Sport Fish Research Studies

Federal Aid Project F-394-R14

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Job Progress Report

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Colorado Parks & Wildlife policy by the Director or the Wildlife Commission.

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State: Colorado **Project No. F-394-R14**

Project Title: Sport Fish Research Studies

Period Covered: July 1, 2014 – June 30, 2015

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 during on-site spawning operations and throughout the rearing process 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 2014, BFRH personnel checked all of the Hofer (GR)[1], 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 18, 2014, 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), and HOF 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 Gaalen fish egg sorter, VMG Industries, Longmont, 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 2014-2015 spawning season**.**

Strain	Date Spawned	No. Spawned Females	No. Green Eggs	No. Eyed Eggs	Shipped To
100% HL	12/18/14-1/15/15	215	31,138	28,644	Fish Research Hatchery
100% GR	11/18/14-12/17/14	178	118,696	106,826	Fish Research Hatchery/CPW Hatcheries
GR×HL	12/10/14	6	18,000	15,660	Fish Research Hatchery
Total	11/18/14-1/15/15	399	167,834	151,130	

The FRH 2014/2015 on-site rainbow trout production spawn started on November 18, 2014, with the last groups of HL females spawned on January 15, 2015. The initial goal was to produce 150,000 eyed eggs; egg take exceeded the production needs with 151,130 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 four 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 2014/2015.

Research Projects

Eggs produced specifically for research projects and brood stock management comprise 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

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. Four 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 of formalin (1,667, 2,000, and 5,000 ppm) for fungal control. The results of this experiment were presented in previous reporting cycles. The second experiment, conducted in 2013, was designed to determine if there is differential sensitivity in fingerlings to varying formalin concentrations used to control external parasite infections as a result of exposure to varying levels of formalin used to treat fungal infections during egg incubation. The results of this experiment were presented in the previous reporting cycle. The third experiment, conducted in 2014 and described below, was designed to determine if certain hatchery conditions, such as

size-at-exposure, crowding, reduced flow, and day-of-feeding, can affect formalin sensitivity in rainbow trout fingerlings. The fourth and final experiment, being conducted in 2015 with the methods described below, is designed to determine the effects of density and multiple exposures for heavy infections on formalin sensitivity in rainbow trout fingerlings. The results of the fourth experiment will be presented in the next reporting cycle.

Experiment 3: Hatchery Practice Effects on Formalin Sensitivity

METHODS

Spawning

Spawning occurred in December 2013. GR egg groups were created by pooling the eggs from 14 pairs of two-year-old GR females spawned with three-year-old GR males. The eggs from 12 pairs of three-year-old HL females spawned with two-year-old HL males were pooled together to create the HL egg groups. The GR×HL 50:50 egg groups were created by pooling the eggs from 12 pairs of three-year-old GR females spawned with three-year-old HL males. The eggs from 12 pairs of two-year-old GR×HL 50:50 males spawned with three-year-old GR females were pooled together to create the GR×HL 75:25 egg groups. 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

One, five gallon per minute (gpm) flow-through egg tray tower was utilized to rear the eggs for the hatchery practice experiment. Seven trays 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 2.1), strains were randomly assigned to an insert using a random number generator (Table 2.1). Each insert contained 500 eggs, providing five, 500 egg replicates per strain. For each strain or cross, 500 eggs were initially counted into a container to quantify egg volume. This known volume was then used to distribute the approximate number of eggs to each insert.

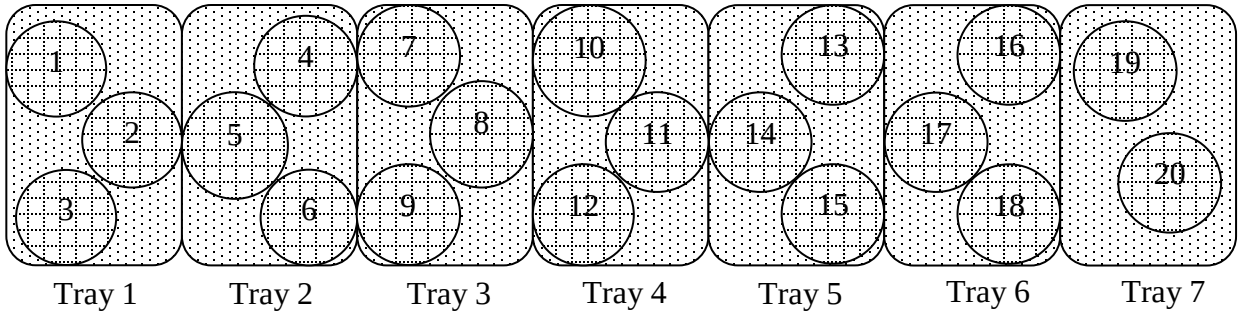

Figure 2.1. Arrangement of the 20 screen-bottomed PVC inserts in the seven trays (from top of tower down) used during egg incubation. Strains were assigned to an insert using a random number generator (Table 2.1).

The formalin concentration to which eggs were exposed during incubation was 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 15 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. Once the eggs eyed, formalin treatments ceased.

Table 2.1. Assignment of strain to PVC insert 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

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 among the strains and crosses were analyzed using a single-factor analysis of variance (ANOVA; $N = 20$). Percentages were arcsine-square root transformed prior to each analysis, and values 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 differences among the strains and crosses.

Rearing of Rainbow Trout Fingerlings

Upon completion of the egg incubation portion of the experiment, replicates were combined into four troughs, one per strain, and fish were reared to fingerling size (approximately 3" total length [TL]) for use in density/flow, crowding and day-of-feeding trials. All four strains were fed 2% body weight per day, and were reared under similar environmental conditions (i.e., flows, temperatures, etc.). For the size-at-exposure trials, a subset of 180 fish per strain was evaluated for formalin sensitivity at 1.5" TL, and an additional subset were evaluated at 5" TL (see below).

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 UV light.

Two weeks prior to initiation of the first formalin sensitivity trial, 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. One VIE color (e.g., GR: red, HL: green, HxH 50:50: orange, HxH 75:25: purple) was used for identification of each of the four strains and

crosses when mortality occurred as all four were included in each experimental replicate (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). In all but the half flow trials, flow was maintained at two gpm achieving three full turnovers during the 30 minute treatment, and allowing us to reach 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).

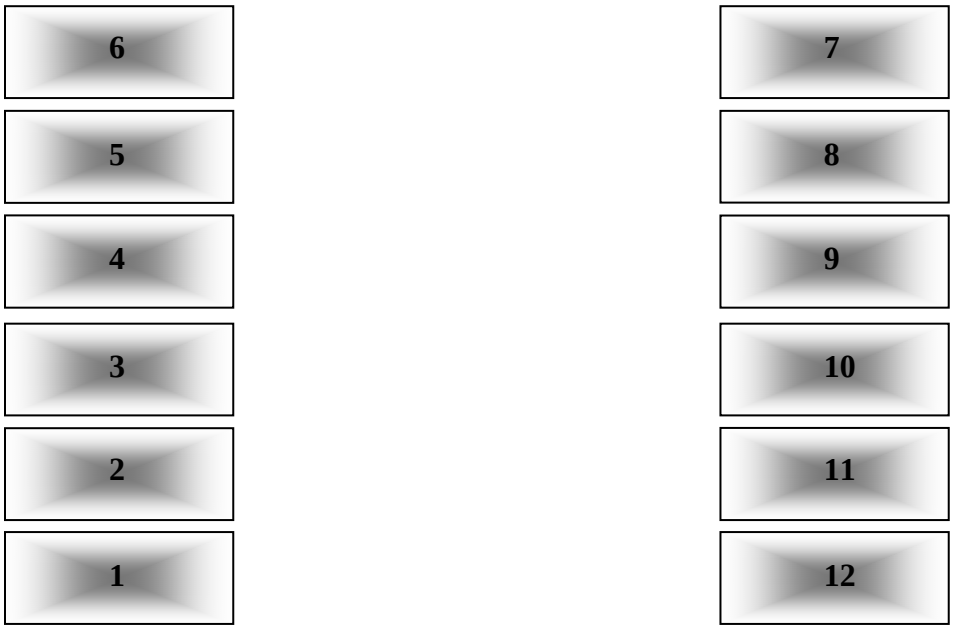

Figure 2.3. Arrangement and numbering of the twelve experimental tanks used in the fingerling formalin sensitivity experiments, housed in FR1 of the BFRH.

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). 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. Mortality occurring during formalin exposure was recorded on a per strain basis, as were the lengths and weights of each fish that died. The time at which mortality occurred in relation to the beginning of the exposure period was also noted. 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, 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 trial.

Overall, the experiment consisted of seven separate trials. Trials 1 and 7 were designed to examine size susceptibility to formalin. Trials 2-6 examined the effects of density, flow, crowding and day-of feeding on the formalin sensitivity. The objective of the density and flow trials was to determine if density or flow conditions affected formalin sensitivity in the four strains. In addition, a feeding trial was designed to determine if feeding the day of treatment increased 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.

Density/Flow Effects on Sensitivity

Table 2.2. 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

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 utilize available 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 assigned to tank, as was the order in which the treatments were applied, using a random number generator (Tables 2.2, 2.3, and 2.4).

Table 2.3. 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 2.4. Assignment of treatment to tank and order in which the treatment was applied in the third 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

For the low flow trials, we were most interested in what effects low flow would have on formalin sensitivity, not the residual effects of maintaining formalin-treated fish under low flow conditions. Essentially, these trials simulated a reduction in water flow prior to treatment (due to pipe clogs, equipment failure, or unintentional reduction at a valve), with flow issues corrected shortly after treatment. As such, flows were reduced prior to exposing the fish to formalin, and increased one hour following the 30 minute treatment.

Crowding Effects on Sensitivity

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 exposed prior to the diffusion of formalin throughout the water column. The crowding trial was designed to determine if keeping fish away from the inflow could prevent mortality during formalin treatment. 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. 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 was removed from the water due to the presence of formalin 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 assigned using a random number generator (Table 2.5).

Table 2.5. Assignment of treatment to tank and order in which the experimental treatment was applied in the 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

Feeding Effects on Sensitivity

The feeding trial, which simulated accidental or unintentional feeding on the day of exposure, was designed to determine if feeding the day of formalin treatment affected formalin sensitivity. Fish in the feeding trial were fed a normal ration of food 30 minutes prior to formalin exposure to ensure they were in the process of digestion during exposure. Due to tank and fish availability, the feeding trial only tested normal densities and normal flows, though all three formalin concentrations were tested. Nine of the twelve experimental tanks were used. The other three tanks served as water controls to determine how much oxygen was removed from the water due to the presence of formalin 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 assigned using a random number generator (Table 2.6). For comparison and statistical

analysis, non-fed controls (i.e., final feeding the day before treatment) came from the normal density, normal flow replicates in Trials 2, 3, and 4, described above.

Table 2.6. Assignment of treatment to tank and order in which the treatment was applied in the 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 Effects on Sensitivity

Table 2.7. Assignment of treatment to tank and order in which the treatment was applied using small fish (1.5-2” TL; 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 differences in gill surface area that is exposed to formalin during treatment. Size susceptibility to formalin was tested with all four strains. The objective of the size trials was to determine if there was differential mortality at different life stages. Therefore, fish at 1.5” TL and fish at 5” TL were tested for their susceptibility to formalin (Trials 1 and 7). In both trials, nine of the twelve experimental tanks were used. The other three tanks served as water controls to determine how much oxygen was removed from the water due to the presence of formalin during a 30 minute treatment in the

absence of fish. Due to tank and fish availability, the feeding trial only tested normal densities and normal flows, though all three formalin concentrations were tested. Treatment concentration and order of treatment was assigned to the tanks using a random number generator (Tables 2.7 and 2.8). For comparison and analysis, data for the medium-sized fish (fingerlings; 3" TL) came from the normal density, normal flow replicates in Trials 2, 3, and 4, described above.

Table 2.8. Assignment of treatment to tank and order in which the treatment was applied using large fish (5" TL; 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

The data for each of the hatchery practices, size-at-exposure, density/flow, crowding, and feeding was analyzed separately using a general linear model implemented in SAS Proc GLM (SAS Institute 2011). An intercept-only model was included in each model set, as were all singular, additive, and interactive combinations of factors suspected to have contributed to mortality. Strain (STRAIN) and formalin concentration (CONC) were included in all model sets, whereas effects of flow or density (FLOW or DEN), crowding (CROWD), feeding (FEED), or size (SIZE) were included only in the model sets examining mortality for those specific hatchery practices. 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 to quantify egg volume. After the initial count, eggs were measured out, not counted out, using this 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 the strains and crosses ($F = 1.47$, $P = 0.262$).

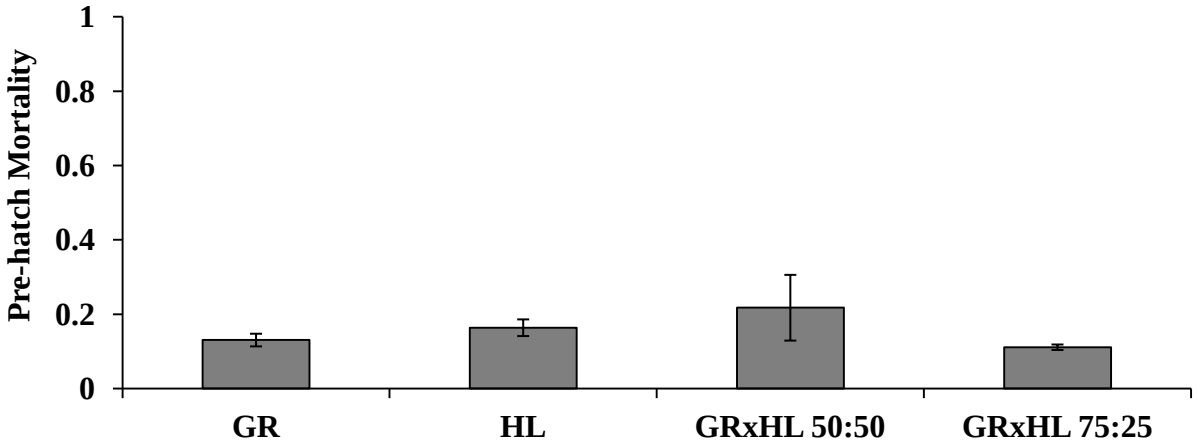

Figure 2.4. Average proportion pre-hatch mortality (SE bars) by strain.

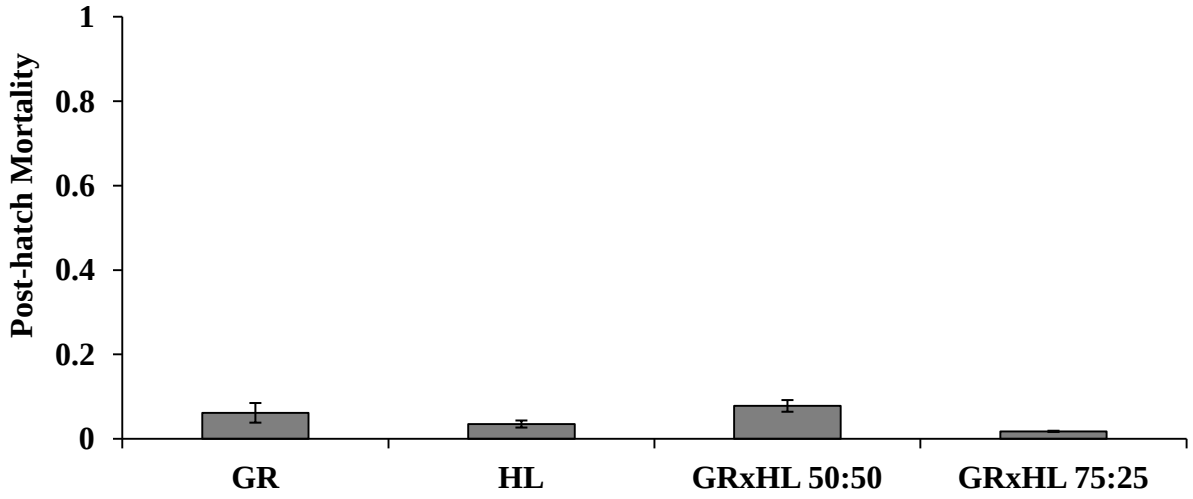

Figure 2.5. Average proportion post-hatch mortality (SE bars) by strain.

Average pre-hatch mortality did not differ among the strains ($F = 0.97$, $P = 0.432$; Figure 2.4). 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 after physically shocking 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 exhibited significantly higher average percent post-hatch mortality (7.8 ± 1.4%) than the GR×HL 75:25 (1.8 ± 0.2%; $P = 0.014$), but did not differ significantly from the GR (6.2 ± 2.3%) or HL (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 2.5). 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 2.6).

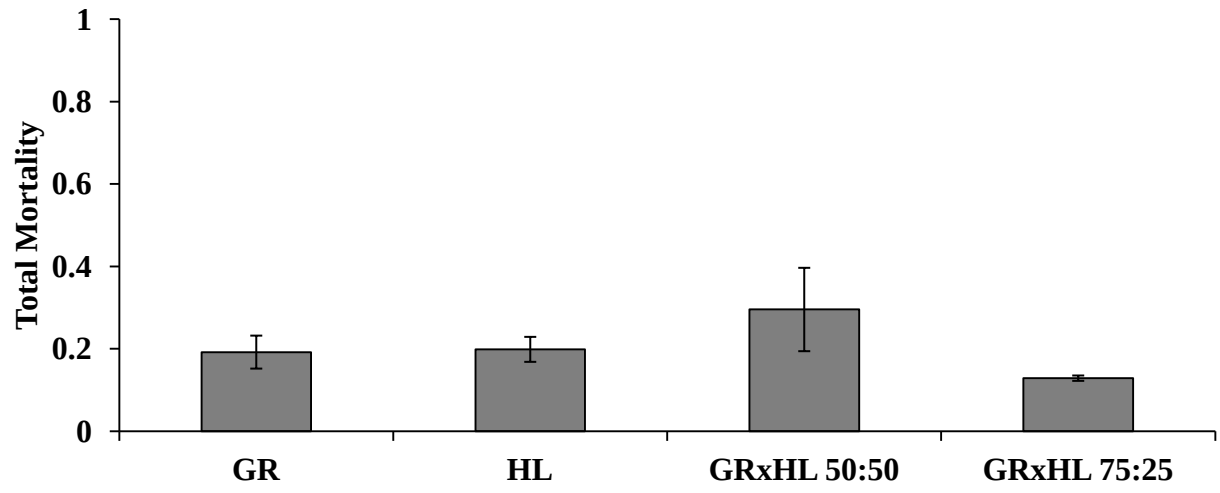

Figure 2.6. Average proportion total mortality (SE bars) by strain.

Density/Flow Effects on Sensitivity

Model selection results for the density and flow trials indicated that the interaction between exposure concentration and flow had a large affect on mortality, appearing in the top model (AICc weight = 0.78), as well as with an additive effect of density in the second best model (AICc weight = 0.11), and with an additive effect of strain in the third best model (AICc weight = 0.05; Table 2.9). Mortality increased with an increase in formalin concentration. In addition, within a formalin concentration, mortality was higher in tanks in which the flow was decreased (1 gpm) versus those tanks that received a normal flow of 2 gpm (Figure 2.7). Although density appeared as an additive effect in the second best model, an effect of density was not apparent. Mortality was increased in the tanks with increased densities (0.035 ± 0.006; 40 fish per strain; 160 fish total), but did not exceed the range of mortality experienced in tanks in which the density was normal (0.030 ± 0.006; 20 fish per strain; 80 fish total).

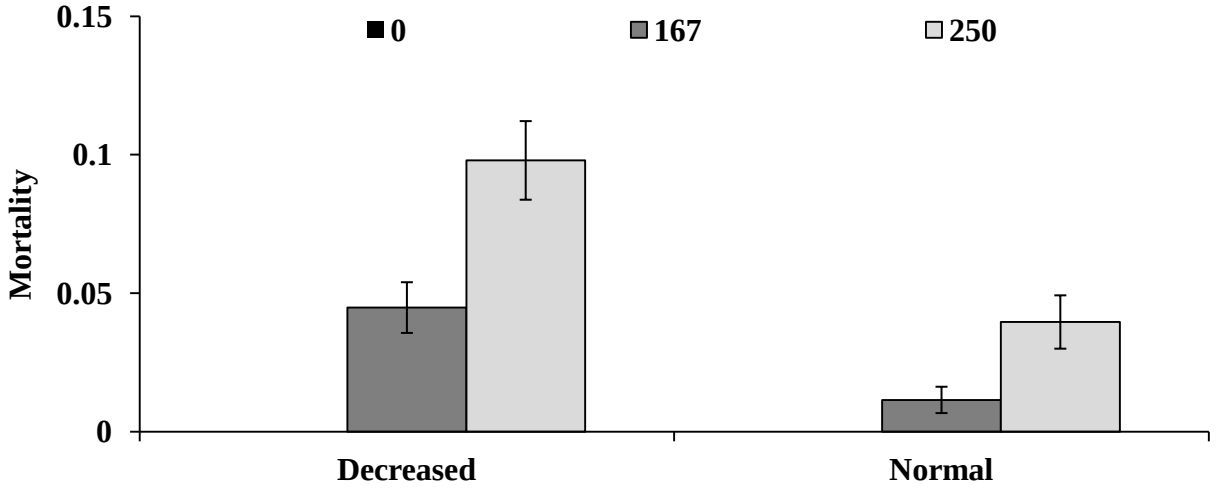

Figure 2.7. Average proportion mortality (SE bars) by flow and formalin concentration. Note that the mortality axis is reduced to show differences among formalin concentrations and flows.

