SALMONID DISEASE INVESTIGATIONS

Federal Aid Project F-394-R8

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Job Progress Report

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State: **Colorado**

Project No. **F-394-R8**

Project Title: Salmonid Disease Studies/ Whirling Disease-Resistant Rainbow Trout Studies

Period Covered: July 1, 2008 - June 30, 2009

Project Objective: Development of rainbow trout brood stocks resistant to *M. cerebralis* for both hatchery and wild fish management applications.

Job No. 1.

Job Title: Breeding and Maintenance of Whirling Disease Resistant Rainbow Trout Stocks

Job Objective:

Rear and maintain stocks of whirling disease resistant rainbow trout stocks.

Hatchery Production

The whirling disease resistant rainbow trout brood stocks reared at the Fish Research Hatchery, Bellvue, CO (FRH) are unique and each requires physical isolation to avoid unintentional mixing of stocks. Extreme caution is used throughout the rearing process and during on-site spawning operations to ensure complete separation of these different brood stocks. All lots of fish are uniquely fin-clipped and some unique stocks are individually marked with Passive Integrated Transponder (PIT) 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 2008, FRH personnel checked all of the Hofer[1] (GR), Harrison Lake (HL), Hofer X Harrison Lake (GRxHL) and Hofer X Colorado River Rainbow (GRxCRR) brood fish (2 and 3 year-olds) weekly for ripeness.

Maturation is indicated by eggs or milt flowing freely with slight pressure 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 13, 2008, the fish from the first group of GRxCRR females were ripe and ready to spawn. Before each fish was spawned, it was examined for the proper identification (fin-clip or PIT tag). This procedure was repeated each time ripe females were spawned throughout the winter.

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

The wet spawning method was used, where eggs from the female are stripped into a bowl along with the ovarian fluid. After collecting the eggs, milt from several males is added to the bowl. Water is poured into the bowl to activate the milt. The bowl of eggs and milt is then covered and not disturbed for several minutes while the fertilization process takes place. The eggs are then rinsed with fresh water to expel old sperm, feces, egg shells and dead eggs. The eggs are then poured into an insulated cooler to water-harden for approximately one hour.

The water-hardened fertilized (green eggs) from all the different crosses of the GR, HL, GRxHL and GRxCRR strains were moved to the FRH main hatchery building. Extreme caution was used to keep each individual cross totally separate from all others. Upon reaching the hatchery the green eggs are tempered and then disinfected (PVP Iodine, Western Chemical Inc., Ferndale, Washington, at 100 ppm for 10 minutes at a pH of 7). Eggs were then put into vertical incubators (Heath Tray, Mari Source, Tacoma, Washington) with 5 gpm of 12.2º C (54º F) of flow-through water. The total number of eggs was calculated using number of eggs per ounce (Von Bayer trough count minus 10%) times total ounces of eggs. Separate 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 minutes) every other day until eye-up.

On the 14th day in the incubator at 12.2º C (54º F), the eggs reach the eyed stage of development. The eyed eggs are removed from the trays and physically shocked to detect dead eggs, which turn white when disturbed. Dead eggs were removed (both by hand and with a Van Galen fish egg sorter, VMG Industries, Grand Junction, Colorado) on the 15th day. The total number of good eyed eggs was calculated using the number of eggs per ounce times total ounces. On the 16th day the eyed eggs were shipped via insulated coolers to other state agency hatcheries. The whole process was repeated throughout the spawning season with separate crosses of GR, HL, GRxHL and GRxCRR rainbow trout.

The FRH 2008/2009 on-site rainbow trout production spawn started on November 13, 2008 with ripe GRXCRR females. The last group of GRXHL females were spawned on January 10, 2009. With a goal in the fall to produce @ 650,000 eyed eggs, the egg take far exceeded the production needs with 853,700 eyed eggs produced (Table 1.1). With the availability of both ripe males and females of several year classes and combinations of previous years crosses (F1 and B2) of GR, HL, GRxHL and GRxCRR strains, FRH personnel produced over 35 different lots during the spawn take. Surprisingly the overall egg quality remained quite good with 1st egg pick-off of only 14% overall. FRH personnel were able to fill all GR, HL, GRxHL and GRxCRR egg requests for Colorado, California, and Nevada for both production and research directed projects in 2008-2009.

Table 1.1. Fish Research Hatchery on-site spawning information for GR, HL, GRxHL, and GRxCRR rainbow trout strains during the winter 2008-2009 spawning season.

STRAIN (CROSSES)	DATE SPAWNED	# OF SPAWNED FEMALES	# OF GREEN EGGS	# OF EYED EGGS	SHIPPED TO
100% GR	11/28/07-12/24/08	48	122,700	121,200	CO Hatcheries/ Research
100% Harrison Lake	12/24/08	5	12,100	10,600	CO Hatcheries/ Research
GRxHL	11/20/08-1/10/09	263	466,200	359,700	CO, CA State Hatcheries/Research
GRxHL*	12/6/08	47	141,000		CO, NV State Hatcheries
GRxCRR*	12/5/08	54	194,500		CO State Hatcheries
GRxCRR	11/13/08-1/9/09	141	389,200	362,200	CO State/USFWS Hatcheries/Research
Total	11/13/08-1/10/09	558	1,325,700	853,700	86% Good Eggs to Eye-up

*Green eggs shipped to Poudre Hatchery, Poudre Canyon, CO.

Research Projects

Fish of the GR, GRxHL (50:50), GRxHL (75:25), and HL strains were reared to fingerling size and marked with coded-wire tags for the Parvin Lake fingerling experiments described in Job 3.

Job No. 2.

Job Title: Whirling Disease Resistance Laboratory Experiments

Job Objective:

Evaluate the inheritability and stability of whirling disease resistance in selected strains of rainbow trout.

Heritability of Myxospore Count, Genetic Correlations, and Effective Number of Genes Involved in Resistance in Whirling Disease Resistant and Susceptible Strains of Rainbow Trout

Quantitative genetics is a form of genetics that operates under the basic idea that phenotypic variation and expression of a trait is dependent on two factors, the underlying genetics of the trait, and the environment in which an individual strain or population exists. Quantitative genetics, as a whole, operates under the idea that trait expression and transmission can be measured without the necessity of DNA, in other words, by examining the phenotypic expression of the trait. It allows the researcher to both understand how the trait works, and how it is passed from generation to generation, without knowing the exact gene or set of genes that control for the trait. The quantitative genetics method is invaluable in situations such as this, where the genes involved in such processes as resistance to whirling disease in the Hofer (GR) strain are still unknown. By examining the phenotypic variability in myxospore count, heritability of myxospore count, genetic correlations between myxospore count and other physical and physiological processes, the effective number of genes involved in resistance can be estimated.

In this experiment, variation in myxospore count was examined in five strains of rainbow trout, the Hofer (GR) trout strain, the Colorado River rainbow (CRR) trout strain, and three intermediate strains, the F1, F2 and B2 strains. The GR strain is a domesticated hatchery strain from Germany that is grown as a food fish for human consumption. For over a century, the GR strain has been exposed to the whirling disease parasite, *Myxobolus cerebralis,* in the Hofer Rainbow Trout farm in Bavaria. Through hatchery selection processes, this strain has developed a resistance to whirling disease, as those individuals that survived exposure to the disease were selected to spawn subsequent generations. However, as a result, domestication selection has also occurred, as individuals that survived well under hatchery conditions were also selected to spawn subsequent generations. Due to this type of selection, the GR strain is considered domesticated, and it is suspected that it no longer possess the characteristics necessary for survival in natural systems. In addition, the GR strain is known to be inbred, and may not possess the genetic variability needed to adapt to changing conditions in the wild. The CRR strain is a wild rainbow trout strain that has historically been used to stock many of Colorado's streams and rivers because of its ability to survive and reproduce in the wild. However, the CRR strain is one of the most susceptible strains of rainbow trout to whirling disease, and has experienced large population declines as a result of exposure to

whirling disease. In addition, little to no natural recruitment has occurred in the wild in areas where a high *M. cerebralis* infection exists.

A selective breeding program was initiated to create several generational strains by crossing the GR and CRR strains, with the ultimate goal of creating a strain of rainbow trout that would have the correct combination of resistant and wild rainbow trout characteristics that would allow it to survive and reproduce in areas where a high *M. cerebralis* infection exists. Three intermediate strains have been created. The F1 strain is the first filial generational cross between the GR and CRR strains, and is created by spawning a GR individual (male or female) with a CRR individual (male or female). Based purely on Mendelian segregation, this strain is 50 percent GR and 50 percent CRR, expressing characteristics of both strains. The F2 strain is the second filial generational cross between the GR and CRR strains, and is created by spawning an F1 male from one family with an F1 female from a different family. This strain is also effectively 50 percent GR and 50 percent CRR. However, any given genotype in this strain has a 25 percent chance of being homozygous GR, expressing only GR-like characteristics, a 50 percent chance of heterozygous GR-CRR, expressing characteristics of both, or a 25 percent chance of being homozygous CRR, expressing only CRR-like characteristics. The B2 strain is the first generational backcross between the F1 and CRR strains, and is created by spawning an F1 individual (male or female) with a CRR individual (male or female). This strain is effectively 25 percent GR and 75 percent CRR, with any given genotype having a 50 percent chance of being heterozygous GR-CRR, expressing characteristics of both, or a 50 percent chance of being homozygous CRR, expressing only CRR-like characteristics. The genetic variation possible due to recombination and linkage characteristics of the genes in these strains leads to a lot of phenotypic variation in myxospore count, which in turn can be used to calculate heritability of myxospore count, and to understand how resistance characteristics are passed on to subsequent generations of these intermediate strains.

Heritability of a character determines the degree of resemblance between relatives, and is calculated using either a full- or half-sibling analysis, or a parent-offspring regression. Heritability estimates are used as a guide to predict which individuals to spawn and how the selected trait will change in subsequent generations. This change can occur either through natural selection in the wild, or through a selective breeding program under hatchery conditions. It is important to understand that heritability calculations are based on the variability seen within a given trait across related individuals within a strain, and therefore, it is the variability seen within the strains that lends an estimate of heritability of myxospore count. Heritability of myxospore count as a result of exposure to *M. cerebralis* was evaluated using a single pair mating design. The development of all the strains from pairs of individuals resulted in unique families containing full sibling offspring for each strain. The full sibling analysis includes both an additive and dominance variance component, and is therefore an estimation of heritability in the broad sense, which measures the extent to which phenotypic variation is determined by genotypic variation. Variance components used in the calculations were estimated using ANOVA. Myxospore count was log transformed prior to analysis. A detailed description of the methods is described in Appendix I.

In addition to heritability, genetic variation within individuals allows estimation of the correlation between characteristics. Deformity development as a result of exposure to whirling disease, growth, and swimming ability of both exposed and unexposed individuals, were previously examined for each of the five strains described above, and correlations were estimated between these characteristics and myxospore count. Three correlations can be estimated from the data: genetic, environmental and phenotypic. Genetic correlations estimate the degree to which two traits are affected by the same genes or pairs of genes, or in other words, the amount to which the two traits covary genetically. Environmental correlations estimate the degree to which two traits respond to variation in the same environmental factors. Phenotypic correlations estimate the degree to which the expressions of two traits covary. Each of these correlations gives information on how different characteristics of interest will respond together in subsequent generations. Variance components from the heritability calculations described above, as well as covariance components between traits estimated from ANCOVA, were used to calculate all three correlations. A detailed description of the methods is described in Appendix I.

A line-cross analysis was used to calculate the effective number of factors (n_e) by which the resistance characteristics in the GR and CRR strains differed, estimated by the Castle-Wright estimator. The quantity n_e is equivalent to the number of freely segregating loci with equal effects that would yield the observed pattern in the two genetic lines, and assumes independent assortment. It explains whether phenotypic variation is caused by a large number of genes with relatively small effects or a few major genes with large effects. It is also an important determinant in artificial selection programs of whether a search for informative markers is likely to be successful. Low values of n_e would suggest that genes responsible for resistance are contained on relatively few chromosomes and higher values suggest that resistance is spread over several or all chromosomes. In addition, the line-cross analysis was used to determine if an additive or additive-dominance model best fit the data. The additive model assumes that all genetic effects are additive within and between loci, where the F1 and F2 lines exhibit median phenotypic expressions between the two parental lines, and the backcrosses exhibit median phenotypic expressions between the F1 and parental line. The additive-dominance model assumes that some genetic effects are the result of dominance in one parent. Dominance results in phenotypes that are more similar to the dominant parent. It was also used to determine if dominance (from the additive-dominance model) accounted for a significant proportion of variance in the strain means. A detailed description of the methods is described in Appendix I.

Variation in myxospore count, both within and between families of the strains, indicated that heritability was estimable for all of the strains. Expectations, based on the variance in myxospore count and response to disease in terms of average myxospore count, for each of the strains were developed based on the predictions of the additive genetic model. The GR strain was expected to have a low variation in myxospore count, and a low response to the disease, because the genes involved in resistance to whirling disease should be approaching fixation in this strain. The CRR strain was expected to

have a low variation in myxospore count, and a high response to the disease, because the development of resistance genes should not have occurred yet for this strain; each individual in this strain was expected to be equally susceptible to the disease. The F1 strain was expected to have a low variation in myxospore count, and an intermediate response to the disease between the GR and CRR strains, because the individuals in this strain should have obtained half of their genes from the GR strain, and the other half from the CRR strain. The F2 strain was expected to have a similar response to the disease as the F1 strain, but the highest variation in myxospore count of all of the strains due to the differences in segregation and recombination of the parental genes in the individuals of this strain. Finally, the B2 strain was expected to have an intermediate variation in myxospore count to the F2 and CRR strains, and an intermediate response to the disease between the F1 and CRR strains, due to the differences in segregation and recombination of genes in the individuals of this strain as a result of the backcrossing between the F1 and CRR strains. The F1 and F2 strains deviated from these expectations, with the F1 strain having a slightly higher variation in myxsopore count and lower response to the disease than expected, and the F2 strain exhibiting a lower variation in myxospore count than expected and differing from the F1 strain in their response to the disease (Figure 2.1).

The F2 strain had a broad sense heritability estimate for myxospore count as a result of exposure to whirling disease of 0.34 ± 0.21; the F1 and GR strains were similarly low in their heritability estimates for myxospore count with estimates of 0.42 ± 0.23 and 0.34 ± 0.21, respectively. The B2 strain had a higher broad sense heritability estimate than the F2 strain, with an estimate of 0.93 ± 0.28. Interestingly, the CRR strain had a higher broad sense heritability estimate than expected at 0.89 ± 0.28 (Table 2.1). A heritability estimate of 0.3 or larger is considered a high heritability estimate.

The heritability estimates for all of the strains are considered high (greater than 0.3), indicating that there is a high selection differential in all of the strains. This means that through selection, whether it occurs through the selective breeding program or by natural selection in the wild, the allele frequencies of the population can be changed in subsequent generations, increasing resistance in future generations. The lower heritability estimate and lack of variability in myxospore count, in the GR strain indicates that selection for resistance has already occurred under hatchery conditions, and that the genes controlling for lowered myxospore count in the GR strain are approaching fixation. The fact that heritability estimates remain low in the F1 and F2 strains indicates that heritability remains similar in the first few generations, meaning that resistance to whirling disease will not be lost as quickly in the first few generational crosses of the GR and CRR strains. Finally, the higher than expected heritability estimate in the CRR strains indicates that either the CRR strain has some innate resistance to the disease, or that over the last two decades of exposure in Colorado, this strain has started to develop a resistance to the disease.

Genetic correlations between myxospore count and deformities were rarely significantly different from zero. Genetic correlations between myxospore count and physiological characteristics were also rarely significantly different from zero. The only

significant genetic correlation with a physiological trait was between myxospore count and swimming performance in CRR, and the correlation was negative. Environmental correlations between myxospore count and deformity were higher than the genetic correlations, and often significantly different from zero, in the F2 and B2 strains. The environmental correlation between myxospore count and weight was also higher than the genetic correlation, and significantly different from zero in the F2 and B2 strains; however, the environmental correlations between myxospore count and length, and myxospore count and swimming ability were low and not significantly different from zero. Phenotypic correlations between myxospore count and deformity were similarly higher than the genetic correlations, and often significantly different from zero, in the F2 and B2 strains. The phenotypic correlation between myxospore count and weight was also higher than the genetic correlation, and significantly different from zero in the F2 and B2 strains; however, the phenotypic correlations between myxospore count and length, and myxospore count and swimming ability were low and not significantly different from zero (Table 2.2).

The low genetic correlations between myxospore count and physiological characteristics indicate that it is possible to select for lowered myxospore count without selecting for/against or changing the other physiological traits. The higher environmental correlations between myxospore count and deformity formation indicate that there is not likely a genetic basis for deformity formation, but that the environmental conditions that the fish is experiencing are more likely responsible for whether or not a certain deformity will be expressed in that individual. The higher phenotypic correlations between myxospore count and deformity formation indicate that a deformity is more likely to occur with increasing myxospore count.

The effective number of factors (n_e) by which the GR and CRR strains differ in relation to myxospore development is 9 ± 5. The test statistic for the likelihood-ratio test between the additive and additive-dominance model was not significant ($P = 0.0836$), indicating that the model of best fit for the data was the additive model. However, there is still some evidence that dominance may play a role in how the resistance characteristics of the GR strain are passed on to the F1 and F2 strains. Dominance appears to break down in the B2 strain, leading to the large amount of variation in myxospore count seen in the families of this strain.

This is the first estimate of the number of genes involved in resistance in the GR strain. Though researchers have been able to make a connection between the interferon system and resistance in the GR strain, the specific genes involved in resistance are till unknown. Since the estimated number of loci involved was low, it is reasonable to believe that a search for informative molecular markers should provide information on the exact location of the loci involved in resistance to whirling disease.

Further work with genetics is planned for the future. Because we have built up a large amount of genetic material from both this and previous experiments conducted throughout the course of the selective breeding program, it may be possible to use AFLPs (Amplified Fragment Length Polymorphisms), SNPs (Single Nucleotide

Polymorphisms), or other sequencing techniques to identify differences in the GR and CRR strains nuclear or mitochondrial genomes, and identify the exact locations of the genes involved in resistance. In addition, it may be possible to track the changes in allele frequencies over time for the CRR strain, both through previous experiments and in the future, to determine if genetic resistance characteristics appear as exposure of this strain to whirling disease in the state of Colorado continues. Finally, the heritability estimates can be used to aid in selecting individuals from the current broodstock of the GR and F1 strains for use as parents to spawn future generations, utilizing the selection potential in these strains to increase resistance in future generations.

References

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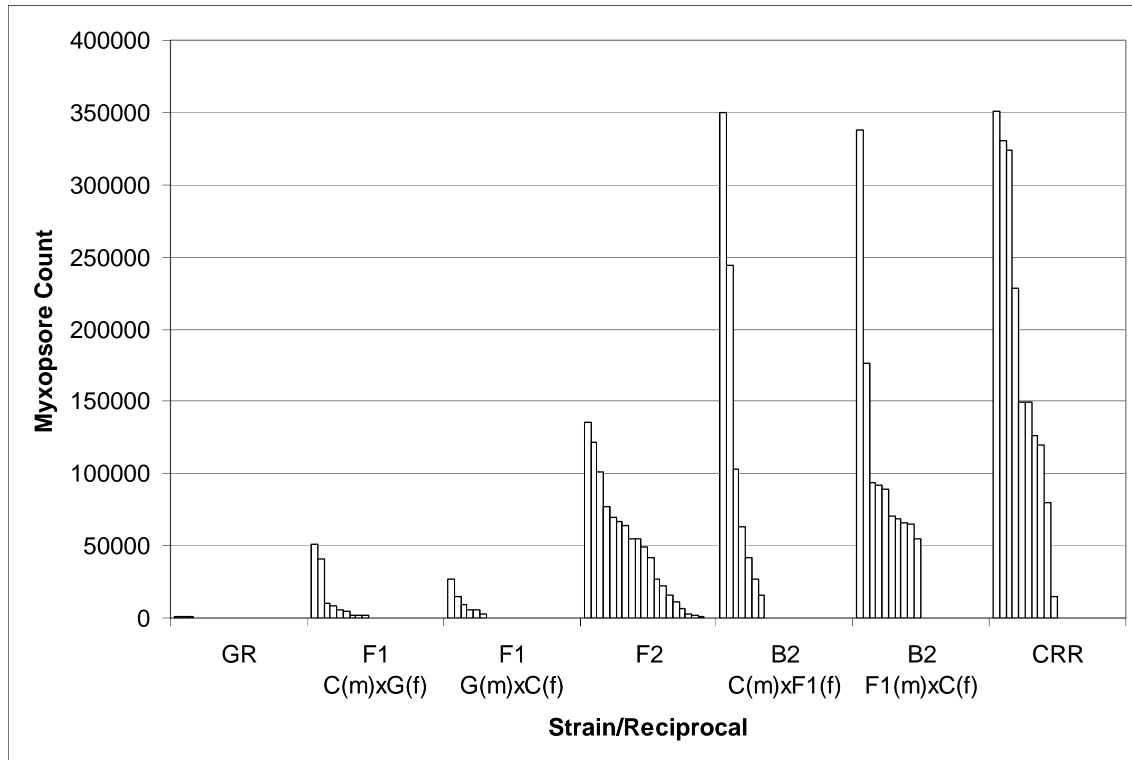


Figure 2.1. Comparison of myxospore count per family for each of five strains (with reciprocal families split out for the F1 and B2 strains) exposed to *M. cerebralis*. Ten families are represented in the GR and CRR strains, as well as in the reciprocals of the F1 and B2 strains, and 20 families are represented in the F2 strain. Notice that despite expectations, variance is low in the F1 and F2 families compared to the B2 families. In addition, variance is higher than expected in the CRR strain.

Table 2.1. Broad sense heritability estimates of myxospore count as a result of exposure to *M. cerebralis*, standard errors (as calculated using the formula from Becker (1992), representing 2 SE), and 95% confidence intervals (for ± 2 SE), for the five strains of rainbow used in the *M. cerebralis* exposure experiment.

Strain	H^2 Myxospore Count	Standard Error	95% Confidence Interval
GR	0.34	0.21	(0.13, 0.55)
F1	0.42	0.23	(0.19, 0.64)
F2	0.34	0.21	(0.13, 0.55)
B2	0.93	0.28	(0.66, 1.21)
CRR	0.89	0.28	(0.61, 1.17)

Table 2.2. Genetic, environmental and phenotypic correlations between myxospore count and deformity or physiological characteristic, and standard errors (in parentheses), for the five strains of rainbow trout used in the *M. cerebralis* exposure experiment. A "------" indicates that the correlation for that deformity or physiological characteristic was inestimable for that strain. A "=====" indicates that there was no heritability for the trait within a given strain, and therefore, genetic correlations could not be estimated. Significance is indicated by an "*".

Deformity/ Characteristic	**GR**	**F1**	**F2**	**B2**	**CRR**
Overall					
Genetic	=====	0.01 (0.02)	-0.001 (0.01)	-0.0001 (0.007)	=====
Environ.	=====	0.23 (0.10)*	0.19 (0.11)*	0.68 (0.38)*	=====
Phenotypic	=====	0.14 (0.07)*	0.14 (0.08)*	0.15 (0.06)*	=====
Cranial					
Genetic	=====	0.02 (0.02)	0.002 (0.01)	0.003 (0.006)	=====
Environ.	=====	0.26 (0.11)*	0.20 (0.11)*	0.78 (0.42)*	=====
Phenotypic	=====	0.15 (0.06)*	0.15 (0.08)*	0.15 (0.06)*	=====
Spinal					
Genetic	-0.04 (0.06)	0.02 (0.03)	0.01 (0.01)	0.005 (0.006)	-0.007 (0.02)
Environ.	0.34 (0.12)*	0.23 (0.10)*	0.20 (0.11)*	0.72 (0.41)*	------
Phenotypic	0.26 (0.10)*	0.16 (0.07)*	0.14 (0.08)*	0.13 (0.06)*	0.45 (0.13)*
Exopthalmia					
Genetic	0.19 (0.13)*	0.01 (0.02)	0.01 (0.02)	0.002 (0.006)	=====
Environ.	0.32 (0.15)*	0.12 (0.10)*	0.08 (0.11)	0.30 (0.37)	=====
Phenotypic	0.27 (0.10)*	0.08 (0.07)*	0.07 (0.09)	0.05 (0.06)	=====
Lower Jaw					
Genetic	0.14 (0.18)	=====	0.006 (0.005)*	0.001 (0.007)	-0.001 (0.01)
Environ.	0.19 (0.14)*	=====	0.12 (0.13)	0.37 (0.33)*	------
Phenotypic	0.18 (0.10)*	=====	0.07 (0.07)	0.08 (0.07)	0.34 (0.13)*
Opercular					
Genetic	0.18 (0.13)*	0.03 (0.02)	0.01 (0.009)	0.01 (0.009)	0.01 (0.01)
Environ.	0.47 (0.13)*	0.20 (0.11)*	0.19 (0.12)*	0.53 (0.33)*	0.98 (0.50)*
Phenotypic	0.38 (0.09)*	0.13 (0.06)*	0.13 (0.08)*	0.13 (0.07)*	0.29 (0.13)*
Blacktail					
Genetic	=====	=====	0.01 (0.01)	0.007 (0.007)	0.02 (0.01)
Environ.	=====	=====	0.04 (0.11)	0.20 (0.31)	------
Phenotypic	=====	=====	0.03 (0.09)	0.05 (0.07)	0.27 (0.11)*
Weight					
Genetic	0.07 (0.13)	0.006 (0.02)	0.005 (0.009)	0.004 (0.01)	0.006 (0.01)
Environ.	0.16 (0.17)	0.15 (0.10)*	0.15 (0.13)*	0.37 (0.31)*	0.58 (0.58)
Phenotypic	0.13 (0.10)*	0.10 (0.07)*	0.09 (0.07)*	0.09 (0.07)*	0.16 (0.15)*
Length					
Genetic	0.05 (0.16)	0.002 (0.02)	0.002 (0.008)	0.001 (0.009)	0.002 (0.01)
Environ.	0.05 (0.15)	0.05 (0.10)	0.05 (0.14)	0.12 (0.29)	0.16 (0.56)
Phenotypic	0.05 (0.11)	0.03 (0.07)	0.03 (0.07)	0.03 (0.07)	0.05 (0.16)
Swimming					
Genetic	=====	0.03 (0.51)	=====	0.01 (0.07)	-0.35 (0.17)*
Environ.	=====	0.06 (0.63)	=====	------	------
Phenotypic	=====	0.03 (0.24)	=====	0.01 (0.21)	------

Job No. 3.

Job Title: Whirling Disease Resistant Domestic Brood Stock Development and Evaluation

Job Objective:

These experiments are focused on the performance of the Hofer (GR) strain and GR-Harrison strain as domestic production fish compared with other commonly used production fish.

Parvin Lake Fingerling Stocking Experiment

Earlier experiments have demonstrated that the GR and GRxHL crosses have excellent growth and return-to-creel when stocked as catchable-sized fish (See Federal Aid Project F-394-R7 Report; Schisler et al. 2008). The Colorado Division of Wildlife is aggressively transitioning its brood facilities to produce larger numbers of GR or GRxHL crosses for catchable production purposes. In addition to catchable stocking, many waters in Colorado are stocked with fingerlings or subcatchable sized fish. These fish are subjected to greater threats from predation than catchable-sized fish and must be able to forage and survive long enough to become available to anglers. Because of the domestic nature of the GR strain, there are reasons to be concerned about the possibility of low survival and returns when fish of the GR strain, or slightly outbred varieties of the strain, are stocked as fingerlings. An experiment was designed to evaluate the survival of these varieties as fingerling plants in a location subjected to high predation pressure.

Parvin Lake, (Figure 3.1) located 45 miles northwest of Fort Collins, Colorado, was used as the test site for this evaluation. The reservoir is stocked annually with fingerling brown trout (*Salmo trutta*), splake (*Salvelinus namaycush x Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*). The reservoir was also stocked in 2000 through 2003 with tiger muskies (*Esox masquinongy x Esox lucius*) to control the abundant white sucker (*Catostomus commersoni*) population. An inlet trap that was historically used for rainbow trout spawning operations has also been operated more recently to remove white suckers from the reservoir in the months of May-July during their annual spawning run up the inlet stream. Numbers of suckers and trout captured in the trap vary from year to year, but appear to have been greatly reduced in recent years (Figure 3.2).

A fall electrofishing survey has been conducted annually since 2002 to monitor species composition and growth in Parvin Lake. A shift from a population dominated by white suckers to one dominated by rainbow trout has occurred since 2006 (Figure 3.3). Average size of white sucker, rainbow trout, splake, and brown trout has remained fairly stable, while average tiger musky length has steadily increased since 2002.

Figure 3.1. Parvin Lake, Colorado, with depth transects.

DEAD END

DEAD END

Red Feather Lakes Road

N

PARVIN RESERVOIR
Larimer County, Colorado
Sections 34 & 35 T10N R73W

CHECK STATION

Figure 3.2. Number of catostomids and salmonids caught at Parvin Lake inlet trap (May-July) for years where data is available.

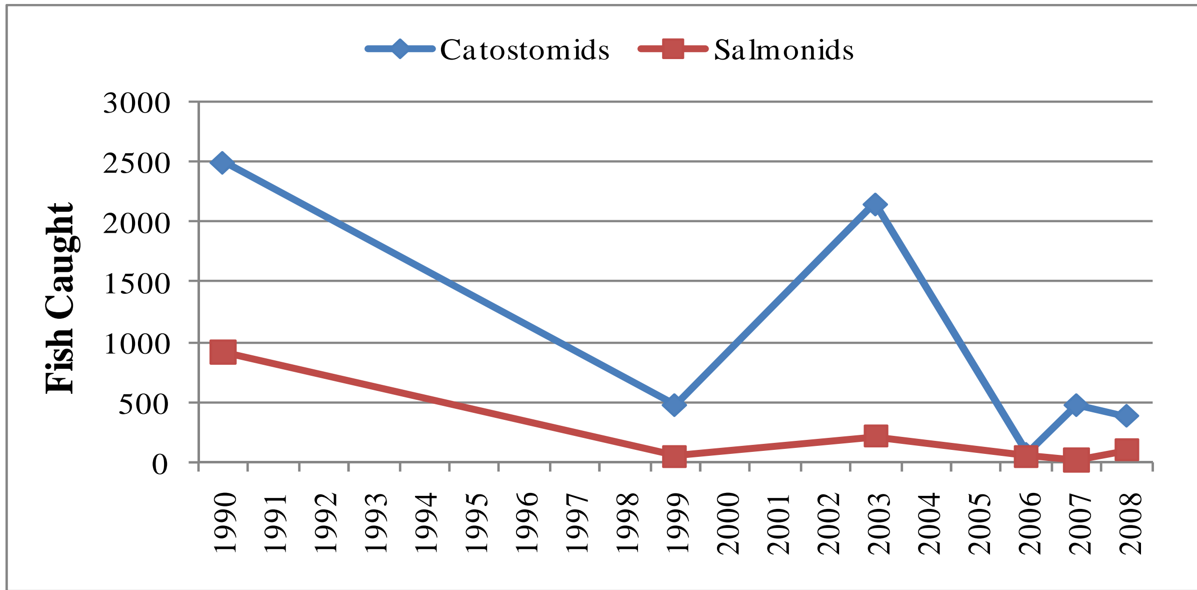


Figure 3.3. Percent of catch by species during fall electroshocking surveys for the years 2002 - 2008.

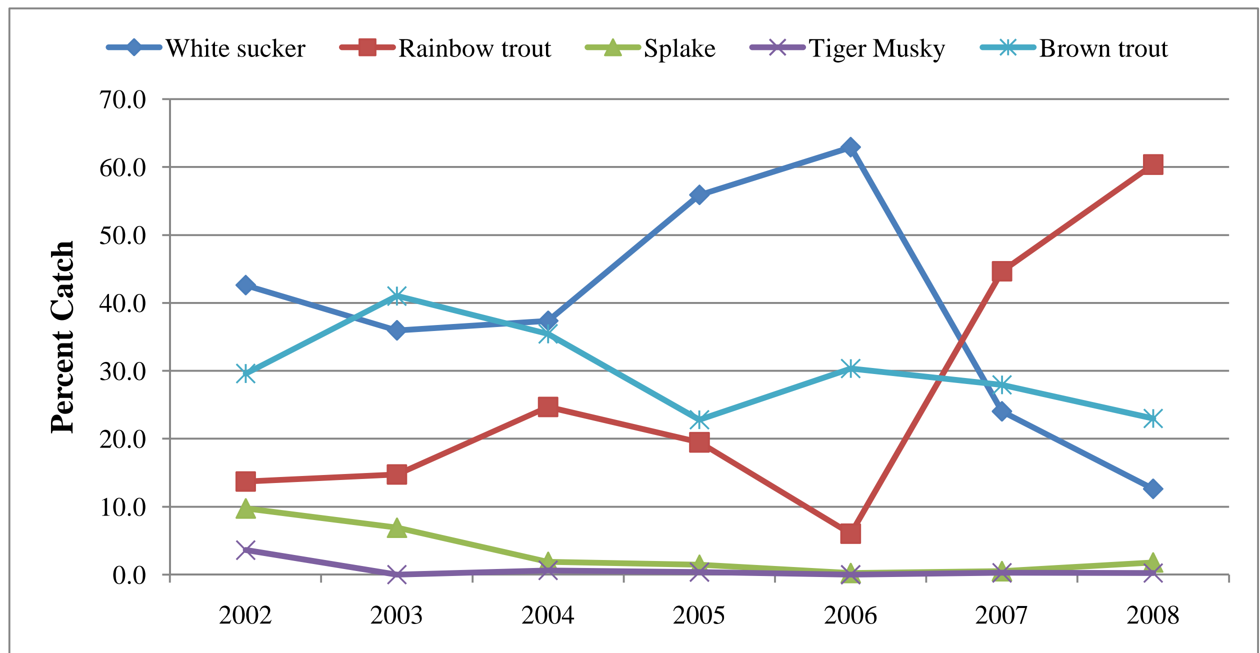
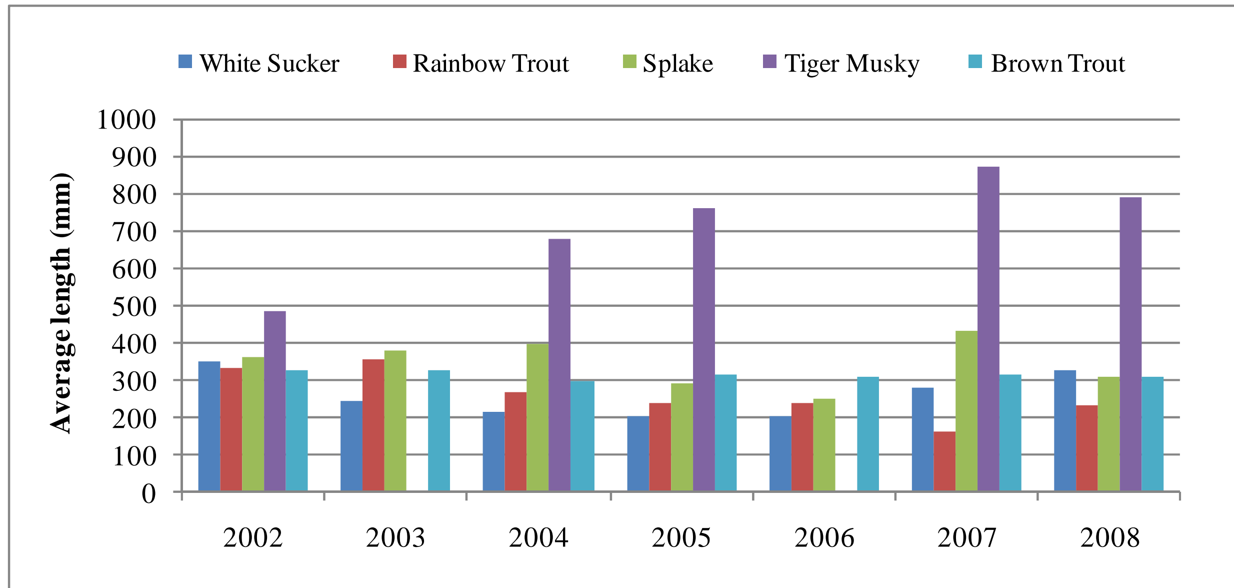


Figure 3.4. Average length of fish by species during fall electroshocking surveys for the years 2002 – 2008.



In 2007, 2,800 fish each of the GR, HL, GRxHL (50:50), GRxHL (75:25), and Bellaire rainbow trout x Snake River cutthroat trout cross RXN (50:50) varieties were batch-marked with coded wire tags to identify returned fish by strain. These fish were reared as closely as possible to the same size before stocking. However, because of the rapid growth of the GR strain, and the very slow growth of the Harrison strain, sizes were not exactly matched (Table 3.1). The fish were all stocked at the same time into Parvin Lake on August 14, 2007.

In 2008, 2,050 fish of each GR, HL, GRxHL (50:50), GRxHL (75:25), and Bellaire rainbow trout x Snake River cutthroat trout cross RXN (50:50) were again batch-marked with coded wire tags. Similar difficulties with matching sizes of the Harrison Lake strain with the other varieties were encountered during the rearing period. These fish were stocked into Parvin Lake on July 31, 2008.

Collections of coded-wire tagged fish were made using electroshocking and gill net sets every two months during the open-water season. Marked fish from the 2007 plant were collected beginning in August, 2007, and marked fish from the 2008 plant were collected beginning in August, 2008. An attempt was made to collect 30 fish per event for each age class of marked fish.

During 2008, a creel survey was conducted during the months of May through September in which catch and angler use was estimated. All fish harvested during the creel sampling days were scanned for coded wire tags to evaluate returns by strain. A winter creel survey was also conducted in the months of January through March, 2009, to evaluate ice-fishing use and harvest, as well as to collect coded wire tags from harvested fish.

Table 3.1. Coded-wire tagged fish stocked in Parvin Lake during 2007 and 2008.

2007 Plants				**2008 Plants**			
Strain	**Lbs**	**Number**	**Length (mm)**	**Strain**	**Lbs**	**Number**	**Length (mm)**
GR	225	2800	147	GR	103	2050	127
HL	64.2	2800	97	HL	38.4	2050	91
GRxHL (50:50)	75.5	2800	104	GRxHL (50:50)	78.2	2050	117
GRxHL (75:25)	76.6	2800	104	GRxHL (75:25)	81.7	2050	117
RXN (50:50)	125	2800	122	RXN (50:50)	103	2050	127

The open-water creel survey from May through September, 2008, resulted in an estimated 4,861 fisherman-hours and 1,829 fishermen. Total estimated catch was 1,168 fish, with 163 kept and 1,005 released. Estimated catch by species was 199 brown trout (22 kept), 751 rainbow trout (87 kept), 87 RXN (12 kept), 122 splake (38 kept), and nine tiger muskies (4 kept) in the open-water period. Fewer than 10 coded-wire tagged fish were collected during the creel survey, so those fish were combined with the August-September, 2008 electrofishing samples as described below and shown in Figure 3.5.

During the ice-fishing period of January through March, 2009, very little fishing pressure occurred, resulting in a total of only 611 fisherman-hours and an estimate of 145 fishermen. Total estimated catch was 212 fish, with only 36 of those being kept and 176 released. Estimated catch by species was three brook trout (none kept), nine splake (none kept), 20 brown trout (none kept), 15 RXN (none kept), 136 rainbow trout (seven kept), and 29 coded-wire tag marked rainbow trout (all kept). The coded wire tagged fish recovered from fishermen during the winter creel survey are reported as the January-March 2009 samples in Figure 3.5.

Very few of the marked fish from the 2007 stocking event and none of the marked fish from the 2008 stocking have been harvested by anglers. The marked fish from the 2007 stocking event are now entering their second summer and average 296 mm (11.6 inches), so should begin to comprise more of the total harvest as they get larger. Creel survey efforts are continuing to recover more of the marked fish that are harvested.

Figure 3.5. Percent of catch for each of the five varieties of fingerling rainbow trout stocked in Parvin Lake in August, 2007. January-March samples are ice-fishing angler returns.

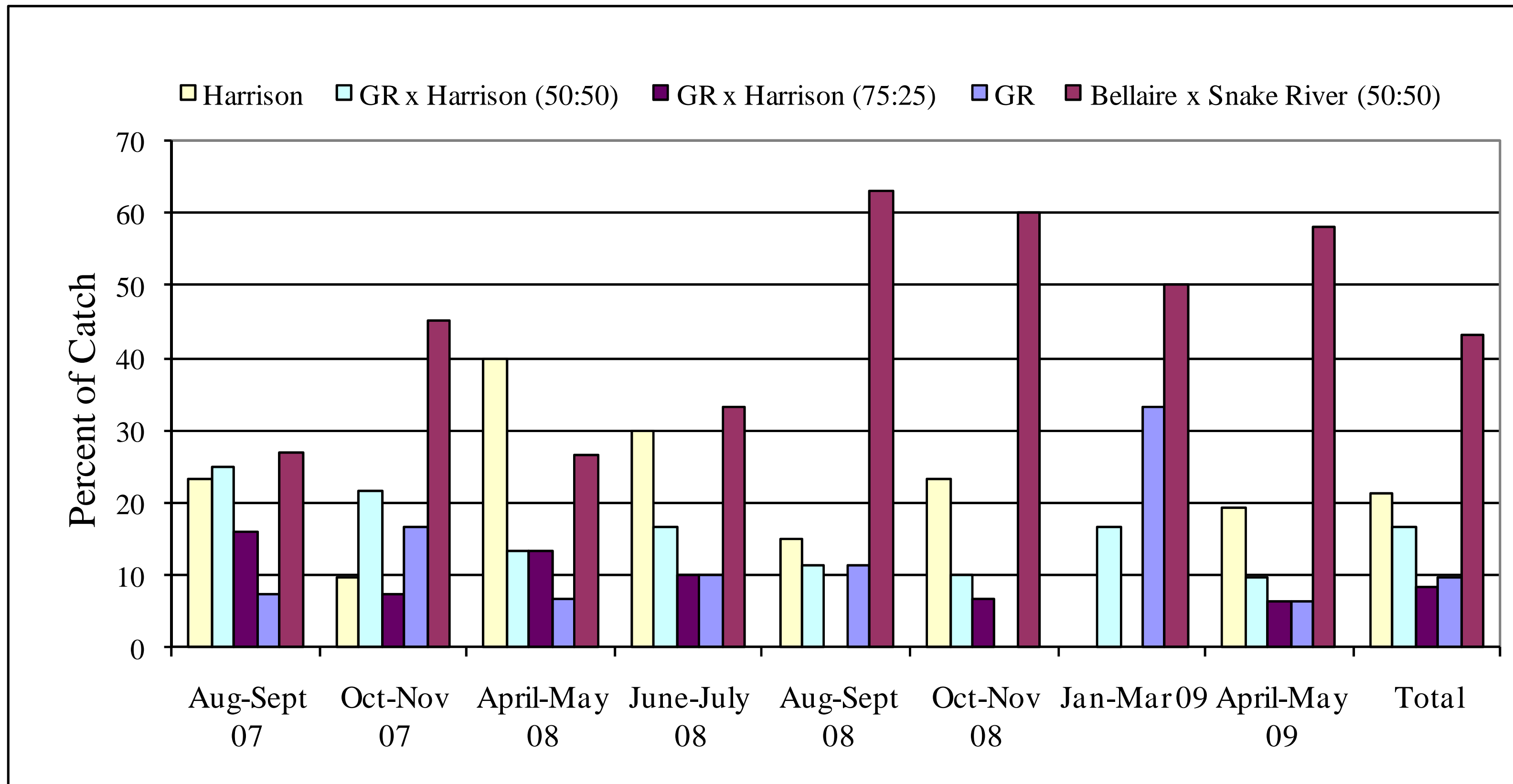
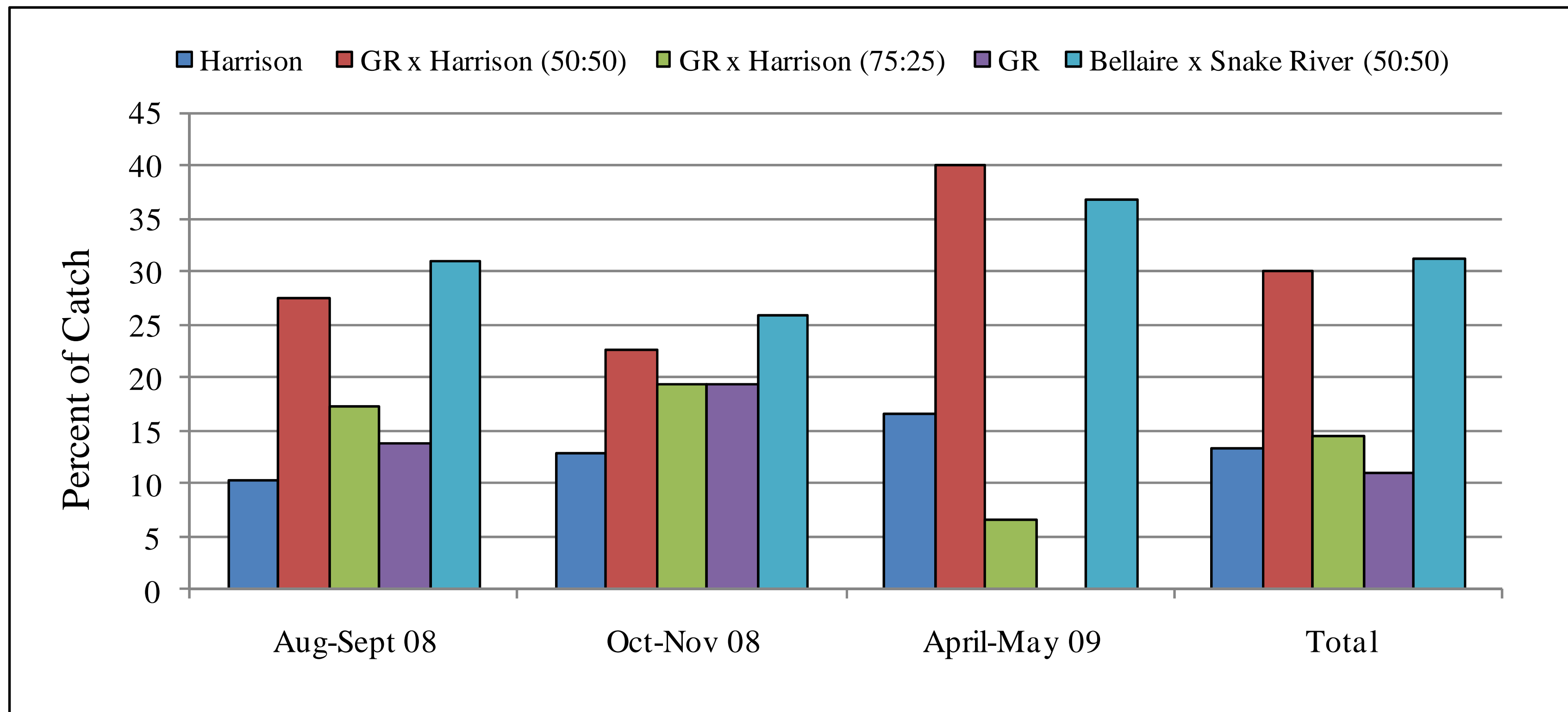


Figure 3.6. Percent of catch for each of the five varieties of fingerling rainbow trout stocked in Parvin Lake in July, 2008.



Collections of fish from the 2007 plant resulted in widely varying results during each time interval (Figure 3.5). Overall, the RXN strain was consistently more abundant in the samples than the other strains, contributing to 43.5% (111 fish) of the overall catch of 255 fish. The Harrison Lake strain contributed to 21.6% (55 fish) of the overall catch. The GRxHL (50:50 cross) contributed to 16.7% (43 fish) of the overall catch. The GRxHL (72:25 cross) contributed to 8.1% (21 fish) of the overall catch, and the pure GR strain contributed to 9.7% (25 fish) of the overall catch.

Collections of fish from the 2008 plant has thus far resulted in relatively consistent results, with the RXN and GRxHL (50:50) cross being more abundant in the samples than the other strains (Figure 3.6). The RXN strain contributed to 31.1% (28 fish) of the overall catch of 90 fish. The Harrison Lake strain contributed to 13.3% (12 fish) of the overall catch. The GRxHL (50:50 cross) contributed to 30.0% (27 fish) of the overall catch. The GRxHL (72:25 cross) contributed to 14.4% (13 fish) of the overall catch, and the pure GR strain contributed to 11.1% (10 fish) of the overall catch.

