for 167 ppm and 1.89 ml per minute for 250 ppm). Oxygen levels were monitored during treatment. Mortality occurring during formalin exposure was recorded on a per strain basis, as were the lengths and weights of each mortality. The time at which the mortality occurred in relation to the beginning of the exposure period was also noted. Fish were retained within the experimental tanks for five days following formalin exposure so that residual mortality could be recorded. Fish were checked in the morning and afternoon during this post-exposure monitoring period, and the time at which mortalities were found, and the strain, length, and weight were recorded. Fish remaining at the conclusion of the post-exposure monitoring period were euthanized using an overdose of MS-222, and fish were counted, measured and weighed. Following removal of fish, tanks were cleaned and prepared for the next round of exposures.

Overall, the experiment consists of seven separate trials. Trials 1 and 7 were designed to examine size susceptibility to formalin. One of these trials was complete at the time of reporting, with the other to be completed in September 2014. Trials 2-6 examine the effects of density, flow, crowding and day-of feeding on the sensitivity to formalin. The objective of the density and flow trials was to determine if density or flow conditions affect sensitivity to formalin in the four strains. In addition, a feeding trial was designed to determine if feeding the day of treatment increases formalin sensitivity, and the crowding trial was designed to determine if moving fish away from the inflow decreased sensitivity to formalin by defusing the formalin throughout the water column prior to exposure. The order in which these three types of trials were conducted was chosen using a random number generator: 1) density/flow, 2) feeding, and 3) crowding. Density/flow trials were conducted as a "group" to maintain proximity of replicates in time. Trials 2-6 began in June 2014, and will be completed in August 2014. The results of these trials will be available in the next reporting cycle.

Density/Flow Trials

Table 2.5. Assignment of treatment to tank and order in which the treatment was applied in the first density/flow trial (Trial 2).

Density	Flow	Treatment (ppm)	Tank	Order
Increased	Decreased	0	3	5
Increased	Decreased	167	5	11
Increased	Decreased	250	12	8
Increased	Normal	0	6	10
Increased	Normal	167	8	2
Increased	Normal	250	2	7
Normal	Decreased	0	7	6
Normal	Decreased	167	11	3
Normal	Decreased	250	10	4
Normal	Normal	0	9	1
Normal	Normal	167	1	9
Normal	Normal	250	4	12

Table 2.6. Assignment of treatment to tank and order in which the treatment was applied in the second density/flow trial (Trial 3).

Density	Flow	Treatment (ppm)	Tank	Order
Increased	Decreased	0	7	6
Increased	Decreased	167	3	1
Increased	Decreased	250	12	2
Increased	Normal	0	2	3
Increased	Normal	167	9	4
Increased	Normal	250	6	12
Normal	Decreased	0	10	7
Normal	Decreased	167	4	8
Normal	Decreased	250	8	9
Normal	Normal	0	1	10
Normal	Normal	167	11	11
Normal	Normal	250	5	5

Table 2.7. Assignment of treatment to tank and order in which the treatment was applied in the second density/flow trial (Trial 4).

Density	Flow	Treatment (ppm)	Tank	Order
Increased	Decreased	0	1	5
Increased	Decreased	167	10	4
Increased	Decreased	250	9	6
Increased	Normal	0	8	11
Increased	Normal	167	4	8
Increased	Normal	250	3	3
Normal	Decreased	0	7	7
Normal	Decreased	167	11	9
Normal	Decreased	250	2	1
Normal	Normal	0	12	2
Normal	Normal	167	5	12
Normal	Normal	250	6	10

Four combinations of density and flow were tested during the density/flow trials: 1) normal density (20 fish/strain) and normal flow (2 gpm), 2) normal density and decreased flow (1 gpm), 3) increased density (40 fish/strain) and normal flow, and 4) increased density and decreased flow. To maximize tank space and minimize the number of trials, one replicate of each combination of density and flow was tested at each of the three formalin concentrations (0, 167, and 250 ppm). Three trials were conducted in the same fashion, providing three replicates for every combination of density, flow, and formalin concentration. For each of the three trials, treatment (density, flow, and concentration) was randomly assigned to tank, as was the order in which the treatments are applied, using a random number generator (Tables 2.5, 2.6, and 2.7).

For the low flow trials, we were most interested in what effects low flow would have on sensitivity to formalin, not the residual effects of maintaining formalin treated fish under low

flow conditions. Essentially, these trials simulate a reduction in water flow prior to treatment (due to pipe clogs, or a mistake made by a hatchery technician), with the flow corrected shortly after treatment. As such, flows were reduced prior to exposing the fish to formalin, and increased one hour following the end of the 30 minute treatment.

Crowding Trial

Table 2.8. Assignment of treatment to tank and order in which the experimental treatment was applied in crowding trial (Trial 5).

Treatment (ppm)	Tank	Order
0	5	3
0	7	4
0	11	8
167	12	1
167	10	7
167	4	9
250	1	2
250	3	5
250	8	6
H_2O Control: 0 ppm	2	10
H_2O Control: 167 ppm	6	11
H_2O Control: 250 ppm	9	12

Hatchery managers have observed that the GR tend to congregate under the water inflow, even during formalin treatments. It is suspected that this crowding below the inflow may cause the GR to be exposed to formalin "hot spots" because they are being exposed prior to the formalin diffusing throughout the water column. The crowding trial was designed to determine if keeping fish away from the inflow can prevent mortality during formalin treatments. Fish were crowded down, using crowding screens, into the lower two-thirds of the tank where they remained throughout the treatment, allowing the formalin to diffuse throughout the water column before contacting the fish. The drawback to this approach is that it increases the density (amount of fish per available water) within the tanks. Therefore, density will be used as a covariate in the analysis. Due to tank and fish availability, the crowding experiment was only tested at normal densities and normal flows, though all three formalin concentrations were tested. Therefore, a total of nine of the twelve tanks were used during the crowding trial, with the other three serving as water controls to determine how much oxygen is removed from the water during a 30 minute treatment in the absence of fish. Assignment of formalin concentration to tank and the order in which treatments were applied was randomly assigned using a random number generator (Table 2.8).

Feeding Trial

The feeding trial was designed to determine if feeding the day of formalin treatment affects formalin sensitivity. The control for this experiment (i.e., the effect of final feeding the day before treatment) will come from the normal density, normal flow replicates in Trials 2, 3, and 4,

described above. Due to tank and fish availability, the feeding trial only tested normal densities and normal flows, though all three formalin concentrations were tested. Assignment of formalin concentration to tank and the order in which treatments were applied was randomly assigned using a random number generator (Table 2.9).

Table 2.9. Assignment of treatment to tank and order in which the treatment was applied in day-of-feeding trial (Trial 6).

Treatment (ppm)	Tank	Order
0	9	2
0	2	5
0	12	8
167	1	3
167	7	4
167	4	7
250	3	1
250	8	6
250	5	9
H_2O Control: 0 ppm	6	10
H_2O Control: 167 ppm	10	11
H_2O Control: 250 ppm	11	12

Size Trials

Table 2.10. Assignment of treatment to tank and order in which the treatment was applied using small fish (1.5-2"; Trial 1).

Treatment (ppm)	Tank	Order
0	6	6
0	8	4
0	11	5
167	5	7
167	10	8
167	12	3
250	1	9
250	2	2
250	9	1
H_2O Control: 0 ppm	3	10
H_2O Control: 167 ppm	4	11
H_2O Control: 250 ppm	7	12

Rainbow trout may exhibit size susceptibility to formalin due to the amount of gill surface area that is exposed to formalin during treatment. Size susceptibility to formalin was tested with all four strains (Trials 1 and 7). Controls (i.e., normal flow and normal density) for the fingerling size (3") came from the crowding and flow trials described above. The objective of the size trials was to determine if there was differential mortality at different life stages. Therefore, in

addition to the fingerlings, fish at 1.5" in length and fish at 5" in length were tested for their susceptibility to formalin. In both of the size-specific trials, nine of the twelve experimental tanks were used, three replicates each of fish exposed to formalin concentrations of 0, 167, and 250 ppm, with the other three tanks serving as water controls to determine how much oxygen is removed from the water during a 30 minute treatment in the absence of fish. All tanks maintained a base flow of two gallons per minute and contained twenty fish of each strain in each tank (i.e, normal flow and normal density). Treatment concentration and order of treatment was assigned to the tanks randomly using a random number generator (Tables 2.10 and 2.11).

Table 2.11. Assignment of treatment to tank and order in which the treatment was applied using large fish (5"; Trial 7).

Treatment (ppm)	Tank	Order
0	2	6
0	4	1
0	8	3
167	1	7
167	7	4
167	10	2
250	3	9
250	6	5
250	11	8
H_2O Control: 0 ppm	5	10
H_2O Control: 167 ppm	9	11
H_2O Control: 250 ppm	12	12

Statistical analyses were conducted for Trial 1 using the GLM procedure in SAS (SAS Institute 2011). Differences in mortality were analyzed using a two-factor ANOVA, with strain/cross and treatment as the factors ($N = 12$). Percentages were arcsine-square root transformed prior to analysis. Values for all analyses were reported from the type III sum of squares. If significant effects were identified ($P < 0.05$), the least-squares means method with a Bonferroni adjustment was used to determine which treatments caused significant differences in mortality within a strain or cross. Statistical results for Experiments 2-7 will be reported in the next reporting cycle.

RESULTS

Egg Formalin Sensitivity

As mentioned in the methods, 500 eggs from each strain or cross were counted by hand and measured to determine how many ounces of eggs constituted 500 eggs. After the initial count, eggs were measured out, not counted out, using this known measurement. Using this procedure to distribute the eggs resulted in an average (± SD) of 511 (± 23) eggs per PVC insert. Average number of eggs did not differ among strains/crosses ($F = 1.47$, $P = 0.262$).

Average pre-hatch mortality did not differ among the strains ($F = 0.97$, $P = 0.432$; Figure 2.10). The greatest pre-hatch mortality was observed in the form of eggs that turned white and were picked off prior to eyeing up (8.8 ± 3.7%), followed by eggs that did not survive to eye-up and were removed following bumping of the eyed eggs (6.7 ± 7.1%). Average post-hatch mortality differed among the strains ($F = 4.91$, $P = 0.013$). The GR×HL 50:50 cross exhibited significantly higher average percent post-hatch mortality (7.8 ± 1.4%) than the GR×HL 75:25 cross (1.8 ± 0.2%; $P = 0.014$), but did not differ significantly from the GR strain (6.2 ± 2.3%) or HL strain (3.5 ± 0.8%; $P > 0.185$). The GR, GR×HL 75:25, and HL did not differ from each other in average percent post-hatch mortality ($P > 0.133$; Figure 2.11). On average, the greatest post-hatch mortality (3.7 ± 3.2%) was observed in the form of crippled fish that were removed either post-mortem, or pre-mortem if it was obvious that the fish was unable to swim up due to deformities. Only a small percentage of post-hatch mortality (0.5 ± 0.4%) occurred in the form of deformed, unhealthy fish that were removed while counting fish at the end of the experiment. Average total mortality did not differ among the strains ($F = 1.58$, $P = 0.233$; Figure 2.12).

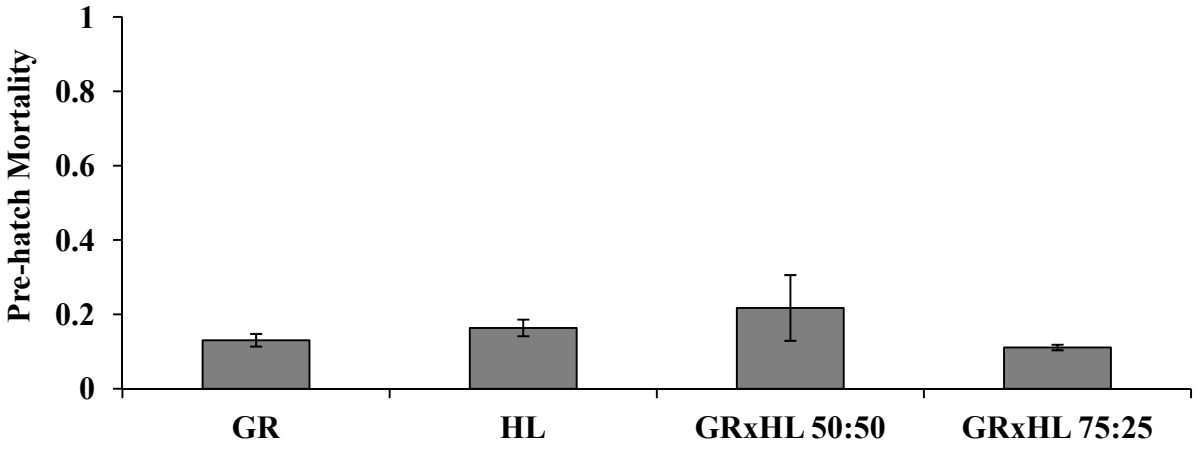

Figure 2.10. Average proportion pre-hatch mortality (SE bars) by strain.

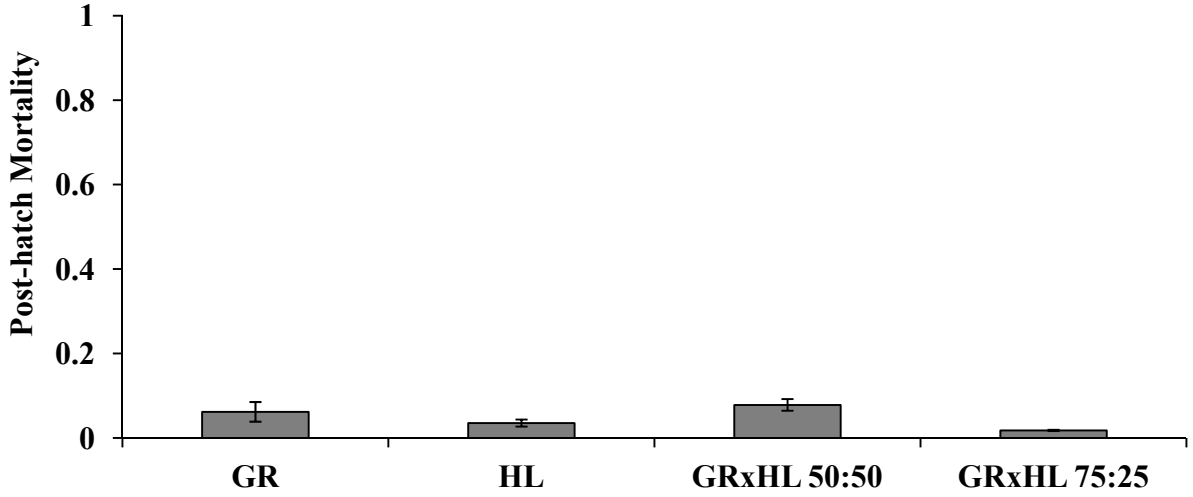

Figure 2.11. Average proportion post-hatch mortality (SE bars) by strain.

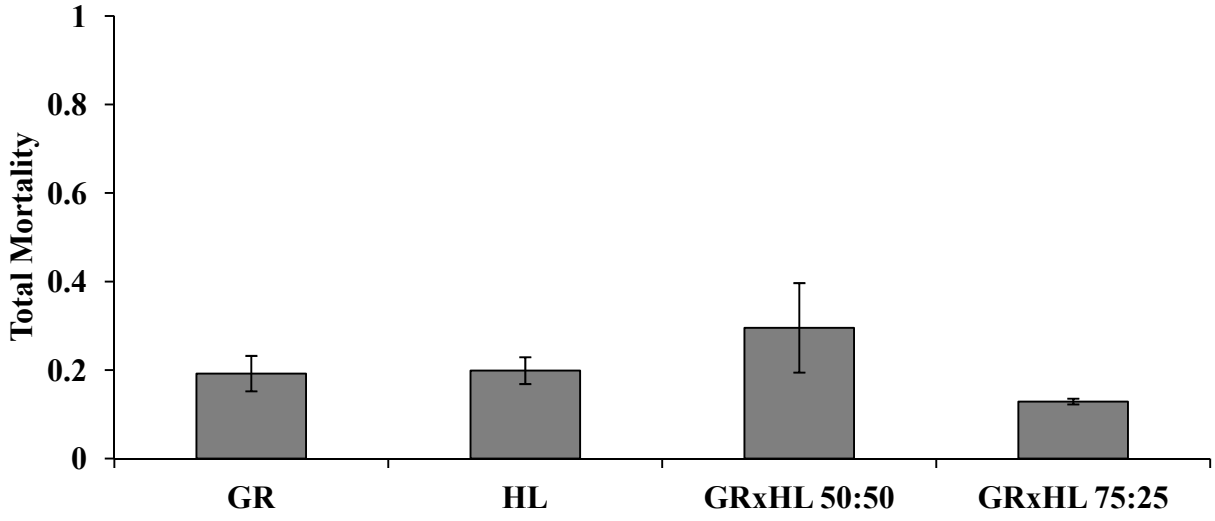

Figure 2.12. Average proportion total mortality (SE bars) by strain.

Fingerling Trial 1

Only four fish died as a result of formalin exposure during the first fingerling trial which examined the susceptibility of the four strains at 1.5" in length. All four fish were crosses between the GR and HL strains, with two of the fish being GR×HL 50:50, and the other two being GR×HL 75:25. Mortality was not high enough to explore statistical differences among the strains. Results from Trial 1 will be compared to results obtained from Trials 2-4 (3" fish), and Trial 7 (5" fish), to determine if fish are more susceptible to mortality following exposure to formalin as they increase in size.

CONCLUSIONS

Unlike in previous years, none of the strains or crosses exhibited higher total mortality rates in the egg life stage. Results from previous years suggested that the crosses may have a genetic predisposition to increased mortality in the egg life stage, a possible result of natural reduction in cripples or other genetically-deficient individuals that would not have otherwise survived post-hatch. However, this was not the case in this experiment. Results from egg formalin exposure experiments conducted in 2012, 2013, and 2014 suggest that egg quality within the year of collection may have the largest impact on mortality in the egg life stage.

Mortality was very low during the first fingerling formalin exposure trial. This is the first trial in which fish smaller than fingerling size have been examined for formalin sensitivity, and initial results, comparing to previous years, suggest that smaller fish are less sensitive than fish fingerling-sized or larger. However, comparisons between other size classes exposed to formalin throughout 2014 will help confirm, and results and conclusions from these trials will be available in the next reporting cycle.

Hatchery Strain Growth Comparisons

INTRODUCTION

The State of Colorado hatchery system produces millions of fry, subcatchable and catchable fish annually. Salmonids, specifically rainbow trout, cutthroat trout, and their crosses, constitute a large proportion of the total number of fish produced. Subcatchable salmonids (5-7 inches in length) are produced for put-grow-and-take fisheries, whereas catchable salmonids (≥ 8 inches) are stocked in put-and-take fisheries where annual return to creel is expected to be high. Because of the numbers and demand for these two size categories, and to meet the stocking requests made by the State's aquatic biologists, it is important for hatchery managers to know how quickly the various salmonid strains grow. This helps the hatcheries know when eggs should be acquired, how often lots need to be split, or food ration and size needs to be increase to meet the needs of the stocking schedule. The following describes the results of salmonid strain growth differences in the CPW Mount Shavano Hatchery (Salida, Colorado) using lots reared by the hatchery between 2006 and 2013.

METHODS

Table 2.12. Hatchery feed sizes, and associated fish size, fed to rainbow and cutthroat trout at the CPW Mount Shavano fish hatchery.

Feed Size	Fish Size (#/lb)
0	<1200
1	1200-600
2	600-200
1/16	200-40
3/32	40-20
1/8	20-9
5/32	>9

The CPW Mount Shavano Hatchery is a production hatchery. As such, it receives fish from the state's brood stock hatcheries in the form of eyed eggs. Fish are reared on the unit from hatch until they reach subcatchable or catchable size, at which time they are stocked. Generally, it takes about five months to reach subcatchable size and eight months to reach catchable size. However, these time frames can vary greatly depending on the strain of fish, the water temperature of the rearing unit, and the amount of feed given to the fish on a daily basis. Mount Shavano hatchery runs on well water, which maintains a fairly constant temperature of 10°C. Small fish (≤ 300 fish/lb; 2") are fed with automatic feeders every hour during daylight hours, equating to 3.0-4.6% of their body weight per day. Fish > 300 fish/lb and ≤ 40 (4") are fed 4-6 times per day by hand, whereas fish > 40 fish/lb are fed 2-3 times per day by hand, equating to 0.5-3.0% of their body weight per day. Hatchery feed sizes are changed based on fish size (Table 2.12).

Ninety lots of fish, consisting of 14 rainbow trout and cutthroat trout strains, and crosses therein, were reared for a minimum of five months (subcatchable size) at the Mount Shavano Hatchery

between 2006 and 2013. The 14 strains and crosses included: 1) the Bellaire rainbow trout strain (BEL), 2) a cross between Bellaire rainbow trout and Snake River cutthroat trout (BXS), 3) the Colorado River rainbow trout strain (CRR), 4) the Erwin rainbow trout strain (ERW), 5) a cross between the Hofer and Harrison Lake rainbow trout strains, secondarily crossed with a Snake River cutthroat trout (HHN), 6) the Hofer rainbow trout strain (HOF), 7) a cross between the Hofer and Colorado River rainbow trout strains (HXC), 8) a cross between the Hofer and Harrison Lake rainbow trout strains (HXH), 9) a cross between the Hofer and Tasmanian rainbow trout strains (HXT), 10) the Puget Sound rainbow trout strain (PUG), 11) a generic cross between rainbow trout and cutthroat trout (RXN), 12) the Snake River cutthroat trout strain (SRN), 13) the Tasmanian rainbow trout strain (TAS), and 14) a cross between the Tasmanian rainbow trout and Snake River cutthroat trout (TXS). Of the 90 lots of fish reared to five months, 64 were reared for an additional three months (eight months total) to reach catchable size. As a result, only 11 strains and crosses were included in the eight month growth data: 1) BXS, 2) ERW, 3) HHN, 4) HXC, 5) HXH, 6) HXT, 7) PUG, 8), RXN, 9) SRN, 10) TAS, and 11) TXS.

Growth analyses, comparing growth rate per month in inches, were conducted in SAS Proc GLM (SAS Institute 2011). Differences in growth were analyzed separately for the five month (N = 14) and eight month (N = 11) growth periods using a single factor ANOVA, with strain/cross as the factor. Values for both analyses were reported from the type III sum of squares. If significant effects were identified ($P < 0.05$), the least-squares means method with a Bonferroni adjustment was used to determine which strains or crosses exhibited differential growth from the others.