Table 2.9. Model selection results for factors influencing formalin sensitivity in *Myxobolus cerebralis* resistant rainbow trout. Model set included singular, additive, and interactive effects of formalin concentration (CONC), strain (STRAIN), flow (FLOW), and density (DEN). The model set included 40 models; only models with weight are shown. 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
CONC*FLOW	0.48	676.61	6.00	-1340.61	0.00	0.78
CONC*FLOW+DEN	0.48	676.85	8.00	-1336.63	3.98	0.11
CONC*FLOW+STRAIN	0.50	678.35	10.00	-1335.05	5.55	0.05
CONC+FLOW	0.44	672.40	5.00	-1334.37	6.24	0.03
CONC*DEN+FLOW	0.46	674.22	8.00	-1331.38	9.23	0.01
CONC*FLOW+DEN+STRAIN	0.51	678.62	12.00	-1330.85	9.75	0.01

Crowding Effects on Sensitivity

Formalin concentration had a large effect on mortality in the crowding trial. All models with weight included formalin concentration in a singular, additive, or interactive fashion (Table 2.10). The top model indicated that formalin concentration alone had a large effect on mortality (AICc weight = 0.62). Mortality increased with an increase in formalin concentration (Figure 2.8). The second best model in the set suggested that crowding may also affect mortality. Initially, it was thought that crowding the fish away from the inlet would reduce exposure to formalin "hot spots", and therefore reduce mortality. However, the results suggest that mortality was higher in tanks where fish had been crowded away from the inlet (0.039 ± 0.012) than in tanks where the fish were not crowded down (0.014 ± 0.004), likely a result of increased densities in the tanks in which fish were crowded.

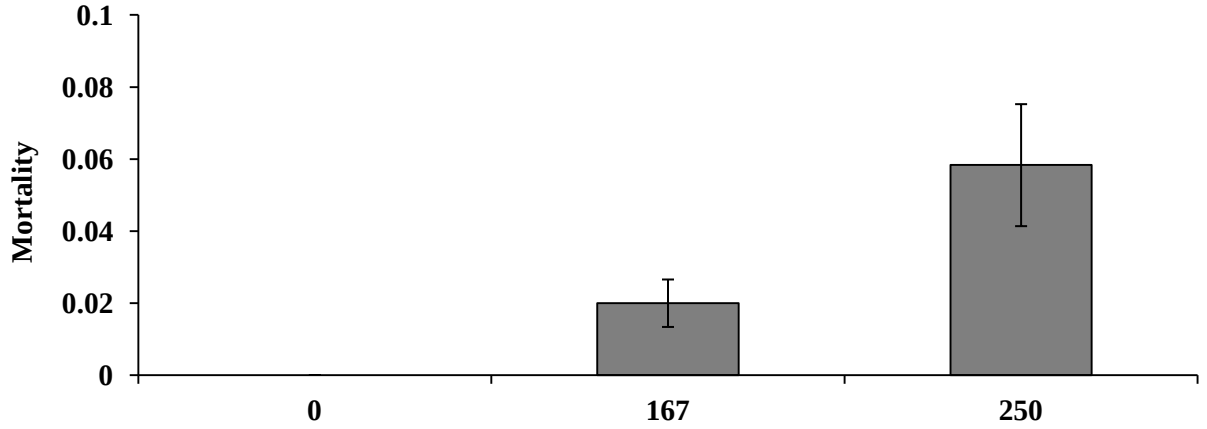

Figure 2.8. Average proportion mortality (SE bars) by formalin concentration. Note that the mortality axis is reduced to show differences among formalin concentrations.

Table 2.10. Model selection results for factors influencing formalin sensitivity in *Myxobolus cerebralis* resistant rainbow trout, including formalin concentration (CONC), strain (STRAIN), and crowding (CROWD) effects. 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
CONC	0.22	300.45	3.00	-594.54	0.00	0.62
CONC+CROWD	0.24	301.19	5.00	-591.47	3.07	0.13
STRAIN+CONC	0.31	303.48	7.00	-591.21	3.33	0.12
CONC*CROWD	0.28	301.98	6.00	-590.66	3.88	0.09
STRAIN+CONC+CROWD	0.34	304.38	9.00	-587.85	6.69	0.02
CONC*CROWD+STRAIN	0.38	305.39	10.00	-587.17	7.37	0.02
STRAIN*CROWD+CONC	0.39	305.42	11.00	-584.45	10.09	0.00
Intercept-only	0.00	292.63	1.00	-583.20	11.34	0.00
CROWD	0.03	293.11	2.00	-582.04	12.50	0.00
STRAIN	0.09	294.65	4.00	-580.69	13.85	0.00
STRAIN+CROWD	0.12	295.21	6.00	-577.12	17.42	0.00
STRAIN*CROWD	0.17	295.60	8.00	-572.92	21.61	0.00
STRAIN*CONC*CROWD	0.61	312.05	24.00	-550.57	43.96	0.00

Feeding Effects on Sensitivity

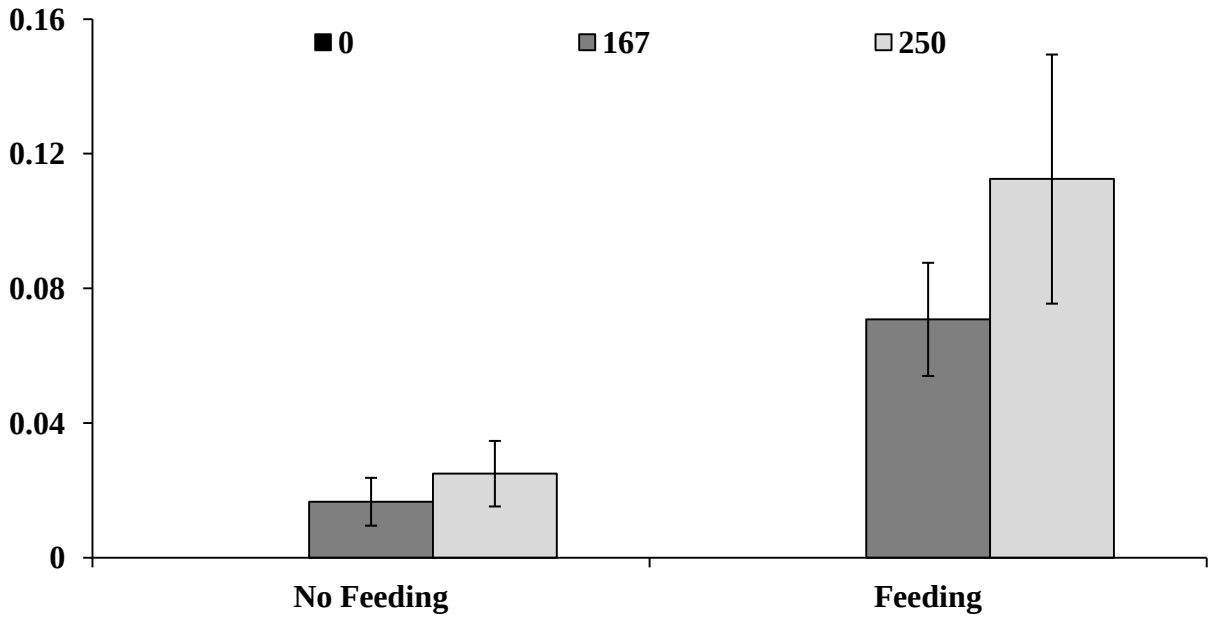

Figure 2.9. Average proportion mortality (SE bars) by feeding status and formalin concentration. Note that the mortality axis is reduced to show differences among formalin concentrations and feeding status.

Model selection results indicated that the interaction between formalin concentration and day-of feeding had a large effect on mortality, appearing in both the top model (AICc weight = 0.48) and the second best model (AICc weight = 0.25) of the set (Table 2.11). Within a formalin concentration, feeding the day of exposure to formalin increased mortality in comparison to tanks that had last been fed the day prior to exposure. Within the fed and not fed groups, there was no difference in mortality between 167 and 250 ppm, however, both concentrations exhibited higher mortality than the controls (Figure 2.9). Strain also had an effect on mortality, appearing additively in the second best model of the set (Table 2.11). The GR (1.7 ± 0.8%) and GR×HL 75:25 (1.7 ± 0.7%) did not differ in mortality, but exhibited lower mortality rates than did the HL (5.6 ± 1.8%) and GR×HL 50:50 (6.1 ± 2.5%).

Table 2.11. Model selection results for factors influencing formalin sensitivity in *Myxobolus cerebralis* resistant rainbow trout, including formalin concentration (CONC), strain (STRAIN), and day-of feeding (FEED) effects. 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
CONC*FEED	0.42	300.31	6.00	-587.33	0.00	0.48
CONC*FEED+STRAIN	0.51	304.80	10.00	-586.00	1.33	0.25
CONC+FEED	0.36	298.05	5.00	-585.19	2.14	0.17
STRAIN+CONC+FEED	0.45	302.00	9.00	-583.10	4.23	0.06
STRAIN*FEED+CONC	0.51	304.23	11.00	-582.06	5.27	0.03
CONC	0.25	292.78	3.00	-579.21	8.11	0.01
STRAIN+CONC	0.34	295.88	7.00	-576.00	11.33	0.00
FEED	0.11	287.23	2.00	-570.28	17.04	0.00
STRAIN+FEED	0.20	289.58	6.00	-565.86	21.47	0.00
Intercept-only	0.00	283.48	1.00	-564.91	22.42	0.00
STRAIN*FEED	0.26	290.58	8.00	-562.87	24.46	0.00
STRAIN	0.09	285.39	4.00	-562.19	25.14	0.00
STRAIN*CONC*FEED	0.71	313.76	24.00	-553.98	33.35	0.00

Size Effects on Sensitivity

Model selection results indicated that formalin concentration and size both had an effect on mortality, with an interaction between the two factors appearing in the top model (AICc weight = 0.64), and both factors appearing additively in the second best model of the set (AICc weight = 0.35; Table 2.12). Small fish (1.5-2" TL) were the least sensitive to formalin exposure, with mortality occurring only in tanks where fish were exposed to 250 ppm of formalin (Figure 2.10). Fingerling fish (medium; 3" TL) exhibited mortality when exposed to both 167 and 250 ppm formalin, but mortality did not differ among the exposures or from the mortality experienced by the small fish exposed to 250 ppm formalin. Mortality increased with an increase in size, with large fish (5" TL) exhibiting higher rates of mortality when exposed to both 167 and 250 ppm formalin relative to the small and medium fish. However, similar to the medium sized fish, there was not a difference in mortality in large fish exposed to either 167 or 250 ppm formalin (Figure 2.10).

Table 2.12. Model selection results for factors influencing formalin sensitivity in *Myxobolus cerebralis* resistant rainbow trout, including formalin concentration (CONC), strain (STRAIN), and size (SIZE) effects. 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
CONC*SIZE	0.44	391.61	9.00	-762.79	0.00	0.64
CONC+SIZE	0.35	387.33	6.00	-761.57	1.22	0.35
CONC*SIZE+STRAIN	0.46	391.20	13.00	-751.20	11.59	0.00
SIZE	0.18	378.62	3.00	-750.93	11.85	0.00
STRAIN+CONC+SIZE	0.36	386.82	10.00	-750.63	12.16	0.00
CONC	0.17	378.31	3.00	-750.32	12.47	0.00
Intercept-only	0.00	371.47	1.00	-740.89	21.90	0.00
STRAIN+SIZE	0.19	377.91	7.00	-740.35	22.43	0.00
STRAIN+CONC	0.19	377.60	7.00	-739.73	23.06	0.00
STRAIN*SIZE+CONC	0.42	387.37	15.00	-737.69	25.10	0.00
STRAIN	0.02	370.65	4.00	-732.79	30.00	0.00
STRAIN*SIZE	0.25	377.68	12.00	-726.96	35.83	0.00
STRAIN*CONC*SIZE	0.63	389.84	36.00	-651.00	111.79	0.00

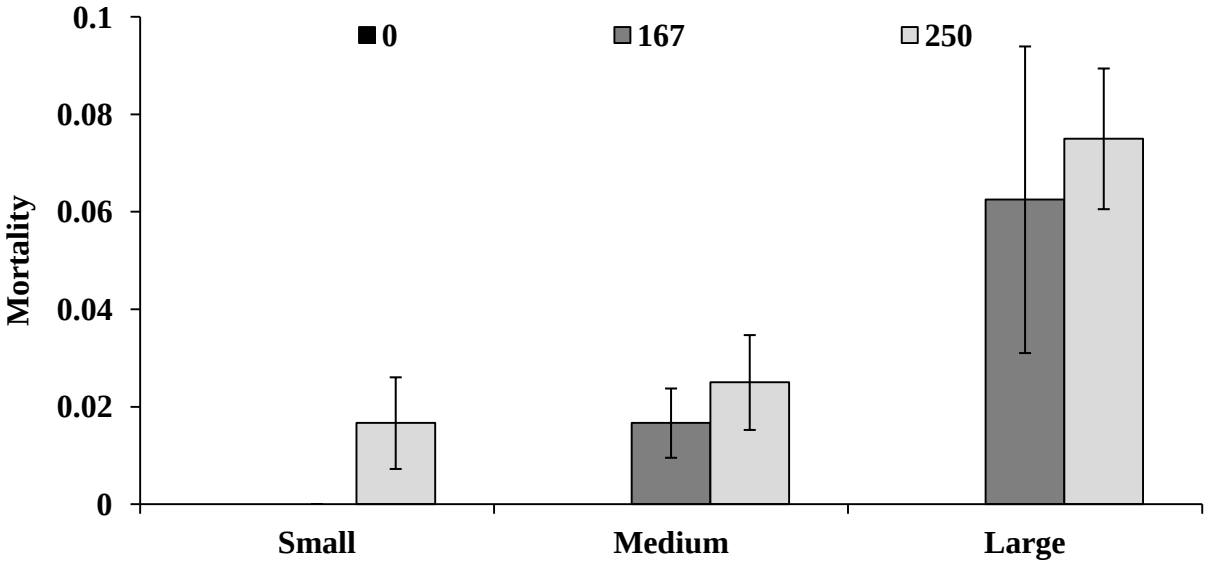

Figure 2.10. Average proportion mortality (SE bars) by size and formalin concentration. Note that the mortality axis is reduced to show differences among formalin concentrations and size.

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. However, formalin concentration and strain of fish being treated are other factors that could contribute to increased mortality. In general, hatchery managers can expect an average loss of 33% in *Myxobolus cerebralis* resistant rainbow trout egg lots.

Formalin concentration appears to be the largest factor contributing to mortality in fingerling rainbow trout, with mortality increasing with an increase in formalin concentration. Mortality is also increased under certain hatchery conditions, such as decreased flow or if the fish are fed the day they are treated. Additionally, fish size has an effect, with smaller fish being less sensitive, exhibiting less mortality when exposed to formalin relative to larger fish. Overall, hatchery managers can expect minimum losses of between 1 and 10% when treating *Myxobolus cerebralis* resistant rainbow trout with formalin. These are considered minimum losses because these experiments were conducted with healthy fish (i.e., no disease outbreaks), and losses are expected to be higher when fish are additionally stressed by the presence of external parasites. In general, it will be important for hatchery managers to consider all factors prior to treating rainbow trout with formalin including severity of disease outbreak, expected minimum losses, strain of fish and life stage being treated, and hatchery conditions, including but not limited to flow, whether the fish to be treated have been fed, temperature, rearing densities, cleanliness, water quality, and type of culture (e.g., raceway versus pond).

Experiment 4 – Density and Effects of Multiple Exposures on Formalin Sensitivity

METHODS

Spawning, Egg Incubation, and Formalin Exposure

The same four strains of whirling disease resistant rainbow trout fingerlings used in Experiments 1, 2, and 3 were used to determine strain sensitivity at various densities and multiple exposures. Multiple exposures are often needed when water temperatures are higher, formalin concentrations are lower, or infestations of external parasites are more severe (Piper at el. 1982).

Spawning occurred in December 2014. GR egg groups were created by pooling the eggs from 21 pairs of two-year-old GR females spawned with three-year-old GR males. The eggs from 12 pairs of three-year-old HL females spawned with two-year-old HL males were pooled together to create the HL strain egg groups for the experiment. The GR×HL 50:50 egg groups were created by pooling the eggs from 12 pairs of three-year-old HL females spawned with two-year-old GR males. The eggs from 18 pairs of two-year-old GR×HL 50:50 males spawned with three-year-old GR females were pooled together to create the GR×HL 75:25 egg groups. 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. Seven trays within one tray tower were used for egg incubation, and each tray contained four screen-bottomed PVC inserts. Inserts within each of the treatments were numbered 1-28 (Figure 2.11), and strains were assigned to an insert using a random number generator (Table 2.13). Each insert contained 500

eggs, providing seven, 500 egg replicates per strain. For each strain or cross, 500 eggs were initially counted into a container to quantify egg volume. This known volume was then used to distribute the approximate number of eggs to each insert.

The formalin concentration to which the eggs were exposed during incubation was 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.

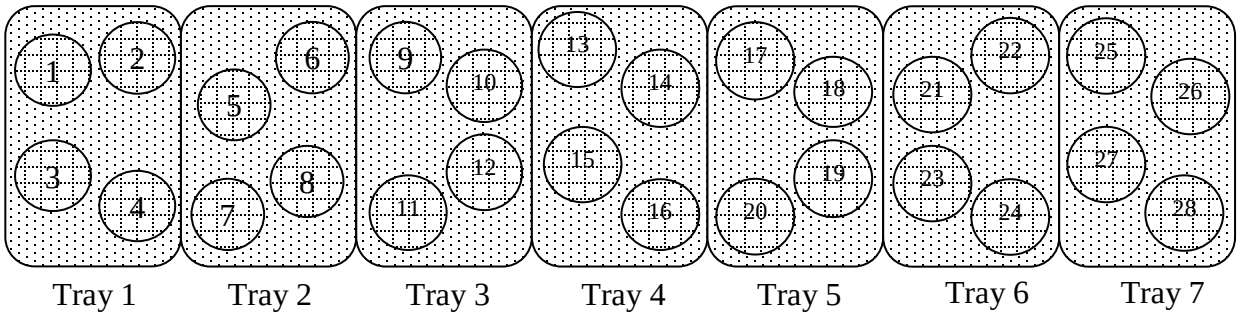

Figure 2.11. Arrangement of 28 screen-bottomed PVC inserts in the seven trays (from top of tower down) used during egg incubation. Strains were randomly assigned to an insert, within a treatment, using a random number generator (Table 2.13).

Table 2.13. Assignment of strain to PVC insert within the tray tower via a random number generator. The tray tower contains four, 500 egg replicates per strain.

PVC Insert	Strain	PVC Insert	Strain
1	Harrison	15	Hofer x Harrison 50:50
2	Hofer x Harrison 50:50	16	Harrison
3	Hofer x Harrison 50:50	17	Hofer
4	Hofer x Harrison 75:25	18	Harrison
5	Hofer x Harrison 50:50	19	Hofer x Harrison 50:50
6	Hofer	20	Hofer
7	Harrison	21	Harrison
8	Hofer x Harrison 75:25	22	Harrison
9	Hofer	23	Hofer x Harrison 75:25
10	Harrison	24	Hofer x Harrison 75:25
11	Hofer x Harrison 50:50	25	Hofer
12	Hofer x Harrison 75:25	26	Hofer x Harrison 75:25
13	Hofer x Harrison 50:50	27	Hofer x Harrison 75:25
14	Hofer	28	Hofer

Similar to Experiments 1, 2, and 3, 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 3.

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 or cross as the factor ($N = 28$). 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 4. All four strains were fed a similar ration of food (i.e., 2% body weight per day), and were reared under similar environmental conditions (i.e., flows, temperatures, etc.), until they reached 3" in length.

Two weeks prior to initiation of the first formalin sensitivity trial, 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 identification upon death as fish from each of the four strains were combined in each replicate. One VIE color was used for each of the four strains (e.g., GR: red, HL: green, HxH 50:50: orange, HxH 75:25: blue; 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). Flow was maintained at two gpm achieving three full turnovers during the 30 minute treatment, and allowing us to reach the desired formalin concentration during the treatments. Treatments were assigned to tanks using a random number generator. Five days prior to the experiment, 10 (half normal density), 20 (normal density), 40 (2x normal density), or 80 (4x normal 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 a trial, and the days in between multiple treatments.

On the first, third, and fifth day of a trial, 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, 1.89 ml per minute for 250 ppm, and 3.78 ml per minute for 500 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 the third and final 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 four different trials with varying densities and multiple exposures. The four trials will be run at four different densities: 1) half normal density or 10 fish per strains per tank, 2) normal density or 20 fish per strain per tank, 3) two times normal density or 40 fish per strain per tank, and 4) four times normal density or 80 fish per strain per tank. The objective of the experiment is to determine if mortality from multiple exposures is additive, occurs with each exposure, or if sensitive fish are lost during the first exposure and more tolerant fish survive the remainder of the exposures. In addition, the objective of this experiment is to determine the effects of rearing density on formalin sensitivity. The results of these trials will be available in the next reporting cycle.

RESULTS

Egg Formalin Sensitivity

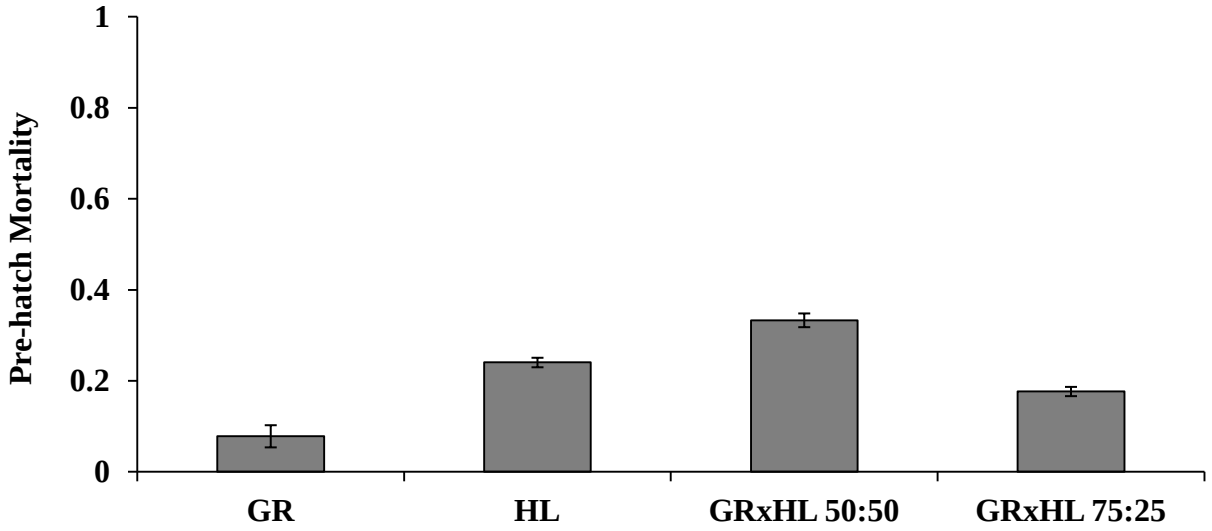

Figure 2.12. Average proportion pre-hatch mortality (SE bars) by strain.

