

## Inheritance of *Myxobolus cerebralis* Resistance among F<sub>1</sub>-Generation Crosses of Whirling Disease Resistant and Susceptible Rainbow Trout Strains

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**Abstract.**—A whirling disease resistant domestic strain of rainbow trout *Oncorhynchus mykiss* (German [GR]) was crossed with a susceptible wild strain of rainbow trout (Colorado River [CR]). Thirty-two families of F<sub>1</sub> crosses, along with five pure GR and two pure CR rainbow trout families, were then evaluated for resistance to whirling disease after experimental exposure to *Myxobolus cerebralis*. The pure domesticated GR strain was verified to have strong resistance to the parasite. In contrast, wild CR rainbow trout were highly susceptible. Crosses of these two strains resulted in offspring with a range of susceptibility. The resistance to whirling disease in some families was similar to that of pure GR rainbow trout, while other families were as susceptible as pure CR rainbow trout. Infection severity was significantly greater in the CR strain than in the pure GR and the GR × CR strains, as measured by both microscopic pathological scores (histology) and myxospore counts. Infection severity in the reciprocal crosses (CR female × GR male) was not significantly different from that of the pure CR rainbow trout as measured by histology but was significantly different with respect to lower myxospore counts. Future studies will examine the stability of inheritance of whirling disease resistance and the potential use of selective breeding to control whirling disease in free-ranging rainbow trout populations.

*Myxobolus cerebralis*, the parasite associated with salmonid whirling disease, has caused severe problems in wild populations of rainbow trout *Oncorhynchus mykiss* throughout the Intermountain West (Nehring and Walker 1996; Vincent 1996). These include wild rainbow trout populations in Beaver Creek on the South Fork of the Rio Grande River, South Cottonwood Creek in the Arkansas River drainage, and the Cache la Poudre, Colorado, Dolores, Fraser, Gunnison, Fryngpan, Roaring Fork, South Platte, Williams Fork, and Rio Grande rivers in the state of Colorado (Nehring and Thompson 2001). Similar effects on rainbow trout populations have been observed in areas of the Madison River and Missouri River basins in Montana (Vincent 1996; Leathe et al. 2002).

In Colorado, wild rainbow trout populations were established with the Colorado River strain (CR) of rainbow trout. This strain originated from stocking programs of rainbow trout from various sources that were reared in federal, state, and public hatcheries in the 1880s (Wiltzius 1985). Recently, a rainbow trout

strain originating from shipments from North America to Germany in the 1880s has been identified as resistant to *M. cerebralis* (Hedrick et al. 2003). This strain has been designated as the German (GR) or “Hofer” strain after the facilities in Bavaria where they were most recently reared (Hedrick et al. 2003). Because the GR strain is a highly domesticated food fish, the survival and viability of the strain in the wild are uncertain. Also, stocking of the strain directly into wild trout waters could have unknown consequences. A breeding program was thus established with the principal aim of incorporating whirling disease resistance from the GR rainbow trout into a strain that retains many of the desired “wild rainbow trout” characteristics. The first steps in this process involved determining whether resistance to *M. cerebralis* could be inherited in crosses between GR and CR rainbow trout. An experiment was therefore designed to test the resistance of pure CR rainbow trout, pure GR rainbow trout, and crosses made from these two strains.

### Methods

**Spawning and rearing.**—Broodstocks of pure GR and CR strains of rainbow trout were spawned as individual male–female pairs to produce offspring for this experiment. Eggs from each mated pair were kept

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separately during incubation. Two families of pure CR rainbow trout and five families of pure GR rainbow trout were created in this manner. Twenty-nine families of GR (female)  $\times$  CR (male) crosses were made. In addition, three reciprocal-cross families of CR (female)  $\times$  GR (male) were created. After swim-up, fingerlings were reduced to approximately 100 per family. The fish were reared at 10–14°C for about 7 weeks and a total of 618 degree-days (°C) before exposure to *M. cerebralis*. All fish were reared under identical conditions; for the duration of the experiment, fish were fed twice daily to achieve a total ration of approximately 2% body weight per day.