RESULTS

The results for the five month growth rate are presented in the order of fastest growing to slowest growing per Figure 2.13. The HOF grew significantly faster than all of the other strains or crosses ($P \leq 0.013$), with the exception of the PUG, HXH, HXT ($P = 1.0$), and ERW ($P = 0.056$), from which the growth rate did not differ. The PUG did not differ in growth rate from the HOF, HXH, or HXT ($P = 1.0$), but grew significantly faster than all other strains and crosses ($P \leq 0.002$). The HXH did not differ in growth rate from the HOF, PUG, HXT, ERW, or HXC ($P \geq 0.115$), but grew significantly faster than all of the other strains or crosses ($P \leq 0.014$). The HXT did not differ in growth rate from the HOF, PUG, HXH, ERW, HXC, or TAS ($P \geq 0.133$), but grew significantly faster than all of the other strains and crosses ($P \leq 0.008$). The ERW did not differ in growth rate from the HOF, HXH, HXT, HXC, TAS, TXS, or BEL ($P \geq 0.056$), but grew significantly slower than the PUG ($P = 0.025$) and significantly faster than all of the other strains and crosses ($P \leq 0.009$). The HXC did not differ in growth rate from the HXH, HXT, ERW, TAS, TXS, or BEL ($P \geq 0.115$), but grew significantly slower than the HOF and PUG ($P \leq 0.013$) and significantly faster than the HHN, SRN, CRR, RXN, and BXS ($P < 0.003$). The TAS grew significantly slower than the HOF, PUG, and HXH ($P \leq 0.014$), but did not differ in growth rate from any of the other strains or crosses ($P \geq 0.133$). The TXS grew significantly slower than the HOF, PUG, HXH, and HXT ($P \leq 0.008$), but did not differ in growth rate from any of the other strains or crosses ($P \geq 0.539$). The BEL grew significantly slower than the HOF, PUG, HXH, and HXT ($P \leq 0.003$), but did not differ in growth rate from any of the other strains or crosses ($P \geq 0.235$). The HHN grew significantly slower than the HOF, PUG, HXH, HXT,

ERW, or HXC ($P \leq 0.009$), and did not differ in growth rate from any of the other strains or crosses ($P \geq 0.748$). The SRN, CRR, RXN, and BXS did not differ in growth rate from each other ($P = 1.0$) or from the TAS, TXS, BEL, or HHN ($P \geq 0.748$), but all grew significantly slower than the HOF, PUG, HXH, HXT, ERW, and HXC ($P \leq 0.002$).

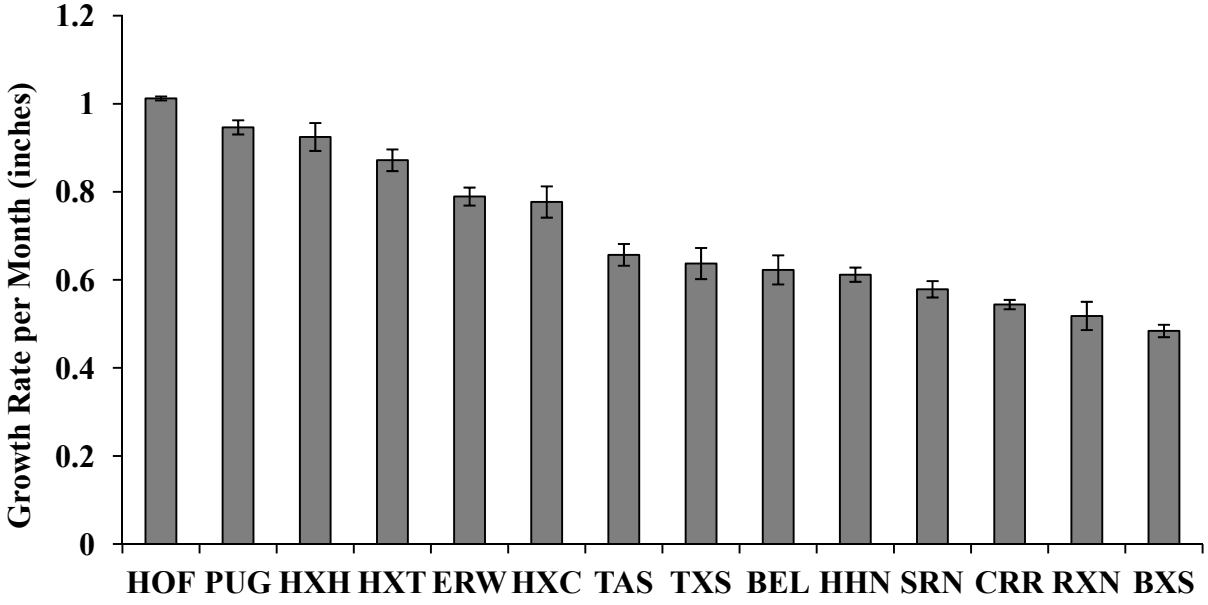

Figure 2.13. Average five month growth rate per month in inches (SE bars) for rainbow trout or cutthroat trout reared at the CPW Mount Shavano Hatchery.

The results for the eight month growth rate are presented in the order of fastest growing to slowest growing per Figure 2.14. The HXH grew significantly faster than all of the other strains and crosses ($P \leq 0.001$). The PUG did not differ in growth rate from the HXT, ERW, and TAS ($P \geq 0.271$), but grew significantly slower than the HXH ($P \leq 0.001$) and significantly faster than the other strains and crosses ($P \geq 0.011$). The HXT did not differ in growth rate from the PUG, ERW, TAS, HXC, or TXS ($P \geq 0.075$), but grew significantly slower than the HXH ($P \leq 0.001$) and significantly faster than the SRN, HHN, RXN, and BXS ($P \leq 0.001$). The ERW did not differ in growth rate from the PUG, HXT, TAS, HXC, or TXS ($P \geq 0.271$), but grew significantly slower than the HXH ($P \leq 0.001$) and significantly faster than the SRN, HHN, RXN, and BXS ($P < 0.048$). The TAS grew significantly slower than the HXH ($P \leq 0.001$) and significantly faster than the RXN and BXS ($P < 0.034$), but did not differ in growth rate from the other strains or crosses. The HXC grew significantly slower than the PUG and HXH ($P \leq 0.012$) and significantly faster than the RXN and BXS, but did not differ in growth rate from the other strains or crosses. The TXS grew significantly slower than the HXH and PUG ($P \leq 0.006$), but did not differ in growth rate from the other strains or crosses. The SRN and HHN did not differ in growth rate from each other ($P = 1.0$), or the TAS, HXC, TXS, RXN or BXS ($P \geq 0.098$), but both grew significantly slower than the HXH, PUG, HXT, and ERW ($P \leq 0.048$). The RXN and BXS both grew significantly slower than the HXH, PUG, HXT, ERW, TAS, and HXC ($P \leq 0.034$), but did not differ in growth rate from the TXS, SRN, HHN or each other ($P \geq 0.098$).

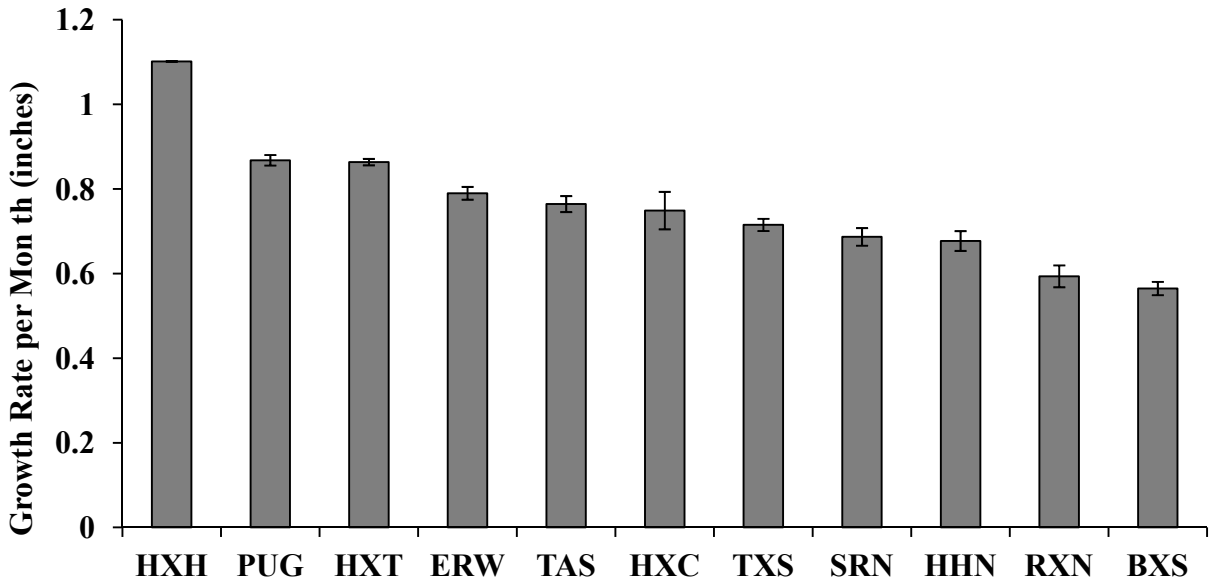

Figure 2.14. Average eight month growth rate per month in inches (SE bars) by rainbow trout or cutthroat trout reared at the CPW Mount Shavano Hatchery.

CONCLUSIONS

Hofer and crosses between the Hofer and other rainbow trout strains such as the HXH, HXT and HXC exhibited the highest growth rates, in terms of inches per month. This was expected as the Hofer has exhibited high growth rates in previous experiments (Schisler et al. 2006; Fetherman et al. 2011). Of the domestic strains of rainbow trout (i.e., the Erwin [ERW], Tasmanian [TAS], and Bellaire [BEL] strains), the Erwin exhibited the highest growth rate, and usually did not differ from the Hofer or Hofer crosses in growth rate. At both five and eight months, Snake River cutthroat trout (SRN) or rainbow trout crosses with the SRN (i.e., TXS, RXN, and BXS) exhibited the slowest growth rates. Interestingly, the Hofer-Harrison-Snake River cutthroat trout cross (HHN) also exhibited fairly low growth rates at both five and eight months, despite having Hofer parental origins. Overall, the data suggests that Hofer and Hofer crosses grow faster, and will therefore, reach subcatchable or catchable size sooner than the cutthroat trout or cutbows, or the domestic Bellaire strain. As such, hatcheries will need to plan accordingly regarding stocking times, or rearing space and time constrains.

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Job No. 3 Whirling Disease Resistant Domestic Brood Stock Development and Evaluation

Job Objective: Identify and propagate whirling disease resistant domestic strains that are useful for catchable put-and-take or fingerling put-grow-and-take fisheries management applications.

INTRODUCTION

Earlier experiments demonstrated that the Hofer (GR) and Hofer × Harrison Lake (GR×HL) crosses have excellent growth and return-to-creel when stocked as catchable-sized fish. Colorado Parks and Wildlife is aggressively transitioning its brood facilities to produce larger numbers of GR or GR×HL crosses for catchable production purposes. In addition to catchable stocking, many waters in Colorado are stocked with fingerlings or subcatchable sized fish. These fish are subjected to greater threats from predation than catchable-sized fish and must be able to forage and survive long enough to become available to anglers. Because of the domestic nature of the GR strain, there are reasons to be concerned about the possibility of low survival and returns when fish of the GR strain, or slightly outbred varieties of the strain, are stocked as fingerlings. An experiment was designed to evaluate the survival of these varieties as fingerling plants in a location subjected to high predation pressure.

Figure 3.1. Parvin Lake, Colorado.

Parvin Lake (Figure 3.1), located 45 miles northwest of Fort Collins, Colorado, was used as the test site for this evaluation. The reservoir is stocked annually with fingerling brown trout (*Salmo trutta*), splake (*Salvelinus namaycush x Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*). The reservoir was also stocked in 2000 through 2003 with tiger muskies (*Esox masquinongy x Esox lucius*) to control the abundant white sucker (*Catostomus commersoni*) population. An inlet trap that was historically used for rainbow trout spawning operations has also been operated more recently to remove white suckers from the reservoir in the months of May through July during their annual spawning run up the inlet stream. Numbers of suckers and trout captured in the trap vary from year to year, but appear to have been greatly reduced in recent years (Figure 3.2). In 2009, 539 white suckers, and 67 salmonids were captured in the inlet trap. In 2010, 176 suckers and 153 salmonids were captured in the inlet trap. In 2011, 121 suckers and 76 salmonids were captured in the inlet trap, although high water in May and June 2011 prevented fish from entering the trap until later than normal. In 2012, only four suckers and 31 salmonids were captured in the trap due to virtually non-existent runoff conditions. In 2013, 310 suckers and 271 salmonids were captured in the trap, with a large proportion of the salmonids consisting of spawning rainbow-cutthroat crosses. In 2014, extremely high runoff conditions through most of the spring prevented many fish from being able to negotiate the run into the trap, and half of the trap screens had to be removed to prevent the structure from being blown out. Nonetheless, 76 suckers and 50 salmonids were caught during the spawning season.

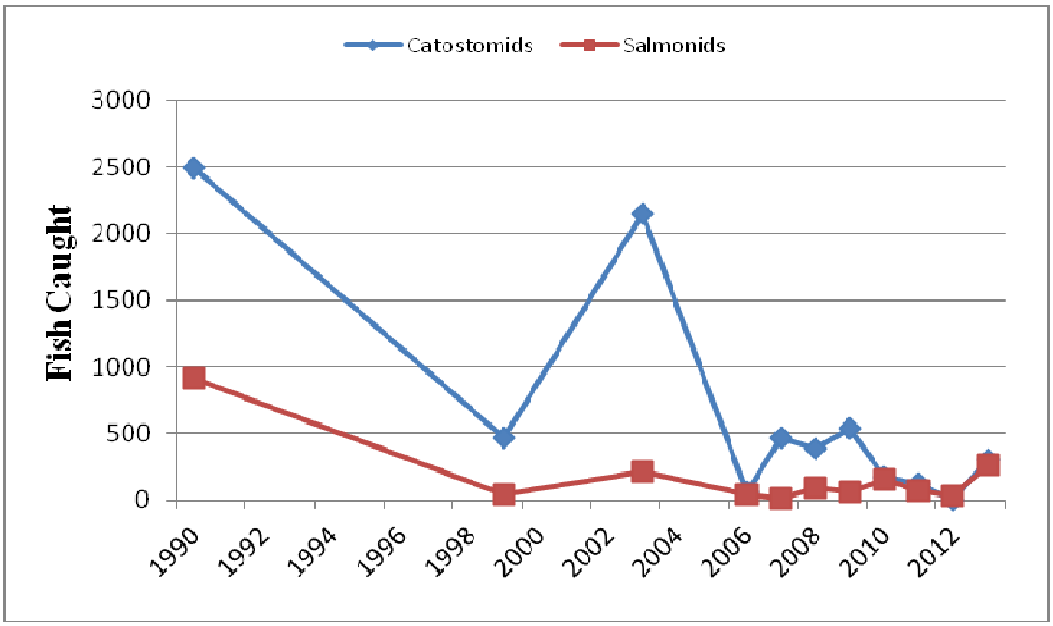

Figure 3.2. Number of catostomids and salmonids captured in the Parvin Lake inlet trap (May-July) in years where data are available.

A fall electrofishing survey has been conducted annually since 2002 to monitor species composition and growth in Parvin Lake. A shift from a population dominated by white suckers to one dominated by rainbow trout has occurred since 2006 (Figure 3.3). In 2009, 69.7% of the total catch was rainbow trout, compared with only 14.4% white suckers. In 2010, the proportions were 76.5% rainbow trout and 3.6% white suckers. In 2011, the proportions were

66.1% rainbow trout and 15.2% white suckers. In 2012, the proportions were 58.9% rainbows and 20.3% white suckers. In 2013, the proportions were 47.5% rainbows and 30.2% white suckers. This compares well with the figures from 2006, when over 60% of the total catch was white suckers, although the trend in the past three years has been an increasing white sucker to rainbow trout ratio.

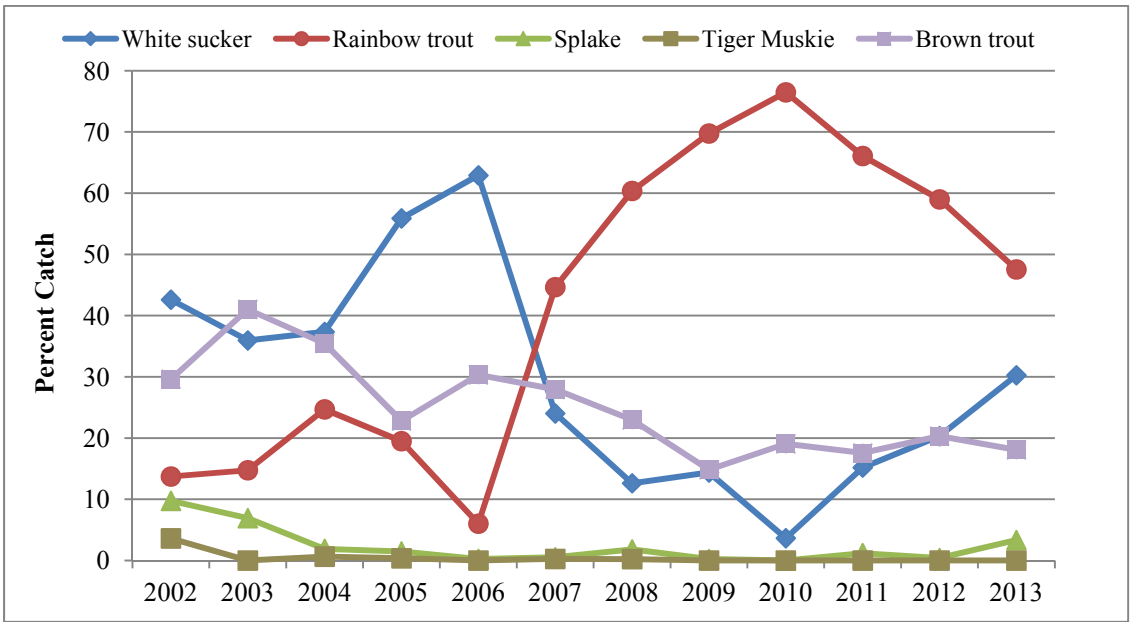

Figure 3.3. Percent of catch by species during fall electroshocking surveys for the years 2002 – 2013.

METHODS

To evaluate survival and growth of multiple varieties of fingerling trout, live-release experiments have been conducted on a yearly basis from 2007 to present. Preliminary returns of the different varieties, as well as fingerling strain availability, were used to determine which varieties would be used for each subsequent plant. In addition, changes to experimental groups stocked each year have been made in response to suggestions by field biologists and hatchery managers to determine if specific strains may be more or less suitable for stocking as fingerlings in lake or reservoir environments. Descriptions of stocking and sampling results from 2007-2009 are provided in the 2013 Federal Aid report (Fetherman et al. 2013).

The experimental stockings described here were primarily focused on evaluation of GR-Snake River cutthroat. Fish stocked in 2010 included two distinct lots, stocked on July 6, 2010. The first lot was the HHN variety, and the second lot was another standard cutthroat-rainbow cross (RXN) produced at the Crystal River Hatchery, created by crossing a Snake River cutthroat trout with a Tasmanian strain rainbow trout (Table 3.1).

Fish stocked in 2011 included four varieties of fish, the HHN, RXN, pure GR, and Hofer × Colorado River (GR×CRR) cross. In this trial, the HHN (a.k.a., HN2) were created using Snake River cutthroats of the spring spawning variety (SR2), and Hofer-Harrisons as described

previously. The RXN were created using Tasmanian rainbows and the spring spawning Snake River cutthroat trout. These fish were stocked on November 3, 2011.

Table 3.1. Coded-wire tagged fish stocked in Parvin Lake in 2010 and 2011.

	2010 Plants		2011 Plants			
Strain	HHN (50:50)	RXN (50:50)	GR	GR×CRR	HHN (50:50)	RXN (50:50)
Lbs	260	219	32.4	32.4	32.4	32.4
Number	7511	7380	3000	3000	3000	3000
Length (mm)	112.4	106.7	76.2	76.2	76.2	76.2

In 2012, four lots of fish were stocked: the pure GR, GR×CRR (HXC) cross, and HN2 were stocked as in the previous year. SR2 (pure spring-spawning Snake River cutthroat trout) were also stocked to determine if the pure Snake River cutthroat would perform as well as the HHN (HN2) variety. These fish were stocked on October 29, 2012, much later in the year than previous plants, so no fish from that plant were collected during the 2012 sampling events (Table 3.2). No fish were stocked for these experiments in 2013 as we transitioned to evaluating spring plants of larger fish in 2014.

Table 3.2. Coded-wire tagged fish stocked in Parvin Lake during 2012 and 2014.

	2012 Plants				2014 Plants			
Strain	GR	GR×CRR (HXC)	HHN (HN2)	SR2	**Strain**	GR	GRXCRR (HXC)	HHN (HN2)
Lbs	105.3	68.9	52.1	40.3	**Lbs**	426.0	426.0	426.0
Number	2,116	2,116	2,116	2,116	**Number**	1,734	1,734	1,734
Length (mm)	126.8	110.1	100.3	92.5	**Length (mm)**	215.9	215.9	215.9

The two previous years in which late fall plants were made, rainbow trout survival was not as good as in previous years when July and August plants were conducted. Therefore, fish reared in at the Bellvue Fish Research Hatchery were grown to larger size and stocked on April 4, 2014. This stocking event consisted of 1,734 pure Hofer, 1,734 GR×CRR (HXC), and 1,734 HN2 fish stocked at an average of 215.9 mm. Half of the fish from each of these groups were adipose clipped as part of a separate trial to evaluate effects of training rainbow trout to help them avoid predation.

Collections of coded-wire tagged fish were made using evening boat electroshocking. Marked sample goals (60-90 fish) could typically be achieved by shocking the entire perimeter of the lake over a three-hour time period. Fish with coded wire tags were identified during the sampling event with a hand-held tag detector. Collected fish were weighed to the nearest gram and measured to the nearest mm. Heads were removed, and coded wire tags extracted and examined with a MagniViewer coded wire tag reader. The remainder of the head tissues were packaged in individually numbered zip-lock bags and frozen for later myxospore count evaluation. Fish length, weight, tag number and myxospore count for each fish was recorded in a database for each individual sampling event.

RESULTS AND DISCUSSION

During the 2013 sampling season, the samples collected produced a representative cross-section of the fish stocked in previous years. Results for each individual year-class of 2010-2012 are listed separately below, along with cumulative catch from previous years of sampling to provide a comprehensive overview of each project year results.

