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Effects of Multiple Stressors on Morbidity and Mortality of Fingerling Rainbow Trout Infected with *Myxobolus cerebralis*

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Abstract.—Myxobolus cerebralis, the causative agent of salmonid whirling disease, has been implicated in year-class losses of rainbow trout *Oncorhynchus mykiss* in several rivers in Colorado. The hypothesis that other factors, such as elevated water temperature, bacterial pathogens, and gas supersaturation, are contributing to these year-class losses was tested in a laboratory setting. Fingerling rainbow trout were exposed to all combinations of these stressors for 6 months. Mortality and morbidity were evaluated for each of the test groups using analysis of variance (ANOVA). Mortality was significantly affected by exposure to *M. cerebralis* ($P = 0.0002$) and elevated water temperature ($P = 0.0002$). Morbidity was significantly affected by exposure to *M. cerebralis* ($P = 0.0001$). A significant linear increase ($P = 0.0020$) in mortality was observed with *M. cerebralis* infection and addition of all combinations of one, two, and three stress factors.

Myxobolus cerebralis, the causative agent of salmonid whirling disease, has been implicated in partial or complete year-class losses of rainbow trout *Oncorhynchus mykiss* in the upper Colorado, Gunnison, Rio Grande, South Platte, Dolores, Frying Pan, Roaring Fork, and Cache la Poudre rivers in Colorado (Nehring et al. 1998). Until recently, the pathogen has not been considered a threat to wild salmonid populations. *Myxobolus cerebralis* exists in at least 21 states (Bergersen and Anderson 1997), but population level effects in these states have been rarely reported. The inconsistent response of wild rainbow trout populations to *M. cerebralis* exposure raises the possibility that other factors may be contributing to year-class losses of rainbow trout in environments positive for *M. cerebralis.*

The presence of an additional stressor or stressors is one explanation for situations where population level effects occur when *M. cerebralis* is present. Three stressors may be acting on wild fish populations in the upper Colorado River in addition to *M. cerebralis*: elevated water temperature, bacterial pathogens, and gas supersaturation.

Elevated water temperature has been shown to increase the virulence and maturation rate of *M. cerebralis.* Halliday (1976) states that the optimum temperature range for growth of the parasite is between 15°C and 17°C. At higher temperatures (20–23°C) the pathogen reportedly begins to lose its virulence (Schaperclaus 1931). The triactinomyxon stage is very short lived at high temperatures, surviving only 2–3 d at temperatures exceeding 19°C (Markiw 1992b). Average water temperatures in the Colorado River during the summer months range from 13°C to 17°C and appear to be ideal for *M. cerebralis* growth. We hypothesized that these temperatures may result in higher mortality among fingerling trout infected with the parasite and could help explain the loss of year-classes in some Colorado rivers.

Gas supersaturation is caused by excessive dissolved gas pressure in water and can result in deleterious effects to young fish. Average gas supersaturations of up to 110% occur in some reaches of the Colorado (Schisler and Bergersen 1999) and Gunnison rivers of Colorado (R. B. Nehring and K. G. Thompson, Colorado Division of Wildlife, unpublished, 1996). Saturations of these levels have been reported to cause a variety of physiological problems for young fish ranging from reduced growth to death (Dennison and Marchyshyn 1973; Schiewe 1974; Bouck 1976; Shrimpton et al. 1989; U.S. National Marine Fisheries Service 1995). Other studies have indicated that gas saturations of up to 110% do not have a serious effect on survival of fish (Dawley and Ebel 1975; Nebeker et al. 1980; Jensen 1988).

Flavobacterium psychrophilum is a widespread fish pathogen that has been known to cause heavy

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mortality among salmonid fry (Lorenzen and Karas 1992; Lorenzen 1994). Several fish examined during routine fish health evaluations on the upper Colorado River exhibited signs of disease that may be related to this pathogen (Walker and Nehring 1995). We hypothesized that *F. psychrophilum* may exacerbate the effects of *M. cerebralis* and that its presence may also contribute to the loss of year-classes of rainbow trout in the Colorado River.

The focus of this study was to test all four of the individual factors simultaneously to identify the relative contributions of each to mortality and to determine if the addition of more than one stress factor increases mortality of fingerling rainbow trout exposed to *M. cerebralis.* This information would help us determine if any or all of these factors are contributing to the loss of rainbow trout year-classes in some Colorado rivers.