***Myxobolus cerebralis* exposure.**—Thirty-five fish were randomly drawn from each family group. At the time of exposure, all fish were relatively uniform in size—about 25–30 mm in length. These fish were then placed into separate 76-L aquaria supplied with 13°C well water at 0.5 L/min for exposures to the triactinomyxon stages (TAMs) of *M. cerebralis*. The production of TAMs and the exposure conditions utilized in the current study were similar to those described previously by Andree et al. (1998) and Hedrick et al. (1999a). Before exposure, the water flow to each aquarium was stopped. During the exposure period, each aquarium received aeration with air stones to ensure full mixing of the TAMs and equal exposure of all fish. Exposures to *M. cerebralis* were conducted by decanting 8.3 mL of filtrate containing 70,000 TAMs into each aquarium, for an average exposure of 2,000 TAMs per fish. After 2 h, water flow to all aquaria was resumed. After a 5-d holding period to account for any potential handling mortality, fish were reduced to 30 per family. Fish were then reared for 5 months (about 2,100 degree-days) to ensure full development of myxospores in each family group.

***Growth, mortality, and infection severity evaluations.***—Dead fish were removed and recorded daily to provide a cumulative mortality for each family group. At the end of the rearing period, 15 fish were randomly chosen from each family for analysis. Fish were weighed and measured. Heads were removed from the fish for pepsin–trypsin digest (PTD) and histological analysis. If used for PTD analysis, heads were placed in individually labeled plastic bags and were held at –20°C until processing. If used for histology, heads were placed into Davidson's solution for 48 h and then were transferred to 70% ethanol.

Infection severity was evaluated with PTD in 10 of the fish from each family. Individual heads were soaked in water at 45°C to soften the tissues. Skeletal elements were separated from soft tissue by agitation in a wrist-action electric shaker that used glass marbles as hammers. The samples were then decanted through

disposable 190- $\mu$ m calculi filters, and rinse water was added back to the skeletal elements for purification and concentration by PTD (Markiw and Wolf 1974) and myxospore quantification (O'Grodnick 1975).

The severity of the microscopic lesions present in stained tissue sections was evaluated by means of the MacConnell–Baldwin scale (Hedrick et al. 1999a; Baldwin et al. 2000). Half-heads from five fish per family were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by standard procedures (Humason 1979). A single midline sagittal section for each fish was evaluated for the presence of microscopic lesions due to *M. cerebralis* on a scale from 0 to 5; 5 represents the most severe lesions and 0 indicates that no abnormalities were seen.

***Statistical analysis.***—Myxospore counts per fish were used to calculate average myxospores per family. Each family group was used as the experimental unit in all analyses to avoid the pseudoreplication that often occurs when individual fish are used as the experimental unit in these types of tank experiments. Analysis of variance (ANOVA) was conducted by use of Statistical Analysis System software (SAS 2000) to determine whether there were significant differences between strains in growth, mortality, and infection severity as measured by both PTD and histology. If significant effects were identified, Duncan's multiple-range test (Duncan 1955) was used to determine which strains had significantly higher myxospore counts and histological scores. The alpha level was set at 0.05 for all tests. The relationship between infection severity values was evaluated by use of simple linear regression between average myxospore counts and average histological scores for each family and by subsequently computing the correlation coefficient.

## Results

### *Growth*

The ANOVA results identified marginally different weights ( $F_{3,35} = 1.18$ ,  $P = 0.1633$ ) and significantly different lengths ( $F_{3,35} = 5.14$ ,  $P = 0.0048$ ) between the strains. Duncan's multiple-range test ( $\alpha = 0.05$ ) showed that GR rainbow trout were significantly heavier than the CR rainbow trout at the end of the experiment, and the crossed families had intermediate weights that were not significantly different from those of either parental strain (Table 1). With regard to length, the CR rainbow trout were significantly shorter than the other strains at the end of the experiment.