Given the relatively large size of the pure GR strain fish in both the 2007 and 2008 stocking events, their low return suggests that they may be more vulnerable to predation pressure than the other strains. The Harrison Lake variety was at a distinct disadvantage during both stocking events due to their smaller size, particularly in the 2007 stocking event, but managed to appear more often in the catch than all the other strains with the exception of the RXN fish. In general, it appears that a higher ratio of HL to GR in the crosses is advantageous to post-stocking survival with fingerling plants. The RXN group has proven to be a more successful fingerling plant than any of the other varieties. This could be due to hybrid vigor, diet preferences, predation avoidance behavior, or a host of other conditions, and warrants more investigation. As more samples are collected in the next couple of years, the long-term survival of the groups will be more definitive.

References

Schisler, G. J., E. R. Fetherman and P. J. Schler. 2008. Salmonid Disease Investigations. Federal Aid Project F-394-R7 Job Progress Report. Colorado Division of Wildlife Fish Research Section, Fort Collins, CO.

Job No. 4.

Job Title: Whirling Disease Resistant Wild Strain Brood Stock Development and Evaluation

Job Objective:

These experiments are designed to 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.

Past Evaluations

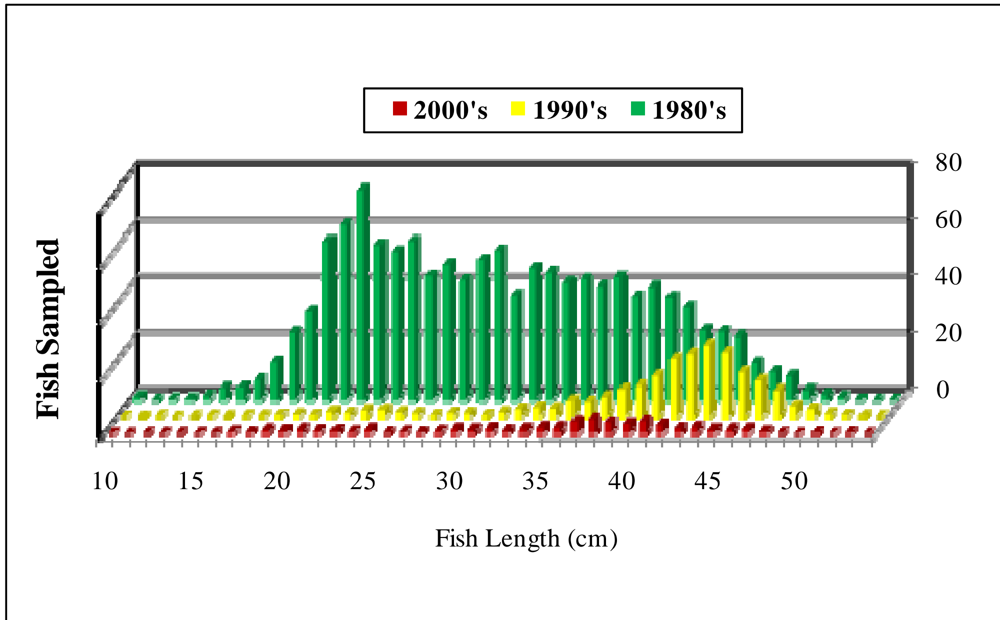
A substantial effort has been exerted in the last several years to incorporate the resistant strains into both domestic and wild rainbow trout programs. An overview of those efforts is summarized in Appendix II- *Resistant Rainbow Trout in Colorado: Current Status and Uses.* Specific work conducted during the 2008-2009 field seasons is presented below.

Upper Colorado River

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

Figure 4.1. Upper Colorado River study area.

Figure 4.2. Upper Colorado River historic rainbow trout length-frequencies averaged by decade.



In 2006, a single lot of GR-CRR 50:50 cross (F1) rainbow trout were stocked in to the upper Colorado River at 23.5 cm (9.4 inches) in length to evaluate the survival of these larger fish in an area dominated by brown trout, and with an extremely high prevalence of *M. cerebralis*. This plant of fish has been monitored during annual population estimates. An extensive population estimate was conducted in spring, 2008. This was designed to evaluate the growth and survival of the F1 fish stocked in 2006, and also to determine what proportions of the fish were sexually mature. The population estimate consisted of a mark-recapture event over a distance of 6.28 river km (3.9 river miles). Brown trout, which have increased dramatically in the river with the decline in rainbow trout numbers, were present in the reach at a density of 1,307.5 fish per kilometer (2,092 fish per mile). Colorado River rainbow trout (residual wild fish and fish present due to repeated stocking of Colorado River rainbow fingerlings) were estimated to exist at a density of 109.4 fish per kilometer (175 fish per mile). The F1 rainbow trout from the 2006 plant were present at a density of 92.5 fish per kilometer (148 fish per mile). They averaged 34.3 cm (13.5 inches) in length, ranging from 30.0 cm to 40.9 cm (11.8 to 16.1 inches). The fish from this single plant of 3,000 F1 fish comprise almost half of the entire rainbow trout population in this stretch of river (Figure 4.3).

Of the 257 F1 fish examined, 32 (12.5 %) were found to be sexually mature. Of these, nine were females and 23 were males. The relatively high proportion of surviving F1 fish and the onset of sexual maturity of many of these fish is very encouraging. Typically, rainbow trout become sexually mature at age two or three under hatchery conditions, and later in natural environments. The identification of sexually mature rainbow trout from the 2006 stocking event is favorable with respect to re-establishing a wild rainbow trout population. Fingerling fish were collected in 2007 and 2008 and

tested for the presence of markers for GR rainbow trout genes using the Amplified Length Frequency Polymorphisms (AFLP) technique. Details of the AFLP technique are presented in Appendix III as conducted by Pisces Molecular in Boulder, Colorado. None of the fish in the 2007 samples contained significant Hofer genetic backgrounds, and only a few individuals from the 2008 collections exhibited high proportions of Hofer markers (Figures 4.4 and 4.5).

Figure 4.3. Hofer-CRR rainbow cross (F1) fish sampled during the spring, 2008 mark-recapture event on the upper Colorado River, compared with pure Colorado River rainbow trout in the same reach.

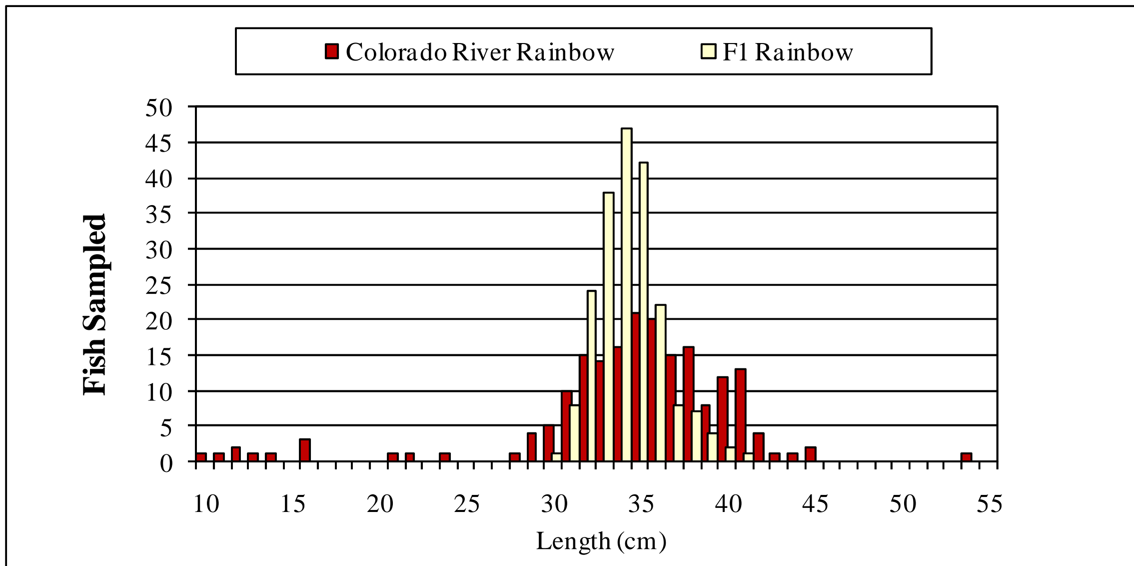


Figure 4.4. AFLP markers for Colorado River rainbow trout (CRR) and Hofer (GR) among rainbow trout fry collected in the upper Colorado River in 2007.

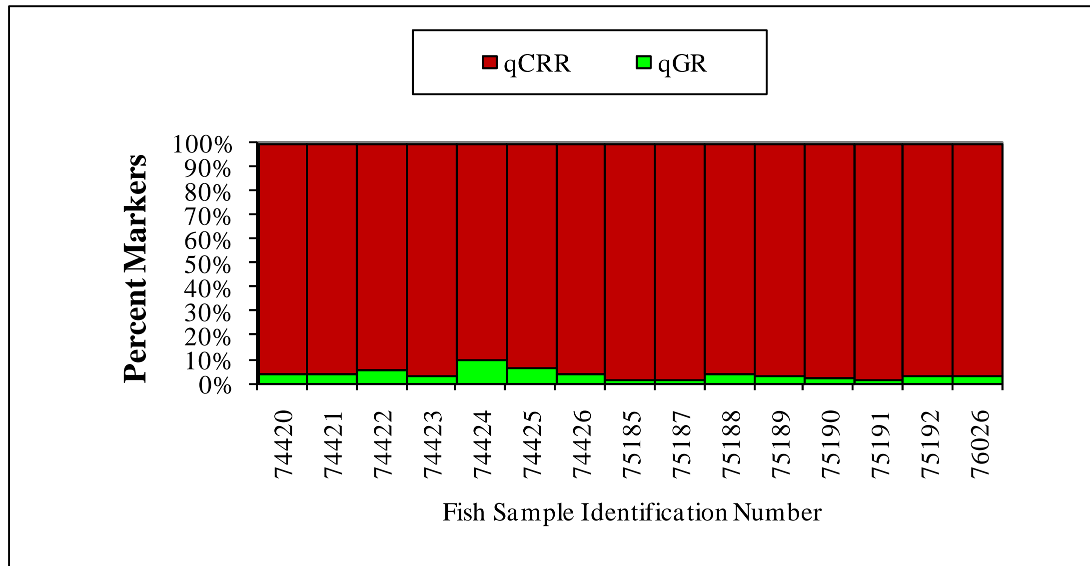
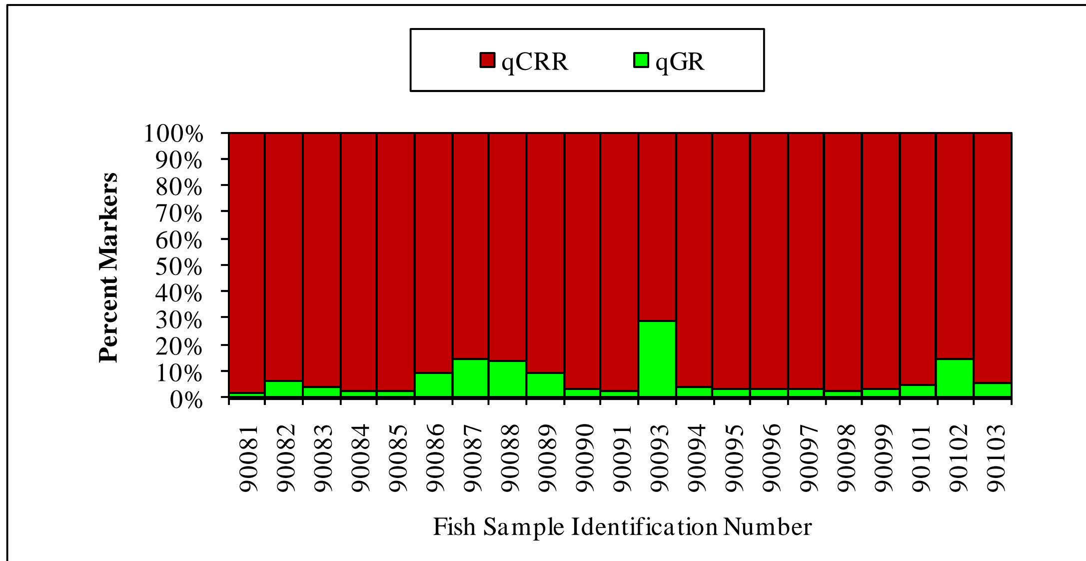


Figure 4.5. AFLP markers for Colorado River rainbow trout (CRR) and Hofer (GR) among rainbow trout fry collected in the upper Colorado River in 2008.



On April 28 and 30, 2009, a population estimate was conducted on the same 6.28 km reach as in 2008. Two raft-mounted electrofishing units, one fixed-boom electrode unit and one throw electrode unit, were used for both the mark and recapture runs. All trout captured during the mark run were given a caudal fin punch for identification on the recapture run. All of the brown trout captured on the mark run were measured to the nearest millimeter. In addition, ten brown trout from each 10 millimeter size class from 150 mm and larger were weighed to the nearest gram. All rainbow trout captured on the mark run were measured to the nearest millimeter and weighed to the nearest gram. If an individual had a Floy tag, the number on the tag and tag color were recorded. If the individual could be identified as one from a previous plant, as evidenced by a missing adipose fin, but did not have a Floy tag, the fish was retagged with a new Floy tag and the number was recorded. In addition, the sex and the reproductive status of each rainbow trout, if easily identifiable, were recorded. On the recapture run, all of the brown trout captured were measured to the nearest millimeter. Weights were recorded to the nearest gram for fish in any of the size classes that had not been completed on the mark run. All rainbows were measured to the nearest millimeter, weighed to the nearest gram, and checked for Floy tag number and color, sex, and reproductive status.

The population estimate was calculated using the Petersen estimator (with the Bailey modification). The brown trout were present in the reach at a density of 1,208.8 fish per km (1,934 fish per mile). Colorado River rainbow trout, including residual wild fish and fish present due to repeated stocking of Colorado River rainbow fingerlings, were estimated to exist at a density of 115.6 per km (185 fish per mile). The F1 rainbow

trout, from the 2006 plant, were present at a density of 160.6 per km (257 fish per mile). Other fish species encountered during the population estimate included speckled dace (*Rhinichthys osculus*), white sucker (*Catostomus commersoni*), longnose sucker (*Catostomus catostomus*), bluehead sucker (*Catostomus discobolus*), and brook trout *(Salvelinus fontinalis*).

Average length of the 2,229 brown trout captured was 327 mm, ranging from 70 to 537 mm. The 92 F1 rainbow trout captured averaged 368 mm in length, ranging from 327 to 440 mm. The 84 CRR trout captured averaging 365 mm and ranging from 140 to 495 mm (Figure 4.6). The F1 rainbows averaged 532 g in weight, ranging from 290 to 1030 g, and the CRR trout averaged 520 g in weight, and ranged from 124 to 1254 g. As with the population estimate in 2008, the F1 fish stocked in 2006 comprised a large proportion of the total rainbow trout population in the study area (Figure 4.7).

Of the 92 F1 fish that were handled during the population estimate, 32 (14 females and 22 males) were found to be sexually mature and ripe. An additional 20 females were sexually mature, but in pre-spawn status (green). Twenty-nine fish were green and unknown sexual status, but appeared that they could be potentially ripe later in the spring. Only seven were clearly immature and did not appear to be potentially sexually mature in 2009.

Eighty-three CRR individuals were handled during the population estimate, and of those, 22 were found to be sexually mature and ripe (14 were females, eight of which were already spent, and eight were males). An additional 16 green females and 39 green fish of unknown sexual status were present. Six sexually immature CRR individuals were also captured.

Figure 4.6. Length-frequency distribution for brown trout, Colorado River Rainbow trout, and F1 (2006 plant) rainbow trout in the upper Colorado River from the Hitchin' Post Bridge, downstream to the Sheriff Ranch, April 2009.

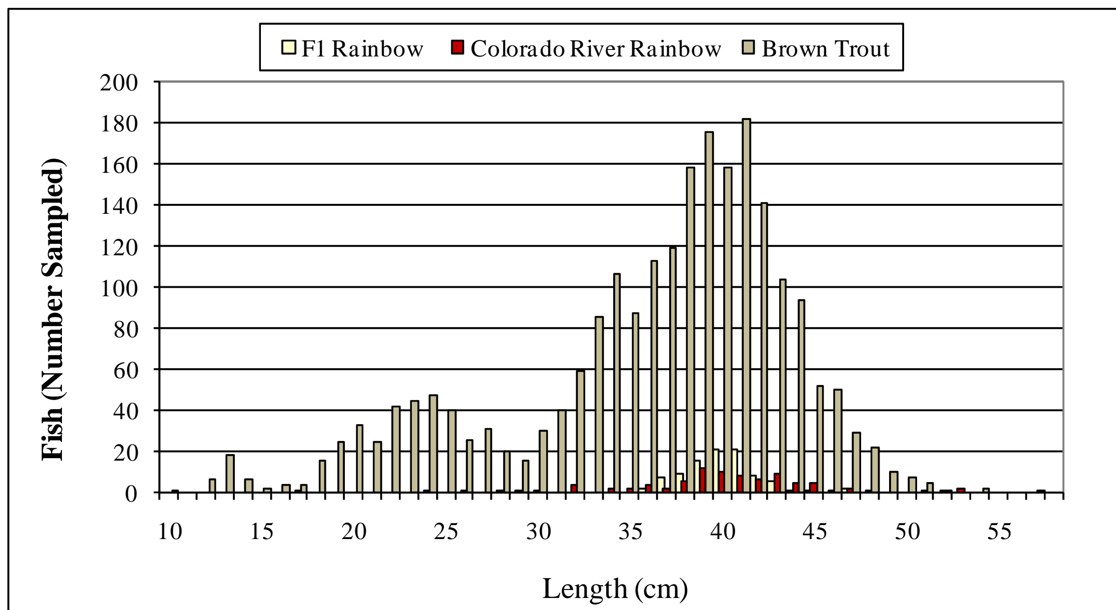
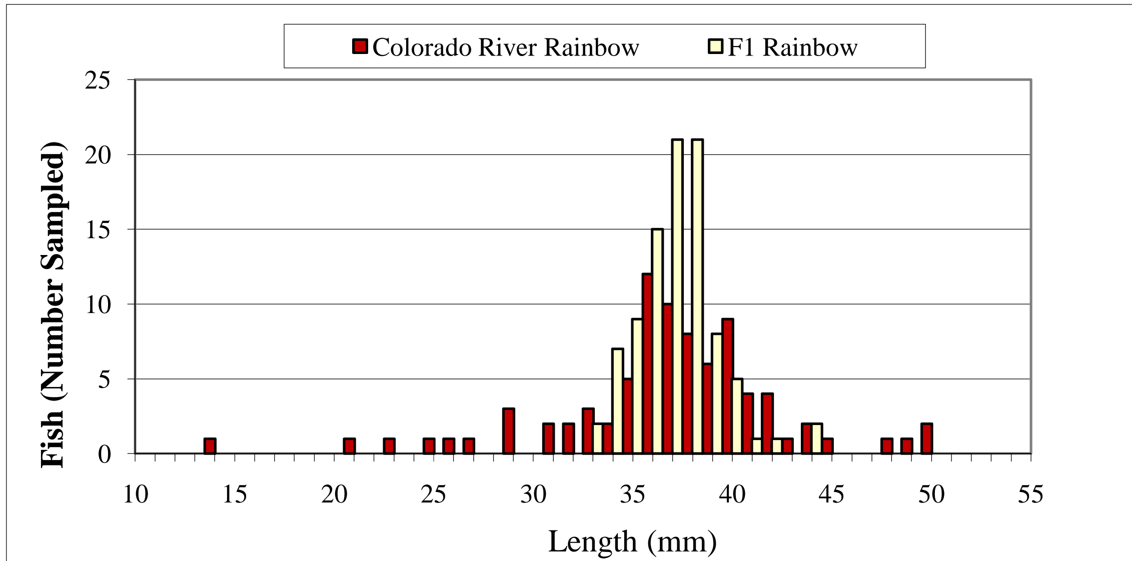


Figure 4.7. Length-frequency distribution for Colorado River Rainbow trout and F1 (2006 plant) rainbow trout in the upper Colorado River from the Hitchin' Post Bridge, downstream to the Sheriff Ranch, April 2009.

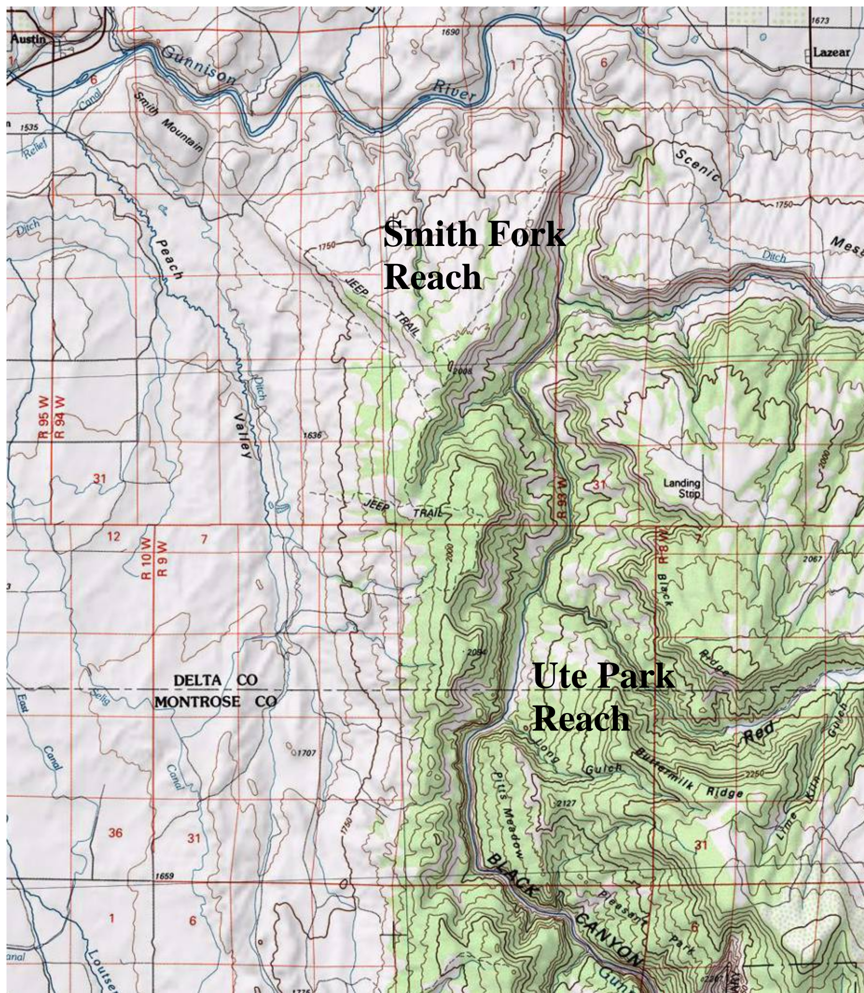


The high survival and good post-stocking growth of the F1 fish stocked as catchable-sized fish in the upper Colorado River has persisted into 2009. Fish from the 2006 plant are as abundant now as in 2008, have gained in average weight and length, and most are now sexually mature. The much higher proportion of sexually mature F1 fish in the population could lead to higher reproductive success of these fish in 2009. Additional evaluations are planned for the upper Colorado River using marked fish. Fry evaluations using the AFLP technique will also be initiated on a large scale to determine if the F1 fish successfully reproduced this season.

Gunnison River

The rainbow trout population in the Gunnison River has dramatically declined since the introduction of whirling disease. Like the upper Colorado River, multiple years of stocking pure Colorado River rainbow trout fingerlings has not resulted in any measurable increase in rainbow trout density or biomass. In fact, rainbow trout numbers have continued to decline, and brown trout numbers have grown to historical highs. A series of stocking events in the Gunnison River have occurred since 2004 in which equal numbers of pure Colorado River rainbow trout and Hofer-CRR cross fish have been differentially marked and stocked together to evaluate relative survival rates of the strains and as an attempt to re-establish a wild self-sustaining population in this location.

Figure 4.8. Gunnison River study area.



In 2004, Hofer-CRR 50:50 cross (F1) fish were marked with red visible implant elastomer (VIE) marks and pure CRR fish were similarly marked with green VIE marks. In this experiment, 10,104 CRR and 10,115 F1 rainbow trout were stocked as 13.6 cm and 11.9 cm fingerlings, respectively, into the Ute Park section of the Gunnison Gorge. The fish were mixed together prior to stocking to prevent bias due to handling, and then spread throughout the stream section using helicopter plants. In 2005, Hofer-CRR 25:75 cross (B2) fish were stocked, rather than F1 fish, along with pure CRR fish. The B2 fish were marked with an adipose clip and pure CRR strain fish were similarly given a right pelvic clip. Stocking was conducted using 5,000 of each variety as 15.2 cm fingerlings. In 2006, B2 fish were stocked again as 17.3 cm fingerlings to determine if the slightly larger B2 fish would perform better than the first (2005) plant of B2 fish. The pure CRR fish were not marked in this plant, while the B2 fish were given an adipose clip and a red VIE mark. In 2007, the number of fish stocked was increased to 20,000 of the pure CRR and 20,000 F1 rainbow trout stocked as 14.7 cm fingerlings. Coded wire tags were used to batch-mark the F1 and the pure CRR fish. Additionally, the F1 fish were adipose clipped to provide a second mark in case the coded wire tag was lost.

Growth, survival, and infection severity of the two strains planted each year were evaluated from samples collected during the annual population estimate conducted the following year. Estimates were conducted using mark-recapture sampling with boat-mounted electroshocking gear. All rainbow trout were carefully examined for evidence of VIE marks, fin clips, and coded wire tags. Subsamples of fish were collected for myxospore evaluation using the PTD method in 2005 and 2006.

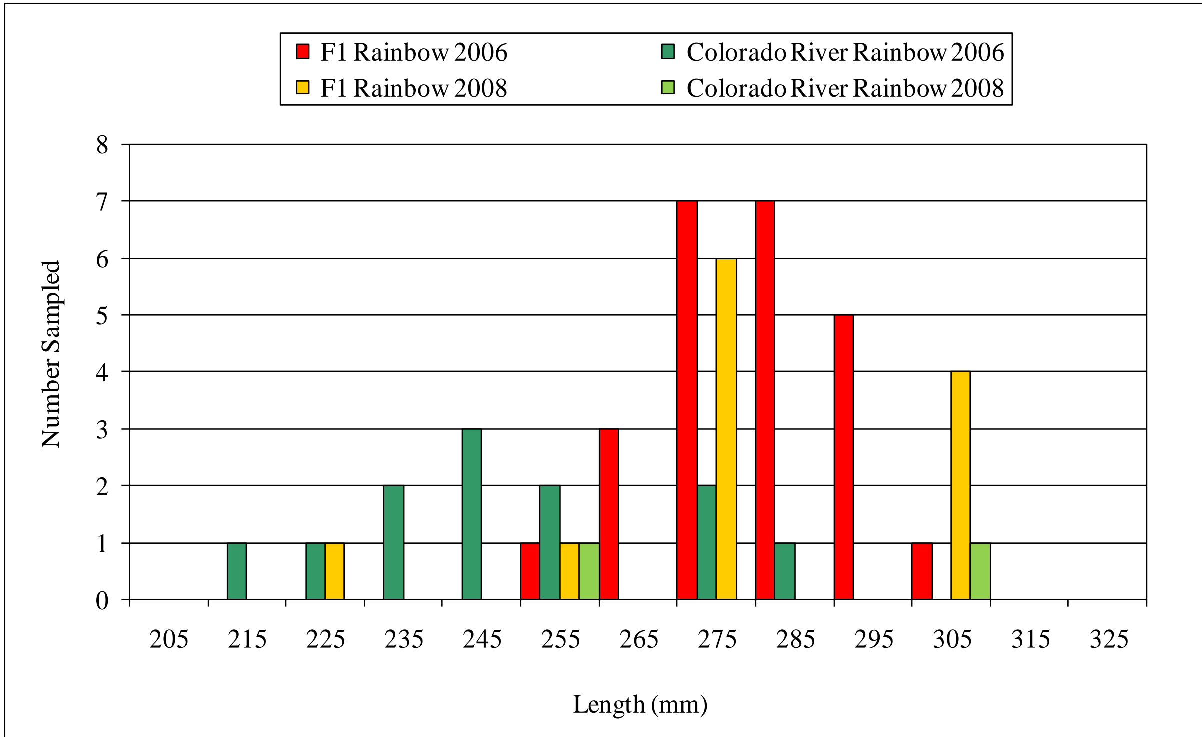
The 2005 population estimate indicated that survival of both varieties of fish stocked in 2004 was relatively low, with only 12 of the pure CRR, and 24 of the F1 fish being found in the 2,375 m sampling area. The sampling resulted in an estimate of 10 pure CRR fish per km (16 fish per mile). The estimates for F1 cross were 14 fish per km (22 fish per mile). The average total length of the CRR fish was 24.8 cm, and 28.3 cm for the F1 fish. All of the pure CRR individuals collected were found to be infected, with an average myxospore count of 124,603 (SD = 129,406). Only six of the 10 F1 individuals collected were found to be infected, with an average myxospore count of 4,055 (SD = 8,336).

Survival and population estimates in 2006 for fish stocked in 2005 were difficult to assess directly because of mark loss (fin regeneration or poor marks) in both the CRR and B2 varieties. AFLP (Amplified Fragment Length Polymorphism) testing, a molecular technique that can help distinguish between individuals of the same species with different genetic lineages, was used to identify a subsample of unmarked fish as either B2 plants or pure CRR fish. Applying the ratio of fish identified as each variety in the subset to the overall population estimate of fish resulted in an estimate of 33 fish per km (53 fish per mile) of the pure CRR strain, and 22 fish per km (35 fish per mile) of the B2 cross. PTD testing identified an average of 83,929 myxospores (SD = 149,719) in the pure CRR fish planted in 2005. The average myxospore count among B2 fish was 40,480 (SD = 48,121).

In 2007, poor mark retention once again made estimating numbers of pure CRR and Hofer-cross fish difficult. The overall population estimate of rainbow trout (over 15 cm in length) was 135 fish per km (217 fish per mile). Of the 144 fish sampled, 16 (11.1%) were identified as either F1 or B2 fish by having either red VIE marks or adipose clips, while only three (2.1%) were identified as pure CRR fish, having green VIE marks. In 2008, the population estimate for rainbow trout (over 15 cm in length) was 111 fish per kilometer (178 fish per mile). Fish stocked in 2007 could be very clearly identified because of the coded wire tags and fin clips. Of the 157 rainbow trout that were sampled, 12 of the F1 fish and two of the pure CRR fish from the 2007 plant were positively identified, producing an estimate of seven F1 and a minimum of two pure CRR fish per kilometer (12 F1 and three CRR fish per mile), respectively. Average length of the F1 fish (27.7 cm) was similar to the pure CRR fish (27.5 cm) in 2008, after the fish had been in the river for one year. Overall, poor survival estimates were quite evident for both the pure CRR and the Hofer-cross fish in each year of stocking. Predation by brown trout, loss of marks, and emigration from the study area were likely contributing factors. However, in both years (2006 and 2008) where definitively identified F1 and CRR fish could be compared directly from the stocking event in the previous year, the F1 fish were much more abundant than the pure CRR fish (Figure 4.9).

Fingerling rainbow trout were collected during fry shocking events in both 2007 and 2008 to be submitted for AFLP testing to determine if offspring had been produced from the F1 and B2 stocking events. The analysis identified a high proportion of the fingerling fish collected in 2007 as having a genetic background consistent with the Hofer strain. In 2008, a lower proportion of fry were identified as having Hofer genetic background. Nonetheless, natural reproduction from the Hofer crosses stocked in the river is now occurring. There is also some evidence that Hofer-cross fry produced in 2007 survived past their first year of life evident from the large number of unmarked age-1 fish in the 2008 samples.

Figure 4.9. Length-frequency and numbers of fish by strain sampled in the Gunnison River in 2006 and 2008 where direct comparisons of pure Colorado River rainbow trout and Hofer-CRR 50:50 (F1) crosses could be made from fish stocked in the previous year.



The results of this field evaluation demonstrate that the F1 fish can survive at least as well as the pure CRR trout when planted as fingerlings. The results also demonstrate that myxospore counts developed after stocking are much lower in the F1 fish than in the pure CRR trout. The myxospore counts in B2 fish released into the wild were similar to those found in the laboratory experiments, and while lower than the spore counts from the pure CRR fish, were also higher than observed in the F1 fish. This reinforces the notion that allowing natural selection of the resistant offspring of the F1 fish to occur in the wild may be a more effective method to producing sufficient resistance and wild behaviors than creating subsequent crosses artificially.

High densities of brown trout continue to contribute to the poor survival of the stocked rainbow trout in the Gunnison River, and poor mark retention has caused problems with producing reliable estimates of survival in B2 fish. However, reproduction from Hofer-cross fish has been confirmed in several locations at, and downstream of, the stocking sites. These results are promising, and could lead to re-establishment of a wild rainbow trout population in the Gunnison River despite the presence *M. cerebralis*. More in-depth genetic analyses of the fry and age-1 fish are planned for 2009 to determine the extent of survival and recruitment from the wild-spawned rainbow trout that are now appearing in the population.

Job No. 5.

Job Title: Technical Assistance

Job Objective:

Provide information on impacts of fish disease on wild trout populations to fisheries managers and hatchery personnel of the Colorado Division of Wildlife and other resource agencies. Provide specialized information or assistance to the Hatchery Section. Contribute editorial assistance to various professional journals and other organizations upon request. Continued work on the new C-SAP computer program has occurred, and assistance to area biologists in operating and conducting analysis with the program has become a routine part of this work. Additional technical assistance with disinfection techniques for aquatic nuisance species has also occurred in this fiscal year (see Appendix IV- *Portable Decontamination Unit for Boat and Equipment Disinfection*). Several evaluations of whirling disease infectivity on mountain whitefish (*Prosopium williamsoni)* have also been directed in the last year.

Technical Assistance Milestones

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

1) Schisler, G. J. 2008. Resistant rainbow trout brood stock development for fisheries management in Colorado. Red Feather Lakes Historical Society, July 16, 2007. Parvin Lake Research Station. Red Feather Lakes, CO.
2) Schisler, G. J. 2008. Resistant rainbow trout brood stock development for fisheries management in Colorado. Colorado State University Student Chapter of the American Fisheries Society. December 3, 2008. Fort Collins, CO.
3) Schisler, G. J. 2008. Resistant rainbow trout brood stock development for fisheries management in Colorado. Chimney Rock Ranch Club, July 24, 2008. Denver, CO.
4) Schisler, G. J. 2009. Resistant rainbow trout brood stock development for fisheries management in Colorado. Colorado Aquaculture Association Meeting. January 24, 2009. Mt. Princeton, CO.
5) Schisler, G.J., J. Ewert, B. Atkinson, K. Rogers, K. Thompson, R. B. Nehring, and E. Fetherman. 2009. Whirling disease resistant rainbow trout Colorado River project update. 15th Annual Whirling Disease Symposium: Conserving coldwater fisheries, Denver, CO, February 5-6, 2009**.**
6) Schisler, G. J., K. B. Rogers, and R. P. Hedrick. 2009. Early development of mountain whitefish *(Prosopium williamsoni*) and effects of *Myxobolus cerebralis* exposure. 15th Annual Whirling Disease Symposium: Conserving coldwater fisheries, Denver, CO, February 5-6, 2009.
7) Schisler, G. J., K. B. Rogers, and R. P. Hedrick. 2009. Early development of mountain whitefish (*Prosopium williamsoni*) and effects of *Myxobolus*

cerebralis exposure. Whitefish summit, Silverthorne, Colorado, January 6, 2009**.**

8) Kowalski, D. A, R. B. Nehring, and G. J. Schisler. 2008. Preliminary results on the introduction of *Myxobolus cerebralis* resistant rainbow trout in the Gunnison River, Colorado. 15th Annual Whirling Disease Symposium: Conserving coldwater fisheries. February 5-6, 2009**,** Denver, CO.
9) Fetherman, E. F., D. L. Winkelman, and G. J. Schisler. 2008. The physiological effects of whirling disease in resistant and susceptible crosses of rainbow trout. 15th Annual Whirling Disease Symposium: Conserving coldwater fisheries 5-6, 2008**,** Denver, CO.
10) Fetherman, E. F., D. L. Winkelman, and G. J. Schisler. 2008. The physiological effects of whirling disease in resistant and susceptible crosses of rainbow trout. Colorado-Wyoming Annual American Fisheries Society meeting, February 23-26, 2009, Loveland, CO.
11) Fetherman, E. F., D. L. Winkelman, and G. J. Schisler. 2008. The physiological effects of whirling disease in resistant and susceptible crosses of rainbow trout. Western Division Annual American Fisheries Society meeting, May 3-7, 2009 Albuquerque, NM .
12) Several popular articles have appeared as a result of interviews this year on this project such as North Forty News (May 2008), TROUT Magazine (Spring 2008), North American Fisherman Magazine (April 2008).

APPENDIX I.

Heritability of myxospore count and the effects of Myxobolus cerebralis exposure on the physiological performance of whirling disease resistant and susceptible strains of rainbow trout

Eric R. Fetherman
Colorado State University

THESIS

HERITABILITY OF MYXOSPORE COUNT AND THE EFFECTS OF *MYXOBOLUS CEREBRALIS* EXPOSURE ON THE PHYSIOLOGICAL PERFORMANCE OF WHIRLING DISEASE RESISTANT AND SUSCEPTIBLE STRAINS OF RAINBOW TROUT

Submitted by

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In partial fulfillment of the requirements

for the degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2008

COLORADO STATE UNVERSITY

April 3, 2009

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY ERIC R. FETHERMAN ENTITLED HERITABILITY OF MYXOSPORE COUNT AND THE EFFECTS OF *MYXOBOLUS CEREBRALIS* EXPOSURE ON THE PHYSIOLOGICAL PERFORMANCE OF WHIRLING DISEASE RESISTANT AND SUSCEPTIBLE STRAINS OF RAINBOW TROUT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Dr. Michael F. Antolin

Dr. Christopher A. Myrick

Advisor, Dr. Dana L. Winkelman

Department Head, Dr. Kenneth R. Wilson

ABSTRACT OF THESIS

HERITABILITY OF MYXOSPORE COUNT AND THE EFFECTS OF *MYXOBOLUS CEREBRALIS* EXPOSURE ON THE PHYSIOLOGICAL PERFORMANCE OF WHIRLING DISEASE RESISTANT AND SUSCEPTIBLE STRAINS OF RAINBOW TROUT

Whirling disease was first introduced to Colorado in 1987, and since its introduction, has caused severe declines in rainbow trout populations across the state. A solution was needed to reduce the effects of the disease on Colorado's rainbow trout populations if the populations were to rebound and stabilize. The solution was discovered at the Hofer Rainbow Trout Farm in Germany, where the German Hofer strain of rainbow trout (GR), a domestic strain reared as a food fish for human consumption, was found to be resistant to whirling disease.

In 2004, the Colorado Division of Wildlife began a selective breeding program using the GR strain and the Colorado River Rainbow (CRR) strain, an extremely susceptible wild rainbow trout strain which had been historically used to establish rainbow trout populations in Colorado. The principle aim of this program was to incorporate the resistance characteristics of the GR strain into a strain that retained many of the desired "wild rainbow trout" characteristics necessary for survival in Colorado.

In this study, five strains, the GR, CRR, F1, F2, and B2 strains, were exposed to whirling disease and evaluated for resistance characteristics, physiological effects of the disease, focusing on growth and swimming, and predator avoidance. The F1 and F2 strains are the first and second filial generational crosses between the GR and CRR strains, and the B2 strain is a backcross between the F1 and CRR strains. In addition, the heritability of myxospore counts as a result of exposure to whirling disease was calculated for each of the five strains. The objective of this study was to determine which of the strains would be the best candidate for use as a brood stock to reestablish rainbow trout populations in Colorado.

In the exposure metrics part of the experiment, the GR strain had a significantly lower mean myxospore count than the B2 or CRR strains, though not differing from the F1 or F2 strains. The CRR strain experienced a higher number and severity of deformities than the other four strains. Differences were seen between the exposures, where both the number of deformities and the number of mortalities were lower in the uninfected treatments.

The F2 strain had a broad sense heritability estimate for myxospore count as a result of exposure to whirling disease of 0.34 ± 0.21; the F1 and GR strains were similarly low in their heritability estimates for myxospore count with estimates of 0.42 ± 0.23 and 0.34 ± 0.21, respectively. The B2 strain had a higher broad sense heritability estimate than the F2 strain, with an estimate of 0.93 ± 0.28. The CRR strain had a higher broad sense heritability estimate than expected at 0.89 ± 0.28. These results indicate that the genes that control for myxospore count are approaching fixation in the GR strain, that these genes remain in subsequent generations, and that the CRR strain may have had some innate resistance to whirling disease or has developed it over the last two decades of exposure.

The effective number of factors (n_e) by which the GR and CRR strains differ in relation to myxospore development is 9 ± 5, which provides the first estimate of the

number of loci used in resistance to whirling disease by the GR strain. Since the number of loci involved was low, it is reasonable to believe that searching for informative molecular markers should provide information on the exact location of the loci involved in resistance to whirling disease.

In the growth experiment, the GR strain reached a significantly higher batch weight than the other four strains, the F1 strain had a significantly higher batch weight than the F2, B2 and CRR strains, and the F2, B2 and CRR strains did not differ from each other in batch weight. There were no differences in batch weight between the exposures in any of the strains. Food conversion ratios were lower in the GR strain, and those strains having a higher percentage of GR genes. There were few differences in percent lipid and protein content among the strains, indicating that exposure to whirling disease does not affect the way that food is processed in any of the strains.

In the swimming experiment, the CRR strain reached significantly higher critical velocities than the GR strain at all of the time periods. The F1, F2 and B2 strains did not differ in swimming ability from each other, nor the GR or CRR strains, indicating that neither pure strain has an advantage in swimming ability over the intermediate strains. There was no difference in swimming ability between infected and uninfected individuals in any of the strains. In the fourth time period, differences in total length affected critical swimming speeds among the strains and between exposures.

The CRR showed the lowest survival rates in the predation experiment, and had the highest rate of susceptibility to predation of all of the strains. The GR, F1, F2 and B2 strains did not differ in their survival rates over the course of the experiment. There was no difference in survival between infected and uninfected individuals in any of the strains.

Based on the results of these experiments, the GR strain seems to be the best candidate for use as a brood stock to reestablish rainbow trout populations in Colorado. However, the F1 strain only differed from the GR strain in batch weight, indicating it may be a good candidate as well. Experiments on the survival and reproduction of these two strains in the wild need to be conducted to determine which strain would be the best candidate for reintroducing rainbow trout to Colorado's waters.

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CHAPTER 1

BACKGROUND AND HISTORY OF WHIRLING DISEASE, *MYXOBOLUS CEREBRALIS*

Whirling disease is caused by the parasite *Myxobolus cerebralis* and was first detected in rainbow (*Oncorhynchus mykiss)* and brook trout (*Salvelinus fontinalis)* that were imported to Germany in 1893 for hatchery production (Höfer 1903). *M. cerebralis* is native to Europe and infects many salmonids, including trout (*Oncorhynchus* spp., *Salmo trutta*, *Salvelinus* spp.), salmon (*Oncorhynchus* spp., *Salmo salar*), and mountain whitefish (*Prosopium williamsoni*). It is a member of the Phylum Cnidaria, based primarily on the structural features of the waterborne infectious triactinomyxon (TAM) stage of the parasite, which has extrusive filaments (cnidocysts) for attachment to the fish host (Siddall et al. 1995; Kent et al. 2001).

Whirling disease has a complex two-host life cycle that was not fully described until the mid-1980's when the oligochaete host *Tubifex tubifex* was discovered to be part of the life cycle (Markiw and Wolf 1983; Wolf and Markiw 1984). The waterborne triactinomyxon (TAM) stage of *M. cerebralis* attaches to a salmonid host (El-Matbouli et al. 1999a; Hedrick and El-Matbouli 2002). After penetrating the epidermis, germ cells from the sporoplasm disperse deeper into the layers of the epidermis, migrating and replicating among nerve bundles in ganglia and the central nervous system (El-Matbouli et al. 1995). The parasite migrates from the central nervous system and undergoes further replication in the host cartilage, eventually undergoing sporogenesis to form the muticellular myxospore stage (Lom and Dyková 1992; El-Matbouli et al. 1995). When the fish host dies, myxospores become available for ingestion by the second host, the oligochaete *T. tubifex* (Hedrick and El-Matbouli 2002). Myxospores undergo several stages of transformation within the intestinal epithelial cells of *T. tubifex* and eventually become the infectious triactinomyxon form of the parasite (El-Matbouli et al. 1998; El-Matbouli and Hoffman 1998; El-Matbouli et al. 1999b). Triactinomyxons are then released into the water by *T. tubifex* where they again can infect salmonid hosts (Markiw 1986; Hedrick and El-Matbouli 2002; Figure 1.1).

After its discovery in Germany in the late 1800s, whirling disease began to spread to many countries around the world. Between 1911 and 1970, whirling disease had been found in several European countries including Denmark, Finland, France, Italy, the USSR, Czechoslovakia, Poland, Bulgaria, Yugoslavia, Sweden, Scotland, and Norway, as well as South Africa and Morocco in Africa (see Bartholomew and Reno 2002 for dates and citations). Hoffman (1970) estimated that the original range of *M. cerebralis* covered an area from central Europe to northeast Asia; however, because it was a disease of brown trout (*Salmo trutta*), in which the infection is usually asymptomatic, it was the introduction of non-native rainbow trout that led to the discovery of the parasite in many locations (Hoffman 1970; Gilbert and Granath 2003). Unrestricted transfers of live infected fish were suspected to be the main route of dissemination outside of the European home range (Hoffman 1970).

Between 1971 and the present, whirling disease has been found in several other European countries including Austria, Belgium, Hungary, England, Ireland, Netherlands, and Spain (see Bartholomew and Reno 2002 for dates and citations). Differences in monitoring and reporting, and inconsistencies in the literature, make it hard to determine whether these introductions were caused by unrestricted transfers of live fish between rearing facilities and into natural populations, or if the original

range of *M. cerebralis* included most of the European countries where the disease had been discovered (Halliday 1976).

In 1971, whirling disease was also discovered in New Zealand, where it was reported to have caused a whirling motion, a condition known locally as "whirly-gig" disease, accompanied by heavy mortality in the rainbow trout populations (Hewitt and Little 1972). Suspected introduction routes included live importation of salmonids, as well as live food for tropical fish which may have included infected tubificids; however, introduction routes are hard to determine because examination of preserved specimens demonstrated that the parasite had been present at least five years before it was identified (Boustead 1993).

Whirling disease was first detected in the United States in brook trout at the Benner Springs Fish Research Station in Bellefonte, Pennsylvania in 1956. It is suspected that it was introduced via infected ground fish tissue fed to the hatchery brook trout (Hoffmann 1962). A second introduction was detected in 1965 in California, where frozen fish from a Danish merchant vessel fed to hatchery fish were implicated in the introduction (Hoffman 1990). Once established at these locations in the eastern and western United States, subsequent spread of the disease has been attributed to transfers of live fish (Hoffman 1970; Hoffman 1990), and has since been found in 22 states: Pennsylvania, Connecticut, Virginia, California, Nevada, New Jersey, Massachusetts, West Virginia, Michigan, Ohio, New Hampshire, New York, Oregon, Idaho, Colorado, Wyoming, New Mexico, Washington, Montana, Maryland, Utah and Arizona (see Bartholomew and Reno 2002 for dates and citations).

In Colorado, whirling disease was detected in rainbow trout at one public and three private aquaculture facilities in November 1987, and by April 1989, had been detected at 11 fish culture facilities and in 40 captive or free-ranging salmonid populations in 11 of the 15 major river drainages (Barney et al. 1988; Nehring and Thompson 2003). Introduction of the disease to Colorado was believed to have occurred accidentally through one or more legal shipments of trout to a private hatchery from an inspected source that subsequently tested positive (Walker and Nehring 1995). The disease became disseminated throughout the state as a result of transfers and planting of infected fish from the affected state and private hatcheries prior to its detection (Barney et al. 1988; Walker and Nehring 1995). Affected watersheds in the state of Colorado include the North and South Platte watersheds, including the Cache la Poudre River, the Colorado River watershed, including the Gunnison River, the Arkansas River watershed, and the Rio Grande watershed (Nehring and Thompson 2001).

