# Evaluation of Chronic Gas Supersaturation on Growth, Morbidity, and Mortality of Fingerling Rainbow Trout Infected with *Myxobolus cerebralis*

GEORGE J. SCHISLER* AND ERIC P. BERGERSEN

*Colorado Cooperative Fish and Wildlife Research Unit,* [1]*Colorado State University,*
*201 Wagar Building, Fort Collins, Colorado 80523, USA*

PETER G. WALKER

*Colorado Division of Wildlife,*
*Post Office Box 128, Brush, Colorado 80723, USA*

*Abstract.*—The effect of environmental stressors on trout infected with *Myxobolus cerebralis,* the causative agent of whirling disease, is of great interest because the parasite has spread to most areas of the United States that support wild trout populations. Gas supersaturation is an environmental stressor found in at least two rivers in Colorado where *M. cerebralis* exists, and year-class losses of rainbow trout *Oncorhynchus mykiss* have occurred. Both of these stress factors may also occur in fish culture operations. A controlled laboratory experiment was conducted to test the hypothesis that chronic low levels of gas supersaturation affect growth, morbidity, and mortality of fingerling rainbow trout infected with *M. cerebralis.* Fingerling rainbow trout, exposed and unexposed to *M. cerebralis,* were held in gas saturations of 110, 107.5, 105, and 100–102.5% for 22 weeks. Significant effects on growth, morbidity, and survival were found due to *M. cerebralis* infection. Gas saturation levels tested did not significantly affect growth, morbidity, or survival of fish in this experiment.

The presence of *Myxobolus cerebralis* has been implicated in rainbow trout year-class losses in the Colorado, Cache la Poudre, Rio Grande, Gunnison and South Platte rivers in Colorado (Walker and Nehring 1995; Nehring 1998). Severe population level effects due to *M. cerebralis* have been rarely reported in other areas where the parasite is known to exist. One possible explanation for this phenomenon is that environmental stressors are acting as exacerbating factors, causing fish to succumb to the effects of *M. cerebralis* infection. Average gas supersaturation levels of 110% have been recorded in the Colorado River (Schisler and Bergersen, in press) and the Gunnison River (Nehring 1998), with occasional peaks of supersaturation of up to 115%. Young-of-the-year rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* sampled from the upper Colorado River by Walker and Nehring (1995) displayed signs of disease normally observed in juvenile salmonids exposed to chronic low levels of supersaturation, including exophthalmia, spinal flexures, and discoloration.

Gas bubbles were also observed in the gill filaments and in the kidneys of some fish. Gas saturation even a few percentage points above 100% has been known to be detrimental to newly hatched fry (Dennison and Marchyshyn 1973). Other signs of gas bubble trauma (GBT) reported between 100% and 110% supersaturation include overinflation of the swim bladder in small fish (Shrimpton et al. 1989), ocular lesions, immunosuppression, reduced growth, bubbles in the intestinal tract (NMFS 1995), loss of swimming ability (Schiewe 1974), and even death (Bouck 1976). The reported threshhold between chronic and acute GBT occurs somewhere between 108% and 110% (Jensen et al. 1986). However, the U.S. Environmental Protection Agency suggested 110% as a safe upper limit for aquatic life (USEPA 1976). This criterion is supported by numerous studies indicating that acute gas saturations below 110% do not significantly affect fish survival (Dawley and Ebel 1975; Nebeker et al. 198; Jensen 1988). The majority of studies have investigated the effect of acute high levels of supersaturation, usually greater than 115%. The effects of chronic low levels of supersaturation on salmonids have been largely ignored.

Fish culture operations are often faced with gas supersaturation problems, and *M. cerebralis* is now present in many fish culture facilities. The com-

* Corresponding author: georges@neota.cnr.colostate.edu

bination of these two stressors has the potential to adversely affect growth and survival of trout in both natural and artificial environments. This study was initiated to determine the effects of chronic gas supersaturation on fingerling rainbow trout infected with *M. cerebralis*. To eliminate the effects of other environmental stressors that may occur in natural systems, a laboratory experiment was designed to test the hypothesis that chronic low levels of gas supersaturation compromise the growth and survival of fingerling rainbow trout infected with *M. cerebralis*.