### *Mortality*

Mortality in fingerling rainbow trout that are reared under experimental conditions similar to those in the current study and that are not exposed to *M. cerebralis*

TABLE 1.—Weight and length characteristics of two strains of rainbow trout at 6.75 months of age (2,718 degree-days) from the Colorado River (CR) and Germany (GR) and their reciprocal crosses 5 months (2,100 degree-days) after experimental exposure to *Myxobolus cerebralis*. The term “Duncan grouping” refers to the classification by Duncan’s multiple-range test.

Strain <sup>a</sup>	Weight (g)			Length (cm)		
	Mean	SD	Duncan grouping	Mean	SD	Duncan grouping
CR	11.5	0.3	A	11.1	0.8	A
GR	28.2	11.2	B	14.6	1.3	B
CR (m) × GR (f)	24.2	8.9	AB	13.5	1.1	B
GR (m) × CR (f)	21.4	0.7	AB	13.0	0.1	B

<sup>a</sup> m = male, f = female.

is typically less than 5%. Average mortality in this experiment was only 2% in the pure GR rainbow trout, 4.0% in the CR (male) × GR (female) cross, and 2.2% in the GR (male) × CR (female) cross when exposed to *M. cerebralis*. The pure CR strain averaged 28.3% mortality. The ANOVA found significant differences in mortality ( $F_{3,35} = 13.0$ ,  $P < 0.0001$ ) between the strains. Mortality was significantly higher in the pure CR rainbow trout families in this experiment than in the pure GR families or  $F_1$  crosses as tested with Duncan’s multiple-range test (Table 2).

#### Myxospore Counts

Significant effects ( $F_{3,35} = 3.27$ ,  $P = 0.0325$ ) on myxospore counts were found due to strain. Infection was significantly more severe, as measured by Duncan’s multiple-range test, in the CR rainbow trout than in the pure GR and the GR × CR rainbow trout families (Table 3). The myxospore counts in the reciprocal crosses were also significantly lower than those in the pure CR rainbow trout families.

#### Histology

Significant effects ( $F_{3,35} = 4.28$ ,  $P = 0.0112$ ) of strain on infection severity as measured by histology were identified. Duncan’s multiple-range test deter-

mined that infection severity values were not significantly different between the pure GR families and the GR × CR crosses (Table 4). The reciprocal crosses were not significantly different from the pure CR rainbow trout in terms of infection severity.

Infection severity, as measured by both PTD and histology, was similar between pure GR rainbow trout and some of the crossed families, while severity in other crossed families was the same as that of the pure CR rainbow trout (Figure 1). These results also revealed a strong relationship between PTD and histology scores for each of the families. Myxospore counts were square-root transformed to linearize the data for the correlation analysis (Figure 2). The linear relationship between the square-root-transformed myxospore counts and histological scores was significant ( $P < 0.0001$ ), and the results of the methods were strongly correlated ( $R^2 = 0.659$ ).

#### Discussion

Family groups resulting from crosses between whirling disease resistant (GR) and susceptible (CR) strains of rainbow trout demonstrated a range of susceptibility to experimental *M. cerebralis* exposure. In contrast, families from the pure parental crosses demonstrated either high or low resistance to whirling disease. Differences in susceptibility to whirling disease were evident in evaluations of total mortality, microscopic lesion severity scores, and myxospore concentrations between strains and family groups. This study demonstrated that inheritance of resistance through selective breeding programs may be one approach for restoration of wild rainbow trout stocks lost to whirling disease.

#### Growth

The excellent growth of the GR rainbow trout has been observed in other lots of this strain that were not involved in the current study. The habit of this strain to be very aggressive surface-oriented feeders makes it a superb production fish. However, these same

TABLE 2.—Cumulative mortality among two strains of rainbow trout from the Colorado River (CR) and Germany (GR) and their reciprocal crosses 5 months after experimental exposure to *Myxobolus cerebralis*. The term “Duncan grouping” refers to the classification by Duncan’s multiple-range test.

Strain <sup>a</sup>	Mortality (%)		Duncan grouping
	Mean	SD	
CR	28.3	11.8	A
GR	2.0	5.4	B
CR (m) × GR (f)	4.0	5.7	B
GR (m) × CR (f)	2.2	1.9	B

<sup>a</sup> m = male, f = female.