As mentioned in the methods, 500 eggs from each strain or cross were counted by hand to quantify egg volume. 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

507 (± 39) eggs per PVC insert. Average number of eggs differed among strains and crosses (F = 19.74, $P < 0.001$). On average, the GR PVC inserts contained a significantly higher number of eggs (561 ± 26 eggs per insert) than did the HL PVC inserts (502 ± 11 eggs per insert), H×H 50:50 PVC inserts (490 ± 6 eggs per insert), or the H×H 75:25 inserts (476 ± 34 eggs per insert; $P < 0.001$). The HL, H×H 50:50, and H×H 75:25 PVC inserts did not differ significantly in average number of eggs per insert ($P \geq 0.166$).

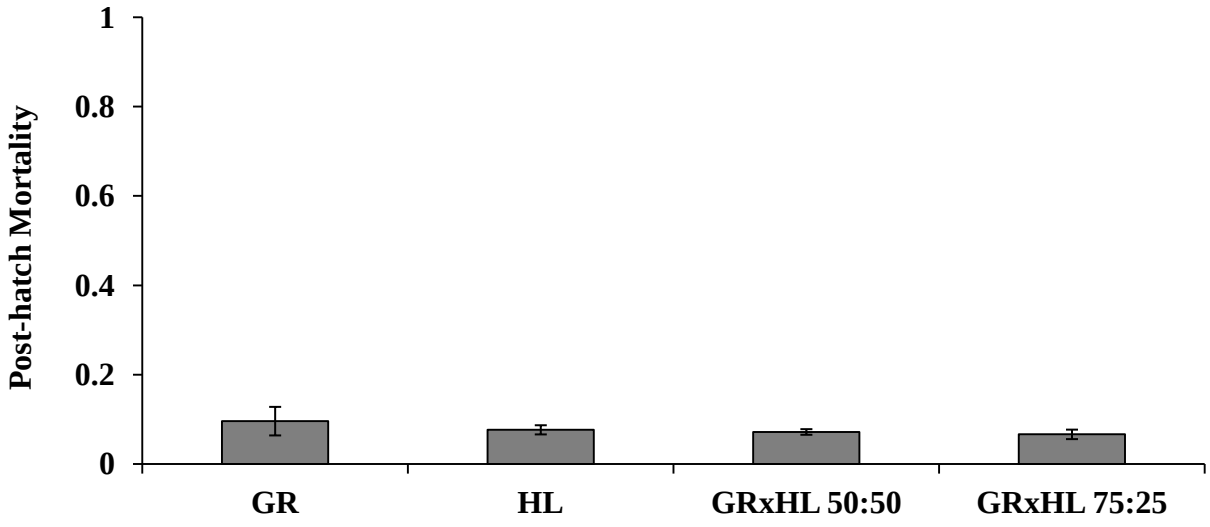

Figure 2.13. Average proportion post-hatch mortality (SE bars) by strain.

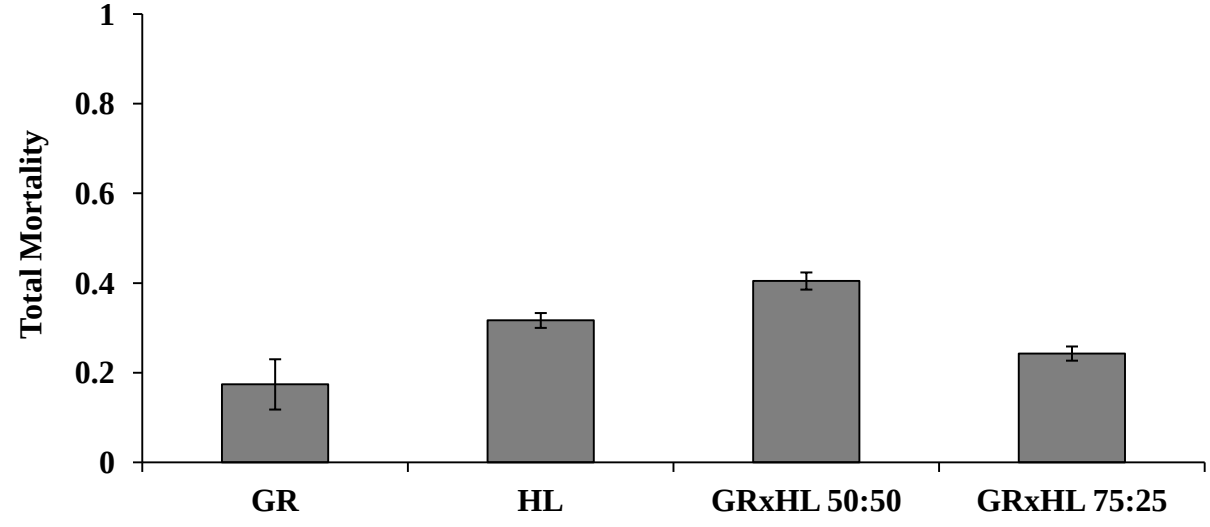

Figure 2.14. Average proportion total mortality (SE bars) by strain.

Average pre-hatch mortality differed significantly among the strains and crosses (F = 41.01, $P <$ 0.001; Figure 2.12). The GR exhibited significantly lower average percent pre-hatch mortality (7.8 ± 2.4%) than any of the other strains or crosses ($P < 0.001$). Average percent pre-hatch

mortality for the GR×HL 50:50 was significantly higher (33.3 ± 1.5%) than any of the other strains or crosses ($P \leq 0.019$). Though significantly different from the GR and GR×HL 50:50, average pre-hatch mortality rates did not differ significantly between the GR×HL 75:25 (17.7 ± 1.0%) and HL (24.0 ± 1.0%; $P = 0.09$). The majority of the pre-hatch mortality occurred in the form of dead eggs that were picked off prior to hatching (17.5 ± 9.6%), with only 3.3 ± 2.1% of the mortality occurring in the form of blank eggs that never produced fish and were picked off post-hatch.

Average post-hatch mortality did not differ among the strains and crosses ($F = 0.40$, $P = 0.751$; Figure 2.13). All of the post hatch mortality (7.8 ± 4.6%) occurred in the form of crippled fish that were pulled out of the tank as swim up was occurring.

Average total mortality differed significantly among the strains and crosses ($F = 10.47$, $P < 0.001$; Figure 2.14). The GR exhibited significantly lower total mortality (17.4 ± 5.6%) than the HL (31.7 ± 1.7%; $P = 0.002$) and the GR×HL 50:50 (40.8 ± 1.9%; $P < 0.001$), but did not differ in total mortality from the GR×HL 75:25 (24.3 ± 1.6%; $P = 0.06$). The GR×HL 50:50 exhibited significantly higher total mortality than the GR and GR×HL 75:25 ($P \leq 0.002$), but did not differ from the HL strain ($P = 0.087$). Similar to pre-hatch mortality, the HL and GR×HL 75:25 did not differ from each other in average total mortality ($P = 0.121$).

CONCLUSIONS

Similar to previous years, total egg mortality was highly variable among the strains and crosses. The GR×HL 50:50 cross exhibited higher rates of mortality than it had in previous years, whereas mortality rates in the GR×HL 75:25 were lower than they had been in previous years. The large majority of mortality occured in the form of dead or unhatched eggs. Crippled fish did contribute to total mortality, however, the rate of cripples that died prior to swim-up was similar among the four strains and crosses, and was generally lower than egg mortality. Therefore, it appears that the majority of the mortality occurs pre- versus post-hatch. Taken together, the egg sensitivity results from the experiments conducted in 2012, 2013, 2014, and 2015 suggest that egg quality within the year of collection has a large impact on mortality in the egg life stage.

Results from the fingerling density and multiple exposures trials were not available at the time of this report. The complete results from Experiment 4 will be available in the next reporting cycle.

DISSOLVED OXYGEN TOLERANCE OF RAINBOW TROUT STRAINS

INTRODUCTION

Whirling disease-resistant rainbow trout strains have been developed for production in Colorado's hatchery system and use in wild reintroductions. However, information on culturing these strains and potential deviations from the norm in the hatchery environment is still being gathered. One of the questions of interest is whether these strains and crosses exhibit differences in dissolved oxygen minimum tolerances, and how these differences may affect hatchery culture practices. There has been little work dedicated to determining if differences in dissolved oxygen tolerances exist among rainbow trout strains; however, dissolved oxygen tolerances have been

examined in stocks of cutthroat trout (Wagner et al. 2001). In addition, Colorado has many high elevation lakes that winterkill fish every couple of years. One proposed strategy for the management of winterkill lakes has been the stocking of salmonids with higher survival rates under low dissolved oxygen concentrations (Ashley and Chan 1992). The objectives of this experiment were to determine the critical dissolved oxygen minimum for four strains of rainbow trout currently cultured in Colorado, and to determine if there are differences in dissolved oxygen tolerance among the strains that could be stocked in lakes that have the potential to winterkill due to low dissolved oxygen concentrations.

METHODS

Strains and Rearing Procedures

Four whirling disease-resistant rainbow trout strains and crosses were used to determine differences in critical dissolved oxygen minima: Hofer (GR), Harrison Lake (HL), Hofer × Harrison Lake 50:50 (GR×HL 50:50), and Hofer × Harrison Lake (GR×HL 75:25). All four of these strains and crosses are maintained as brood stock at the BFRH. Rainbow trout were spawned at the BFRH in December 2013 and reared in conjunction with the fish used in the formalin sensitivity experiments described previously. Fish were reared for approximately 7 months and averaged 178 ± 35 mm total length and 74 ± 48 g at the time of experimentation.

Experimental Procedures

A hatchery trough (366 cm long × 48 cm wide × 30 cm deep), enclosed in plastic tarps to prevent inducing a physiological stress response to movement or light, was used to conduct the dissolved oxygen experiments at the BFRH. The trough was divided using a perforated screen. Fish for the following day experiments were housed in the upper half of the trough to prevent accidental feeding or handling stress in the 24 hours prior to an experiment. Dissolved oxygen experiments were conducted in the lower half of the trough in two, 41.6 liter plastic containers. Water (12°C) was run through the trough at a rate of 18.93 liters per minute to maintain temperature within the plastic containers during the experiments. Fluorescent lighting was hung above the tanks to provide constant and consistent lighting within the tarps during the experiments. In addition, a Logitech web camera was mounted above each tank so that end points of the experiment could be determined after the experiment had been conducted, preventing observer movement from increasing fish stress levels and affecting dissolved oxygen tolerances.

At the start of a dissolved oxygen trial, both of the plastic containers were filled with water directly from the trough. Fish from the upper portion of the trough were transferred to both containers, one fish each, allowing two dissolved oxygen trials to be conducted at one time. A perforated lid allowing dissolved oxygen exchange at the water surface was placed on top of each container and secured for the pre-experiment acclimation period of one hour. In addition, oxygen was introduced to both tanks through a Sweetwater fine pore diffuser at a rate of 3-4 ml per minute for the period of one hour to allow the fish to acclimate to the tanks, reduce the physiological effects of handling that could affect dissolved oxygen tolerances, and increase oxygen levels so that all trials started at 100% saturation. Saturation was confirmed using two YSI ProODO dissolved oxygen meters.

Following the one hour acclimation period, the perforated lid was removed from the tanks and replaced with a clear Plexiglas lid. The Plexiglas lid had only two holes, one for the air hose and one for the dissolved oxygen meter. Both holes were cut to fit equipment exactly, allowing the plastic containers to become sealed chambers and prevent oxygen exchange at the water surface. To conduct the dissolved oxygen trial, nitrogen gas was introduced to the tank at a rate of 50 ml per minute. The rate of nitrogen introduction had been determined prior to experimentation as the rate that would reduce dissolved oxygen levels in the tanks to less than 1.0 ppm after four to six hours. Trials were terminated once mortality occurred.

Oxygen depletion was confirmed using the logging function of the dissolved oxygen meters which produced dissolved oxygen curves that could be analyzed after each trial. In addition, the logging function of the dissolved oxygen meters was synchronized with the time function in the Logitech cameras so that dissolved oxygen endpoints could be determined post-experimentation. Three endpoints were observed: 1) initial loss of equilibrium (ILOE), 2) final loss of equilibrium (FLOE), an 3) mortality. Initial loss of equilibrium was defined as the first time at which the fish lost its equilibrium, turning upside down for a second or two, but recovering shortly thereafter. Final loss of equilibrium was the point at which a fish could not recover it's equilibrium for longer than 10 seconds. Mortality was considered the point where movement of the fish, including its operculum, fins, or tail, was no longer visible. At each of these endpoints, the time at which the endpoint occurred, the dissolved oxygen concentration (in both ppm and % saturation), and temperature were recorded. Following a trial, the fish were removed from tank, measured, and weighed. Only one trial was run per tank per day. Ten replicate trials were conducted for each strain or cross, for a total of 40 trials.

In addition to determining dissolved oxygen tolerance endpoints, additional trials were conducted to determine if fish could recover once they had reached the average concentration at which FLOE had occurred in the dissolved oxygen trials (13% saturation). All experimental procedures were similar to those described above. However, once tanks reached 13% saturation, fish were immediately removed from the experimental tank and placed in a separate trough with fresh, flowing, well-oxygenated water. Fish were held for 24 hours to determine if they would recover. At the end of 24 hour monitoring period, fish were measured, weighed, and their status (dead or alive) was recorded. Four replicate trials were conducted for each strain or cross, for a total of 12 additional trials.

All statistical analyses were conducted using the GLM procedure in SAS (SAS Institute 2011). Differences in dissolved oxygen endpoints (ILOE, FLOE, and mortality) were analyzed using a repeated measures analysis of covariance (RM ANCOVA), with strain or cross and endpoint as the factors (including interaction) and weight as the covariate. Recovery status was compared using an ANCOVA with strain or cross as the factor and weight as the covariate. Values for all analyses were reported from the type III sum of squares. If significant ($P < 0.05$) effects were identified, the least squares means method was used to determine significant differences in dissolved oxygen minimums among the strains or crosses and endpoints, and mortality among the strains or crosses.

Weight had a significant effect on minimum dissolved oxygen concentrations tolerated by a given individual ($F = 7.52$, $P = 0.007$), and results suggest that the larger the fish, the less tolerant it is of low dissolved oxygen concentrations. Dissolved oxygen concentrations differed among the strains and crosses ($F = 11.41$, $P < 0.001$). The GR×HL 50:50 appeared to be more tolerant of lower dissolved oxygen concentrations, reaching significantly lower dissolved oxygen concentrations than either the HL ($P < 0.001$) and GR×HL 75:25 ($P < 0.001$) before exhibiting signs of distress or mortality. The GR×HL 50:50 did not differ from the GR in dissolved oxygen concentration ($P = 0.085$). Dissolved oxygen concentrations did not differ among the GR, HL, or GR×HL 75:25 ($P \geq 0.075$; Figure 2.15). Although these results suggest differences in tolerance to low dissolved oxygen concentrations exist among the strains and crosses, dissolved oxygen concentrations are averaged across endpoints and are therefore not biologically informative with regards to the physiological signs of stress exhibited by the fish.

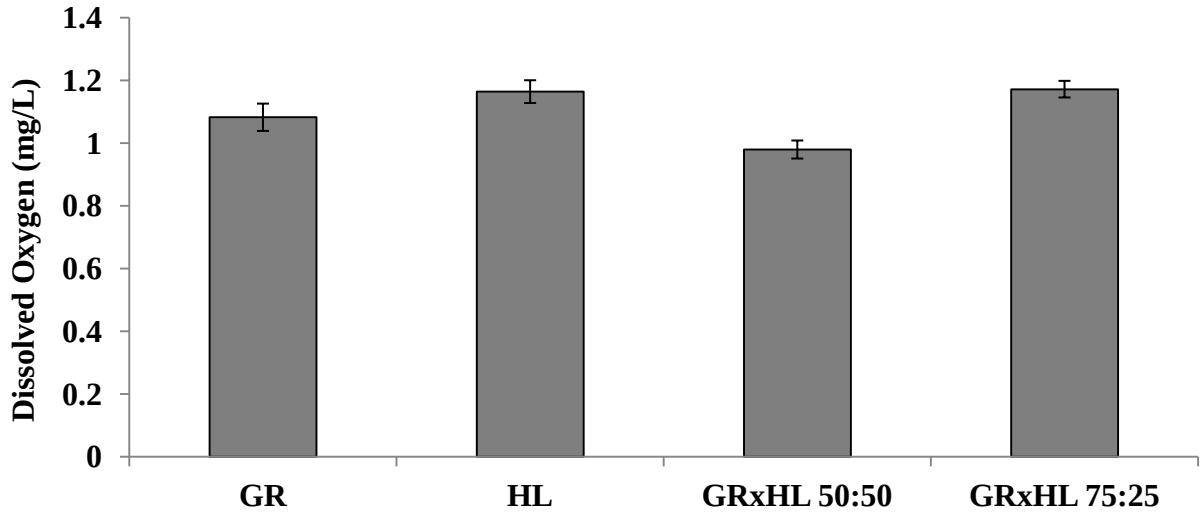

Figure 2.15. Differences in average minimum dissolved oxygen concentrations (mg/L) among the strains and crosses.

Dissolved oxygen concentrations also differed among the endpoints ($F = 89.99$, $P < 0.001$). Initial loss of equilibrium (ILOE) and final loss of equilibrium (FLOE) did not occur at significantly different dissolved oxygen concentrations ($P = 0.070$). However, mortality occurred at significantly lower dissolved oxygen concentrations than either ILOE or FLOE ($P < 0.001$; Figure 2.16). These results suggest that although fish may be exhibiting signs of stress (i.e., loss of equilibrium) as dissolved oxygen concentrations drop, there may be chance to recover if dissolved oxygen concentrations are returned to a tolerable range. To support this, the results of the experiment examining the ability of the fish to recover suggested that fish could recover after being exposed to low dissolved oxygen concentrations. Of the 12 fish tested, only two, a GR and a GR×HL 75:25, died after being exposed to dissolved oxygen concentrations at 13% of saturation. The ability to recover from dissolved oxygen concentrations at 13% of saturation did not differ among the strains and crosses ($F = 0.39$, $P = 0.767$), and weight did not contribute significantly to the ability of fish to recover from low dissolved oxygen concentrations ($F = 0.17$, $P = 0.694$).

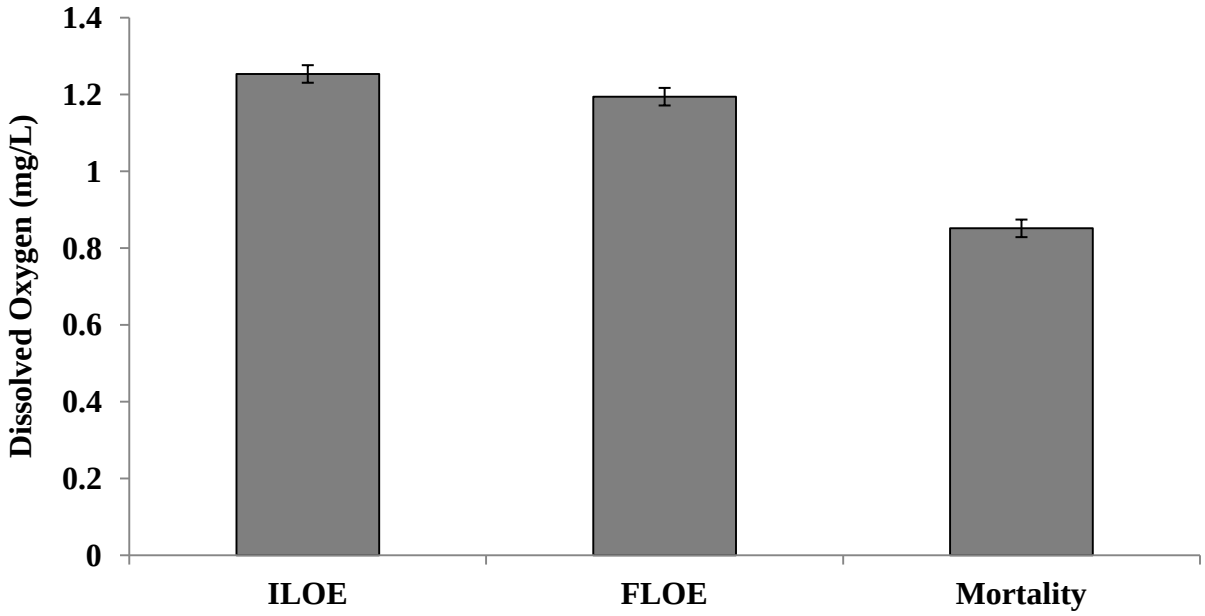

Figure 2.16. Differences in average dissolved oxygen minimums (mg/L) among the endpoints.

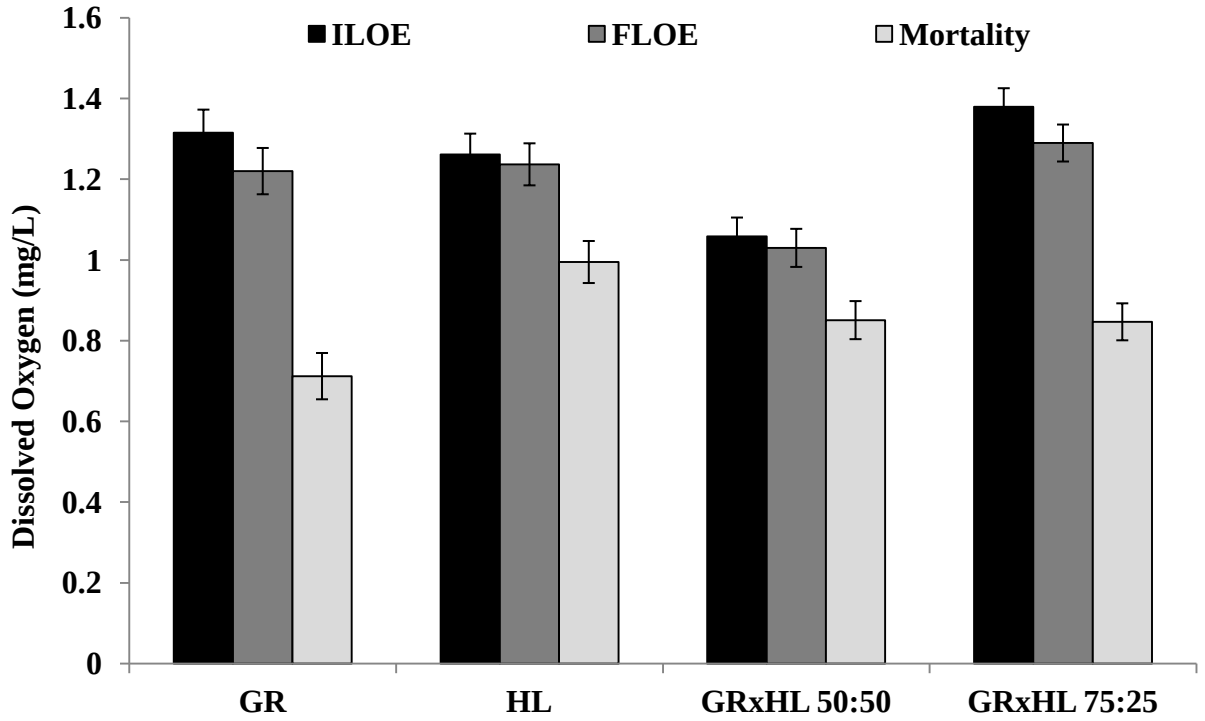

Figure 2.17. Average dissolved oxygen concentrations (mg/L) at which initial loss of equilibrium (ILOE), final loss of equilibrium (FLOE), and mortality occurred among the strains and crosses.