Methods

Certified disease-free rainbow trout eggs were incubated in a 38-L aquarium at 12.5°C with a flow of 3 L well water/min (pH 7.2, $CaCO_3$ 450 mg/L). Treatment and control fish were placed in separate aquaria 2 days before swim-up as 18-d-old sac fry. All sac fry in the treatment group were exposed to an average of 485 triactinomyxons, the infective stage of *M. cerebralis,* for 3 h. This exposure level was chosen by using data from yearling rainbow trout disease testing in the Colorado River. Of surviving yearling rainbow trout sampled, 83% were infected, and myxospore loadings averaged 71,250 per fish (Walker and Nehring 1995). Markiw (1992a) found that infectivity rates and spore loadings of this magnitude could be produced by exposing fish to anywhere from 100 to 1,000 infective units.

A battery of 48 aquaria were set up to allow for a four-factor experimental design conducted in triplicate. Each factor occurred at two levels, with each level occurring with each level of the other three factors, for a complete 24-factorial treatment design. Flow rate to each aquarium was set at 0.5 L well water/min. Twenty fish exposed to *M. cerebralis* were placed in each of 24 aquaria. Fish were distributed five at a time to ensure their random allocation to treatment groups. Twenty unexposed fish were placed in each of the remaining 24 aquaria in a similar manner.

Water temperatures were adjusted to 17°C in 12 of the *M. cerebralis*-positive aquaria and in 12 of the *M. cerebralis*-negative aquaria to test the effect of summer water temperatures observed in the upper Colorado River. The remaining 24 aquaria were maintained at 12.5°C.

Gas saturations were elevated to 110% in half of the aquaria for each treatment group. The remaining 24 aquaria were maintained at 100% (nominal) saturation. Saturations used in the experiment were chosen to test the upper bound of gas saturations found in Colorado rivers. Nominal saturations were achieved by rigorously aerating water in 300-L headboxes before delivery to the aquaria. Supersaturation was achieved by forcing atmospheric gasses together with water in a VMG Industries In-line Oxygenator at a rate of 18 L air and water/min. This produced saturations of approximately 118%. The water was then aerated in 300-L headboxes to reduce supersaturation to 110% before it was gravity-fed into the aquaria. Water temperatures and gas saturations were closely monitored with Common Sensing TBO saturometers throughout the entire experiment to ensure consistent treatment and control levels.

Half of the aquaria in each treatment group were exposed to *F. psychrophilum* at a dosage of 9.9×10^7 colony-forming units per aquarium 12 weeks after the beginning of the experiment. The culture used was isolated from the Colorado State Fish Hatchery, Bellvue, and is thought to have been the cause of a particularly serious epizootic occurring at that facility earlier in 1997. To prevent possible infection in treatment groups designated as *F. psychrophilum*-negative, Terramycin-343 (75.6% oxytetracycline) was added to the food of these fish at 1-month intervals at a rate of 2.5 g active ingredient/45 kg fish daily for 10 d. Fish were fed daily using guidelines in Piper et al. (1982).

Dead fish were removed daily from each aquarium for the duration of the experiment. At the end of the experiment, signs of disease were recorded for all fish remaining in each aquarium. Deformities and clinical signs of disease including blacktail, spinal deformities, cranial deformities, deformed mandibles, deformed opercules, and exophthalmia were recorded. Four-factor analysis-of-variance (ANOVA) tests were conducted to determine if differences in mortality and morbidity existed due to the treatment effects used in the experiment. All two-, three-, and four-way interactions were tested in the ANOVA. Arcsine square root transformations were used in the analyses to stabilize variances. However, results of the ANOVA with and without transformations were very similar, so results are reported from the ANOVA without transformations to simplify interpretation. Linear regression was used to test if

TABLE 1.—Mortality and morbidity of fingerling rainbow trout exposed to gas supersaturation of 110.0% (S), *Flavobacterium psychrophilum* (F), *Myxobolus cerebralis* (M), and elevated water temperature (17°C, T) for 6 months; $N = 3$ aquaria for each treatment.

	Mortality (%)		Morbidity (%)	
Treatment	Mean	SD	Mean	SD
Control	11.7	10.4	14.8	13.9
S	23.3	12.6	4.4	7.7
F	11.7	11.5	14.6	6.0
T	23.3	7.6	17.6	4.8
M	41.7	7.6	95.1	4.3
F, S	15.0	0.0	43.8	28.6
M, F	23.3	5.8	97.6	4.1
T, S	30.0	34.6	20.2	12.2
M, S	38.3	32.1	100.0	0.0
M, T	60.0	22.9	100.0	0.0
F, T	46.7	15.3	29.7	4.3
F, T, S	45.0	26.5	41.0	52.4
M, S, F	43.3	36.9	100.0	0.0
M, T, S	46.7	15.3	97.4	4.4
M, T, F	76.7	7.6	100.0	0.0
M, F, T, S	70.0	20.0	91.7	14.4

addition of multiple stress factors caused a subsequent increase in mortality and morbidity of *M. cerebralis*-infected fish. Individual aquaria were used as the experimental unit, and alpha level was set at 0.05 for all statistical tests.