2010 Year Class

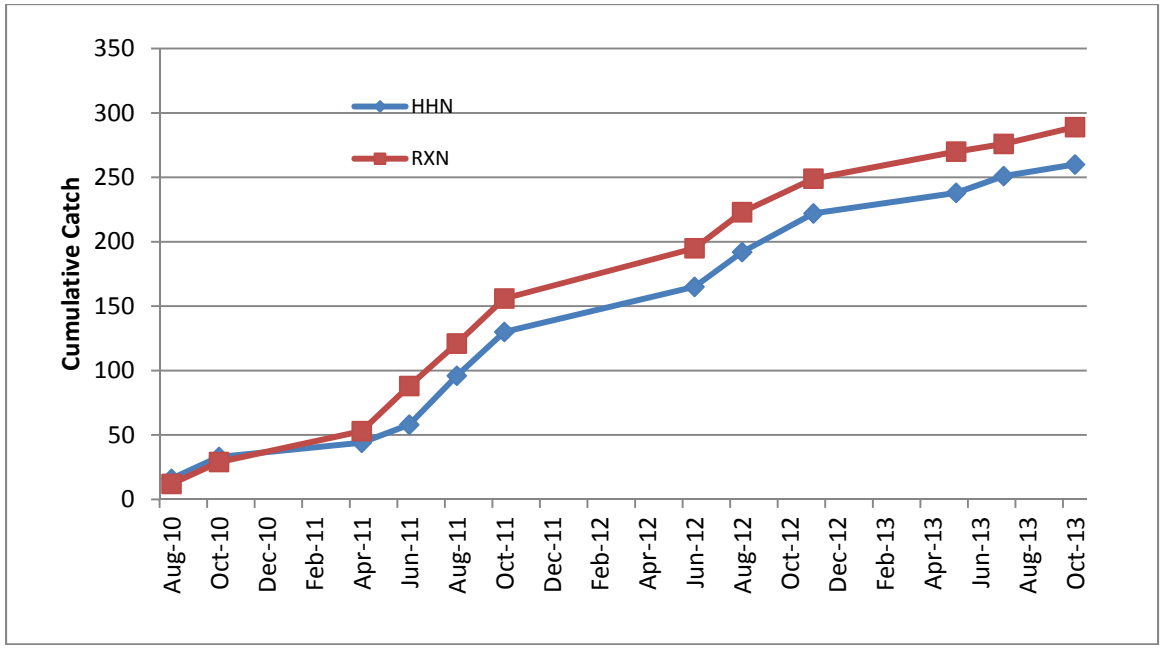

Figure 3.4. Cumulative catch for two varieties of fingerling rainbow trout stocked in Parvin Lake in July 2010.

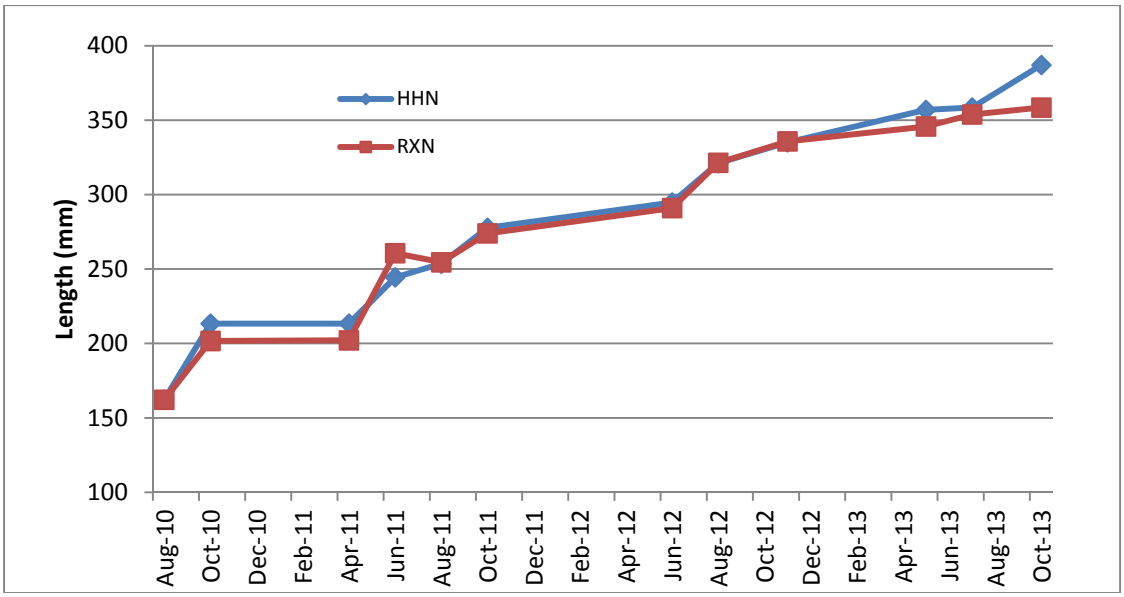

Figure 3.5. Fish length from 2010 to 2012 from the two varieties stocked in Parvin Lake in July 2010.

Sampling of the 2010 year class from 2010 through 2013 resulted in relatively equal numbers for fish of the HHN and RXN varieties. Sample numbers collected in 2010 were nearly identical, consisting of 33 HHN and 29 RXN fish. During 2011, 97 HHN and 127 RXN fish were collected. During 2012, 92 HHN and 93 RXN fish were collected. In 2013, 38 HHN and 40 RXN were collected. Collective sums were 260 HHN (47.4%) and 289 RXN (52.6%), for a total of 549 fish (Figure 3.4). Growth was also nearly identical between the two strains. Average length at the end of the 2013 sampling season was 289 mm for the HHN and 260 mm for the RXN (Figure 3.5).

For sampling conducted in 2013, none of the HHN variety fish were found to contain myxospores (n= 38), whereas myxospore counts among the RXN fish averaged 7,027 (n = 21), 9,454 (n = 6) and 10,509 (n = 13), respectively, in the May, July, and October samples.

2011 Year Class

Sampling of the 2011 year class did not begin until the following spring (April 2012) due to the late fall stocking of that year class. Very few fish were found in the initial sampling event (Figure 3.6). However, divergence of catch rates had already occurred by fall of 2012, with only one pure GR, and five HXC fish being found, whereas 17 and 27 HN2 and RXN fish had been collected, respectively. Average lengths of the four strains were very similar, averaging around 250 mm in November 2012 (Figure 3.7). No HXC or GR strain fish were found in the 2013 sampling events. Growth of the RXN and HN2 varieties were very similar in length, with the HN2 reaching an average of 352 mm and the RXN reaching an average of 321 mm. Weight among the HN2 (492 g) was greater than that of the RXN (412 g).

For sampling conducted in 2013, none of the HN2 variety fish were found to contain myxospores (n= 16), whereas myxospore counts among the RXN fish averaged 1,092 (n = 10), 6,435 (n = 10) and 2,127 (n = 7), respectively, in the May, July, and October samples.

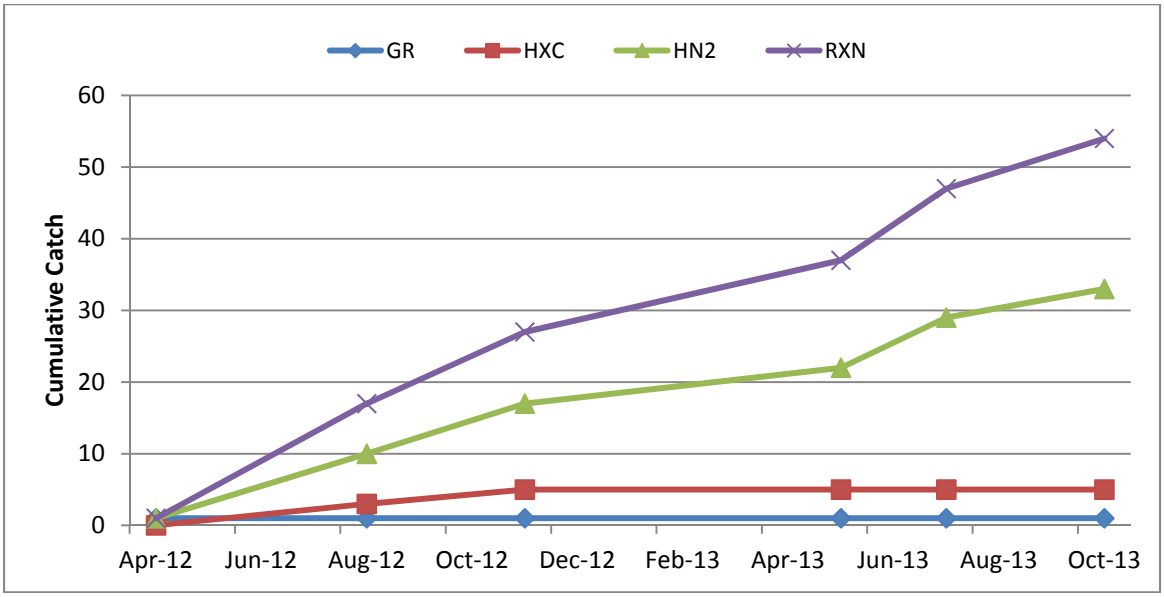

Figure 3.6. Cumulative catch for four varieties of fingerling rainbow trout stocked in Parvin Lake in November 2011.

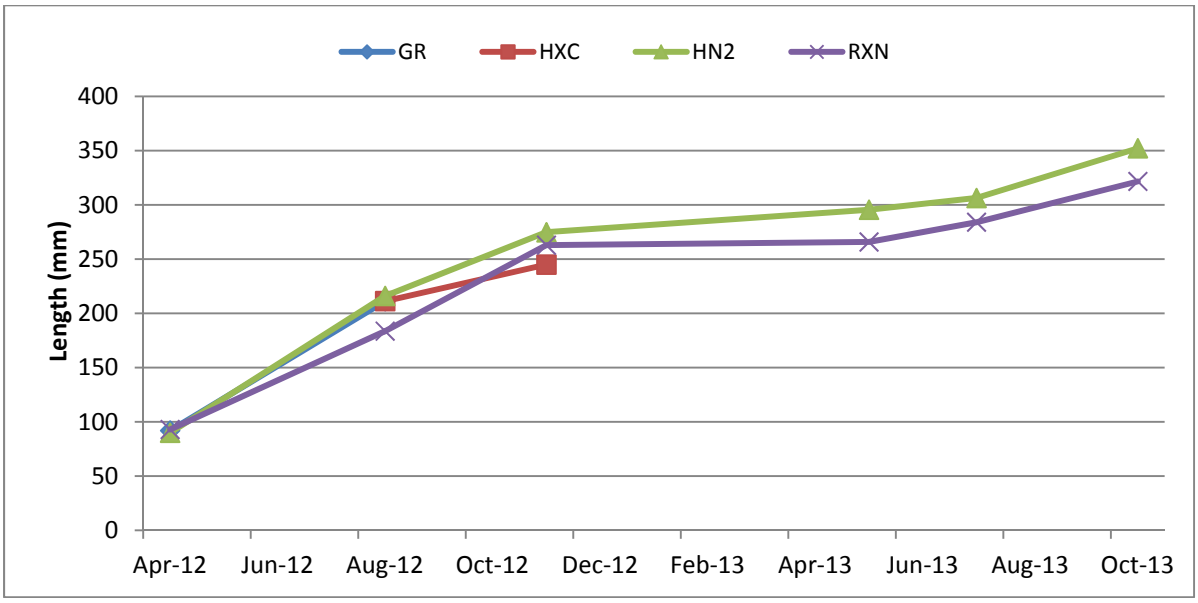

Figure 3.7. Fish length for each of four varieties stocked in Parvin Lake in November 2011.

2012 Year Class

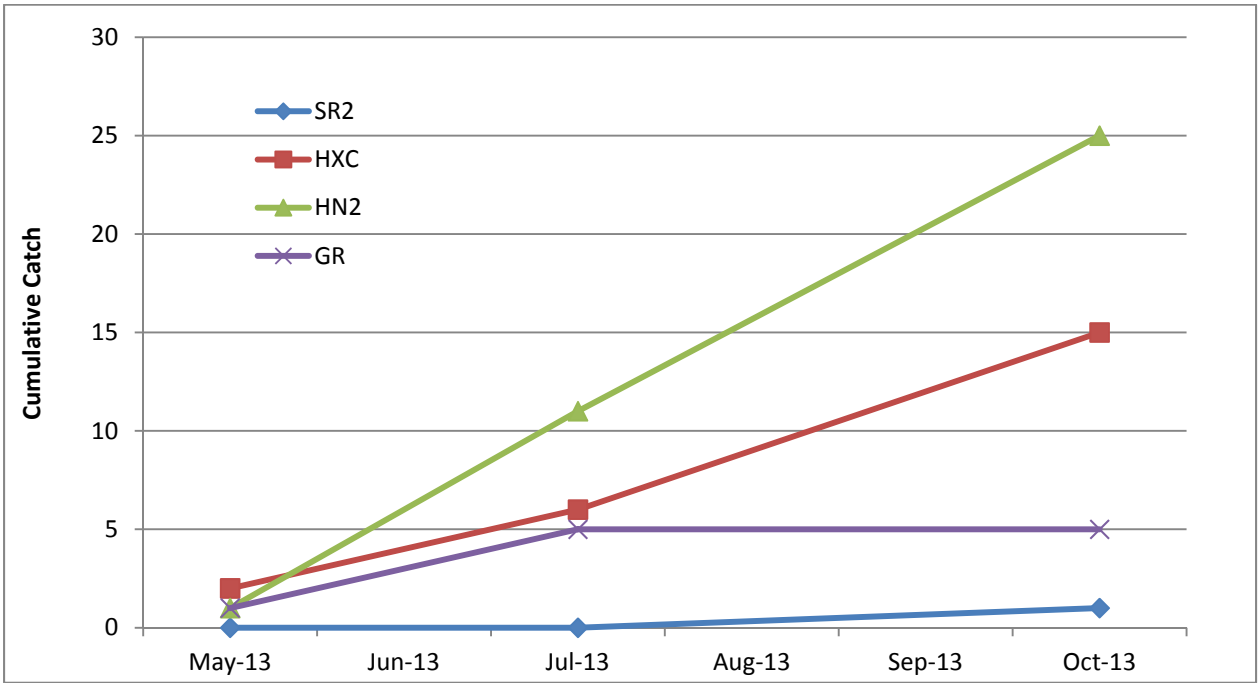

Figure 3.8. Cumulative catch for four varieties of fingerling rainbow trout stocked in Parvin Lake in October, 2012.

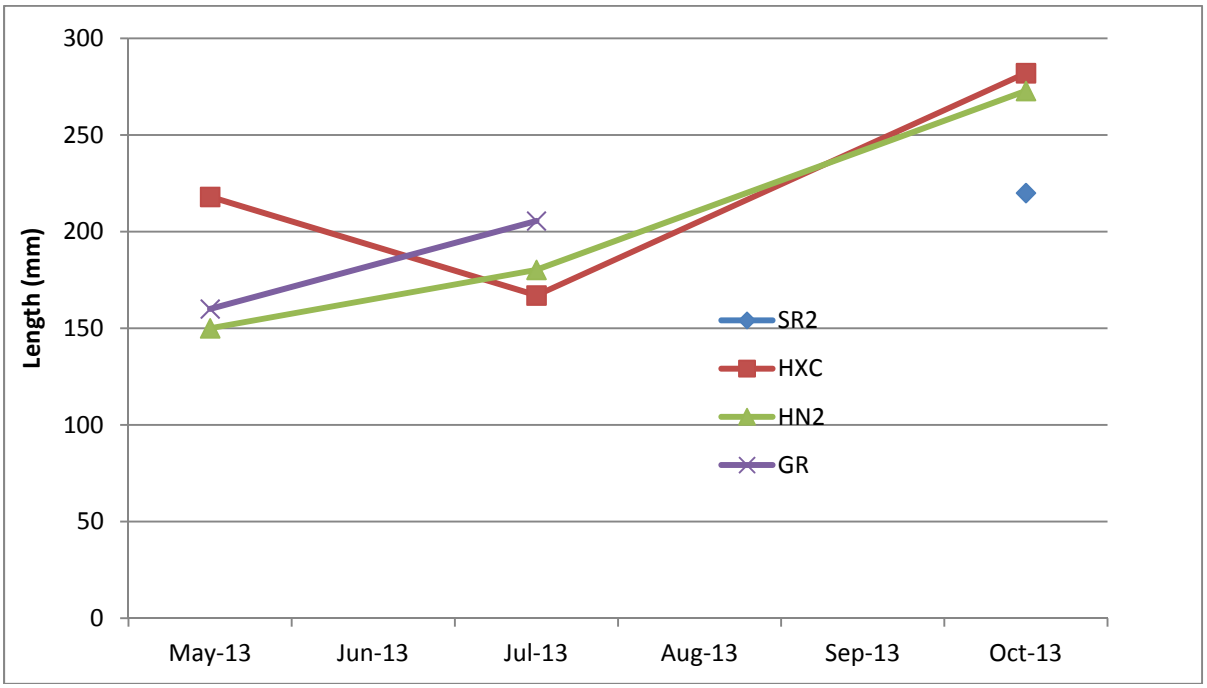

Figure 3.9. Fish length for each of four varieties stocked in Parvin Lake in October, 2012.

Collection of the 2012 year class first occurred during the 2013 sampling season. The GR and SR2 fish were poorly represented in the samples, with only five and one, respectively, of these strains found during the entire season. The HXC variety fared better, with 15 fish found in the samples. The HN2 were far better performers than the others, with 25 fish being found in the samples (Figure 3.8). Low numbers of the SR2 and GR fish made growth estimates unreliable for those strains. The HXC and HN2 varieties grew at nearly identical rates in this first year, with an average of 274.9 mm for the HXC and 288.1 mm for the HN2 by October of 2013 (Figure 3.9). Myxospore counts, like growth rates, were difficult to reliably quantify due to the low sample numbers in the GR and SR2 fish. No myxospores were found in any of the two strains, except for a single GR fish with 2,056 myxospores in the May 2013 sample. No myxospores were found in any of the HXC fish over the entire season. No myxospores were found in any of the HN2 fish until the last sample event in October, which resulted in an average myxospore count of only 538 in those fish.

Samples collected for these stocked year-classes of fish suggest that the HHN or HN2 variety is a very good option for these types of environments, and will provide a whirling disease-resistant alternative for cutbow stocking that also demonstrates high survival. Because of the resistance to whirling disease, the high survival of the variety, and the general appeal of cutbows overall, these varieties seems to be emerging as an optimal variety for lake and reservoir plants. The performance compares favorably with that of the typical RXN variety with the added benefit of whirling disease resistance. Given that this variety can be easily produced from Hofer-Harrison and pure Snake River cutthroat brood fish in the hatchery system, this variety has the potential for great utility for fingerling plants throughout the state.

Field Performance Evaluations: Poudre Ponds Fingerling Stocking Experiment

INTRODUCTION

The concept of stocking fish reared in a whirling-disease positive facility versus those stocked from a clean facility has been a topic of debate ever since the implementation of the Colorado Division of Wildlife D-9 stocking policy. The argument has been made that fish produced in a clean facility will ultimately become infected and produce myxospores when stocked into an infected environment, so the benefit of producing fish at a clean facility is negated. The goal of this study was to quantify infection levels in fish reared to catchable size in both infected and uninfected environments, and subsequent myxospore production of those fish. Both susceptible and resistant strains of fish were used to determine if using resistant strains would produce a better outcome in either scenario. This long-term experiment was conducted over a period of three years in three separate phases to evaluate overall growth, survival, and infection severity among the various varieties from fingerling to catchable size.

METHODS

The first phase of this experiment began in 2009 with an evaluation of growth, survival, and infection severity of eight varieties of rainbow trout held in two earthen ponds at the Poudre Rearing Unit. This experiment was conducted to determine infection level and growth of the eight varieties reared together in a natural setting known to have high ambient levels of *M.*

cerebralis. One thousand fish of pure GR, pure Harrison Lake, pure Tasmanian, RXN, HHN, GR×HL (50:50), GR×HL (75:25), and GR×HL (87.5:12.5) were marked with coded wire tags and stocked as fingerlings (35-70 fish lb^{-1}) into each of the two ponds, for a total of 8,000 fish per pond on June 23, 2009. Samples were collected at eight months and 12 months post-release. All fish collected from the ponds were weighed and measured, and coded wire tags were extracted for variety identification. Fish were then numbered, individually bagged, and a subset was submitted for PTD testing.

The second phase of the experiment involved stocking fish from the first phase that had been grown to catchable size, along with catchable-size fish each of two varieties previously reared in a *M. cerebralis*-negative environment (Rifle Falls Hatchery) and stocked into an infected pond. The objectives of the second phase of the experiment was to determine the level of infection developed by both susceptible and resistant fish reared initially in both infected and non-infected environments, and then exposed to the parasite. The first variety of fish brought to the facility was the susceptible Bellaire rainbow strain (1.97 fish lb^{-1}), which had been created for the experiment in 2005. The second variety of fish was the resistant GR×HL (87.5:12.5) cross (2.12 fish lb^{-1}), which had also been created in 2005 and reared at the same facility. These clean, catchable-size fish were stocked into a third infected pond on the Poudre Rearing Unit, along with all remaining fish from the first phase of the experiment, on October 5, 2010.

Fish from the second phase of the experiment were reared at the Poudre Rearing Unit for another year, after which the third phase of the experiment was initiated. The third phase of the experiment consisted of stocking these fish into a put-and-take fishery to determine final growth, infection level, and return to creel of the ten varieties of fish reared at the Poudre Rearing Unit over the duration of the experiment. The location for this portion of the evaluation was Douglas Reservoir, a typical put-and-take fishery north of Fort Collins, Colorado.

RESULTS AND DISCUSSION

Phase I

Table 3.3. Total catch for the eight month post-release sample at Poudre Ponds.

	Pure HL	Pure TAS	Pure GR	GR×HL 50:50	GR×HL 75:25	GR×HL 87.5:12.5	HHN	RXN
Pond 1								
Hook and Line	0	0	7	2	13	6	2	2
Gill Net	0	1	6	0	3	4	9	7
Pond 2								
Hook and Line	0	3	5	3	4	10	5	8
Gill Net	0	1	6	1	6	6	4	1
TOTAL	0 (0.0%)	5 (4.0%)	24 (19.2%)	6 (4.8%)	26 (20.8%)	26 (20.8%)	20 (16.0%)	18 (14.4%)

Catch results for the eight-month sample are summarized by gear type in Table 3.3. No Harrison Lake rainbow trout were found among the 125 fish collected during the eight-month post-release sample. Only five pure Tasmanian strain fish were found, and six GR×HL (50:50) crosses. The other strains were relatively uniform in catch, ranging from 18 (14.4%) to 26 (20.8%).