There was a significant interaction between strain and cross and endpoint ($F = 5.11$, $P < 0.001$). Within a strain, dissolved oxygen concentrations resulting in ILOE did not differ from those resulting in FLOE in any of the strains ($P \geq 0.145$; Figure 2.17). These results suggest that

dissolved oxygen concentration does not need to drop much before the fish can no longer maintain their equilibrium. As such, though ILOE was measured with the intention of having an early warning sign that there may be an issue with dissolved oxygen concentrations in the hatchery, hatchery personnel are more likely to observe fish that have permanently lost their equilibrium if dissolved oxygen concentrations become an issue. Dissolved oxygen concentrations resulting in ILOE and FLOE were significantly lower in the GR×HL 50:50 than any of the other strains or crosses ($P \leq 0.012$). The GR, HL, and GR×HL 75:25 did not differ in dissolved oxygen concentration resulting in either ILOE or FLOE ($P \geq 0.092$) This is consistent with the results presented above suggesting that the GR×HL 50:50 is more tolerant of low dissolved oxygen concentrations than the other strains or crosses.

Dissolved oxygen concentrations resulting in mortality differed among the strains and crosses (Figure 2.17). Mortality in the HL strain occurred at significantly higher dissolved oxygen concentrations than in the GR, GR×HL 50:50, or GR×HL 75:25 ($P \leq 0.032$). However, the GR, GR×HL 50:50, and GR×HL 75:25 did not differ in dissolved oxygen concentrations that resulted in mortality ($P \geq 0.067$). Therefore, the HL strain is likely to be the first to exhibit mortality if dissolved oxygen concentrations become a problem in the hatchery. Within all of the strains and crosses, dissolved oxygen concentrations resulting in mortality were significantly lower than the concentrations resulting in ILOE or FLOE ($P \leq 0.006$). However, differences between concentrations resulting in ILOE, FLOE, and mortality were larger in the GR and GR×HL 75:25 than in the HL and GR×HL 50:50. Therefore, hatchery managers may have more time to correct issues with low dissolved oxygen concentrations after observing loss of equilibrium in the GR and GR×HL 75:25 than they would after observing loss of equilibrium in the HL or GR×HL 50:50. As a result, higher losses would be expected in the HL and GR×HL 50:50 if actions were not taken quickly to resolve issues with low dissolved oxygen concentrations.

One of the objectives of this study was to determine if there are differences in dissolved oxygen tolerances between the strains that would lead to one strain being stocked over another in bodies of water in which winterkills occur. Although the results suggest that there are statistical differences between the strains and crosses, due to the relatively small scale of the differences in dissolved oxygen concentrations among the strains and endpoints, these differences are not likely biologically significant with regards to tolerances in the wild. It is unlikely that one strain or cross would exhibit increased survival in lakes in which winterkills occur. Therefore, these results should mainly be used as a guide to assist with correcting issues with low dissolved oxygen concentrations in the hatchery environment.

TRIPLOID WALLEYE PRODUCTION

The CPW Pueblo Hatchery has been working on methods to optimize Colorado's triploid walleye production using pressure shock treatments. The objective was to maximize triploid induction rates while minimizing the egg pick-off rates. We worked with David Harris, CPW Pueblo Hatchery manager, to analyze data collected between 2010 and 2014 to determine the optimal time to initiation of pressurization to optimize induction rates and minimize pick-off. The results of the analysis were presented in a CPW white paper produced by the aquatic wildlife research section in 2015, and can be found in Appendix A of this report.

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

Job Objective: These experiments are focused on the performance of the Hofer and Hofer × Harrison Lake strain as domestic production fish compared with other commonly used production fish. Segment objectives described below will occur over the course of more than one fiscal year.

Parvin Fingerling Plant Experiments

INTRODUCTION

Evaluation of the various strains of Hofer and Harrison crosses to optimize stocking efficiency and survival are an important part of the long-term strategies for CPW hatchery production. Several years of data from previous live-release experiments in Parvin Lake, Colorado, have demonstrated that certain resistant strain fish are more desirable from a growth and survival standpoint than others (Fetherman and Schisler 2013, 2014). The focus of this segment of these evaluations is to continue to evaluate survival and growth of fish from previous stocking events. 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.

METHODS

In addition to the lots of fish stocked in previous years, three additional lots were stocked in April of 2014 and 2015 (Table 3.1). Numerous acronyms have been developed over the past few years to describe these varieties. Table 3.2 contains the formal Colorado Parks and Wildlife acronyms of the various strains and their definitions.

Table 3.1. Coded-wire tagged fish stocked in Parvin Lake during 2014 and 2015.

	2014 Plants			2015 Plants		
Strain	GR	HXC	HN2	GR	HXC	GBN
Lbs	426.0	426.0	426.0	212.3	140.6	32.9
Number	1,734	1,734	1,734	1,125	1,125	1,125
Length (mm)	215.9	215.9	215.9	195.6	172.7	106.7

Table 3.2. Acronyms for various strains used in these experiments.

HAR	Pure Harrison Lake rainbow trout, also described as HL in this document.
HOF	Pure Hofer rainbow trout, also described as GR in this document.
HXN	Pure Hofer rainbow trout (described above) crossed with fall spawning Snake River cutthroat trout (see SRN below).
HXH	Hofer rainbow trout crossed with Harrison Lake strain rainbow trout. Proportion of Hofer to Harrison is typically provided with parentheses. For example, (75:25) would be 75% Hofer and 25% Harrison Lake. If no parentheses or other designation are provided, as with the Colorado Parks and Wildlife species codes, these fish are typically from Crystal River Hatchery brood stock. That stock originated from a mixture of HXH year classes consisting of (75:25) and (87.5:12) crosses.
HHN	Crystal River Hatchery HXH brood stock (as described above) crossed with fall spawning Snake River Cutthroat (see SRN below).
HH1	Crystal River Hatchery HXH brood stock (as described above) crossed with SR1.
HH2	Crystal River Hatchery HXH brood stock (as described above) crossed with SR2.
HN1	Pure Hofer rainbow trout crossed with SR1.
HN2	Pure Hofer rainbow trout crossed with SR2.
SRN	Fall spawning Auburn strain Snake River cutthroat trout housed at Crystal River Hatchery
SR1	Pure Wyoming spring spawning Snake River cutthroat trout, brought to Crystal River Hatchery to increase genetic diversity of SRN.
SR2	Cross of any form of SRN with SR1, including 50:50, 75:25, and other back-crosses. This is the lot created to increase Snake River cutthroat brood stock diversity, but intended to be as close to old fall spawn timing as possible.
RXN	Standard hatchery rainbows (usually Bellaire or Tasmanian) crossed with Snake River Cutthroat (SRN, SR1 or SR2).
HXC	Hofer rainbow trout crossed with Colorado River rainbow trout. As with the HXH strains, parentheses or other designations are typically used to delineate the proportional crosses of these fish. Early crosses used in these experiments were 50:50 crosses from the Glenwood Springs Hatchery. After 2012 these fish are subsequent generational crosses (matings of the original HXC year classes with other HXC year classes).
GBN	Recreational greenback cutthroat trout (not pure, used for recreational stocking opportunities).

Collections of coded-wire tagged fish for this project were made using evening boat electro-fishing in May, July, September, and October. Additional samples were collected for two separate graduate student projects, including an investigation of susceptibility of rainbow trout strains to gill lice and another project focused on pre-stocking predator avoidance training. Only those samples explicitly collected for myxopore counts for this particular project are reported here. Marked sample goals (40-60 fish) could typically be accomplished by sampling 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.

Table 3.3. Average length, weight and myxospores for *M. cerebralis* sampling at Parvin Lake.

May 2014	Count	Length (mm)	Weight (g)	Myxospores
HXH (50:50) 2009	2	334	417	0
RXN 2009	2	379	733	5,294
HAR 2009	1	345	374	0
HHN 2010	10	378	567	0
RXN 2010	2	372	563	0
HN2 2011	6	337	432	0
RXN 2011	5	320	353	0
SR2 2012	1	273	186	0
HXC 2012	4	307	316	0
HN2 2012	3	300	284	0

July 2014	Count	Length (mm)	Weight (g)	Myxospores
RXN 2009	1	386	560	0
HHN 2010	1	400	681	0
RXN 2010	4	375	558	0
HN2 2011	3	351	410	0
RXN 2011	5	346	399	0
HXC 2012	2	309	286	0
HN2 2012	4	304	271	0
HN2 2014	1	252	148	0

September 2014	Count	Length (mm)	Weight (g)	Myxospores
HHN 2010	1	372	534	0
RXN 2010	2	374	502	0
HXC 2011	2	387	577	0
HN2 2011	4	350	397	0
RXN 2011	4	340	393	0
SR2 2012	5	273	200	0
HN2 2012	9	331	367	0
HXC 2014	15	276	222	0
HN2 2014	12	266	199	0
GR 2014	6	266	194	0

October 2014	Count	Length (mm)	Weight (g)	Myxospores
HXH (50:50) 2009	1	369	407	0
HHN 2009	1	376	501	0
HHN 2010	7	397	611	0
RXN 2010	2	421	870	292,317
HN2 2011	3	390	575	0
RXN 2011	3	369	508	0
SR2 2012	2	309	275	0
HXC 2012	1	338	382	0
HN2 2012	5	348	404	0
HXC 2014	9	289	266	1,348
HN2 2014	19	298	282	465
GR 2014	3	281	225	1,337

During the 2014 sampling season, forty-four fish were collected for this project in the May sample (seven with no tags), 21 in the July sample (all with tags), 62 in the September sample (all with tags) and 62 in the October sample (six with no tags). The samples collected included a variety of the fish stocked in previous years. Results for each individual year-class up to 2012 are provided in previous reports. Sampling results for 2014 are listed in Table 3.3. The table contains only strains that were recovered in the sampling events. See previous reports (Fetherman et al. 2014) for total numbers of fish stocked of each strain stocked prior to 2014. A high proportion of fish with Snake River cutthroat trout were found in the samples, especially among the groups stocked prior to 2014. Further data analysis will be needed to determine the extent and significance of these findings, but these results indicate that the Snake River cutthroat trout crosses are better suited to long-term survival than pure rainbow trout strains. Sample results from the 2014 plant indicate that the HN2 strain had better initial survival than the GBN or the HXC strains. Another interesting observation in the sampling is the low proportion of fish found to be infected with *M. cerebralis.* A total of only seven fish were found to be infected with *M. cerebralis* during the 2014 sampling season. One of these was an RXN from the 2010 plant, caught in the May sampling event. The other six were captured in the October sampling event. This included two RXN from the 2010 plant, along with one HXC, two HN2, and one GR from the 2014 stocking event. This is a substantial reduction in both proportion and severity of infected fish relative to previous years.

Poudre Ponds Hatchery Evaluation

INTRODUCTION

The goal of this study was to quantify infection levels in fish reared to catchable size in an infected environment. Both susceptible and resistant strains of fish were used to determine if using resistant strains would produce a better outcome in either scenario. A 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 (Fetherman and Schisler 2014.).

Resistant strains of fish in those previous experiments compared quite well with susceptible fish reared in environments free of the parasite. However, the rate of harvest and specific variety of fish in question weighed heavily on the final outcome. The focus of the experiment described in this segment was a follow-up to this original experiment in which four strains were evaluated, including pure GR, HXC, SR2, and HN2.

METHODS

The work conducted for this objective is a similar effort to that performed from 2009-2014. This experiment was conducted to determine infection level and growth of the four varieties reared together in a natural setting known to have high ambient levels of *M. cerebralis*. The first phase of this experiment began in 2012. The four strains of fish were reared at the Fish Research hatchery in Bellvue, Colorado. When fish reached 3-4 inches in length, they were marked with coded wire tags to distinguish between each of the four varieties. Twenty-five hundred fish of

each strain were transported and stocked into an earthen pond (Pond #3) at the Poudre Rearing Unit as fingerlings (24-97 fish lb^{-1}), for a total of 10,000 fish on October 18, 2012.

Random samples of fish were collected to determine growth rates and relative survival of each of the groups at one year (October 2013) and two years (October 2014). An additional sample was collected at 29 months (March 2015) during an annual hatchery inspection. 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 into a high use recreational fishery to evaluate survival and return to creel. On May 26, 2015, fish were harvested from the earthen pond and trucked to Boyd Lake, in Loveland, Colorado.

A standardized creel survey was being implemented on this reservoir, so this provided an opportunity to evaluate post-stocking survival and return to creel of the various lots of fish. The creel survey was ongoing at the time of this writing, and results of that phase of this experiment will be reported in the next reporting cycle.

RESULTS AND DISCUSSION

Table 3.4. Length, weight, and proportions of each of the four varieties of fish in Pond 3 of the Poudre Rearing Unit during random sampling events at one year and two years post-stocking.

October 2013	Number	Percent of catch	Length (mm)	Weight (g)
SR2	8	8.1	208.6	89.8
HXC	33	33.3	246.0	168.1
HN2	27	27.3	253.6	172.7
GR	31	31.3	262.6	199.0

October 2014	Number	Percent of catch	Length (mm)	Weight (g)
SR2	5	8.3	300.9	258.4
HXC	8	13.3	352.7	464.0
HN2	12	20.0	357.0	468.9
GR	35	58.3	361.7	505.7

At the time of this report, results were only available for the first two sampling events. Catch for pure SR2 fish was very low, making up only 8.1 and 8.3% of the catch in the first and second year of sampling (Table 3.4). HXC , HN2, and GR fish were very comparable in the first year sampling event, ranging from 27.3% in the HN2, to 33.3% in the HXC. In the second year sampling event, proportional catch of HXC (13.3%) and HN2 (20%) was much lower than that of the pure GR strain fish, which made up 58.3% of the catch. Growth results show the pure SR2 were much smaller in both length and weight than the other groups during both sampling events. Growth among HXC and HN2 were very comparable during both sampling events, and

pure GR growth was superior to the other varieties in both sampling events. These results also show very clearly the lower myxospore count among the HN2 and pure GR fish in this experiment compared to the SR2 and the HXC varieties (Figure 3.1).

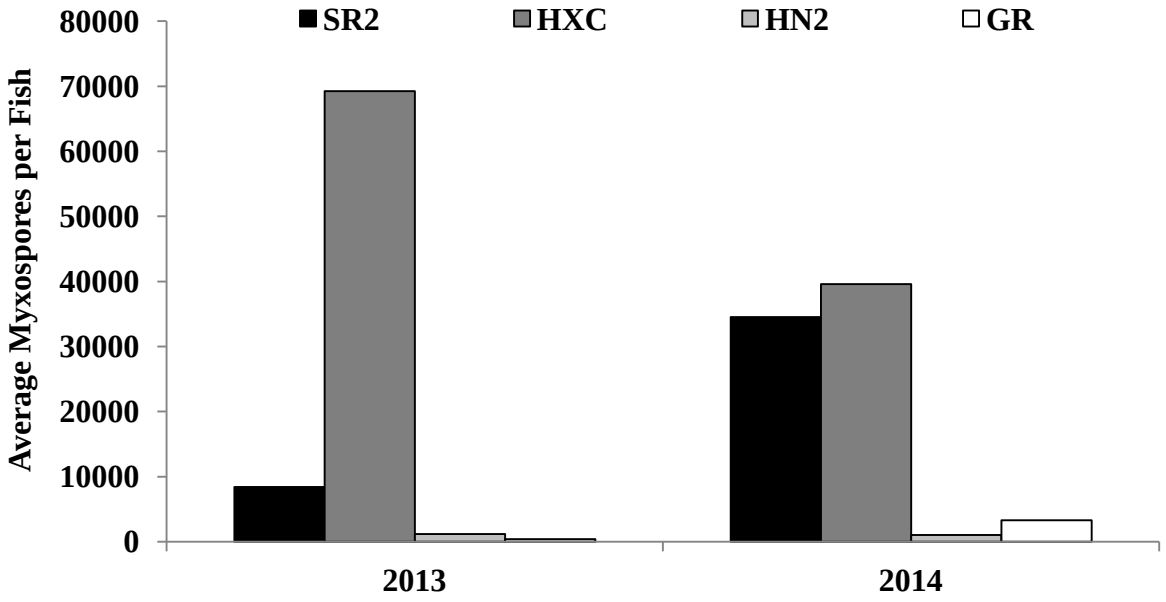

Figure 3.1. Myxospore count for four varieties of fingerling rainbow trout reared in an earthen pond at the Poudre Rearing Unit.

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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).

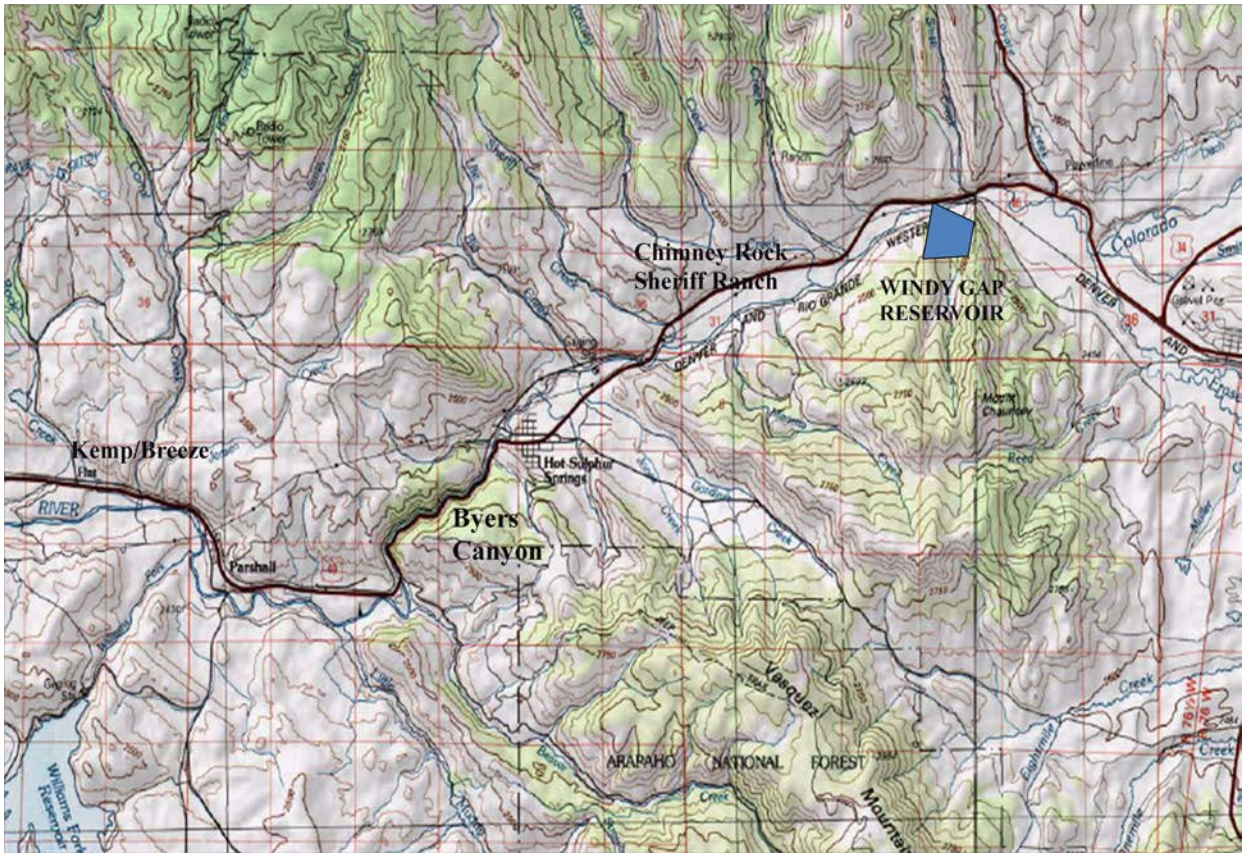

Figure 4.1. Upper Colorado River study area.