Results

Mortality

Mortality was strongly affected by exposure to factors tested in this study (Table 1). Cumulative mortality was higher ($P = 0.0002$) among fish exposed to *M. cerebralis* than among their unexposed counterparts (Table 2). Average mortality across all treatments was 50.0% ($N = 24$, SD = 24.6) for *M. cerebralis*-exposed fish and 25.8% ($N = 24$, SD = 20.0) for unexposed fish. Mortality among fish across all treatment groups at 17.0°C (mean = 49.79%, $N = 24$, SD = 24.6) was much higher ($P = 0.0002$) than those at 12.5°C (mean = 26.0%, $N = 24$, SD = 20.3).

Treatment groups exposed to *F. psychrophilum* suffered slightly higher mortality (mean = 41.5%, $N = 24$, SD = 27.9) than those unexposed (mean = 34.4%, $N = 24$, SD = 22.6) to the bacterium, although the effect was not significant ($P = 0.2249$). A significant interaction effect was observed for *F. psychrophilum* and elevated water temperature ($P = 0.0364$). Mortality among fish in water at 110% saturation (mean = 38.9%, $N = 24$, SD = 26.5) was not significantly different ($P = 0.7183$) than those in water at 100% saturation (mean = 36.9%, $N = 24$, SD = 24.7).

Although the only significant interaction effect was *F. psychrophilum* and water temperature, treatment groups with combinations of factors often experienced higher mortality (Table 1), which was due to the additive nature of *M. cerebralis* exposure and additional stressors to mortality. Regression analysis revealed a significant linear increase ($F = 11.97$, df = 1, 25; $P = 0.0020$) in mortality with the addition of stress factors. When no factors were present, average mortality was 11.7%. When

TABLE 2.—Results of ANOVA for mortality and morbidity of rainbow trout exposed to four different stressors for 6 months: gas supersaturation at 110% (S), water temperature elevated to 17°C (T), *Flavobacterium psychrophilum* (F), and *Myxobolus cerebralis* (M).

	Mortality			Morbidity		
Source[a]	Mean square	*F*-value	*P*	Mean square	*F*-value	*P*
S	52.08	0.13	0.7183	158.81	0.59	0.4481
F	602.08	1.53	0.2249	889.33	3.30	0.0785
T	6,768.75	17.21	0.0002	599.04	2.23	0.1456
M	7,008.33	17.82	0.0002	66,517.35	247.9	0.0001
F, S	33.33	0.08	0.7728	297.85	1.11	0.3007
M, F	2.08	0.01	0.9424	1,062.67	3.95	0.0556
T, S	408.33	1.04	0.3158	99.27	0.37	0.5480
M, S	102.08	0.26	0.6139	246.38	0.92	0.3459
M, T	102.08	0.26	0.6139	222.27	0.83	0.3703
F, T	1,875.00	4.77	0.0364	39.55	0.15	0.7040
F, T, S	52.08	01.3	0.7183	216.45	0.80	0.3766
M, S, F	408.33	1.04	0.3158	599.04	2.23	0.1456
M, T, S	133.33	0.34	0.5644	33.82	0.13	0.7253
M, T, F	8.33	0.02	0.8852	0.86	0.00	0.9552
M, F, T, S	52.08	0.13	0.7183	142.45	0.53	0.4723

[a] Degrees of freedom = 1, 32.