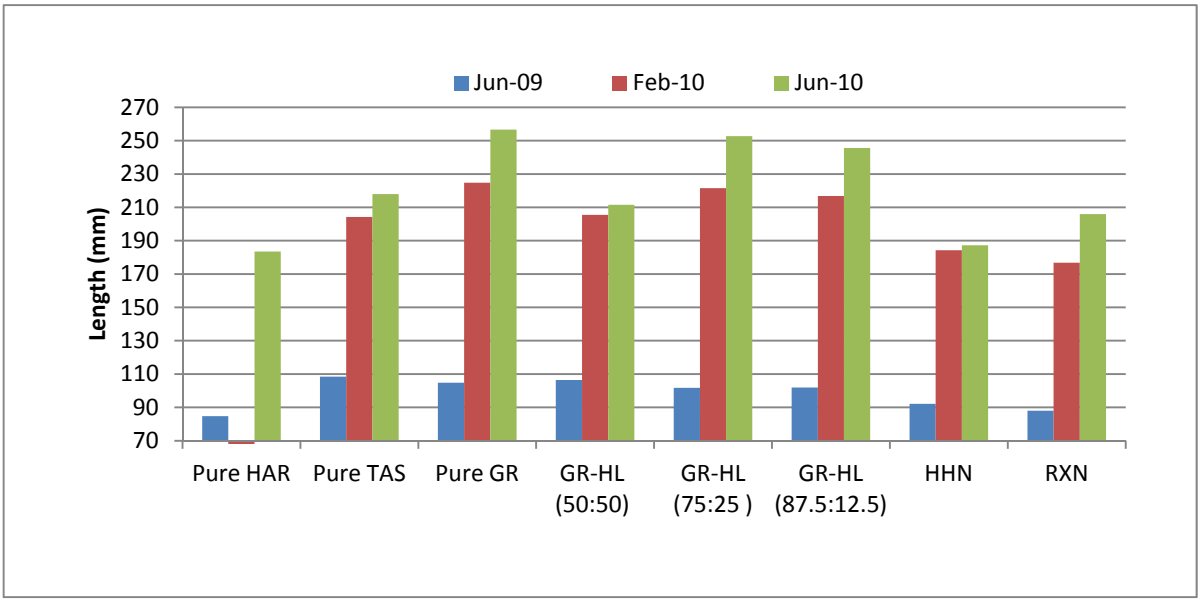

Figure 3.10. Lengths of eight rainbow and rainbow-cutthroat trout cross varieties upon release, eight and 12 months post-release at the Poudre Rearing Ponds.

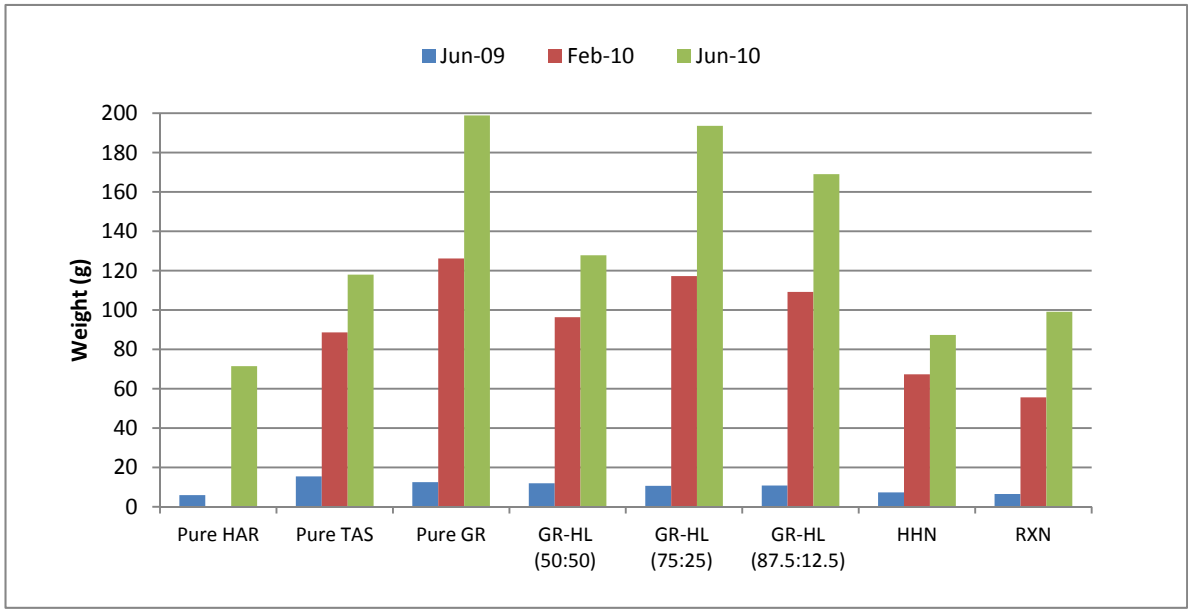

Figure 3.11. Weights of eight rainbow-cutthroat trout cross varieties upon release, eight and 12 months post-release at the Poudre Rearing Ponds.

The eight-month length results suggest that the GR strain and high proportion GR crosses, such as the GR×HL (75:25) and GR×HL (87.5:12.5) had slightly better growth compared to the other varieties (Figure 3.10). Each variety averaged over 210 mm in length at eight months. Weight measurements demonstrated an even greater advantage for the GR strain and high proportion GR crosses, with all three averaging over 100 grams (Figure 3.11).

Table 3.4. Total catch for the 12 month post-release sample at Poudre Ponds.

	Pure HL	Pure TAS	Pure GR	GR×HL 50:50	GR×HL 75:25	GR×HL 87.5:12.5	HXN	RXN
Pond 1								
Seine	1	4	14	2	8	23	6	7
Pond 2								
Seine	2	3	10	2	12	16	2	3
TOTAL	3 (2.6%)	7 (6.1%)	24 (20.9%)	4 (3.5%)	20 (17.4%)	39 (33.9%)	8 (7.0%)	10 (8.7%)

The 12-month results were very similar, with the GR and high proportion GR crosses exhibiting the best growth as measured by both length and weight. The high proportion GR varieties were also present in the sample at higher rates than the other varieties (Table 3.4). The exception was the HHN variety, in which growth (both length and weight) was more comparable to the RXN variety.

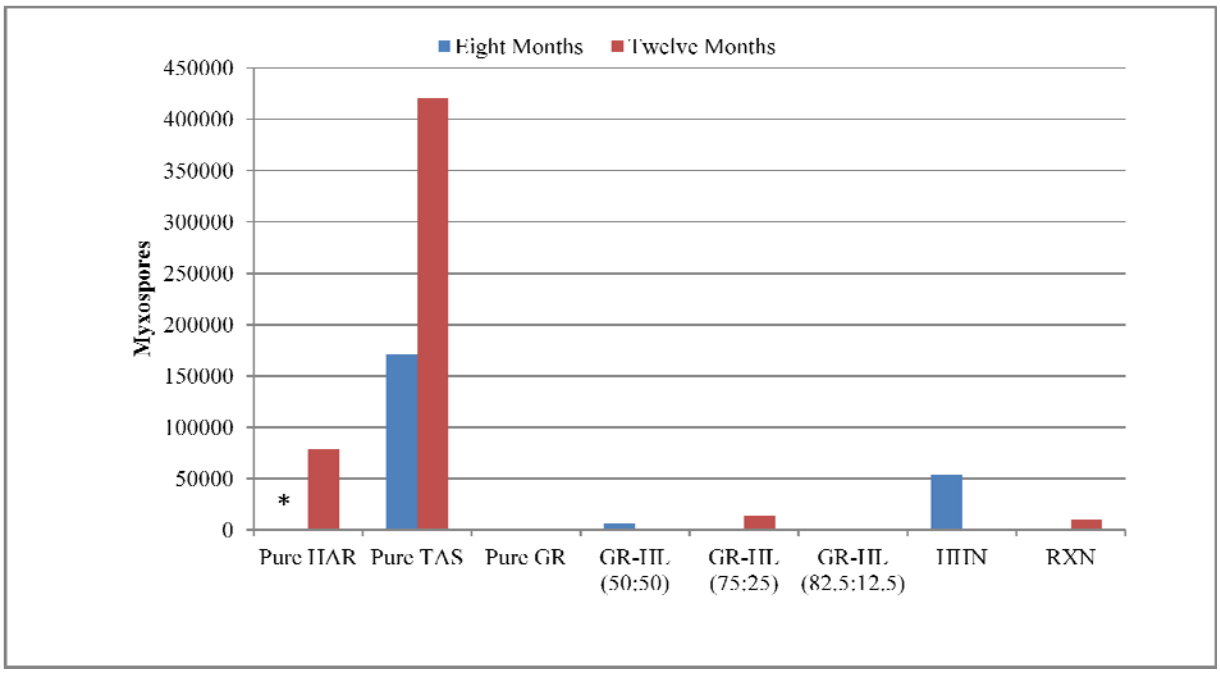

Figure 3.12. Myxospore count by strain at the Poudre Rearing Unit at eight months and 12 months post-release. No Harrison Lake variety fish were found in the eight month collection.

Myxospore count results were very similar to the other experiments in which these varieties were evaluated. At both eight months and twelve months, the Tasmanian strain exhibited much higher parasite loads than the other varieties. Average myxospore count for the Tasmanian strain at twelve months was over 400,000 myxospores per fish (Figure 3.12). This high myxospore level, as observed in a highly infected natural environment, would unquestionably lead to amplification of *M. cerebralis* in waters stocked with this suceptible strain.

Phase II

Myxospore counts in the fish reared from fingerling size on the infected facility doubled between June 2010 and June 2011 among more suseptible groups such as the Tasmanian and GR×HL (50:50) varieties. Pure GR, HHN, HXN, and high proportion GR crosses such as the GR×HL (75:25) and GR×HL (82.5:12.5) maintained realtively low myxospore counts. No Harrison Lake strain fish were found during either of these collections, so myxospore counts and growth are not shown for that strain (Figure 3.13).

For fish that were brought to the facility as clean catchable-sized fish, the Bellaires developed an average myxospore count of 182,908 myxospores per fish in the first year, while the resistant fish (GR×HL (82.5:12.5)) exposed as catchables developed an average myxospore count of only 1,027 myxospores per fish. The myxospore count among the Bellaires exceeded the myxospore count observed in all other varieties reared in the exposed environment, with the exception of the susceptible Tasmanian strain, and the GR×HL (50:50) cross.

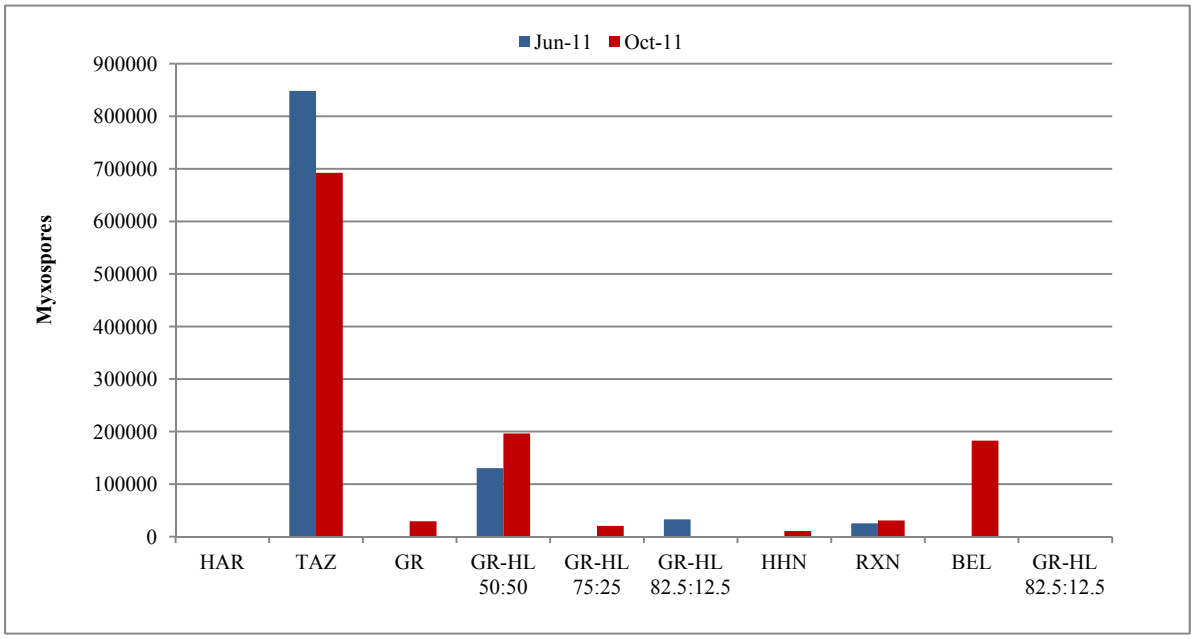

Figure 3.13. Myxospore counts among fish collected at 24 and 28 months post-exposure. The results for the Bellaire (BEL) and GR×HL (82.5:12.5) groups on the far right of the graph are for eight months and 12 months post-stocking into the infected environment as clean catchables. No Harrison Lake variety fish were found in these collections.

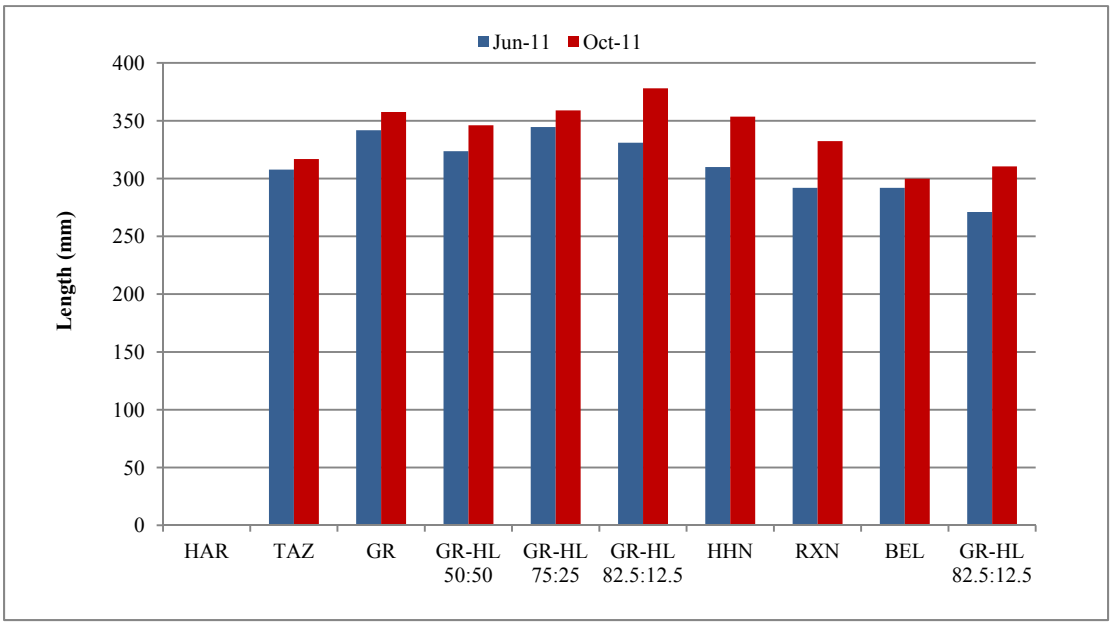

Figure 3.14. Length of fish collected at 24 and 28 months post-stocking. The results for the Bellaire (BEL) and GR×HL (82.5:12.5) groups on the far right of the graph are for eight months and 12 months post-stocking into the infected environment as clean catchables. No Harrison Lake variety fish were found in these collections.

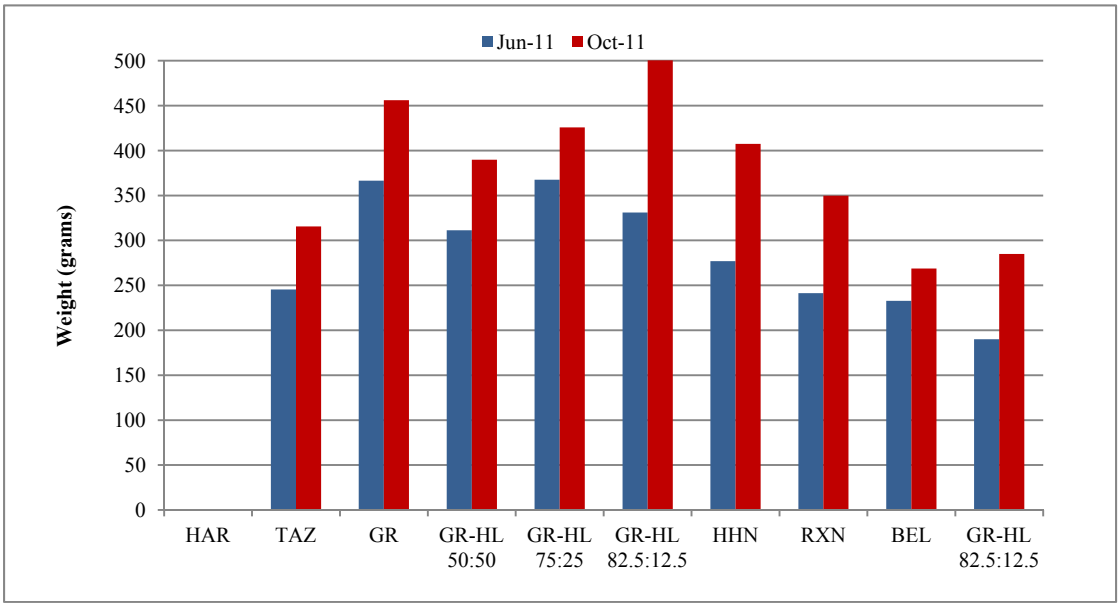

Figure 3.15. Weight of fish collected at 24 and 28 months post-stocking. The results for the Bellaire (BEL) and GR×HL (82.5:12.5) groups on the far right of the graph are for eight months and 12 months post-stocking into the infected environment as clean catchables. No Harrison Lake variety fish were found in these collections.

Growth in this phase of the experiment followed the same pattern as that in Phase I, with high proportion GR crosses exhibiting the greatest growth as measured by both length and weight. The GR×HL (82.5:12.5) cross brought from the Rifle Falls Hatchery at the beginning of Phase II started out smaller than the Bellaire strain brought over at the same time. By the end of Phase II the GR×HL (82.5:12.5) cross had outgrown the Bellaire strain (Figures 3.14, 3.15).

Phase III

The fish remaining in 'Pond 3', where the fish were reared for one year after reaching catchable size (Phase II), were stocked into Douglas Reservoir on June 4, 2012. Samples were collected immediately prior to stocking for both growth and myxospore counts. Lengths of fish of the various varieties immediately prior to stocking were similar to those obtained while the fish were in the pond phase of the experiment (Figure 3.16). Pure GR, GR×HL (82.5:12.5), and GR×HL (75:25) were the three largest varieties. All varieties were larger in the creel samples than immediately prior to stocking. This could be possibly due to some post-release growth, but since the creel samples were from harvested fish, it could also be due to harvest selection for larger fish by anglers.

Total fish stocked, based on average weight of 1.21 per lb, was 13,107 fish. Given that 20,000 fish were stocked into the ponds originally, this was a fairly high rate of survival over the course of the captive rearing period. While 2,000 fish per strain were stocked into the ponds initially in Phase I, not all strains were stocked into the reservoir in equal numbers due to differential mortality in Phase 1 and Phase II. It is important to calculate the number of fish of each strain to evaluate catch rates of each variety. To do this, we took the average ratio of each of the strains found in the June 2011, October 2011, and June 2012 samples and multiplied that figure by the total fish stocked. The estimated counts for the various strains at the end of Phase II, and beginning of Phase III ranged from 44 for the pure Harrison strain, up to 1,979 for the Bellaire strain (Figure 3.20).

A creel survey consisting of two weekdays and two weekend days per week was conducted from the beginning of June through the end of August 2012 at Douglas Reservoir. An estimated 8,890 (SE = 977) rainbow trout were caught during the first three months post-stocking, of which 3,730 (SE = 474) were reported to have been kept, and 5,160 (SE = 735) were reported to have been released. Catch per hour was highest in June, with 0.7109 (SE = 0.1168) being reported on June weekdays, and 0.5632 (SE = 0.0826) being reported on June weekends. July weekdays had a catch rate of 0.1636 (SE = 0.0449), and July weekends had a catch rate of 0.1899 (SE = 0.0623). Catch rates declined dramatically in August, with a catch rate of 0.0041 (SE < 0.001) on weekdays and 0.0071 (SE < 0.001) on weekend days. This was likely due to the much warmer water temperatures in the late summer.

Lengths and heads were collected from fish harvested during the creel surveys through the month of June and the beginning of July. Fish were identified to strain by extracting the coded wire tag from each individual. Five hundred eighty-nine fish were collected with tags. Every third fish collected was submitted for myxospore testing to the Aquatic Animal Health Lab. The ratios of the various strains in the fish collected during the creel survey were used to extrapolate total harvest by strain over the survey period.

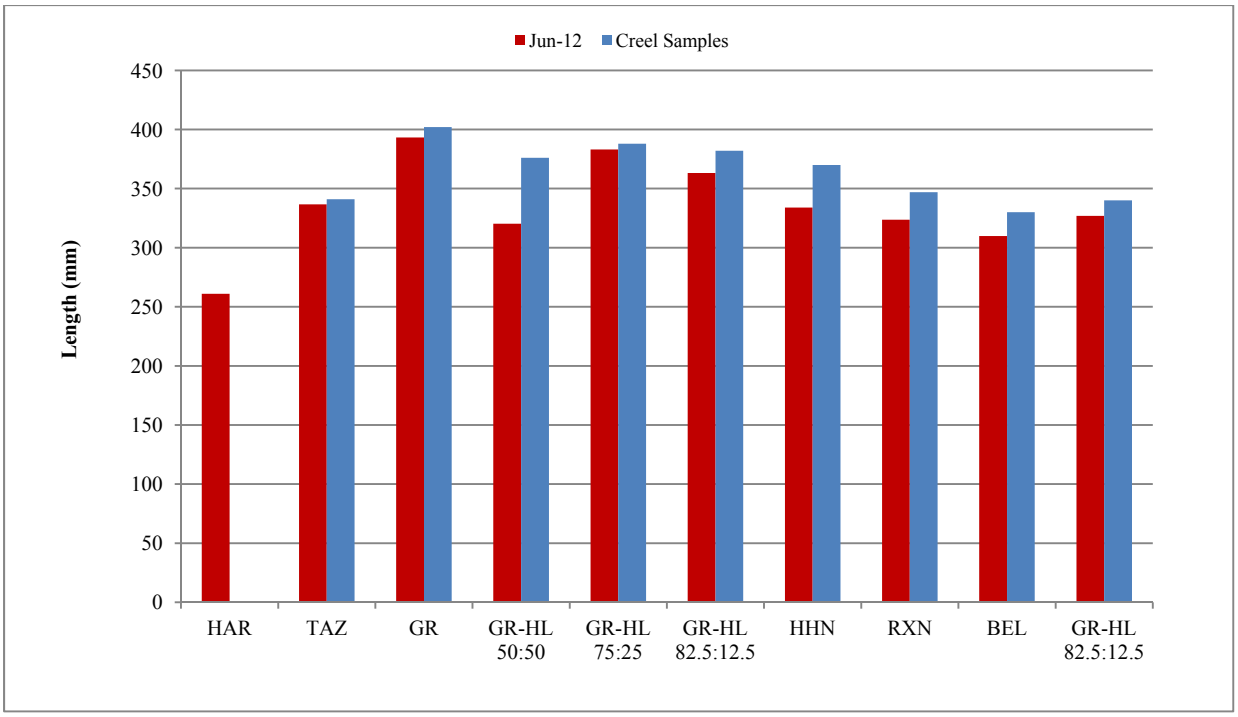

Figure 3.16. Length of fish immediately prior to stocking in Douglas Reservoir, and lengths of fish collected during creel survey efforts in June and July, 2012. Only one Harrison Lake variety fish was found in the June 2012 (pre-stocking) collection.