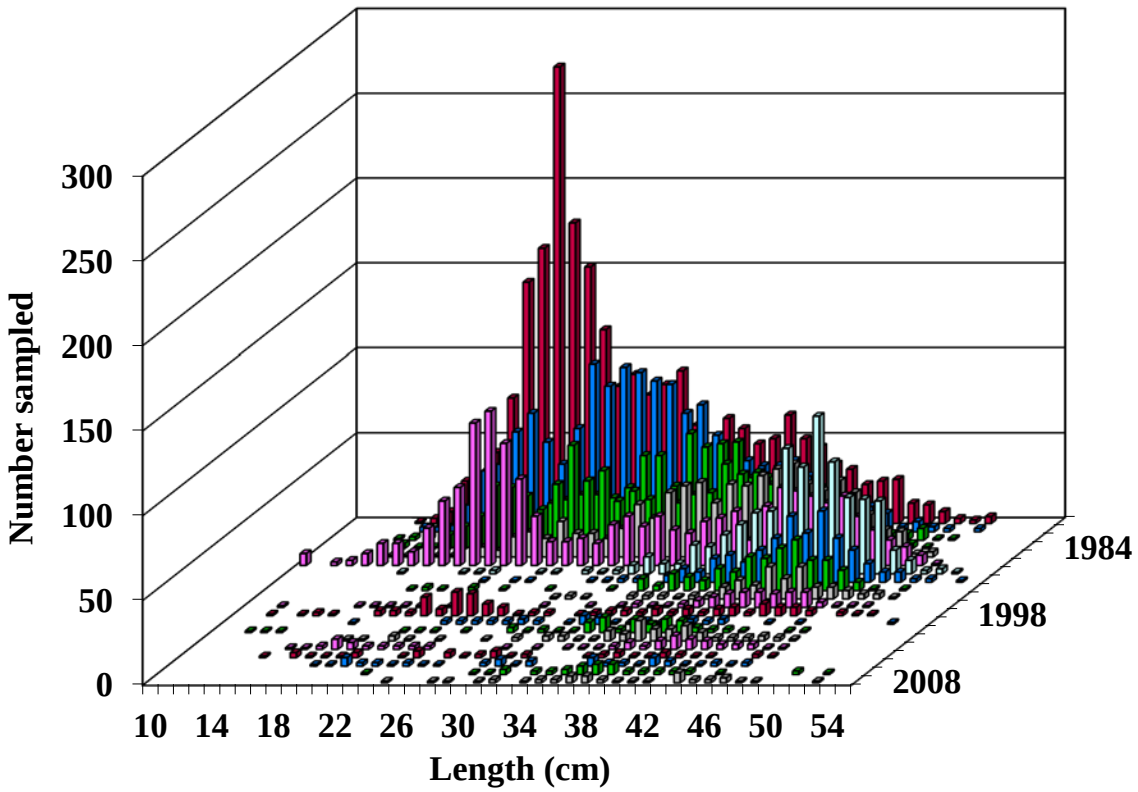

Figure 4.2. Upper Colorado River historic rainbow trout length-frequencies at Kemp-Breeze State Wildlife Area.

Adult GR×CRR Introductions

Whirling disease resistant rainbow trout introductions (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 population estimates conducted in 2011 suggested 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 number of fry surviving through the fall was 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).

Due to the positive results observed below Byers Canyon, GR×CRR fry stocking began in the Chimney Rock/Sheriff Ranch section of the upper Colorado River in 2013. The effects of the fry

stocking are monitored on an annual basis using both fry population estimates that are conducted once a month, June through October, and adult population estimates conducted in the spring of each year (see below).

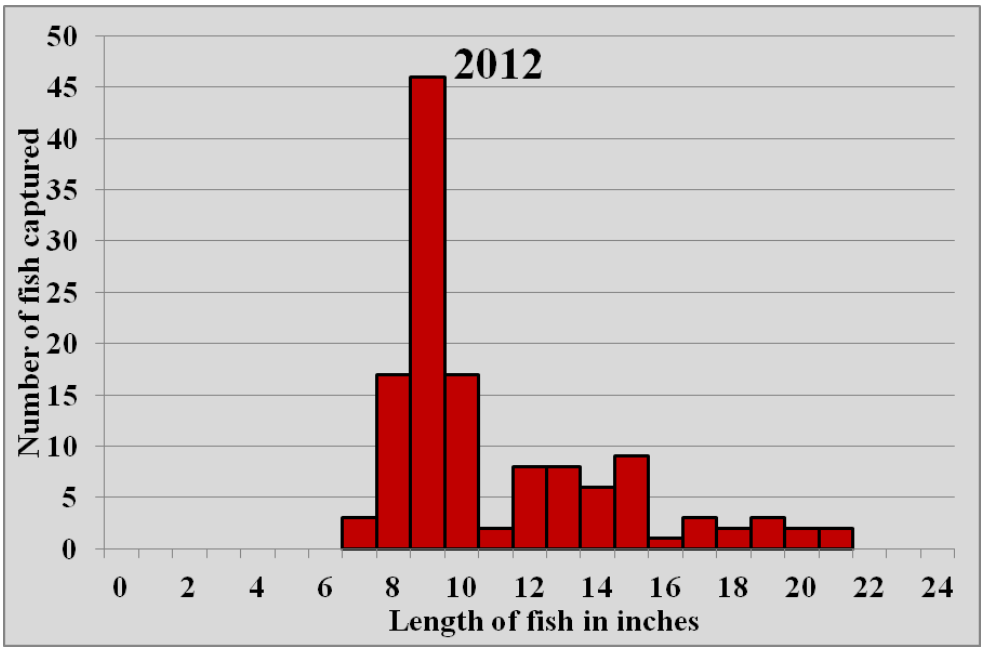

Figure 4.3. Number of rainbow trout captured in each length class in the Parshall-Sunset reach of the upper Colorado River in 2012. Courtesy of Jon Ewert, CPW Aquatic Biologist, Hot Sulphur Springs.

RESULTS AND DISCUSSION

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, 2014, and 2015. 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 adult rainbow trout and brown trout abundance 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 complete population estimate was conducted in May 2014, although flow conditions again presented a challenge as the river was higher (935 cubic feet per second [cfs]) than in any other year in which estimates were conducted previously. 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 31 (± 1) 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 recruitment from the fry stocking that occurred in the summer of 2013.

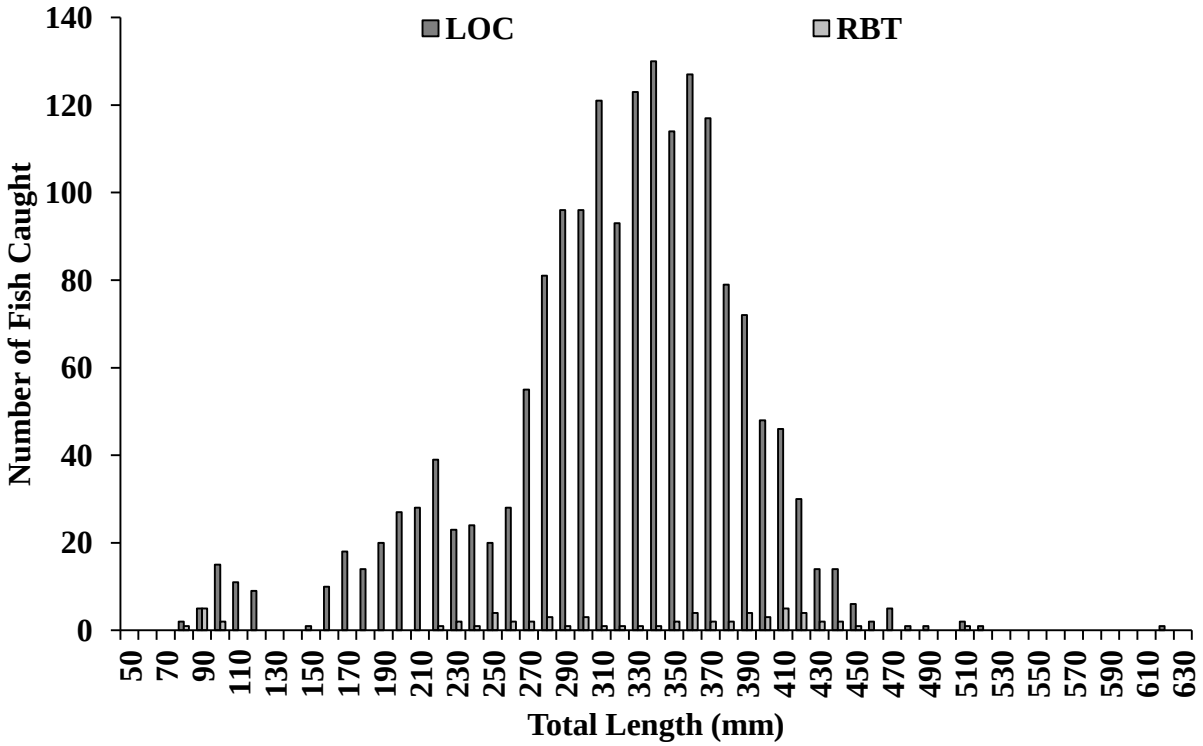

Figure 4.4. Number of brown trout (LOC) and rainbow trout (RBT) caught by total length (mm) during the 2015 adult population estimates in the Chimney Rock/Sheriff Ranch study section.

A population estimate was also completed in 2015 despite high spring flow conditions (1,235 cfs). An estimated 4,812 (± 347) adult brown trout, averaging 320 (± 61) mm TL and 346 (± 176) g, were present in the Chimney Rock/Sheriff Ranch study section. All age classes of brown trout were represented in the sample, including several juvenile (≤ 150 mm TL) brown trout, but the majority of the brown trout captured were age 3+ (Figure 4.4).

Rainbow trout densities increased between 2014 and 2015, with an estimated 113 (± 31) adult rainbow trout, averaging 343 (± 71) mm TL and 461 (± 210) g, present in the study section. Of the 57 adult rainbow trout captured during the estimates, 20 (35%) were female, the majority of which had already spawned, 16 (28%) were male, and 21 (37%) were immature. Age 1, 2 and 3+ rainbow trout were represented in the estimates (Figure 4.5). Fry stocked in 2014 were represented in the smaller length classes (70-110 mm TL), the presence of which suggested that fry stocked in 2014 survived the winter and were recruiting. Growth of the rainbow trout in the Chimney Rock/Sheriff Ranch appeared to be comparable to the population downstream of Byers

Canyon. A gap in length classes was observed between 110 and 210 mm TL suggesting that fish grow at least 100 mm TL between age 1 and age 2. Age 2 fish (150-300 mm TL) were more prevalent in the population than in previous years, and also made up a larger proportion of the total rainbow trout population (Figure 4.6).

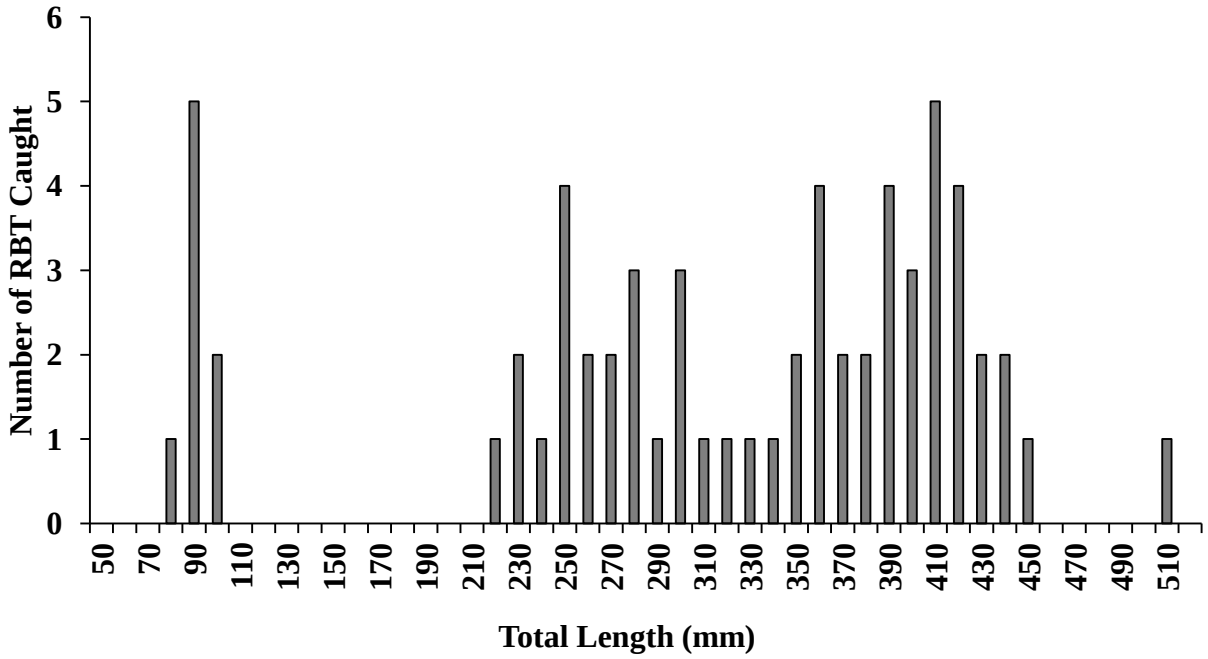

Figure 4.5. Number of rainbow trout (RBT) caught by total length (mm) during the 2015 adult population estimates in the Chimney Rock/Sheriff Ranch study section.

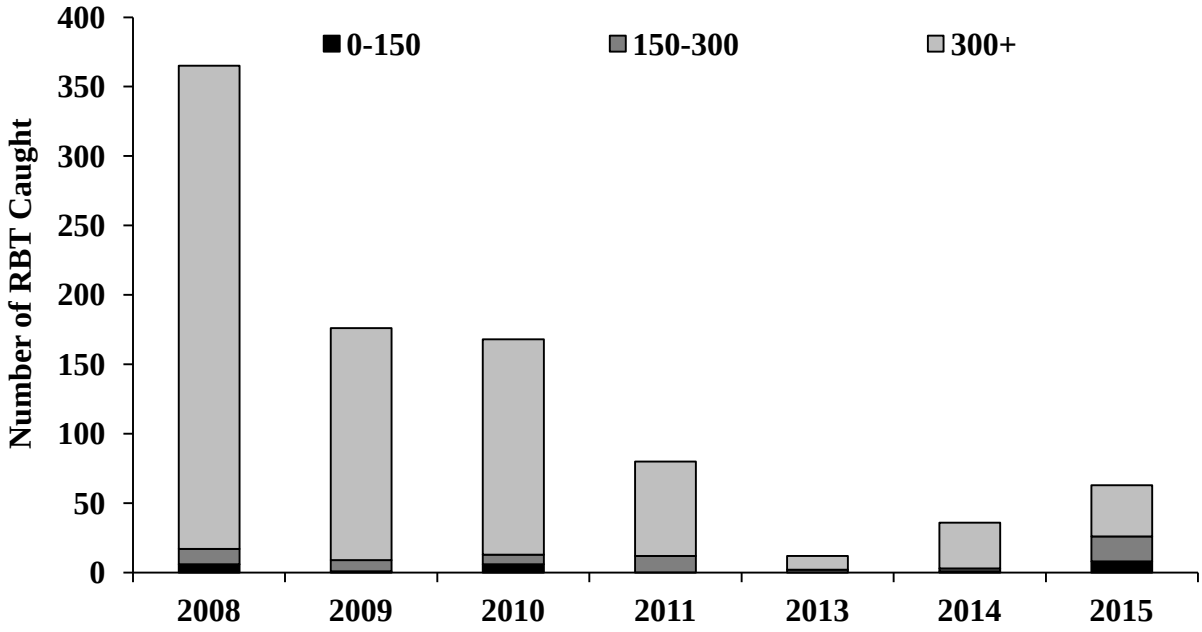

Figure 4.6. Number of age 1 (0-150 mmTL), age 2 (150-300 mm TL) and age 3+ (300+ mm TL) rainbow trout captured in the Chimney Rock/Sheriff Ranch study area every year between 2008 and 2015.

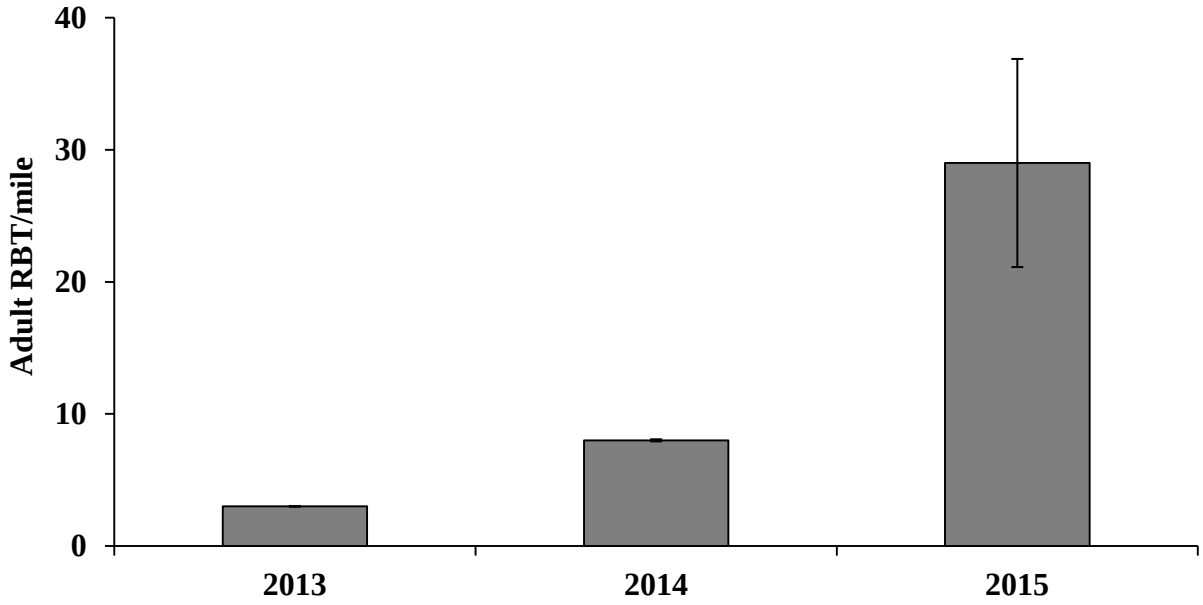

Figure 4.7. Estimated number of adult rainbow trout (RBT) per mile in the Chimney Rock/Sheriff Ranch study section in 2013, 2014, and 2015.

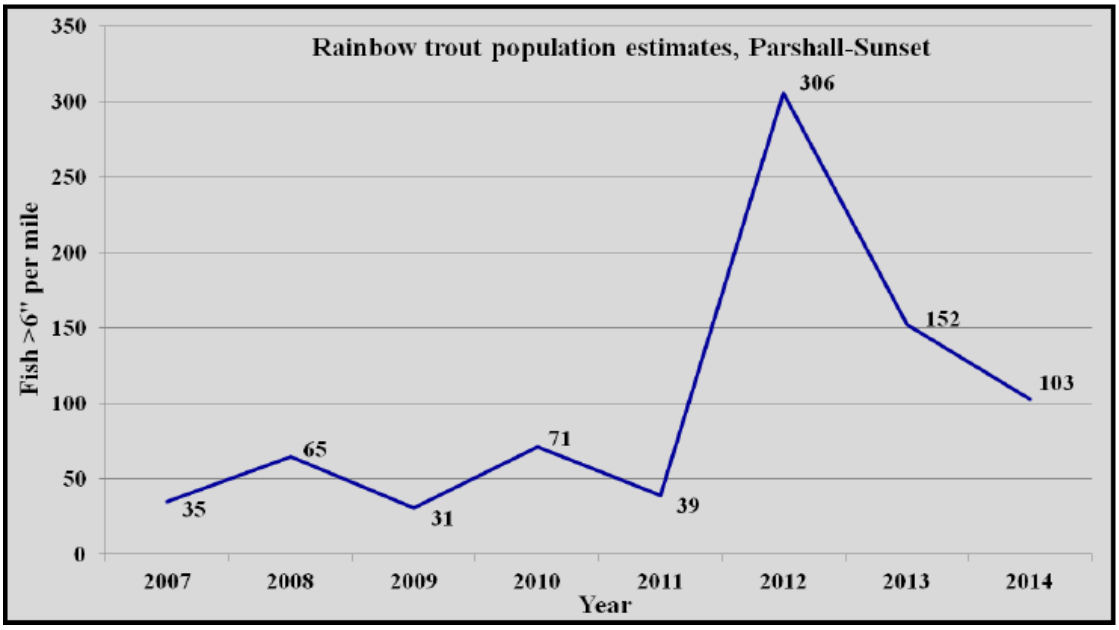

Figure 4.8. Estimated number of adult (> 6" TL) rainbow trout per mile in the Parshall-Sunset reach of the upper Colorado River for the years 2007 to 2014. Rainbow trout fry stocking first occurred in 2010. Courtesy of Jon Ewert, CPW Aquatic Biologist, Hot Sulphur Springs

Overall, stocking rainbow trout as fry has resulted in increases in the estimated number of adult rainbow trout per mile between 2013 and 2015 (Figure 4.7). Although the increase in the adult rainbow trout population is slower than what had occurred in the Parshall-Sunset reach below Byers Canyon, the change in the population between 2013 and 2015 represents the first positive

growth of the rainbow trout population in the Chimney Rock/Sheriff Ranch study section since stocking of whirling disease resistant rainbow trout commenced in 2006. The presence of both age 1 and age 2 fish in the population suggests that fish are recruiting, and will potentially continue to result in an increase in the adult rainbow trout population. Interestingly, the rainbow trout population increased in 2015 despite the adult brown trout population doubling between 2014 and 2015, likely increasing predatory and competitive interactions between the two species. In addition, the rainbow trout population in the Chimney Rock/Sheriff Ranch study section has continued to increase unlike the population below Byers Canyon, which has experienced declines in recent years despite fry stocking efforts (Figure 4.8). Fry will be stocked for a final time in 2015 with the final adult population estimates being conducted in 2016. However, given the positive results, we suggest that fry stocking continue to be used as a management option for increasing the adult rainbow trout population until significant natural reproduction is observed and fish produced by wild spawns survive and recruit to the adult population.

Salmonid Fry Population

The salmonid fry population in the upper Colorado River was sampled once a month, June through October in 2013 and 2014. The June 2013 and June and July 2014 samples provided a baseline of the number of naturally occurring rainbow trout fry in the river prior to stocking the GR×CRR fry. On July 16, 2013, approximately 100,000 rainbow trout fry each were introduced to the upstream half of the Chimney Rock Ranch study section. Rainbow trout fry (approximately 100,000) were stocked again on August 6, 2014 and were introduced throughout the entire length of the 3.9 mile study section. Fry were stocked by raft into the margins on both sides of the river to increase post-stocking survival. Sampling events occurring after fry stocking in both years 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. 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 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 (Figure 4.9).