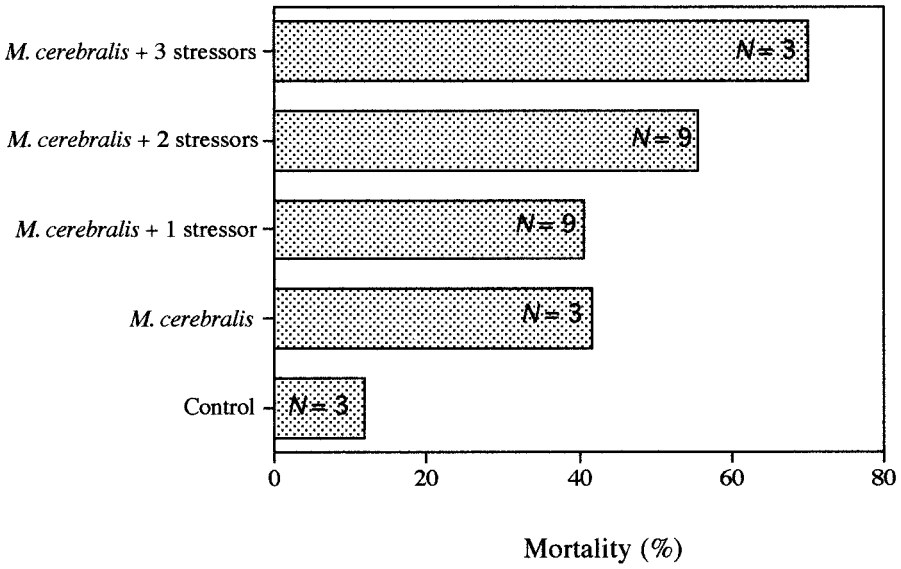

FIGURE 1.—Mortality (%) of fingerling rainbow trout as a function of stress and disease (*Myxobolus cerebralis*) factors after 6 months; N = number of aquaria.

M. cerebralis was the only stress factor, mortality was 41.7%. *Myxobolus cerebralis* combined with one stress factor resulted in an average of 40.5% mortality. Addition of two stress factors increased average mortality to 55.6%. Addition of three stress factors elevated mortality to 70.0% (Figure 1). The relationship of higher mortality with addition of stress factors clearly illustrates the effect that multiple stress factors can have on survival of trout.

Morbidity

Fish exposed to *M. cerebralis* began exhibiting clinical signs of disease within 60 d. By the end of the experiment, 97.7% (N = 24, SD = 5.6) of fish surviving *M. cerebralis* exposure exhibited at least one sign of disease (P = 0.0001; Table 2) whereas only 23.3% (N = 24, SD = 22.9) of those not exposed to *M. cerebralis* did the same.

Exposure to *F. psychrophilum* had an effect on clinical signs of disease only when other stressors were present. Overall percentage of fish exhibiting signs of disease when exposed to *F. psychrophilum* was 64.8% (N = 24, SD =38.9), whereas 56.2% (N = 24, SD = 43.5) of those not exposed to the bacteria showed signs of disease (P = 0.0785). The interaction effect of *M. cerebralis* and *F. psychrophilum* on signs of disease was nearly significant (P = 0.0556).

Water temperature (P = 0.1456) and gas saturation (P = 0.4481) did not have significant effects on morbidity of fish. Nearly the same percentage of fish not exposed to gas supersaturation exhibited signs of disease (mean = 58.7%, N = 24, SD = 41.0) as did exposed fish (mean = 62.3%, N = 24, SD = 41.9). Signs of disease occurred in 58.8% (N = 24, SD = 42.8) of fish held at low water temperature, and 62.2% (N = 24, SD = 40.1) of those held in higher water temperature were afflicted.

Deformities occurred in 14.8% (N = 3, SD = 13.9) of fish not exposed to stress factors (Table 1). Deformities in these fish could have been caused by genetic factors or by confinement to the relatively small aquaria used in this study. Exposure to *M. cerebralis* as a single stress factor resulted in morbidity among 95.1% (N = 3, SD = 4.3) of fish tested. Because of the high proportion of fish exhibiting signs of disease with exposure to *M. cerebralis*, subsequent addition of stress factors did not increase morbidity in a linear manner as was observed with the mortality data (Figure 2).

Discussion

Addition of stressors increased mortality in varying amounts. The most substantial increase was due to *M. cerebralis* infection. Water temperature also increased mortality quite drastically. *Flavobacterium psychrophilum* exposure was a serious concern only when other stressors were present. Gas supersaturation had a fairly weak effect on mortality.