## Methods

A battery of 24 aquaria (96-L) was set up in the aquatic toxicology laboratory at the Colorado Division of Wildlife Research Station in Fort Collins, Colorado. Cooled well water (12.5°C) was mixed under pressure with atmospheric gasses to produce water saturated at 118%. The water was then mixed in 300-L headboxes with unsaturated well water to produce total gas saturations of 110% ($O_2$ = 103%, $N_2$ + Ar = 112%), 107.5% ($O_2$ = 103%, $N_2$ + Ar = 108%), 105% ($O_2$ = 97%, $N_2$ + Ar = 107%), and 100–102.5% ($O_2$ = 91.6−95%, $N_2$ + Ar = 102.3−103%) total dissolved gas. Saturations were chosen based on saturation levels observed in the upper Colorado River, which ranged from 99.3% difference in pressure between total gas pressure and local barometric pressure, ($\Delta P$ = −4) to 113.4% ($\Delta P$ = 77) below Windy Gap Reservoir during the months of June through October 1995. Saturations were monitored with Common Sensing TBO saturometers throughout the entire experiment to ensure consistent saturation levels. The water was gravity fed at a rate of 1 L/min to each aquarium, which would provide adequate flows to maintain 35 fish in each aquarium. Three aquaria were designated for fish exposed to *M. cerebralis* and three for unexposed fish at each saturation level.

Colorado River rainbow trout fry were obtained from the Glenwood Springs rearing unit, a facility free from *M. cerebralis,* immediately after swim-up for use in the experiment. The number of infective units (triactinomyxons) and exposure time were decided by using data from yearling rainbow trout disease testing in the Colorado River. Of surviving yearling rainbow trout sampled, 83% were found to be infected, and myxospore loadings averaged 71,250 per fish (Walker and Nehring 1995). Markiw (1992) found that infectivity rates and spore loadings of this magnitude could be produced by exposing fish to between 100 to 1,000 infective units. Fry designated for the *M. cerebralis* treatment groups were all exposed at once to an estimated 100–500 triactinomyxons per fish for 3 h in standing water to ensure infection. Both the treatment and control groups were held for 10 d after the treatment fish were infected to allow fish to recover from handling. After the 10-d recovery period, 35 fish were placed into individual aquaria, 5 fish at a time, to ensure random assignment to aquaria.

Fish were fed several times daily using guidelines in Piper et al. (1982), and dead fish were removed daily throughout the entire 5 months of the experiment. Midway through the experiment (10 weeks postexposure) three fish from each aquarium were removed for evaluation. These fish were euthanatized one at a time with an overdose of tricaine methanesulfonate (MS-222), weighed, measured, and examined for external signs of whirling disease and gas bubble trauma. Lengths and weights were compared to determine if there were any growth effects due to different treatments. Morbidity data consisted of proportions of fish exhibiting abnormalities, including cranial, spinal, opercular, or lower jaw deformaties, black-tail, and exophthalmia, in each test group. Because of its transient nature, whirling behavior was not recorded in the morbidity data. All four gill arches from the right side of the fish were removed and examined under a compound microscope (40× magnification) for presence and severity of gas emboli in the gill filaments. Ten filaments from each gill arch were categorized as either containing emboli or not containing emboli. The ratio of filaments containing emboli to those not containing emboli was calculated for each fish in this manner.