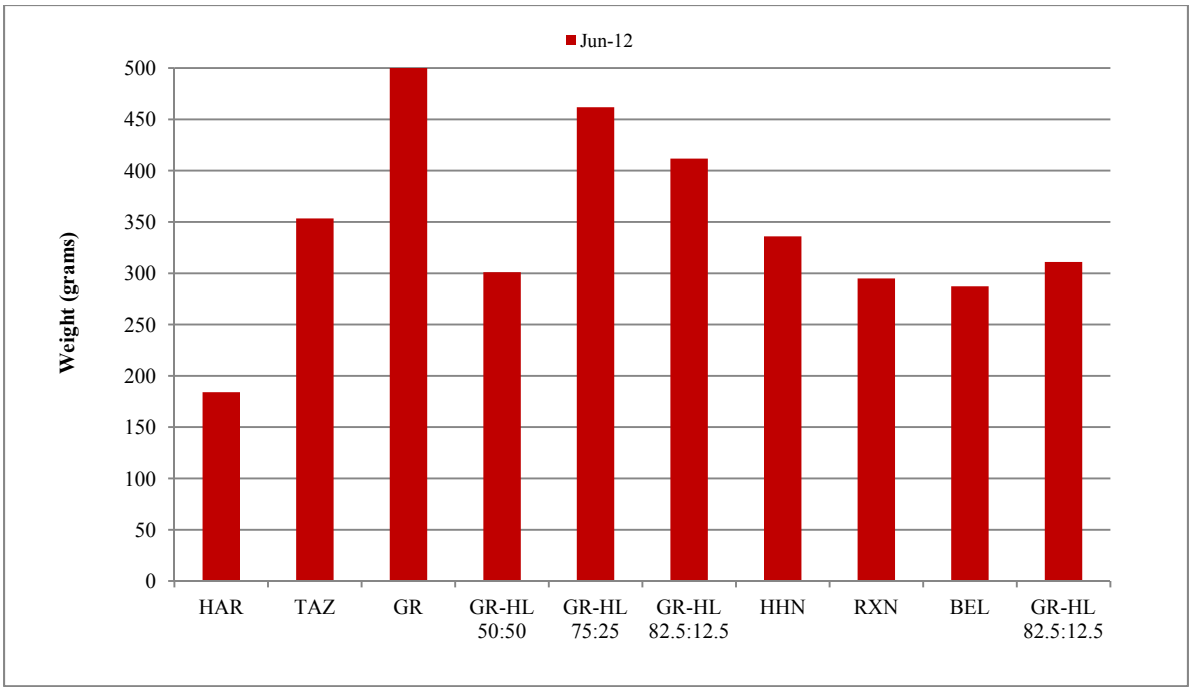

Figure 3.17. Weight of fish immediately prior to stocking in Douglas Reservoir. Weights were not evaluated in the post-stocking creel survey.

Final myxospore counts for the third phase of the experiment (pre-release and creeled fish) are presented in Figure 3.18. Note that the graphs provided herein are slightly different than those provided in the 2013 report (Fetherman et al. 2013) due to the previous omission of a single Harrison Lake rainbow trout found in the June 2012 samples just prior to stocking. The results for the Harrison Lake fish should be interpreted with caution, as they do represent only one individual in this sample.

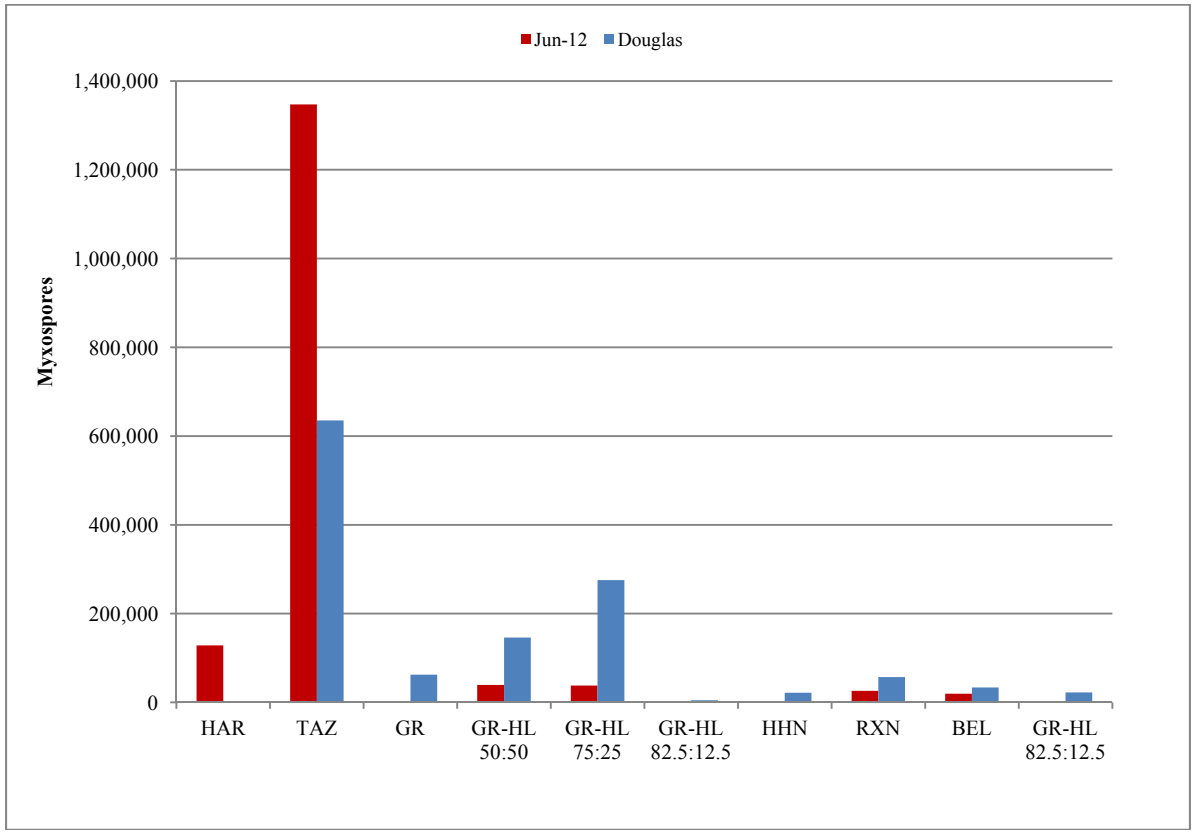

Figure 3.18. Myxospore counts among fish immediately before release to Douglas Reservoir (36 months post-exposure), and for fish captured during the creel survey period. The results for the Bellaire (BEL) and GR×HL (82.5:12.5) groups on the far right of the graph are 20 months post-stocking into the infected environment as clean catchables. Only one Harrison Lake variety fish was found in the June 2012 (pre-stocking) collection.

End-of-season electrofishing, gill netting, and trap netting was conducted in Douglas Reservoir in October, 2012 to determine which varieties were remaining after the intensive harvest period. No marked rainbow trout from this experiment were found in 30 minutes of boat electroshocking on October 2, 2012, or in a 20 hour trap net and 20 hour gill net set the following day. Water temperatures in Douglas Reservoir are very high in the late summer (Figure 3.19), precluding survival of fish that had not been caught by anglers.

Totals of fish stocked and subsequently harvested for each of the strains in Phase III at Douglas Reservoir are provided in Figure 3.20. High proportion GR crosses and HHN reared at Poudre Ponds in Phase I and II had the highest return of the varieties evaluated.

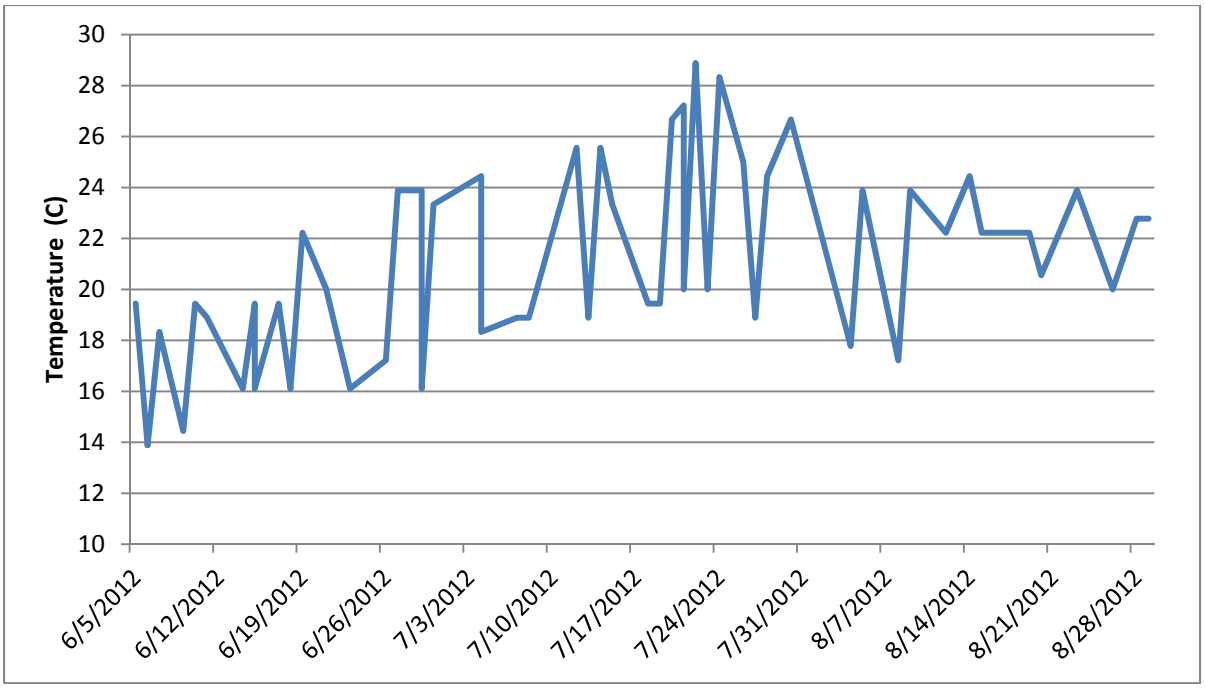

Figure 3.19. Water temperature in Douglas Reservoir, June through August, 2012.

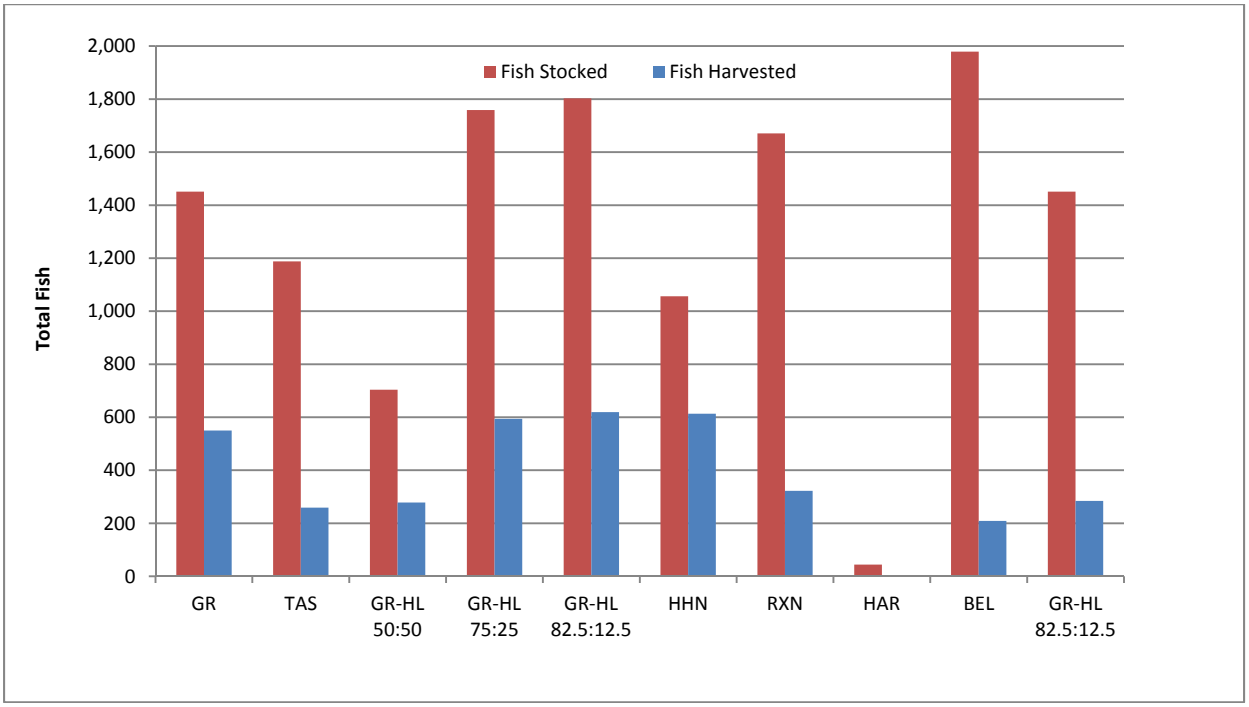

Figure 3.20. Fish stocked and harvested in Douglas Reservoir. The Bellaire and GR×HL (82.5:12.5) groups on the right end of the graph are those brought to the Poudre Ponds as clean catchables. No Harrison Lake variety fish were found in the harvest collections.

Table 3.5. Total number of fish stocked by strain, based on samples collected prior to stocking. Total fish harvested, based on creel survey results. Average spore counts and total spores contributed to the system based on samples collected prior to stocking.

STRAIN	Total Stocked	Percent Harvested	Total Harvested	Average Spore Count	Total Spores Contributed
GR	1,451	38	550	28,511	25,687,760
TAS	1,188	22	259	820,407	761,991,090
GR-HL 50:50	704	40	278	134,212	57,151,438
GR-HL 75:25	1,759	34	594	126,301	147,105,959
GR-HL 82.5:12.5	1,803	34	620	10,777	12,753,921
HHN	1,056	58	613	12,545	5,554,650
RXN	1,671	19	322	38,029	51,285,199
HAR	44	0	0	128,288	5,644,672
BEL	1,979	11	209	33,458	59,232,385
GR-HL 82.5:12.5	1,451	20	284	22,349	26,069,736

Table 3.6. Normalized spore contribution results based on equal stocking numbers, using percent harvest and average spore count from this experiment.

STRAIN	Total Stocked	Percent Harvested	Total Harvested	Average Spore Count	Total Spores Contributed
GR	1000	38	379	28,511	17,703,487
TAS	1000	22	218	820,407	641,406,641
GR-HL 50:50	1000	40	395	134,212	81,181,019
GR-HL 75:25	1000	34	338	126,301	83,630,449
GR-HL 82.5:12.5	1000	34	344	10,777	7,073,722
HHN	1000	58	581	12,545	5,260,085
RXN	1000	19	193	38,029	30,691,322
HAR	1000	0	0	128,288	128,288,000
BEL	1000	11	105	33,458	29,930,462
GR-HL 82.5:12.5	1000	20	196	22,349	17,966,738

Weight and length gains were superior in the GR and high proportion GR crosses compared to the other strains in every phase of this experiment. This trend has been observed in many of our previous lab experiments, reinforcing the body of evidence for excellent growth rates in the GR crosses. The high proportion GR and HHN varieties raised in an infected environment from the outset of the experiment had the highest returns to creel, demonstrating that GR crosses are excellent for put-and-take fisheries. This was especially true of the HHN variety, of which 58% were harvested.

While total catch in this reservoir was relatively high, the majority of caught fish were returned to the water. The contribution of myxospores to a system due to the stocking event is the final endpoint to be evaluated. We can arrive at this value for each strain by estimating the fish that are not harvested and multiplying by their average myxospore count. Average myxospore counts used in this analysis are the overall average of myxospore counts in fish collected in the June 2011, October 2011, June 2012, and Douglas Reservoir creel samples (Table 3.5). With 13,107 fish stocked, and only 3,730 harvested, 9,377 fish of the various strains potentially died in the

reservoir without being harvested. The overall contribution of the various strains to spore loading in the system varies widely depending on harvest rate and average myxospore count.

Calculations can also be made to compare relative contribution of spore counts to the system given hypothetical equal stocking rates of 1,000 per each strain (Table 3.6). This exercise provides a more easily interpreted evaluation of the potential effects of stocking the various strains of fish. The HHN strain had both low spore counts and high return to creel, resulted in a very low contribution of spores to the system under both scenarios. Tasmanian strain fish, with poor returns and high spore counts, contributed a very high number of spores to the system.

This exercise can be repeated given a wide variety of circumstances to predict myxospore burden added to a system where myxospore load of stocked fish and harvest are known. The underlying question of the differences between stocking fish exposed to whirling disease versus stocking clean catchable-sized fish is very site and circumstance specific. Resistant strains of fish do in fact compare quite well with susceptible fish reared in environments free of the parasite. However, the rate of harvest and specific variety of fish in question weighs heavily on the final outcome. Strain such as the pure GR, GR×HL (82.5:12.5) strain or HHN are the varieties consistently providing low spore burdens that would be relatively equivalent over the long term, whether reared in whirling disease positive or negative environment prior to stocking. However, if the goal is to protect environments from further myxospore loading, the best alternative is to use resistant strain fish reared in a whirling disease negative environment. These fish require at least 3.5 months for myxospores to develop after they are stocked. A large proportion of the fish are likely to be harvested before development of mature myxospores, which greatly reduces the potential for perpetuating the parasite in these systems. If fish are anticipated to remain in these systems over long periods of time, and the locations are already heavily infected with the parasite, there may be some merit in utilizing resistant fish reared in positive environments relative to cost of production and risk of parasite amplification associated with those locations.

REFERENCES

Fetherman, E. R., and G. J. Schisler. 2013. Sport Fish Research Studies. Federal Aid Project F-394-R12. Federal Aid in Fish Restoration Job Progress Report. Colorado Parks and Wildlife. Aquatic Research Section. Fort Collins, CO. 132 pp.

Job No. 4 Whirling Disease Resistant Wild Strain Establishment, Brood Stock Development and Evaluations

Job Objective: These experiments are designed to establish, develop, and evaluate "wild" strain whirling disease resistant rainbow trout for reintroduction into areas where self sustaining populations have been lost due to whirling disease.

Upper Colorado River

INTRODUCTION

The upper Colorado River downstream of Windy Gap Reservoir is known to be one of the most heavily infected river segments with whirling disease in the state of Colorado. The 26 km (16.2 mi) reach, downstream of the reservoir to the Kemp-Breeze State Wildlife area (Figure 4.1) has been an area of particular interest with respect to whirling disease investigations. Historically, prior to the introduction of whirling disease, this area had been used as a source of eggs to maintain Colorado River Rainbow (CRR) trout brood stock. However, since the introduction of whirling disease, no natural recruitment of rainbow trout has occurred in the upper Colorado River, leading to severe population declines (Figure 4.2).

Figure 4.1. Upper Colorado River study area.

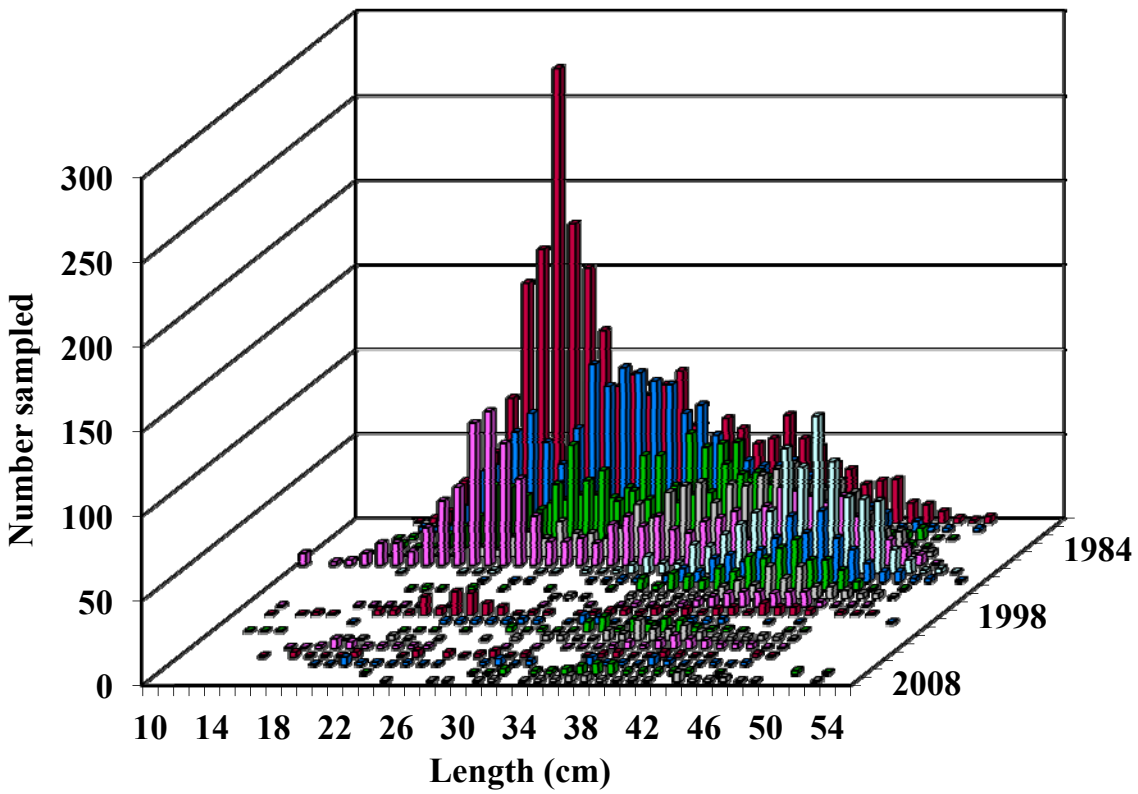

Figure 4.2. Upper Colorado River historic rainbow trout length-frequencies at Kemp-Breeze State Wildlife Area.