M. cerebralis has caused severe problems in wild rainbow trout populations in the intermountain west (Nehring and Walker 1996). Walker and Nehring (1995) examined several possible factors to explain the decline in young-of-year rainbow trout and identified whirling disease as the primary factor causing the declines in recruitment. Additional laboratory and field studies demonstrated that whirling disease was the primary factor explaining the loss of juvenile rainbow trout in many stream segments throughout Colorado (Schisler et al. 1999a; Schisler et al. 1999b; Nehring and Thompson 2001).

Since the introduction of *M. cerebralis* into Colorado, several management strategies have been considered to reintroduce and manage rainbow trout. Although

many of these management options work well in hatchery situations, they are not applicable to wild populations. The most promising potential management option for wild populations is the use of resistant hosts (Schisler et al. 2006). A whirling disease resistant strain of rainbow trout was discovered at the Hofer Rainbow Trout Farm in Germany (El-Matbouli et al. 2002). These rainbow trout had been imported into Germany in the late 1800s for hatchery production and presumably these trout were exposed to *M. cerebralis* and developed resistance to whirling disease; however, few details are available regarding the potential selection mechanisms. El-Matbouli et al. (2002) found that the German Hofer strain of rainbow trout (GR) showed a resistance to whirling disease similar to that found in brown trout, and showed that the GR strain had similar levels of clinical signs, as well as similar myxospore concentrations and average histological scores, to those found in brown trout. Hedrick et al. (2003) also found that the GR strain was much more resistant to whirling disease than other North American rainbow trout strains tested during laboratory exposures of *M. cerebralis*. However, because the GR strain is a domesticated fish, their survival and viability in the wild is questionable, and the consequences of stocking them directly into wild trout waters is unknown (Schisler et al. 2006). The GR strain is also known to be inbred, exhibiting low levels of heterozygosity (El-Matbouli et al. 2006). Therefore, the GR may lack the genetic diversity necessary for survival and adaptation in natural conditions.

The genes responsible for resistance to *M. cerebralis* are relatively unknown. Severin and El-Matbouli (2007) examined the expression of several immune regulatory genes that may be used in the process of resisting whirling disease, and found that the expression of transforming growth factor beta (TGF-beta) was more highly elevated in the non-susceptible strain. However, they concluded that TGF-beta was not important for resistance. Baerwald et al. (2008) examined several genes in the skin of susceptible and non-susceptible rainbow trout, and found that the response to infection may be linked with the interferon *Metallothionein B*. More research is needed to identify the genes and mechanisms responsible for resistance; however, it is possible to use selective breeding and quantitative genetic techniques to understand the heritability of resistance.

In 2004, the Colorado Division of Wildlife began a selective breeding program using the GR and Colorado River rainbow (CRR) trout strains. The CRR is a wild rainbow trout strain that had been historically used to establish rainbow trout populations in Colorado (Schisler et al. 2006). Wood and Schisler (2005) bred resistant GR fish with susceptible CRR fish to evaluate if whirling disease resistance could be incorporated into a rainbow trout strain that was resistant but retained the desirable wild characteristics of the CRR (Schisler et al. 2006). Wood and Schisler (2005) directly selected for *M. cerebralis* resistance by exposing progeny to *M. cerebralis* and selecting those individuals which survived and showed no pathology for spawning and brood stock development. Resistant progeny were screened using Amplified Fragment Length Polymorphisms (AFLPs) to identify individuals with genetic characteristics most similar to CRR. Families created with this approach may be used as brood stock to reestablish rainbow trout populations in Colorado, with the goal that natural reproduction will occur so that stocks may become self-sustaining (Czapla 1999).

The ultimate goal is to develop a strain of rainbow trout that will be able to reproduce in the presence of *M. cerebralis*. Development of a *M. cerebralis* resistant strain is still being evaluated; however, other selective breeding programs have been successful using similar techniques. A selective breeding program was developed to get Arctic char (*Salvelinus alpinus*) to grow larger and faster in frigid spawning and rearing conditions in the Yukon, Canada (McGowan et al. 2005). Common carp (*Cyprinus carpio*) have been selectively bred for a number of characteristics including shape and disease resistance (Vandeputte 2003). In salmonids, selective breeding programs have been used to increase the resistance to salmon louse in Atlantic salmon (*Salmo salar*) (Kolstad et al. 2005), and increase the resistance to furunculosis in brook trout (Cipriano 2002). My experiments were designed to evaluate the resistant rainbow trout developed through the selective breeding and brood stock development program begun by the Colorado Division of Wildlife. The objective was to examine the resistance characteristics and physiological performance of rainbow trout strains created from the resistant GR strain and the susceptible CRR strain and make recommendations regarding the appropriate strain to use as a brood stock to reestablish wild rainbow trout populations in Colorado.

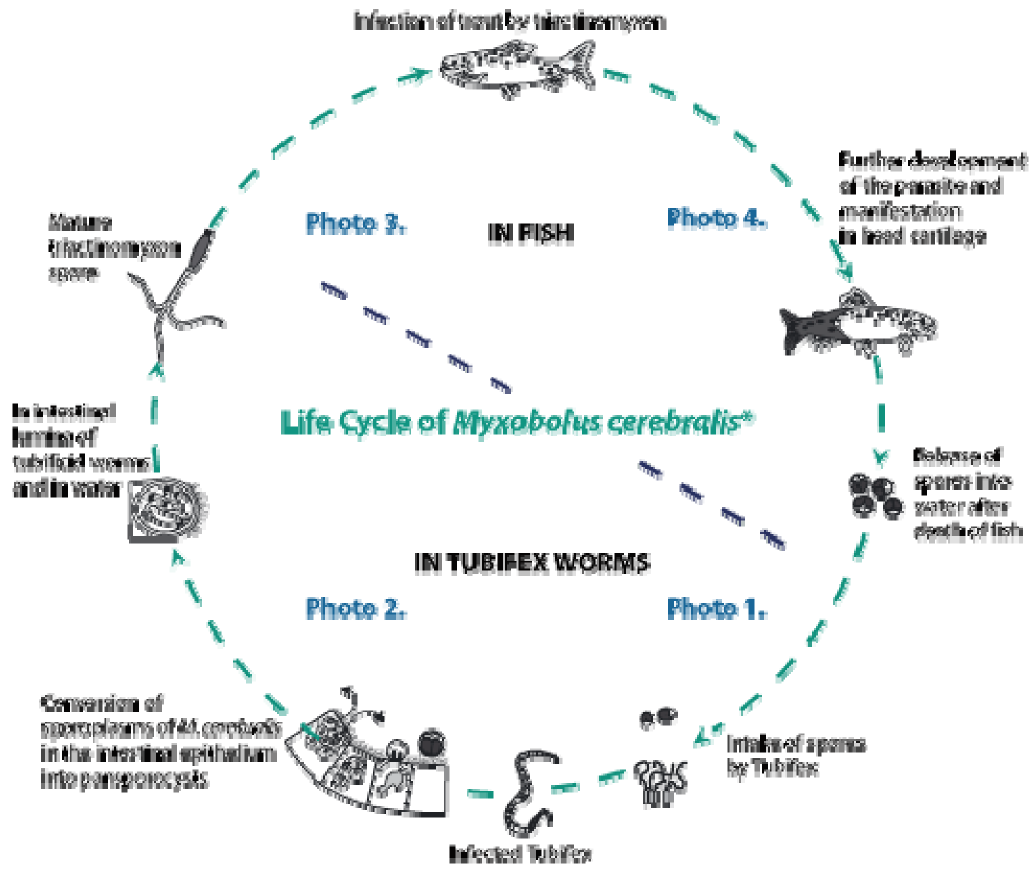

Figure 1.1. Life cycle of *Myxobolus cerebralis* (adapted by the Whirling Disease Initiative from El-Matbouli et al. 1992).

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CHAPTER 2:

HERITABILITY OF MYXOSPORE COUNT AS A RESULT OF EXPOSURE TO *MYXOBOLUS CEREBRALIS* IN SUSCEPTIBLE AND RESISTANT STRAINS OF RAINBOW TROUT

INTRODUCTION

The parasite *Myxobolus cerebralis,* responsible for salmonid whirling disease, is the primary factor causing recruitment failure and population collapses in rainbow trout fisheries throughout the intermountain west (Nehring and Walker 1996; Walker and Nehring 1995; Schisler et al. 1999*a*; Schisler et al. 1999*b*; Nehring and Thompson 2001). Due to the complex life cycle of the parasite and its durability in the environment, control of the parasite in wild trout populations is problematic. One promising avenue for managing trout populations in the presence of the parasite is the use of resistant rainbow trout (Price 1985; Schisler et al. 2006).

Intentionally and unintentionally, hatchery managers have increased disease resistance in fish stocks by the continued use of survivors of disease as brood stock (Herman 1970), and presumably this has occurred in strains of rainbow trout that are resistant to *M. cerebralis*. El-Matbouli et al. (2002) found that, under experimental laboratory conditions, a German strain of rainbow trout (GR) was at least as resistant to whirling disease as brown trout (*Salmo trutta*). Development of this resistance is presumed to be a result of growth and reproduction of the GR strain under continuous exposure to the parasite in Bavarian hatcheries (El-Matbouli et al. 2002). Hedrick et al. (2003) also found that the GR strain was more resistant to whirling disease than other North American rainbow trout strains in laboratory exposures.

Resistance to disease in animal species is a complex trait involving many genes (Grenfell and Dobson 1995) and results from a series of complex interacting variables among the host, pathogen and environment (Snieszko 1974; Hedrick 1998). The mechanisms for resistance to whirling disease seen in the GR strain, like those seen in trout resistant to a similar myxosporean, *Ceratomyxa shasta*, are suspected to be polygenic and at least partly additive (Hedrick et al. 2001). However, the genes involved in *M. cerebralis* resistance are relatively unknown. Severin and El-Matbouli (2007) found that the expression of transforming growth factor beta (TGF-beta) was more highly elevated in the non-susceptible strain of rainbow trout when exposed to the parasite. Baerwald et al. (2008) found that the response to infection seemed to be linked with the interferon system, specifically *Metallothionein B*, which was differently expressed in two strains of rainbow trout. However, it is likely that other genes are involved in resistance. Until the specific genetic and immune mechanisms of resistance are known, we must rely on other approaches to predict how resistance is conferred to subsequent generations, such as the development of disease resistant strains, and the use of cross-breeding and quantitative genetic techniques to genetically manipulate fish stocks (Price 1985).

We used a quantitative genetics approach (Becker 1992; Falconer and MacKay 1996; Conner and Hartl 2004) and estimated heritability to evaluate how resistance to *M. cerebralis* is acquired. Heritability of a character determines the degree of resemblance between relatives and includes environmental circumstances in which the individuals live and the way in which the phenotype is measured by the researcher. Heritability estimates are used as a guide to predict which individuals to breed and how the selected traits will change in subsequent generations (Falconer and MacKay 1996). Statistical and experimental techniques to estimate heritability are well known (Turner and Young 1969; Becker 1992; Falconer and MacKay 1996;

Conner and Hartl 2004), and are often used in selective breeding programs (Turner and Young 1969). Genetic correlations (Becker 1992; Falconer and MacKay 1996; Conner and Hartl 2004) between myxospore count, deformities and physiological characteristics important for survival, as well as the number of genes involved in myxospore formation (Lynch and Walsh 1998), were also estimated using the quantitative genetics approach. My overall goal was to determine which rainbow trout strain would be the best candidate for use as brood stock to reestablish rainbow trout populations in Colorado. My objective was to gain a better understanding of how resistant trout characteristics are inherited by subsequent generations and to see if traits of resistant strains of rainbow trout were inherited when spawned with rainbow trout strains showing little natural resistance.

METHODS

Brood Stock Development

In 2004, the Colorado Division of Wildlife, began a selective breeding program using the GR strain and the Colorado River rainbow (CRR) strain, a wild rainbow trout strain that had been historically used to establish rainbow trout populations in Colorado (Schisler et al. 2006). Wood and Schisler (2005) crossed resistant GR fish with susceptible CRR fish to incorporate whirling disease resistance from the GR strain into a strain that retained many of the desired "wild rainbow trout" characteristics (Schisler et al. 2006). Crossing the GR and CRR strains was done due to concerns that the GR strain was highly domesticated and may have lost the ability to survive and reproduce in the wild (Schisler et al. 2006). Besides being domesticated, the GR strain is inbred, exhibiting low levels of heterozygosity (El-Matbouli et al. 2006). Therefore, genetically, the GR may lack the genetic diversity necessary for survival and adaptation in natural conditions. Brood stock developed during these studies were used to create the strains that we evaluated.

Strain Development

The five strains of rainbow trout used in these experiments were spawned at the Colorado Division of Wildlife Bellevue Fish Research Hatchery (BFRH) from mid-November 2006 through the end of January 2007. Each family of each strain consisted of a unique male-female pairing and offspring were kept separate throughout the rearing process. The strains tested were designated GR, CRR, F1, F2, and B2. GR and CRR were pure families of German rainbow (GR) or Colorado River rainbow (CRR) trout. F1 families consisted of crosses of GR with CRR (Figure 2.1). F2 families consisted of crosses of F1 individuals (Figure 2.1). B2 families consisted of F1 individuals backcrossed with CRR individuals (Figure 2.1). GR and F1 brood stock were held at the BFRH. CRR brood stock was held at the Colorado Division of Wildlife Glenwood Springs Hatchery (GWSH). CRR males were spawned at the GWSH and their milt was transported in individual, numbered containers back to the BFRH for spawning. Live male and female CRR were also transported to BFRH for spawning (Figure 2.1). Reciprocal F1 and B2 families were created in the same manner (Figure 2.1).

F1 individuals were tagged with Passive Integrated Transponder (PIT) tags prior to spawning, and identified by their 10 digit alpha-numeric code. GR and CRR individuals were not tagged and were numbered in the order that they were spawned for fin clip and parental identification. An anal fin clip was taken from each spawned individual for genetic analysis. Eggs were placed in incubators at the BFRH or Quonset hut and held until they were eyed. Once eyed, eggs were placed in 20 gallon (76 liter) flow through (0.5 L/min) tanks, supplied by a combination of well water and charcoal-filtered city water, at the Colorado Cooperative Fish and Wildlife Research Unit Quonset hut wet lab where they were hatched.

Exposure Metrics

One hundred tanks were used in the exposure metrics experiment, each containing one family per tank (Figure 2.2). Eighty families were infected with whirling disease and were composed of ten GR families, ten CRR families, 20 F1 families, 20 F2 families, and 20 B2 families (Figure 2.2). The 20 F1 families were composed of ten CRR male by GR female families, and reciprocally, ten GR male by CRR female families (Figure 2.2). The 20 B2 families were composed of ten CRR male by F1 female families, and reciprocally, ten F1 male by CRR female families (Figure 2.2). Reciprocal families were included to test whether there was a difference in performance, based on directionality of spawning, when exposed to whirling disease. Because reciprocal families cannot be created in the F2 strain, 20 F2 families were used to have an equal number of families in each of the generational strains. Due to restriction on the number of brood stock available for spawning, only 80 total families could be created. Ten infected families per strain were needed to have a large enough sample size to calculate heritability, and space constraints limited the number of tanks that could be accommodated. Therefore, prior to infection, four families from each strain were split and used as uninfected families, for a total of 20 uninfected families.

Uninfected families were placed together on the top shelf of a two-tier shelving unit to avoid potential contamination due to overflow and spills from infected tanks. Otherwise infected and uninfected families from the five strains were randomly assigned to tanks using a random number generator.

Each tank contained 25 fish at exposure, and the fish were infected at an average of 678 (±44) degree-days (ºC) post-hatch. TAMs were supplied by Dr. Ron Hedrick's lab at U.C. Davis and R. Barry Nehring with the Colorado Division of Wildlife in Montrose, Colorado. Cultures of TAMs in both cases were produced from Mt. Whitney *T. tubifex* worms. TAMs were counted by mixing 1,000 µl of filtrate containing the TAMs and 60 µl of crystal violet; 84.6 µl of this mixture was then placed on a slide and the number of TAMs per slide was counted. Ten counts were conducted in this fashion to account for a possible uneven distribution of the TAMs within the filtrate. An average of the ten counts was taken, and this number was used to calculate TAMs per ml. Fish were infected with 2,000 TAMs per individual, a total of 50,000 TAMs per tank.

Prior to the addition of TAMs, water flow to each aquarium was stopped for one hour and each aquarium received aeration to ensure mixing of the TAMs and equal exposure of all fish. The amount of filtrate needed to deliver 2,000 TAMs per

fish was placed in each aquarium in two doses, each dose containing half of the necessary filtrate. Using two doses helped ensure equal distribution of TAMs in the tank and accounted for a possible unequal distribution of TAMs within the filtrate. Twenty tanks, four tanks of each of the five strains, were not infected with whirling disease, but were treated in the same manner as the infected tanks. After infection, fish were reared for approximately six months to ensure the full development of myxospores. During this time, developing signs of disease and mortalities were recorded daily. Over the course of the exposure experiment, the effects of whirling disease exposure on growth and swimming performance were also evaluated (Chapter 3).

Exposure evaluations began when the fish reached approximately 2,240 (±38) degree-days (ºC) post-exposure. The first individuals were sacrificed for exposure evaluation on August 8th, 2007, and the evaluations concluded on October 24th, 2007. At the time of evaluation, 15 individuals from each tank were removed and sacrificed. Ten individuals were used for myxospore enumeration (O'Grodnick 1975) using the pepsin-trypsin digest (PTD) method (Markiw and Wolf 1974), and five individuals were kept for histological analysis if necessary (Humason 1979; Hedrick et al. 1999b; Baldwin et al. 2000). The heads were severed from the body just behind the operculum and pectoral fins, and were placed into individually labeled bags. The bodies were also placed into similarly labeled bags to be used for later protein and lipid analyses (Chapter 3). Heads to be used for myxospore enumeration were sent to the Colorado Division of Wildlife Brush Fish Health Laboratory in Brush, Colorado. Heads to be used for histological analysis were placed in 50 ml tubes containing 10 percent neutral buffered formalin and kept on-site; however, histological analysis was not conducted because PTD tests were effective in determining the severity of infection.

Lengths (cm), weights (g), and deformities were recorded for each individual at the time of evaluation. Deformities were recorded as 0 if absent and 1 if present, and included cranial, spinal, lower jaw and opercular deformities, exopthalmia, and blacktail. Cranial deformities were categorized by sunken facial features and indentations in the cranium. Spinal deformities were categorized by unusual bends or curves of the spine. Lower jaw deformities were categorized by shortened lower jaws, or lower jaws that were extended to one side or the other. Opercular deformities were categorized by the operculum being indented or pulled back exposing the gills. Exopthalmia was categorized by the eyes being inflated in their sockets, extending past the orbitals. Blacktail was categorized by the posterior quarter of the fish turning black and was identified prior to sacrificing individuals because it disappears upon death and a loss of circulation. Fish that remained in each tank after the exposure evaluations were concluded were kept alive for predator avoidance experiments conducted in spring 2008 (Chapter 3).

Percent mortalities for each of the strains was calculated using the equation,

$$m = 1 - \frac{S}{N}$$

where m is the percent mortality experienced by a strain, S is the number of fish surviving at the conclusion of the exposure metrics part of the experiment in a given strain, and N is the number of fish starting on the day of exposure in a given strain.

Mortality was unusually high in one of the uninfected GR families due to feeding problems, so this family was removed prior to analysis. Total percent deformities was calculated for each strain by adding up the number of individuals showing any clinical sign of disease, and dividing this by the total number of individuals in a strain. The percentage of fish showing a given deformity within a strain was calculated by adding up the number of individuals showing that deformity, and dividing it by the number of individuals showing any kind of deformity.

Statistical Analyses

Myxospore counts were analyzed using a two-factor ANOVA, with exposure and strain as the factors, in SAS Proc GLM (SAS Institute, Inc. 2007-2008). Type III sum of squares were used to account for the unbalanced design of the experiment. If significant effects were identified, the least squares means method, adjusted using a Bonferroni adjustment, was used to identify the strains that had significantly higher mean myxospore counts. The influences of myxospore counts on final weight were also evaluated using regression.

Mortality and deformity percentages were transformed prior to analysis using an arcsine-square root transformation. Transformed percentages were then analyzed using a two-factor ANOVA in SAS Proc GLM (SAS Institute, Inc. 2007-2008). Values were reported from the Type III sum of squares to account for the unbalanced design of the experiment. If significant effects were identified, the least squares means method, adjusted using a Bonferroni adjustment, was used to identify the strains with significantly higher mortalities or deformities.

Quantitative Genetics Analyses

Heritability

Heritability of myxospore count as a result of exposure to *M. cerebralis* was evaluated using a random single pair mating design (Becker 1992). The development of all the strains from pairs of individuals resulted in unique families containing full sibling offspring for each strain. Heritability was estimated using the covariance in traits for full sibling (FS) families,

$$COV_{FS} = \frac{1}{2}V_A + \frac{1}{4}V_D + V_{EC.}$$

where V_A represents the additive variance, V_D represents the dominance variance, and V_{EC} represents the common environmental variance (Becker 1992; Falconer and MacKay 1996; Conner and Hartl 2004). For our experiments, common environmental variance is a result of raising full sibling families in a single tank, with all siblings sharing the same environment. However, environmental variance can be considered small because environmental conditions, including temperature, water quality, and feed amount were kept constant throughout the experiment. Therefore, our estimates of heritability include both additive and dominance variance components and are an estimation of heritability in the broad sense (Lush 1940). Broad sense heritability measures the extent to which phenotypic variation is determined by genotypic variation (Conner and Hartl 2004).

Heritability of myxospore count was estimated using the equation,

$$H^2 = \frac{2\sigma_S^2}{\sigma_S^2 + \sigma_W^2}$$

where the variance components σ^2_w and σ^2_S were calculated as $\sigma^2_w = MS_W$, and $\sigma^2_S = (MS_S - MS_W)/k$. MS_S is the mean squared error of myxospore counts associated with variance among families, MS_W is the mean squared error of myxospore counts associated with variance among progeny within a family, and k is the number of individuals represented from each family (Becker 1992). In our case k=10. Both MS_S and MS_W were estimated using ANOVA (Table 2.1a). The standard error and 95 percent confidence intervals for the heritability estimates were calculated using the equations in Becker (1992). Myxospore count was log transformed prior to analysis.

Heritability estimates for weight, length and swimming ability (Chapter 3) were calculated using the single pair mating design method described above (Becker 1992).

Deformity data were categorized by a zero if the deformity was absent and a one if the deformity was present, and traits categorized in this manner are known as threshold traits (Roff 2001). Threshold traits are believed to have an underlying continuous genetic variation that controls for the expression of the trait, where above a certain threshold, the trait will be expressed, and below the threshold, the trait will not be expressed (Roff 2001; Voordouw and Anholt 2002). Raw heritability estimates for deformity were calculated using the single pair mating design method describe above (Becker 1992), and then were transformed using the threshold model to get the estimates of heritability on the underlying continuous scale (Roff 2001), using the equation,

$$H^2 = H^2_{0,1} \frac{p(1-p)}{z^2}$$

where $H^2_{0,1}$ is the heritability estimate of the dichotomous distribution of the deformity, *p* is the mean proportion per family displaying that deformity (number of ones divided by the total number of observations), and *z* is the ordinate on the standard normal curve that corresponds to a probability *p*.

Correlations between Myxospore Count and Deformity/Physiological Characteristic

The variance components (described above) for myxospore count ($\sigma^2_{w(X)}$ and $\sigma^2_{S(X)}$) and other phenotypic traits ($\sigma^2_{w(Y)}$ and $\sigma^2_{S(Y)}$) were obtained from ANOVA and used to estimate genetic correlations. In addition, ANCOVA (SAS Institute, Inc. 2007-2008) was used to obtain the covariance components between traits (cov_w and cov_s) to estimate genetic correlations, where $cov_w = MCP_w$ and $cov_s = (MCP_s - MCP_w)/k$. MCP_S is the mean squared error of the cross products associated with variance among families and MCP_W is the mean squared error of the cross products associated with variance among progeny within a family (Becker 1992; Table 2.1b).

Three correlations can be estimated from the data: genetic, environmental and phenotypic. Genetic correlations estimate the degree to which two traits are affected by the same genes or pairs of genes, or in other words, the amount to which the two traits covary genetically (Conner and Hartl 2004). Genetic correlations were estimated using the equation (Becker 1992),

$$r_G = \frac{2\,\mathrm{cov}_s}{\sqrt{2\sigma^2_{s(X)}\,2\sigma^2_{s(Y)}}}$$

Environmental correlations estimate the degree to which two traits respond to variation in the same environmental factors (Conner and Hartl 2004). Environmental correlations were estimated using the equation (Becker 1992),

$$r_E = \frac{\mathrm{cov}_w - \mathrm{cov}_s}{\sqrt{(\sigma^2_{w(X)} - \sigma^2_{s(X)})(\sigma^2_{w(Y)} - \sigma^2_{s(Y)})}}$$

Phenotypic correlations estimate the degree to which the expression of two traits covary (Conner and Hartl 2004). Phenotypic correlations were estimated using the equation (Becker 1992),

$$r_P = \frac{\mathrm{cov}_w + \mathrm{cov}_s}{\sqrt{(\sigma^2_{w(X)} + \sigma^2_{s(X)})(\sigma^2_{w(Y)} + \sigma^2_{s(Y)})}}$$

The standard errors for the genetic, environmental and phenotypic correlation estimates were calculated using the equation in Becker (1992).

Number of Independently Segregating Genes

A line-cross analysis was used to calculate the effective number of factors (n_e) by which the resistance characteristics in the GR and CRR strains differed. The quantity n_e is equivalent to the number of freely segregating loci with equal effects that would yield the observed pattern in the two genetic lines, and assumes independent assortment It explains whether phenotypic variation is caused by a large number of genes with relatively small effects or a few major genes with large effects (Lynch and Walsh 1998). It is also an important determinant in artificial selection programs of whether a search for informative markers is likely to be successful (Lynch and Walsh 1998). Low values of n_e would suggest that genes responsible for resistance are contained on relatively few chromosomes and higher values suggest that resistance is spread over several or all chromosomes.

The line-cross analysis consists of several steps. First, composite effects were calculated using the log transformed myxospore counts. Composite effects evaluate additive, dominance, and, epistatic interactions among genes of the parental strains (Lynch and Walsh 1998). Secondly, a joint-scaling test was used to determine if an additive or additive-dominance model best fit the data (Lynch and Walsh 1998). The additive model assumes that all genetic effects are additive within and between loci, where the F1 and F2 lines exhibit median phenotypic expressions between the two parental lines, and the backcrosses exhibit median phenotypic expressions between the F1 and parental line. The additive-dominance model assumes that some genetic effects are the result of dominance in one parent. Dominance results in phenotypes that are more similar to the dominant parent. Thirdly, a likelihood-ratio test statistic was used to determine if dominance (from the additive-dominance model) accounted for a significant proportion of variance in the strain means (Lynch and Walsh 1998). Finally, the variance terms calculated from the composite effects were used to estimate n_e using the Castle-Wright estimator,

$$n_e = \frac{[z(P_1) - z(P_2)]^2 - Var[z(P_1)] - Var[z(P_2)]}{8Var(S)}$$

where $z(P_i)$ are the observed means, $Var[z(P_i)]$ are the sampling variances of the means for the *i*th parental line, and $Var(S)$ is the segregational variance estimate

(Lynch and Walsh 1998). Standard error for the estimate of n_e from the Castle-Wright estimator was calculated using the methods described in Lynch and Walsh (1998). The assumption of independent assortment was tested using haploid chromosome number (Hartley and Horne 1982; Ocalewicz et al. 2004; Thorgaard 1983) and genetic linkage maps (Nichols et al. 2003; Sakamoto et al. 2000; Young et al. 1998) for rainbow trout to estimate c_{bar}, or linkage distance. The maximum value of 0.5 for c_{bar} indicates independent assortment, with lower values indicating linkage among genes on a chromosome (Lynch and Walsh 1998).

RESULTS

Exposure Metrics

There were significant differences in myxospore count among strains ($P < 0.0001$; Table 2.2). CRR individuals had a significantly higher mean myxospore count, than any of the other strains ($P < 0.0001$). B2 individuals had a significantly higher mean myxospore count, than the F2, F1 or GR strains, but were significantly lower than the CRR strain ($P \leq 0.0133$). The F2, F1 and GR strains did not differ significantly from each other in mean myxospore count ($P \geq 0.7235$) but all had significantly lower mean myxospore counts than the CRR or B2 strains ($P \leq 0.0133$; Table 2.2). Uninfected families did not show any myxospores.

The GR strain showed the lowest range of variability, ranging from 0 to 1,177 mean myxospores per family. The F1 strain showed slightly higher variation, ranging from 0 to 51,418 mean myxospores per family. Variation doubled between the F1 and F2 strains, with the F2 strain ranging from 0 to 135,064 mean myxospores per family. The largest variation in mean myxospore count was seen in the B2 and CRR strains, with the B2 strain ranging from 0 to 338,128 mean myxospores per family, and the CRR strain ranging from 15,090 to 350,423 mean myxospores per family (Figure 2.3; Figure 2.4).

Significant differences were seen among myxospore counts of the families of the F1, F2, B2, and CRR strains ($P \leq 0.0002$). There was no significant difference in myxospore counts of the families in the GR strain ($P = 0.1831$), indicating that resistance to whirling disease is constant throughout the individuals within this strain. No significant differences were seen between the myxospore counts in the reciprocal families of the F1 ($P = 0.1169$) or B2 ($P = 0.2331$) strains, indicating directionality of spawning does not have an effect on resistance to whirling disease.

There were significant differences in average final wet weight per individual between the exposures ($P = 0.0006$) and among the strains ($P < 0.0001$), however, there was not a significant interaction between exposure and strain ($P = 0.2326$). Within the F1, F2, B2 and CRR strains, there was no significant difference in average weight per individual between the infected and uninfected families ($P \geq 0.6389$). In the GR strain, the individuals in uninfected families weighed significantly more than did the individuals in infected families ($P = 0.0277$). In addition, individuals in the GR strain weighed significantly more than all of the other strains ($P < 0.0001$). Individuals in the CRR strain weighed significantly less than all of the other strains ($P \leq 0.0114$). Individuals in the F1 and F2 strains weighed significantly more than individuals in the B2 strain ($P \leq 0.0196$; Figure 2.5).

There was not a significant correlation between myxospore count and weight in the GR (R^2 = 0.0074, P = 0.3943) or F1 strain (R^2 = 0.0023, P = 0.4968). There was a significant negative correlation between myxospore count and weight in the F2 (R^2 = 0.0549, P = 0.0009), B2 (R^2 = 0.1198, P < 0.0001) and CRR strains (R^2 = 0.0356, P = 0.0615; Figure 2.6).

There were no significant differences in mortality between the exposures (P = 0.1198), among the strains (P = 0.2032), or a significant interaction between exposure and strain (P = 0.2545; Figure 2.7).

There were significant differences in percent deformities between the exposures (P < 0.0001), among the strains (P < 0.0001), and a significant interaction between exposure and strain (P = 0.0002). The F2 strain exhibited significantly fewer deformities than the GR, B2 or CRR strains (P ≤ 0.0081). There was a significant difference in percent deformities between infected and uninfected families of the F2, B2 and CRR strains (P ≤ 0.0032). There was not a significant difference in percent deformities between the infected and uninfected families of the GR or F1 strain (P ≥ 0.2141; Figure 2.8).

Cranial deformities were the most common. There was not a significant difference in cranial deformities between exposures (P = 0.1207), however, there were significant differences in the percent of individuals with cranial deformities among the strains (P < 0.0001), and a significant interaction between exposure and strain (P < 0.0001). The CRR strain exhibited significantly lower cranial deformities than all of the other strains (P ≤ 0.0005). The other four strains did not differ significantly in the number of cranial deformities exhibited (P ≥ 0.0870; Figure 2.9).

Spinal and opercular deformities were also common. There was not a significant differences in the percent of spinal deformities among the strains (P = 0.2644), or between the exposures (P = 0.1400), however, there was a significant interaction between exposure and strain (P = 0.0798; Figure 2.9). There were significant differences in the percent of opercular deformities between the exposures (P < 0.0001), however, there was not a significant difference among the strains (P = 0.1971) or a significant interaction between exposure and strain (P = 0.5633; Figure 2.9).

Other deformities were less common. Exopthalmia occurred in 5.5 percent of the individuals and was significantly different between the exposures (P = 0.0015), but was not significantly different among the strains (P = 0.4504), and there was not a significant interaction between exposure and strain (P = 0.2794; Figure 2.9). Lower jaw deformities were seen in 13 percent of the individuals and were not significantly different between exposures (P = 0.1100), among the strains (P = 0.4448), and there was not a significant interaction between exposure and strain (P = 0.3269, Figure 2.9). Blacktail was seen only in infected individuals in the CRR, F2 and B2 strains. It was significantly different both among strains (P = 0.0472) and between exposures (P = 0.0018), and there was a significant interaction between exposure and strain (P = 0.0472). The CRR strain experienced a significantly higher occurrence of blacktail than did the F2 strain (P = 0.0166). The B2 strain did not exhibit a significantly higher or lower occurrence of blacktail than either the CRR or F2 strains (P ≥ 0.1235; Figure 2.9).

Quantitative Genetics
Heritability

Variation in myxospore count, both within and between families of the strains, indicated that heritability was estimable for all of the strains. Expectations, based on the variance in myxospore count and response to disease, in terms of average myxospore count, for each of the strains were developed based on the predictions of the additive genetic model (Lynch and Walsh 1998). The GR strain was expected to have a low variation in myxospore count, and a low response to the disease, because the genes involved in resistance to whirling disease should be approaching fixation in this strain. The CRR strain was expected to have a low variation in myxospore count, and a high response to the disease, because the development of resistance genes should not have occurred yet for this strain; each individual in this strain was expected to be equally susceptible to the disease. The F1 strain was expected to have a low variation in myxospore count, and an intermediate response to the disease between the GR and CRR strains, because the individuals in this strain should have obtained half of their genes from the GR strain, and the other half from the CRR strain. The F2 strain was expected to have a similar response to the disease as the F1 strain, but the highest variation in myxospore count of all of the strains due to the differences in segregation and recombination of the parental genes in the individuals of this strain. Finally, the B2 strain was expected to have an intermediate variation in myxospore count to the F2 and CRR strains, and an intermediate response to the disease between the F1 and CRR strains, due to the differences in segregation and recombination of genes in the individuals of this strain as a result of the backcrossing between the F1 and CRR strains. The F1 and F2 strains deviated from these expectations, with the F1 strain having a slightly higher variation in myxsopore count and lower response to the disease than expected, and the F2 strain exhibiting a lower variation in myxospore count than expected and differing from the F1 strain in their response to the disease (Figure 2.10).

No significant differences were seen between the myxospore counts in the reciprocal families of the F1 ($P = 0.1169$) or B2 ($P = 0.2331$) strains, indicating directionality of spawning does not have an effect on resistance to whirling disease. Therefore, all 20 families of the F1 and B2 strains were used in heritability calculations.

The F2 strain had a broad sense heritability estimate for myxospore count as a result of exposure to whirling disease of 0.34 ± 0.21; the F1 and GR strains were similarly low in their heritability estimates for myxospore count with estimates of 0.42 ± 0.23 and 0.34 ± 0.21, respectively. The B2 strain had a higher broad sense heritability estimate than the F2 strain, with an estimate of 0.93 ± 0.28. Interestingly, the CRR strain had a higher broad sense heritability estimate than expected at 0.89 ± 0.28 (Table 2.3).

Heritability and correlations are discussed in terms of the F2 and B2 strains, unless otherwise stated, because the furthest filial strain from the parental strains, as well as the backcross, provides the most information on factors such as dominance, epistasis, correlations and number of genes controlling trait expression (Hercus and Hoffmann 1999). Broad sense heritability estimates of deformities, corrected for heritability on the continuous underlying scale, were close to zero for most of the

deformities measured (0.001 – 0.68). Heritability estimates of overall and spinal deformities were low (0.02-0.49), and were significantly different from zero in both the F2 and B2 strains. Heritability of lower jaw deformities and blacktail were low (0.03) and significantly different from zero in the F2 strain. Heritability of exopthalmia was very close to zero (0.006) but significantly different from zero in the B2 strain (Table 2.4).

Broad sense heritability estimates of physiological characteristics, especially weight and length, were higher than those estimates for heritability of deformity (0.10-0.57). In the F2 strain, heritability estimates of both weight and length were significantly different from zero (0.48 and 0.57, respectively), however, were lower and not significantly different from zero in the B2 strain. Estimates of heritability of swimming ability were also higher and significantly different from zero in the B2 strain (0.17-0.45); estimates of heritability of swimming ability were inestimable for the F2 strain (Table 2.5).

Correlations between Myxospore Count and Deformity/Physiological Characteristic

Genetic correlations between myxospore count and deformities were rarely significantly different from zero (Table 2.6). Interestingly, genetic correlations between myxospore count and exopthalmia , and myxospore count and opercular deformities, were higher and significantly different from zero in the GR strain. Genetic correlations between myxospore count and physiological characteristics were rarely significantly different from zero. The only significant genetic correlation with a physiological trait was between myxospore count and swimming performance in CRR, and the correlation was negative (Table 2.6)

Environmental correlations between myxospore count and deformity were higher than the genetic correlations, and often significantly different from zero, in the F2 and B2 strains. The environmental correlation between myxospore count and weight was also higher than the genetic correlation, and significantly different from zero in the F2 and B2 strains; however, the environmental correlations between myxospore count and length, and myxospore count and swimming ability were low and not significantly different from zero (Table 2.6).

Phenotypic correlations between myxospore count and deformity were similarly higher than the genetic correlations, and often significantly different from zero, in the F2 and B2 strains. The phenotypic correlation between myxospore count and weight was also higher than the genetic correlation, and significantly different from zero in the F2 and B2 strains; however, the phenotypic correlations between myxospore count and length, and myxospore count and swimming ability were low and not significantly different from zero (Table 2.6).

Number of Independently Segregating Genes

The effective number of factors (n_e) by which the GR and CRR strains differ in relation to myxospore development is 9 ± 5. The test statistic for the likelihood-ratio test between the additive and additive-dominance model was not significant (P = 0.0836), indicating that the model of best fit for the data was the additive model. The

estimate of c_{bar}, providing an estimate of linkage distance, was 0.48 (0.50 is considered independent segregation).

DISCUSSION

Heritability is calculated based on the variance between full or half sibling families, or between parents and offspring, of a quantitatively measurable trait (Falconer and MacKay 1996; Conner and Hartl 2004). In fish disease studies, the number of mortalities that occur after exposure to a particular pathogen is often the only measurable response (Price 1985), and is therefore, the only available characteristic with which to measure and discuss resistance to disease. However, with whirling disease, exposure to *M. cerebralis* causes the development of myxospores within infected individuals, a response that is quantitatively measurable within every exposed individual, making it not only the best and easiest measure for use in calculating heritability, but also to measure and discuss resistance to *M. cerebralis*. Because the genetic mechanisms of both susceptibility and resistance to *M. cerebralis* are unknown, we referred to the heritability estimates as heritability of myxospore count, since this was the characteristic being directly measured and estimated. However, myxospore development, or the lack thereof, does correspond to varying levels of susceptibility or resistance in the strains, and therefore, it is relevant to discuss the heritability estimates in relation to both.

Heritability of myxospore count as a result of exposure to *M. cerebralis* was low in the GR strain, which was expected, because they have displayed low variability in myxospore count in other studies (El-Matbouli et al. 2002; Hedrick et al. 2003; Schisler et al. 2006; Schisler et al. 2007). Heritability was also low, and about equal, in the F1 and F2 strains. This was unexpected because the F1 strain was expected to show very little between family variation, and therefore, a low heritability of myxospore count, whereas the F2 strain was expected to show more beteen family variation, and therefore and estimable heritability of myxospore count, because of the recombination of genes from the two parental strains. The B2 strain showed the highest heritability of the intermediate strains, and was the furthest removed from the GR strain, containing on average only 25 percent GR genes. A high heritability in the CRR strain was unexpected, because it was assumed that no development of the resistance genes has occurred in this strain, leading to very little between family variation in myxospore count. However the CRR strain has shown a high variability in myxospore count in other laboratory experiments (Schisler et al. 2006; Schisler et al. 2007). The higher than expected heritability estimates for the CRR strain indicate that this strain of rainbow trout may have some innate resistance to the parasite, or has developed resistance over the last 20 years of exposure.

When disease resistance involves many genes the simplest and most practical approach is to investigate variation between different strains of the same species or between different related species (Price 1985). Challenge tests and selective breeding experiments, similar to this one, used to study the effects of a similar myxosporean, *Ceratomyxa shasta*, on susceptible and resistant rainbow trout strains, indicated that resistance to *C. shasta* is a heritable trait (Hemmingsen et al. 1986; Ibarra et al. 1992a; Ibarra et al. 1992b). In general, similar to the results seen in this experiment,

crosses between resistant and susceptible strains of salmonids yield progeny with intermediate resistance to the parasite (Hedrick et al. 2001). Resistance in the hybrids may be exhibited through hybrid vigor, or heterosis, for resistance, or the resistance may be dominant in the hybrid (Price 1985). In addition, heritabilities have been estimated for the resistance of Atlantic salmon to the salmon louse using a similar selective breeding program (Kolstad et al. 2005).

Development of a *M. cerebralis* resistant rainbow trout strain is still being evaluated; however, other selective breeding programs have been successful using similar techniques. A selective breeding program was developed to get Arctic char to grow larger and faster in frigid spawning and rearing conditions in the Yukon, Canada (Mcgowan et al. 2005). Common carp have been selectively bred for a number of characteristics including shape and disease resistance (Vandeputte 2003). In salmonids, selective breeding programs have been used to increase the resistance to salmon louse in Atlantic salmon (Kolstad et al. 2005), and increase the resistance to furunculosis in brook trout (Cipriano 2002).

Heritability estimates for both weight and length were high for all strains except the B2 strain, indicating that there is a high selection potential for growth in these strains. Physiological traits, such as growth, often possess heritability estimates intermediate between life history traits, which tend to have lower heritabilities, and morphological traits, which tend to have higher heritabilities (Mousseau and Roff 1987). In addition, phenotypic variability in weight and length in fish, which are indeterminate growers, is at least in part by the action of many minor genes which individually have small effects, the expression of which can be affected by the environmental circumstances, including temperature, food availability and nutrition, in which the individual is living (Nakjima and Fujio 1993).

Weight was negatively correlated with myxospore counts in the F2, B2 and CRR strains and wasn't correlated in the GR or F1 strains. The pattern of correlations between myxospore count and growth suggests that growth is related to susceptibility to the parasite. Susceptibility is higher in strains that share fewer genes with the parental GR strain or have no GR genes at all. Reduced growth in more heavily exposed individuals has been expressed in channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*), and their hybrids when exposed to enteric septicemia of catfish (Bilodeau-Bourgeois et al. 2008) and Atlantic salmon when exposed to infectious pancreatic necrosis virus (Damsgård et al. 1998). Exposure to disease elicits a high level of stress on fish, which can affect several biochemical and physiological processes resulting in reduced growth in highly infected individuals (Wedemeyer 1970).

Both growth and resistance are important traits when considering reestablishing rainbow trout populations and selecting brood stock. The low heritability of myxospore count coupled with the growth potential of the GR strain indicate that GR strain would be a good candidate for use in managing wild rainbow trout populations that are exposed to whirling disease. The high growth potential of the GR strain may improve survival and would be attractive to anglers. However, the growth potential of the GR is probably a result of artificial selection in a hatchery environment and this may have negative consequences for survival and reproduction in the wild (Schisler et al. 2006). We suggest that further research in a natural setting

is needed to assess the performance of these fish and their potential for establishment of rainbow trout fisheries.

Genetic correlations estimate the degree to which two traits are affected by the same genes or pairs of genes, or in other words, the amount to which the two traits covary genetically (Conner and Hartl 2004). Low genetic correlations between myxospore count and the growth characteristics suggest that it is possible to improve both the growth characteristics and resistance to *M. cerebralis* simultaneously in rainbow trout using a selective breeding program, because variation of one trait does not affect the other. In a similar study, genetic correlations between body weight and number of lice in Atlantic salmon, suggest that that it is possible to improve both body weight and resistance to salmon louse through selection (Kolstad et al. 2005). In addition, intentional selection for both growth and disease resistance is being used to improve domestic catfish stocks (Peterson et al. 2008).

Little is known about the genetic mechanisms of resistance to whirling disease, though studies have found some evidence that the interferon system is involved (Severin and El-Matbouli 2007; Baerwald et al. 2008). The genes involved are largely unknown and it is also unknown if their interaction is polygenic or additive, or if alleles have dominant or recessive characteristics. The line-cross analysis used in this study does offer more insight into these questions. Based on the results of the joint-scaling test, the likelihood-ratio test statistic between the additive and additive-dominance models was not significant, indicating that the additive model was the best fit to the data, and therefore, that dominance does not account for a significant proportion of the variation in the line means (Lynch and Walsh 1998). The number of loci involved in resistance to myxospore development estimated using the line-cross analysis was 9, and provides the first estimate of the number of loci involved in resistance to whirling disease by the GR strain. The number of freely segregating loci cannot exceed the number of independently segregating chromosomal segments (104; Hartley and Horne 1982; Ocalewicz et al. 2004; Thorgaard 1983), or two to three times the haploid chromosome number (29; Hartley and Horne 1982; Ocalewicz et al. 2004; Thorgaard 1983). Since our estimate (9) is far lower than either of these cutoffs, it meets the assumption of additive gene action, as well as the assumption that the loci are unlinked (Lynch and Walsh 1998). Our estimate of c_{bar} (0.48) confirms that independent segregation does occur, since independent segregation occurs at a value of 0.5 (Falconer and MacKay 1996). Therefore, our estimate of the number of loci should be fairly accurate. In addition, since the number of loci involved was low, it is reasonable to believe that searching for informative molecular markers should provide information on the exact location of the loci involved in resistance to whirling disease.

The cellular mechanisms of resistance are also relatively unknown; however, recent research has shown that replication of the parasite is reduced in the skin, muscle, and cartilage of the GR strain, and it is thought that the parasite is exposed to the immune system while in these tissues (Hedrick et al. 2008). However, quantitative measurements, such as heritability, allow researchers to predict what will occur in future generations based on the phenotypic expression of traits in the current generation, without knowledge of the genes involved (Falconer and MacKay 1996). The heritability estimate for the CRR strain is an interesting result that suggests

resistance may have always existed in this strain, or has developed from almost two decades of exposure to whirling disease in Colorado. It is interesting to note that the CRR strain does show more variability in myxospore count than expected after 20 years of exposure, indicating that genes controlling for reduced myxospore count may be beginning to evolve in at least a few of the CRR individuals. We suggest that heritability of myxospore count for the CRR strain be reevaluated in the future, using a paternal half-sib mating design, to determine if resistance is indeed increasing in this strain as it continues to be exposed to whirling disease.

The genetic mechanisms of resistance have been examined and identified for several pathogens in other fish species. Non-specific defense mechanisms linked to differences in disease resistance have been associated with the complement system, specifically complement haemolytic activity, in several fish species including rainbow trout (Røed et al. 1990), Atlantic salmon (Røed et al. 1992; Røed et al. 1993), and common carp (Weigertjes et al. 1993). Variations in the MHC class I and class II polymorphisms have been identified to explain the susceptibility to infectious salmon anemia virus and furunculosis in Atlantic salmon (Grimholt et al. 2003). Resistance to bacterial kidney disease in coho salmon (*Oncorhychus kisutch*) was found to be a result of different genotypes (Suzumoto et al. 1977). Price (1985) suggests that once a superior genotype has been identified for resistance, one could take measures to increase its frequency in the population; however, he cautions that extrapolation of the results from one environment to another should be done with caution because the genotype may not confer fitness in the same manor in different environments. Resistance is never absolute (Price 1985), and more research is needed to understand the complexities of resistance.