TABLE 3.—Mean myxospore counts (average spore count per head) of families from two strains of rainbow trout from the Colorado River (CR) and Germany (GR) and their reciprocal crosses 5 months after experimental exposure to *Myxobolus cerebralis*. The term “Duncan grouping” refers to the classification by Duncan’s multiple-range test.

Strain <sup>a</sup>	Families (N)	Myxospore count per head		Duncan grouping
		Mean	SD	
CR	2	210,983	83,735	A
GR	5	3,593	3,195	B
CR (m) × GR (f)	29	84,400	91,102	B
GR (m) × CR (f)	3	42,376	40,137	B

<sup>a</sup> m = male, f = female.

behaviors can be detrimental in wild rainbow trout, which must balance predator avoidance with feeding activity. Typically, domesticated salmonids are less likely to forego feeding behavior in the presence of predators, making them more vulnerable to predation and less likely to survive and reproduce in the wild (Johnsson and Abrahams 1991). The CR rainbow trout, like many wild strains, have slow growth, longevity, and fitness in natural stream situations. These are quite different from the fast-growth and low-survival characteristics seen in many domestic stocks (Vincent 1960). Other naturalized wild rainbow trout populations have demonstrated survival advantages over domestic strains that may be related to localized adaptation, predator avoidance, and migratory behavior (Miller et al. 2004). Incidental observations during this experiment revealed that the F<sub>1</sub> crosses had an intermediate fright response. Growth was likewise better than that of the pure CR fish but less than that of the pure GR rainbow trout. Continued back-crossing of resistant families with CR strain rainbow trout will be important for maximizing the wild behavior and genetic diversity in the ensuing broodstock used to reintroduce the CR rainbow trout into areas where it has been eliminated due to *M. cerebralis*.

TABLE 4.—Mean lesion severity scores for families from two strains of rainbow trout from the Colorado River (CR) and Germany (GR) and their reciprocal crosses 5 months after exposure to *Myxobolus cerebralis*. The term “Duncan grouping” refers to the classification by Duncan’s multiple-range test.

Strain <sup>a</sup>	Families (N)	Histology score		Duncan grouping
		Mean	SD	
CR	2	3.3	0.1	A
GR	5	0.2	0.4	B
CR (m) × GR (f)	29	1.6	1.2	B
GR (m) × CR (f)	3	1.8	1.2	AB

<sup>a</sup> m = male, f = female.

Mortality

The greater mortality in pure CR rainbow trout than in other trout strains after *M. cerebralis* exposure in our study was expected. Prior live-cage studies in the upper Colorado River by Thompson et al. (1999) found that CR rainbow trout experienced mortality from 7% to 39%. In that study, total *M. cerebralis* exposure was not known, but the highest mortality was observed in fish exposed earlier in the summer and at younger ages. The laboratory and field studies combined with complete year-class losses in many Colorado rivers, such as the Poudre, Colorado, Fryingpan, Dolores, Gunnison, Rio Grande, South Platte, and Williams Fork rivers (Nehring and Thompson 2001), are all evidence of the susceptibility of wild CR rainbow trout to whirling disease.

Infection Severity

Lesion severity scores and myxospore concentrations have become the standard method for assessing the severity of whirling disease among trout exposed to *M. cerebralis* in both field and laboratory studies (Hedrick et al. 1999a, 1999b; Thompson et al. 1999; Baldwin et al. 2000; Densmore et al. 2001; MacConnell and Vincent 2002). In the present study, lesion severity scores for pure CR rainbow trout were generally high (3.3) and exceeded the value (2.7) deemed as indicating the potential for population impacts in wild trout surveys (MacConnell and Vincent 2002). The lesion scores and myxospore concentrations for the CR rainbow trout in the current study are very similar to those obtained in earlier studies and those found in a second highly susceptible rainbow trout strain (the TL strain) exposed to similar experimental conditions (Thompson et al. 1999; Hedrick et al. 2003). The strong correlation between lesion severity scores and myxospore concentrations observed in the current study is similar to that found in several studies, including most recently that of Kelley et al. (2004), and suggests that the scores are good measures of susceptibility to *M. cerebralis* infection. Significant