Adult GR×CRR Introductions

Whirling disease resistant rainbow trout introductions (adult Hofer [GR] × Colorado River Rainbow [CRR], known as GR×CRR; > 150 mm) first occurred in the upper Colorado River in June of 2006, with a second introduction occurring in January of 2009, and a third introduction occurring in June of 2010. Following these introductions, the population in the upper Colorado River, specifically within the Chimney Rock/Sheriff Ranch study area, was monitored on a yearly basis. Adult population estimates were conducted in the spring to determine the abundance and survival rate of the stocked GR×CRRs. In addition, fry shocking was used to evaluate the rainbow trout and brown trout fry populations in the upper Colorado River, and to determine if rainbow trout offspring were being produced by the stocked GR×CRRs. The majority of this work was conducted as part of a Ph.D. project through Colorado State University (CSU) and has since been published (Fetherman et al. 2014).

In summary, apparent survival of the introduced GR×CRR over the entire study period (2007 to 2011) was estimated to be 0.007 (± 0.001), and the most recent population estimates conducted in 2011 estimated that there were less than ten adult GR×CRR remaining in the study section. Despite low survival of the GR×CRRs, age-0 progeny of the GR×CRR were encountered in all

years of the study. Genetic assignments revealed a shift in the genetic composition of the rainbow trout fry population over time, with CRR fish comprising the entirety of the fry population in 2007, and GR-cross fish comprising nearly 80% of the fry population in 2011. A decrease in average infection severity (myxospores fish^{-1}) was observed concurrent with the shift in the genetic composition of the rainbow trout fry population, decreasing from an average of 47,708 (± 8,950) myxospores fish^{-1} in 2009 to 2,672 (± 4,379) myxospores fish^{-1} in 2011. Results from this experiment suggested that the GR×CRR could survive and reproduce in rivers with a high prevalence of *M. cerebralis*, although survival was low. In addition, reduced myxospore burdens in the age-0 fish indicated that stocking this cross may ultimately lead to an overall reduction in infection prevalence and severity in the salmonid populations of the upper Colorado River. Despite these positive results, a self-sustaining rainbow trout population was still not present in the upper Colorado River at the end of this introduction experiment. Therefore, other management options needed to be explored to increase resistant rainbow trout survival and recruitment.

GR×CRR Fry Introductions

Although reproduction was occurring, and the fry being produced were better able to survive exposure to whirling disease in the upper Colorado River, the numbers of fry surviving through the fall were still fairly low. As a result, recruitment to the adult population was low and the rainbow trout population as a whole was expected to exhibit a very slow rate of increase, if at all. Therefore, we initiated a project introducing whirling disease resistant rainbow trout (GR×CRR) fry into the Chimney Rock/Sheriff Ranch section of the river, an approach that has shown promising results, both in terms of fry survival and recruitment to the adult population, in the Colorado River below Byers Canyon.

Prior to the fry introduction experiment initiated in the Chimney Rock/Sheriff Ranch study section in 2013, GR×CRR fry were introduced to the upper Colorado River below Byers Canyon, from the Paul Gilbert State Wildlife Area downstream to below the Kemp-Breeze State Wildlife Area. In 2010, 2011, and 2012, up to 200,000 rainbow trout fry were stocked in this section of the river in late July or early August. As a result, the rainbow trout fry population exceeded the brown trout fry population in the months following their introduction. Although abundance was reduced in the fall, similar numbers of rainbow trout and brown trout fry were encountered in these lower study sections in October of each of these years. In addition, the number of rainbow trout fry remaining in October was up to five times higher than the numbers of naturally produced fry remaining in the Chimney Rock Ranch section of the river.

As a result of these fry introductions, and the increased survival rates of the introduced fry, these fish began recruiting to the adult (≥ 6”) population, with an increase from 71 adult rainbow trout per mile in 2010 to 306 in 2012. Additionally, results from this section suggested that the GR×CRR fry exhibit extraordinary growth rates, gaining an average of up to six inches each year post stocking. For example, during the September 2012 population estimates in the Parshall-Sunset reach of the Colorado River, a large number of the fish stocked in 2011 appeared in the population estimate as average 9” in length, with the fish stocked in 2010 appearing in the population sample between 12 and 14” in length (Figure 4.3).

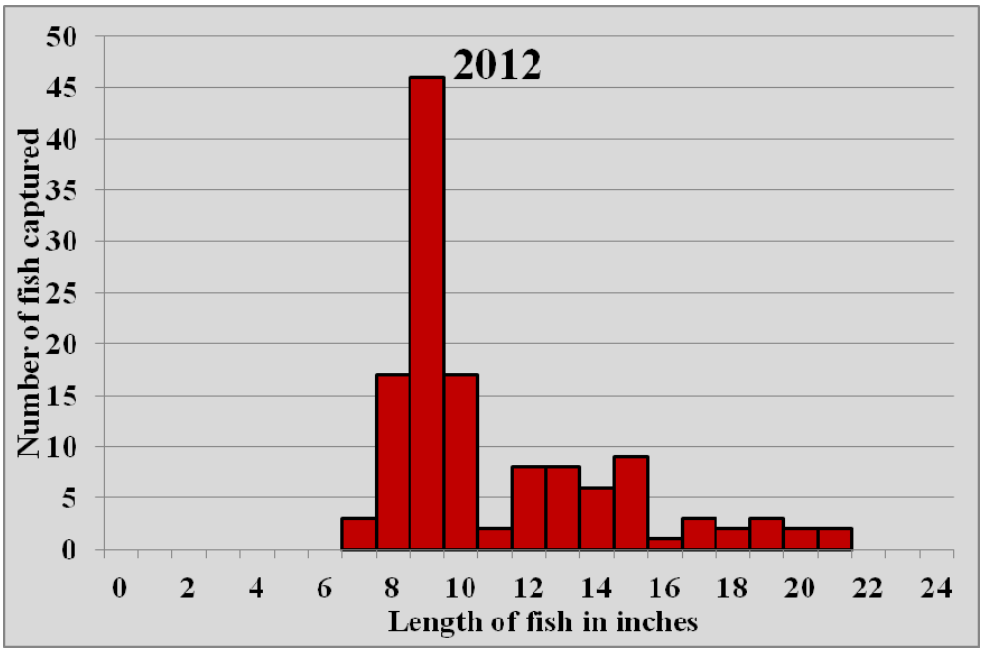

Figure 4.3. Number of rainbow trout captured in each length class in the Parshall-Sunset reach of the upper Colorado River in 2012.

CURRENT RESULTS

The GR×CRR rainbow trout fry introduction experiment is still in the early stages of completion, with a project end date of spring 2016. However, sampling of both the adult and fry populations occurred in 2013 and 2014. The summary below provides the most current information regarding the populations in the upper Colorado River. Additional population sampling data and implications will be presented in future reporting cycles.

Adult Salmonid Population

The adult salmonid population in the upper Colorado River was sampled in April 2013 to provide a baseline estimate of the adult population prior to GR×CRR fry introductions. Unfortunately, low flow conditions precluded a recapture run from being accomplished in 2013, so only count data, not estimates, are available for that year. A total of 464 adult brown trout and 12 rainbow trout were captured during the estimates, suggesting that rainbow trout numbers continued to remain low following the adult introduction experiments that concluded in 2012.

A full population estimate was conducted in May 2014, although flow conditions again presented a challenge as the river was higher than in any other year in which estimates were conducted. As such, the data for the 2014 population estimates is biased towards larger fish that were both easier to see through the low water clarity, and easier to catch during high flows. An estimated (± SD) 1,958 (± 218) adult brown trout, averaging 318 (± 68) mm total length (TL) and 334 (± 175) g, and 65 (± 35) adult rainbow trout, averaging 372 (± 63) mm TL and 550 (± 179) g, were present in the 3.9 mile Chimney Rock/Sheriff Ranch study section. Although conditions were not conducive to capturing smaller rainbow trout, one rainbow trout 88 mm TL was captured

during the estimates, representing the first documented recruitment in this section of the upper Colorado River since whirling disease resistant rainbow trout introductions began in 2006.

Salmonid Fry Population

The salmonid fry population in the upper Colorado River was sampled once a month, June through October in 2013. The June sample provided a baseline of the number of naturally occurring rainbow trout fry in the river prior to stocking the GR×CRR fry, which occurred in July 2013. On July 16, 2013, approximately 100,000 rainbow trout fry each were introduced to the upstream half of the Chimney Rock Ranch study section, and the reference section below Byers Canyon. The July through October samples were used to examine the post-stocking survival of the introduced GR×CRR fry.

Although this current study focuses on the survival of the GR×CRR fry introduced to the Chimney Rock/Sheriff Ranch study section, GR×CRR fry have been stocked on an annual basis below Byers canyon, and as such, three reference sites below Byers Canyon were used to compare survival in the two stocked sections of the river. Sampling sites (n = 3) below Byers Canyon include the Kemp-Breeze, Lone Buck, and Paul Gilbert State Wildlife Areas, and sampling sites (n = 4) in the Chimney Rock/Sheriff Ranch study section include the Sheriff Ranch, upper and lower Red Barn, and the Hitching Post Bridge.

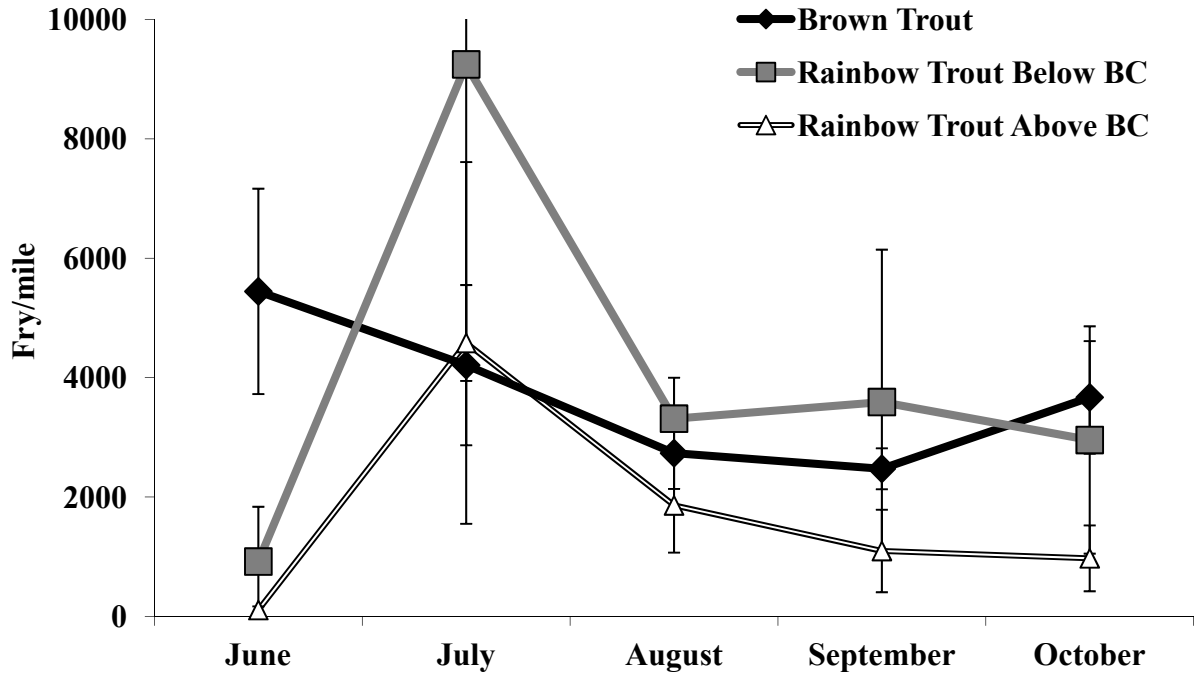

Figure 4.4. Upper Colorado River brown trout density estimates (fry/mile; SE bars), and rainbow trout density estimates above and below Byers Canyon (BC), for the months of June to October 2013. Note that these estimates represent the total number of fry per mile, including both sides of the river.

Fry density estimates were calculated using the three-pass removal equations of Seber and Whale (1970). Brown trout fry densities were highest in June 2013, with an estimated 5,444 (± 1,720) brown trout per mile. However, brown trout densities did not change by much throughout the summer and into the fall, with an estimated 3,668 (± 942) brown trout per mile still present in October 2013. Prior to the introduction of the GR×CRR fry, an estimated 917 (± 917) and 105 (± 60) naturally produced rainbow trout fry were present per mile below and above Byers Canyon, respectively. Rainbow trout fry densities peaked in July, following the introduction of GR×CRR to the Chimney Rock Ranch study section and reference section below Byers Canyon, with an estimated 9,247 (± 5,303) rainbow trout fry per mile below Byers Canyon, and an estimated 4,580 (± 3,030) rainbow trout fry per mile in the Chimney Rock/Sheriff Ranch study section above Byers Canyon. Following a large, initial decline between July and August 2013, rainbow trout densities remained fairly stable through late summer into the fall, with final estimates of 2,954 (±1,904) rainbow trout fry per mile Below Byers Canyon and 971 (± 551) rainbow trout fry per mile above Byers Canyon in October 2013. In the months of August through October, rainbow trout fry densities did not differ from brown trout fry densities.

A maximum of five brown trout and five rainbow trout fry were collected from each sampling site in October 2013 to estimate infection rates. Brown trout averaged 3,362 (± 1,393) myxospores per fish, whereas rainbow trout averaged 4,936 (± 3,705) myxospores per fish throughout the upper Colorado River. Myxospore counts differed in rainbow trout below versus above Byers Canyon, with rainbow trout below Byers canyon averaging 339 (± 339) myxospores per fish, and rainbow trout above Byers Canyon averaging 8,384 (± 3,210) myxospores per fish.

CONCLUSIONS

Adult rainbow trout densities in the upper Colorado River were higher in 2014 than they had been in previous years, suggesting that either naturally produced rainbow trout had begun recruiting to the adult population, or that the population was supplement by rainbow trout moving into the Chimney Rock/Sheriff Ranch study section from other locations in the river. The inclusion of the juvenile rainbow trout in the adult population estimates suggests that at least some of the GR×CRR fry stocked in 2013 overwintered in the section and had begun recruiting to the adult population. This represents the first recruitment observed in this section of the river since whirling disease resistant rainbow trout began being introduced to the river in 2006.

Stocking GR×CRR fry had a positive effect on the rainbow trout fry populations in the Chimney Rock/Sheriff Ranch study section. Rainbow trout fry densities in the Chimney Rock/Sheriff Ranch study section in October 2013 were much higher than they have been in previous years when natural production was the only source of rainbow trout fry in the section. In previous years, rainbow trout densities in October rarely exceeded 100 fry per mile, whereas over 900 fry per mile were present in the section in 2013. This suggests that stocked GR×CRR were surviving through the fall, and had the potential to recruit to the adult population in 2014. Sampling in future years will help confirm whether the stocked GR×CRR are overwintering in the Chimney Rock/Sheriff Ranch study section, and whether these fish are recruiting to the adult population. This data will be available in future reporting cycles.

East Portal of the Gunnison River H×C Brood Stock

INTRODUCTION

The East Portal of the Gunnison River is currently being managed as a wild brood stock location for the H×C rainbow trout. H×C fingerlings have been stocked in the East Portal of the Gunnison River every year since 2006. In 2009, a population estimate was conducted in the East Portal to determine the size and age distribution of the introduced rainbow trout. In 2011, 60 rainbow trout were collected for a disease inspection. Fins were collected from all 60 age-1 fish used for the disease inspection. In addition, fins were collected from adult fish (ranging in size from 150 to 510 mm) captured during the electrofishing efforts used to obtain the 60 fish disease sample. Finally, the shoreline just downstream of the boat ramp was shocked, and fin clips were obtained from the 40 rainbow trout fry encountered.

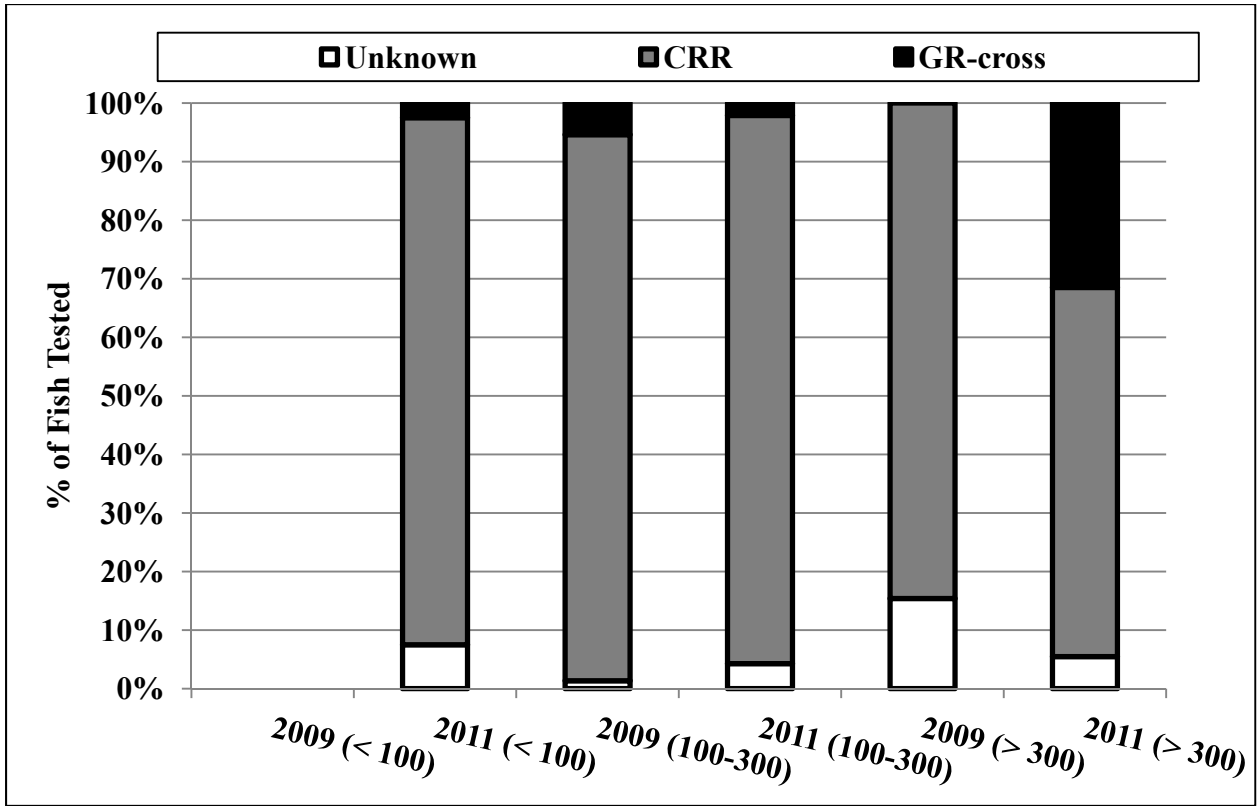

Figure 4.5. Percent of fry (< 100 mm), juvenile, and adult (100-300 and > 300 mm) rainbow trout, encountered during the East Portal of the Gunnison River population estimate in 2009 and disease inspection in 2011, categorized as unknown, pure CRR, and GR-cross fish.

Less than 3% of the fry encountered in 2009 were identified as GR-cross fish, with the majority of the fry encountered (90%) identified as pure CRR. In the 100-300 mm size class, GR-cross fish only comprised 5% or less of the population in 2009 and 2011; the majority of the fish in this size class (> 90%) were identified as pure CRR. In 2009, none of the fish encountered over 300 mm were identified as GR-cross fish. However, over 30% of the rainbow trout greater than 300 mm in length encountered in 2011 were identified as GR-cross fish (Figure 4.5).

The genetic results described above were unexpected for this location. GR-cross fish had been the only rainbow trout stocked into the East Portal of the Gunnison River since 2006 in an effort to create a wild GR-cross brood stock. However, even with the 2011 results for the 300+ mm size class showing an increase of GR-cross fish in the population, the population as a whole could not be classified as a GR-cross brood stock. Therefore, egg collection for hatchery production, which was scheduled to begin in 2012, was postponed until further research could be conducted on the genetic and resistance characteristics of the East Portal rainbow trout.

In 2012, eggs were collected from the East Portal rainbow trout during the spring spawning season. The objectives of this experiment were to determine which strains of rainbow trout were spawning in the East Portal of the Gunnison River, and to determine if offspring produced by these fish exhibited increased resistance characteristics when exposed to *Myxobolus cerebralis* in the laboratory.

SPAWNING AND REARING

Rainbow trout in the East Portal were captured via boat electrofishing unit at three time points within the spawning period: 1) April 17, 2012, 2) May 1, 2012, and 3) May 15, 2012. Eggs were collected over these three time periods to obtain a range of families over the course of the spawning period in case CRR or GR-cross fish attained spawn-ready status at different times. On each spawning occasion, fish were captured the day prior to the spawn, separated by gender, and held in two live cages overnight. Fish were spawned in the morning of the dates listed above. Following spawn, eggs were water hardened in five gallon water coolers for one hour; eggs were also disinfected using iodine during water hardening. Once eggs had water hardened, the iodine was rinsed out of the coolers, and clean water was added to the coolers for transport to the CPW Aquatic Toxicology Lab in Fort Collins, Colorado. In the Aquatic Toxicology Lab, eggs were held at different temperatures so that eggs collected at each of the time points would hatch at the same time. Fish were reared in the Aquatic Toxicology Lab until swim-up.

One hundred and twenty rainbow trout were captured via electrofishing on April 16 and 17 for spawning and genetic sample collection. Of these, 102 were green or spent females (69), or immature fish (33); genetic samples were collected from all of these fish to determine whether they were pure CRR or GR-cross fish. Of the 69 females, 65 were green and only four were spent. These fish averaged 408 mm in length, ranging from 297 to 557 mm. Four fish were used to create "Group 1" for the exposure experiment. Group 1 consisted of two male-female pairs. The first pair was a 496 mm female spawned a 527 mm male. The second pair was a 416 mm female spawned with a 415 mm male. Genetic samples were taken from each of the fish for comparison to offspring genetics following the exposure experiment. The remaining eight fish captured were ripe males. These fish were not used during the spawning operations, and no genetic samples were collected from these fish. Upon arriving at the Aquatic Toxicology Lab, eggs were held at a temperature of 6.9°C to prolong hatching, so that these fish and fish collected later in the spawning period would hatch at the same time.