Myxospore count, mortality, and deformity data confirmed that the CRR strain is very susceptible to whirling disease. In addition, the more CRR genes a strain had, the lower the resistance and the higher the number of mortalities and deformities that strain showed when exposed to whirling disease. This is apparent in the CRR, F2 and B2 strains, the only strains to develop blacktail, a deformity that only appears in the most highly infected individuals within a strain. Conversely, the more GR genes a strain had the more resistance to whirling disease it showed. However, the GR, F1 and F2 strains did not differ from each other in mean myxospore count, though they did differ in the variability of myxospore counts among families within the strains. This is likely a result of the distribution of GR and CRR genes in each of these strains. Dominance in the resistant alleles of the GR strain may also be a factor, suggesting a positive effect, where even though only approximately half of the GR genes are represented in the F1 and F2 strains, both strains still show a large amount of resistance compared to the CRR strain.

The number of deformities was lower in the uninfected individuals than the infected individuals for F2, B2 and CRR strains, but not the F1 or GR strain, indicating that exposure to whirling disease caused deformities to develop within these strains. In addition, these results reinforce the increased resistance of the F1 and GR strains to whirling disease, indicating that exposure to the disease does not cause an increase in deformities that would not normally be expressed by these strains due to genetic or morphological differences between individuals.

The low heritability estimates for each of the deformities confirm this conclusion. Low heritability estimates for the deformities indicate that there is little genetic variation controlling for the formation of deformities. In addition, the low genetic correlation between myxospore count and deformity indicates that the two traits are not genetically related. However, the higher environmental correlations, which estimate the degree to which the two traits respond to variation in the same environmental factors (Conner and Hartl 2004), indicate that environment has a significant effect on the formation of deformities in conjunction with myxospore count, especially in the B2 strain. In addition, the higher phenotypic correlations, which estimate the degree to which the expression of the two traits covary (Conner and Hartl 2004), indicate that an increase in myxospore count results in the increased likelihood of the expression of a deformity.

The high incidence of cranial deformities in the GR, F1, F2 and B2 strains, are likely a result of the morphological differences in the head shape of the GR strain, as well as the fact that when feeding or surprised, fish often ran into the sides of the tank. Because of the high incidence of cranial deformities in both exposed and unexposed individuals in this experiment, cranial deformities were not considered a sign of disease; however, cranial deformities are considered a sign of disease when examining infected wild fish because exposure to whirling disease is one of the only factors that cause cranial deformities in wild fish populations.

The GR strain is known to be inbred, exhibiting low levels of heterozygosity (El-Matbouli, et al. 2006). Inbreeding depression in fish relates to an increase in deformities as well as a decrease in survival rate of young fish where the cause of death is unknown (Price 1985). Inbreeding may explain the morphological differences in head shape seen in the GR strain as well as account for the unusual mortality experienced in the uninfected GR family at the beginning of the experiment. In addition, there is a higher genetic correlation between myxospore count and exopthalmia and myxospore count and opercular deformities in the GR strain. These higher correlations indicate that some of the same genes controlling for lowered myxospore count in the GR strain are also controlling for the development of these other deformities, which may be a result of the selection of resistant individuals leading to inbreeding and higher incidences of genetically related deformities in this strain.

The lack of differences in mortality between the exposures and among the strains indicates that mortality was not necessarily attributable to whirling disease in any of the strains in this experiment. However, other experiments have shown that exposure to whirling disease does result in increased mortality in the CRR strain (Schisler et al. 2006).

Resistance is often measured by mortality over a given period of time following exposure to infection, an unfortunate necessity, since there may be no other way of measuring infection levels in fish (Price 1985). This is not the best method to use, if possible, since it may be impossible to exclude death from other causes (Kirpichnikov et al. 1979). Purdom (1974) suggests that a better response to selection could probably be achieved by selecting for some specific, measurable character, such as myxospore count, rather than focusing on the vague ideal of disease resistance identified by mortality.

The results of this experiment suggest that the GR strain is a good candidate for use as a brood stock to reestablish rainbow trout populations in Colorado. The low myxospore counts of this strain demonstrate its high resistance to the whirling disease parasite. A lower myxospore count in the fish host translates to lower infectivity in the drainages to which those individuals are introduced, reducing the overall myxospore load of the drainage. In addition, the low heritability of myxospore count estimates for this strain suggests that resistance to whirling disease may not be lost in subsequent generations, as it may be in the intermediate strains, because the genes controlling for lower myxospore counts are approaching fixation in this strain. Finally, the high growth potential of the GR strain may improve survival, as well as be an attractive quality to anglers.

Other studies have suggested that the F1 strain may be the best candidate for reintroducing rainbow trout to Colorado because it has the correct combination of resistant and wild rainbow trout characteristics that may be necessary for survival in the wild (Schisler et al. 2006; Schisler et al. 2007). The results of this experiment similarly suggest that the F1 strain may be a good candidate for this purpose. This strain also exhibited low myxospore counts, not differing from the GR strain. In addition, the heritability of myxospore count was low, and similar to the heritability estimates for the F2 strain, indicating that resistance to whirling disease is not lost in subsequent generations. The effect of whirling disease on the performance of important survival characteristics such as growth, swimming ability and predator avoidance on both strains needs further evaluation, as does the survival and reproductive characteristics of these strains in a natural setting.

Table 2.1a. Example of how results of the ANOVA test run on the infected families within a strain are displayed and the outputs used to calculate heritability of resistance.
S is the number of matings (10 for the GR and CRR strains, and 20 for the F1, F2 and B2 strains), n_i is the number of individuals within the i-th mating, k is the n_i expected in the mean squares, and n. is the total number of individuals (Becker 1992).

Source of Variation	d.f.	SS	MS	EMS
Between Matings	S – 1	SS_S	MS_S	$\sigma^2_W + k\sigma^2_S$
Between progeny, within matings	n. – S	SS_W	MS_W	σ^2_W

Table 2.1b. Example of how results of the ANCOVA test between two traits are displayed. The ANCOVA gives a measure of how much the two traits covary together and allow for an estimate of the genetic, environmental and phenotypic correlations between the two traits. S is the number of matings (10 for the GR and CRR strains, and 20 for the F1, F2 and B2 strains), n_i is the number of individuals within the i-th mating, k is the n_i expected in the mean squares, and n.. is the total number of individuals (Becker 1992).

Source of Variation	d.f.	SS	MS	EMS
Between Matings	S – 1	SS_S	MCP_S	$cov_W + kcov_S$
Between progeny, within matings	n.. – S	SS_W	MCP_W	cov_W

Table 2.2. Mean myxospore counts and confidence intervals, per strain, for the *M. cerebralis* exposure experiment.
*, ** = significantly higher average myxospore count

Strain	Myxospore Count	Confidence Interval
CRR** (N=10)	187,209	(171,222, 203,196)
B2* (N=20)	97,588	(83,402, 111,774)
F2 (N=20)	46,227	(40,621, 51,883)
F1 (N=20)	9,566	(7,603, 11,529)
GR (N=10)	275	(211, 339)

Table 2.3. Broad sense heritability estimates of myxospore count as a result of exposure to *M. cerebralis*, standard errors (as calculated using the formula from Becker (1992), representing 2 SE), and 95% confidence intervals (for ± 2 SE), for the five strains of rainbow used in the *M. cerebralis* exposure experiment.

Strain	**H^2 Myxospore Count**	**Standard Error**	**95% Confidence Interval**
GR	0.34	0.21	(0.13, 0.55)
F1	0.42	0.23	(0.19, 0.64)
F2	0.34	0.21	(0.13, 0.55)
B2	0.93	0.28	(0.66, 1.21)
CRR	0.89	0.28	(0.61, 1.17)

Table 2.4. Broad sense heritability estimates of deformities corrected for heritability on the continuous underlying scale, and standard errors (in parentheses), for the five strains of rainbow trout used in the *M. cerebralis* exposure experiment. A "------" indicates that the heritability for that deformity was inestimable for that strain. Significance is indicated by an "*".

Deformity	GR	F1	F2	B2	CRR
Overall	------	0.03 (0.02)*	0.02 (0.01)*	0.26 (0.13)*	------
Cranial	------	0.09 (0.05)*	0.03 (0.03)	------	------
Spinal	0.001 (0.003)	0.04 (0.03)*	0.49 (0.29)*	0.006 (0.003)*	0.01 (0.02)
Exopthalmia	0.01 (0.008)*	0.005 (0.003)*	0.002 (0.003)	0.006 (0.004)*	------
Lower Jaw	0.001 (0.001)	------	0.03 (0.01)*	0.68 (0.84)	0.01 (0.01)
Opercular	0.05 (0.03)*	0.05 (0.03)*	0.45 (0.29)*	0.01 (0.01)	0.06 (0.05)*
Blacktail	------	------	0.03 (0.02)*	------	------

Table 2.5. Broad sense heritability estimates of the physiological characteristics (Chapter 3), and standard errors (in parentheses), for the five strains of rainbow trout used in the *M. cerebralis* exposure experiment. A "------" indicates that the heritability for that physiological characteristic was inestimable for that strain. Significance is indicated by an "*".

Physiological Characteristic	**GR**	**F1**	**F2**	**B2**	**CRR**
Weight	0.44 (0.23)*	0.22 (0.18)*	0.48 (0.24)*	0.10 (0.14)	0.33 (0.21)*
Length	0.19 (0.17)*	0.21 (0.17)*	0.57 (0.25)*	0.12 (0.14)	0.29 (0.19)*
Swimming Ability	0.28 (0.19)*	0.03 (0.11)	------	0.45 (0.23)*	0.17 (0.16)*

Table 2.6. Genetic, environmental and phenotypic correlations between myxospore count and deformity or physiological characteristic, and standard errors (in parentheses), for the five strains of rainbow trout used in the *M. cerebralis* exposure experiment. A "------" indicates that the correlation for that deformity or physiological characteristic was inestimable for that strain. A "=====" indicates that there was no heritability for the trait within a given strain, and therefore, genetic correlations could not be estimated. Significance is indicated by an "*".

Deformity/ Characteristic	GR	F1	F2	B2	CRR
Overall					
Genetic	=====	0.01 (0.02)	-0.001 (0.01)	-0.0001 (0.007)	=====
Environ.	=====	0.23 (0.10)*	0.19 (0.11)*	0.68 (0.38)*	=====
Phenotypic	=====	0.14 (0.07)*	0.14 (0.08)*	0.15 (0.06)*	=====
Cranial					
Genetic	=====	0.02 (0.02)	0.002 (0.01)	0.003 (0.006)	=====
Environ.	=====	0.26 (0.11)*	0.20 (0.11)*	0.78 (0.42)*	=====
Phenotypic	=====	0.15 (0.06)*	0.15 (0.08)*	0.15 (0.06)*	=====
Spinal					
Genetic	-0.04 (0.06)	0.02 (0.03)	0.01 (0.01)	0.005 (0.006)	-0.007 (0.02)
Environ.	0.34 (0.12)*	0.23 (0.10)*	0.20 (0.11)*	0.72 (0.41)*	------
Phenotypic	0.26 (0.10)*	0.16 (0.07)*	0.14 (0.08)*	0.13 (0.06)*	0.45 (0.13)*
Exopthalmia					
Genetic	0.19 (0.13)*	0.01 (0.02)	0.01 (0.02)	0.002 (0.006)	=====
Environ.	0.32 (0.15)*	0.12 (0.10)*	0.08 (0.11)	0.30 (0.37)	=====
Phenotypic	0.27 (0.10)*	0.08 (0.07)*	0.07 (0.09)	0.05 (0.06)	=====
Lower Jaw					
Genetic	0.14 (0.18)	=====	0.006 (0.005)*	0.001 (0.007)	-0.001 (0.01)
Environ.	0.19 (0.14)*	=====	0.12 (0.13)	0.37 (0.33)*	------
Phenotypic	0.18 (0.10)*	=====	0.07 (0.07)	0.08 (0.07)	0.34 (0.13)*
Opercular					
Genetic	0.18 (0.13)*	0.03 (0.02)	0.01 (0.009)	0.01 (0.009)	0.01 (0.01)
Environ.	0.47 (0.13)*	0.20 (0.11)*	0.19 (0.12)*	0.53 (0.33)*	0.98 (0.50)*
Phenotypic	0.38 (0.09)*	0.13 (0.06)*	0.13 (0.08)*	0.13 (0.07)*	0.29 (0.13)*
Blacktail					
Genetic	=====	=====	0.01 (0.01)	0.007 (0.007)	0.02 (0.01)
Environ.	=====	=====	0.04 (0.11)	0.20 (0.31)	------
Phenotypic	=====	=====	0.03 (0.09)	0.05 (0.07)	0.27 (0.11)*
Weight					
Genetic	0.07 (0.13)	0.006 (0.02)	0.005 (0.009)	0.004 (0.01)	0.006 (0.01)
Environ.	0.16 (0.17)	0.15 (0.10)*	0.15 (0.13)*	0.37 (0.31)*	0.58 (0.58)
Phenotypic	0.13 (0.10)*	0.10 (0.07)*	0.09 (0.07)*	0.09 (0.07)*	0.16 (0.15)*
Length					
Genetic	0.05 (0.16)	0.002 (0.02)	0.002 (0.008)	0.001 (0.009)	0.002 (0.01)
Environ.	0.05 (0.15)	0.05 (0.10)	0.05 (0.14)	0.12 (0.29)	0.16 (0.56)
Phenotypic	0.05 (0.11)	0.03 (0.07)	0.03 (0.07)	0.03 (0.07)	0.05 (0.16)
Swimming					
Genetic	=====	0.03 (0.51)	=====	0.01 (0.07)	-0.35 (0.17)*
Environ.	=====	0.06 (0.63)	=====	------	------
Phenotypic	=====	0.03 (0.24)	=====	0.01 (0.21)	------

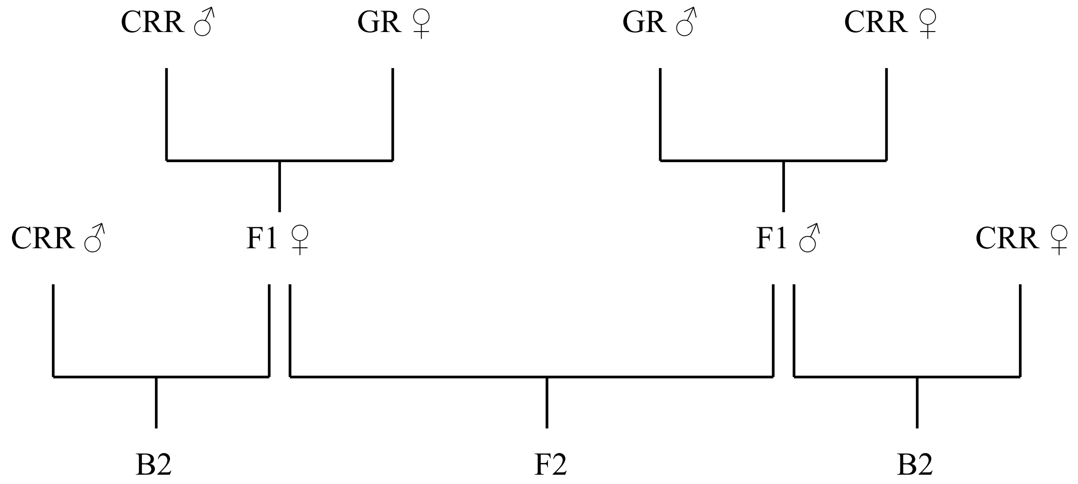

Figure 2.1. Spawning structure of unique male-female pairings used to create the five strains, and their reciprocals, for the *M. cerebralis* exposure and physiological experiments.

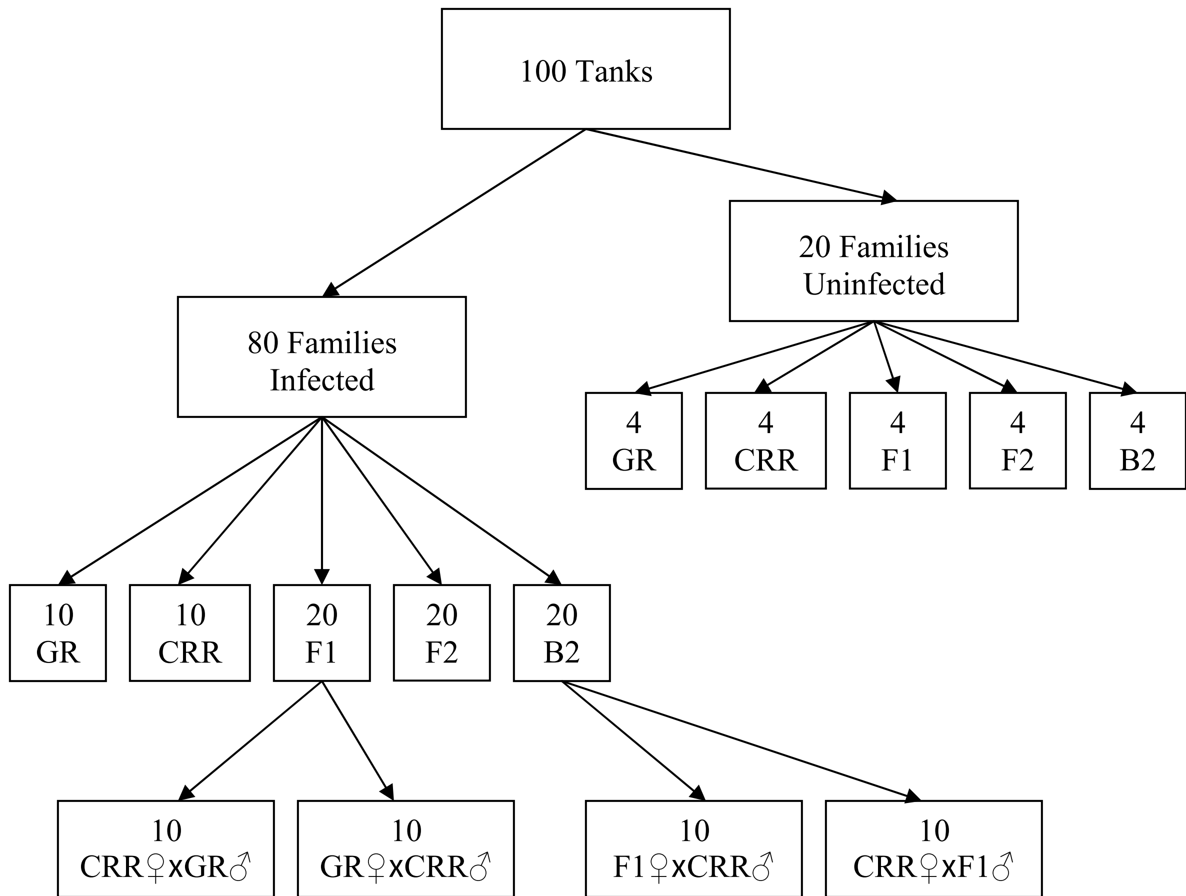

Figure 2.2. Design of the exposure metrics experiment, and the distribution of the infected and uninfected families of the five strains of rainbow trout used in the exposure metrics experiment.

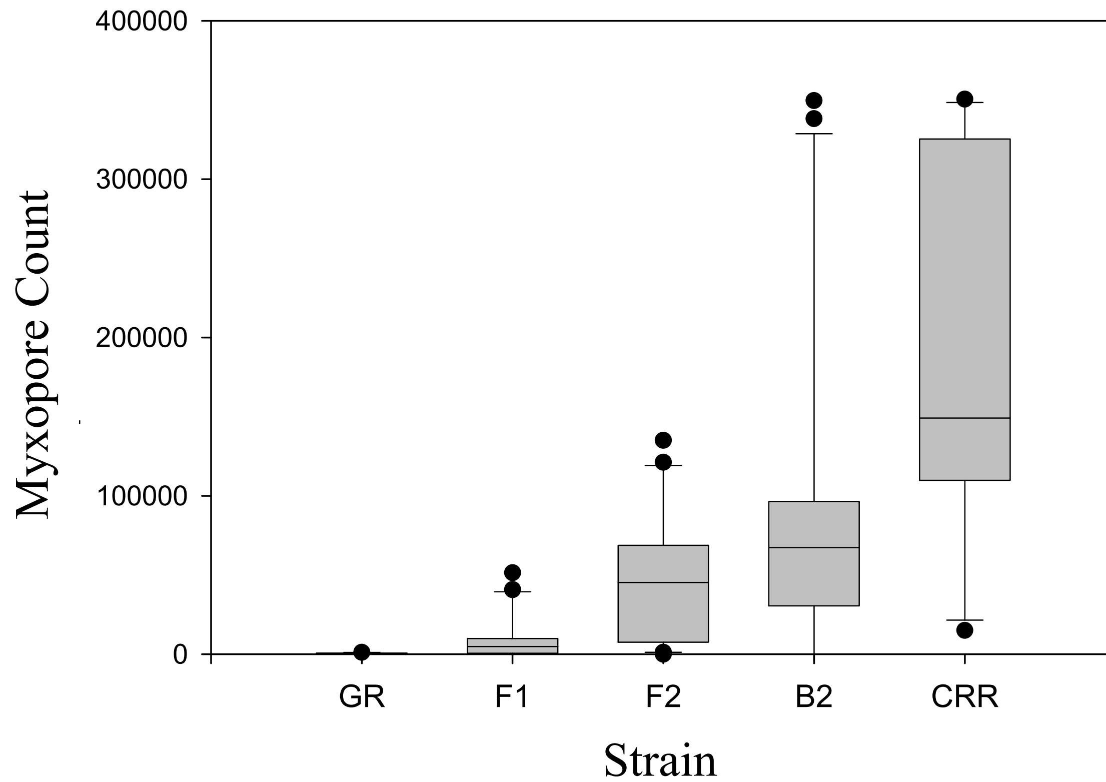

Figure 2.3. Myxospore count distribution for each of the five strains exposed to *M. cerebralis*. The box plots display the distribution of the mean myxospore counts per family within a strain, with 10 families represented in the GR and CRR strains and 20 families represented in the F1, F2 and B2 strains.

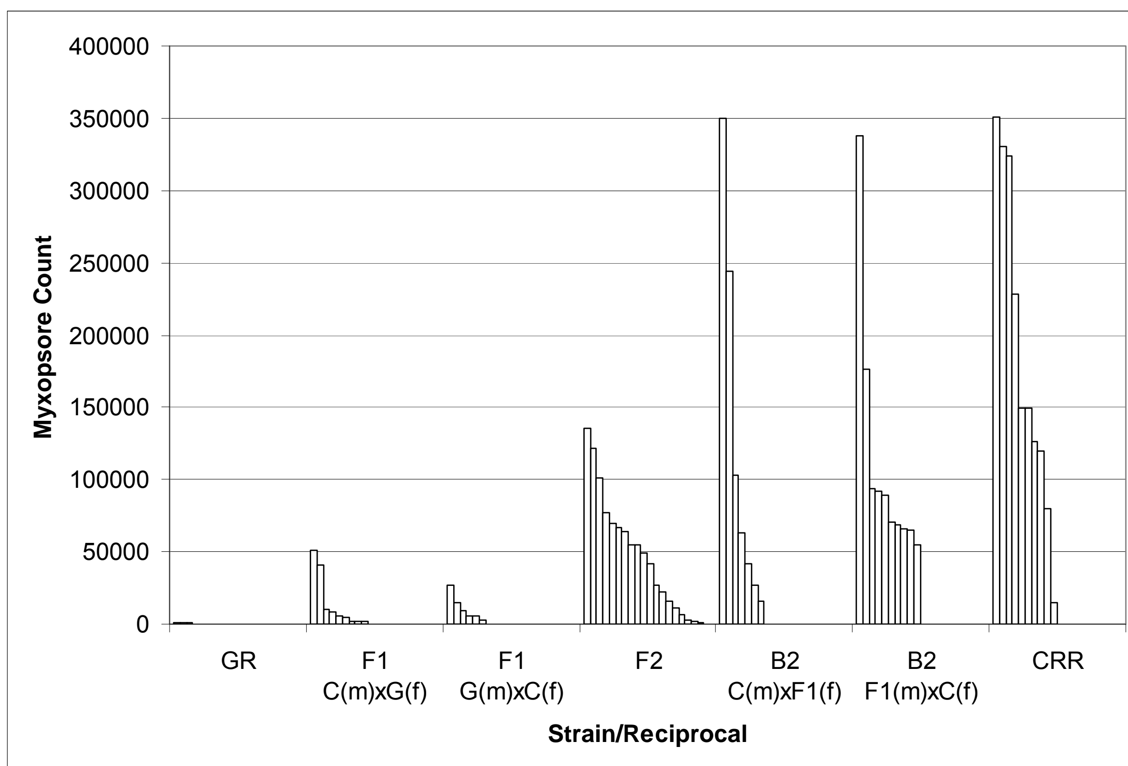

Figure 2.4. Comparison of myxospore count per family for each of five strains (with reciprocal families split out for the F1 and B2 strains) exposed to *M. cerebralis*. Ten families are represented in the GR and CRR strains, as well as in the reciprocals of the F1 and B2 strains, and 20 families are represented in the F2 strain. Notice that despite expectations, variance is low in the F1 and F2 families compared to the B2 families. In addition, variance is higher than expected in the CRR strain.

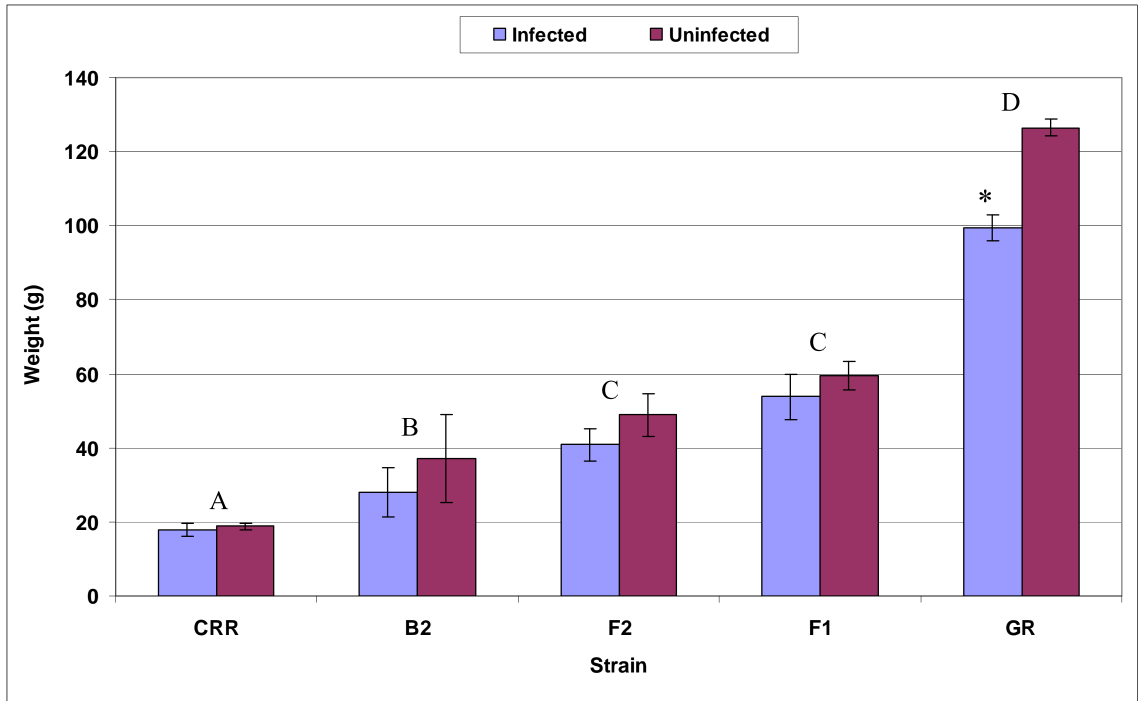

Figure 2.5. Average weight, in terms of grams per fish, for both infected and uninfected individuals within a strain at the end of the *M. cerebralis* exposure experiment.
* = significantly smaller within a strain.
A-D = significant differences in strain average.

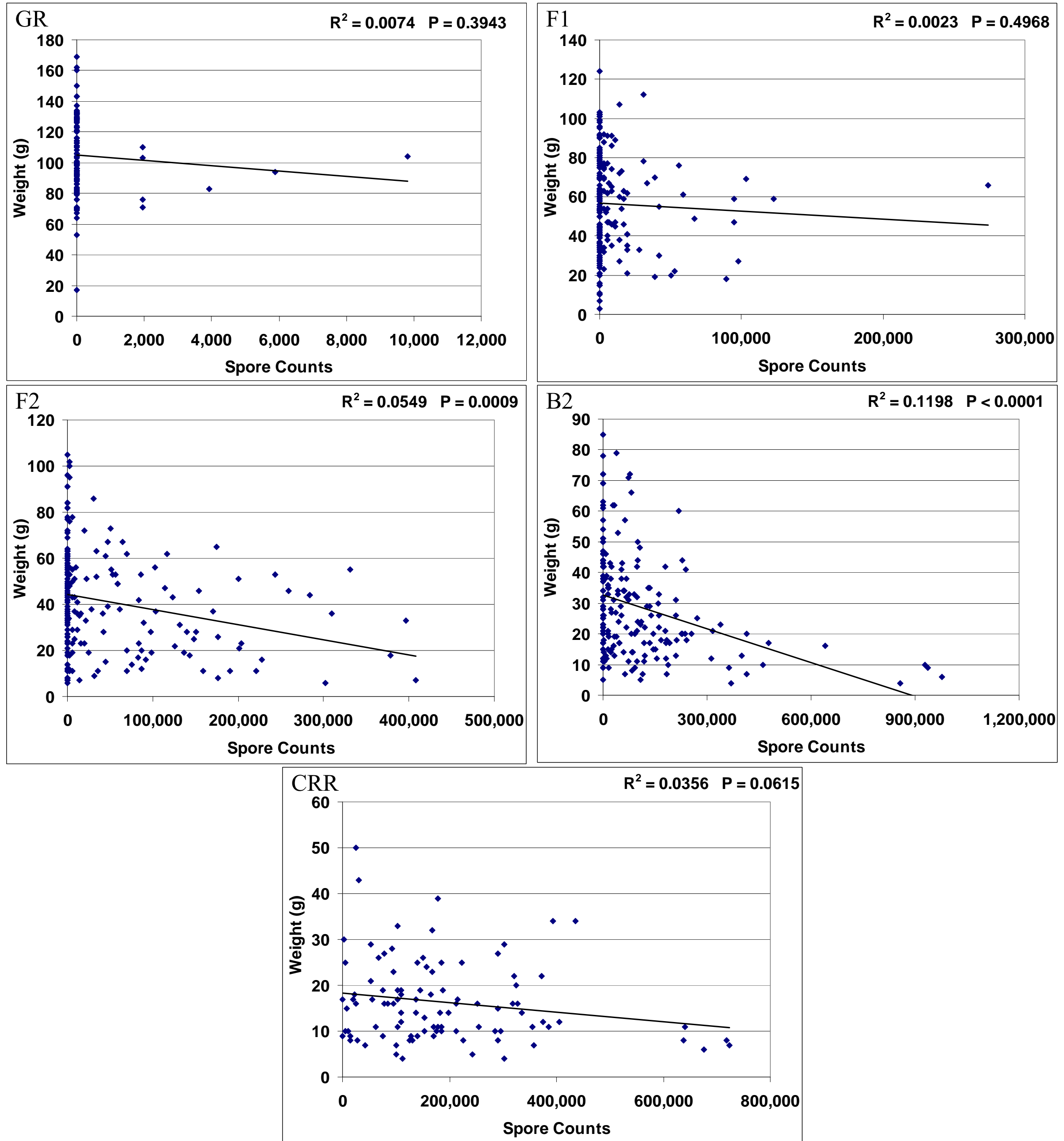

Figure 2.6. Correlation between myxospore count and weight (g) for the GR, F1, F2, B2, and CRR strains in the *M. cerebralis* exposure experiment.

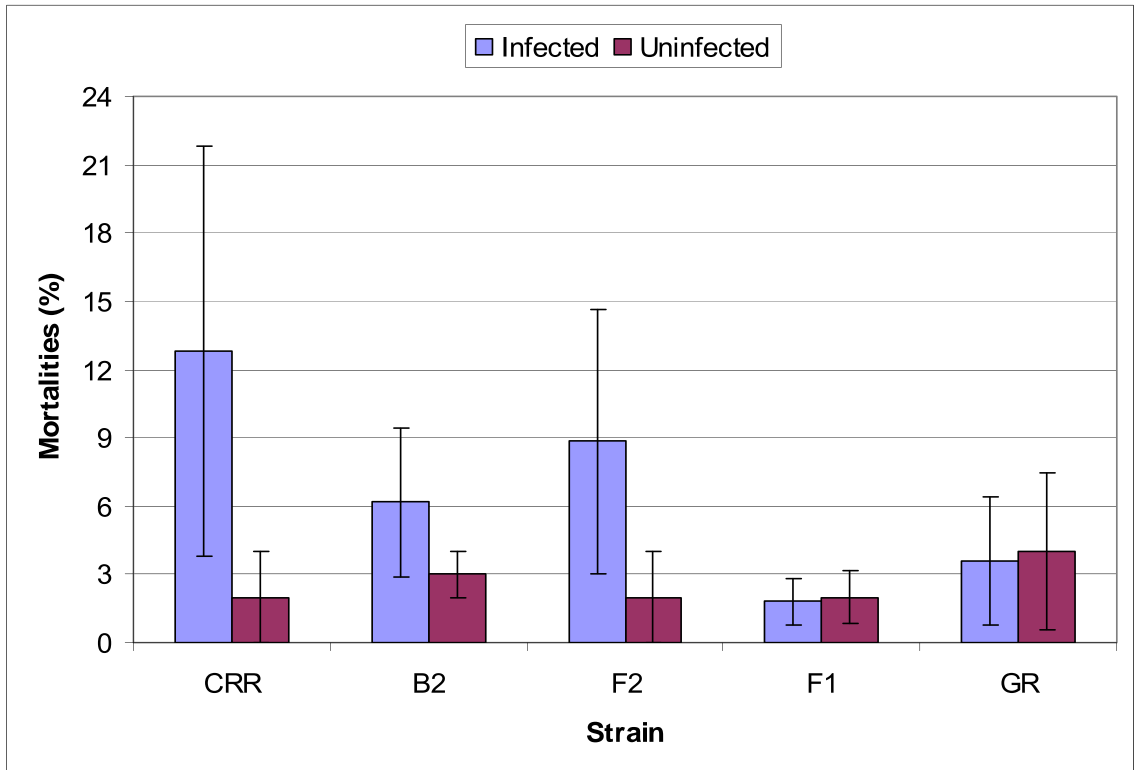

Figure 2.7. Percent mortalities of infected and uninfected individuals for each of the strains in the *M. cerebralis* exposure experiment.

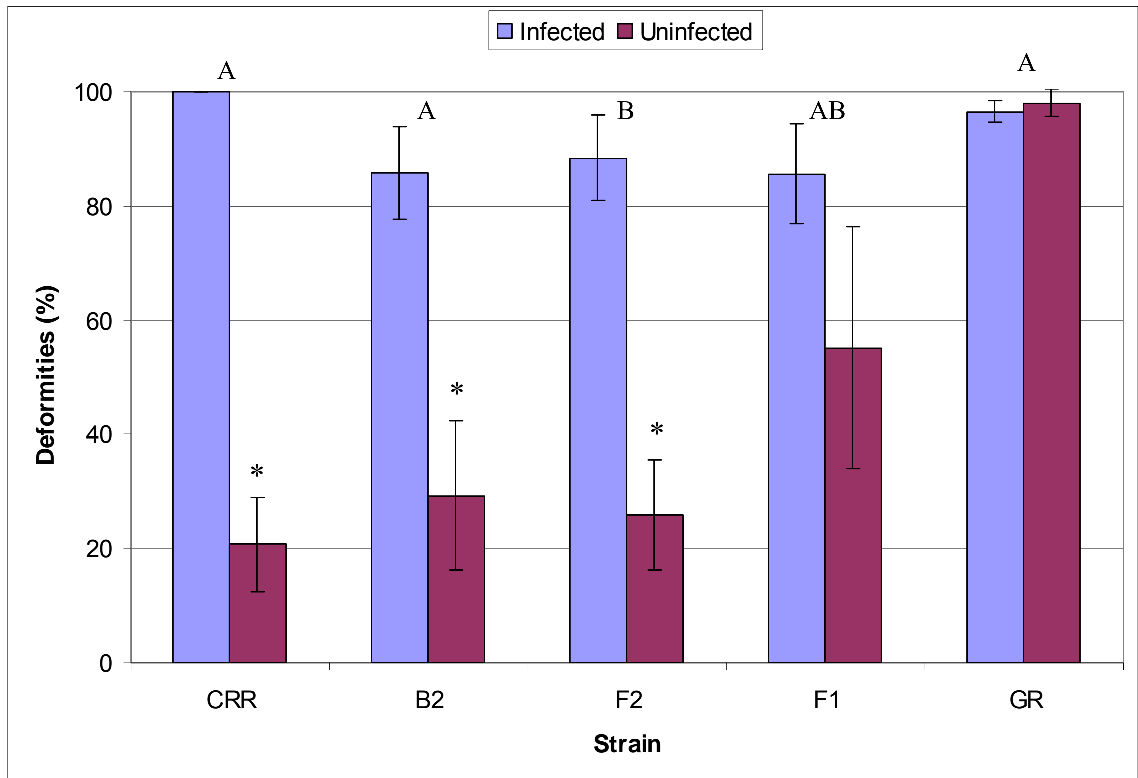

Figure 2.8. Percent of individuals with deformities in infected and uninfected individuals within a strain in the *M. cerebralis* exposure experiment.
* = significantly fewer deformities within a strain.
A-B = significant differences in strain average.

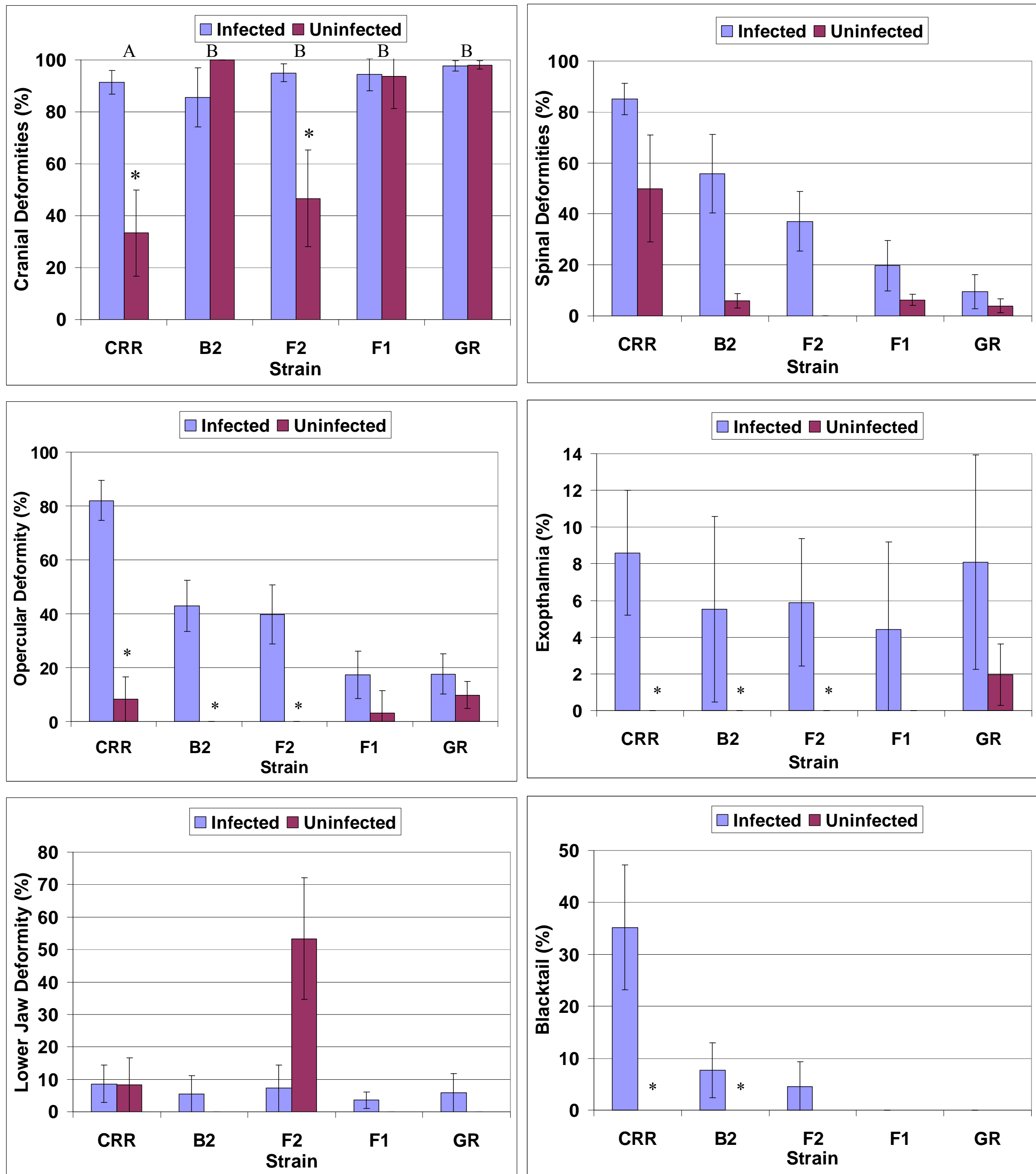

Figure 2.9. Percent of deformed individuals exhibiting cranial deformities, spinal deformities, opercular deformities, exopthalmia, lower jaw deformities and blacktail in infected and uninfected individuals within a strain in the *M. cerebralis* exposure experiment.
* = significantly fewer within a strain.
A-B = significant differences in strain average.

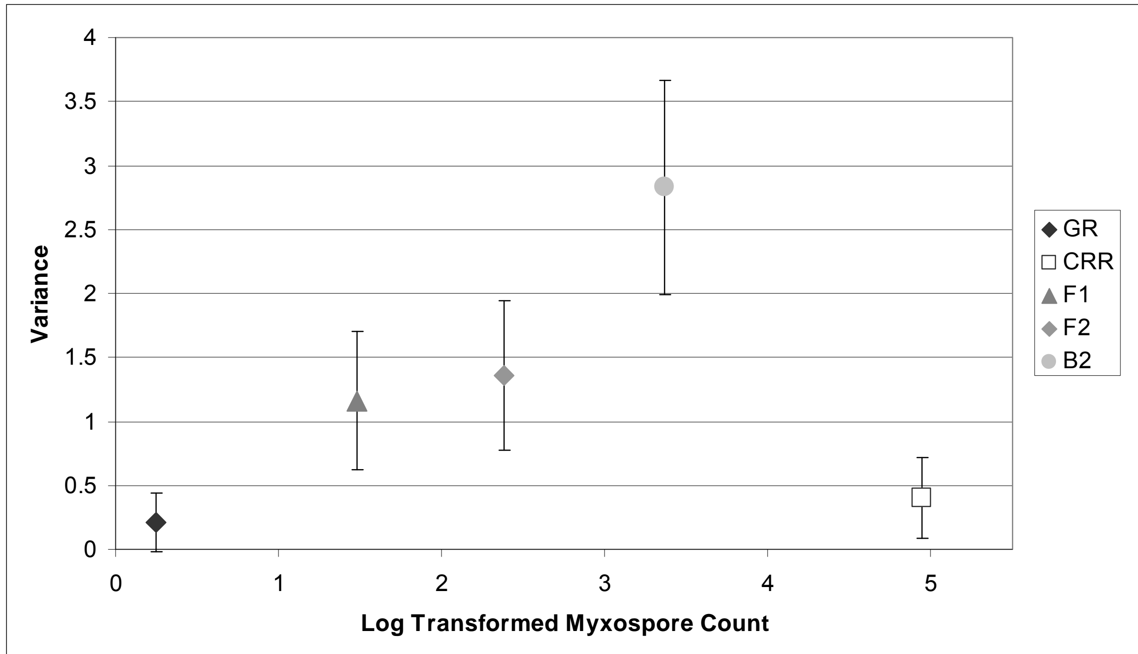

Figure 2.10. Variance versus log transformed myxospore counts for each of the five strains exposed to *M. cerebralis.* Notice that the variances plotted against myxospore counts form a triangle, a common result of responses to disease of forward and backcrosses similar to those used in the *M. cerebralis* exposure experiment, based on the predictions of the additive genetic model (Lynch and Walsh 1998).

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CHAPTER 3:

EFFECTS OF *MYXOBOLUS CEREBRALIS* ON THE PHYSIOLOGIAL PERFORMANCE OF WHIRLING DISEASE RESISTANT AND SUSCEPTIBLE STRAINS OF RAINBOW TROUT

INTRODUCTION

Disease is an integral part of existence in both cultured and wild fish populations (Hedrick 1998), influencing host abundance through long-term impacts on physiological processes affecting growth and reproduction, as well as survival (Arkoosh et al. 1998). By examining the physiological processes that fish undergo when exposed to a stressor such as disease, including factors associated with growth, swimming ability and predator avoidance characteristics, we can understand how exposure to whirling disease (*Myxobolus cerebralis*) affects critical functions necessary for survival.

Disease affects growth in many ways, including general impairment of the appetite, malfunctions in digestion or metabolism, or inhibition of feeding (Damsgård et al. 1998). In addition, the higher the susceptibility of a strain of fish to a disease, the more feeding activity, and therefore growth, is reduced (Bilodeau-Bourgeois et al. 2008). Growth is one of the three major components of the fish energy budget developed by Kitchell et al. (1977) that follows the thermodynamic principle that energy input (consumption) and outputs (respiration, waste and growth) must balance. If more energy is allocated to one of the output components, then there is less energy reserved for the other output components. Fish undergo many biological, biochemical, and physiological changes when exposed to disease that affect how energy is directed and used (Wedemeyer 1970). For this reason, growth can be a good indicator of the effects whirling disease has on the physiological processes occurring in strains of rainbow trout that vary in their susceptibility to the disease.

Swimming performance is considered a primary character in determining survival in many species of fish (Plaut 2001), including rainbow trout (*Oncorhynchus mykiss*). Swimming speed limits and endurance are directly related to food capture, escape from predators and reproduction (Videler 1993). Critical swimming velocity (Brett 1964) is a well-established method of measuring aerobic swimming performance and is a common method used to determine how other factors, including exposure to a disease, affect the overall physiological performance of an individual (Plaut 2001). The critical swimming velocity is an ecologically relevant measurement, giving at least a rough estimate of the maximum aerobic swimming velocity in fishes, and providing a relative index by which the physical status of the fish can be quantified and compared (Plaut 2001).

Critical swimming velocity tends to decrease with an increase in parasite load (Butler and Milleman 1971; Wagner et al. 2003). *Myxobolus cerebralis* targets and digests cartilage, destroying the structural framework needed for healthy bone formation (El-Matbouli et al. 1992). An increase in parasite load leads to an increase in destroyed cartilage, causing a larger number of deformities from permanent skeletal disfiguration (Rice et al. 2005). Such deformities have been shown to significantly decrease critical swimming velocities (Basaran et al. 2007). In this experiment, the critical swimming velocity was used to compare the physical status and test the limits of individuals of five strains of rainbow trout that were, and were not, exposed to whirling disease.

Previous research has also shown a relationship between swimming performance and vulnerability to predation (Bams 1967). Predation is a powerful

evolutionary force that can determine not only the kinds and numbers of potential prey species in an environment, but also the kinds and numbers of species at lower trophic levels (Li and Moyle 1999). Piscine predators search for prey actively or passively (Helfman et al. 1997). Two such predators of rainbow trout in Colorado are the brown trout (*Salmo trutta*) and the northern pike (*Esox lucius*).

Exposure to disease has been shown to increase susceptibility to predation (Seppālā et al. 2004). Prey in substandard condition are often eaten in higher than expected proportions owing to either increased prey vulnerability or active predator selection (Mesa and Warren 1997). Parasites also lower the energy reserves of their host (Poulin 1993), and parasitized fish often take more chances in order to feed in the presence of a predator than unparasitized fish (Milinski 1985; Godin and Sproul 1988). In this experiment, the effect of whirling disease on the predator recognition and avoidance of five strains of rainbow trout was examined.

M. cerebralis has caused severe problems in wild rainbow trout populations in the intermountain west (Nehring and Walker 1996). Affected watersheds include the North and South Platte, the Colorado River and the Rio Grande watershed (Nehring and Thompson 2001). Typically rainbow trout recruitment has been low or non-existent in these populations since the introduction of whirling disease. Researchers believed that establishment of a sustainable rainbow trout fishery in Colorado's rivers could be accomplished using resistant strains of rainbow trout, particularly if other methods failed to reduce infectivity in the infected drainages (Schisler et al. 2006).