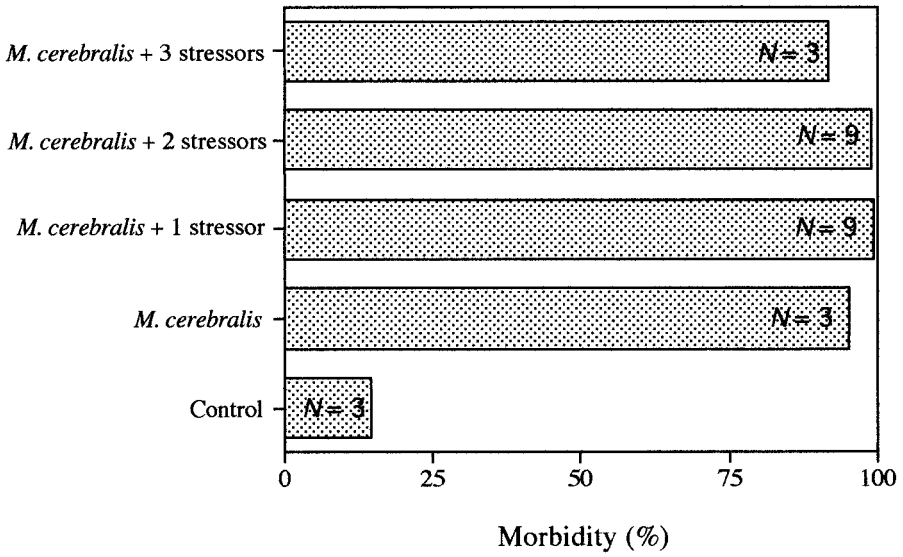

FIGURE 2.—Morbidity (percent of fish exhibiting at least one deformity or sign of disease) of fingerling rainbow trout as a function of stress and disease (*Myxobolus cerebralis*) factors after 6 months; N = number of aquaria.

A prior study investigating the chronic effects of low levels of gas supersaturation had similar results, in which gas supersaturations of 110% or less had very little effect on the growth or survival of fingerling rainbow trout (Schisler et al. 1999). Although gas supersaturation exists in the Colorado and Gunnison rivers, the effect is probably minor on rainbow trout year-class losses.

Mortality and morbidity results were very similar for *F. psychrophilum*-infected and uninfected groups at 12.5°C. Noticeable differences in mortality were, however, observed at 17°C, leading to the significant interaction effect observed for *F. psychrophilum* and water temperature. Generally considered to be more virulent at colder water temperatures, *F. psychrophilum* affects fish most dramatically when water temperature is less than 15°C (Holt et al. 1989). Our results indicate that this particular strain of the pathogen was more damaging to fish at 17°C than at 12.5°C. Morbidity results show that addition of any factor caused an increase in signs of disease among *F. psychrophilum*-exposed fish.

Elevated water temperatures contributed greatly to mortality of fingerling rainbow trout in this study. Although the effect was relatively minor when temperature was the only factor, it was quite noticeable with the addition of other factors, especially when exposures to *M. cerebralis*, *F. psychrophilum*, and elevated water temperature were combined. It is possible that increased metabolic rate of both the fish and pathogens at higher temperatures contributed to this effect.

Myxobolus cerebralis was the most important contributor to mortality tested in this experiment at the challenge dose administered. Fish in the exposed test groups experienced higher mortality than their unexposed counterparts in every case. These results tend to support the hypothesis that whirling disease is the predominant cause of declines in fingerling rainbow trout survival in some Colorado rivers, with other stressors playing an important but secondary role. However, other untested factors may be present in these drainages that could result in year-class losses of rainbow trout. Further studies should be conducted in the wild to verify the role of *M. cerebralis* and other stressors on declining trout populations.

Given the results of this study, the argument could be made that high infectivity levels of *M. cerebralis* and optimal temperatures for *M. cerebralis* are important contributors to losses of rainbow trout in rivers of Colorado. In many rivers of Colorado, water use dictates flows, which can have an effect on water temperatures. Shallow, silty-bottomed impoundments, such as Windy Gap Reservoir on the Colorado River, further contribute to the problem by acting as point sources of *M. cerebralis* infection. Alteration of natural rivers may not be a problem for healthy fish populations, but once a pathogen such as *M. cerebralis* is added to the system, the effects of the alterations may be

realized. According to Snieszko's model of fish disease (Snieszko 1974), a fish must be exposed to a pathogen and a physiological stressor for disease to occur. In other words, the mere presence of the pathogen is not enough to cause disease. If we accept this theory, then it is not unusual to see the majority of fish populations incurring no obvious harm from the presence of *M. cerebralis.* The populations within an altered ecosystem such as the Colorado River may not show any deleterious effects in the absence of a serious pathogen, but the arrival of the pathogen can make the other problems a population is facing more apparent. This experiment has shown that addition of stressors can exacerbate the effects of *M. cerebralis*, and addition of multiple stress factors results in higher mortality and morbidity among fingerling rainbow trout. Whereas the presence of bacterial pathogens in natural systems is virtually impossible to control, factors such as point sources of infection from *M. cerebralis* and elevated water temperatures may be manipulated in highly regulated systems such as the Colorado River to reduce their effects on trout populations.

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