Fifty eight rainbow trout were captured via electrofishing on April 30 and held in net pens overnight for spawning. Of these, 44 (76%) were females and 14 (24%) were males. Of the females, ten (23%) were ripe, 24 (55%) were green, and ten (23%) were spent. All of the males

were ripe. Four groups were created using the ripe males and females. All groups consisted of two male-female pairs. Group 2 consisted of a 437 mm female spawned with a 495 mm male, and a 425 mm female spawned with 415 mm male. The 425 mm female was a previously green female that had been captured and from which genetic information had been collected on April 17 (evidenced by the fin clip on the upper caudal fin). Group 3 consisted of a 445 mm female spawned with 378 mm male, and a 507 mm female (recapture; previously green) spawned with a 430 mm male. Group 4 consisted of a 468 mm female spawned with a 440 mm male, and a 411 mm female spawned with a 483 mm male. Group 5 consisted of a 507 mm female (recapture; previously green) spawned with a 373 mm male, and a 435 mm female spawned with a 362 mm male. The first female used to create this group was mostly spent, containing only a few eggs. The eggs were discarded and not included in the group; however, a genetic sample was collected from this fish. Similarly, the first male used to create this group did not produce enough milt for fertilization. The milt was discarded and not included in the group. However, a genetic sample was collected from this fish. Five ripe males were remaining following the spawning operations. A genetic sample was collected from each, and the fish were returned to the river. Upon arriving at the Aquatic Toxicology Lab, eggs were held at a temperature of 9.2°C.

In addition to the spawn, 60 rainbow trout and 60 brown trout were collected for PTD sampling on April 30. Genetic samples were collected from the rainbow trout, and genetic sample number and head number were paired to facilitate matching of myxospore count to strain.

Two hundred five rainbow trout were captured via electrofishing on May 15. Of these, 102 (50%) were females, 20 (10%) were males, and 83 (40%) were immature. Of the 102 females, 30 (29%) were green, 11 (11%) were ripe, and 61 (60%) were spent. Of the 20 males, 18 (90%) were ripe, and two (10%) were spent. All ripe females and males were kept in separate net pens for spawning. All green, spent, and immature fish were returned to the river. Two groups were created using ripe males and females. Group 6 consisted of a 426 mm female spawned with a 397 mm male, and a 378 mm female spawned with a 286 mm male. The first female spawned was mostly spent, and the few remaining eggs were overripe. The eggs were discarded and not included in the group but, a genetic sample was collected from this fish. A third pair of fish was then spawned to created eggs for this group: a 440 mm female spawned with a 248 mm male. Only a small number of eggs were produced by this female, and though they looked good, it was decided that another female should be used to obtain more eggs. The next female/male combination produced a high number of quality eggs, which were retained to make up the remainder of group 6. Group 7 consisted of a 516 mm female spawned with a 292 mm male, and a 437 mm female spawned with a 349 mm male. Eight ripe males were remaining following the spawning operations. A genetic sample was collected from each, and the fish were returned to the river. Upon arriving at the Aquatic Toxicology Lab, eggs were held at a temperature of 15.5°C.

Eggs from all groups began to hatch on June 4. By June 9, all groups had finished hatching. All groups were maintained in the Aquatic Toxicology Lab through swim-up; fish were transported from the Aquatic Toxicology Lab to the Parvin Lake Research Station on July 16 for the *Myxobolus cerebralis* exposure experiment. No mortalities occurred during transport.

MYXOBOLUS CEREBRALIS EXPOSURE EXPERIMENT

The seven groups were maintained in separate 76-L flow through tanks within the Parvin Lake Research Station Lab. One week prior to exposure to *Myxobolus cerebralis*, family groups were split into control tanks and exposure tanks; numbers of fish were reduced to 25 fish per tank. Tanks containing control fish were maintained in a separate row from the exposure tanks so that no cross contamination could occur during the exposure experiment.

Unfortunately, the *Tubifex tubifex* worm cultures maintained at the Parvin Lake Research Station did not produce any triactinomyxons for the exposure experiment. As a result, exposure fish were transported from their tanks at the Parvin Lake Research Station to the CPW Poudre Rearing Unit for exposure. Fish were put in 3-in diameter PVC cages, designed to allow water to flow in through a grate in the top of the cages and out of the bottom of the tube, which was covered with fine mesh netting to prevent fish escape. Cages were placed in the inlet of Pond 5, which receives water from the Cache la Poudre River, known to be a *Myxobolus cerebralis*-infested water source. Fish remained in the cages in Pond 5 for one month prior to being transported back to the Parvin Lake Research Station. Control and exposure fish were held at the Parvin Lake Research Station through May 2013 to allow full development of myxospores within the exposed fish.

On May 9, 2013, all remaining rainbow trout within the control and exposure tanks were sacrificed using an overdose of MS-222. Lengths, weights, and signs of infection (cranial, spinal, lower jaw, and opercular deformities, and blacktail) were recorded from each individual. Heads were removed, placed in individually labeled bags, and sent to the Brush Fish Health Lab for myxospore enumeration using the Pepsin-Trypsin Digest method. Fin clips were also taken from each individual to determine genetic background relating to the parents spawned in the East Portal in the spring of 2012.

RESULTS AND DISCUSSION

Exposed fish in the *Myxobolus cerebralis* exposure experiment averaged 17,028 (± 7,671) myxospores per fish. In addition, all rainbow trout spawned to create the family groups used in the *Myxobolus cerebralis* exposure experiment were found to be pure CRR individuals. As such, all offspring contained within the exposure experiment were also found to be pure CRR. This was unexpected as the East Portal of the Gunnison River has only been stocked with GR-cross fish since 2006. Knowing this, myxospore counts were very low for pure CRR fish compared to other exposure experiments where myxospore counts averaged over 100,000 myxospores per fish (Schisler et al. 2006; Fetherman et al. 2012).

The genetic test also suggested that there is some amount of differentiation between the pure CRR individuals encountered in the East Portal, and hatchery CRR stocks that had been used in 2008-2010 to develop the GR versus CRR differentiation test. The CRR in the Gunnison River have maintained a self-sustaining rainbow trout population despite the presence of *Myxobolus cerebralis,* although, infection levels in the East Portal are lower than many other rivers in Colorado, and were never high enough to result in a collapse in the East Portal rainbow trout population. The combination of low infection levels and natural recruitment in this location

created conditions that may be leading to the development of *Myxobolus cerebralis*-resistance in the East Portal CRR population. The myxospore count results support this conclusion. However, the results of this experiment were confounded by the fact that exposure rates at the Poudre Hatchery were unknown compared to previous exposure experiments where fish were exposed to 2,000 TAMs per fish. Therefore, we cannot currently determine if myxospore counts were low due to exposure rates or the development of resistance.

Because the results of this experiment were inconclusive, a second experiment was initiated in 2014 to determine if natural resistance has developed in the East Portal CRR. On May 2, 2014, ten families were spawned in the East Portal using the same techniques described above. Eggs were transported back to Fort Collins and hatched in the CPW Aquatic Toxicology Laboratory. Sac-fry were transported to Parvin Lake for use in this exposure experiment on June 13, 2014, where they were reared for approximately 650 degree-days prior to exposure, at which point each family was divided into control and exposure tanks and reduced to 20 fish per tank. Due to the poor condition of two families consisting of a high number of fry that did not transition to feed upon swim-up, eight families were used in the 2014 exposure experiment, along with two control families of the Puget Sound rainbow trout strain obtained from Troutlodge, Inc. (Sumner, WA). All ten exposure families were exposed to a dose of 2,000 TAMs per fish on July 28, 2014. Triactinomyxons were obtained from worm cultures maintained at the Parvin Lake Research Station. Fish will be reared for approximately eight months to ensure the full development of myxospores. Myxospore counts and genetic results for this experiment should be available in the next reporting cycle.

The results of this experiment, and the genetic testing that occurred in 2011, suggest that the GR-cross fish are not surviving well in the East Portal, and are not contributing to the offspring being naturally produced in the river. As such, we are suggesting that this location not be considered as a wild GR-cross brood stock location at this time. However, if it is determined that the CRR in the East Portal have developed some natural resistance to *Myxobolus cerebralis*, this may be considered a wild CRR brood stock in the future.

Lake Catamount H×H Brood Stock

Hofer × Harrison Lake (H×H) rainbow trout crosses have been stocked into Lake Catamount and the Yampa River near Steamboat Springs since 2007 with the objectives of reducing infection levels within the Yampa River and establishing a wild H×H brood stock in Lake Catamount. Previous exposure experiments have shown a reduction in infection severity in the rainbow trout in the Yampa River and its tributaries between 2002 (no H×H present in the system) and 2010 (three years post-introduction of H×H to the system). In addition, H×H stocked into Harrison Creek, a tributary to Lake Catamount, have exhibited a fidelity to Harrison Creek during the spawning period, suggesting that a wild egg take from the fish returning to Harrison Creek could be used to replace hatchery brood stocks of H×H in Colorado hatcheries.

An exposure experiment, similar to that conducted on the East Portal of the Gunnison River H×C brood stock, is being used to assess the resistance characteristics of the offspring produced by fish returning to Harrison Creek to spawn. In May 2013, rainbow trout were captured in Harrison Creek via electrofishing to obtain eggs for an exposure experiment. Five family groups

were created from the fish in Harrison Creek, each consisting of two male-female pairs. In addition, three families groups were created using rainbow trout (presumed to be H×Hs) captured via trap nets in Lake Catamount that had not run up Harrison Creek. All eight family groups were spawned on the same day and transported back to the Aquatic Toxicology Lab in Fort Collins for rearing. Eggs were maintained at 12°C and held until they eyed up. Upon eye up, eggs were transported to the Parvin Lake Research Station where they hatched.

The fish were reared until they reached 650 degree-days post-hatch. At that time, family groups were split into control and exposure tanks. Fish within the exposure tanks were exposed to a dose of 2,000 triactinomyxons per fish. Triactinomyxons were obtained from worm cultures maintained at the Parvin Lake Research Station. Following exposure, fish were held for approximately nine months to allow full development of myxospores. Similar to the East Portal exposure experiment, fish were euthanized at the end of the experiment with an overdose of MS-222. Heads were sent to the Brush Fish Health Lab for myxospore enumeration, and genetic samples were sent to the Genomic Variation Laboratory at the University of California Davis to determine and compare the genetic backgrounds of the offspring to the parental brood stock in Lake Catamount. At the time of this report, myxospore enumeration and genetic testing had not been complete for any of the families in the experiment. Results will be available within the next reporting cycle.

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Fetherman, E. R., D. L. Winkelman, G. J. Schisler, and M. F. Antolin. 2012. Genetic basis of differences in myxospore count between whirling disease-resistant and -susceptible strains of rainbow trout. Diseases of Aquatic Organisms 102:97-106.

Fetherman, E. R., D. L. Winkelman, M. R. Baerwald, and G. J. Schisler. 2014. Survival and reproduction of *Myxobolus cerebralis* resistant rainbow trout in the Colorado River and increased survival of age-0 progeny. PLoS ONE 9(5):e96954.

Schisler, G. J., K. A. Myklebust, and R. P. Hedrick. 2006. Inheritance of *Myxobolus cerebralis* resistance among F1-generation crosses of whirling disease resistant and susceptible rainbow trout strains. Journal of Aquatic Animal Health 18(2):109-115.

Seber, G. A. F., and J. F. Whale. 1970. The removal method for two and three samples. Biometrics 26(3):393-400.

Job No. 5 Technical Assistance

Job Objective: Provide information on impacts of fish disease on wild trout populations to the Management and Hatchery Sections of Colorado Parks and Wildlife and other resource agencies. Provide specialized information or assistance to the Hatchery Sections. Contribute editorial assistance to various professional journals and other organizations upon request.

Technical Assistance Milestones

Major contributions in the area of technical assistance included various public and professional meeting presentations and posters, including the following:

1. Fetherman, E. R., J. M. Lepak, C. J. Kopack, E. D. Broder, and L. M. Angeloni. 2014. Chemical cues of predation induce anti-predator behavior in Hofer rainbow trout. 2014 Annual Meeting of the Great Plains Fisheries Workers Association. Loveland, CO. February 4, 2014.

2. Kopack, C. J., E. D. Broder, J. M. Lepak, E. R. Fetherman, and L. M. Angeloni. 2014. Chemical cues of predation induce anti-predator behavior in naïve rainbow trout: implications for training hatchery-reared fish. Poster, Front Range Student Ecology Symposium. Fort Collins, CO. February 19, 2014. Best undergraduate poster award.

3. Kopack, C. J., E. D. Broder, J. M. Lepak, E. R. Fetherman, and L. M. Angeloni. 2014. Chemical cues of predation induce anti-predator behavior in naïve rainbow trout: implications for training hatchery-reared fish. Poster, 2014 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, WY. March 4-6, 2014.

4. Wardell, J. A., E. R. Fetherman, and S. F. Brinkman. 2014. Dissolved oxygen and formalin tolerance of whirling disease-resistant strains of rainbow trout. 2014 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, WY. March 4-6, 2014.

5. Avila, B. W., E. R. Fetherman, and D. L. Winkelman. 2014. Raft and floating antenna systems for detecting PIT-tagged fish in rivers. 2014 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, WY. March 4-6, 2014.

6. Broder, E. D., C. J. Kopack, J. M. Lepak, E. R. Fetherman, and L. M. Angeloni. 2014. Chemical cues of predation induce anti-predator behavior in naïve rainbow trout: implications for training hatchery-reared fish. Poster, 2014 Annual Meeting of the Western Division of the American Fisheries Society. Mazatlan, Mexico. April 7-11, 2014.

In addition to public and professional meeting presentations, two presentations were given to the fisheries management class at Front Range Community College in Fort Collins, CO. The first, an informal presentation/laboratory, was presented at the BFRH. During this lab, students

learned about the various fish tagging methods used in research and management across Colorado, and were given a chance to try the various tagging methods on live fish. The second, a formal presentation, was given to the class in March 2014:

- Fetherman, E. R. 2014. Salmonid disease research in Colorado. Front Range Community College, Fisheries Management class. Fort Collins, Colorado. March 10, 2014.

Technical assistance milestones included the peer review of two manuscripts:

- Anonymous. 2013. Survival and growth of tiger shrimp (*Penaeus monodon*) in inland saline water supplemented with potassium. Submitted to the Proceedings of the National Academy of Sciences, India Section B: Biological Sciences.

- Schmidt, T., C. Löb, B. Schreiber, and R. Schulz. 2013. A pitfall with PIT tags: reduced detection efficiency of passive integrated transponders in groups of marked fish. Submitted to the North American Journal of Fisheries Management.

Technical assistance milestones also included the publication of two peer-reviewed journal articles:

- Fetherman, E. R., and J. M. Lepak. 2013. Addressing depletion failure and estimating gear efficiency using back-calculation of capture probabilities. Fisheries Research 147: 284-289.

- Fetherman, E. R., D. L. Winkelman, M. R. Baerwald, and G. J. Schisler. 2014. Survival and reproduction of *Myxobolus cerebralis* resistant rainbow trout in the Colorado River and increased survival of age-0 progeny. PLoS ONE 9(5):e96954.

In addition to those manuscripts published in peer-reviewed journals, two other manuscripts were submitted for publication:

- Fetherman, E. R., B. W. Avila, and D. L. Winkelman. In press. Raft and floating RFID antenna systems for detecting and estimating abundance of PIT-tagged fish in rivers. North American Journal of Fisheries Management.

- Kopack, C. J., E. D. Broder, J. M. Lepak, E. R. Fetherman, and L. M. Angeloni. In review. Chemical cues of predation induce anti-predator behavior in naïve rainbow trout: implications for training hatchery-reared fish. Fisheries Research.

Lastly, the CPW Pueblo Hatchery asked for assistance in writing up the methods used to create triploid walleye. Writing for this paper is in process, with expected completion in the fall of 2014. Two publications are expected: 1) a CPW white paper with a focus on the detailed description of the production methods, intended for both internal distribution and distribution to other states (Kansas, Nebraska, and Montana) that are interested in replicating the methods, and 2) a peer-reviewed journal article intended for submission to the North American Journal of Aquaculture.

APPENDIX

Job 2

Supplemental Analyses

Formalin Sensitivity in Rainbow Trout

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ABSTRACT

Formalin is a commonly used prophalyctic antifungal and antiparasitic treatment of fish and fish eggs, yet little is known about the differential sensitivity among strains after exposure as eggs. This study seeks to determine the sensitivities (measured by mortality) of four rainbow trout strains, after first exposure to formalin as eggs, and a later exposure as fingerlings. The data is analyzed using logistic regression and a Cox proportional hazard model. Both models yield consistent conclusions; the different strains do die at different rates as fingerlings, but the egg treatment does not contribute to these differences.

1 INTRODUCTION

1.1 Background

Formalin is among the most effective and commonly used antifungal and antiparasitic treatments in fish and fish eggs (Bills et. al 1977). As such, a better understanding of the sensitivities of various strains to treatment conditions commonly used in hatcheries has commercial relevance. Past research has demonstrated different sensitivities among strains exposed to formalin post-hatch (Piper and Smith 1973), yet little to no research has explored the effects of exposure as eggs. Therefore, the purpose of this study is to determine whether different formalin exposure levels as eggs affects mortality later as fingerlings, after secondary exposure conditions. Four strains of rainbow trout are considered here: pure Hofer, pure Harrison Lake, 50:50 cross, and 75:25 Hofer:Harrison cross.

1.2 Questions of Interest

1. Does dosage as eggs affect mortality as fingerlings?

2. Are there sensitivity differences among the different strains?

3. Does dosage as fingerlings affect mortality, among fish previously exposed as eggs?

4. Does duration of exposure as fingerlings affect mortality, among fish previously exposed as eggs?

5. How does fish size affect sensitivity to formalin?

1.3 Experimental Design

The experiment is composed of two stages. In the first stage, eggs are treated with for- malin for 15 minutes, at two different levels: 1667 or 5000 ppm. Subsequently, the surviving eggs are allowed to grow to the fingerling stage (approx. 3 inches in size), whereupon they are re-exposed to one of eight treatment conditions, according to a complete randomized de- sign. The eight fingerling treatment conditions consist of the combinations of four exposure dosages (0, 167, 250, 500 ppm) at two possible durations (30 or 60 minutes). These levels are chosen to be in line with common hatchery treatment conditions, which, according to a survey of Colorado Parks and Wildlife hatchery managers, range from 130-250 ppm, with 167 ppm for 30 minutes being the most common. The 500 ppm condition is included to test for toxicity at extraordinarily high dosages. After treatment, the fish are observed over five days, and time of death is recorded. Following the observation period to test for delayed mortality effects, the fish are sacrificed (i.e., the data are censored), and the weight, length, and strain of each fingerling is recorded.

2 EXPLORATORY DATA ANALYSIS

We begin by viewing the overall structure of the data, and then move on to visualizations that highlight the effects of different explanatory variables. We consider all possible explanatory variables that were measured, except length. Length was excluded due to its high colinearity with weight (r = .958). Weight serves as a general proxy for size. For ease of reference, the explanatory variables under consideration follow:

X_1 = Duration of exposure
X_2 = Exposure concentration as fingerlings
X_3 = Weight as fingerlings
X_4 = Indicator for pure Hofer strain
X_5 = Indicator for 50:50 cross
X_6 = Indicator for 75:25 Hofer:Harrison hybrid
X_7 = Exposure concentration as eggs

The measured response variable is survival time. It will also be convenient to create a response vector of zeros and ones, where a one corresponds to a fish that survived and a zero corresponds to a fish that died. Figure A1.1 shows that fish tended to either die quickly or survive until censored, suggesting that we are not losing as much information if we replace time of death with this binary response vector. Doing so will allow us to analyze the data with a logistic regression model.

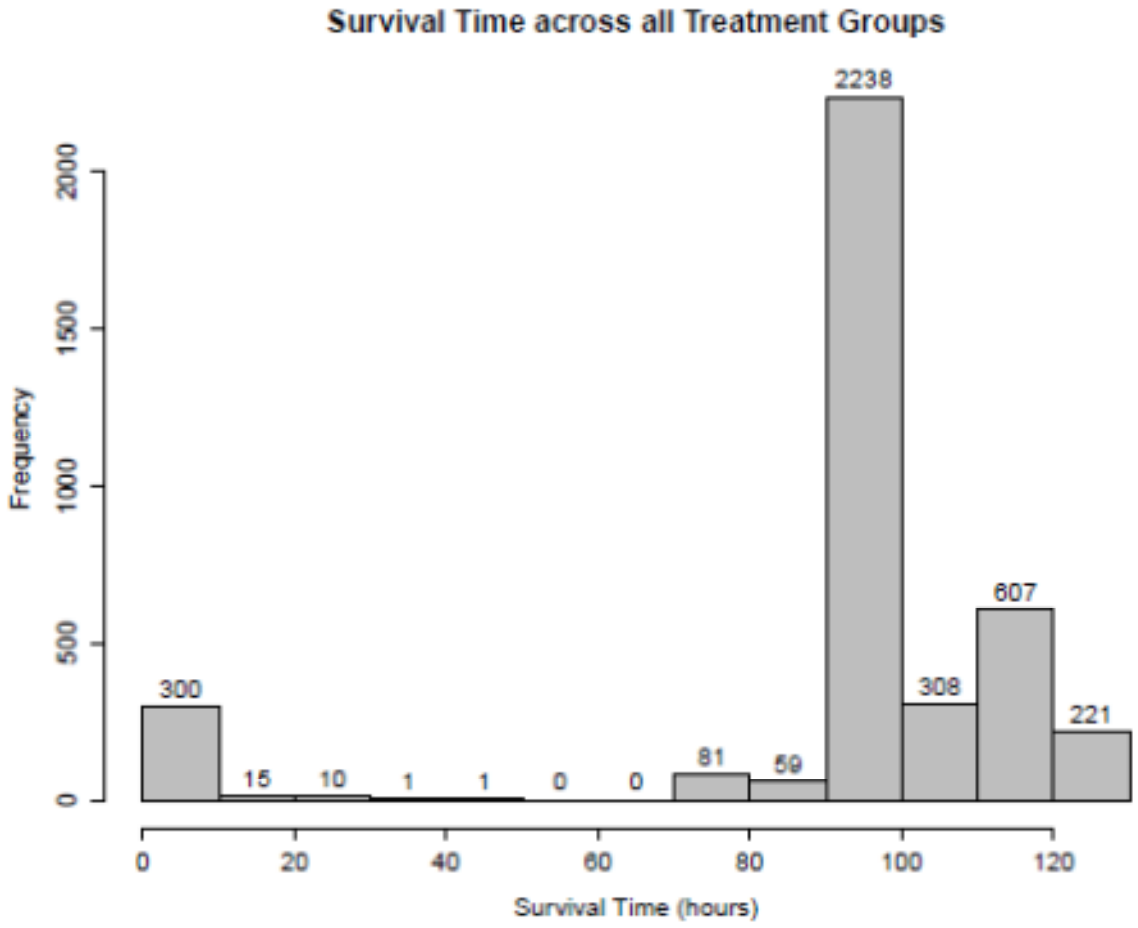

Figure A1.1. Note that all fingerlings in the > 70 buckets survived the duration of the experiment. Therefore, all those on the right hand side of the histogram can be treated equally as survivors without great loss of fidelity. The discrepancy is due to the physical constraint of recording one fish tank at a time at the end of the observation period

While the majority of fish survived the full duration of the experiment, a significant fraction did not. To get an idea of which treatments were having an effect on the proportion of survivors, figure 2 shows a bar graph broken down by treatment.