Intentionally and unintentionally, innate, non-specific disease resistance has been increased at many hatcheries by the continued use of survivors of diseases as brood stock (Herman 1970). El-Matbouli et al. (2002) found that under experimental laboratory conditions a German strain of rainbow trout (GR) was at least as resistant to whirling disease as brown trout. Development of this resistance is presumed to be a result of growth and reproduction of the GR strain under continuous exposure to the parasite in Bavarian hatcheries (El-Matbouli et al. 2002). Hedrick et al. (2003) also found that the GR strain was more resistant to whirling disease than other North American rainbow trout strains in laboratory exposures. The GR strain has been crossed with the susceptible Colorado River rainbow (CRR) strain to create several strains with varying levels of susceptibility to whirling disease.

In this experiment, the swimming ability and growth potential of five rainbow trout strains were examined to determine what effects whirling disease may have on these important survival characteristics. The predator recognition and avoidance characteristics of the different strains were also evaluated.

The objectives of the growth experiment were to determine if there were differences in growth, in terms of weight and total length, and condition among each strain for both exposed and unexposed treatments. The objective of the swimming experiment was to estimate the difference in swimming ability among each strain for both exposed and unexposed treatments. The objectives of the predation experiment were to identify the strain(s) that can better avoid predation, and determine if exposure to whirling disease makes individuals more susceptible to predation. The overall objective of these experiments was to identify the best candidate strain for use as brood stock to reestablish rainbow trout populations in Colorado.

METHODS

Spawning and Rearing

The five strains of rainbow trout used in these experiments were spawned at the Colorado Division of Wildlife Bellevue Fish Research Hatchery (BFRH) from mid-November 2006 through the end of January 2007. Each family of each strain consisted of a unique male-female pairing and offspring were kept separate throughout the rearing process. The strains tested were designated GR, CRR, F1, F2, and B2. GR and CRR were pure families of German rainbow (GR) or Colorado River rainbow (CRR) trout. F1 families consisted of crosses of GR with CRR (Figure 3.1). F2 families consisted of crosses of F1 individuals (Figure 3.1). B2 families consisted of F1 individuals backcrossed with CRR individuals (Figure 3.1). GR and F1 brood stock were held at the BFRH. CRR brood stock were held at the Colorado Division of Wildlife Glenwood Springs Hatchery (GWSH). CRR males were spawned at the GWSH and their milt was transported in individual, numbered containers back to the BFRH for spawning. Live male and female CRR were also transported to BFRH for spawning (Figure 3.1). Reciprocal F1 and B2 families were created in the same manner (Figure 3.1).

F1 individuals were tagged with Passive Integrated Transponder (PIT) tags prior to spawning, and identified by their 10 digit alpha-numeric code. GR and CRR individuals were not tagged and were numbered in the order that they were spawned for fin clip and parental identification. An anal fin clip was taken from each spawned individual for genetic analysis. Eggs were placed in incubators at the BFRH or Quonset hut and held until they were eyed. Once eyed, eggs were placed in 20 gallon (76 liter) flow through (0.5 L/min) tanks, supplied by a combination of well water and charcoal-filtered city water, at the Colorado Cooperative Fish and Wildlife Research Unit Quonset hut wet lab where they were hatched.

One hundred tanks were used in the exposure metrics experiment, each containing one family per tank (Figure 3.2). Eighty families were infected with *M. cerebralis* and were composed of ten GR families, ten CRR families, 20 F1 families, 20 F2 families, and 20 B2 families (Figure 3.2). The 20 F1 families were composed of ten CRR male by GR female families, and reciprocally, ten GR male by CRR female families (Figure 3.2). The 20 B2 families were composed of ten CRR male by F1 female families, and reciprocally, ten F1 male by CRR female families (Figure 3.2). Reciprocal families were included to test whether there was a difference in performance, based on directionality of spawning, when exposed to whirling disease. Because reciprocal families cannot be created in the F2 strain, 20 F2 families were used to have an equal number of families in each of the generational strains. Due to restriction on the number of brood stock available for spawning, only 80 total families could be created. Ten infected families per strain were needed to have a large enough sample size to calculate heritability, and space constraints limited the number of tanks that could be accommodated. Therefore, prior to infection, four families from each strain were split and used as uninfected families, for a total of 20 uninfected families.

Uninfected families were placed together on the top shelf of a two-tier shelving unit to avoid potential contamination due to overflow and spills from

infected tanks. Otherwise infected and uninfected families from the five strains were randomly assigned to tanks using a random number generator.

Myxobolus cerebralis **Exposure**

Each tank contained 25 fish at exposure, and the fish were infected at an average of 678 (±44) degree-days (ºC) post-hatch. TAMs were supplied by Dr. Ron Hedrick's lab at U.C. Davis and R. Barry Nehring with the Colorado Division of Wildlife in Montrose, Colorado. Cultures of TAMs in both cases were produced from Mt. Whitney *T. tubifex* worms. TAMs were counted by mixing 1,000 µl of filtrate containing the TAMs and 60 µl of crystal violet; 84.6 µl of this mixture was then placed on a slide and the number of TAMs per slide was counted. Ten counts were conducted in this fashion to account for a possible uneven distribution of the TAMs within the filtrate. An average of the ten counts was taken, and this number was used to calculate TAMs per ml. Fish were infected with 2,000 TAMs per individual, a total of 50,000 TAMs per tank.

Prior to the addition of TAMs, water flow to each aquarium was stopped for one hour and each aquarium received aeration to ensure mixing of the TAMs and equal exposure of all fish. The amount of filtrate needed to deliver 2,000 TAMs per fish was placed in each aquarium in two doses, each dose containing half of the necessary filtrate. Using two doses helped ensure equal distribution of TAMs in the tank and accounted for a possible unequal distribution of TAMs within the filtrate. Twenty tanks, four tanks of each of the five strains, were not infected with whirling disease, but were treated in the same manner as the infected tanks. After infection, fish were reared for approximately six months to ensure the full development of myxospores. During this time, developing signs of disease and mortalities were recorded daily (Chapter 2). Over the course of the exposure metrics experiment (Chapter 2), the effects of whirling disease exposure on growth and swimming performance were also evaluated.

Swimming Experiment

The swimming experiment was conducted with the same families used for the exposure metrics (Chapter 2) and growth experiments. Five fish from four families of each strain (20 fish/strain), both infected and uninfected, were evaluated for swimming performance during each of four time periods: 14 days post-exposure, 30 days post-exposure, 74 days post-exposure and 134 days post-exposure (Figure 3.3). All four uninfected families from each strain were used, and four infected families from each strain were chosen at random. A total of 735 trials were conducted over the course of the six month swimming experiment, which included repeated measures on each of the individuals within a family at all four time periods (Figure 3.3).

Three days prior to the first swimming trial, five fish were chosen randomly from each of the tanks to be used in the swimming experiment. Each fish was marked with a Visual Implant Elastomer (VIE) tag for individual identification. The five unique identification colors used were green, red, pink, orange, and green/orange. Fish were marked in both the adipose fin and in the adipose tissue behind the right eye. Green/orange fish were marked with orange in the adipose fin, green along the base of the dorsal fin, green in the adipose tissue behind the right eye, and orange in

the adipose tissue behind the left eye. Identification of the tags was possible without visual aid for the first two swimming periods. As fish got larger, identification of the colors was made using a UV light and UV reflection filtering glasses. All tags were present and identifiable in the first three time periods. Twenty five, approximately 13 percent, of the tags were no longer visible at 134 days post-exposure. If a tag was lost, another individual was randomly chosen from the same tank to be used in place of the missing individual to keep sample sizes consistent at each of the four time periods. Untagged replacements were only used in the fourth time period.

Two Loligo® Model-32 swimming flumes were used to conduct the swimming experiments; one was used for infected individuals and the other for uninfected individuals. A fish was identified, removed from the tank, and placed in the swimming flume chamber. The time and water temperature were recorded when the fish was placed in the chamber. The flume was then set to the lowest velocity of 2 cm/sec and run for one hour to allow the fish to acclimate. At the conclusion of the acclimation period, flume speed was increased to 5 cm/sec and the swimming trial began. Flume speed was increased by 5 cm/sec every ten minutes, and the swimming trial was considered complete when fish became impinged on the screen at the back of the swimming chamber. At the conclusion of the trial, the flow was stopped and the fish was removed. Final flume speed, and length of time the fish swam at that speed, was recorded. Fish weights and lengths were recorded and fish were placed into an aerated bucket of water, allowed to recover for approximately five minutes, and returned to their tank.

The number of deformities for each individual was ranked and a rating of one indicated no visual deformities and no whirling behavior. A rating of two indicated one deformity or whirling behavior. A rating of three indicated two deformities, a spinal deformity between 0 and 15 degrees, or whirling behavior. A rating of four indicated three deformities, a spinal deformity between 15 and 45 degrees, or whirling behavior. A rating of five indicated four or more deformities, a spinal deformity that was greater than 45 degrees, multiple spinal deformities of varying severity, or whirling behavior.

The critical swimming velocity (U_{crit}), or fatigue speed, was calculated for each individual using the equation,

$$U_{crit} = V_p + \frac{t_f}{t_i} \times V_i$$

where V_p is the penultimate velocity reached at fatigue (cm/s), t_f is the time elapsed from the velocity increase to fatigue, t_i is the time between velocity increments (in this case, ten minutes), and V_i is the velocity step (in this case, 5 cm/sec) (Brett 1964). The U_{crit} was divided by the total length of the individual to calculate relative swimming velocity (body lengths per second).

Statistical Analyses

Swimming data at all four time periods was analyzed using a two-factor ANCOVA, with exposure and strain as the two factors, in SAS Proc GLM (SAS Institute, Inc. 2007-2008), using individual length as a covariate. Values were reported from the Type III sum of squares to account for the unbalanced design of the experiment. If significant effects were identified, the least squares means method,

with a Bonferroni adjustment, was used to determine which strains were significantly different from the others.

To determine if clinical signs of disease affected swimming ability, swimming data for all four time periods was analyzed in a second two-factor ANCOVA, with exposure and strain as the two factors, which included three treatment exposures: uninfected, infected showing no clinical signs, and infected showing clinical signs. This ANCOVA was run using SAS Proc GLM (SAS Institute, Inc. 2007-2008). Absolute critical swimming velocity (m/s) was used in place of relative U_{crit}, and individual length was used as a covariate to determine if variations in length among the strains affected critical swimming velocities.

Regression was used to analyze the relationship between deformity rating and swimming velocity.

Regression analysis was used to analyze the relationship between myxospore numbers and swimming velocity. Myxospore count was also plotted against deformity rating at the last swim, and a regression analysis was used to determine if there was a correlation between the two, indicating that the rating scale was a good predictor of myxospore count.

Growth Experiment

Upon swim-up, fish were fed size zero trout diet (Rangen, Inc.). After approximately 340 (±11) degree-days (ºC), fish were fed size one trout diet and families were reduced to 50 fish per family. Each family of 50 was batch weighed and fed four percent of the total batch weight. An additional 50 fish from four families from each strain were placed in uninfected tanks (Figure 3.2). The day before infection, all families were reduced to 25 fish. Each family of 25 fish was batch weighed and fed four percent of the total batch weight. The day of infection was considered the beginning of the growth experiment.

Fish were reweighed every two weeks over the course of the growth experiment and the amount of feed was adjusted to four percent of the batch weight for each family. Following hatchery guidelines, when batch weights reached 75 g, 162.5 g, and 500 g, feed size was increased to a size two, three, or four trout pellet, respectively. Adjusting feed size at these batch weights helped avoid mortalities related to gape limitation of small fish. If mortality occurred in a tank, fish were reweighed and feed was readjusted to four percent of the total biomass of that tank.

The growth experiment was concluded at four months post-exposure because fish were becoming too large to be held in the tanks. When a family reached the four month post-exposure point, the family was batch weighed, and this was the final weight used for analysis.

Fish were kept alive past the conclusion of the growth experiment for use in the exposure (Chapter 2), swimming, and predation experiments. These fish were fed a maintenance diet of two percent of their batch weight until the conclusion of the exposure experiment (Chapter 2). During this period, if batch weight exceeded 1,875 grams, the tank was put on size 5 trout diet.

Food conversion ratios were calculated for each of the strains. The food conversion ratio was calculated by summing the total amount of feed fed over the course of the growth experiment for a given family and dividing this by the total

weight that family gained over the course of the growth experiment. The food conversion ratio shows how many grams of feed are required by an individual to gain one gram of weight (Avault 1996).

Protein and lipid analyses were run on 100 fish, ten infected and uninfected individuals of each strain, to determine if there were differences in the way the strains process their food. A range of fish sizes were selected for each strain. Fins were removed to facilitate grinding the tissue. Standard lengths, minus the heads (previously removed to assess myxospore numbers), were taken on each individual after fin removal. Frozen fish were ground in a food processor, and alcohol (95 percent ETOH) was added during the grinding process to help homogenize the tissue and remove tissue that was fixed to the processor. Samples were dried for approximately five days at 60˚F until they reached a constant dry weight. The dried sample was ground to a fine powder using a food chopper and mortar and pestle and placed into individually labeled bags.

Lipid analyses were conducted in the Animal Science Laboratory at Colorado State University. Two replicates were run per individual. The fat extraction filter bags (ANKOM ID # XT4) for each replicate were labeled and filled with approximately one gram of sample (plus or minus 0.1 g). Petroleum ether was used to remove the lipids from the sample, and samples were run for 30 minutes. Bags were removed from the ANKOM XT 20 fat analyzer, placed under a flume hood to cool and dry for two hours, and then dried for two hours at 100˚F. After drying, the samples were placed in a desiccator to keep the samples from absorbing moisture and cooled to room temperature. Bags were then weighed and total weight recorded.

Total lipid content was calculated using the equation,

$$TL = \frac{W_{sample} - (W_{final} - W_{bag})}{W_{sample}} \times 100$$

where W_{sample} was the weight of the sample put into a bag, W_{final} was the final weight of the bag containing the sample after a run, and W_{bag} was the initial weight of the bag previous to containing the sample. If replicates for an individual deviated by more than 15 percent, samples were rerun. Two replicates for each individual were averaged to estimate the total lipid content for each individual.

After lipid extraction samples were analyzed for protein, nitrogen and carbohydrates. Four samples did not have enough material for analysis after the lipid extraction and another fish from the same family was processed to replace these samples. Aluminum tins were filled with approximately a tenth of a gram of sample and placed in wells in a Leco® gas chromatograph to analyze the sample. Results are expressed in percent protein, percent nitrogen, and percent carbohydrate. Two replicates for each individual were averaged to estimate the parameters.

Statistical Analyses

Growth data was analyzed using a two-factor ANOVA, with exposure and strain as the factors, in SAS Proc GLM (SAS Institute, Inc. 2007-2008). Values were reported from the Type III sum of squares to account for the unbalanced design of the experiment. If significant effects were identified, the least squares means method, with a Bonferroni adjustment, was used to determine which strains were significantly different from the others.

Food conversion ratios, as well as lipid, protein, nitrogen and carbohydrate percentages, were arcsine-square root transformed prior to analysis. Transformed ratios and percentages were analyzed using a two-factor ANOVA in SAS Proc GLM (SAS Institute, Inc. 2007-2008). Values were reported from the Type III sum of squares to account for the unbalanced design of the experiment. If significant effects were identified, the least squares means method, adjusted with a Bonferroni adjustment, was used to determine which strains were significantly different from the others.

Quantitative Genetics Analyses

Broad sense heritability estimates of length, weight and swimming ability, as well as genetic, environmental and phenotypic correlations between myxospore count and length, weight and swimming ability, were calculated using the methods from Becker (1992; Chapter 2). In addition, genetic, environmental and phenotypic correlations between the physiological characteristics were also estimated using the methods in Becker (1992).

Predation Experiment

The predation experiment was conducted at the Foothills Fisheries Laboratory on the Colorado State University Foothills Campus in Fort Collins, Colorado. After the conclusion of the exposure, growth, and swimming experiments, individuals within a strain were divided into multiple tanks with fish about the same size. Each group of fish was then fed an amount of food appropriate for their size (two percent of batch weight for tanks containing large individuals, three percent of batch weight for tanks containing medium individuals, and four percent of batch weight for tanks containing small individuals). The goal of dividing fish into size groups was to grow each strain to about equal sizes before the predation experiment. The GR strain was significantly larger at the end of the exposure experiment than all other strains; therefore, GR individuals were kept in cooler water (average of 4˚C) and fed less than the other strains (0.5 percent of batch weight). The CRR strain was significantly smaller than the other strains and was kept in larger round tanks in warmer water (average 10.5˚C) and fed more feed to promote growth (5 percent of batch weight). They were also fed live feed, including eggs, fry and fingerlings. The F1, F2 and B2 strains were similar in size to each other but smaller than the GR strain and larger than the CRR strain. These three strains were held in cool water (average of 7˚C) and fed intermediate amounts of food. Fish were held for about three months.

Two weeks prior to the start of the predation experiment, individuals from all five strains were counted, weighed, and measured. Only 36 uninfected individuals per strain were available; therefore, to keep sample sizes consistent, 36 infected individuals per strain, which had approximately the same average weights and lengths as uninfected individuals, were chosen for use in the experiment.

Four ponds were used for the predation experiment, two uninfected and two infected. The locations of the infected and uninfected ponds were chosen randomly. Ponds one and four contained uninfected rainbows and ponds two and three contained infected rainbows. Each pond contained 18 individuals of each strain, a total of 90 rainbows per pond.

There was large variation in size within a strain for both infected and uninfected individuals and large variation in size among the strains. We wanted to keep the average fish size about equal among the strains within a pond to reduce the potential effect of rainbow trout size on predation rate. Therefore, we divided the 36 trout within each strain and exposure into two groups of 18. The smallest 18 individuals from each strain and exposure were placed in two ponds and the largest 18 from each strain and exposure were placed in the other two ponds. This procedure resulted in average weight and length being approximately equal among the strains in each pond (Table 3.1).

All rainbow trout individuals were marked using VIE tags. CRR individuals were marked with a pink VIE tag in the right eye, GR individuals with a red VIE tag in the left eye, F1 individuals with a green VIE tag in the right eye, F2 individuals with an orange VIE tag in the left eye, and B2 individuals with a green VIE tag in the left eye and an orange VIE tag in the right eye. The rainbows were placed in their respective ponds and allowed to acclimate five days before the introduction of predators.

Northern pike for the experiment were caught using volunteer anglers out of Lake Lodora on the Rocky Mountain Arsenal National Wildlife Refuge in Denver, Colorado. Only pike over 66 cm were used in the experiment to maintain at least a 3:1 predator to prey ratio. Twelve northern pike ranging between 71.1 and 81.3 cm were brought back to Fort Collins for use in the experiment. Three pike were placed in net pens in each of the four ponds prior to introduction to allow them to acclimate and digest whatever food was in their stomachs (Table 3.2). The two largest of the three pike were introduced into the ponds two days later which marked the beginning of the predation experiment (Table 3.3). Pike size for each of the ponds was chosen based on the average size of the rainbows which had been previously introduced to the pond. The four remaining pike were held for a short period of time after the beginning of the experiment in case predator mortality occurred.

Starting the second week of the experiment, ponds were seined every two weeks to determine the number of rainbows remaining per strain per pond. Two passes were made through each pond to obtain removal estimates of trout population size. Lengths and weights of individual rainbow trout were taken during the last four seining events to determine if consumption was affected by the size of a given strain. The number of individuals of each strain was also recorded for each of the ponds and the proportion of each strain left in the ponds was estimated.

The pond experiment was concluded after ten weeks due to rising water temperatures and decreasing water quality. The ponds were drained and any rainbows that were left in the pond were counted and measured. Secchi disk depth, temperature, and dissolved oxygen where measured in each pond every day to determine if differences in predation rate throughout the experiment could be accounted for by differences in pond environment.

Statistical Analyses

Survival data at all five sampling periods was analyzed using a two-factor ANOVA, with exposure and strain as the two factors, in SAS Proc GLM (SAS Institute, Inc. 2007-2008). Values were reported from the Type III sum of squares to

account for the unbalanced design of the experiment. If significant effects were identified, the least squares means method, with a Bonferroni adjustment, was used to determine which strains were significantly different from the others. Raw estimates of survival are reported without any additional analyses because they are thought to be representative.

RESULTS

Swimming Experiment

Critical swimming velocity reached, in terms of body lengths per second, decreased within all five strains as fish length increased. There was not a significant difference in critical swimming velocity (BL/s) between the exposures at 14, 30, 74 or 134 days post exposure ($P \geq 0.0845$). Therefore, analyses of swimming data combined infected and uninfected individuals from a strain into an overall representation of the strain at all four time periods, which was used for a comparison across the strains.

There was a significant difference in combined swimming ability among the strains at all four time periods ($P < 0.0001$). At all time periods, the CRR strain reached significantly higher critical swimming velocities (BL/s) than did the GR strain ($P \leq 0.0011$). In the first time period, 14 days post-exposure to whirling disease, the CRR and F2 strains reached significantly higher critical swimming velocities than the F1 strain ($P < 0.0007$). In addition, the F2 strain reached significantly higher critical swimming velocities than the GR or B2 strains ($P \leq 0.0443$). In the second time period, 30 days post-exposure, the CRR strain reached significantly higher velocities than did the F1, F2, B2 or GR strains ($P \leq 0.0289$). The F1, F2, B2 and GR strains did not differ significantly from each other in this time period ($P \geq 0.3075$). In the third time period, 74 days post-exposure, the CRR strain reached significantly higher critical swimming velocities than the F1 and B2 strains ($P \leq 0.0071$), and the F2 strain reached significantly higher critical swimming velocities than the GR strain ($P \leq 0.0010$). In the fourth time period, 134 days post exposure, the F2, B2 and CRR strains reached significantly higher critical swimming velocities than did the GR strain ($P \leq 0.0006$; Figure 3.4).

In the fourth time period, we divided the exposed fish into those showing clinical signs of the disease and those that did not and compared those fish to the uninfected treatments (Table 3.4). For the analysis with these three treatment exposures, length explained a significant amount of the variation in critical swimming velocity ($P < 0.0001$). Critical swimming velocity was significantly different among the exposures ($P = 0.0264$), strains ($P = 0.0686$), and the interaction between exposures and strain was also significant ($P < 0.0001$). Critical swimming velocity did not differ among uninfected individuals, infected individuals showing no clinical signs, or individuals showing clinical signs among the GR, F1 or F2 strains ($P \geq 0.8971$). Critical swimming velocity of infected individuals showing clinical signs was significantly slower than that of uninfected individuals within the B2 and CRR strains ($P \leq 0.0106$). Critical swimming velocity did not differ between infected individuals not showing clinical signs and uninfected individuals in the B2 and CRR strains ($P \geq 0.6291$; Figure 3.5).

There was a significant negative correlation between deformity rating and critical swimming velocity ($R^2 = 0.0390$, $P < 0.0001$), indicating that critical swimming velocity is reduced by the number and severity of deformities. In addition, there was a significant negative correlation between deformity rating and critical swimming velocity in the CRR ($R^2 = 0.1111$, $P = 0.0182$), B2 ($R^2 = 0.1442$, $P = 0.0157$), and F2 ($R^2 = 0.1114$, $P = 0.0314$) strains. There was not a significant correlation between rating and critical swimming velocity in the F1 ($R^2 = 0.0385$, $P = 0.1870$) or GR ($R^2 = 0.0002$, $P = 0.9775$) strains (Figure 3.6).

The GR strain individuals did not develop any myxospores, and therefore, myxospore count did not affect critical swimming velocity in this strain. There was not a significant correlation between myxospore count and critical swimming velocity for any of the other strains ($R^2 \leq 0.2967$, $P \geq 0.2749$).

There was a significant positive correlation between the deformity rating and myxospore counts ($R^2 = 0.3065$, $P < 0.0001$; Figure 3.7), indicating that deformity rating can be used as an indicator of the severity of infection.

Broad sense heritability estimates of swimming ability were high and significantly different from zero in the B2 strain (0.45); estimates of heritability of swimming ability were inestimable for the F2 strain (Table 3.5).

Genetic correlations between weight and swimming ability were low (0.24), and length and swimming ability were high (0.82), and were significantly different from zero, in the B2 strain; estimates between both weight and length and swimming ability were inestimable for the F2 strain. Environmental and phenotypic correlations between weight and length and swimming ability were low (-0.09-0.22) and not significantly different from zero for both the F2 and B2 strains (Table 3.6).

Growth Experiment

There was a significant difference in batch weight between the exposures ($P = 0.0504$) and among the strains ($P < 0.0001$), however, there was not a significant interaction between exposure and strain ($P = 0.3549$). The GR strain weighed significantly more than any of the other strains ($P < 0.0001$). The F1 strain weighed significantly more than the F2, B2 or CRR strains ($P \leq 0.0004$). The F2, B2 and CRR strains did not differ significantly in weight from each other ($P \geq 0.1137$). Once probabilities were corrected for pair-wise comparisons, there were no significant differences in batch weight between infected and uninfected individuals within a strain for any of the strains ($P \geq 0.2485$; Figure 3.8). There was not a significant effect of exposure on the batch weights of the reciprocal families of the F1 or B2 strains ($P = 0.9909$). Therefore, directionality of spawning does not appear to affect the growth of these strains when exposed to whirling disease.

There was a significant difference in food conversion ratio among the strains ($P < 0.0001$), but no difference between the exposures ($P = 0.4460$) or the interaction between exposure and strain ($P = 0.1271$). The GR strain had a significantly lower food conversion ratio than all of the other strains ($P \leq 0.0143$) and the others did not differ ($P \geq 0.1838$; Table 3.7).

There was a significant difference in lipid content among the strains ($P = 0.0078$), but no difference in lipid content between the exposures ($P = 0.7830$) or interaction between exposure and strain ($P = 0.8221$). The GR strain had

significantly lower lipid content than the F2 or B2 strains ($P \leq 0.0875$) and the other strains did not differ ($P \geq 0.1010$; Figure 3.9).

There was a significant difference in protein content between the exposures ($P = 0.0009$) and among the strains ($P < 0.0001$), however, there was not a significant interaction between exposure and strain ($P = 0.2176$). The GR strain had significantly higher protein content than any of the other strains ($P \leq 0.0062$). There was not a significant difference in protein content among the F1, F2, B2 or CRR strains ($P \geq 0.5870$). The infected families within the F2 strain had a significantly lower protein content than the uninfected families ($P = 0.0331$). However, there was not a significant difference in protein content between infected and uninfected families within the GR, F1, B2 or CRR strains ($P \geq 0.6397$).

There was a significant difference in nitrogen content between the exposures ($P = 0.0009$) and among the strains ($P < 0.0001$), however there was not a significant interaction between exposure and strain ($P = 0.2157$). The GR strain had significantly higher nitrogen content than any of the other strains ($P \leq 0.0058$). There was not a significant difference in nitrogen content among the F1, F2, B2 or CRR strains ($P \leq 0.5837$). The infected families within the F2 strain had a significantly lower nitrogen content than the uninfected families ($P = 0.0331$). However, there was not a significant difference in nitrogen content between infected and uninfected families within the GR, F1, B2 or CRR strains ($P \geq 0.3982$).

Broad sense heritability estimates of weight and length were low and significantly different from zero in the B2 strain (0.12), but were higher and significantly different from zero in the F2 strain (0.57; Table 3.5).

Genetic correlations between weight and length were high and significantly different from zero in both the F2 and B2 strains (0.18-0.99). Environmental and phenotypic correlations between weight and length were low (-0.04-0.10) and not significantly different from zero for both the F2 and B2 strains (Table 3.6).

Predation Experiment

Although efforts were made to begin the experiment with similar sized fish for each strain and exposure treatment, the CRR individuals were significantly shorter, by 5.8 cm, than the other strains ($P < 0.0001$).

The condition, in terms of sechhi disk depth, dissolved oxygen levels, and temperature, of all four ponds was kept as constant as possible throughout the course of the experiment ($P \geq 0.0880$; Table 3.8).

There was not a significant difference in the proportion of infected or uninfected individuals within a strain remaining in any of the ponds at any of the sampling periods ($P \geq 0.9898$). Therefore, proportions of infected and uninfected individuals remaining from a strain were combined for each of the sampling times to compare the strains.

There was a difference in survival among the strains across sampling times ($P \leq 0.0125$, Figure 3.10). CRR individuals showed significantly lower survival rates in the first three sampling occasions than the other strains ($P \leq 0.0124$), dropping to less than 10 percent of the individuals remaining by the third sampling occasion, while the other strains still had over 50 percent of the individuals remaining, and did not differ from each other in survival ($P \geq 0.6537$). In the fourth sampling occasion the number

of CRR individuals remaining did not differ from the other four strains ($P \geq 0.5932$). Upon completion of the experiment, no CRR individuals remained and the GR and B2 strains also had very few individuals remaining, and did not differ from the CRR strain or each other in survival ($P \geq 0.4928$). The F1 and F2 strains showed significantly higher survival than the CRR strain ($P \leq 0.0353$), however, they did not differ from the GR and B2 strains ($P \geq 0.4928$; Figure 3.10).

DISCUSSION

As expected, the CRR strain had a significantly higher critical swimming velocity (BL/s) than GR strain individuals. The GR has been selected to do well in a hatchery environment and we predicted that they would have a slower swimming velocity because they have not experienced flows over those of a raceway for over a century. In contrast, the CRR strain is a wild strain that has not been selected to perform well in hatchery conditions, and has adapted to the swifter, changing flows of a more natural environment. The other three strains had intermediate critical swimming velocities to the GR and CRR strains.

The critical swimming velocities exhibited by the GR, F1, F2 and B2 strains in the first three time periods of this experiment, are similar to those of the Shelton and Aberdeen cutthroat trout strains (Hawkins and Quinn 1996) and Eagle Lake and Mt. Shasta rainbow trout strains (Myrick and Cech 2000) of similar size, however, were slightly higher in the forth time period than those exhibited by rainbow trout of similar size (Schneider and Connors 1982). The CRR strain exhibited higher critical swimming velocities at all four time periods than those exhibited by cutthroat and rainbow trout strains of similar size (Hawkins and Quinn 1996; Myrick and Cech 2000; Schneider and Connors 1982).

Differential critical swimming velocity between wild and hatchery-reared individuals has been seen for juvenile brown trout (Pedersen et al. 2008), juvenile and yearling Atlantic salmon (McDonald et al. 1998; Pedersen et al. 2008), and juvenile Coho salmon (Brauner et al. 1994). Many of the differences, especially between yearlings, are attributable to rearing environment, and the morphological changes that occur as a result of rearing environment, including fin condition, growth, and muscle biochemistry (McDonald et al. 1998).

Overall, as the days post-exposure increase in this experiment, so does the length of the fish, resulting in the downward trend in critical swimming velocity experienced in all of the strains. A decrease in critical swimming velocity with an increase in fish length is typical for fish, where the smaller individuals of a species are favored due to their reduced hydrodynamic drag (Beamish 1978), which outweighs the advantage of the increased metabolic scope and body musculature of the larger fish (Brett 1965). Differences in length among the strains did have an effect on the critical swimming velocities reached by the strains, especially in the fourth time period. The greatest difference in size was seen between the GR and CRR strains. In the Coho salmon, similar to this study, many of the differences in critical swimming velocity between wild and hatchery-reared fish could be accounted for by the fact that the hatchery-reared fish were significantly larger than their wild counterparts (Brauner et al. 1994). Had the GR and CRR individuals been the same

size in the fourth swimming time, the differences between the two strains may not have been as apparent. However, the GR strain reached significantly slower velocities than the CRR strain at the beginning of the experiment as well, where length did not explain the variability among the strains, suggesting that, based on their hatchery-reared life history, there may be a maximum velocity that the GR strain can reach before exhaustion, which could cause them to be unable to survive in high flow conditions in the wild.

There were differences in critical swimming velocities (cm/s) between critically infected and uninfected individuals within the B2 and CRR strains, especially in the fourth time period, when length was introduced as a covariate; however, the other strains did not differ among treatment exposures. These results indicate that critical swimming velocity was affected by severity of infection in those strains that are more susceptible to whirling disease. These results also indicate that that in those strains, such as the GR or F1 strain, that are more resistant to whirling disease, exposure does not affect swimming ability.

Other studies have shown that critical swimming velocity tends to decrease with an increase in parasite load (Butler and Milleman 1971; Wagner et al. 2003). In addition, deformities that developed due to exposure to whirling disease were significantly higher in infected individuals than the uninfected individuals within the B2 and CRR strains, though did not differ between exposures in the GR and F1 strains (Chapter 2). The increase in deformities in the B2 and CRR strains may account for the lower critical swimming velocities reached by these individuals, as has been seen in a similar study on the affects of deformities on the critical swimming velocities of juvenile sea bass (Basaran et al. 2007).

We found no correlation between myxospore count and critical swimming velocity in any strain. These results suggest that exposure to whirling disease does not affect an individual's ability to reach a certain critical swimming velocity. However, Rice et al. (2005) conducted an endurance swimming experiment with exposed and unexposed rainbow trout in which fish were exposed to constant velocity until they were exhausted. Rice et al. (2005) showed that exposed individuals did not have the endurance of the unexposed individuals. Although our protocol differed from that of Rice et al. (2005), the two experiments attempted to measure similar effects and we do not know why these experiments differed.

We believe that the design of the swimming experiment could be altered to make a more realistic challenge. Whirling disease can result in an uncontrollable whirling motion when and individual is startled (Höfer 1903; Hoffman 1970; Hedrick et al. 1998). A swimming experiment that included some element of surprise would better represent the demands that exposed individuals would experience and demonstrate if individuals are to recover once startled.

Genetic correlations estimate the degree to which two traits are affected by the same genes or pairs of genes, or in other words, the amount to which the two traits covary (Conner and Hartl 2004). The low genetic correlation between myxospore count and the swimming ability suggest that it is possible to improve both the swimming ability and resistance to *M. cerebralis* simultaneously in rainbow trout using a selective breeding program, because variation of one trait does not affect the other (Chapter 2). Similarly, the low genetic correlations between weight and length

and swimming ability also suggest that it is possible to improve both the swimming ability and growth simultaneously in rainbow trout using a selective breeding program.

Growth did differ among the strains. The GR strain had the highest batch weight, highest protein levels, and the lowest lipid levels at the end of the growth experiment. In addition, the GR strain also had the lowest feed conversion ratio of all the strains. The growth potential of the GR strain is likely a result of selection in the Hofer fish hatchery where the GR strain was cultivated as a food fish (Höfer 1903). Domestication selection probably selected for larger and faster growing individuals, resulting in a fish that performs well in hatchery and other artificial environments. In contrast, the CRR individuals were the slowest growing individuals, having a fairly high feed conversion ratio. The CRR strain has been cultured for stocking in Colorado; however, brood stocks were collected from wild populations and haven't been selected for rapid growth in a hatchery environment. Gregory and Wood (1998) suggest that rapid growth may occur to the detriment of aerobic swimming performance. This may explain the poor swimming ability of the GR strain in comparison the CRR strain. Selected for rapid growth under hatchery conditions, the GR strain may have traded growth ability for swimming ability based on their hatchery-reared life history. In contrast, for the wild CRR strain, a better swimming ability may have been more necessary for survival than growth, leading to their superior swimming performance and poor growth performance.

The other strains show intermediate growth, and the F1 strain, which has more GR genes, was significantly larger than the F2 or B2 strains. Therefore, the F1 strain, which shows an intermediate swimming ability to the two pure strains, and good growth performance in comparison to the CRR strain, may have the correct combination of swimming and growth characteristics preferable for survival and angler satisfaction in Colorado's rivers. In the wild, growth is ultimately limited by several physiological and ecological aspects including increased predation risk associated with the heightened appetite and foraging behavior stimulated by high levels of growth hormone, increased occurrence of developmental errors associated with rapid growth, and the investment in growth occurring at the expense of somatic maintenance and repair (Johnsson and Björnsson 1994).

Growth has been shown to differ between hatchery-reared and wild fish in Atlantic salmon (Einum and Flemming 1997). This was suspected to be a result in genetic differences in consumption rate, metabolism, assimilation efficiency, or a combination of the three, all of which have probably developed in the GR strain through selection. In addition, in a study on rainbow trout where differences in growth between hatchery-reared and wild fish were seen, the F1 hybrids displayed phenotypic values for growth very close to the mean of the parental values (Tymchuk and Devlin 2005), similar to what was seen in this experiment. Analysis of the size-frequency distributions of the backcrosses in the experiment conducted by Tymchuk and Devlin (2005) supported an additive model that more than one gene is involved in growth enhancement of the domesticated strain relative to wild stock.

Exposure to the parasite did not result in significantly lower batch weights in any strain; however, other experiments have shown that strains that are more highly susceptible to a disease show reduced feeding efficiency and growth as a result

(Bilodeau-Bourgeois et al. 2008). The lack of differences between infected and uninfected families within a strain in this experiment was probably a result of high variability within families. A lot of variation was seen in the size of individuals within any given family. Therefore, larger individuals within a family, those with the higher growth potential that could have been switched to a larger feed size sooner than the smaller individuals, may have been held back by the smaller individuals within that family. This may also have caused the lack of differences in batch weight among the F2, B2 and CRR strains. Had the individuals with the higher growth potential been fed the larger feed size when they were able to consume it, differences between exposures and among the strains may have been more apparent. A difference in weight among the strains has been seen in previous experiments (Schisler et al. 2006, Schisler et al. 2007). However, this experiment is the first experiment with these strains where they were given a set percentage of their body weight per day throughout the entire experiment.

Despite the lack of differences seen in batch weights, there was a negative correlation when comparing myxospore count to either weight or length in the F2, B2 and CRR strains. There was no correlation between myxospore count and weight or length in GR or F1 strains. These negative correlations suggest that susceptibility to the parasite is higher in those strains that are further from the parental GR strain or have no GR genes at all, suggesting that growth is related to susceptibility to the parasite (Chapter 2).

The low genetic correlation between myxospore count and the growth characteristics suggest that it is possible to improve both the growth characteristics and resistance to *M. cerebralis* simultaneously in rainbow trout using a selective breeding program, because variation of one trait does not affect the other (Chapter 2). In a similar study, genetic correlations between body weight and number of lice in Atlantic salmon, suggest that that it is possible to improve both body weight and resistance to salmon louse through selection (Kolstad et al. 2005), reflecting the unintentional selection that may have occurred in the GR strain. In addition, intentional selection for both growth and disease resistance is being used to improve domestic catfish stocks (Peterson et al. 2008). High genetic correlations between weight and length indicate that it is not possible to select for one trait without selecting for the other. However, this result is not unexpected as many relationships between weight and length have been described for fish and are used in the calculation of growth characteristics for management purposes (Anderson and Neumann 1996).

Reduced growth in more heavily exposed individuals has been seen in channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*), and their hybrids when exposed to enteric septicemia of catfish (Bilodeau-Bourgeois et al. 2008) and Atlantic salmon when exposed to infectious pancreatic necrosis virus (Damsgård et al. 1998). This is because exposure to disease incurs a high level of stress on fish, which can affect several biochemical and physiological processes resulting in reduced growth in highly infected individuals (Wedemeyer 1970).

In the predation experiment, raw survival estimates suggest that the CRR strain had significantly lower survival than the other strains. The CRR strain may have been more active in the pond environment because of the need to replenish

depleted energy stores. Parasites lower the energy reserves of their host (Poulin 1993), and parasitized fish often take more chances in order to feed in the presence of a predator than unparasitized fish (Milinski 1985; Godin and Sproul 1988).

We expected that infected individuals would be less likely to escape a predator because of reduced swimming performance since this phenomenon had been seen in previous studies (Bams 1967). In addition, exposure to disease has been shown to increase susceptibility to predation (Seppālā et al. 2004), and prey in substandard condition are often eaten in higher than expected proportions owing to either increased prey vulnerability or active predator selection (Mesa and Warren 1997). However, we saw few differences related to parasite exposure. One explanation may be that pike are an ambush predator and not actively searching for prey (Horn 1998). Therefore, the most active individuals would be consumed, whether they were swimming normally or whirling. Additionally, highly infected individuals may have been easier to capture, but may not have been actively swimming near the pike.

Size may also have been a factor. Prey selection in the pike is based on the length of prey species in comparison to the length of the pike (Nilsson and Brönmark 2000). However, prey weight, translating to body depth based on prey length, has more of an effect on prey selectivity because it increases handling time, incurring a higher capture cost, a loss of energy in comparison to the energy gained in consumption of the prey, and also makes the pike more susceptible to cannibalism and kleptoparasites. Therefore, the pike more selectively choose smaller prey when it is available (Hart and Connellan 1984; Nilsson and Brönmark 1999; Nilsson and Brönmark 2000). The CRR individuals were significantly smaller in both length and weight than the other strains. Therefore, they may have been more selectively preyed upon based on their size. In contrast, the GR individuals were larger, and weighed more in relation to their length, than the other strains, which may have caused them to be more actively selected against for consumption by the pike. The fact that the CRR was present in the lowest proportions in both the infected and uninfected ponds supports this conclusion. Also, the other strains surviving in equal proportions, and being close in size, indicates that one strain was not more susceptible to predation than another because of behavior.

The results of the pond experiment do suggest that there is a minimum stocking size for susceptibility to predation. The majority of the individuals consumed in the first half of the experiment were on the smaller range of those stocked for each of the strains, whereas the larger individuals were consumed at a much slower rate. Larger size at stocking is an important concept to recognize, not only in the case of waters that contain pike as the top predator, but also in waters that have predators, such as brown trout, that can be just as voracious. In terms of hatchery rearing and stocking, it makes more sense to stock fewer, larger individuals that will have a high survival rate than to stock hundreds of thousands of smaller fish whose survival rate could be close to zero due to predation, especially if the cost of these two options is the same. As further introduction to wild situations occurs, predation will be a major component in the survival of these fish, in addition to their ability to survive exposure to whirling disease.

Though there were differences in growth when correlated with myxospore count, the differences in batch weight between the infected and uninfected families were fewer than expected. In addition, the fewer differences in performance between infected and uninfected individuals in the swimming and predation experiments were unexpected and we predicted more differences based on two reasons. First, whirling disease has caused complete extirpation of wild rainbow trout populations throughout Colorado (Walker and Nehring 1995). Second, other studies have shown effects of exposure on growth and swimming performance (Schisler et al. 2006; Schisler et al. 2007; Rice et al. 2005). We feel that the relatively benign tank environment may explain the lack of exposure effects because fish were fed a high level of food that was easily available. Infected fish were often seen missing food particles on the first strike but were able to consume the food on a second try. However, if infected individuals miss feeding opportunities in the wild it is less likely they would get additional opportunities at the same food item. Additionally, fish did not have other competing demands such as avoiding predation, swimming in a current, or other external stressors.

The overall objective of this study was to determine which of the strains would be the best candidate for use as a brood stock to reintroduce rainbow trout to Colorado's rivers and streams. The GR strain was the largest of the strains in the growth experiment. Though it did not reach the critical swimming velocities of the CRR strain, the GR strain did not show a difference in swimming ability from the F1 strain. The abundance estimates for the GR strain in the pond experiment did not differ from the F1, F2 or B2 strains, all of whose abundance estimates were higher than the CRR strain. Based on these results, we conclude that the GR strain is the best candidate for use as a brood stock in Colorado.

The GR strain was not originally considered a good candidate for stocking into Colorado for several reasons, the largest of which was its history of domestication (Schisler et al. 2006). It was thought to be a negative to introduce the domesticated genes of the GR into a natural, wild population. The consequences of doing so, if the GR strain was not able to survive well in the wild, could cause a complete collapse of the wild rainbow trout population. However, due to the losses caused by the introduction of whirling disease, there are very few rainbow trout populations left in Colorado that would be affected by the introduction of the GR strain.

Because of the concerns expressed above, the Colorado Division of Wildlife started using the F1 strain to experimentally stock Colorado's waters because it was thought to have the best combination of the GR resistance characteristics and the CRR wild strain survival characteristics. In this study, the GR and F1 strains did not differ from each other in performance in any of the experiments but the growth experiment. Under laboratory conditions, the GR strain displayed the same physiological and survival characteristics as the F1 strain, and therefore, may survive just as well as the F1 strain in the wild. In addition, the faster growth of the GR strain will be appealing to anglers across the state. However, rapid growth has been shown to occur at the detriment of aerobic swimming performance (Gregory and Wood 1998), which may cause the GR strain to show lower survival due to an inferior swimming performance compared to wild rainbow trout strains. The F1 strain,

therefore, may have the correct combination of swimming and growth characteristics preferable for survival and angler satisfaction in Colorado's rivers.

The F1 strain has been experimentally stocked into the Gunnison and Colorado Rivers in the state since 2004 and 2006, respectively. Preliminary survival data indicates that individuals from these stocking events remain in the population, and recent genetic analysis indicates that some of the fry contain GR genetics meaning that natural reproduction has occurred. At this time, survival of these individuals past the fry stage is still unknown. The next step is to experimentally stock the GR strain and F1 strain into a river or stream where their survival and reproductive characteristics can be monitored side by side. Only then can it be determined which of these two strains can be used to return rainbow trout populations to Colorado.

Table 3.1. Average length (cm), and standard deviation (in parentheses), of the five rainbow trout strains that were used in the four ponds in the predation experiment: Pond 1 (uninfected), Pond 2 (infected), Pond 3 (infected), and Pond 4 (uninfected). 18 individuals per strain were stocked into each pond for a total of 90 rainbow trout per pond. * = strain significantly shorter on average than other strains.

Strain	Pond 1	Pond 2	Pond 3	Pond 4
GR	27.8 (0.56)	25 (0.93)	27.4 (0.49)	25.1 (0.87)
F1	26.5 (0.93)	22.8 (1.68)	26.3 (1.14)	22.9 (1.51)
F2	27.3 (1.39)	21.7 (2.67)	26.9 (1.20)	21.9 (2.97)
B2	25.8 (1.51)	21.1 (2.52)	25.7 (1.26)	21.1 (2.38)
CRR	20.1* (1.17)	15.3* (1.93)	20* (1.10)	15.1* (2.06)

Table 3.2. Lengths (cm) of northern pike brought back from the Rocky Mountain Arsenal National Wildlife Refuge and placed in the net pens in each of the four ponds used in the predation experiment on March 10, 2008.

Pond 1	**Pond 2**	**Pond 3**	**Pond 4**
80.6	73	73.7	70.5
77.5	72.4	73.7	72.4
79.4	71.8	74.3	72.4

Table 3.3. Lengths (cm) of northern pike released into each of the four ponds used in the pond predation experiment on March 12, 2008.

Pond 1	Pond 2	Pond 3	Pond 4
80.6	73	74.3	72.4
79.4	72.4	73.7	72.4

Table 3.4. Results of the ANCOVA with three treatment exposures: uninfected, infected showing no clinical signs, and infected showing clinical signs. Length was used as the covariate. Results are displayed for all four time periods: 14, 30, 74 and 134 days post-exposure to whirling disease.

Main Effects	14 Days PE	30 Days PE	74 Days PE	134 Days PE
Exposure	NS	NS	NS	0.0264
Strain	0.0076	0.0167	< 0.0001	0.0686
Exposure*Strain	NS	NS	0.0017	0.0332
Length	0.0101	NS	< 0.0001	< 0.0001

Table 3.5. Broad sense heritability estimates of the physiological characteristics, and standard errors (in parentheses), for the five strains of rainbow trout used in the swimming and growth experiments. A "------" indicates that the heritability for that physiological characteristic was inestimable for that strain. Significance is indicated by an "*".

Physiological Characteristic	GR	F1	F2	B2	CRR
Weight	0.44 (0.23)*	0.22 (0.18)*	0.48 (0.24)*	0.10 (0.14)	0.33 (0.21)*
Length	0.19 (0.17)*	0.21 (0.17)*	0.57 (0.25)*	0.12 (0.14)	0.29 (0.19)*
Swimming Ability	0.28 (0.19)*	0.03 (0.11)	------	0.45 (0.23)*	0.17 (0.16)*

Table 3.6. Genetic, environmental and phenotypic correlations between the physiological characteristics, and standard errors (in parentheses), for the five strains of rainbow trout used in the swimming and growth experiments. A "------" indicates that the correlation for that deformity or physiological characteristic was inestimable for that strain. A "=====" indicates that there was no heritability for the trait within a given strain, and therefore, genetic correlations could not be estimated. Significance is indicated by an "*".