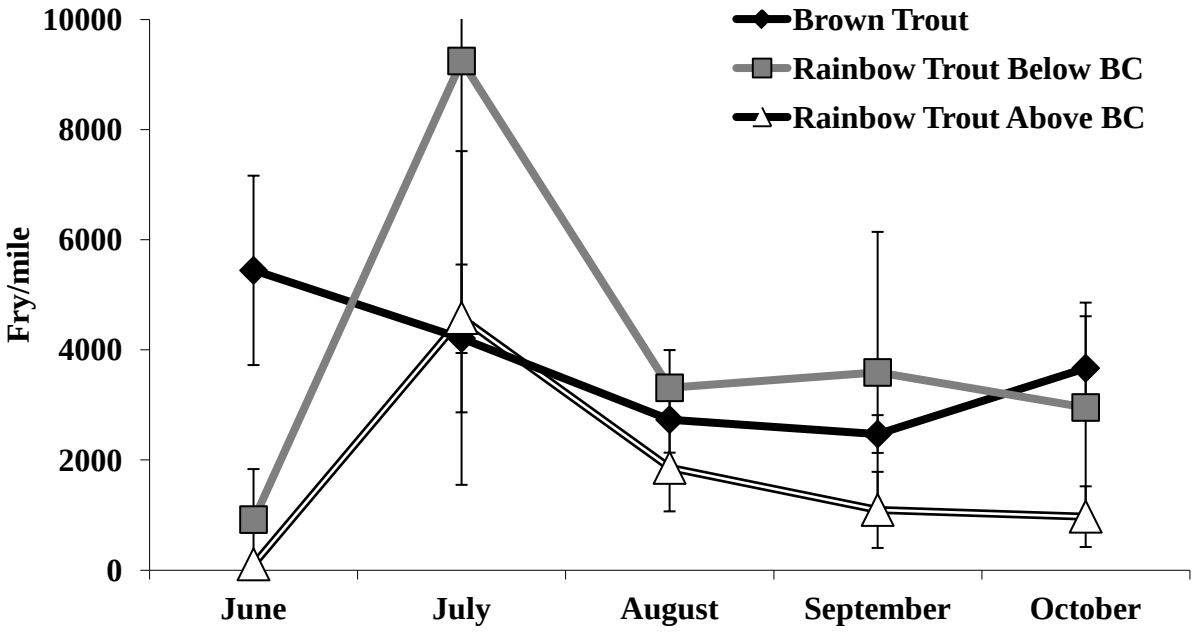

Figure 4.9. Upper Colorado River brown trout density estimates (fry/mile; SE bars), and rainbow trout density estimates above and below Byers Canyon (BC), for the months of June to October 2013. Note that these estimates represent the total number of fry per mile, including both sides of the river.

In 2014, brown trout fry densities were highest in July, with an estimated 1,871 (± 789) brown trout per mile. Although there was some variation in the estimates, brown trout fry numbers decreased only slightly throughout the summer and into the fall, with an estimated 1,353 (± 773) brown trout per mile still present in October. Prior to the introduction of rainbow trout fry in early August, no rainbow trout fry were observed in the Chimney Rock/Sheriff Ranch study section. High water likely delayed emergence in this section, so rainbow trout fry may have been present, but undetectable in low numbers, or may have emerged shortly after the July fry shocking events. Rainbow trout fry were only observed in one of the three sections below Byers Canyon in July, where water temperatures are slightly warmer, and averaged 443 (± 190) rainbow trout per mile. Rainbow trout fry densities were highest in August, following fry stocking, with an estimated 10,597 (± 8,486) rainbow trout fry per mile below Byers Canyon, and an estimated 4,336 (± 2,952) rainbow trout fry per mile in the Chimney Rock/Sheriff Ranch study section. Similar to 2013, rainbow trout fry declined after stocking, with final estimates of 5,699 (± 5,626) rainbow trout fry per mile below Byers Canyon and 1,029 (± 951) rainbow trout fry per mile in the study section. Although rainbow trout fry densities did not differ from brown trout fry densities in the study section, rainbow trout fry densities were significantly higher than brown trout densities in the section of river below Byers Canyon (Figure 4.10).

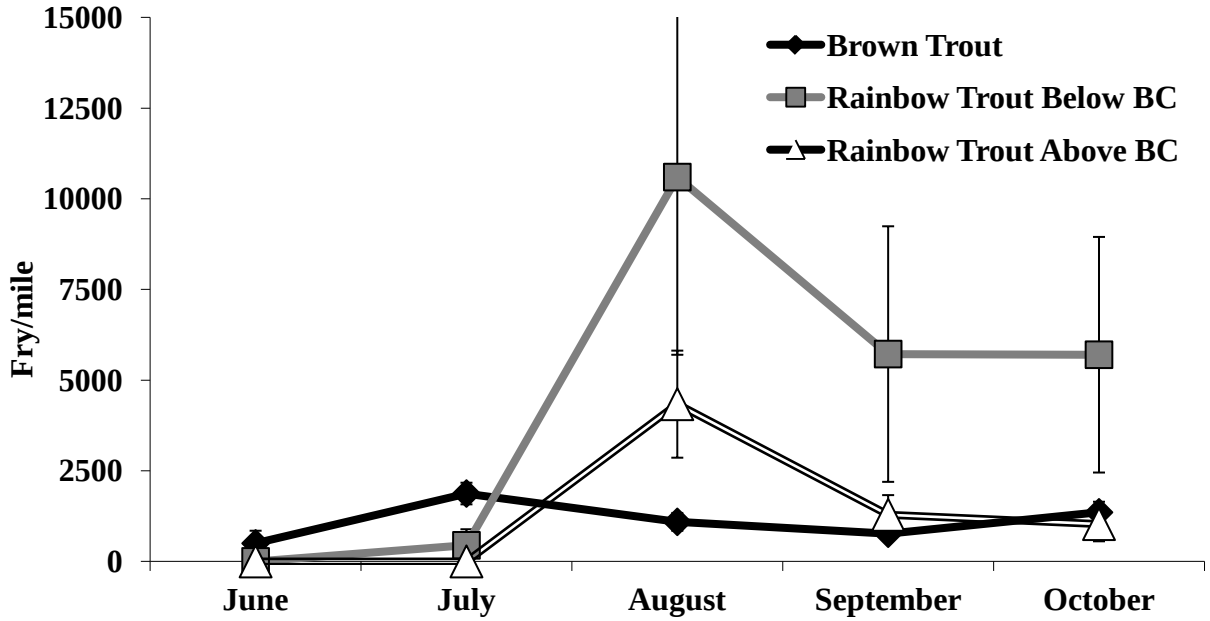

Figure 4.10. Upper Colorado River brown trout density estimates (fry/mile; SE bars), and rainbow trout density estimates above and below Byers Canyon (BC), for the months of June to October 2014. Note that these estimates represent the total number of fry per mile, including both sides of the river.

Stocking GR×CRR fry has 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 and 2014 were much higher than they have been in previous years when natural production was the only source of rainbow trout fry (Figure 4.11). 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 and just over 1,000 fry per mile were present in the section in 2014. In addition, rainbow trout fry densities did not differ from brown trout fry densities in October of either year. This suggests that stocked GR×CRR were surviving through the fall, and had the potential to recruit to the adult population. 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.

One potential benefit of using the GR×CRR for fry introductions is the reduction in infection severity, both in the rainbow trout fry, and the river as a whole. A maximum of five brown trout and five rainbow trout fry were collected from each sampling site in October 2013 and 2014 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 in October 2013. For comparison, rainbow trout fry averaged 123,355 (± 35,931) myxospores per fish in 2012. Average myxospore counts were further reduced in the rainbow trout fry in 2014, with fry averaging only 39 (± 39) myxospores per fish. Brown trout fry myxospore counts were similar in to 2013 in 2014, with fry averaging 3,860 (± 2,256) myxospores per fish. Interestingly, brown trout, which traditionally averaged 17,000 myxospores per fish (Fetherman et al. 2014), have also started to exhibit lower myxospore counts in recent years.

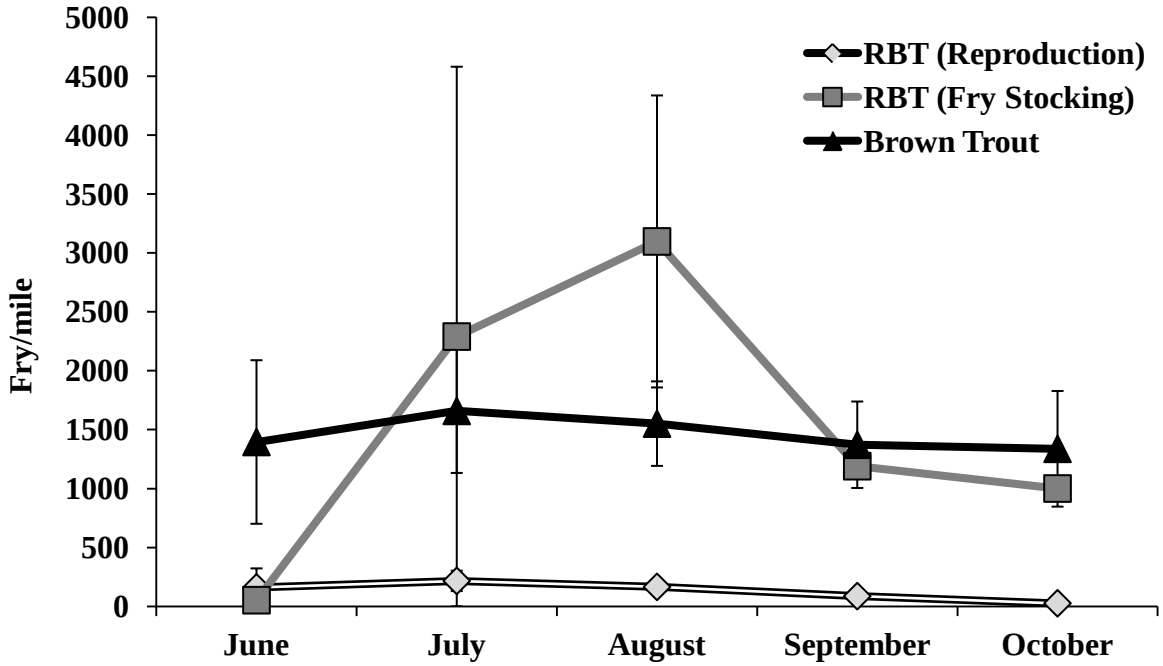

Figure 4.11. Upper Colorado River rainbow trout densities (fry/mile; SE bars) resulting from natural reproduction and occurring prior to the fry stocking experiment (RBT[Reproduction]), rainbow trout fry densities resulting from fry stocking, and brown trout fry densities for the months of June through October. Data represented was collected between 2008 and 2014.

Lower infection rates in the Chimney Rock/Sheriff Ranch study section may be attributable to a number of causes. First, *M. cerebralis*-resistant rainbow trout are less susceptible to infection and develop fewer myxospores as a result. Lower myxospore production in these fish ultimately results in lower infection rates overall in the river. However, lower infection rates in the brown trout fry suggest that additional environmental factors are driving changes in myxospore count.

Windy Gap used to be one of the most heavily infected locations in the state due to the presence and density of susceptible, Lineage III *Tubifex tubifex* worms and continuous inputs of high numbers of myxospores from upstream of Windy Gap. Both of these variables have recently changed. Nehring et al. (2013) showed that the *T. tubifex* lineages in Windy Gap reservoir have changed from having a high proportion of the susceptible lineage III worms in the late 1990s, which produce high numbers of actinospores, to higher densities of more resistant lineages (I, V, and VI) that produce lower numbers of actinospores in the early to mid-2000s. In addition, *M. cerebralis*-resistant rainbow trout have been stocked and established upstream of Windy Gap in the Fraser River. These fish are likely contributing fewer myxospores to Windy Gap annually. Therefore, this combination of factors upstream of the study section has likely resulted in fewer actinospores coming out of Windy Gap reservoir and infecting fish downstream. Annual variation in actinospore production along with annual flow fluctuations could also contribute to lower infection levels, and more years of disease collections are needed to determine if infection levels observed in 2013 and 2014 represent annual variation or reflect a more permanent change in infection levels in the upper Colorado River.

East Portal of the Gunnison River H×C Brood Stock

INTRODUCTION

The East Portal of the Gunnison River was being managed as a wild brood stock location for the GR×CRR rainbow trout until recently. GR×CRR fingerlings were stocked in the East Portal of the Gunnison River every year between 2006 and 2012. 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.

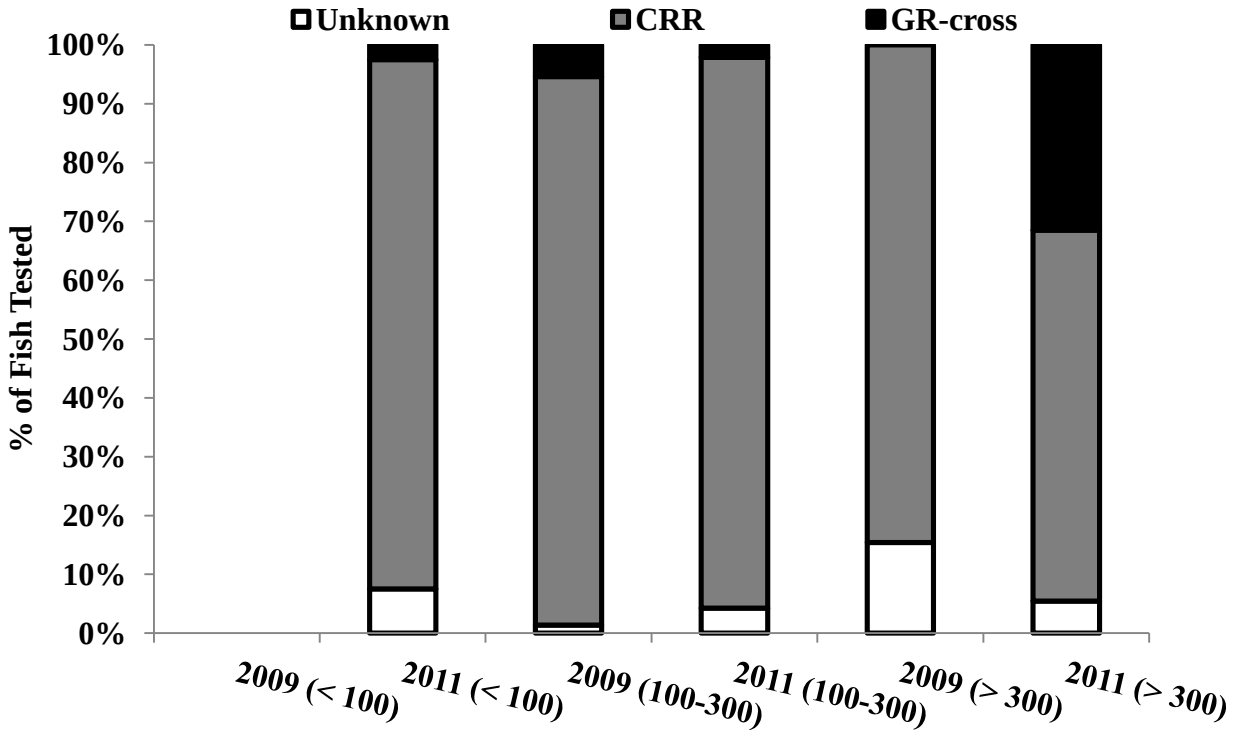

Figure 4.12. Percent of fry (< 100 mm), juvenile, and adult (100-300 and > 300 mm) rainbow trout, encountered during the East Portal of the Gunnison River population estimate in 2009 and disease inspection in 2011, categorized as unknown, pure CRR, and GR-cross fish.

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 4.12).

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 (2012 EXPERIMENT)

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. Eggs collected on April 17 were held at 6.9°C, eggs collected on May 1 were held at 9.2°C, and eggs collected on May 15 were held at 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 (2012 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 would 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, exophthalmia, 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 (2012 EXPERIMENT)

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 had 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 was 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 could not determine if myxospore counts were low due to exposure rates or the development of resistance.

The results of this experiment, and the genetic testing that occurred in 2011, suggested 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 suggested that this location not be considered

as a wild GR-cross brood stock location at the time, and that more testing was needed to determine if the CRRs in the East Portal had developed a resistance to *Myxobolus cerebralis*.

MYXOBOLUS CEREBRALIS EXPOSURE (2014 EXPERIMENT)

Because the results of the 2012 exposure experiment were inconclusive, a second experiment was initiated in 2014 to determine if natural resistance had 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 the 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 were held at the Parvin Lake Research Station through May 2015 to allow full development of myxospores within the exposed fish.

On May 12, 2015, 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, exophthalmia, 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 2014.

RESULTS AND DISCUSSION (2014 EXPERIMENT)

Survival averaged 95.6 ± 5.0% for East Portal families exposed to *Myxobolus cerebralis*, whereas average survival was much lower (57.5 ± 17.7%) for the exposed Puget Sound rainbow trout. Conversely, only 15.6 ± 15.1% of the exposed East Portal fish exhibited signs of infection, whereas signs of infection were found in 100% of the exposed Puget Sound rainbow trout. On average, East Portal fish exhibiting signs of infection exhibited only 1.4 ± 0.8 signs of disease per fish (out of a possible 6 common signs of disease), whereas Puget Sound rainbow trout exhibited an average 1.6 ± 0.8 signs of disease per fish. Taken together, the initial results suggest that the fish originating from the East Portal of the Gunnison River are more resistant to infection by *Myxobolus cerebralis* than the Puget Sound rainbow trout. Myxospore counts and genetic results for this experiment should be available in the next reporting cycle. In addition to comparisons made among strains within the 2014 experiment, myxospore counts from the East Portal rainbow trout will be compared to the myxospore results obtained from the 2012 experiment, as well as previous laboratory experiments conducted with known CRR individuals. The combined comparisons will provide valuable data as to whether resistance has begun to develop in the East Portal rainbow trout population. 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

INTRODUCTION

Hofer × Harrison Lake (GR×HL) 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 GR×HL 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 GR×HL present in the system) and 2010 (three years post-introduction of GR×HL to the system). In addition, GR×HL 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 GR×CRR brood stock, was used to assess the resistance characteristics of the offspring produced by fish returning to Harrison Creek to spawn.

SPAWNING AND REARING

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 family groups were created using rainbow trout (presumed to be GR×HLs) captured via trap nets in Lake Catamount that had not run up Harrison Creek. Fin clips were taken from all fish spawned to create each family group. 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 and reared until they reached 650 degree-days post-hatch. Unfortunately, between swim-up and the day of exposure, two family groups were lost (families 4 and 7). Therefore, only 6 total family groups were used in the exposure experiment.

MYXOBOLUS CEREBRALIS EXPOSURE

Once fish reached 650 degree-days post-hatch, 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, measured, weighed, and examined for signs of disease. 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.

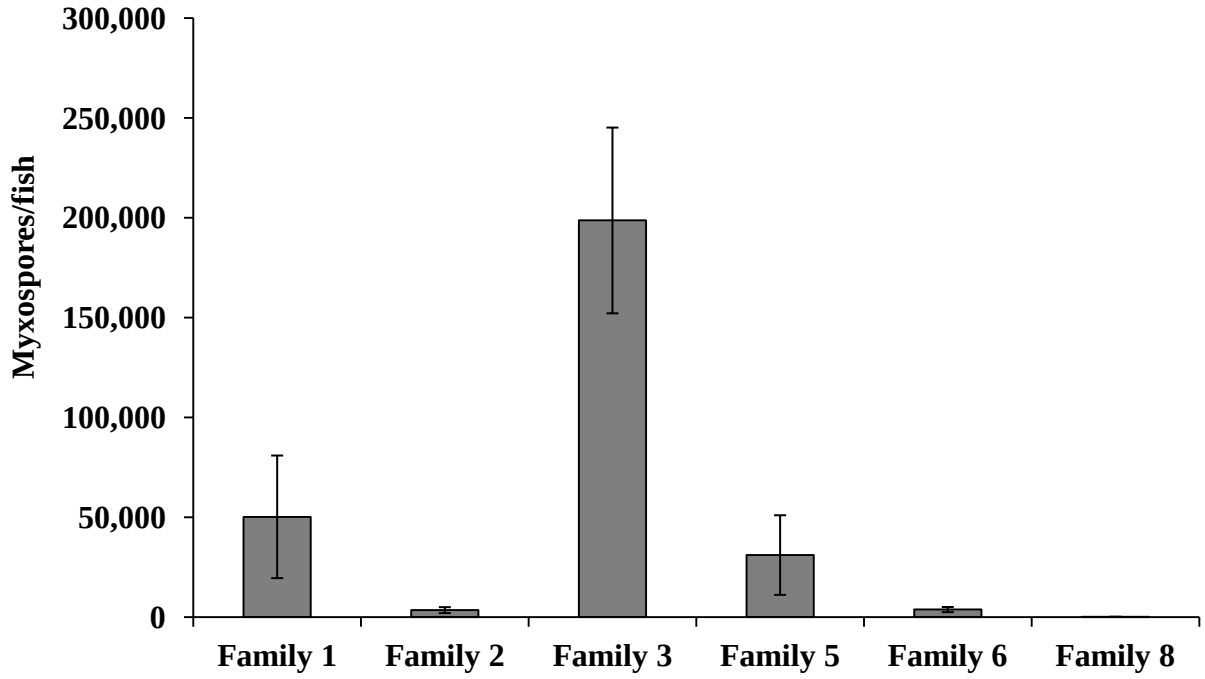

Figure 4.13. Average number of myxospores per fish (SE bars) for each of the six family groups created from fish captured in Harrison Creek (families 1-3 and 5) or in trap nets in Lake Catamount (families 6 and 8) in May of 2013.

There was a large amount of variability in average number of myxospores per fish both within and among families (Figure 4.13), a large part of which can be attributed to genetics of the parents and resulting genetics of the offspring. Family 3 was produced by two parents in which the proportion of GR was ≥ 75%, and two parents in which the proportion of HL was ≥ 50%. All ten of the resulting offspring exhibited a genetic composition that was ≥ 50% HL, and as a result, average myxospore counts of this family were higher than for any other family included in the experiment. It appeared that the GR resistance characteristics from two of the parents were not passed on to the offspring when eggs and spermatozoa were pooled to create the family group. In contrast, families 6 and 8 were both produced by four parents in which the proportion of GR was > 75%, resulting in 100% of the offspring exhibiting a genetic composition that was ≥ 75% GR. Average myxospore counts from both families were lower than any of the other families included in the experiment with the exception of family 2. Family 2 illustrates some of the issues with predicting resistance outcomes of offspring when pooling eggs and spermatozoa from multiple parents. Three of the parents that were used to create family 2 had a genetic composition that was ≥ 75% GR, with one parent, a male, which was 100% HL. Interestingly, all 10 of the resulting offspring exhibited a genetic composition that was ≥ 75% GR, resulting in low average myxospore counts, and suggesting that at least one parent, the HL male, had not contributed to the offspring genetically.