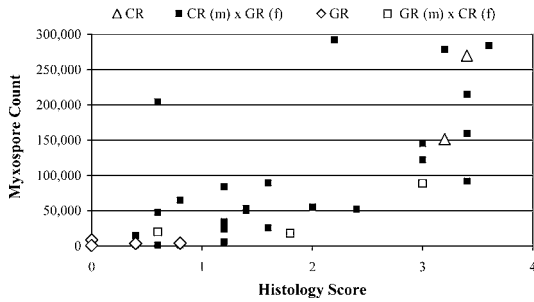


FIGURE 1.—Myxospore counts and histological scores for pure rainbow trout from Germany (GR), pure Colorado River rainbow trout (CR), and GR (female)  $\times$  CR (male) and GR (male)  $\times$  CR (female) crosses after experimental exposure to *Myxobolus cerebralis*.

differences in myxospore counts, but not lesion severity, between the CR strain and the reciprocal crosses (CR female  $\times$  GR male) may in part be a function of the large variation seen in the few family groups ( $n = 3$ ) analyzed from these crosses. Variability between lesion severity scores and subsequent myxospore counts has been observed in prior studies with rainbow trout (Ryce et al. 2005). Such variability may reflect differences in the onset of key immune responses, some of which may still be maturing, in young rainbow trout (Manning et al. 1982).

The GR strain's resistance to *M. cerebralis* infections in the current study was similar to that observed in prior studies (Hedrick et al. 2003) and provides further confirmation of the unique properties of this strain of rainbow trout. The low lesion severity and myxospore counts observed in nine families of  $F_1$  crosses resemble those seen in naturally and experimentally infected resistant trout species, including the brown trout *Salmo trutta*. Field and experimental exposures of brown trout routinely result in low lesion scores ( $\leq 1$ ) and myxospore counts ( $10^{3-4}$  per half-head) than those seen in most rainbow trout strains ( $\geq 2.5$  and  $\geq 10^5$ ) (Hedrick et al. 1999a). If the inherited *M. cerebralis* resistance observed in several  $F_1$  families is as stable as that observed in brown trout, then these  $F_1$  rainbow trout might also possess the capability to withstand environmentally relevant concentrations of TAMs in natural systems and avoid population declines. However, it is critical to stress that resistance to whirling disease is just one of many factors that would likely determine survival and reproduction of rainbow trout bred for disease resistance in natural systems.

Selective breeding approaches have been used successfully for disease control with fish, most often among fish species reared for aquaculture (Chevassus

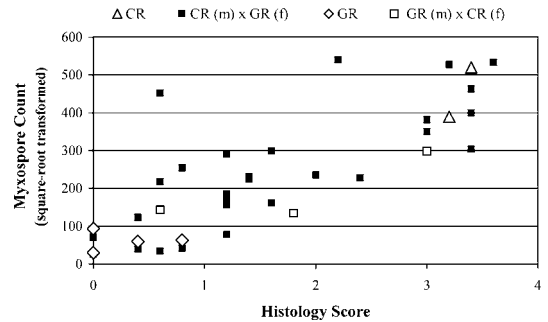


FIGURE 2.—Square-root-transformed myxospore counts and histological scores for pure rainbow trout from Germany (GR), pure Colorado River rainbow trout (CR), and GR (female)  $\times$  CR (male) and GR (male)  $\times$  CR (female) crosses after experimental exposure to *Myxobolus cerebralis*. The two variables are highly correlated, as measured by linear regression ( $R^2 = 0.659$ ).

and Dorson 1990). These include strains of trout and cyprinids with resistance to bacterial, viral, and parasitic diseases (Ehlinger 1977; Bartholomew 1998; Osaki et al. 2001). Disease resistance to myxosporean pathogens in salmonids has been limited to studies on *Ceratomyxa shasta* (reviewed by Bartholomew 1998). This resistance has been exploited in the propagation of both anadromous and resident rainbow trout populations in the Pacific Northwest of North America. Unfortunately, early trials demonstrated that *C. shasta* resistant rainbow trout were not resistant to whirling disease, indicating that the evolution of host defense mechanisms to myxosporean diseases are specific for each host-parasite relationship (Hedrick et al. 2001).