Physiological Characteristic Comparison	**GR**	**F1**	**F2**	**B2**	**CRR**
Weight x Length					
Genetic	0.47 (------)	0.61 (0.008)*	0.18 (0.006)*	0.99 (0.02)*	0.44 (------)
Environ.	0.19 (0.19)	-0.03 (0.11)	-0.04 (0.17)	-0.006 (0.11)	0.10 (0.21)
Phenotypic	0.26 (0.12)*	0.11 (0.09)*	0.08 (0.08)	0.10 (0.10)	0.20 (0.14)*
Weight x Swimming					
Genetic	------	0.18 (0.34)	=====	0.24 (0.22)*	------
Environ.	-0.03 (0.63)	0.02 (0.60)	=====	-0.03 (0.88)	0.13 (0.55)
Phenotypic	0.04 (0.63)	0.03 (0.48)	=====	0.02 (0.66)	0.16 (0.50)
Length x Swimming					
Genetic	------	0.42 (0.41)*	=====	0.82 (0.27)*	------
Environ.	-0.08 (0.69)	0.23 (0.67)	=====	-0.09 (0.87)	------
Phenotypic	0.001 (0.58)	0.22 (0.56)	=====	0.07 (0.67)	------

Table 3.7. Feed conversion ratios (FCR) (g feed/g gain), and standard deviation (in parentheses), for infected and uninfected families within each of the strains in the growth experiment. * = strain average FCR smaller than other strains

Strain	F.C.R	
	Control	**Infected**
GR*	1.05 (0.08)	1.08 (0.18)
CRR	1.39 (0.13)	1.88 (0.85)
F1	1.31 (0.12)	1.24 (0.17)
F2	1.53 (0.35)	1.26 (0.11)
B2	1.42 (0.28)	1.45 (0.14)

Table 3.8. Average and standard deviation (in parentheses) of the secchi disc depths (cm), dissolved oxygen levels (ppm) and temperatures (˚C) for each of the four ponds used in the predation experiment. Ponds 1 and 4 contained uninfected individuals, and ponds 2 and 3 contained infected individuals.

Pond	Secchi Disk Depth	Dissolved Oxygen	Temperature
Pond 1	103 (41.88)	7.9 (1.58)	8.86 (3.13)
Pond 2	136.75 (31.28)	7.24 (1.68)	8.98 (3.14)
Pond 3	118.82 (36.35)	7.51 (1.68)	9.26 (3.21)
Pond 4	128.31 (33.94)	7.63 (1.50)	9.47 (3.34)

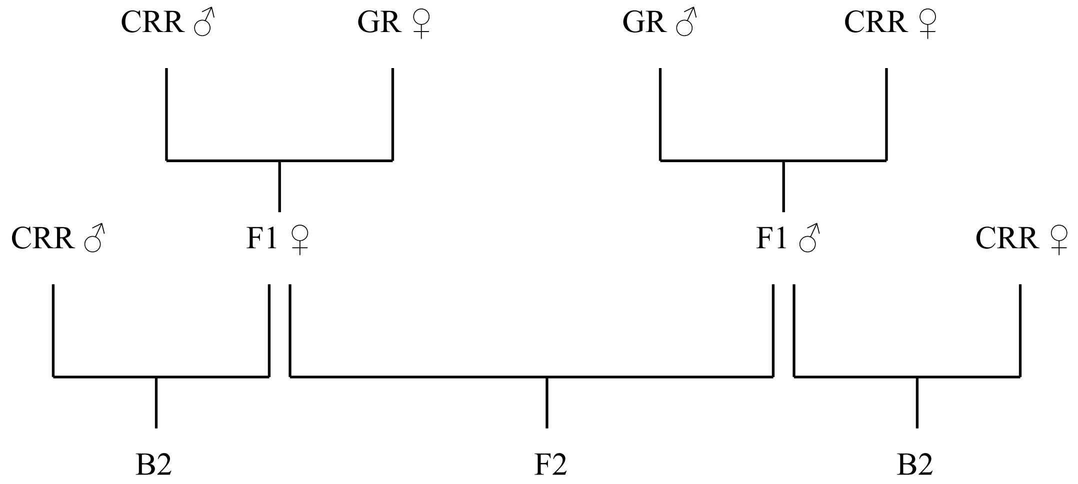


Figure 3.1. Spawning structure of unique male-female pairings used to create the five strains, and their reciprocals, for the *M. cerebralis* exposure and physiological experiments.

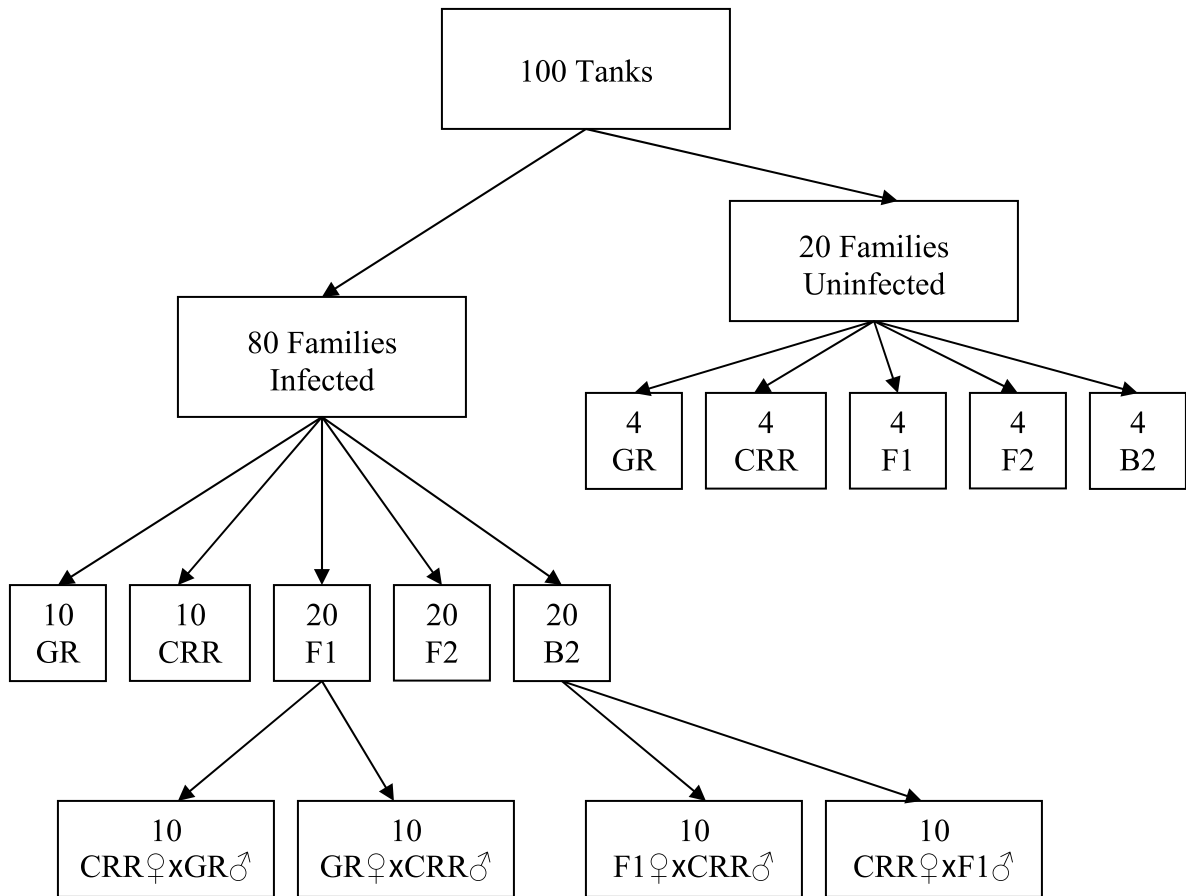


Figure 3.2. Design of the growth experiment, and the distribution of the infected and uninfected families of the five strains of rainbow trout used in the growth experiment.

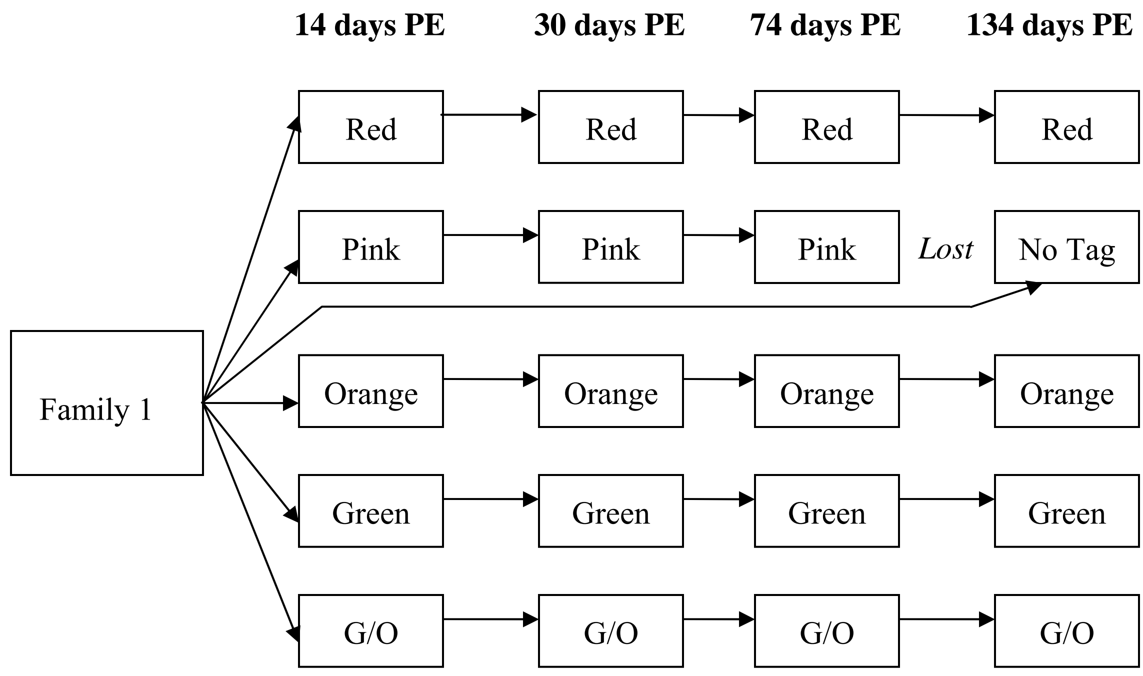


Figure 3.3. Example of repeated swimming measures of the five individuals swum out of a single family within a given strain in the swimming experiment. Each of the five individuals, identified by different colored VIE tags, was swum at each of the four time periods: 14, 30, 74 and 134 days post-exposure. If a tagged individual was lost or unable to be identified, that individual was replaced by an untagged individual from that same family so that five fish from each family were swum at each time period.

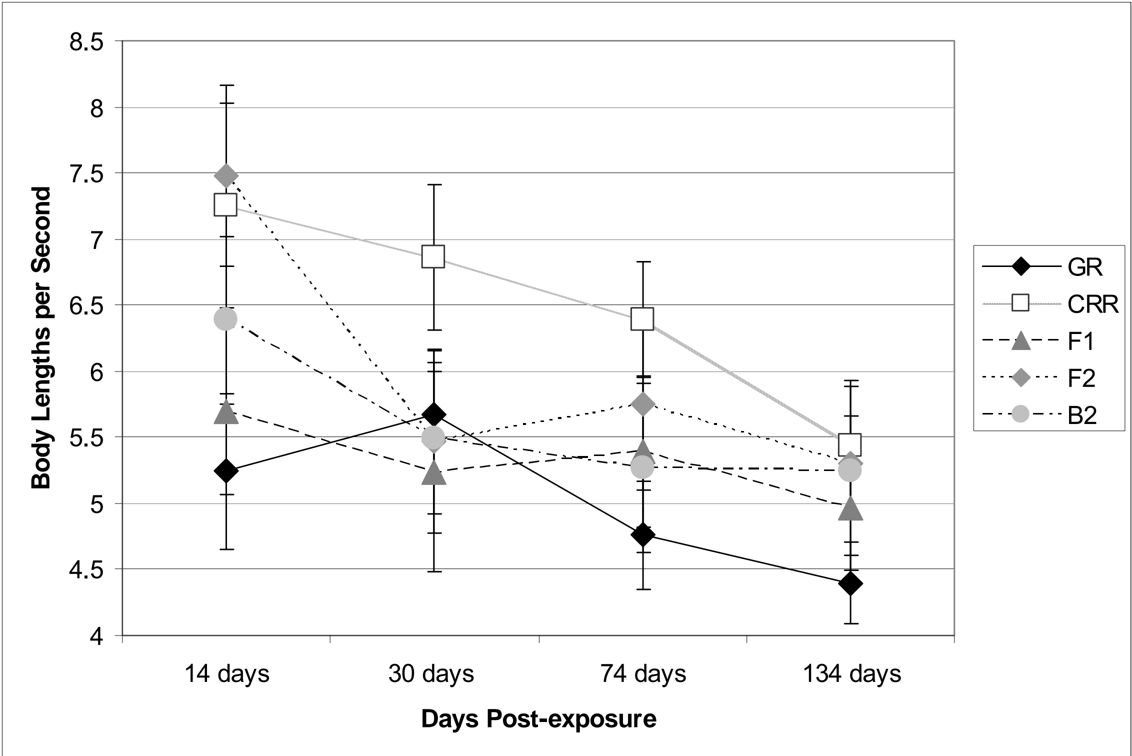


Figure 3.4. Average critical swimming velocity (BL/s) of the five strains, at each of the four time periods. Each point on the graph represents a combined average of the infected and uninfected individuals because there were no significant differences between the two in any of the strains at any of the four time periods.

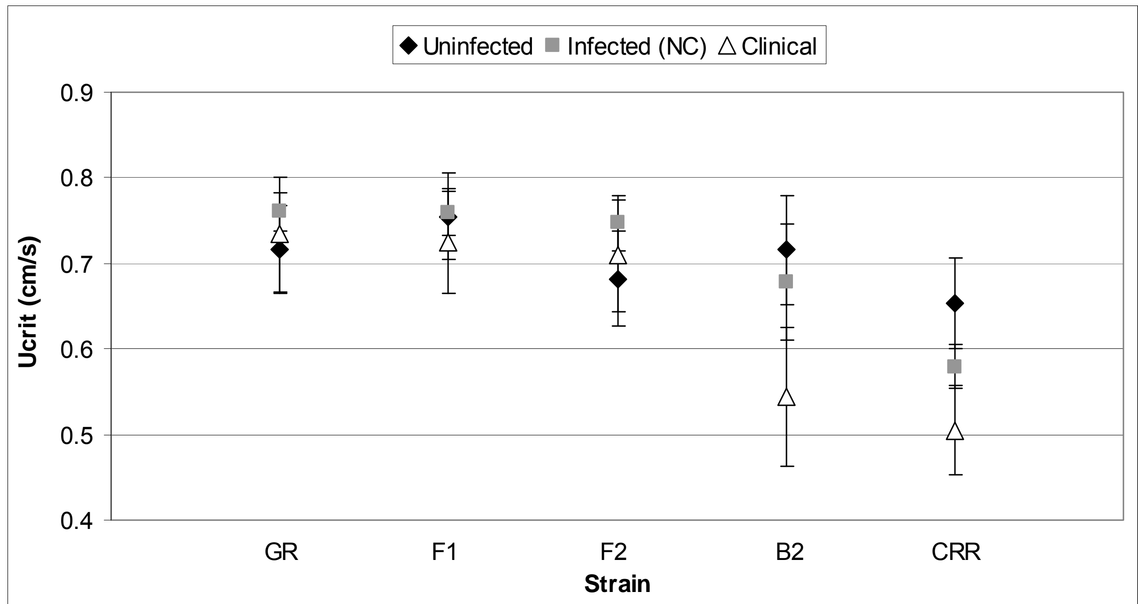


Figure 3.5. Differences in average critical swimming velocity among uninfected individuals, infected individuals showing no clinical signs (rating of 1), and infected individuals showing clinical signs (rating of 2 or greater) as a result of to exposure to *M. cerebralis* in each of the five strains at 134 days post-exposure.

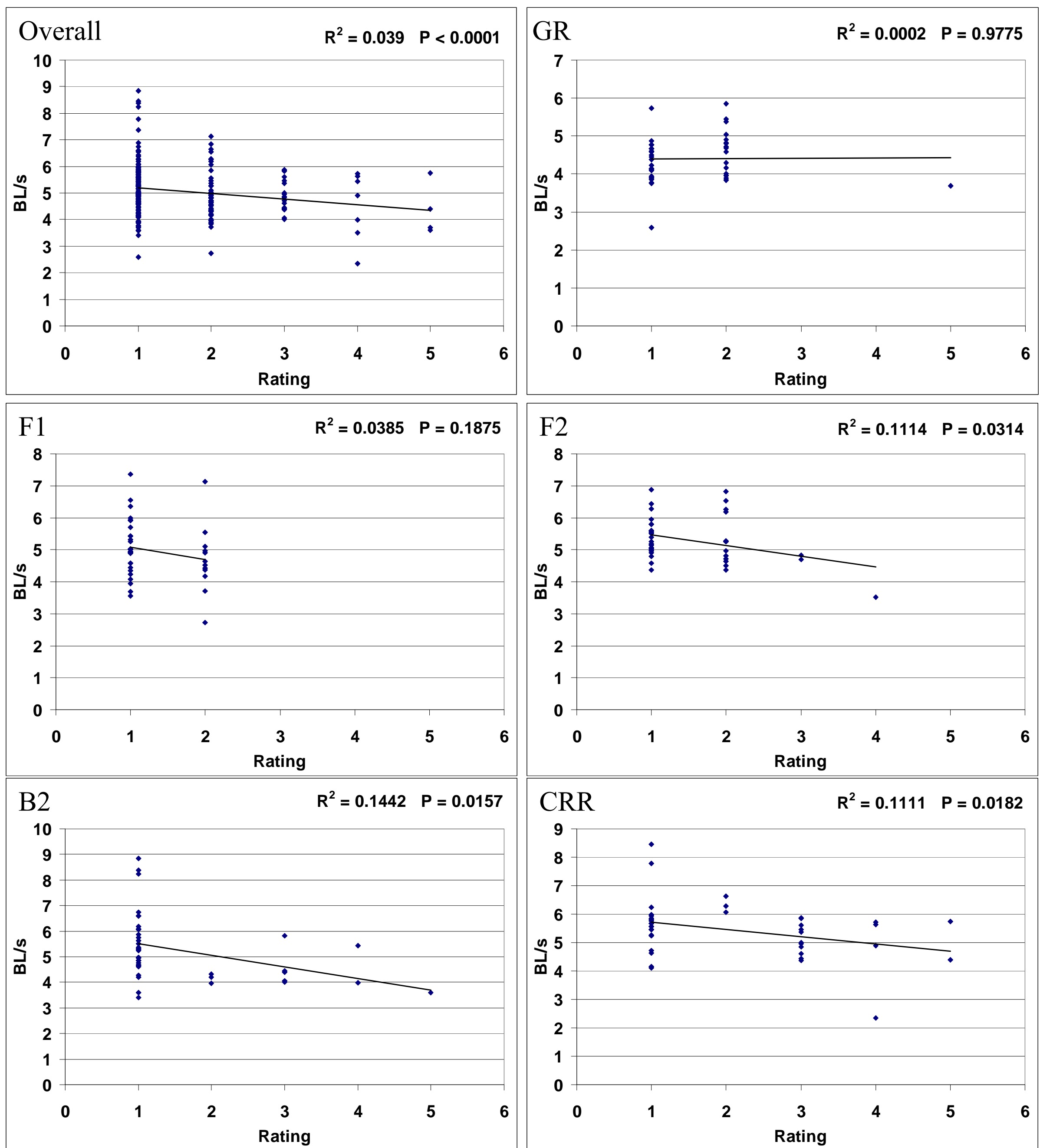


Figure 3.6. Overall correlation between deformity rating scale and critical swimming speed across all strains. Correlation between deformity rating scale and critical swimming speed for the GR, F1, F2, B2 and CRR individuals used in the swimming experiment.

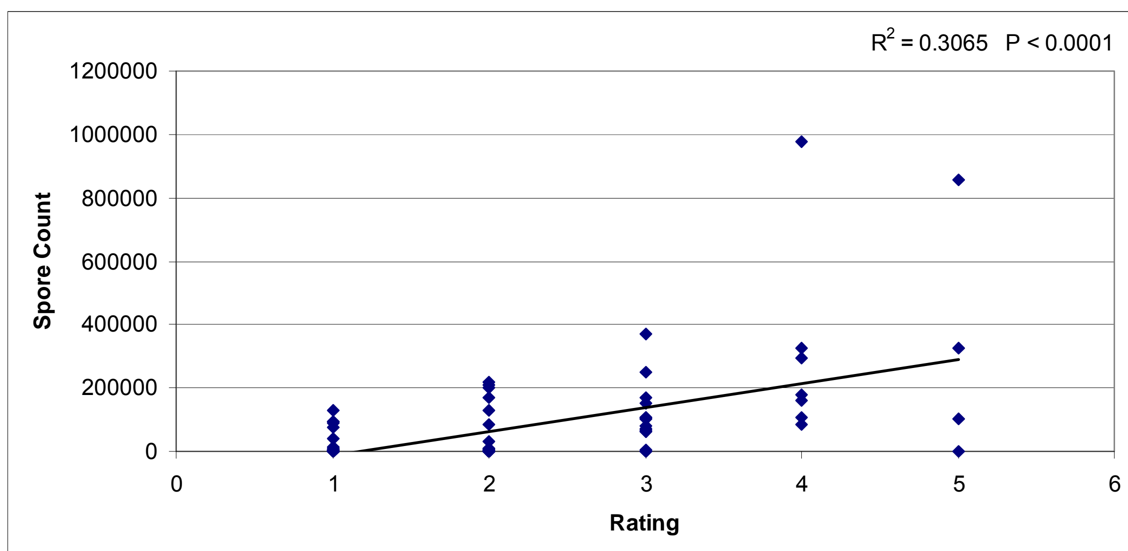
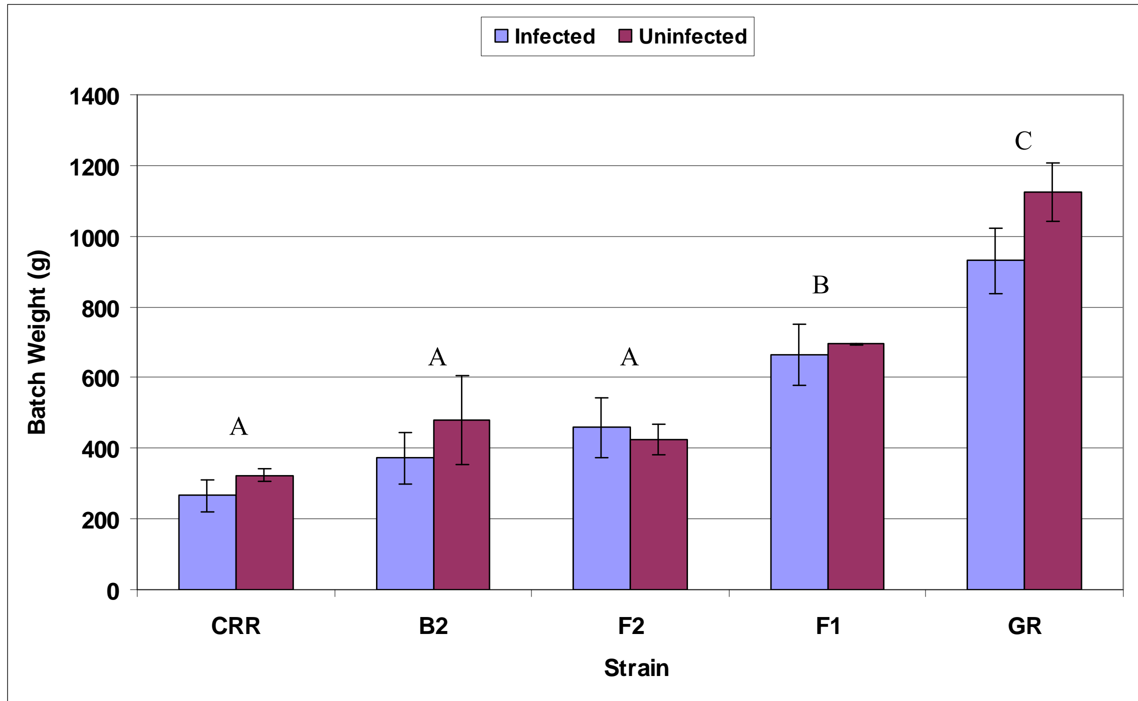


Figure 3.7. Correlation between myxospore count and deformity rating scale for the swimming experiment. This correlation indicates that as deformity rating increases, in general, myxospore count increases, meaning that the developed deformity rating scale can be a good predictor of myxospore count.

Figure 3.8. Average batch weight (g), representing 25 fish per family, for both



infected and uninfected individuals within a strain at the conclusion of the growth experiment.
A-C = significant differences in strain average.

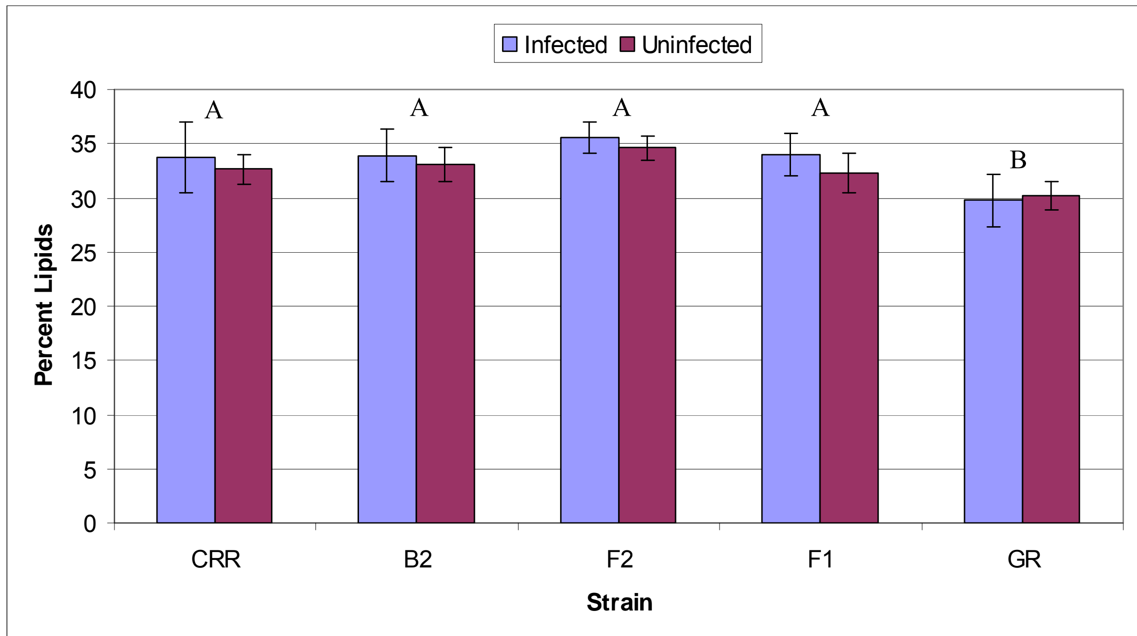


Figure 3.9. Average percent lipids for infected and uninfected individuals within a strain after the conclusion of the growth experiment.
A-B = significant differences in strain average.

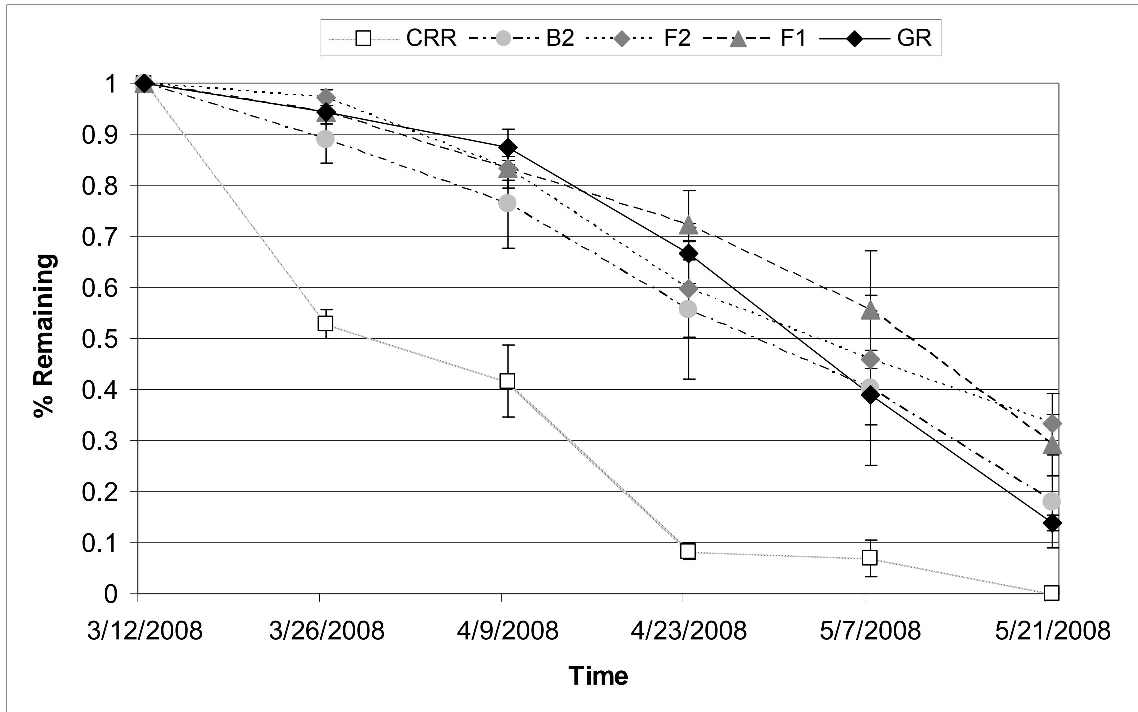


Figure 3.10. The proportion of individuals remaining per strain at each of the sampling periods during the predation experiment. Each point on this graph represents the combined proportion of infected and uninfected individuals remaining for that strain at that time period, and is a compilation of the raw data collected for each strain at each of the sampling periods. No difference in survival was seen between infected and uninfected individuals within a strain at any of the sampling periods.

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APPENDIX II.

Resistant Rainbow Trout in Colorado: Current Status and Uses

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Resistant Rainbow Trout in Colorado: Current Status and Uses

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Salmonid whirling disease, caused by the myxozoan parasite, *Myxobolus cerebralis,* has caused considerable difficulty for fisheries managers and fish rearing facilities in Colorado since its introduction in 1987. Loss of year-classes of wild rainbow trout, leading to population collapse, has occurred in many previously robust rainbow trout fisheries in the state, including the Gunnison, Colorado, Rio Grande, Cache la Poudre, South Platte, Dolores, Fryingpan, Fraser, Roaring Fork, and others. Over a decade of stocking large numbers of fingerling rainbow trout in these locations has failed to remedy the problem. Even with the stocking measures in place, the rainbow trout component of these fisheries has remained at less than 10% of historical densities and biomass. *M. cerebralis* has become established in many fish culture facilities as well. For example, fish in 10 of Colorado's 14 state-operated trout rearing facilities were identified as infected with the parasite as recently as 1997. Presently, five state-operated facilities are considered *M. cerebralis*-positive. Due to the surface-water influences at these facilities, complete eradication of the parasite is not feasible. Fish reared at these facilities are currently subjected to the Colorado Division of Wildlife D-9 policy, which restricts the stocking of fish from these facilities to non-salmonid habitat.

One reason for the severe problems with whirling disease in Colorado is the vulnerability of rainbow trout to *M. cerebralis* infection. Laboratory and field trials have demonstrated that Colorado River rainbow (CRR) trout, which are used as a wild riverine strain in Colorado, are extremely vulnerable to the effects of the parasite. Similarly, varieties such as the Tasmanian and Bellaire strains, used for put-and-take or put-grow-and-take fisheries in Colorado are also very susceptible to infection and can develop very high parasite loads. Until recently, it was thought that all rainbow trout strains were equally vulnerable to the effects of the parasite. In 2000, Richard Vincent of the Montana Department of Fish, Wildlife and Parks evaluated ten separate varieties of rainbow trout including the Arlee, DeSmet, DeChutes, Erwin, Eagle Lake, Finger Lake, Firehole River, Madison River, Missouri River, and Randolf strains. He found that only the DeSmet variety showed some reduced infection severity compared to the others (Vincent 2001). A wild rainbow trout spawning run at Willow Creek, a tributary of Harrison Lake in Montana, is comprised of a stock originating in part from the DeSmet strain. Subsequent testing determined that this "Harrison Lake" strain exhibited increased resistance to the parasite. In 2001, Dr. Mansour El-Matbouli of the University of Munch, while evaluating infection in various varieties of domestic strains of rainbow trout in Germany, also found that fish from the Hofer Trout Farm in Bavaria appeared to have a strong resistance to infection (El-Matbouli et al. 2002). Laboratory experiments conducted concurrently by Dr. El-Matbouli and Dr. Ron Hedrick in Germany and at the University of California-Davis determined that the infection prevalence and severity in the "Hofer" rainbow trout strain was significantly lower than in the Mt. Lassen and Trout Lodge rainbow trout strains (Hedrick et al. 2003). The identification of rainbow trout strains with potential resistance to the parasite in both Montana and Germany were promising findings, and the Colorado Division of Wildlife immediately began the process of importing both strains for follow-up evaluations. Eyed eggs of the Harrison Lake variety were imported to the Colorado Division of Wildlife Fish Research Hatchery (FRH) in Bellvue in the spring of 2002. These fish were obtained from a wild rainbow trout spawning operation at Harrison Lake. Hofer rainbow trout were transported to the FRH as 1-year old fish from the University of California-Davis in the spring of 2003. These fish were originally imported to the University of California-Davis from the Hofer Trout Farm in Germany as eyed eggs.

Two separate objectives were set for the use of the resistant strains. The first was to establish brood stocks of domestic strains to be used in put-and-take, and put-grow-and-take fisheries that would be potential replacements for more susceptible varieties in use by the Colorado Division of Wildlife. The second objective was to develop brood stocks for use in wild rainbow trout fisheries that would be capable of reproducing in the wild and have a sufficient amount of resistance to *M. cerebralis* to re-establish recruitment in these locations. Verification of the testing conducted by previous investigators was an important component of this work. Another important consideration is the evaluation of the strains in various management situations to determine which varieties are best suited for the desired applications.

Three strains of rainbow trout have been used in these evaluations as components in the overarching goal of identifying functional varieties for the aforementioned purposes. These include the Colorado River rainbow (CRR) trout, the Harrison Lake rainbow trout, and the Hofer rainbow trout strains. The Colorado River rainbow trout has a long history, prior to the introduction of *M. cerebralis*, as a highly successful "wild" rainbow trout strain in Colorado. The CRR strain has characteristics typical of other wild varieties, such as exhibiting slow growth, long lifespan, and natural spawning behavior. Another characteristic that has been considered beneficial is the tendency of the fish to take up permanent residency near their natal spawning areas. Rainbow trout are not native to Colorado, and the Colorado River rainbow trout, therefore, is not a native strain. The strain was derived from a combination of stocking events by private, State, and Federal hatcheries in the late 1800's and early 1900's. However, the excellent reproduction and recruitment success of the strain suggest that it was quite well adapted to rivers in Colorado, and it is considered a naturalized strain. As a result, an effort has been made to integrate the resistant strains into the wild rainbow trout recovery effort without completely abandoning the Colorado River rainbow trout strain, in order to maintain some of the desired characteristics that made it successful in Colorado.

The Harrison Lake rainbow trout strain is considered a "wild" variety best suited for lake and reservoir environments. Typical of most other wild varieties, the Harrison Lake strain is slow-growing, long-lived, has a fusiform body conformation, and is a prolific spawner in natural environments. More specifically, the strain is an inlet spawner, and the fry tend to migrate downstream out of spawning areas and into downstream lakes or reservoirs very soon after emergence. The strain is reported to feed primarily on zooplankton and tends to occupy open water areas rather than the shoreline of these water bodies.

The Hofer rainbow trout strain is a highly domesticated variety that has been reared in a hatchery environment for over 100 years, principally as a food fish. As a result, the strain is extremely fast-growing and early maturing. The ability of the strain to survive and reproduce in the wild is unknown. Cross-breeding of the Hofer strain with wild-type strains, such as the Colorado River and the Harrison Lake rainbow trout strains would presumably make this strain better adapted to reproduction and survival in natural systems.

Several laboratory, hatchery, and field studies have been conducted by the Colorado Division of Wildlife on these strains and their crosses over the course of the last few years. There are also ongoing projects for which results are not yet available. The following descriptions of these experiments are short summaries of more detailed narratives available in the Federal Aid in Fish Restoration Project F-394, Salmonid Disease Studies, for the years 2005-2008, and published laboratory experiments in Schisler et al. (2006).

Pure Harrison Lake Strain

Laboratory and hatchery experiments conducted in Colorado have substantiated the earlier work by the Montana Department of Fish, Wildlife and Parks, indicating that the Harrison Lake strain is more resistant to infection and develops lower parasite loads than other strains of rainbow trout. The observed resistance is not as dramatic as that observed in the Hofer strain, but the Harrison Lake strain does demonstrate a marked advantage over other strains.

In one laboratory experiment, the Harrison Lake strain was compared to Colorado River cutthroat trout, and the Colorado River and Big Thompson River rainbow trout strains for susceptibility to *M. cerebralis*, exposed as two-month old fingerlings. Three replicates of thirty fish from each strain were exposed to 2,358 triactinomyxons (TAMs) per individual. TAMs are the stage of the parasite that infects the fish, and an exposure of 2,000 - 3,000 TAMs per individual at this age is considered a relatively high exposure level. This level of exposure results in infections similar to that seen in the wild where population-level impacts would be observed. The fish were reared for five months and then evaluated for infection prevalence and severity using the pepsin-trypsin digest (PTD) method. Fifteen fish from each of three replicate groups for each strain were evaluated with PTD. The Harrison Lake strain had the lowest infection prevalence, with 77.7% of fish found to be infected (Table 1). Severity of infection is determined by the enumeration of mature parasites (myxospores) present in the head cartilage of a fish. In this experiment, an average of 137,523 myxospores was found in the Harrison Lake strain, which was the lowest of the strains tested. Growth in the unexposed Harrison Lake strain individuals was similar to the other unexposed "wild" varieties tested in this experiment. However, growth in the Harrison Lake strain, as measured by weight, was significantly better than the other strains when exposed to *M. cerebralis* (Table 2).

A second experiment was conducted at the Poudre Rearing Unit (a facility known to harbor *M. cerebralis*) to evaluate the effects of chronic long-term exposure to the parasite on the Harrison Lake strain compared with a commonly used hatchery strain, the Tasmanian rainbow trout strain. Seven hundred-fifty fish of each variety, approximately 3-inches in length and five months of age, were transported to the facility and reared together in a single raceway for one year. The Harrison Lake strain was adipose-clipped to distinguish between the two strains. Sixty-fish samples were collected from each strain once the fish had been at the facility for four months, and at subsequent two month intervals, to test for infection due to *M. cerebralis*. No myxospores were found in either strain during the first three collections. On the fourth collection (at 10 months), an average of 26,104 myxospores were found in the Harrison Lake strain, and 109,402 were found in the Tasmanian strain. On the fifth collection (at 12 months), an average of 38,857 myxospores were found in the Harrison Lake strain, and 161,276 were found in the Tasmanian strain. The differences were highly significant for both sampling events (Figure 1). Growth of the Harrison Lake strain was much slower than the Tasmanian strain throughout the rearing period (Figure 2). The Harrison Lake strain did have the potential to produce much lower parasite loads than other strains currently used in Colorado. The downside of the Harrison Lake strain from a production standpoint was the slow growth that was evident for this strain of rainbow trout. Use of the Harrison Lake strain in some capacity, either as a wild strain or crossed with other varieties remained a possibility.

Table 1. PTD and PCR results, at five months post-exposure, of Colorado River cutthroat, Colorado River rainbow, Harrison Lake rainbow, and Big Thompson River rainbow trout exposed to *M. cerebralis* at a dose of 2,358 TAMS per individual as two month-old fry.

			PTD Results	
Strain	Replicates	N	Myxospore counts (Infection Severity)	Percent positive (Infection Prevalence)
Colorado River Cutthroat	3	45	204,572	100.0
Colorado River Rainbow	3	45	335,327	95.5
Harrison Lake Rainbow	3	45	137,523	77.7
Big Thompson Rainbow	3	45	675,633	100.0

Table 2. Weight and length information, at five months post-exposure, for Colorado River cutthroat, Colorado River rainbow, Harrison Lake rainbow, and Big Thompson River rainbow trout, both exposed and not exposed to *M. cerebralis*. Subscripts 'a', 'b' and 'c' indicate significant differences.

	Not Exposed to *M. cerebralis*		Exposed to *M. cerebralis*	
Strain	Weight (grams)	Length (cm)	Weight (grams)	Length (cm)
Colorado River Cutthroat	8.0 a	9.6 a	6.5 b	8.8 a
Colorado River Rainbow	7.5 a	9.1 a	7.0 b	9.0 a
Harrison Lake Rainbow	7.3 a	9.2 a	7.7 a	9.1 a
Big Thompson Rainbow	5.7 b	8.1 b	5.6 c	8.0 b

Figure 1. Myxospore counts found in pure Harrison Lake and Tasmanian rainbow trout strains reared at the Poudre Rearing Unit for 10 and 12 months.

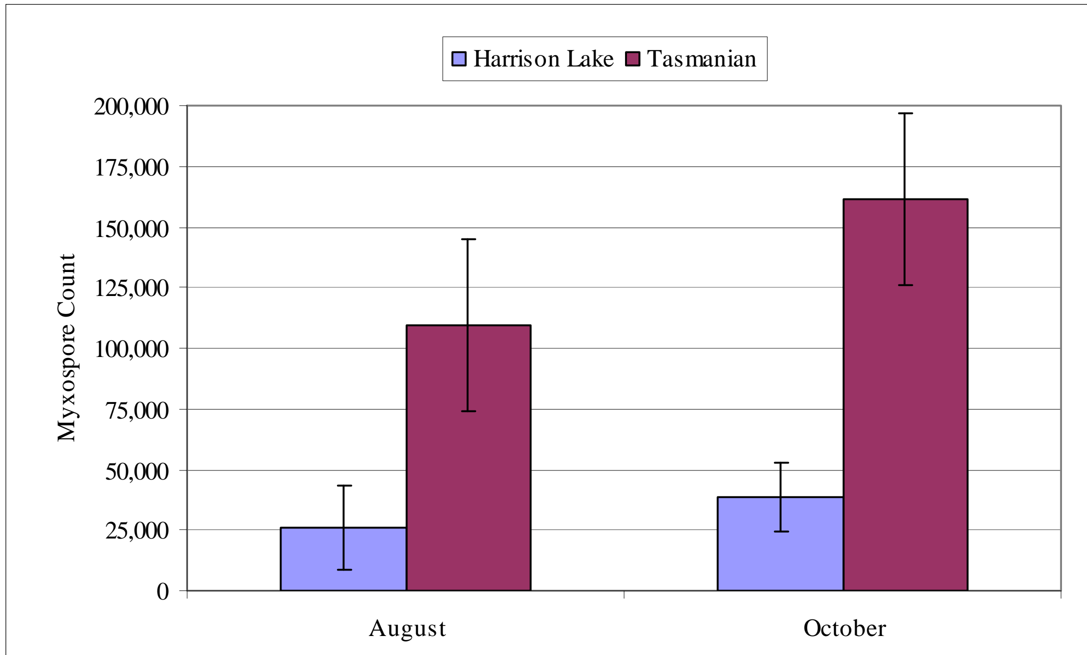
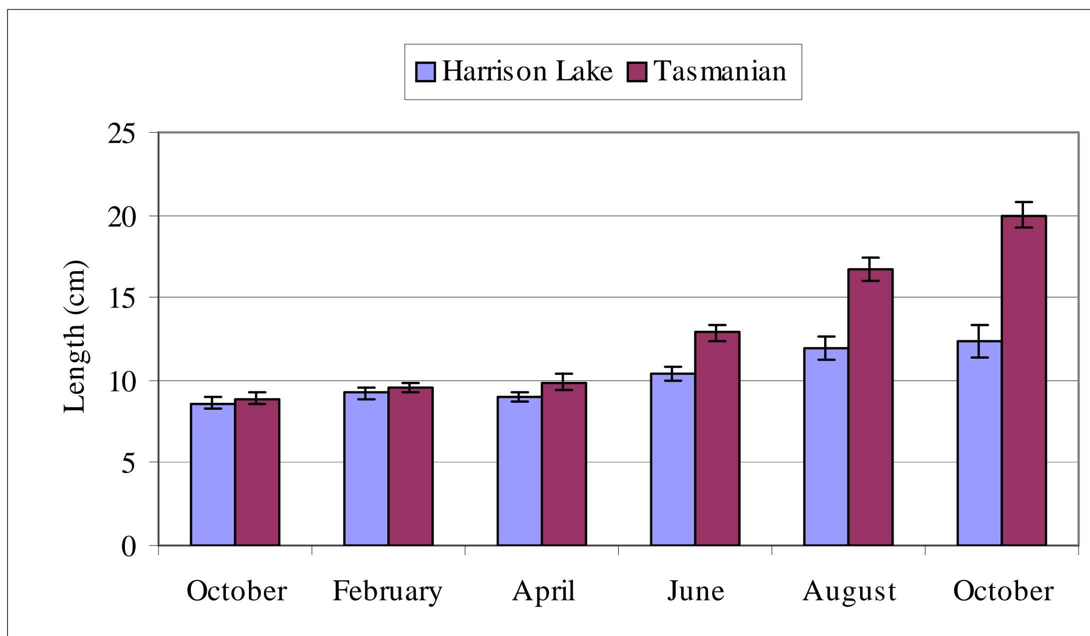


Figure 2. Average lengths of Harrison Lake and Tasmanian rainbow trout strains reared over the course of one year, in the Poudre Rearing Unit raceways.



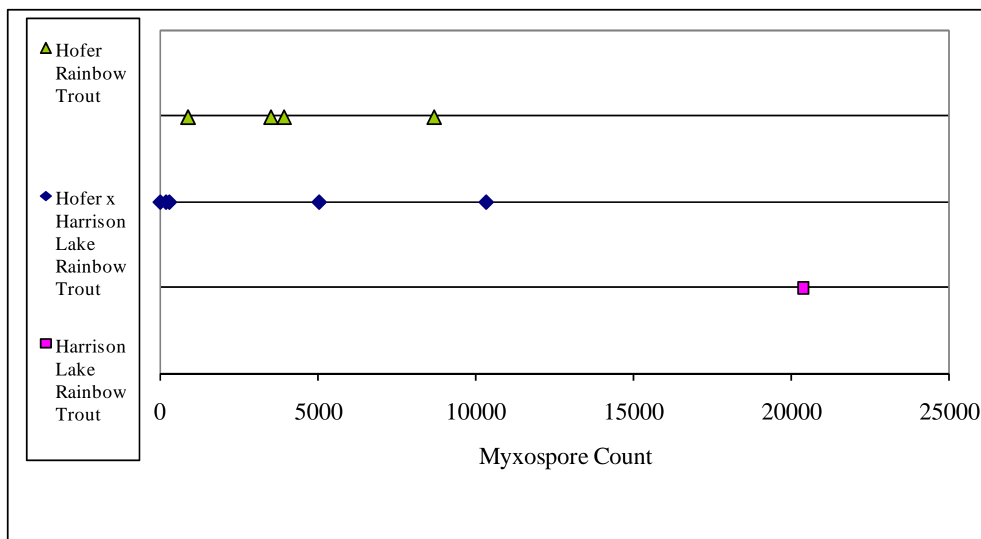
Hofer and Hofer-Harrison Lake Crosses

While the Harrison Lake strain appeared to have some promise as a resistant strain, the Hofer strain was reported to be much more resistant to the effects of *M. cerebralis*. An experiment was conducted in a laboratory setting, in which the Harrison Lake strain was evaluated against the Hofer strain. In addition, a 50:50 cross of the two strains was created by fertilizing Hofer eggs with Harrison Lake strain milt, which was evaluated in conjunction with the two pure strains. The objective was two-fold; to determine if the Hofer strain was substantially more resistant to *M. cerebralis* than the Harrison Lake strain, and to determine how a cross of the two strains would perform when exposed to the parasite. Five replicate groups of the pure Hofer, one replicate of the pure Harrison Lake, and five replicates of the Hofer-Harrison (50:50) cross were used in this experiment. Thirty fish per each replicate group were exposed to 2,000 TAMs per individual as two-month old fingerlings. The fish were reared for five months post-exposure. At the end of the rearing period, ten fish from each family were evaluated for infection using the PTD method. The Harrison Lake strain performed fairly well again in this experiment, producing an average of only 20,398 myxospores per fish (Table 3). However, the pure Hofer strain was even more resistant to the parasite, developing an average of 3,593 myxospores per fish. The Hofer-Harrison (50:50) cross developed a very low myxospore count as well, with an average of 3,168 per fish. These results indicated that out-crossing the Hofer strain with the Harrison Lake strain would not significantly dilute the resistance. The resistance found in the two strains may actually be somewhat enhanced in the Hofer-Harrison (50:50) cross.