Families 1 and 5 had myxospore counts that were greater than families 2, 6, and 8, but were less than family 3 (Figure 4.13). Family 5 illustrates how offspring genetic composition and myxospore count are related in rainbow trout. Created from two parents in which the proportion of GR was ≥ 75%, one parent in which the proportion of GR and HL were about equal (50:50), and one parent that was 100% HL, four of the resulting offspring exhibited a genetic composition that was ≥ 75%, whereas the remaining six offspring exhibited a genetic composition that was about equal between the GR and HL (50:50). Although the average myxospore count (± SE) for the family as a whole was 31,137 ± 19,930 myxospores per fish, offspring that were a 50:50 mix of the GR and HAR exhibited significantly higher myxospore counts (57,464 ± 37,899) than did the offspring in which the GR component was ≥ 75% (6,164 ± 3,201). These results confirm that offspring in which the GR component is ≥ 75% are less likely to develop high numbers of myxospores relative to those offspring (individuals or entire families) in which the HL component is ≥ 50%.

Variability in the adult genetics results in variability in the fry genetics, with a wide range of myxospore counts. Pooling the adults makes it hard to predict the genetic and resistance outcomes of the progeny. In future spawning events, especially if being used to supplement hatchery brood stocks, families should be created from a single male-female pair and maintained separately until genetic results can be obtained for the adults. Based on the above results, it appears that only families that arise from a male-female pair consisting of a high proportion (≥ 75%) GR should be retained to maintain resistance in future brood stocks.

Comparison of Pure GR and GR×CRR Fry

The following describes an experiment being conducted by Colorado State University (CSU) Master's Candidate Brian Avila. The work described here is in the process of being completed, with a full summary of results expected in the fall of 2016.

INTRODUCTION

In the early 2000s, Colorado Division of Wildlife (CDOW; now Colorado Parks and Wildlife, CPW) imported the German Rainbow (GR), a *Myxobolus cerebralis*-resistant rainbow trout strain, for use in Colorado's production hatcheries (Schisler et al. 2009). However the survival of the pure GR in the wild was questionable due to its history of domestication (Schisler et al. 2006). Therefore, in 2004, CPW started a selective breeding program using the GR and the wild, susceptible Colorado River Rainbow (CRR) trout strains to produce a cross (known as the H×C or GR×CRR) that was resistant to the disease and could survive in Colorado's rivers. Crosses created under this approach were used to begin reestablishing rainbow trout populations in Colorado shortly thereafter, and the GR×CRR has since been stocked into a large proportion of Colorado's drainages. GR×CRR were initially stocked at > 6" with the goal of establishing a naturally reproducing, self-sustaining population. However, wild evaluations of the GR×CRR showed that post-stocking survival of fish stocked at these larger sizes was low (Fetherman et al. 2014). As a result, stocking programs were changed in many locations to stock the GR×CRR as fry to overcome the effects of domestication that may arise from maintaining fish in hatchery environments for up to a year to reach stocking size. GR×CRR stocked as fry have recently

started to show recruitment into older age classes in the Colorado, Gunnison and Arkansas River systems (Eric Fetherman, Jon Ewert, Dan Brauch, Greg Policky, pers. comm.).

CPW has been producing and stocking primarily resistant crosses of the GR and CRR. However, Fetherman et al. (2011) concluded that there is little difference between the GR×CRR cross and the pure GR strain physiologically. In addition, continued stocking and use of further filial generations of the GR×CRR could result in a loss of resistance due to outcrossing, a problem that could be avoided by stocking the pure GR. Both the GR×CRR and GR are reared in the CPW hatchery system, and there is a possibility that despite questions regarding the survival of pure GR (Schisler et al., 2006), post-stocking survival of the GR could be equivalent to the GR×CRR in the wild. One concern when stocking either the GR or GR×CRR is that, generally, hatchery-reared rainbow trout are naïve and particularly susceptible to predation. The ability of stocked fish to avoid predation is generally inferior to wild fish, and therefore, hatchery-reared fish often have elevated mortality rates compared to wild fish (Olla et al. 1994, 1998, Weber and Fausch 2003). Also, when comparing hatchery-reared fish to wild fish, it has been suggested that lifetime fitness may be linked to established early life dominance due to stream positioning following emergence (Fausch 1984). By stocking rainbow trout as fry, instead of larger sizes later in the year, the problems of establishing dominance and avoiding predation could be alleviated and might ultimately increase survival. The objectives of this study are to: (1) evaluate the survival of pure GR and GR×CRR stocked as fry into streams across Colorado, and (2) compare predation susceptibility of the GR and GR×CRR when stocked in the presence of brown trout in the laboratory.

METHODS

Stream Survival Evaluations

Stream survival evaluations were conducted in nine streams, three streams in each of three drainages: 1) Lone Pine Creek, the North Fork of the Cache la Poudre River, and Sheep Creek in the Cache la Poudre drainage, 2) Jefferson Creek, Michigan Creek, and Tarryall Creek in the Middle Fork of the South Platte drainage, and 3) the East Fork of Troublesome Creek, Rock Creek, and Willow Creek in the Colorado River drainage. Streams were selected based on access, similarity of habitat, width of stream, and fish assemblage. All streams contain brown trout as the dominant predator in the system, but brook trout are present in several streams as well. Prior to the introduction of rainbow trout fry, two sampling sites were established in each stream, and population estimates were conducted in July 2014 to provide baseline data on fish density and biomass.

Rainbow trout fry (45-50 mm total length) were tagged at the CPW Rifle Falls Fish Hatchery prior to being socked so that the two strains could be differentiated in the field. The GR was initially expected to exhibit lower survival rates than the GR×CRR. As such, to prevent conferring a further survival disadvantage due to tagging, the GR were not tagged, whereas all of the GR×CRR (45,000) were tagged using coded wire tags. Tag retention is generally high when using coded wire tags, so the assumption was made that fish encountered in the stream that were tagged were GR×CRR, whereas untagged fish were pure GR. Coded wire tags were injected into the nose of anesthetized fish (tricane methanosulfonate; MS-222) using a Mark IV automatic

tag injector (Northwest Marine Technology, Inc., Shaw Island, WA). Fish were monitored for mortality for a 24 hour period following tagging, and prior to stocking, a subset of fish were scanned to determine tag retention.

Ten thousand rainbow trout fry, 5,000 each of the GR and GR×CRR, were stocked into each of the streams August 4-6, 2015. Fish were transported in hatchery trucks to each stream from the Rifle Falls Fish Hatchery. Once fish arrived at the streams, they were acclimated to the conditions of the stream by allowing an exchange of hatchery and stream water in five gallon buckets. Once acclimated, fish were stocked out of five gallon buckets throughout the study section to allow even spread of the fish and stocking of fish in fry habitat.

Population estimates were conducted at two sites in each of the study streams in October 2014 and April 2015. Estimates were conducted using two to three LR-24 backpack electrofishing units (depending on stream width), and fish were removed on each of three passes. All fish captured during the estimates were measured to the nearest mm and weighed to the nearest gram. The October 2014 estimates were used to estimate the abundance of both the GR and GR×CRR in the study sections and determine short-term post-stocking survival. April 2015 estimates were used to estimate abundance and determine over-winter survival. Another population estimate is scheduled for August 2015. These estimates will be used to determine the survival of the GR and GR×CRR to age 1. At the time of writing this report, only the data for the October 2014 estimates were available for reporting.

Fish abundance was estimated using a closed capture-recapture model in Program MARK. Estimates were compared among strains within a stream and across streams using overlapping confidence intervals (CIs) to determine if there was a difference in GR versus GR×CRR abundance. In addition, estimates from MARK were compared using a two factor analysis of variance for differences in strain and stream (no interaction) using Program R.

Laboratory Predator Susceptibility Evaluations

Two sets of laboratory trials were conducted at the Colorado State University Foothills Fisheries Laboratory (FFL) to determine rainbow trout fry susceptibility to predation. The first experiment was conducted in September 2014, and the second experiment was conducted in May 2015. The experiment was conducted twice because results from the experiment conducted in 2014 suggested that brown trout spawning activity may have affected predation results. In addition, the experiments conducted in 2014 did not include an element of cover, which was added to the 2015 experiment because it was thought that the use of cover could affect survival among the two strains. At the time that this report was written, only the results from the trials conducted in 2014 were available for reporting.

Brown trout, the predator selected for the predation susceptibility experiments, were collected from Parvin Lake (Red Feather Lakes, CO) using a boat-mounted electrofishing unit. Using wild brown trout for the experiments ensured that brown trout had switched to piscivorous behavior and could identify the rainbow trout fry as prey prior to use in the experiment. All of the brown trout captured for the experiments were a minimum of 300 mm total length so that rainbow trout

fry were not larger than the gape limit of the predators used. Rainbow trout for the experiment were reared at the CPW BFRH.

Prior to experimentation, it was estimated that a single brown trout predator could consume up to 12 rainbow trout fry in a 24 hour period. Therefore, in both sets of trials, 15 fish each of the GR and GR×CRR were included in a predator arena with a single brown trout predator to determine if one strain was more susceptible to predation than the other. Visual implant elastomer (VIE) tags were used to differentiate the two strains within a tank. Brown trout were starved for 48 hours prior to conducting a trial to ensure that all food eaten previously had been evacuated. Brown trout were placed in the tanks first and allowed to acclimate to the predator arenas. Once acclimated, a 50:50 mix of the GR and GR×CRR (15 of each strain) of known size were stocked into the tank with the brown trout. Trials (N = 12) ran for 24 hours. At the end of a trial, all remaining fish in the tank were removed, identified to strain, measured to the nearest mm and weighed to the nearest g. Lengths and weights were used as covariates to determine if predation susceptibility was size selective. Brown trout, which were only used once and euthanized after being used in a trial, were identified to sex to determine if consumption rates varied by sex, especially in the September 2014 trials which were conducted during the brown trout spawning season. A t-test was run in Program R to determine if there were differences in the number of GR and GR×CRR remaining in the predator arenas at the end of the 24 hour trials.

Results and Discussion

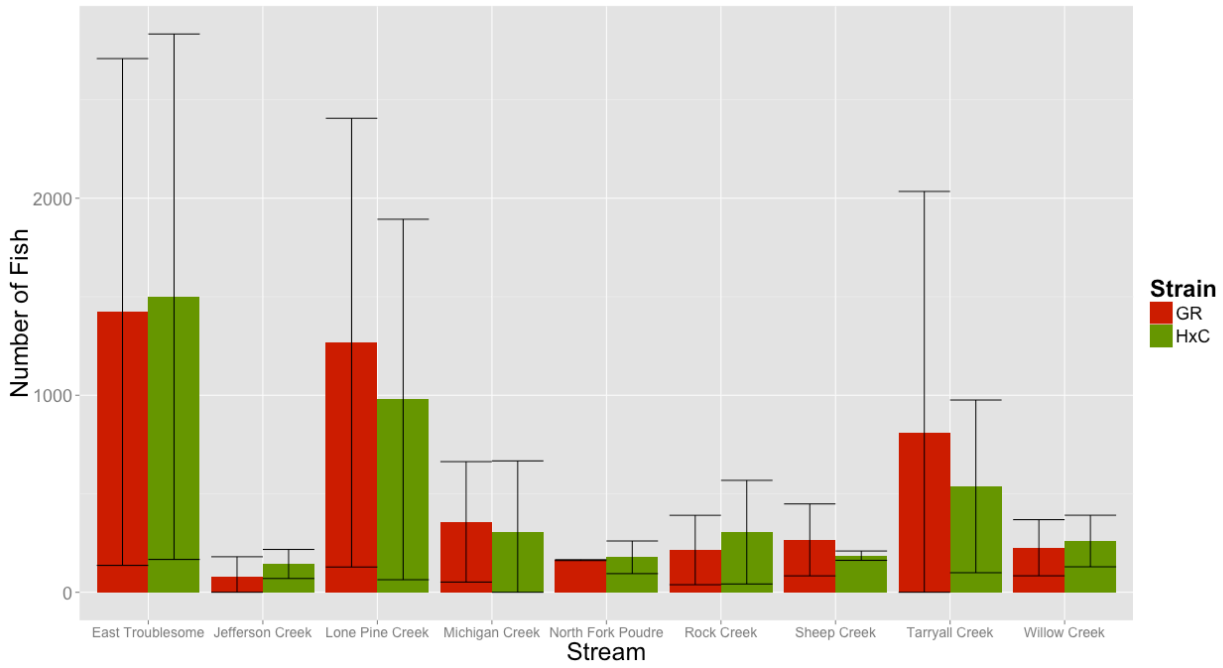

Figure 4.14. Average (95% CI bars) GR and GR×CRR (H×C) abundance, by stream, in October 2014, 2.5 months post-stocking.

Rainbow trout were present in all nine of the streams in October 2014, 2.5 months after they were stocked. Both the GR and GR×CRR were encountered in all nine of the streams and overlapping confidence intervals indicated that they were found in roughly equal proportions in

every stream (Figure 4.14). Strain did not significantly affect survival (ANOVA; $F = 0.115$, $P = 0.737$), however, there were significant differences in average rainbow trout fry abundance among the streams ($F = 5.706$, $P < 0.001$). Streams in which the rainbow trout survival was highest were also streams that had a large diversity of other fish species as well as higher total fish biomasses, suggesting that they were more productive, and therefore more conducive to higher post-stocking survival rates. Overall, against pre-experiment expectations, the GR appeared to survive just as well as the GR×CRR in the short term.

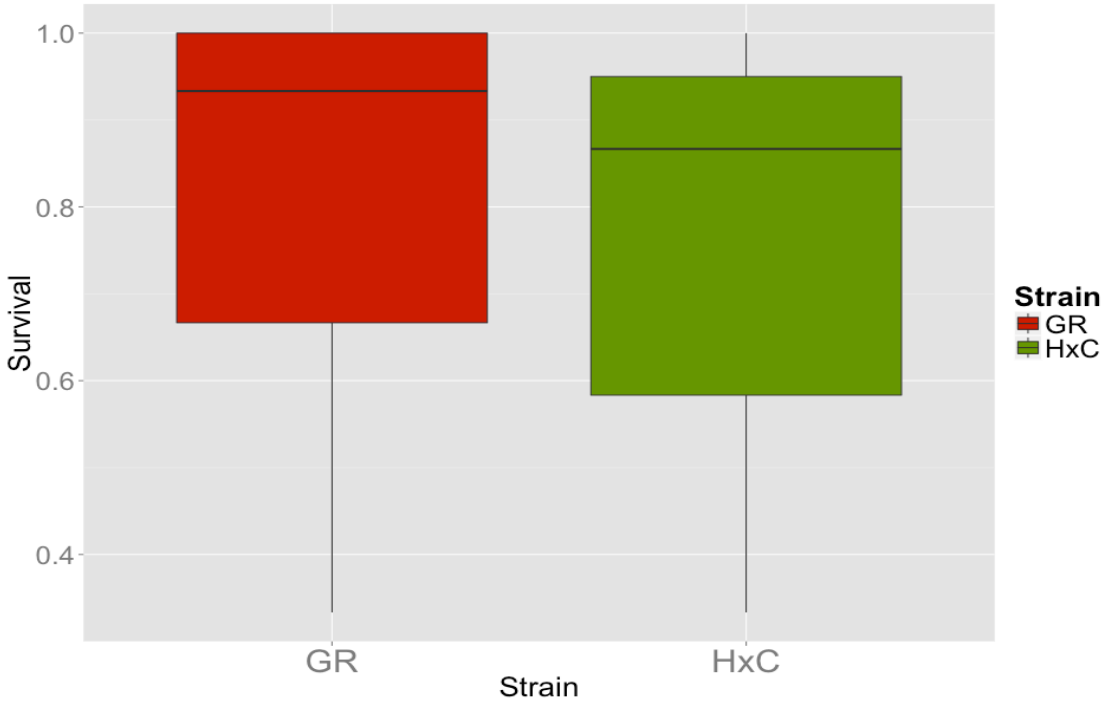

Figure 4.15. Box plot showing the average and range of survival of the GR and GR×CRR in the brown trout predation susceptibility trials conducted in September 2014.

The laboratory experiment helped confirm that the GR were not any more susceptible to predation than the GR×CRR (Figure 4.15). There was not a significant difference in survival between the strains (t-test; $t = 0.335$, df = 21.774, $P = 0.741$). Taken together, the field and laboratory results suggest that GR stocked as fry may have the ability to survive and recruit in small Colorado streams. However, these conclusions are based on one of two laboratory experiments, and only one of three sampling occasions. Due to the stochastic nature of the events that occur in these streams, the results obtained for short-term survival of the two strains may not necessarily translate to long-term survival or recruitment. Further sampling is needed to determine the fate of the two strains in the streams. The results of this sampling will be available in the next reporting cycle.

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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. B. Stout, and B. W. Avila. 2015. Replacement of Sparquat for New Zealand mudsnail disinfection. Colorado Parks and Wildlife Aquatic Biologist Meeting. Cripple Creek, Colorado. January 22, 2015.

2. Fetherman, E. R., and B. W. Avila. 2015. Genetic trends in Hofer-cross survival and reproduction: preliminary results. Colorado Parks and Wildlife Aquatic Biologist Meeting. Cripple Creek, Colorado. January 22, 2015.

3. Richer, E. E., E. R. Fetherman, and M. C. Kondratieff. 2015. RFID-GPS system development. Colorado Parks and Wildlife Aquatic Biologist Meeting. Cripple Creek, Colorado. January 22, 2015.

4. Kondratieff, M. C., E. E. Richer, D. Kowalski, E. R. Fetherman, and R. B. Nehring. 2015. Influence of boulder habitat structures on giant stonefly abundance. Colorado Parks and Wildlife Aquatic Biologist Meeting. Cripple Creek, Colorado. January 22, 2015.

5. Fetherman, E. R., B. Neuschwanger, and C. Praamsma. 2015. Formalin sensitivity of whirling disease-resistant rainbow trout. 2015 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Fort Collins, Colorado. February 25, 2014.

6. Avila, B. W., D. L. Winkelman, and E. R. Fetherman. 2015. Evaluation of resistant rainbow trout fry stocking in Colorado. 2015 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Fort Collins, Colorado. February 26, 2014.

7. Kondratieff, M., C., E. E. Richer, D. Kowalski, E. R. Fetherman, and R. B. Nehring. 2015. Influence of stream habitat enhancement on trout and giant stonefly abundance on the Wason Ranch, Rio Grande River, CO. 2015 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Fort Collins, Colorado. February 25, 2014.

8. Barnes, T., E. Richer, E. Fetherman, and M. Kondratieff. 2015. Incorporating GPS and radio-frequency identification (RFID) technology to evaluate fish movement and habitat utilization. 2015 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Fort Collins, Colorado. February 24-27, 2014.

9. Kopack, C. J., E. D. Broder, J. M. Lepak, E. R. Fetherman, and L. M. Angeloni. 2015. Chemical cues of predation induce anti-predator behavior in naïve rainbow trout: implications for training hatchery-reared fish. 2015 Annual Meeting of the Colorado/Wyoming Chapter of the American Fisheries Society. Fort Collins, Colorado. February 24-27, 2014.

10. Fetherman, E. R., G. J. Schisler, and B. W. Avila. 2015. Post-stocking survival of whirling disease resistant rainbow trout, and changes in fish health before and after stocking whirling disease resistant rainbow trout. Continuing Education session of the Western Fish Disease Workshop. Steamboat Springs, Colorado. June 2, 2015.

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 2015:

- Fetherman, E. R. 2015. Salmonid disease research in Colorado. Front Range Community College, Fisheries Management class. Fort Collins, Colorado. March 30, 2015.

Technical assistance milestones included the peer review of three manuscripts:

- Anonymous. 2014. 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.

- Hodge, B. W., R. Henderson, K. B. Rogers, and K. D. Battige. 2014. Tracking movement of Colorado River cutthroat trout in a small stream using portable PIT detectors. Submitted to the North American Journal of Fisheries Management.

- Sarker, S., G. Kumar, M. Saleh, and M. El-Matbouli. 2014. Differential expression of protease-activated receptor-2 and innate immune response genes in salmonid whirling disease. Submitted to Diseases of Aquatic Organisms.

In addition to professional reviews for scientific journals, technical assistance milestones also included the friendly review of two white papers/technical reports produced by CPW researchers, and one dissertation chapter produced by Ashley Ficke, Ph.D. candidate at Colorado State University.

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

- Fetherman, E. R., B. W. Avila, and D. L. Winkelman. 2014. Raft and floating radio frequency identification (RFID) antenna systems for detecting and estimating abundance of PIT-tagged fish in rivers. North American Journal of Fisheries Management 34:1065-1077.

- Fetherman, E. R., D. L. Winkelman, L. L. Bailey, G. J. Schisler, and K. Davies. 2015. Brown trout removal effects on short-term survival and movement of *Myxobolus cerebralis*-resistant rainbow trout. Transactions of the American Fisheries Society 144:610-626.

- Kopack, C. J., E. D. Broder, J. M. Lepak, E. R. Fetherman, and L. M. Angeloni. 2015. Behavioral responses of a highly domesticated, predator naïve rainbow trout to chemical cues of predation. Fisheries Research 169:1-7.

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

- Fetherman, E. R., J. M. Lepak, B. L. Brown, and D. J. Harris. *In press*. Optimizing time of initiation for triploid walleye production using pressure shock treatment. Submitted to North American Journal of Aquaculture.

- Stout, J. B., B. W. Avila, and E. R. Fetherman. *In review*. Efficacy of commercially available quaternary ammonia compounds for controlling New Zealand mudsnails. Submitted to North American Journal of Fisheries Management.

Lastly, the CPW Pueblo Hatchery asked for assistance in writing up the methods used to create triploid walleye. A CPW white paper was completed and released for distribution in 2015:

- Fetherman, E. R., J. M. Lepak, and D. J. Harris. 2015. Optimizing triploid walleye production in Colorado. Colorado Parks and Wildlife, Aquatic Wildlife Research Section. Fort Collins, Colorado. (See Appendix A).