Selective breeding for disease resistance may also result in the appearance of unwanted or deleterious traits, as demonstrated in programs with certain bacterial and viral diseases. In certain cases, breeding has resulted in resistance to a particular disease but increased susceptibility to other agents (Chevassus and Dorson 1990; Allendorf et al. 2001). Both field and laboratory trials with selectively bred whirling disease resistant rainbow trout will be required to assess this potential. Fortunately, trials with the pure GR strain to evaluate susceptibility to selected pathogens that may not have been routinely experienced in Germany demonstrated that this strain was no more susceptible than currently used domesticated strains in North America (Bartholomew et al. 2004).

Future research will be required to determine whether this resistance can be carried through multiple generations. However, it is very encouraging that the average resistance was unquestionably greater in the  $F_1$  crosses than in the pure CR rainbow trout groups and

that some  $F_1$  families demonstrated resistance as strong as that of the pure GR rainbow trout families.

Additional characteristics of  $F_1$  crosses with CR  $\times$  GR that might be important to survival in natural systems were observed in the laboratory study. The aggressive feeding behavior and lack of fright response were more subdued in these crosses than in pure GR rainbow trout. Thus, these  $F_1$  crosses displayed a more "wild" behavior of diving to the bottom of the tanks to avoid surface disturbance, as described by Vincent (1960). This intermediate behavior in crosses of wild and domestic fish has also been observed in anadromous rainbow trout (Negus 1999). Field studies to further evaluate survival of  $F_1$  crosses are planned, as are studies of  $F_1 \times$  CR back-crosses that may retain resistance to *M. cerebralis* while also instilling additional and important traits needed for survival in natural systems.

As field trials and later applications for the use of these strains are considered, additional factors must be evaluated, including the presence of residual wild rainbow trout or native salmonid populations. Allendorf et al. (2001) pointed out several drawbacks of replacing susceptible wild stocks with resistant strains. Among these are loss of genetic variation in natural populations and competition and introgression with native strains. They also correctly point out that the use of resistant strains may result in failure to address the underlying source of elevated *M. cerebralis* infection in a given drainage, such as habitat degradation and point sources of infection. Thus, fisheries management approaches should consider this range of potential implications prior to the stocking of any waters.

Certain areas in Colorado and the Intermountain West are candidates for the responsible use of rainbow trout with a resistance to *M. cerebralis*. In Colorado, rainbow trout are not native, and wild populations that have been established in the past are unique only in their ability to survive and reproduce naturally in many popular fisheries throughout the state. These populations have been introgressed with other domestic and wild stocks accidentally and intentionally throughout their history. Unfortunately, domestic hatchery strains are routinely stocked into or upstream of these wild populations, in some cases on an annual basis. These populations may be genetically divergent from other stocks, but their history and proximity to domestic fish dictate that this divergence is not as unique as that of some naturalized stocks occurring in other western states (Allendorf et al. 2001). The argument has also been made that populations affected by *M. cerebralis* will rebound after an initial decline (Allendorf et al. 2001). In cases where complete population collapse has occurred, there is no possibility of natural rebound of

the population. Wild rainbow trout populations in most affected rivers in Colorado have been completely decimated by *M. cerebralis*. Rainbow trout remaining in those rivers are either very old fish that hatched prior to the outbreak of whirling disease or fish that have been stocked to replace the missing year-classes (R. B. Nehring, Colorado Division of Wildlife, personal communication). Natural selection cannot occur when there are no surviving members of the population. In these cases, replacement of the rainbow trout component of these recreational fisheries with a resistant strain of rainbow trout is appropriate, particularly if attempts to reduce infectivity in the drainage by habitat modification or other means have failed and if there is no endangerment to naturally reproducing populations of native trout. In conclusion, our studies provide initial evidence that the use of rainbow trout with a genetic resistance to *M. cerebralis* may be one management strategy for restoration of naturally reproducing populations of rainbow trout in selected areas of the Intermountain West.

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