Table 3. Overall myxospore counts and prevalence of infection in Hofer, Harrison Lake, and Hofer-Harrison (50:50) crosses exposed to 2,000 TAMs per individual.

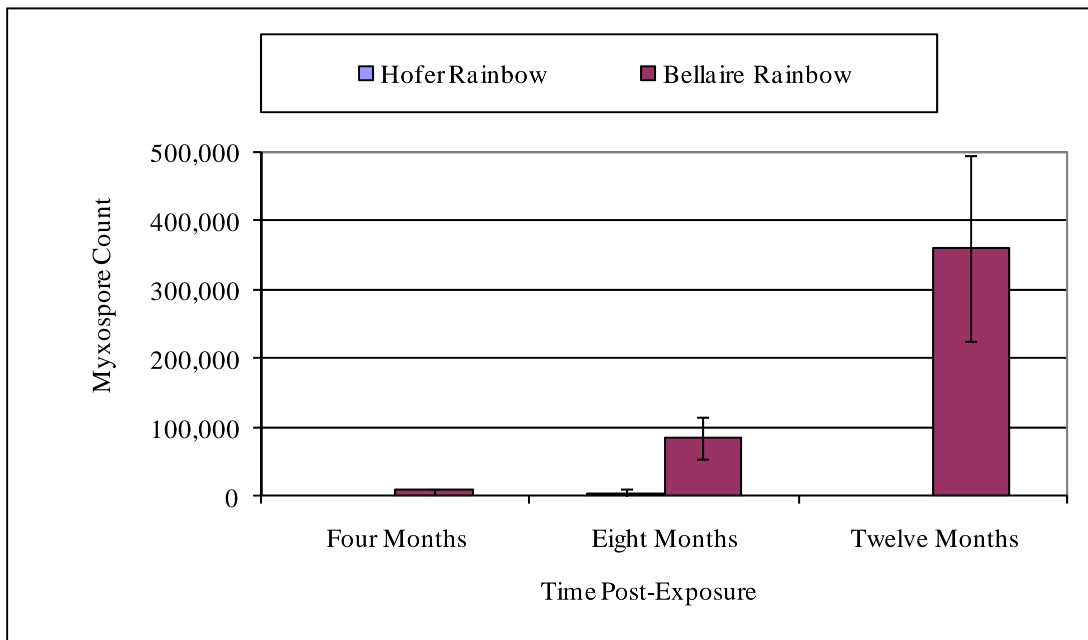
Strain	Families	N	Spore Count Mean	PTD Infected (%)
Hofer Rainbow	5	50	3,593	30.0
Hofer- Harrison Lake (50:50) Cross	5	50	3,168	30.0
Harrison Lake Rainbow	1	10	20,398	40.0

Figure 3. Average myxospore counts for pure Hofer, Harrison Lake, and Hofer-Harrison (50:50) crosses. Each point represents the average myxospore count for each individual family.



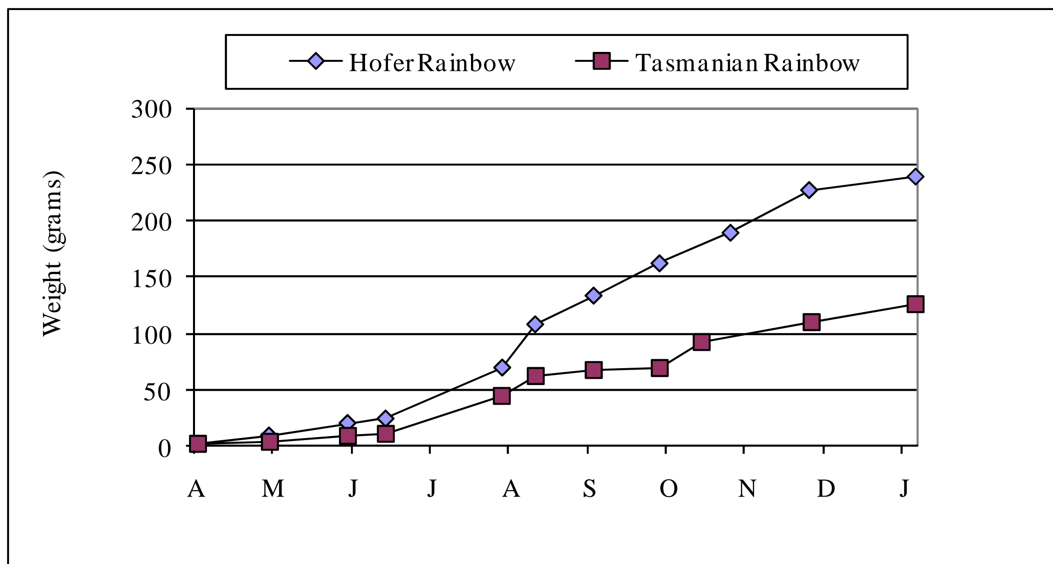
A follow-up evaluation of the Hofer strain was then conducted in a hatchery setting. An experiment similar to the earlier Harrison Lake and Tasmanian rainbow trout experiment was performed at the Colorado Division of Wildlife Poudre Rearing Unit, using pure Hofer rainbow trout. In this experiment, another commonly used hatchery strain, the Bellaire rainbow trout strain, was used for comparison. Seven month-old Hofer and 9.5 month-old Bellaire rainbows were brought to the facility as 5-inch long fingerlings. The difference in age was due to the faster growth of the Hofer strain, making it necessary to use younger Hofer strain fish to size-match with the Bellaire strain fish. This situation provided an advantage to the Bellaire strain with regard to infection, since rainbow trout become more resistant as they get older and larger. The Hofer strain fish were adipose clipped for identification purposes, and the fish were reared in the same raceway for one year. Thirty fish per strain were sampled at four, eight, and twelve months after being brought to the facility. At four months, no myxospores were found in any of the Hofer strain rainbow trout, while the Bellaire strain rainbow trout had an average of 7,314 myxospores (Figure 4). At eight months, the Hofer strain had an average myxospore count of 3,440, with only three individuals in the sample identified as infected. The Bellaire strain had an average myxospore count of 84,993, and every fish was identified as infected. At 12 months, no infected fish were found in the sample from the Hofer strain, while the average myxospore count among Bellaire strain was 361,099.

Figure 4. Myxospore counts found in pure Hofer and Bellaire rainbow trout reared at the Poudre Rearing Unit for 4, 8, and 12 months.



A second hatchery experiment was conducted to evaluate the pure Hofer strain at the Colorado Division of Wildlife Chalk Cliffs Rearing Unit, a *M. cerebralis*-positive facility, in which Tasmanian rainbow trout were used as the comparison group. The two strains were brought to the facility as eyed eggs, hatched during the same week, and reared in parallel throughout the production cycle. Samples collected at 3 months post-hatch were identified as negative with histology for both the Hofer and Tasmanian rainbow trout strains. Samples collected at 5 months post-hatch also resulted in negative results for both histology and PTD in both strains. At 9.5 months post-hatch, infection prevalence in the Hofer strain was 73.3%, and prevalence in the Tasmanian strain was 96.7% Average myxospore count in the Hofer strain was 5,175 (N = 30, SD = 7,643), compared to 48,883 (N = 30, SD = 50,825) in the Tasmanian strain. Growth, as measured by weight, was much faster in the Hofer strain than the Tasmanian strain (Figure 5). Growth, as measured by length, was also quite different between the strains. At 9.5 months post-hatch, average length for Hofer strain was 23.6 cm (N = 60, SD = 1.5), compared to 18.5 cm (N = 60, SD = 2.4) for the Tasmanian strain. At 12 months post-hatch, the Hofer strain averaged 28.4 cm (N = 50, SD = 2.8), while the Tasmanian strain averaged 22.3 cm (N = 50, SD = 3.3).

Figure 5. Average weights of pure Hofer and Tasmanian rainbow trout reared at Chalk Cliffs for one year (data shown for last 10 months of growth).



These results for the Hofer strain, in both growth and resistance to *M. cerebralis,* suggested that the pure Hofer rainbow trout strain may be an ideal strain for hatchery production purposes. However, there were some characteristics of the pure Hofer strain, perhaps due to their long period of domestication, which we felt could eventually cause problems with the stock. For instance, the strain tends to be very surface-oriented and has been observed in raceways to swim for extended periods of time with their backs completely out of the water. The strain also has very little fright response to disturbance, and some hatchery managers have reported that the strain has a sensitivity to formalin. Finally, the strain has been shown to have low heterozygosity

(El-Matbouli et al. 2006) and therefore may lack genetic diversity. The Harrison Lake strain, on the other hand, appears to be free of these limitations. Given that the Hofer-Harrison crosses produced myxospore counts similar to the pure Hofer strain, and exhibited intermediate characteristics to the Hofer and Harrison strains in the laboratory experiment, producing a Hofer-Harrison blended stock seemed to be a logical approach for long-term domestic strain production. A higher proportion of Hofer to Harrison genetics would be desirable from a production standpoint, to maintain the high growth and superior *M. cerebralis* resistance of the Hofer strain. To test this theory, a Hofer-Harrison (75:25) cross was created by crossbreeding Hofer-Harrison (50:50) strain fish, with pure Hofer strain fish.

A second hatchery experiment was conducted at the Chalk Cliffs Rearing Unit using this Hofer-Harrison (75:25) cross. As with the previous experiment, Tasmanian rainbow trout were used as a comparison group. The two strains were brought to the facility as eyed eggs and reared in parallel throughout the production cycle. Tasmanian rainbow trout developed an average myxospore count of 5,106 (SD = 8,999) after eight months on the facility. No myxospores were found in any of the Hofer-Harrison (75:25) strain fish tested at eight months. The Tasmanian rainbow trout developed an average myxospore count of 158,437 (SD = 239,901) after 16 months of growth at the Chalk Cliffs rearing facility. Again, no myxospores could be found in any of the Hofer-Harrison (75:25) rainbow trout, even after 16 months at the facility. Growth of the Hofer-Harrison (75:25) cross was substantially greater than the Tasmanian strain. Average length was 145 mm (SD = 19.1) in the Tasmanian strain compared with 182 mm (SD = 28.9) in the Hofer-Harrison (75:25) cross after eight months. At 16 months, average length of the Tasmanian strain was 221 mm (SD = 37.0), and average length of the Hofer-Harrison (75:25) cross was 315 mm (SD = 28.6). Weight differences were even more dramatic. Average weight at eight months for the Tasmanian strain was 35.8 g (SD = 13.5) compared to 75.7 g (SD = 27.1) for the Hofer-Harrison (75:25) cross. At 16 months, average weight was 123.6 g (SD = 51.7), compared with 332.4 g (SD = 94.20) for the Hofer-Harrison (75:25) cross. These results were quite similar to the results observed in the previous hatchery experiment with the pure Hofer strain. Growth and resistance to *M. cerebralis* did not appear to be compromised by outbreeding the Hofer strain with the Harrison Lake strain in a 75:25 ratio.

At this time, brood stocks of pure Hofer strain and Hofer-Harrison crosses have been established at the Colorado Division of Wildlife Fish Research Hatchery, Poudre Rearing Unit, and the Crystal River Hatchery. Hofer and Hofer-Harrison eggs have been transported to the majority of Colorado Division of Wildlife trout rearing facilities for production purposes. The Hofer strain and Hofer-Harrison crosses have consistently demonstrated superior growth rates compared to other domestic strains of fish reared in these facilities. For instance, growth records at Bellvue-Watson from 2006 and 2007 show pure Hofer rainbow trout averaging 130 mm at 6 months of age, compared to less than 100 mm for strains such as Erwin rainbow trout and Bellaire-Snake River cutthroat crosses. At Crystal River Hatchery, Hofer-Harrison (75:25) crosses at eight months post-eye-up were nearly 180 mm, compared to strains such as Bellaire rainbow trout, Tasmanian rainbow trout, and Snake River cutthroat trout that were less than 140 mm.

Hofer and Harrison Lake Field Trials

Two separate field trials were conducted using the Hofer strain and Hofer-Harrison (75:25) cross fish produced at the Chalk Cliffs Rearing Unit. Fish of the pure Hofer strain were evaluated in 2006, and fish of the Hofer-Harrison (75:25) cross were evaluated in 2007. In both cases the Hofer or Hofer-Harrison cross rainbows were compared with the Tasmanian rainbow trout strain with respect to return-to-creel and angler satisfaction. Two front range reservoirs, Flatiron and Pinewood reservoirs, were used as study locations. Both reservoirs are typical of coolwater reservoirs on the front range of Colorado in which fish are stocked for immediate recreational angling and harvest, and managed as put-and-take fisheries.

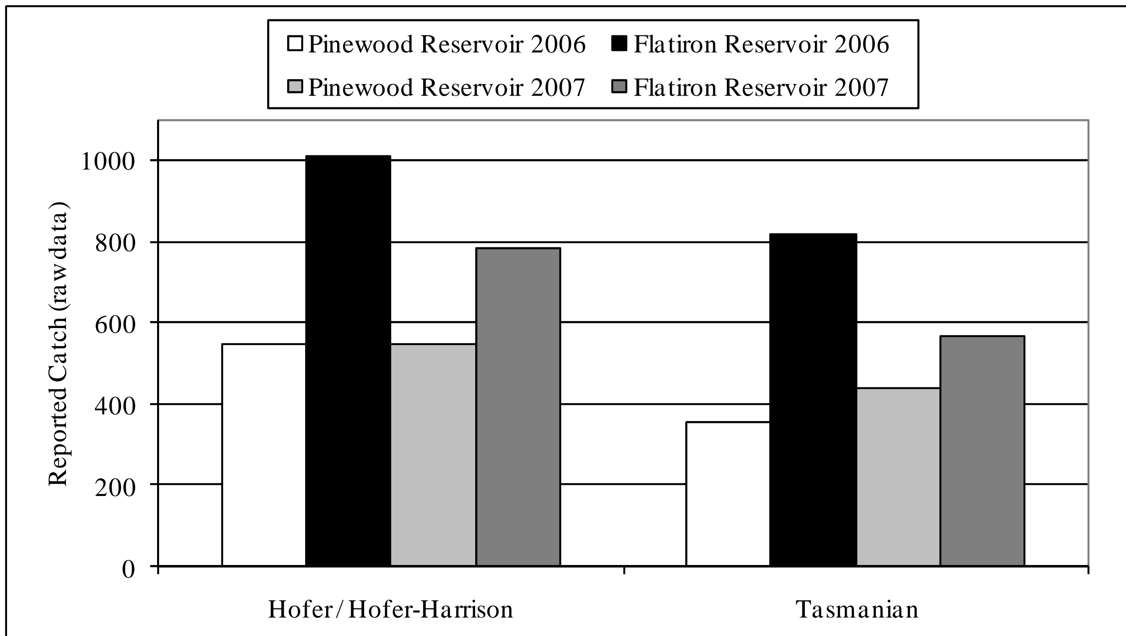
One half of the fish released on each scheduled stocking occasion were Hofer strain or Hofer-Harrison (75:25) crosses, and the other half were of the Tasmanian strain. The fish had been marked prior to stocking with fin clips to identify the fish by strain. Hofer strain or Hofer-Harrison (75:25) crosses were marked with adipose clips, and Tasmanian strain fish were marked with pelvic fin clips.

A creel schedule was created in which anglers were surveyed on both weekend days of every week, and two randomly chosen weekdays per week during the majority of the open water fishing season. Angler counts were conducted five times daily throughout the daylight hours. Angler interviews were conducted between count times. Because the strains were differentially marked with fin clips, the creel clerk could easily distinguish between the two, and catch estimates were made for both strains. During the angler interviews, additional questions were asked to determine if there was an angler preference between the strains. If there was a preference, the anglers were asked to describe which characteristics were most important in making that determination.

In 2006, a much higher proportion of the Hofer rainbow trout were captured than the Tasmanian rainbow trout. Total reported catch was 34.6% higher for the Hofer strain than the Tasmanian strain in Pinewood Reservoir. Total reported catch was 19.2% higher for the Hofer strain than the Tasmanian strain in Flatiron Reservoir (Figure 6). When asked about strain preference based on the fin clip marks, 22.6% of the 1,831 respondents chose the Hofer strain, compared to 3.2% that chose the Tasmanian strain. The remaining 74.2% of respondents had no preference.

In 2007, the results followed the same pattern for the Hofer-Harrison (75:25) cross as was observed with the pure Hofer strain in the previous year. At Flatiron Reservoir, 27.7% higher catch was reported for the Hofer-Harrison (75:25) cross than for the Tasmanian strain. At Pinewood Reservoir, a 24.7% higher catch was reported for the Hofer-Harrison (75:25) cross than for the Tasmanian strain. When asked about strain preference based on the fin clip marks, 9.5% of the 2,441 respondents chose the Hofer-Harrison (75:25) cross, compared with 1.1% that chose the Tasmanian strain. The remaining 89.3% had no preference. These results show that the Hofer strain and Hofer-Harrison (75:25) cross both perform better as a catchable plant than the Tasmanian strain with respect to return-to-creel. Additionally, anglers tend to have no preference with regard to strain, but slightly favor the Hofer strain and Hofer-Harrison (75:25) cross over the Tasmanian strain.

Figure 6. Reported catch by strain for Flatiron and Pinewood Reservoirs in 2006 and 2007.



Fish stocked as catchable size for immediate harvest have a different role than fish stocked as fingerlings, which are expected to have good survival in the face of predation and other natural conditions as they grow in the wild to catchable size. Presumably, fish with more Hofer background would be at a disadvantage as a fingerling plant compared to crosses containing more Harrison Lake background, because of the domesticated history of the Hofer strain. However, this theory needs to be thoroughly tested to determine which combination of the two would be best suited for fingerling plants. An ongoing evaluation is being conducted of all crosses of the Hofer and Harrison Lake strains that are currently available for testing. These include the pure Hofer, pure Harrison, Hofer-Harrison (50:50) and Hofer-Harrison (75:25) crosses. A fifth cross made from Hofer-Harrison (75:25) cross, backcrossed with pure Hofer strain rainbow trout, which is essentially 7/8 Hofer strain and 1/8 Harrison Lake strain (87.5:12.5), is also included in these evaluations. These live-release studies are currently being conducted at Parvin Lake, Northwest of Fort Collins, to determine if any particular strain or cross is better adapted as a fingerling plant in a reservoir setting. Additional work is being conducted to evaluate infection severity of all of these Hofer-Harrison varieties in combination, when exposed to high doses of *M. cerebralis* parasites as fingerlings in a pond setting at the Poudre Rearing Unit. The creation of a wild brood stock of Hofer-Harrison crosses is also being attempted at Catamount Reservoir, near Steamboat Springs, Colorado, using plants of the Hofer-Harrison crosses.

Such enthusiasm for the Hofer-Harrison crosses has been generated that suggestions have been made that they may be useful as a riverine strain. As a result, two river plant evaluations of the Hofer-Harrison (87.5:12.5) cross are in progress. Given that the Harrison Lake strain has a tendency to migrate downstream, it is quite possible that the crosses will not remain as resident

fish in these situations. The domestic background of the Hofer strain may also put the Hofer-Harrison crosses at a disadvantage in wild riverine environments. Nonetheless, in 2008, 2,200 fish of the Hofer-Harrison (87.5:12.5) cross (>200 mm) were adipose clipped, Floy tagged, and stocked in the Cache la Poudre River. Two thousand fish of this cross were similarly marked and stocked in the Middle Fork of the South Platte River, and 1,000 fish of the cross were similarly marked and stocked in the South Fork of the South Platte River upstream of Antero Reservoir. Both of these locations are known to be heavily infected with *M. cerebralis*. Survival and infection severity will be evaluated for these plants in the coming year to determine if this variety would be a possible candidate for this purpose.

Colorado River and Hofer Rainbow Crosses

Evaluations of the Colorado River rainbow (CRR) trout and their crosses have followed a different research program than the Hofer-Harrison crosses. The CRR has been a preferred strain for wild rainbow trout populations in rivers in Colorado because of the historical characteristics of the strain that have led to successful reproduction and survival. However, because the strain is extremely susceptible to *M. cerebralis*, it is now at a distinct disadvantage in rivers where it used to thrive. With the strain virtually eliminated in these waters, little hope for natural selection of resistance exists. In locations such as the Colorado River and Gunnison River, very large numbers (30,000 - 60,000) of fingerling CRR trout have been stocked annually for over a decade in an attempt to maintain the rainbow trout component of these fisheries. No natural recruitment has occurred, and the stocked fingerlings have had extremely low survival, resulting in rainbow trout biomass of less than 10% of historic levels in these locations. Increasing survival by integrating some resistance to *M. cerebralis* into these CRR populations was a possible solution to the problem. The original intent of this research was to enhance the resistance of the CRR strain through crossbreeding with the Hofer strain, while retaining as much of the CRR genetic background as possible in this stock destined for wild rainbow population recovery efforts.

To test the resistance of the Hofer-CRR crosses, the first experiment consisted of the pure CRR and Hofer strains, and an F1 (50:50) cross of the Hofer and CRR strains. Five families of Hofer rainbows, two families of pure CRR, 29 families of Hofer (female) x CRR (male), and three reciprocal cross families of CRR (female) x Hofer (male) were created. Eggs from each mated pair were kept separate during incubation. Thirty-five fish from each family were exposed to 2,000 *M. cerebralis* TAMs per individual as two-month old fingerlings and reared for five months post-exposure. Ten fish from each family were evaluated for infection from *M. cerebralis* using the PTD method. Infection was significantly more severe in the CRR strain than in the pure Hofer strain and the F1 rainbow trout families (Table 4). The myxospore counts in the reciprocal crosses were also lower than in the pure CRR families. Individual families of F1 crosses produced a wide range of myxospore counts (Figure 7). These results demonstrated that the resistance to *M. cerebralis* infection could be inherited in some individuals in the F1 cross between the pure strains. Even more interesting was the tendency of some families to inherit more resistance than others. While some individuals and families developed parasite loads similar to the pure CRR parental strain, others showed a high resistance to the parasite. Those individuals could presumably survive in the wild in areas where *M. cerebralis* had eliminated natural recruitment in previously pure CRR populations.

Because one of the original goals of this research was to maintain as much of the CRR genetic background as possible, a second laboratory experiment was conducted to determine if further out-breeding of the F1 cross with pure CRR rainbow trout would dilute the resistance in the offspring. In this experiment, three pure CRR families, three pure Hofer families, and 10 F1 families were created. In addition, 16 B2 cross families were made. The B2 (25:75) cross was an F1 cross, backcrossed with the pure CRR strain. As with the first experiment, these fish were exposed to *M. cerebralis* at a rate of 2,000 TAMs per individual at two months of age. The fish were then reared for five months to allow full development of the myxospores. The results of this experiment were very similar to the first experiment (Table 5). The pure Hofer families

developed very low myxospore counts, the pure CRR families developed very high myxospore counts, and the F1 families produced intermediate myxospore counts. The B2 families developed myxospore counts intermediate to the pure CRR and F1 families (Figure 8).

The results of these first two experiments showed that continued out-breeding of the Hofer-CRR crosses with the pure CRR strain results in a loss of resistance. Some individuals and some families in the B2 cross maintained a high level of resistance, which could still provide enough resistance in natural situations to eventually overcome the effects of *M. cerebralis*. However, the resistance is rapidly lost in most individuals and families due to dilution of the Hofer strain genetic background. This loss is substantial enough that further back-crossing of the Hofer-CRR cross with the pure CRR strain may be counterproductive towards the goal of reestablishing wild rainbow trout populations where the parasite exists.

A third experiment was conducted to validate the results of the first two experiments, and to account for another possible outcome of these crosses in the wild, the F2 (50:50) cross. This cross is a result of an F1 (50:50) cross spawning with another F1 (50:50) cross. One would expect a large proportion of offspring produced in a natural setting where F1 fish have been stocked to be of this variety. In this experiment, 10 pure Hofer families, 10 pure CRR families, 20 F1 families, 20 B2 families, and 20 F2 families were all exposed to *M. cerebralis* as 2-month old fingerlings and reared for five months. Ten fish from each family were evaluated using the PTD method, as in the previous experiments. The results determined that the resistance to *M. cerebralis* infection in the F2 cross fish was intermediate to the B2 cross and the F1 cross (Table 6). In addition, the distribution of myxospore counts, by family, was not as wide as seen in the B2 cross (Figure 9).

Because of the rapid decrease in resistance found in the B2 cross compared with the F1 cross, the laboratory results suggest that the F1 cross would be a much better candidate for reintroduction efforts in rivers where rainbow trout populations have been lost. While B2 individuals have not been crossed with other B2 individuals, or with pure CRR fish, in laboratory studies, the assumption is that an even greater loss of resistance would occur if these fish were to spawn with those strains in the wild. Some individual offspring would still retain the resistance, and heavy selection pressure would strongly favor those individuals for survival. This approach could eventually bring back wild populations in the presence of whirling disease. However, the alternative of using the F1 cross for stocking is the preferred method if more rapid re-population is the goal. This approach assumes that the loss of wild characteristics in the F1 cross does not outweigh the benefits of enhanced resistance.

The evaluation of the F2 cross shows that if exclusively F1 fish were stocked, and F2 fish were generated as offspring of those stocking events, the loss in resistance would not be overwhelming. A high proportion of the offspring in the F2 generation would retain resistance to *M. cerebralis* and would provide a relatively rapid recovery of the population if infection from the parasite was the only limiting factor.

Table 4. Average myxospore counts and prevalence of infection in the Hofer and Colorado River rainbow trout strains, and crosses of those strains (male CRR x Hofer female and reciprocal cross), exposed to 2,000 TAMs per fish.

Strain	Families	N	Spore Count Mean	PTD Infected (%)
Hofer Rainbow	5	50	3,593	30.0
Hofer (f) x Colorado River Rainbow (m)	29	290	84,400	82.4
Colorado River (f) x Hofer Rainbow (m)	3	30	42,376	86.7
Colorado River Rainbow	2	20	210,982	100.0

Figure 7. Average myxospore counts of the Hofer and Colorado River rainbow trout strains, and the F1 (50:50) cross of these strains. Each point represents the average myxospore count for each individual family.

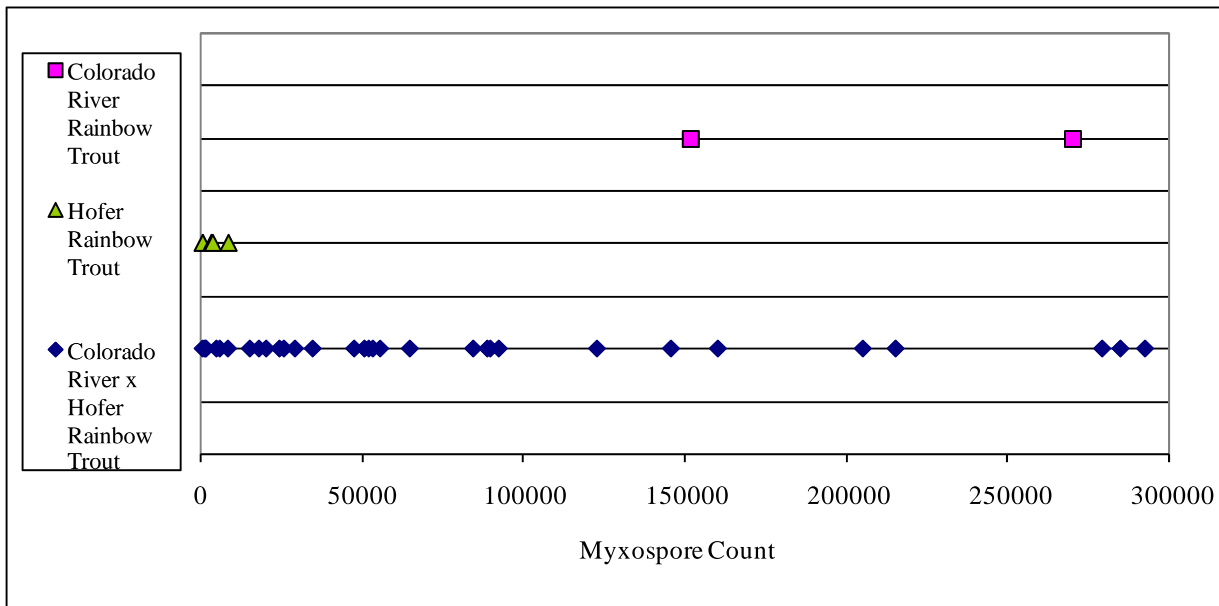


Table 5. Average myxospore counts and prevalence of infection for the Hofer and Colorado River (CRR) rainbow trout strains, and the F1 (50:50) and B2 (75:25) crosses.

Strain	Families	N	Spore Count Mean	PTD Infected (%)
Hofer Rainbow	3	30	1,482	49.6
F1 (50:50)	10	100	47,128	77.0
B2 (25:75)	16	160	125,167	93.0
Colorado River Rainbow	3	30	232,973	100.0

Figure 8. Average myxospore counts for the three Hofer, three Colorado River rainbow (CRR) ten F1 [Hofer-CRR (50:50)] and 16 B2 [Hofer-CRR (25:75)] families. Each point represents the average myxospore count for each individual family.

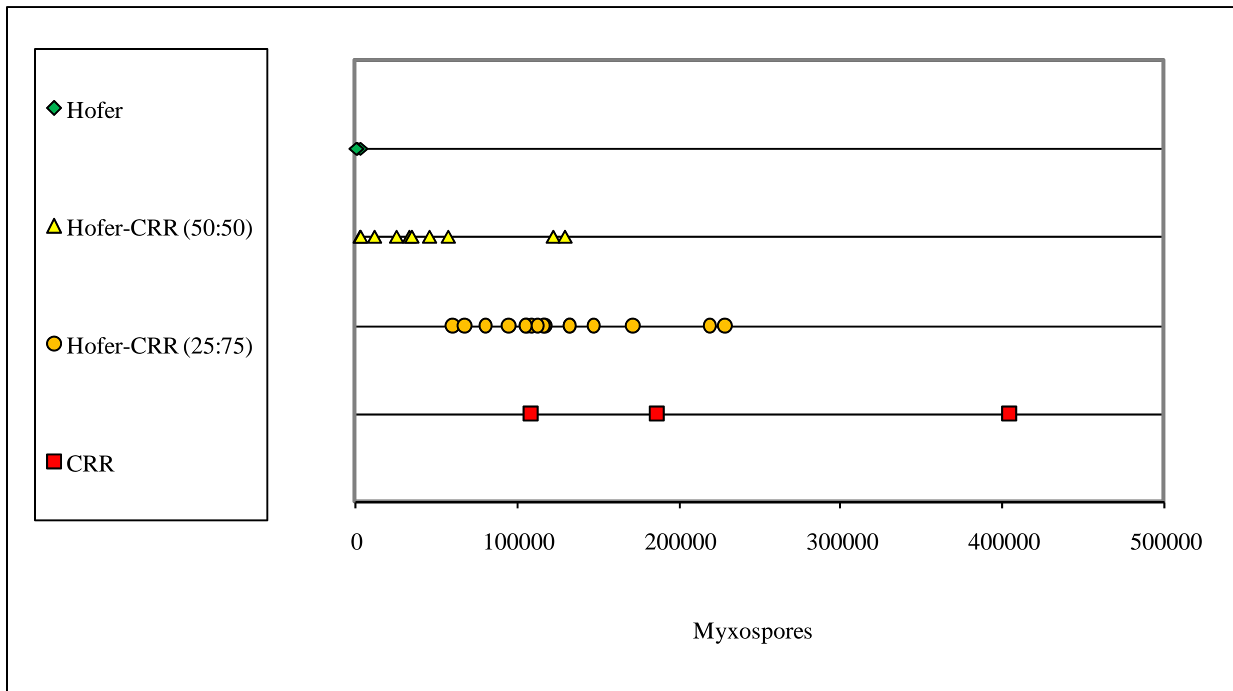
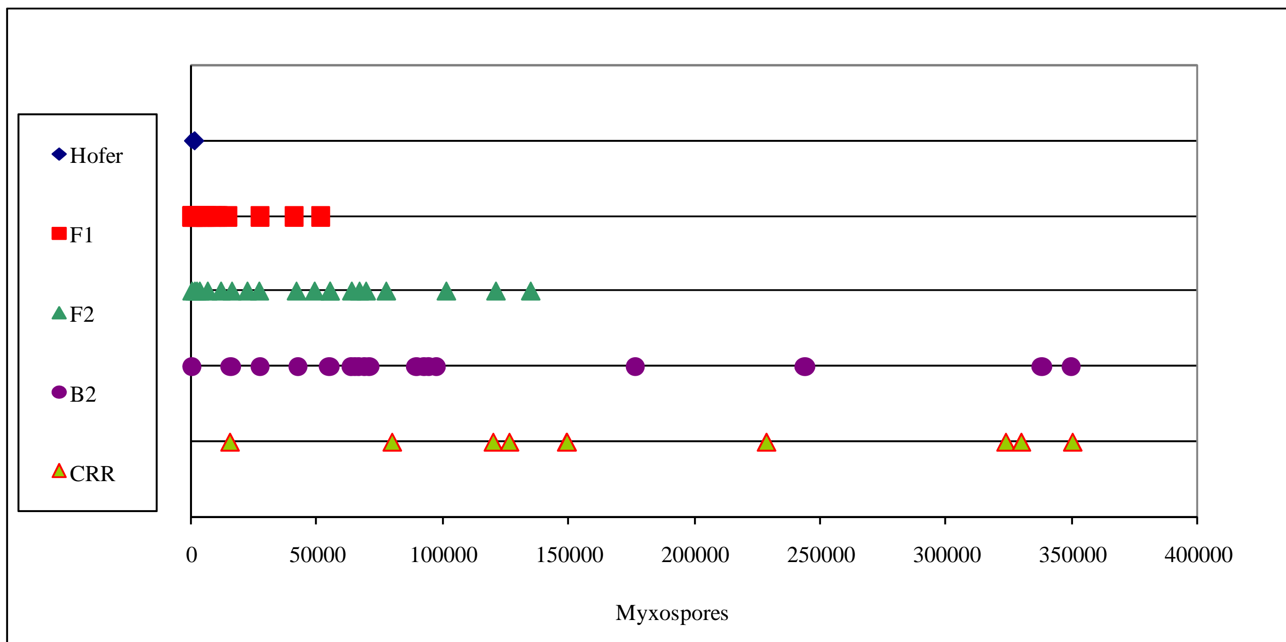


Table 6. Average myxospore counts and prevalence of infection for the Hofer, F1, F2, B2, and pure Colorado River rainbow trout.

Strain	Families	N	Spore Count Mean	PTD Infected (%)
Hofer Rainbow	10	100	275	7.0
F1 (50:50)	20	200	9,566	36.0
F2 (50:50)	20	200	46,227	52.0
B2 (25:75)	20	200	97,588	69.0
Colorado River Rainbow	10	10	187,209	100.0

Figure 9. Average myxospore counts for the10 Hofer, 10 Colorado River rainbow, 20 F1 [Hofer-CRR (50:50)], 20 B2 [Hofer-CRR (25:75)] and 20 F2 [Hofer-CRR $(50:50)^2$] families. Each point represents the average myxospore count for each individual family.



Hofer-Colorado River Rainbow Field Trials

Formal field testing of the Hofer-CRR crosses in comparison to the pure Colorado River rainbow trout strain was conducted in a limited manner over the same time period as the laboratory evaluations. The study areas of primary focus up to this point have been the Gunnison River and Colorado River. Both of these locations had very strong wild populations of rainbow trout in the past, but have experienced complete losses of rainbow trout year-classes and lack of natural recruitment since the mid-1990's. Both locations have high ambient levels of *M. cerebralis*. Brown trout numbers have increased over the past decade in both rivers, and fingerling plants of Colorado River rainbow trout to augment the rainbow trout populations have exhibited extremely low survival.

Gunnison River

A series of stocking events in the Gunnison River have occurred since 2004 in which equal numbers of pure Colorado River rainbow trout and Hofer-CRR cross fish have been differentially marked and stocked together to evaluate relative survival rates of the strains and as an attempt to re-establish a wild self-sustaining population in this location.

In 2004, Hofer-CRR 50:50 cross (F1) fish were marked with red visible implant elastomer (VIE) marks and pure CRR fish were similarly marked with green VIE marks. In this experiment, 10,104 CRR and 10,115 F1 rainbow trout were stocked as 13.6 cm and 11.9 cm fingerlings, respectively, into the Ute Park section of the Gunnison Gorge. The fish were mixed together prior to stocking to prevent bias due to handling, and then spread throughout the stream section using helicopter plants. In 2005, Hofer-CRR 25:75 cross (B2) fish were stocked, rather than F1 fish, along with pure CRR fish. The B2 fish were marked with an adipose clip and pure CRR strain fish were similarly given a right pelvic clip. Stocking was conducted using 5,000 of each variety as 15.2 cm fingerlings. In 2006, B2 fish were stocked again as 17.3 cm fingerlings to determine if the slightly larger B2 fish would perform better than the first (2005) plant of B2 fish. The pure CRR fish were not marked in this plant, while the B2 fish were given an adipose clip and a red VIE mark. In 2007, the number of fish stocked was increased to 20,000 of the pure CRR and 20,000 F1 rainbow trout stocked as 14.7 cm fingerlings. Coded wire tags were used to batch-mark the F1 and the pure CRR fish. Additionally, the F1 fish were adipose clipped to provide a second mark in case the coded wire tag was lost.

Growth, survival, and infection severity of the two strains planted each year were evaluated from samples collected during the annual population estimate conducted the following year. Estimates were conducted using mark-recapture sampling with boat-mounted electroshocking gear. All rainbow trout were carefully examined for evidence of VIE marks, fin clips, and coded wire tags. Subsamples of fish were collected for myxospore evaluation using the PTD method in 2005 and 2006.

The 2005 population estimate indicated that survival of both varieties of fish stocked in 2004 was relatively low, with only 12 of the pure CRR, and 24 of the F1 fish being found in the 2,375 m sampling area. The sampling resulted in an estimate of 10 pure CRR fish per km (16 fish per mile). The estimates for F1 cross were 14 fish per km (22 fish per mile). The average total length of the CRR fish was 24.8 cm, and 28.3 cm for the F1 fish. All of the pure CRR

individuals collected were found to be infected, with an average myxospore count of 124,603 (SD = 129,406). Only six of the 10 F1 individuals collected were found to be infected, with an average myxospore count of 4,055 (SD = 8,336).

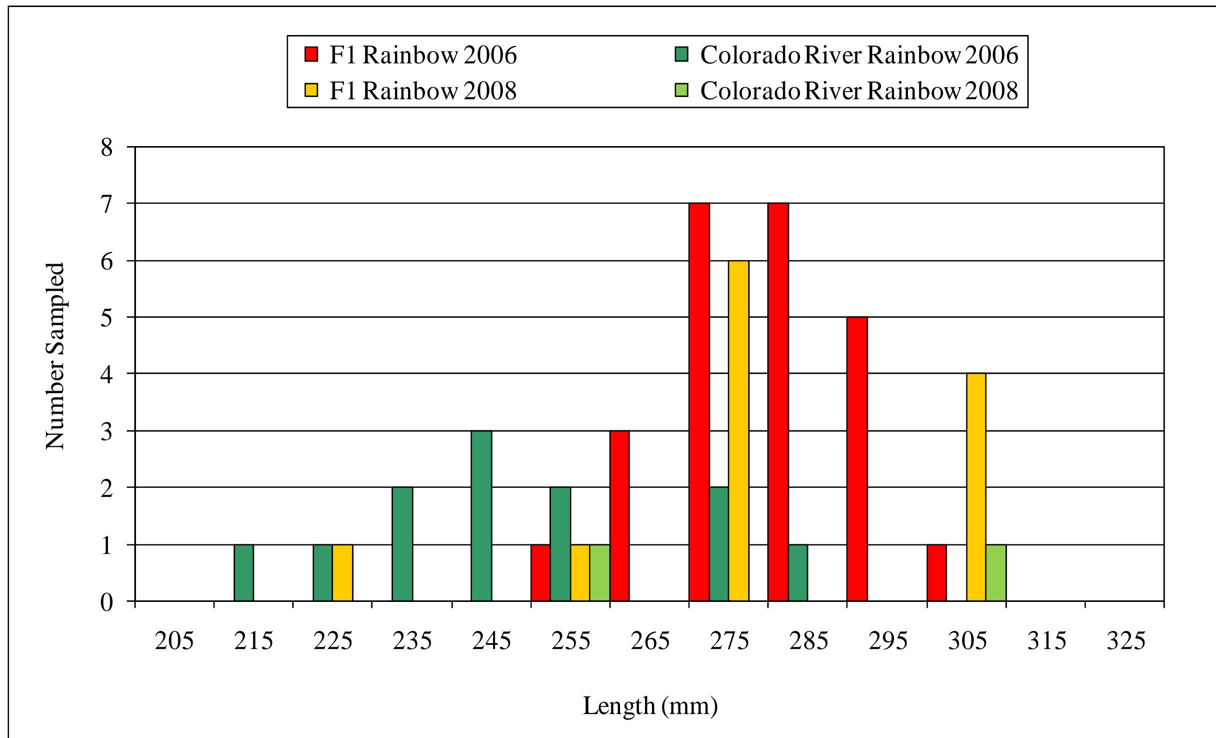
Survival and population estimates in 2006 for fish stocked in 2005 were difficult to assess directly because of mark loss (fin regeneration or poor marks) in both the CRR and B2 varieties. AFLP (Amplified Fragment Length Polymorphism) testing, a molecular technique that can help distinguish between individuals of the same species with different genetic lineages, was used to identify a subsample of unmarked fish as either B2 plants or pure CRR fish. Applying the ratio of fish identified as each variety in the subset to the overall population estimate of fish resulted in an estimate of 33 fish per km (53 fish per mile) of the pure CRR strain, and 22 fish per km (35 fish per mile) of the B2 cross. PTD testing identified an average of 83,929 myxospores (SD = 149,719) in the pure CRR fish planted in 2005. The average myxospore count among B2 fish was 40,480 (SD = 48,121).

In 2007, poor mark retention once again made estimating numbers of pure CRR and Hofer-cross fish difficult. The overall population estimate of rainbow trout (over 15 cm in length) was 135 fish per km (217 fish per mile). Of the 144 fish sampled, 16 (11.1%) were identified as either F1 or B2 fish by having either red VIE marks or adipose clips, while only three (2.1%) were identified as pure CRR fish, having green VIE marks.

In 2008, the population estimate for rainbow trout (over 15 cm in length) was 111 fish per kilometer (178 fish per mile). Fish stocked in 2007 could be very clearly identified because of the coded wire tags and fin clips. Of the 157 rainbow trout that were sampled, 12 of the F1 fish and two of the pure CRR fish from the 2007 plant were positively identified, producing an estimate of seven F1 and a minimum of two pure CRR fish per kilometer (12 F1 and three CRR fish per mile), respectively. Average length of the F1 fish (27.7 cm) was similar to the pure CRR fish (27.5 cm) in 2008, after the fish had been in the river for one year. Overall, poor survival estimates were quite evident for both the pure CRR and the Hofer-cross fish in each year of stocking. Predation by brown trout, loss of marks, and emigration from the study area were likely contributing factors. However, in both years (2006 and 2008) where definitively identified F1 and CRR fish could be compared directly from the stocking event in the previous year, the F1 fish were much more abundant than the pure CRR fish (Figure 10).

Fingerling rainbow trout were collected during fry shocking events in both 2007 and 2008 to be submitted for AFLP testing to determine if offspring had been produced from the F1 and B2 stocking events. The analysis identified a high proportion of the fingerling fish collected in 2007 as having a genetic background consistent with the Hofer strain. In 2008, a lower proportion of fry were identified as having Hofer genetic background. Nonetheless, natural reproduction from the Hofer crosses stocked in the river is now occurring. There is also some evidence that Hofer-cross fry produced in 2007 survived past their first year of life evident from the large number of unmarked age-1 fish in the 2008 samples.

Figure 10. Length-frequency and numbers of fish by strain sampled in the Gunnison River in 2006 and 2008 where direct comparisons of pure Colorado River rainbow trout and Hofer-CRR 50:50 (F1) crosses could be made from fish stocked in the previous year.



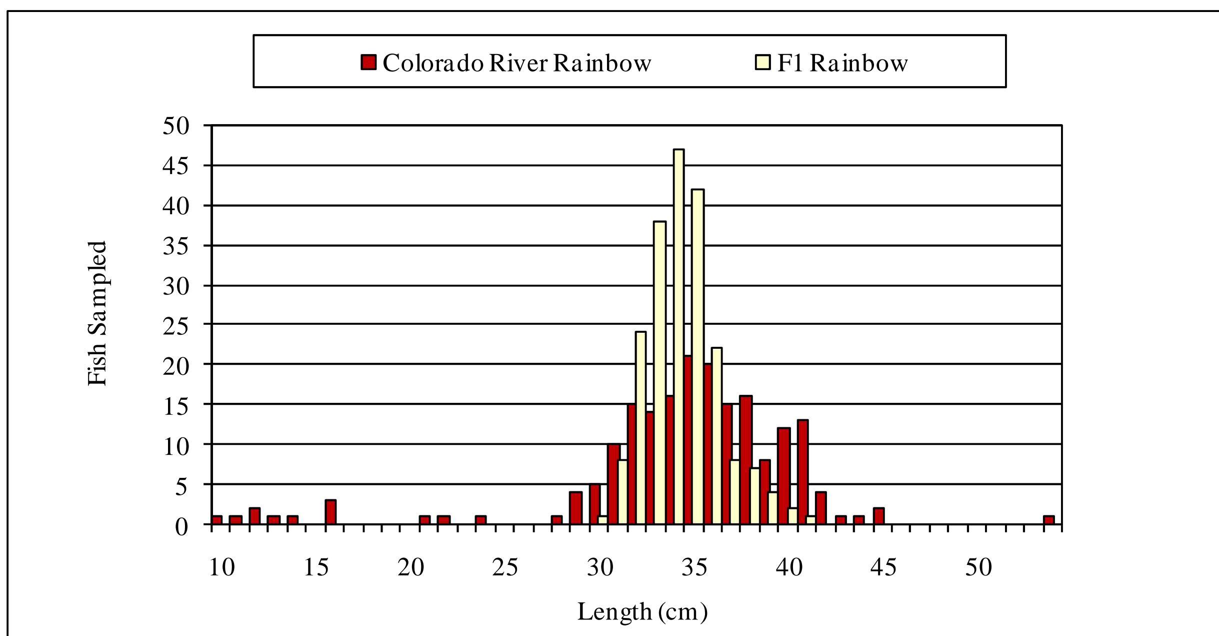
The results of this field evaluation demonstrate that the F1 fish can survive at least as well as the pure CRR trout when planted as fingerlings. The results also demonstrate that myxospore counts developed after stocking are much lower in the F1 fish than in the pure CRR trout. The myxospore counts in B2 fish released into the wild were similar to those found in the laboratory experiments, and while lower than the spore counts from the pure CRR fish, were also higher than observed in the F1 fish. This reinforces the notion that allowing natural selection of the resistant offspring of the F1 fish to occur in the wild may be a more effective method to producing sufficient resistance and wild behaviors than creating subsequent crosses artificially.