Appendix A

Optimizing Triploid Walleye Production in Colorado

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Abstract

Walleye (*Sander vitreus*) provide important recreational and commercial fisheries throughout the United States and Canada. In Colorado, more walleye are stocked every year than any other species of fish. Use of sterile triploid fish in Colorado is an important management strategy for protecting native endangered fish species from naturally reproducing populations of introduced predators like walleye. Triploid fish are created by exposing eggs to hydrostatic pressure shock, temperature shock, or chemical treatments after fertilization to prevent the extrusion of the second polar body. This results in sterile triploids because chromosomes are unable to synapse correctly during division. Hydrostatic pressure has been one of the more effective methods for inducing triploidy in walleye, and the technique is currently evolving. A 2.7-L capacity electric pressure chamber manufactured by TRC Hydraulics was used to produce triploid walleyes using hydrostatic pressure with eggs collected from Pueblo Reservoir, Colorado. The TRC hydraulic press has many advantages over manually-operated pressure chambers used to produce triploid fish including its portability, large capacity, safety, and increases in hatching survival and induction rates. Here, the methods used to create triploid walleye using the TRC hydraulic press are described. In addition, times to initiation (TIs; time between fertilization to when eggs were subjected to pressure) were varied between 4 minutes and 8.5 minutes to determine the optimal TI for maximizing induction and hatching success rates. Our results suggest that triploidy in walleye is maximized with a TI of approximately 7.5 minutes, whereas hatching success is maximized with a TI of just over 8 minutes. Because the goal of the triploid walleye production program in Colorado is focused on maximizing induction rates, we recommend inducing triploidy at approximately 7.5 minutes to meet this objective.

Introduction

Throughout the United States and Canada, walleye provide important recreational and commercial fisheries (Becker 1983). In Colorado, more walleye (*Sander vitreus*) are typically stocked every year than any other species of fish. For example, in 2013 over 40 million walleye were stocked in Colorado in contrast with about 11 million rainbow trout (*Oncorhynchus mykiss* and their crosses) and 9 million kokanee salmon (*Oncorhynchus nerka*). Walleye are one of the most sought after species of fish in the state due to their availability, palatability, and potential to reach trophy size. Although this popular sportfish species is targeted by anglers, walleye are not native to Colorado. Walleye natural reproduction now occurs in some systems in Colorado. However, in other systems where walleye are desirable, natural reproduction is limited or absent. In these cases, walleye populations are maintained and enhanced through stocking.

Despite their popularity, walleye represent a novel predator in Colorado and they can have impacts on native fish populations (USFWS 2009). More specifically, walleye escapement from reservoirs has occurred in the past, and movement of naturally reproducing predators from reservoirs into rivers has been problematic for some native endangered and threatened species. In these instances and in others where control of walleye populations (maintaining appropriate densities) is desirable, there has been growing interest to create and make use of sterile triploid walleye to curb natural reproduction and help regulate walleye populations.

Creating sterile fish for management purposes has been practiced for decades, and more recently Colorado Parks and Wildlife (CPW) has begun to use sterile triploid walleye to address targeted management issues within the state. Triploid fish are created by exposing eggs to treatments (e.g., hydrostatic pressure shock, temperature shock, chemical) after fertilization to prevent the extrusion of the second polar body. This causes sterility because chromosomes are unable to synapse correctly during division (Thorgaard 1983; Strickberger 1985). The result is an extra set of chromosomes, or triploidy instead of diploidy.

Hydrostatic pressure shock has been used to induce triploidy in walleye. This technique is currently evolving, and walleye are a relatively recent addition (Malison et al. 2001) to the list of species for which this approach has been formally described. The success of hydrostatic pressure to produce viable triploid fish is dependent on the timing and magnitude of pressurization, which is species-specific, so control of these factors is crucial (Abiado et al. 2007). The objective of this study was to determine the most effective time between fertilization and when eggs were pressurized to 9,500 PSI to optimize induction rates, increase survival rates, and produce viable triploid walleye.

Source of Gametes – Pueblo Reservoir

Pueblo Reservoir (Pueblo, Colorado) is a 1,880 hectare reservoir managed primarily for sport fishing and recreational opportunities in southern Colorado. Sportfish in the reservoir include walleye, wiper (*Morone chrysops* x *Morone saxatillis*), largemouth bass (*Micropterus salmoides*), smallmouth bass (*Micropterus dolomieu*), spotted bass (*Micropterus punctulatus*), channel catfish (*Ictalurus punctatus*), flathead catfish (*Pylodictis olivaris*), blue catfish (*Ictalurus furcatus*), black crappie (*Promoxis nigormaculatus),* white crappie (*Promoxis annularis*),

bluegill (*Lepomis macrochirus*), yellow perch (*Perca flavescens*), and rainbow trout. A number of other species can be found in the reservoir, including green sunfish (*Lepomis cyanellus*), common carp (*Cyprinus carpio*), gizzard shad (*Dorosoma cepedianum*), and white sucker (*Catostomus commersonii*). Walleye spawn in the reservoir from mid-March to mid-April, and spawning operations conducted by CPW in the reservoir support over 50% of the state's walleye production on an annual basis.

Eggs and spermatozoa used to produce triploid walleye for the years 2010 to 2014 were collected from spawning walleyes caught in gill nets set parallel to the shoreline once reservoir temperatures reached 6.1°C, generally corresponding to the last week of March. Walleye captured in gill nets were transported by boat in aerated hauling tanks to a centrally-located boathouse for spawning. Each day of the spawning operation, male walleye were sorted into a separate holding tank where they were held until stripped (number of days held varied), whereas female walleye were sorted into ripe and green groups. Ripe fish were spawned on the day captured. Green females were held for up to three days to allow them to become ripe, and spawning status was checked on a daily basis. Therefore, gametes collected on any given day, including the days in which triploid walleye were produced, were from a combination of males and ripe females captured from the lake that day, males held over from previous capture days, and females that had ripened in the boathouse holding tanks.

Spawning Operations

Females were spawned into a plastic spawning container, with two to four females spawned per batch. Males (two to four) were used to fertilize the eggs according to the dry method (Piper et al. 1982). Eggs and sperm were mixed for 90 seconds in reservoir water filtered through a 25 micron sock filter. A stock solution of 800 mg/L tannic acid (Sigma Chemical Co., St. Louis, Missouri) was added to filtered reservoir water at an equal volumetric rate to attain a 400 mg/L tannic acid wash concentration. Fertilized eggs were washed for 90 seconds to reduce egg adhesiveness, and goose (*Branta* sp.) feathers were used to stir the eggs to prevent clumping. Tannic acid was decanted from the spawning container, and filtered reservoir water was used to rinse the eggs one to two times to remove acid and other spawning debris. Note that the use of Fuller's Earth for preventing adhesion is not recommended as it has been attributed to lower hatch rates in triploid walleye, and requires more time to decant during the rinsing process, potentially delaying target times for egg pressurization. Diploid eggs were poured into a fine-mesh, screen-bottomed basket and placed in a bath of filtered reservoir water for water hardening (1 hour). Eggs used to create triploids were retained in the spawning container while the pressure chamber was prepared for use.

Pressure Shock Treatment Methods

A 2.7-L capacity electric pressure chamber manufactured by TRC Hydraulics (New Brunswick, Canada) was used to pressure shock the walleye eggs. To prepare the chamber for use, filtered reservoir water was run through the chamber for several minutes to acclimate chamber temperature to that of the reservoir as the chamber generally cooled to the ambient air temperature overnight. The top and bottom valves of the chamber were closed prior to egg transfer to ensure that water and eggs would not be lost. A 3 mm mesh egg basket (standard

from TRC Hydraulics) was placed in the chamber to hold the walleye eggs. Note that a basket with 3 mm mesh does allow the passage of some eggs from the basket during treatment. This can be prevented by using a basket with finer mesh (e.g., 1.5 mm; can be custom ordered from TRC Hydraulics). The chamber was filled half full with filtered reservoir water so that eggs were not exposed to air or damaged by impacting the bottom of the egg basket at any point while being transferred.

Walleye eggs were transferred to the chamber using a 2.8 L wide-spout plastic pitcher with equal parts filtered reservoir water and eggs. Eggs were poured directly into the seated egg basket within the chamber and residual eggs were rinsed from the pitcher. Care was taken to prevent eggs from being caught on the upper lip of the chamber. Once eggs had settled within the egg basket, filtered reservoir water was used to fill the chamber so that there was no air inside the chamber once the plug was inserted (air would compress during pressurization, potentially preventing the chamber from reaching full pressure). To complete chamber preparation, the plug was inserted, the top valve of the chamber was opened to allow the plug to seat, and the plug was rotated clockwise to seat it within the locking arms, preventing accidental release while under pressure. Finally, the top valve was closed to create the vacuum needed for pressurization.

Hydrostatic pressures exceeding 8,000 PSI have produced higher rates of triploid induction in walleye and other fish species (Malison et al. 2001; Kozfkay et al. 2005; Abiado et al. 2007); CPW used a pressure of 9,500 PSI to induce triploidy in walleye. Pressurization of the chamber to 9,500 PSI required 45 to 50 seconds, depending on temperature. As such, the chamber operator timed the initiation of pressurization to correspond with the goal time of initiation (TI; time between fertilization and when eggs were under 9,500 PSI) for a given trial or year. Eggs remained under 9,500 PSI for ten minutes. Following pressure shock treatment, the hydraulic valve was opened to depressurize the chamber, and the top valve was opened to release the vacuum so that the plug could be removed after pressure was released. The egg basket containing the eggs was removed from the chamber, and the lower plug was opened to drain the chamber through a fine mesh filter that caught any remaining eggs that had made it through the egg basket during treatment. Eggs were transferred to a fine-mesh, screen-bottomed basket and placed in a bath of filtered reservoir water to complete the water hardening procedure (1 hour). Following water hardening, diploid and triploid eggs were transferred to a five gallon bucket for transport back to the main hatchery building for incubation, hatching, and rearing.

Methods of preparation and timing differed slightly from year to year. In 2010, TI occurred at approximately four minutes after fertilization. In 2011, TI was increased with each batch of eggs to determine the TI needed to optimize both induction and hatch rates. Batches were run at a TI of 5.5, 6.5, 7.5 and 8.5 minutes with a water temperature of 6.1°C. Batches with shorter TIs (i.e., 5.5 and 6.5 minutes) were generally only rinsed once following tannic acid treatment, whereas eggs were rinsed twice with the longer TIs. Aside from these exceptions, methodology remained the same among TIs. In 2012, 2013, and 2014, a TI of 7.5 minutes at a water temperature of 6.1°C was used to pressure shock walleye egg batches.

Egg Incubation and Rearing

Egg incubation occurred at the CPW Pueblo Hatchery (Pueblo, Colorado). The hatchery is supplied by well water, with temperatures ranging between 8.9 and 14.4°C. Upon arriving at the hatchery, egg size was assessed using a Von Bayer trough (Piper et al. 1982). The number of eggs per liter was calculated to determine the initial number of eggs per hatching jar where they were held for the duration of the incubation period (approximately 300 degree days; Piper et al. 1982). Eggs were treated with hydrogen peroxide at a concentration of 500 ppm on a daily basis to prevent fungal infections. Dead eggs were allowed to float out of the jars during the incubation period, or were siphoned off when they floated to the top.

Once eggs were eyed, the remaining eggs in a jar were stirred using increasing and decreasing flows until they were uniformly mixed. A 6.3 mm glass tube was inserted into the center of the rolling mass of eggs and used to pull out between 600 and 800 eggs to determine the ratio of live to dead eggs, providing the hatching success percentage for each trial. Egg volume from each jar was recalculated to account for the dead eggs removed during incubation, and the hatching success rate was applied to this final volume. Following this procedure, jars were transferred to hatching tanks to complete incubation and hatch.

Ploidy Analysis

One-day-old walleye fry were shipped live from the CPW Pueblo Hatchery to Virginia Commonwealth University where the ploidy analysis was performed using batches of approximately 100 fry. Each trial or year was analyzed separately. Ploidy was determined using the flow cytometry method in which the florescence of a dye used to stain the samples is quantified into channels indicating diploidy or triploidy (Thorgaard et al. 1982; Ewing et al. 1991; Harrel et al. 1998). Ploidy determination took only one day to complete, and the results were used to guide fry stocking upon receipt. Groups in which induction was high were stocked in locations where triploidy was imperative to management, whereas those groups in which induction was low were used in locations where triploidy was not required to meet management objectives.

Statistical Analyses

In 2010, CPW used the TRC hydraulic press to increase hydrostatic pressure to 9,500 PSI approximately 4 minutes after fertilization (n = 4 batches). In 2011, the same process was used, but eggs were pressurized at 5.5, 6.5, 7.5 and 8.5 minutes (n = 4 batches). In 2012, 2013 and 2014, eggs were pressurized 7.5 minutes after fertilization in all cases (single batches each year). Based on data collected from these trials, statistical analyses were conducted to: 1) determine the optimal TI to maximize triploidy induction, 2) determine the optimal TI to maximize egg hatching success, 3) determine if there was a difference in triploidy induction rates between walleye eggs with a TI of 4 minutes (2010) versus those with a TI of 7.5 minutes (2011 – 2014), and 4) determine if there was a difference in egg hatching success between walleye eggs with a TI of 4 minutes (2010) versus those with a TI of 7.5 minutes (2011 – 2014). Mean inductances and hatching success (by batch of pressurized eggs) were compared to avoid pseudoreplication associated with sample duplicates.

The TIs needed to optimize triploidy inductance (analysis 1) and egg hatching success (analysis 2) were determined by solving the first derivative of second order polynomial equations (quadratics) of the best fit lines for egg hatching success and triploidy inductance (respectively) as functions of TI (5.5, 6.5, 7.5 and 8.5 minutes). This was done with data collected in 2011. A comparison of triploidy induction rate (analysis 3) between walleye eggs with a TI of 4 minutes (2010; n = 4) versus those with a TI of 7.5 minutes (2011 – 2014; n = 1 for each year) was conducted using a two-sample *t* test assuming unequal variance. The same was done to compare the egg hatching success of these two groups (analysis 4).

Results

Based on the data collected in 2011 using the TRC hydraulic press, the optimal TI for walleye eggs to maximize triploidy induction (analysis 1) was approximately 7 minutes and 33 seconds. Further, the optimal TI for walleye eggs to maximize egg hatching success (analysis 2) was approximately 8 minutes and 10 seconds (Figure 1).

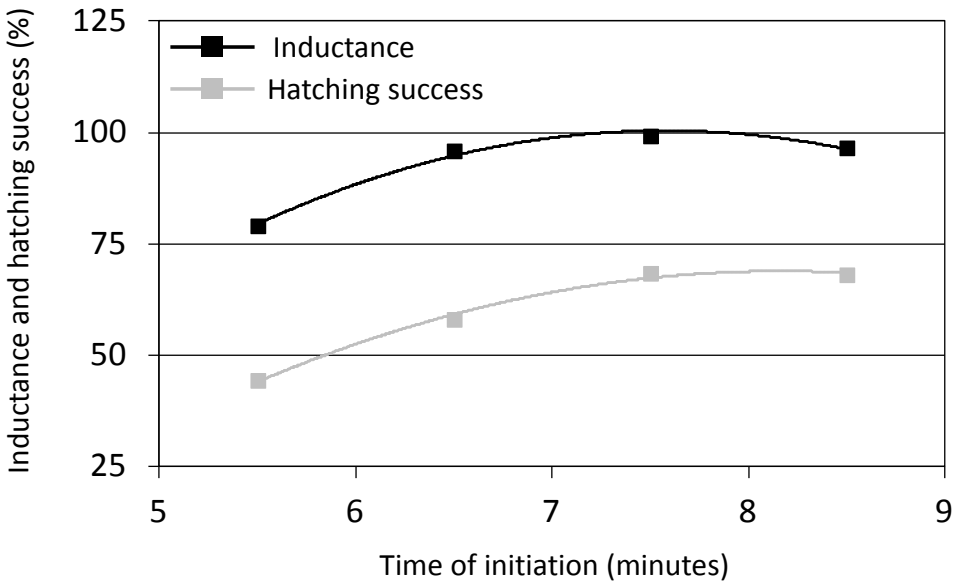

Figure A1.1. Percent walleye triploidy inductance and egg hatching success as functions of time of initiation (TI). Inductance is represented in black and egg hatching success is represented in gray. Solid lines are best fit quadratic equations.

The comparison of triploidy induction rates between walleye eggs with a TI of 4 minutes (2010) versus those with a TI of 7.5 minutes (2011 – 2014) indicated that triploidy induction rates were approximately 12% lower for eggs pressurized 4 minutes versus 7.5 minutes after fertilization (85 and 97%, respectively; one –tail *t* test, *t* statistic = 3.19, n = 4, 4, p = 0.02; analysis 3). The comparison of hatching success between walleye eggs with a TI of 4 minutes (2010) versus those with a TI of 7.5 minutes (2011 – 2014) indicated that hatching success rates were 30-35% lower for eggs pressurized 4 minutes versus 7.5 minutes after fertilization (23 and 56%, respectively; one –tail *t* test, *t* statistic = 4.82, n = 4, 4, $p < 0.01$; analysis 4).

Discussion

The TRC hydraulic press used in this study has many advantages over manually-operated pressure chambers used to produce triploid fish in previous studies including its portability, large capacity, and increases in hatching survival and induction rates (Abiado et al. 2007). Abiado et al. (2007) showed that triploid saugeye (*Sander vitreus* x *S. canadensis*) hatching success increased from 59.3 to 81.6%, and induction rates increased from between 80 and 96.7% to 100% when using the 2.7-L TRC hydraulic press compared to a 1-L manually-operated pressure chamber. Due to the advantages of the TRC hydraulic press, it has also been used to successfully induce triploidy in Atlantic salmon (*Salmo salar*; O'Flynn et al. 1997), lake trout (*Salvelinus namaycush*; Kozfkay et al. 2005), and now, walleye.
The success of hydrostatic pressure to produce viable triploid fish is dependent on the timing and magnitude of pressurization, which is species-specific, so control of these factors is crucial (Abiado et al. 2007). For example, pressures ranging from 9,000 to 9,500 PSI have been used to successfully induce triploidy in lake trout (Kozfkay et al. 2005), whereas pressures as low 8,000 PSI have been used to induce triploidy in walleye (Malison et al. 2001). Similarly, duration at which eggs are under pressure have varied greatly among species, from five minutes in lake trout (Kozfkay et al. 2005), to up to 30 minutes in walleye (Malison et al. 2001). As such, protocols used to develop triploid fish are constantly evolving.

As the production of triploid walleye is relatively new compared to other species (Malison et al. 2001), techniques for maximizing induction and hatching success rates continue to advance. Unfortunately, induction of triploidy through hydrostatic pressure tends to lower hatching success regardless of time to initiation (Garcia-Abiado et al. 2001). In Colorado, diploid hatching success rates are higher than maximized hatching success rates observed in triploid walleye (85 versus 56%, respectively). However, if maximization of hatching success is the goal of a particular triploid walleye production program, our results suggest that hatching success is maximized at a TI of 8 minutes and 10 seconds using 9,500 PSI. In addition, hatching success appears to increase with an increase in TI, with hatching success rates increasing by 12% with an increase in TI from 4 to 7.5 minutes. Similar increases have been observed by other states using similar protocols. For example, the Kansas Department of Wildlife, Parks and Tourism (KDWPT) achieved an increase in hatching success from 29 to 46% when increasing time of initiation from 4 to 7.5 minutes at 9,500 PSI (pers. comm.). Survival at a time of initiation of four minutes has ranged from 7.6 to 18.2% for triploid saugeye (Abiado et al. 2007) to 63.3 to 73.3% for triploid walleye (Malison et al. 2001). Therefore, it is likely that hatching success of triploid walleye is dependent upon a number of different factors including, but not limited to, time to initiation, duration of pressurization, temperature, egg quality, and hatchery rearing conditions.

One consistency among percid triploid induction methodology up to this point has been the initiation of pressurization at 4 minutes post-fertilization (Malison et al. 2001; Abiado et al. 2007). Success regarding triploid induction rates has varied within the percids at this TI. For example, Abiado et al. (2007) achieved 100% triploid induction rates in saugeye treated in the TRC hydraulic press at 9,000 PSI for durations of 5, 12, and 16 minutes, whereas Malison et al. (2001), using 8,000 PSI, achieved induction rates in walleye of 72.2% and 100% at durations of

15 and 30 minutes, respectively. Our results suggest, however, that induction rates were 30-35% lower at a TI of 4 versus 7.5 minutes when using higher pressures and lower durations. In fact, induction rates are maximized at a TI of 7 minutes and 33 seconds when using 9,500 PSI for a ten minute duration. Other states have noted a change in successful induction rates when increasing from a TI of 4 to 7.5 minutes. For example, KDWPT obtained induction rates of only 93% at a TI of 4 minutes, but increased induction rates to over 99% when increasing the TI to 7.5 minutes at 9,500 PSI (pers. comm.). Likewise, the Montana Fish, Wildlife and Parks Fort Peck Hatchery found that induction rates of 98.5% were achieved when using a TI of 8 minutes at 9,500 PSI (pers. comm.).

Triploid fish are used in a variety of management situations, and have been found to be useful for the control of overpopulation (due to sterility), for increasing growth in juveniles, and for extending survival and improving growth in mature fish (Tiwary et al. 2004). Although maximizing both triploidy induction rates and hatching success is desirable, the management goals for many agencies require that triploid induction rates be as high as possible to guarantee that sterile fish are being stocked. For example, triploid walleye are used in reservoirs on the western slope of Colorado where escapement and reproduction in rivers could present a risk to endangered native species (USFWS 2009). Under controlled culture situations, triploid fish tend to exhibit similarities in growth relative to diploids (Myers and Hershberger 1991; Galbreath et al. 1994). However, triploid saugeye have exhibited differences in foraging behavior, capturing smaller prey with lower reward, and exhibiting lower capture efficiencies and greater foraging times, all of which can affect growth, and ultimately survival, if similar behaviors are seen in other percid species (Czesny et al. 2002). Despite this, triploid walleye appear to survive and grow well in Narraguinnep Reservoir in the San Juan drainage of southern Colorado, where biologists found up to three year classes of triploid walleye, with the largest being 593 mm total length, in 2013, four years after triploid walleye stocking had commenced in the reservoir (pers. comm.). Therefore, it appears that triploid walleye are surviving after being stocked, providing important recreational fishing opportunities while meeting the management objectives of protecting threatened and endangered native fish species in the event of escapement.

Our results suggest that triploidy in walleye is maximized with a time of initiation of approximately 7 minutes and 33 seconds, whereas hatching success is maximized with a time of initiation of 8 minutes and 10 seconds. We acknowledge that there is error in the interpolation of these values and they are specific to the circumstances described here. However, these values could be used as targets or starting points, and refinement and development of situation-specific ranges for these values are encouraged. It is important to note that techniques were selected to optimize triploidy induction due to its relative importance for the management objectives of the State of Colorado, compared to hatching success. Therefore, we recommend inducing triploidy at approximately 7 minutes and 30 seconds to meet this objective.

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