There are three main things to notice from Figure A1.2:

1. The blueish blocks tend to show substantially higher mortality rates than the reddish blocks, suggesting that the 60 min group experienced higher rates of death than the 30 min group (i.e. longer duration of exposure as fingerlings appears to increase the probability of death.)

2. The mortality rate tends to increase within each block as fingerling dosage increases, suggesting that increased dosage as fingerlings is associated with higher mortality.

3. The two reddish blocks look roughly the same, as do the two blueish blocks. This suggests that egg treatment may have no significant effect on mortality rate as fingerlings.

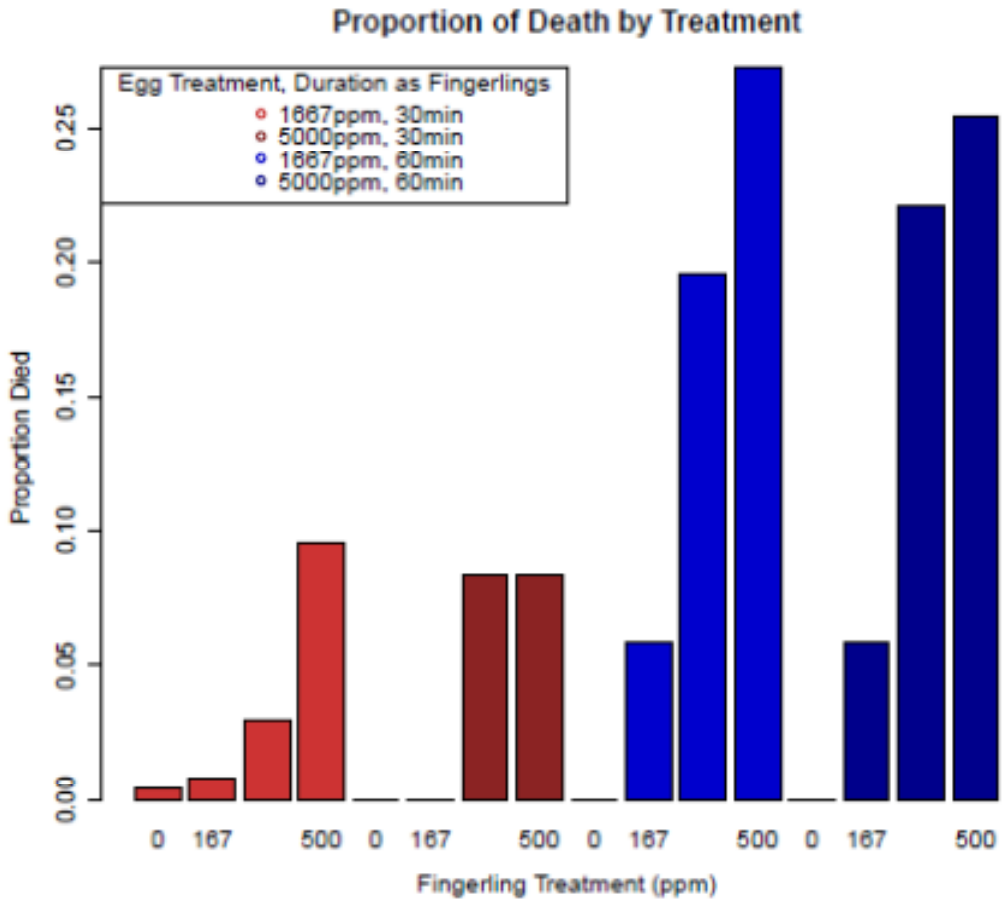

Figure A1.2. Mortality rates broken down by treatment group. There is one bar for each of the sixteen possible treatment combinations. To generate this plot, an fingerling surviving to > 70 hours was coded as having survived, and all others as having died.

Figure 3 attempts to illuminate the other two research questions (2 and 5). There are couple things to notice from Figure A1.3. The points on the left side (representing fish that died) are somewhat more densely clumped near the low weight side of the spectrum, and get sparser as weight increases, possibly suggesting that increased weight tends to reduce mortality. However, this requires that the points on the right side of the plot (those that survived) are not also more clumped on the low weight side of the spectrum, which is difficult to tell from this plot. Secondly, there appears to be relatively fewer green points, and relatively more purple and blue points toward the left side of the plot, suggesting that Harrison Lake strain may be less sensitive, and Hofer may be relatively more sensitive.

We have now provided suggestive answers to our questions of interest. Next we turn to formal analysis to quantify our results.

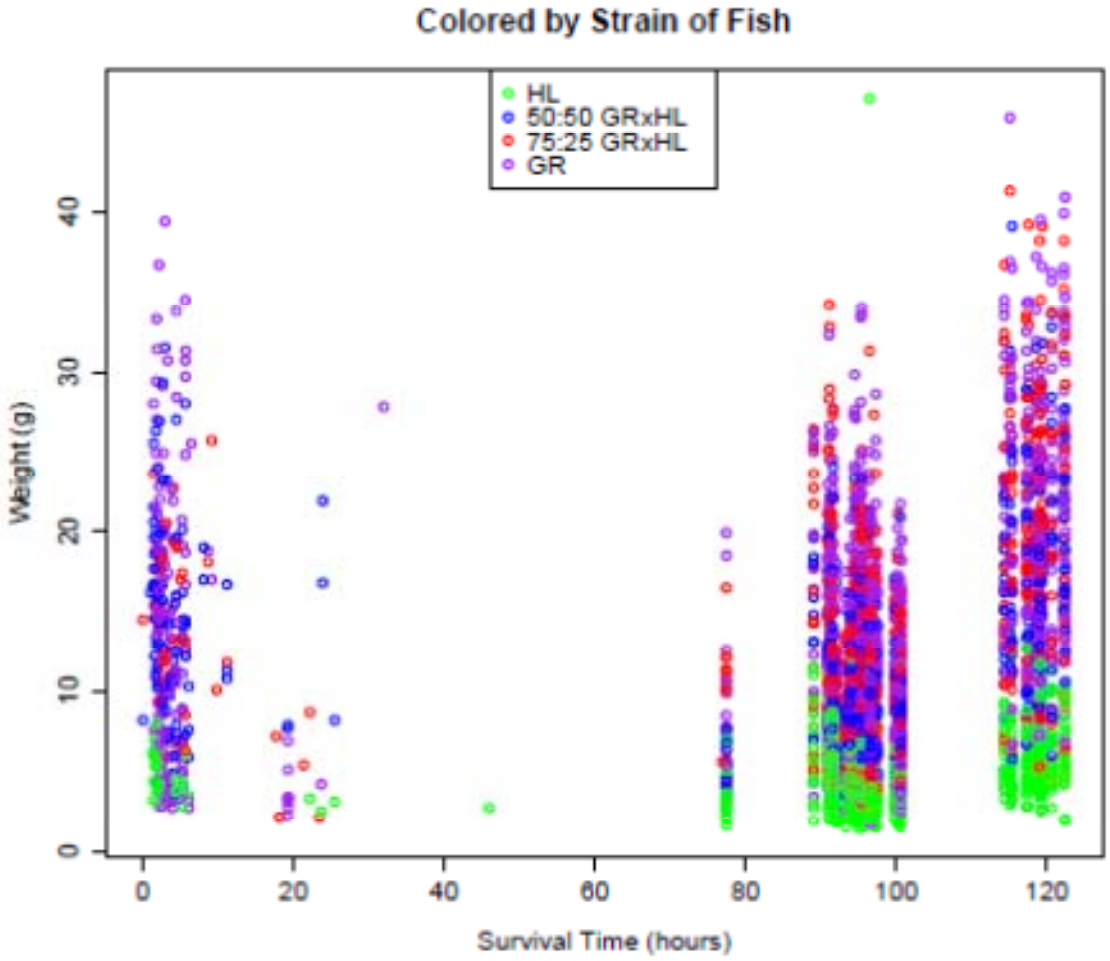

Figure A1.3. HL: Harrison Lake, GR: Hofer.

3 FORMAL ANALYSIS

We built two models to analyze the data, both commonly used in survival analysis. First we will describe a logistic regression model, and follow up with a Cox proportional hazard (PH) model. The logistic regression model has advantage of simplicity and more intuitive interpretations, but at the cost of ignoring time of death, and instead treating survival as an indicator variable. The Cox PH model has the advantage of accounting for the time of death information, while still appropriately handling the censored nature of the data. Both models yield consistent results, providing additional confidence for our conclusions.

3.1 Logistic Regression Model

The logistic regression model treats survival as an indicator variable, where any fingerling that survived for more than 70 hours was coded as "success" (Y=1) and all others as "failure" (Y=0). The choice of the 70 hour time cutoff is appropriate because all fish that survived past 70 hours, did in fact survive until censored (see Figure A1.1).

In this model, each Y_i is assumed to be a Bernouli random variable with probability of survival p_i that depends on the values of the covariates for the i^{th} fish. The value of p_i depends on the covariates according to the following relation:

$$logit(p_i) = ln(\frac{p_i}{1-p_i}) = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_3 x_{3i} + \beta_4 x_{4i} + \beta_5 x_{5i} + \beta_6 x_{6i} + \beta_7 x_{7i}$$

where p_i = probability of surviving, and the covariates are those outlined above.

In other words, the ln(odds of survival) are assumed to follow a linear relationship with the covariates.

Equivalently, the model can be stated as:

$$E[Y_i] = p_i = logit^{-1}(\beta_0 + \beta_1 X_1 + ... + \beta_7 X_7) = \frac{1}{1 + e^{-(\beta_0 + \beta_1 X_1 + ... + \beta_1 X_7)}}$$

This function has a sigmoidal shape and assymptotically approaches 0 and 1, making it an appropriate choice for modeling a probability measure.

For model selection, we started with all the listed covariates and used successive log- likelihood ratio tests to check for significant effects (i.e., H_a: $\beta_i = 0$). By this procedure, exposure concentration as eggs did not have a statistically significant effect ($p = .1297$ in the model with all covariates), while all other covariates were significant at the 0.05-level (see Table A1.1).

Table A1.1. Here, the reported p-values are actually generated using the Wald test, which approximates the likelihood ratio test for one covariate. The results are very close to what is given by the likelihood ratio test where the full model has these six covariates, and the reduced model excludes just the covariate of interest.

	Estimate	**Std. Error**	**exp(coef)**	**p-value**
(Intercept)	7.53	0.36	1863.106	0.00
fingerling concentration	-0.01	0.00	0.990	0.00
50:50 Hofer:Harrison	-1.63	0.22	0.196	0.00
75:25 Hofer:Harrison	-0.53	0.25	0.589	0.03
Hofer	-1.74	0.23	0.176	0.00
weight	0.03	0.01	1.030	0.00
duration	-0.06	0.01	0.942	0.00

It is important to note that the likelihood ratio test works by comparing the goodness-of-fit of a full model to a reduced model that drops the covariate(s) of interest. As such, the results of likelihood-ratio test depend critically on which covariates are included in the full model. Nonetheless, these conclusions were robust to model selection effects, as long as interaction terms were not considered. Specifically, concentration of exposure as eggs was consistently not significant and all the other covariates consistently were, across many choices of full model. Interaction terms were ignored for three reasons: because there are so many possible interactions that could potentially be considered (almost 2^7), because they complicate the interpretation of the model and in many cases have no straightforward interpretation at all, and most importantly,

because they are not necessary to answer our questions of interest. The best model, according to our criteria of parsimony and significant covariates is given in Table A1.1.

The estimated β_is indicate the estimated change in ln(odds of survival) associated with an increase of one unit in X_i, while holding all other covariates constant. For ease of interpretation, it is convenient to take $e^{\beta i}$ which gives the estimated change in odds of survival associated with an increase of one unit in X_i. Thus, if β_i is significantly less than zero, then increasing X_i tends to harm the odds of survival, while if β_i is significantly greater than zero, then increasing X_i tends to improve odds of survival. Note, though, that we did not standardize the covariates. Therefore, the magnitude of the β_is can only be compared directly across the three indicator variables. Other direct comparisons do not make sense, because the meaning of a one unit increase differs across the variables.

This model allows us to make predictions of the probability of survival for a fingerling at any level of the covariates. For example, a roughly average weight (10g) Hofer strain fingerling, treated at 167 ppm for 30 min, has an estimated probability of survival given by:

$$p_i = logit^{-1}(7.53 + (-.01 * 167) + (-1.74) + (.03 * 10) + (-.06 * 30)) = .932$$

3.2 Cox Proportional Hazard Model

The Cox proportional hazard (PH) model is concerned with modeling the time to some event (in our case, the time to death). The model utilitizes the concept of a hazard function, which intuitively, can be thought of as the instantaneous risk of death at time t. If we define a random variable T to be the time to death, with a probability density function f(t), and cumulative density function F(t) = P(T<t), then the hazard function is given by:

$$h(t) = \lim_{\delta t \to 0} \frac{P(t<T\leq t+\delta t|T>t)}{\delta t} = \frac{f(t)}{1-F(t)} = \frac{f(t)}{S(t)}$$

where S(t) = 1 - F(t) = P(T ≥ t) is the survivor function.

The Cox PH model makes the assumption that the hazard function at each level of the covariates is proportional to some baseline hazard $h_0(t)$. Specifically, the Cox PH model is:

$$h(t|X_1,...,X_7) = h_0(t)e^{(\beta_0+\beta_1X_1+\cdots+\beta_7X_7)}$$

assuming we consider the same covariates as before.

Equivalently, we can say that the natural log of the hazard ratio is a linear combination of the covariates. That is,

$$ln\left(\frac{h(t|X_1,...,X_7)}{h_0(t)}\right) = \beta_0 + \beta_1X_1 + \beta_2X_2 + \cdots + \beta_7X_7$$

The Cox PH model is known as a semiparametric model because it does not require specification of the baseline hazard; it only assumes that the baseline hazard is nowhere negative (because a negative hazard would imply immortality). This is acceptable as long as we only

care about the hazard ratio between two levels of the covariates, because in calculating the ratio, the baseline hazard cancels out, as shown:

$$\frac{h(t|x_1,...,x_7)}{h(t|z_1,...,z_7)} = \frac{h_0(t)e^{(\beta_0+\beta_1 x_1+\cdots+\beta_7 x_7)}}{h_0(t)e^{(\beta_0+\beta_1 z_1+\cdots+\beta_7 z_7)}}$$

where x and z represent two different levels of the covariates.

Since no underlying distribution for the hazard function is assumed, the βs must be estimated using non-parametric methods (specifically, the estimates can be calculated by using Newton's method to maximize the partial log-likelihood function).

Like before, we can use likelihood ratio tests for significance of the covariates. Doing so tends to yeild p-values very close to that given by the logistic regression model. Again we find that egg dosage is not significant (p = 0.1493 in the model with all other covariates). By the same criteria as before, we get the same six covariates in the best model. The model is given in Table A1.2.

Table A1.2. Here again the p-values are actually given by the Wald test, but are very close to that given by the likelihood ratio test where the full model has all six covariates and the reduced model has all but the covariate of interest.

	coef	**exp(coef)**	**se(coef)**	**z**	**Pr(>|z|)**
dur	0.05	1.05	0.00	10.73	0.00
treat ppm	0.00	1.00	0.00	14.14	0.00
factor(color)O	1.47	4.37	0.20	7.44	0.00
factor(color)P	0.48	1.62	0.23	2.11	0.04
factor(color)R	1.55	4.72	0.20	7.62	0.00
weight	-0.03	0.97	0.01	-3.09	0.00

Here, the interpretation of the estimated βs is slightly different than before. In this case, β_i corresponds to the change in the ln(hazard ratio) (instead of ln(odds of survival)) that is associated with an increase in one unit of X_i, while holding all other covariates constant. Therefore, unlike before, in the Cox PH model, positive βs correspond to an increase in sensitivity, and negative βs correspond to a decrease in sensitivity. Note that this would have been the case in the logistic regression model too if we had instead considered death as "success" and survive as "failure". In any case, the magnitude of the βs should not be compared directly across the models, because they mean different things.

Again, it is convenient for interpretation to take $e^{\beta i}$, which corresponds to the change in the hazard ratio for every increase of one unit in X_i, while holding other covariates constant.

An advantage of the Cox PH model is that it allows us to generate survival curves with associated confidence intervals. In all cases, we see a steep drop in survival early and then a leveling off. Non-overlaping confidence intervals indicate a significant difference between the groups. Figure A1.4 emphasizes the decreased survival among fish in the 60 minute condition

versus the 30 minute condition. It also shows that the Harrison Lake strain is less sensitive than the Hofer strain. Figure A1.5 emphasizes the effect of increased dosage as fingerlings.

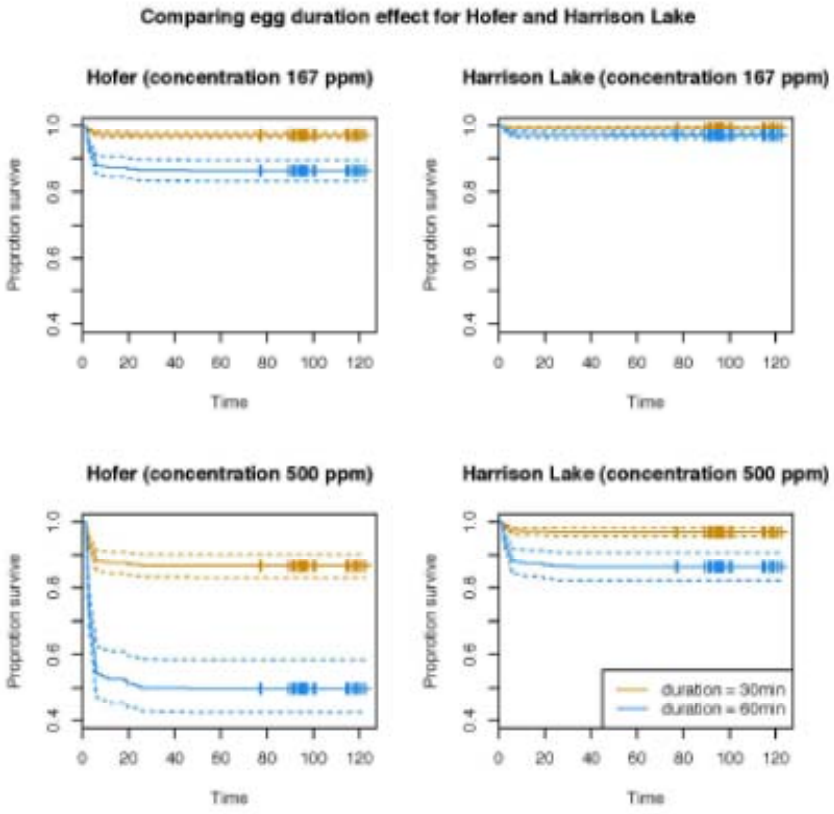

Figure A1.4. Note that the data for only the two hybrid strains and the data for the two fingerling dosage groups are not shown in this figure.

4 CONCLUSIONS

Based on these results, we can return to our questions of interest, and conclude the following:

1. There is not sufficient evidence to suggest that the exposure dosage as eggs has any effect on mortality as fingerlings, within the range tested (p=.1296). This suggests that hatchery managers do not need to be particularly concerned about the dosage at which they treat eggs, assuming that they are only concerned with risk of death later on. Note, though, that this does not imply that dosage as eggs had no effect on mortality as eggs. That question was not tested in this experiment.

2. The different strains do express differential sensitivities to formalin treatment conditions. Specifically, pure Hofer is the most sensitive (i.e., least likely to survive), followed by the 50:50 cross, then the 75:25 Hofer:Harrison cross, and finally the pure Harrison Lake strain is the least sensitive. This result is surprising in that the 75:25 Hofer:Harrison cross reacts more like the Harrison Lake strain, despite being genetically more similar to the Hofer strain.

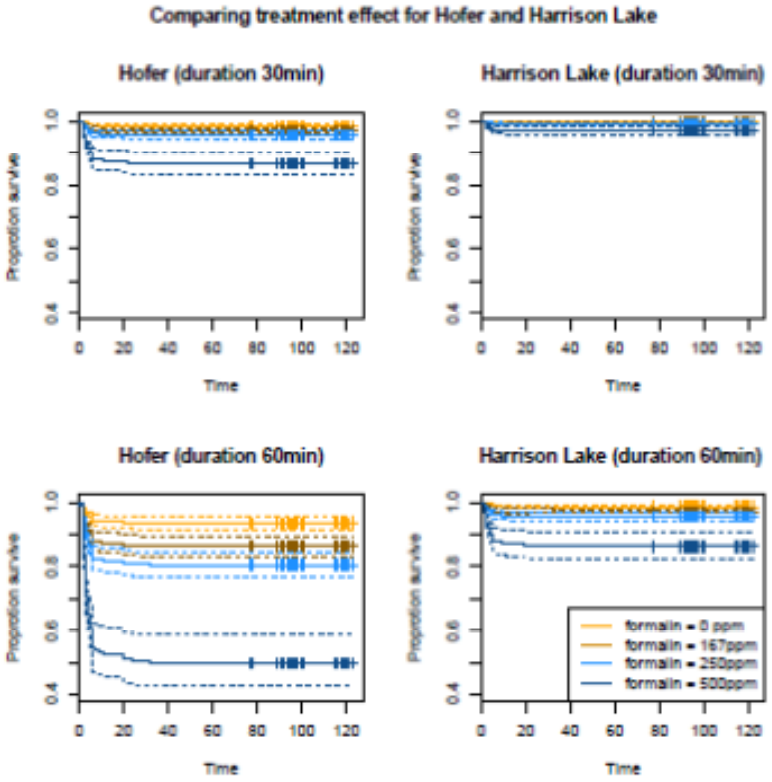

Figure A1.5. Note that the data for the two hybrid strains is not shown in this figure.

3. Duration of exposure affects mortality, among fingerlings previously exposed to formalin as eggs ($p < 2e^{-16}$). Specifically, longer durations of exposure increase the probability of death.

4. Formalin dosage as fingerlings affects mortality in fingerlings previously exposed as eggs. Specifically, increased dosage increases the mortality rate.

5. Increased size (as measured by weight) increases probability of survival ($p = 0.0004$). That is, larger fingerlings tend to be less sensitive.

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