High densities of brown trout continue to contribute to the poor survival of the stocked rainbow trout in the Gunnison River, and poor mark retention has caused problems with producing reliable estimates of survival in B2 fish. However, reproduction from Hofer-cross fish has been confirmed in several locations at, and downstream of, the stocking sites. These results are promising, and could lead to re-establishment of a wild rainbow trout population in the Gunnison River despite the presence *M. cerebralis*. More in-depth genetic analyses of the fry and age-1 fish are planned for 2009 to determine the extent of survival and recruitment from the wild-spawned rainbow trout that are now appearing in the population.

Upper Colorado River

In 2006, a single lot of Hofer-CRR 50:50 cross (F1) rainbow trout were stocked in to the upper Colorado River at 23.5 cm (9.4 inches) in length to evaluate the survival of these larger fish in an area dominated by brown trout, and with an extremely high prevalence of *M. cerebralis*. This plant of fish has been monitored during annual population estimates. An extensive population estimate was conducted in spring, 2008. This was designed to evaluate the growth and survival of the F1 fish stocked in 2006, and also to determine what proportions of the fish were sexually mature. The population estimate consisted of a mark-recapture event over a distance of 6.28 river km (3.9 river miles). Brown trout, which have increased dramatically in the river with the decline in rainbow trout numbers, were present in the reach at a density of 1,307.5 fish per kilometer (2,092 fish per mile). Colorado River rainbow trout (residual wild fish and fish present due to repeated stocking of Colorado River rainbow fingerlings) were estimated to exist at a density of 109.4 fish per kilometer (175 fish per mile). The F1 rainbow trout from the 2006 plant were present at a density of 92.5 fish per kilometer (148 fish per mile). They averaged 34.3 cm (13.5 inches) in length, ranging from 30.0 cm to 40.9 cm (11.8 to 16.1 inches). The fish from this single plant of 3,000 F1 fish comprise almost half of the entire rainbow trout population in this stretch of river (Figure 11).

Figure 11. Hofer-CRR rainbow cross (F1) fish sampled during the spring, 2008 mark-recapture event on the upper Colorado River, compared with pure Colorado River rainbow trout in the same reach.



Of the 257 F1 fish examined, 32 (12.5 %) were found to be sexually mature. Of these, nine were females and 23 were males. The relatively high proportion of surviving F1 fish and the onset of sexual maturity of many of these fish is very encouraging. Typically, rainbow trout become sexually mature at age two or three under hatchery conditions, and later in natural environments. The identification of sexually mature rainbow trout from the 2006 stocking event is favorable with respect to re-establishing a wild rainbow trout population. Fingerling fish were collected in 2007 and 2008 and tested for the presence of markers for Hofer genes using the AFLP technique. None of the fish in the 2007 samples contained significant Hofer genetic backgrounds, and only a few individuals from the 2008 collections exhibited high proportions of Hofer markers. More of the F1 fish from the 2006 plant will be sexually mature in spring 2009, and have the potential to produce a large year-class of offspring. Further monitoring and evaluation of the marked fish and any new reproduction in the upper Colorado River is necessary to determine if the strategy of using the F1 cross will be successful in returning natural recruitment to locations where wild rainbow trout populations have been lost due to *M. cerebralis*.

The high survival and good post-stocking growth of the F1 fish stocked as catchable-sized fish in the upper Colorado River is particularly encouraging, as it is quite possible that these fish are capable of surviving and reproducing in large numbers when they reach sexual maturity. These results also demonstrate that stocking larger fish increases survival in the presence of predatory brown trout. Additional evaluations are planned for the upper Colorado River using marked fish. Fry evaluations using the AFLP technique will also be initiated on a large scale in 2009 to determine if the F1 fish are reproducing in this location.

The resistant strain evaluations are still in the early stages with regard to re-establishment of wild rainbow trout populations. Work conducted over the next several years will be very important in determining which combinations of the Hofer and wild strains are effective for establishing self-sustaining rainbow trout populations.

Summary

The current philosophy for use of resistant strains continues to be to use the Hofer-Harrison strain as a replacement for other varieties typically used as catchable plants in lakes and reservoirs. Pure Hofer strain fish will be maintained as a broodstock for catchable plants, and to replenish the Hofer-Harrison stock in the event that a decline in resistance is observed over time. Hofer, Harrison Lake, and several varieties of their crosses are currently being evaluated to determine which variety is best suited as a fingerling plant for lakes and reservoirs. Hofer-Colorado River 50:50 (F1) crosses appear to be a useful replacement for the Colorado River rainbow trout strain. Further dilution of the Hofer genetics by back-crossing the F1 cross with pure Colorado River rainbow trout is detrimental due to the rapid loss of resistance in the back-crosses. The increased proportion of Colorado River rainbow trout genetic background in the crosses does not appear to improve survival of the fish in the wild. Ongoing field evaluations will provide more information as to the long-term viability of the Hofer-Colorado River cross with regard to reproduction and recruitment. Additional studies to evaluate the Hofer-Harrison cross as a possible river plant will influence those decisions as well. It is unlikely that a single variety will be best suited as a catchable, subcatchable, and wild strain. Further refinement of applications for the different varieties will occur as more information becomes available from field trials in the next few years.

Acknowledgements

Phil Schler and Art Avalos of the Colorado Division of Wildlife Fish Research Hatchery were instrumental in accomplishing the research described in this document. Hatchery managers such as Chris Hertrich, Arlene Ganek, Doc Capwell, and others contributed considerable time and resources to these projects. Area biologists Dan Kowalski, Billy Atkinson, and Jon Ewert provided substantial field support to these efforts.

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APPENDIX III.

CRR vs. Hofer Progress Report

J. Wood and Patrick Power
Pisces Molecular

CRR vs. Hofer AFLPs progress report

J. Wood, Patrick Power
Pisces Molecular
May 16, 2009

Between July 2008 and May 2009 140 fin clip samples, from (approximately) 20 populations from locations along the Colorado, Gunnison, Poudre and Yampa Rivers, were received. Total DNA was extracted from all samples and analyzed by AFLP marker analysis for the presence or absence of Colorado River Rainbow (CRR), Hofer (HO) & Tasmanian (TAS) genetic markers. The populations analyzed are shown in the following table.

Date Rcvd	N	Source ID	Source Info/ Population name	Pisces #s
10/17/2008	11	CR-SR (1-11)	site # 4 - Sheriff Ranch 10/14/2008	90081 - 90091
	10	CR-RB/RBC (1-10)	site # 5 - Red Barn 10/14/2008	90092 - 90101
	1	CR-HP1-1	site # 6 - Hitching Post 10/15/2008	90102
	1	CR-WGD-2	site # 6 - Below Windy Gap Dam 10/15/2008	90103
11/12/2008	3	CAP K (1-1 – 2-1)	Cap K Ranch	90290 - 90292
11/21/2008	16	G (1-16)	Gunnison River Below Smith Fork Confluence	90293 -90308
10/16/2008	42	GR1, BC (1-10), CHK (1-10), UP (1-21)	Gunnison River	90309 - 90350
12/30/2008	2	PL-RH15-1, 20-1	Gunnison R	90940 - 90941
	4	BSFMBC (1C-4C)	Gunnison 8-6-08 (on bag)	90942 - 90945
	4	UPA, UPB	Gunnison 8-6-08 (on bag)	90946 - 90949
	1	Site 1 RBT-1	Poudre Sleeping Elephant 8-14-08 (on bag)	90950
	1	Poudre site 6	Poudre RBT 8-15-08 (on bag)	90951
	1	HGTB-1	Poudre River Hewlett Gulch Trailhead Bridge 8/21/08 (on bag)	90952
	14	SPR (1-10), SPC (1-4)	Poudre River Stove Prairie Campground 8/26/08 (on bag)	90953 - 90966
	3	DR (1-3)	Poudre River Diamond Rock 8/26/08 (on bag)	90967 - 90969
1/19/2009	26	Site 1, 2, 3, 4	Yampa 8-22 & 11-4-08	91387 - 91412

Aliquots of all sample DNAs were carried through an AFLP fragment amplification procedure using +3 selective primers ACA and CAG. The amplified fragments were separated by capillary electrophoresis. Presence or absence of a set of 143 previously determined markers fragments (CRR x HO bin set) was scored with GeneMapper 4.0 and exported as a presence (1) or absence (0) genetic "fingerprint" for each sample. The genetic fingerprints of these test samples were then compared, by population, to similarly derived genetic fingerprints for reference sets of CRR, HO, and/or TAS populations using the program STRUCTURE 2.2 to determine whether and how much of reference populations genetic fingerprints could be recognized in each of the

test samples. The average overall amount of genetic background in each population was computed and 95% confidence intervals determined with the program QSTRAP 2.0.

Although samples collected from several identical or nearby populations last year (July 2007 through June 2008) did show significant quantities of HO genetic background, nearly all of the samples tested this year exhibited low to zero amounts of HO genetic background. Based on the previous year's results, this was unexpected; therefore several possible causes for the differences were explored. First, for historical reasons the reference sample sets used in 2007 was smaller than the total amount of reference data available, so a larger reference data set was assembled and the 2008 samples compared in STRUCTURE against this "full" reference set. Second, because many of the 2008 populations were relatively small in total number, which could possibly diminish the power of STRUCTURE to distinguish between intra-population genetic diversity and genetic background differences, they were analyzed as larger groups, combined with the 2007 samples from the same locations. Additionally several of the 2008 populations were also analyzed against a Tasmanian rainbow trout (TAS) reference set (either TAS+HO or CRR, TAS & HO). Finally, the genetic fingerprint data for several populations were reformatted and analyzed with a different population genetic assignment program, NEWHYBRID, which was designed expressly to recognize and assign individuals as early generation hybrids between two reference populations.

The additional STRUCTURE analyses, either using a larger reference set, or using larger test population sample numbers, produced little or no significant changes in the amount of HO background scored in any of the 2008 sample populations. Adding or changing the reference population to TAS for sample population which were expected to have TAS backgrounds, also gave little change to the amount of HO background scored. Results using the NEWHYBRID program were inconclusive – the documentation explaining the use of the program and the interpretation of results is obtuse.

Given the 2007 sample analysis results and some of the field-collected evidence, the very low levels of HO genetic background in the 2008 samples is somewhat discouraging and inevitably leads to questions about the accuracy and/or power of the AFLP analysis method being used. Although there are a huge variety of different genetic marker types available, in the absence of definitive linkage between <u>any</u> genetic markers and the Whirling Disease resistant phenotype exhibited by HO trout (even the preliminary connection between higher metallothionein gene expression after infection by *M.c.* and HO background described by Baerwald, et al. does not provide or prove a direct genetic marker linked to WD resistance), the only way to discern incorporation of HO genetic background into riverine populations of trout after stocking a river with HO-derived fish is to use and analyze anonymous, genome-wide genetic marker sets. AFLPs markers are far easier to generate and tailor to specific, narrow questions such as the genetic differences between Colorado River and Hofer rainbow trout that have been separated for only one hundred years, than alternatives genetic markers like microsatellites , SNPs (Single Nucleotide Polymorphisms), VNTRs (Variable Number Tandem Repeats), or STRs (Short Tandem Repeats). Although the number of AFLP markers being scored in these analyses (143) was sufficient to distinguish CRR and HO genetic backgrounds using known CRR, HO and F1 hybrids in a blind test, as well as apparently in the 2007 samples, it is possible that this marker set may not have sufficient power to discern F2 or later generation hybrids. One of the unique advantages of the AFLP technique or over types of genomic markers is that additional markers in almost unlimited number can be generated using different selective primer pairs, so the power of the marker set could be increased if necessary. However, while generating and scoring

additional AFLP marker is technically straightforward, the time and expense necessary for this is not negligible, therefore it would be useful to examine methods for calculating the power of different size marker sets to distinguish and quantify two parental backgrounds at different hybridization levels (F1, F2, B1, B2, etc) given different values for the similarity or dissimilarity of the parental backgrounds and the average heterozygosity of any single AFLP marker (one possible example of such a method is the program WHICHLOCI). We will explore this in the coming year. An additional area for exploration will be a more thorough comparison of the population assignment computer programs we have used or are available (STRUCTURE, NEWHYBRID, AFLPOP, WHICHRUN are examples we currently know of). Although we originally chose STRUCTURE as appropriate and the easiest to use program for the CRR x HO analyses (and continue to successfully use it in numerous other population assignment projects such as cutthroat trout subspecies and boreal toad population relatedness studies), as our understanding of the subtleties of the CRR versus HO problem have increased (limitations with dominant markers like AFLPS, multiple stocking events, variations in the CRR parent used for producing the stocked fish or present in a particular river location, likely differences in the genetic diversity of the different parental populations, departures from Hardy-Weinberg equilibrium caused by Whirling Disease mortality, etc.), reconsidering the difference between the programs, and even reanalyzing the existing data with the different programs could provide either greater confidence in the results obtained with STRUCTURE, or insights into more accurate analysis methods.

APPENDIX IV.

Portable Decontamination Unit for Boat and Equipment Disinfection

George J. Schisler and Mikael Catanese
Colorado Division of Wildlife and
Colorado Cooperative Fish and Wildlife Research Unit

Portable Decontamination Unit for Boat and Equipment Disinfection

George J. Schisler, Colorado Division of Wildlife
Mikael Catanese, Colorado Cooperative Fish and Wildlife Research Unit
July 16, 2008

The following is a description and parts list for a portable decontamination unit used to disinfect boats and gear used for field sampling that may have been exposed to invasive species such as Zebra mussels *Dreissena polymorpha* or New Zealand mud snails *Potamopyrgus antipodarum*. Many other variations of this sort of unit using other, possibly less expensive parts are certainly available. This description is intended to provide a general guideline for others who may need to build a similar unit. This particular unit was constructed using parts readily available at most hardware or farm-and-ranch stores. The primary components are a towable flatbed trailer, a water tank, a gas-powered water pump, and a diesel-heated pressure washer.

The unit is used in two ways to disinfect boats. The first is with the pressure-washer wand. This is used to spray the bottom of the boat, and inside live wells or bilges of the boat. The second function is to connect motor “muffs” to the water outlet for disinfection of boat motors. The muffs are normally used to run boat motors out of the water, so they provide a convenient way to run disinfectant and hot water into the water intake of the motor. This unit is equipped with quick-releases on all of the hose fittings, so switching from the wand to the muffs takes just a matter of seconds. There are several varieties of boat motor muffs, so having a full set of the various types with quick-releases is necessary if disinfecting more than one type of boat.

Self-heating pressure washers can produce water heated up to 82.2° C (180.0° F) or more. Actual water temperature as it is released from the end of the pressure washer wand or out of the muffs on this particular unit is about 60.0° C (140.0° F). Adult Zebra mussels from 5.0 - 20.2 mm in shell length have been shown to reach 100% mortality in an average of five minutes or less with temperatures as low as 38.0° C (100.4° F) (Rajagopal et al. 1997). The high temperature alone may be sufficient for a complete disinfection. For added assurance that Zebra mussels or New Zealand mud snails are not spread on sampling gear or boats, chemical disinfectant can be added to the water.

Quaternary ammonia compounds such as SPARQUAT (active ingredients: 5% alkyl (C_{14}-50%, C_{12}-40%, C_{16}-10%) dimethyl benzyl ammonium chloride, 3.75% octyl decyl dimethyl ammonium chloride, 1.875% dioctyl dimethyl ammonium chloride, 1.875% didecyl dimethyl ammonium chloride) are highly effective for killing New Zealand mud snails. Schisler et al. (2008) found that 4 ounces of SPARQUAT per gallon of water (3.9 g/L (ppT) or 3,900 mg/L (ppm)) active ingredient) was 100% effective with a 10 minute exposure. Compounds specifically used for Zebra mussel control such as CALGON H-139M, CLAMTROL, MACROTROL 9210, and VeliGON contain the same or similar active ingredients (quaternary or polyquaternary ammonia compounds) as SPARQUAT (See Appendix). Toxicity of these compounds to Zebra mussels has been demonstrated in the past, typically with very low doses and longer exposure. Zebra mussels, especially early life stages, are quite susceptible to these chemicals. For instance, Fisher et al. (1994) found 24 hour LC_{50} with CLAMTROL for the preveliger, D-stage, post-D stage, plantigrade, and adult stages to be 0.048 mg/L (ppm), 0.095 mg/L, 0.179 mg/L, 8.8 mg/L, and >13.0 mg/L active ingredient, respectively. The active

ingredient concentration used for New Zealand mud snails is considerably higher than that found to be effective for the Zebra mussel preveliger through adult stages, although the exposure duration is much shorter for the currently-recommended New Zealand mud snail disinfection. Verification of concentrations adequate for rapid decontamination (ten minutes or less), for Zebra mussels, may be necessary. Various durations and concentrations of quaternary and polyquaternary ammonia compounds for specific Zebra mussel control applications are listed in the Appendix. In cases where adult stages of the organism may be present, extra effort should be used to ensure thorough disinfection due to the decreasing sensitivity of Zebra mussels to the chemicals as they mature. However, hot water in combination with quaternary ammonia disinfectant will greatly reduce the likelihood of unwanted organisms surviving in the bilge and motor of a treated boat. In addition, the disinfectant will continue to act on the organisms after the cleaning procedure is complete if it is not rinsed out of the boat. Please note that care should be taken to prevent waste water from these disinfecting procedures from draining into adjacent bodies of water.

The disinfection unit we constructed has a 200 gallon water tank that can be used to mix disinfectants with water at proper concentrations, and to haul clean water to a remote site if none is readily available. The tank should not be filled to capacity when towing. Alternatively, a smaller tank could be used with a 2000 lb axle trailer to avoid overloading the trailer. Prices are shown for both a 2000 lb axle trailer and 3500 lb axle trailer in parts list.

The unit is also equipped with a 4-stroke gasoline-driven water pump that can be used to draw water from either the water tank or from any other open source. Note that when purchasing quick-release fittings for the hoses, always use high-pressure fittings on the pressurized side of the washer. Standard garden hose-style quick releases will burst under pressure.

If tap water is available, the attached hose reel will allow the unit to run from a spigot within 100 feet of the trailer. Most pressure washers are equipped with a detergent tube that can be placed into a disinfectant container to meter in disinfectant at the appropriate concentration if fresh water is used rather than pre-mixed water and disinfectant from the storage tank. The attached storage bin is used to hold bottles of disinfectant, spare fittings, and the various sets of muffs.

Complete assurance that a boat or equipment is free of unwanted organisms such as Zebra mussels and New Zealand mud snails is never a guarantee. However, thorough decontamination with this sort of disinfection unit will greatly reduce the risk that your boats and field equipment are acting as vectors for distribution of these organisms.

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2008 Zebra Mussel Disinfecting Trailer Supply List (3500 lb).

Material Description	Quantity	Price Each	Total Cost
Chore Master 2000 heated pressure washer	1	$ 2,400.00	$ 2,400.00
Honda WX 10 Water Pump	1	$ 349.95	$ 349.95
5'X10' trailer 3500lb Axle	1	$ 1,300.00	$ 1,300.00
Steel Hose Reel	1	$ 129.00	$ 129.00
200 gal. Water Tank	1	$ 259.95	$ 259.95
5 gal. Industrial Steel Diesel Can	1	$ 39.95	$ 39.95
2 gal. Plastic Gas Can	1	$ 8.50	$ 8.50
Diesel Auto Stop Funnel	1	$ 8.95	$ 8.95
Boat Motor Muffs	3	$ 20.00	$ 60.00
50'x3/4" Heavy Duty Garden Hose	2	$ 33.00	$ 66.00
Folding Lid Rubbermaid Storage Container	1	$ 13.99	$ 13.99
2"x27' 10,000lb Ratchet Strap (plate hook)	1	$ 14.99	$ 14.99
2"x27' 10,000lb Ratchet Strap (rod hook)	1	$ 14.99	$ 14.99
4 pack 15' 500lb Ratchet Straps	1	$ 19.99	$ 19.99
Spare Tire Carrier	1	$ 6.95	$ 6.95
Trailer D-Ring Bolt on 400lb Capacity	2	$ 7.95	$ 15.90
Package of Trailer Wood Screws 1/4"x1 1/2"	1	$ 9.95	$ 9.95
Curved Pivot Hooks	2	$ 10.00	$ 20.00
6" Straight Metal Brackets	4	$ 1.89	$ 7.56
3/4" Goose Neck Hose Adapter	1	$ 4.69	$ 4.69
3/4" Garden Hose Quick Disconnect Couplers (sets)	5	$ 10.95	$ 54.75
3/4" Female Garden Hose Quick Disconnect	1	$ 6.19	$ 6.19
Male 3/4" to 3/4" Garden Hose Joint	1	$ 1.95	$ 1.95
Package of 3/4" Garden Hose Washers	1	$ 1.79	$ 1.79
3/4" Fem. Hose x 1/2" MIP Adapter	2	$ 1.99	$ 3.98
1/2" Female Pipe Coupling	1	$ 1.99	$ 1.99
3/4" Male Hose x 3/8" Female Bushings	3	$ 1.99	$ 5.97
3/8" Male NPT Tru-Flat I/M Style Quick Disconnect	5	$ 4.89	$ 24.45
3/8" Fem. NPT Tru-Flat I/M Style Quick Disconnect	2	$ 6.49	$ 12.98
Teflon Tape	3	$ 2.50	$ 7.50
21" Rubber Tarp Strap	2	$ 1.59	$ 3.18
9" Rubber Tarp Strap	2	$ 0.99	$ 1.98
1/2" Pressure Treated Plywood	1	$ 33.99	$ 33.99
Pressure Treated 2"x4" 12'	2	$ 6.99	$ 13.98
Pressure Treated 2"x6" 12'	1	$ 9.62	$ 9.62
5lb Box 3" Deck Screws	1	$ 19.89	$ 19.89
4"x3" "L" Bracket	2	$ 2.49	$ 4.98
300lb 1/2" x 8" Eye Bolts	2	$ 3.19	$ 6.38
1/2" Flat Washer	2	$ 0.32	$ 0.64
1/2" lock Washer	2	$ 0.32	$ 0.64
1 1/8"x 3 1/2" U-Bolt	5	$ 1.49	$ 7.45
1 1/4"x3 1/4" Flat Ended Snap Hook	1	$ 2.89	$ 2.89
		Total	**$ 4,978.48**

2008 Zebra Mussel Disinfecting Trailer Supply List (2000 lb).

Material Description	Quantity	Price Each	Total Cost
Chore Master 2000 heated pressure washer	1	$ 2,400.00	$ 2,400.00
Honda WX 10 Water Pump	1	$ 349.95	$ 349.95
5'X8' trailer 2000lb Axle	1	$ 900.00	$ 900.00
Steel Hose Reel	1	$ 129.00	$ 129.00
125 gal. Water Tank	1	$ 199.95	$ 199.95
5 gal. Industrial Steel Diesel Can	1	$ 39.95	$ 39.95
2 gal. Plastic Gas Can	1	$ 8.50	$ 8.50
Diesel Auto Stop Funnel	1	$ 8.95	$ 8.95
Boat Motor Muffs	3	$ 20.00	$ 60.00
50'x3/4" Heavy Duty Garden Hose	2	$ 33.00	$ 66.00
Folding Lid Rubbermaid Storage Container	1	$ 13.99	$ 13.99
2"x27' 10,000lb Ratchet Strap (plate hook)	1	$ 14.99	$ 14.99
2"x27' 10,000lb Ratchet Strap (rod hook)	1	$ 14.99	$ 14.99
4 pack 15' 500lb Ratchet Straps	1	$ 19.99	$ 19.99
Spare Tire Carrier	1	$ 6.95	$ 6.95
Trailer D-Ring Bolt on 400lb Capacity	2	$ 7.95	$ 15.90
Package of Trailer Wood Screws 1/4"x1 1/2"	1	$ 9.95	$ 9.95
Curved Pivot Hooks	2	$ 10.00	$ 20.00
6" Straight Metal Brackets	4	$ 1.89	$ 7.56
3/4" Goose Neck Hose Adapter	1	$ 4.69	$ 4.69
3/4" Garden Hose Quick Disconnect Couplers (sets)	5	$ 10.95	$ 54.75
3/4" Female Garden Hose Quick Disconnect	1	$ 6.19	$ 6.19
Male 3/4" to 3/4" Garden Hose Joint	1	$ 1.95	$ 1.95
Package of 3/4" Garden Hose Washers	1	$ 1.79	$ 1.79
3/4" Fem. Hose x 1/2" MIP Adapter	2	$ 1.99	$ 3.98
1/2" Female Pipe Coupling	1	$ 1.99	$ 1.99
3/4" Male Hose x 3/8" Female Bushings	3	$ 1.99	$ 5.97
3/8" Male NPT Tru-Flat I/M Style Quick Disconnect	5	$ 4.89	$ 24.45
3/8" Fem. NPT Tru-Flat I/M Style Quick Disconnect	2	$ 6.49	$ 12.98
Teflon Tape	3	$ 2.50	$ 7.50
21" Rubber Tarp Strap	2	$ 1.59	$ 3.18
9" Rubber Tarp Strap	2	$ 0.99	$ 1.98
1/2" Pressure Treated Plywood	1	$ 33.99	$ 33.99
Pressure Treated 2"x4" 12'	2	$ 6.99	$ 13.98
Pressure Treated 2"x6" 12'	1	$ 9.62	$ 9.62
5lb Box 3" Deck Screws	1	$ 19.89	$ 19.89
4"x3" "L" Bracket	2	$ 2.49	$ 4.98
300lb 1/2" x 8" Eye Bolts	2	$ 3.19	$ 6.38
1/2" Flat Washer	2	$ 0.32	$ 0.64
1/2" lock Washer	2	$ 0.32	$ 0.64
1 1/8"x 3 1/2" U-Bolt	5	$ 1.49	$ 7.45
1 1/4"x3 1/4" Flat Ended Snap Hook	1	$ 2.89	$ 2.89
		Total	**$ 4,518.48**

Appendix:

Quaternary and Polyquaternary Ammonium Compounds Used for Zebra Mussel Control

SOURCE:

ARMY CORPS OF ENGINEERS ZEBRA MUSSEL INFORMATION SYSTEM

BULAB 6002

This compound has the following characteristics:

a. *Chemical name:*
   - poly[oxyethylene(dimethyliminio)ethylene(dimethyliminio)ethylene dichloride
   - $C_{10}H_{24}N_2OCl_2$
   - Also known as PQ1 or WSCP

b. *BULAB 6002:*
   - 60 percent polymeric quaternary ammonium
   - Water-soluble liquid; U.S. Environmental Protection Agency Reg. No. 1448-42

c. *Source*:

Buckman Laboratories, Inc.
1256 McLean Boulevard
Memphis, TN 38108
(901) 278-0330
1-800-BUCKMAN

To control mollusks in recirculating or once-through cooling water and industrial systems using continuous or intermittent application, add BULAB 6002 at dosage rates of 0.2 to 2.2 fluid ounces of BULAB 6002 per 1,000 gallons of water (15.6 ml to 172 ml to 10,000 L), or 2 to 20 ppm product. Addition should be made continuously or intermittently to the intake water. Continuous addition is required for noticeably fouled systems.

Intermittent feeding is used to maintain control. Mollusk fouling has been prevented by concentrations as low as 2 ppm. Initial concentrations of 2 to 5 mg L-1 can be used up to 21 days. The long-term limit is 0.5 mg L-1.

The following tabulation summarizes laboratory studies showing efficacies of this product and the relationship of exposure time to concentration. Martin, Mackie, and Baker (1993a) showed that toxicity was temperature dependent.

Activity of BULAB 6002 on Zebra Mussel

Concentration mg L^{-1}	LT_{50}, hr		LT_{100}, hr		MTD, hr	
	MMB	MSL	MMB	MSL	MMB	MSL
1.0	168	499	250	680	514	175
2.0	148	216	250	313	231	166
4.0	108	174	196	244	189	123
8.0	96	124	144	197	147	107

Note: MMB = Martin. Mackie, and Baker (1993b).
MSL = McMahon, Shipman, and Long (1993).
LT_{50}, LT_{100} = time to percent mortality.
MTD = mean time to death.

References

Martin, I. D., Mackie, G. L., and Baker, M. A. (1993a). "Acute toxicity tests and pulsed-dose delayed mortality at 12 and 22 °C in the zebra mussel Dreissena polymorpha," *Archives of Environmental Contamination and Toxicology* 24, 389-398.

Martin, I. D., Mackie, G. L., and Baker, M. A. (1993b). "Control of the biofouling mollusc, *Dreissena polymorpha* (Bivalvia: Dreissenidae), with sodium hypochlorite and with polyquaternary ammonia and benzothiazole compounds," *Archives of Environmental Contamination and Toxicology* 24, 381-388.

McMahon, R. F., Shipman, B. N., and Long, D. P. (1993). "Laboratory efficacies of nonoxidizing molluscicides on the zebra mussel (*Dreissena polymorpha*) and the Asian clam (*Corbicula fluminea*)." *Zebra mussels: Biology, impacts and control*. T. F. Nalepa and D. W. Schloesser, ed., Lewis Publishers, Boca Raton, FL, 575-598.

CALGON H-139M

This compound has the following characteristics:

a. *Chemical Name*: Didecyldimethylammonium chloride, known as DDMAC.
b. *Formulation*: H-130M; 50 percent DDMAC; liquid, U.S. Environmental Protection Agency Registration No. 6836-203-10445.
c. *Source*:

Calgon Corporation
P.O. Box 1346
Pittsburgh, PA 15230-1346
(412) 777-8000
Health and Environmental Affairs: (412) 494-8000

Apply 1 to 10 ml H-130M per 1,000 L (1-10 ppm) water (0.15 to 1.5 fluid ounces per 1,000 gal of water) to give 1 to 10 mg L-1 (ppm) (Calgon Corporation 1996).

Treatment is typically a 24-hr feed period at 1.5 ppm to the inlet of the plant to maintain a residual at the discharge of 0.5 ppm. Actual treatment durations may vary from site to site dependent on water temperature and other site-specific conditions.

Do not apply H-130M more than four times per year. The duration of the treatment must not exceed 120 hr per application.

Refer to the following tabulation for efficacy data.

Toxicity of Active Ingredient (DDMAC) to Target Organisms

	Median Lethal Concentration	
Organism/Size	48-hr Test mg L^{-1}	48-hr Post Exposure mg L^{-1}
Zebra mussel, 20- to 25-mm diameter	0.85	0.38
Zebra mussel, 5- to 8-mm diameter	1.12	0.59
Threehorn wartyback	6.12	3.72

Note: from Waller et al. 1993; Fisher et al. 1994.

References

Calgon Corporation. (1996). “H-130M: Label.” Pittsburgh, PA. 1 p.

Fisher, S.W., Dabrowska, H., Waller, D. L., Babcock-Jackson, L., and Zhang, X. (1994). “Sensitivity of zebra mussel (*Dreissena polymorpha*) life stages to candidate molluscicides,” *J. Shellfish Res.* 13: 373-377.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). “Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms,” J. *Great Lakes Res.* 19, 695-702.

CLAMTROL

The active ingredients of these products have the following chemical names:

a. *ADBAC, Quat*: n-Alkyl (C12, C14, and C16) dimethylbenzyl ammonium chloride
b. *DGH*: Dodecylguanidine hydrochloride

Their formulations are as follows:

a. Clam-Trol CT-1:
    - 8 percent n-alkyl (C12-40 percent, C14-50 percent, C16-10 percent) dimethylbenzyl ammonium chloride
    - 5 percent dodecylguanidine hydrochloride
    - Liquid
    - U.S. Environmental Protection Agency Registration No. 3876-145
b. Clam-Trol CT-2:
    - 50 percent n-alkyl (C12-50 percent, C14-40 percent, and C16-10 percent) dimethylbenzyl ammonium chloride
    - Liquid
c. Clam-Trol CT-4
    - 10 percent n-alkyl (C12-50 percent, C14-40 percent, and C16-10 percent) dimethylbenzyl ammonium chloride
    - Liquid
d. *Source*:

    BetzDearborn, Inc.
    4636 Somerton Road
    Trevose, PA 19053
    Information: (215) 355-3300
    Emergency: 1-800-877-1940

Rates vary by product formulation and system. See accompanying tabulations (BetzDearborn, Inc., 1988, 1993a, 1993b). Badly fouled systems must be cleaned before treatment is begun.
Clam-Trol CT-1 is applied as follows:

a. Warmer water temperatures and longer contact times reduce the concentration of CT-1 needed for effective kills.
b. Product weight is 1 kg L-1 (8.5 lb/gal), and concentrations are based on product.
c. Apply as follows for recirculating cooling water systems:
    1. Intermittent or slug method: Initially when fouled, 360 g to 2.4 kg per 10,000 L water (0.3 to 2.0 lb per 1,000 gal of water), 36 to 240 ppm. Repeat until control is achieved. Subsequently, apply 180 g to 1.8 kg per 10,000 L water (0.15 to 1.5 lb per 1,000 gal of water), 18 to 180 ppm, every 3 days, or as needed to maintain control.
    2. Continuous feed method: Initially when fouled, 360 g to 2.4 kg per 10,000 L water (0.3 to 2.0 lb per 1,000 gal of water), 36 to 240 ppm. Subsequently, maintain 60 to 600 g per 10,000 L water (0.05 to 0.5 lb per 1,000 gal water), 6 to 60 ppm, in system.
d. Apply as follows for once-through industrial cooling water systems:
    1. Intermittent or slug method: Initially when fouled, 240 g to 1.2 kg per 10,000 L water (0.2 to 1.0 lb per 1,000 gal of water), 24 to 120 ppm, at minimum treatment intervals of 15 min. Repeat until control is achieved. Subsequently, 60 to 600 g per 10,000 L water (0.05 to 0.5 lb per 1,000 gal of water), 6 to 60 ppm, as needed to maintain control.

2. Continuous feed method: Initially when fouled, 240 to 1.2 kg per 10,000 L water (0.2 to 1.0 lb per 1,000 gal of water), 24 to 120 ppm. Continue until control is achieved. Subsequently, 24 to 240 g per 10,000 L water (0.02 to 0.2 lb per 1,000 gal of water), 2.4 to 24 ppm.

e. *Apply as follows for auxiliary water and wastewater systems:*
   1. Intermittent or slug method: 1.8 to 4.8 kg in 10,000 L water (1.5 to 4.0 lb per 1,000 gal of water), 180 to 480 ppm, in system water or in water being added to system, for 4 to 8 hr, 1 to 4 times per week or as needed to achieve control. Subsequently, 900 g to 2.4 kg in 10,000 L water (0.75 to 2.0 lb per 1,000 gal of water), 90 to 240 ppm.
   2. Can be sprayed onto a waste pile.

CT-2 is applied as follows:

a. Rates are given as weight of product, at 960 g L-1 (8.0 lb per gal). Concentrations are based on product.
b. Apply as follows for recirculating or once-through cooling water systems: add 20 to 200 g per 10,000 L water (0.016 to 0.166 lb per 1,000 gal of water), 2 to 20 ppm, based on water in the system or on flow rate through the system. Maintain this concentration for 3 to 48 hr.
   1. Intermittent or slug method: 360 - 1.56 kg/10,000 L (0.3 to 1.3 lb per 1,000 gal) of water in system or being added to system, 36 to 156 ppm, for 4 to 8 hr, 1 to 4 times per week or as needed to achieve control. Subsequently, use 180 to 780 g per 10,000 L (0.15 to 0.65 lb per 1,000 gal) of water, 18 to 78 ppm.
   2. Can be sprayed onto a waste pile.

CT-4 is applied as follows:

a. Rates are given as volume of product. Concentrations are based on active ingredient (ai) of quaternary compound (10 percent of product).
b. Apply as follows for once-through freshwater cooling water systems: 100 ml to 1 L per 10,000 L (1.28 to 12.8 fluid ounces per 1,000 gal) of water, 1 to 10 ppm at no more than 4 times per year and for no more than 120 hr per application.

References

BetzDearborn, Inc. (1988). “Label: Clam-Trol CT-1.” Trevose, PA. 2 pp.

BetzDearborn, Inc. (1993a). “Label: Clam-Trol CT-2.” Trevose, PA. 2 pp.

BetzDearborn, Inc. (1993b). “Label: Clam-Trol CT41.” Trevose, PA. 1 p.

Application Methods for Clam-Trol Formulations			
Product/System	Intermittent or Slug	Continuous Feed	Other
CT-1			
Recirculating cooling water	Initial: 0.3 - 2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.15 - 1.5 lb/1,000 gal 180 - 1.8 kg/10,000 L (18 - 180 ppm) Every 3 days/as needed	Initial: 0.3 - 2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm)	
Once-through industrial cooling water	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm) As needed	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.02 - 0.2 lb/1,000 gal 24 - 240 g/10,000 L (2.4 - 24 ppm)	
Auxiliary water/wastewater	Initial: 1.5 - 4 lb/1000 gal 1.8 - 4.8 kg/10,000 L for 4 to 8 hr 1 to 4 times/week (180 - 480 ppm) Subsequent: 0.75 - 2 lb/1000 gal 900 g - 2.4 kg/10,000 L (90 - 240 ppm)		Spray onto waste pile
CT-2			
Recirculating or once-through Industrial/commercial cooling water systems	Initial: 0.016 - 0.166 lb/1,000 gal 20 - 200 g/10,000 L (2 - 20 ppm) Maintain 3 - 48 hr		
Auxiliary water/service water and wastewater systems	0.3 to 1.3 lb/1,000 gal 360 g - 1.56 kg/10,000 L (36 - 156 ppm) for 4 - 8 hr 1 - 4 x/week Subsequent: 0.15 - 0.65 lb/1,000 gal 180 - 780 g/10,000 L 18 to 78 ppm		Spray onto waste pile
CT-4			
Once-through freshwater cooling systems	1.28 - 12.8 fl oz/1,000 gal 100 ml - 1 L/10,000 L 1 - 10 ppm ai Treat #120 hr #4 times per year		

Application Rates of Clam-Trol Formulations for Various Water-Handling Systems			
Water/System Application Method	Clam-Trol Product		
	CT-1	CT-2	CT-4
Recirculating Cooling Water			
Intermittent or slug	Initial: 0.3 - 2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.15 - 1.5 lb/1,000 gal 180 - 1.8 kg/10,000 L (18 - 180 ppm) Every 3 days/as needed	Initial: 0.016 - 0.166 lb/1,000 gal 20 - 200 g/10,000 L (2 - 20 ppm) Maintain 3 - 48 hr	
Continuous feed:	Initial: 0.3 -2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm)		
Once-Through Freshwater Cooling			
Intermittent or slug	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm) As needed	Initial: 0.016 - 0.166 lb/1,000 gal 20 - 200 g/10,000 L (2 - 20 ppm) Maintain 3 - 48 hr	1.28 - 12.8 fl oz/1,000 gal 100 ml - 1 L/10,000 L 1 - 10 ppm ai Treat #120 hr #4 times per year
Continuous feed	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.02 - 0.2 lb/1,000 gal 24 - 240 g/10,000 L (2.4 - 24 ppm)		
Auxiliary Water/Service Water and Wastewater			
Intermittent or slug	Initial: 1.5 - 4 lb/1,000 gal 1.8 - 4.8 kg/10,000 L for 4 to 8 hr 1 to 4 times/week (180 - 480 ppm) Subsequent: 0.75 - 2 lb/1,000 gal 900 g - 2.4 kg/10,000 L (90 - 240 ppm)	Initial: 0.3 to 1.3 lb/1,000 gal 360 g - 1.56 kg/10,000 L (36 - 156 ppm) for 4 - 8 hr 1 - 4 x/week Subsequent: 0.15 - 0.65 lb/1,000 gal 180 - 780 g/10,000 L 18 to 78 ppm	
Other	Spray onto waste pile	Spray onto waste pile	

MACROTROL 9210

These products have the following characteristics:

a. *Chemical name*: Ammonium chlorides
b. *Formulations*:
   1. MACROTROL™ 9210
      - 5 percent alkyl (60 percent C14, 30 percent C16, 5 percent C12, 5 percent C-18) dimethyl benzyl ammonium chloride
      - 5 percent alkyl (68 percent C12, 32 percent C14) dimethyl ethylbenzyl ammonium chlorides
      - Liquid
      - EPA Reg. No. 6836-57-1706
   2. NALCO® 9380
      - 40 percent alkyl (60 percent C14, 30 percent C16, 5 percent C12, 5 percent C-18) dimethyl benzyl ammonium chloride
      - 40 percent alkyl (68 percent C12, 32 percent C14) dimethyl ethylbenzyl ammonium chloride
      - Liquid
      - EPA Reg. No. 6836-234-1706
c. *Source*:

   Nalco Chemical Company
   One Nalco Center
   Naperville, IL 60563-1198
   (630) 305-1000
   Emergencies: 1-800-462-5378

MACROTROL 9210. Note: Excess residual MACROTROL 9210 must be detoxified prior to discharge to a receiving stream by using the proprietary compound NALCO 1315 or by using bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product (Nalco 1995a).

a. *In recirculating, auxiliary cooling water, and wastewater systems:*
   1. *Initial dose: Add 0.08 to 0.8 lb per 1,000 gal water (10 to 100 ppm: 100 to 1,000 kg per 10,000 L). Repeat as necessary to achieve control.*
   2. *Subsequent dose: When control is evident, add 0.08 to 0.4 lb per 1,000 gal (10 to 50 ppm: 100 to 500 kg per 10,000 L), as needed to maintain control.*
b. *In once-through cooling water systems:*
   1. *Intermittent feed:*
      - *Initial dose: When the system is noticeably fouled, add 0.08 to 0.8 lb per 1,000 gal water (10 to 100 ppm: 100 to 1000 kg per 10,000 L), based on system flow rates. The minimum treatment period should be 6 to 24 hr. Repeat as necessary to achieve control.*
      - *Subsequent dose: When control is evident, add 0.04 to 0.4 lb per 1,000 gal (5 to 50 ppm: 50 to 500 kg per 10,000 L), based on system flow rates on an as-needed basis to maintain control. Frequency of feed should be tied to a monitoring program.*
   2. *Continuous feed:*
      - *Initial dose: When the system is noticeably fouled, add 0.04 to 0.4 lb per 1,000 gal water (5 to 50 ppm: 50 to 500 kg per 10,000 L), based on system flow rates. Continue to feed until needed control is achieved.*

- *Subsequent dose: Maintenance control can be effective through continuous feed at 0.016 to 0.16 lb per 1,000 gal (2 to 20 ppm: 20 to 200 kg per 10,000 L), based on system flow rates.*

***NALCO 9380**. Note: NALCO 9380 must be deactivated prior to discharge from the system by using bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product or by using the proprietary compound NALCO 1315 (Nalco 1995b).*

a. *In recirculating, auxiliary cooling water, and wastewater systems:*
   1. *Initial dose: Add 0.2 to 1.7 fluid ounces per 1,000 gal water (1 to 12 ppm: 10 to 120 ml per 10,000 L). Repeat as necessary to achieve control.*
   2. *Subsequent dose: When control is evident, add 0.2 to 0.9 fluid ounces per 1,000 gal (1 to 6 ppm: 10 to 60 ml per 10,000 L), as needed to maintain control.*

b. *In once-through cooling water systems:*
   1. *Initial dose: When the system is noticeably fouled, add 0.2 to 1.7 fluid ounces per 1,000 gal water (1 to 12 ppm: 10 to 120 ml per 10,000 L) based on system flow rates. The minimum treatment period should be 6 to 24 hours. Repeat as necessary to achieve control.*
   2. *Subsequent dose: When control is evident, add 0.1 to 0.9 fluid ounce per 1,000 gal (0.6 to 6 ppm: 6 to 60 ml per 10,000 L), based on system flow rates on an as-needed basis to maintain control. Frequency of feed should be tied to a monitoring program.*

c. *Continuous feed:*
   1. *Initial dose: When the system is noticeably fouled, add 0.1 to 0.9 fluid ounce per 1,000 gal (0.6 to 6 ppm: 6 to 60 ml per 10,000 L), based on system flow rates. Continue to feed until needed control is achieved.*
   2. *Subsequent dose: Maintenance control can be effective through continuous feed at 0.03 to 0.3 fluid ounces per 1,000 gal (0.2 to 2.5 ppm: 2 to 25 ml per 10,000 L), based on system flow rates.*

References

Nalco. (1995a). “MACROTROL 9210: Label,” Nalco Chemical Company, Chicago, IL, 1 p.

Nalco. (1995b). “Nalco 9380: Label,” Nalco Chemical Company, Chicago, IL, 1 p.

VeliGON

This compound has the following characteristics:

a. *Active ingredient*: poly (dimethyl diallyl ammonium chloride)
b. *Synonyms*: DMDAAC, pDADMAC, DDDMAC, DMDACC
c. *Formulations*:
    1. VeliGON CL-M
        - 39.8 percent poly (dimethyl diallyl ammonium chloride); liquid
        - EPA Registration No. 10445-115
    2. VeliGON DL-M
        - 17.5 percent poly (dimethyl diallyl ammonium chloride); liquid
        - EPA Registration No. 10445-121
    3. VeliGON L-M
        - 19.8 percent poly (dimethyl diallyl ammonium chloride); liquid
        - EPA Registration No. 10445-116
    4. VeliGON LS-M
        - 10 percent poly (dimethyl diallyl ammonium chloride); liquid
        - EPA Registration No. 10445-117
    5. VeliGON TL-M
        - 19.8 percent poly (dimethyl diallyl ammonium chloride); liquid
        - EPA Registration No. 10445-118
    6. VeliGON T-2-M
        - 33 percent poly (dimethyl diallyl ammonium chloride); liquid
        - EPA Registration No. 10445-122
d. *Source*:

Calgon Corporation
P.O. Box 1346
Pittsburgh, PA 15230-1346
(412) 777-8000
Health and Environmental Affairs: (412) 494-8000
Emergency: (412) 494-8000
For information, jim.farmerie@ecc.com.

Apply at a rate of 1 to 5 ppm on a continuous basis during the spawning/breeding season.

The various VeliGON compounds differ in molecular weight and cationic charge density. Choice of the most suitable product and determination of the application rate required for local water treatment systems is usually based on local veliger monitoring and optimal clarification effect in an on-site bench-scale test (jar test) under local water and site conditions.

DMDAAC has been shown to have a median lethal concentration LC50 at 96 hr for adult zebra mussels at between 1.5 and 3.0 mg L-1 (ppm) (Blanck, Mead, and Adams 1996).

The following tabulation lists the treatment rates for VeliGON formulatons:

Treatment Rates for VeliGON Formulations[1]		
Product	Treatment to Intake Water	Concentration of Active Ingredient, ppm
VeliGON CL-M	0.3 to 3.3 fluid ounces per 1,000 gal 9 to 98 ml per 378,500 L	1 to 10
VeliGON DL-M	0.68 to 6.8 fluid ounces per 1,000 gal 20 to 200 ml per 378,500 L	1 to 10
VeliGON L-M	0.6 to 6.6 fluid ounces per 1,000 gal 18 to 195 ml per 378,500 L	1 to 10
VeliGON LS-M	1.2 to 13.2 fluid ounces per 1,000 gal 35.5 to 390 ml per 378,500 L	0.1 to 1
VeliGON TL-M	0.6 to 6.6 fluid ounces per 1,000 gal 18 to 195 ml per 378,500 L	1 to 10
VeliGON T-2-M	0.36 to 3.6 fluid ounces per 1,000 gal 11 to 106 ml per 378,500 L	1 to 10
[1]From product labels (Calgon Corporation 1995a, b, c, d, 1996a, b)		

References

Blanck, C. A., Mead, D. F., and Adams, D. J. (1996). "Effective control of zebra mussels using a high molecular weight polymer." Abstract from *the Sixth International Zebra Mussel and Other Aquatic Nuisance Species Conference*, Dearborn, MI, March 1996.

Calgon Corporation. (1995a). "Label: VeliGON CL-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995b). "Label: VeliGON L-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995c). "Label: VeliGON LS-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995d). "Label: VeliGON TL-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1996a). "Label: VeliGON DL-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1996b). "Label: VeliGON T-2-M," Pittsburgh, PA. 1 p.

Conversions:

1 fluid ounce (oz) = 29.57 milliliters (ml)

1 gallon = 128 ounces (oz) = 3.785 liters (L)

1 liter aqueous solution (at 4 °C) = 1 kilogram (Kg)

1 milliliter aqueous solution (at 4 °C) = 1 gram (g)

1 gram per liter (g/L) = 1 part per thousand (ppT)

1 milliliter per liter (ml/L) = 1 part per thousand (ppT)

1 milligram per liter (mg/L) = 1 part per million (ppm)

1 microgram per liter (µg/L) = 1 part per billion (ppb)

1 pound (lb) = 453.6 grams (g)