Mortalities were evaluated at the end of the experiment to determine if exposure to chronic gas supersaturation and *M. cerebralis* infection affected survival. A sample of three fish from the survivors in each aquarium was weighed, measured, and examined for external signs of gas bubble trauma and whirling disease in a similar manner as in the midexperiment evaluations. Two-factor analysis of variance (ANOVA) with aquaria as the experimental units was used to compare the mortalities, weights, lengths, proportion of gill filaments containing gas emboli, and numbers of signs of disease in the experiment. Four additional fish from each of the aquaria containing fish exposed to *M. cerebralis* were sacrificed for myxospore evaluations. Evaluations were conducted using the pepsin–trypsin digest method described by Markiw and Wolf (1974). A one-way ANOVA was
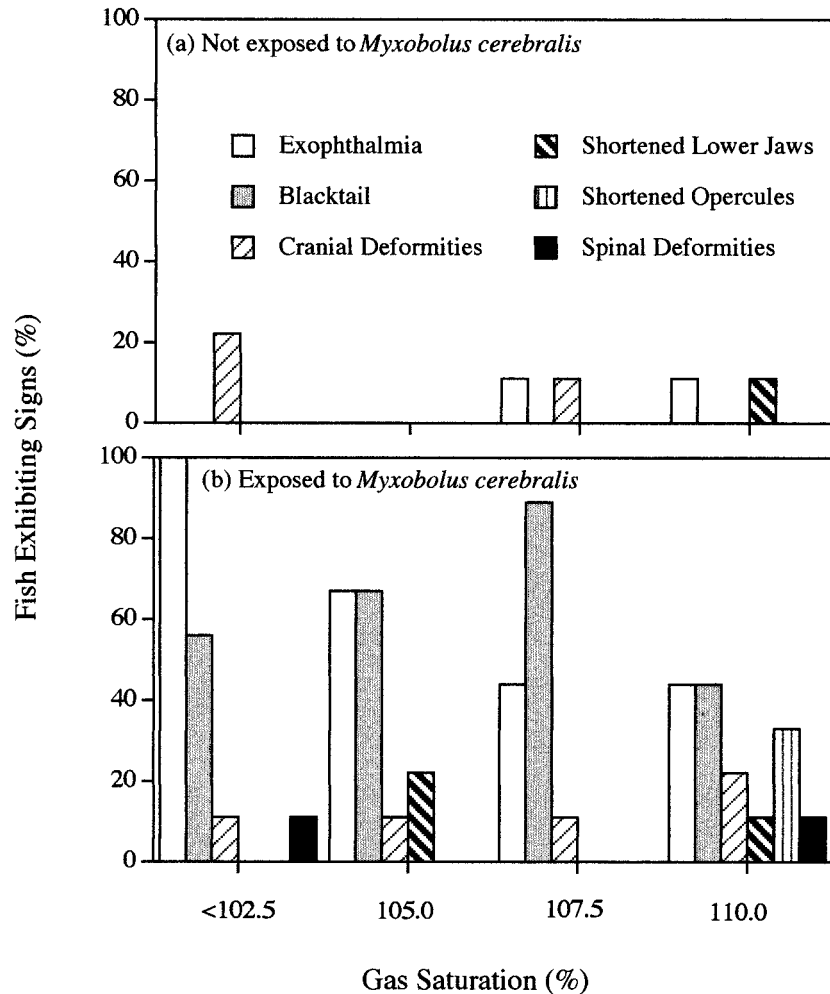
FIGURE 1.—Percentage of fingerling rainbow trout **(a)** not exposed and **(b)** exposed to *M. cerebralis* exhibiting signs of disease after 10 weeks at four gas supersaturation levels.

conducted to determine if myxospore numbers were affected by gas saturation level.

## Results

Overt signs of whirling disease were apparent in some fish as early as 1 month after exposure to *M. cerebralis.* Clinical signs of disease were observed in the majority of fish exposed to *M. cerebralis* by 7 weeks after exposure. A small proportion of fish in the groups not exposed to *M. cerebralis* exhibited signs of disease (Figure 1). We later discovered that the fish used in this experiment had been exposed to *Flavobacterium psychrophilum,* the causative agent of bacterial coldwater disease, before arrival at our facility. A subsample of fish taken from each aquaria at the end of the experiment all tested pos-

itive for the presence of the organism. Signs of disease observed in the groups not exposed to *M. cerebralis* were probably caused by bacterial coldwater disease. Average frequency of signs of disease was 0.14 (SD = 0.22) per fish among fish not exposed to *M. cerebralis.* Fish exposed to *M. cerebralis* averaged 1.9 (SD = 0.68) signs per fish. Because of the nonuniformity of variances observed between fish exposed and fish not exposed to *M. cerebralis,* a square-root transformation was conducted before analysis. During the first morbidity evaluation event, frequency of signs of disease was significantly affected by exposure to *M. cerebralis* ($F$ = 79.23; df = 1, 16; $P$ = 0.0001). Gas supersaturation ($F$ = 0.34; df = 3, 16; $P$ = 0.7979) and interaction effects ($F$ = 0.47; df = 3, 16; $P$ = 0.7078) were not sig-
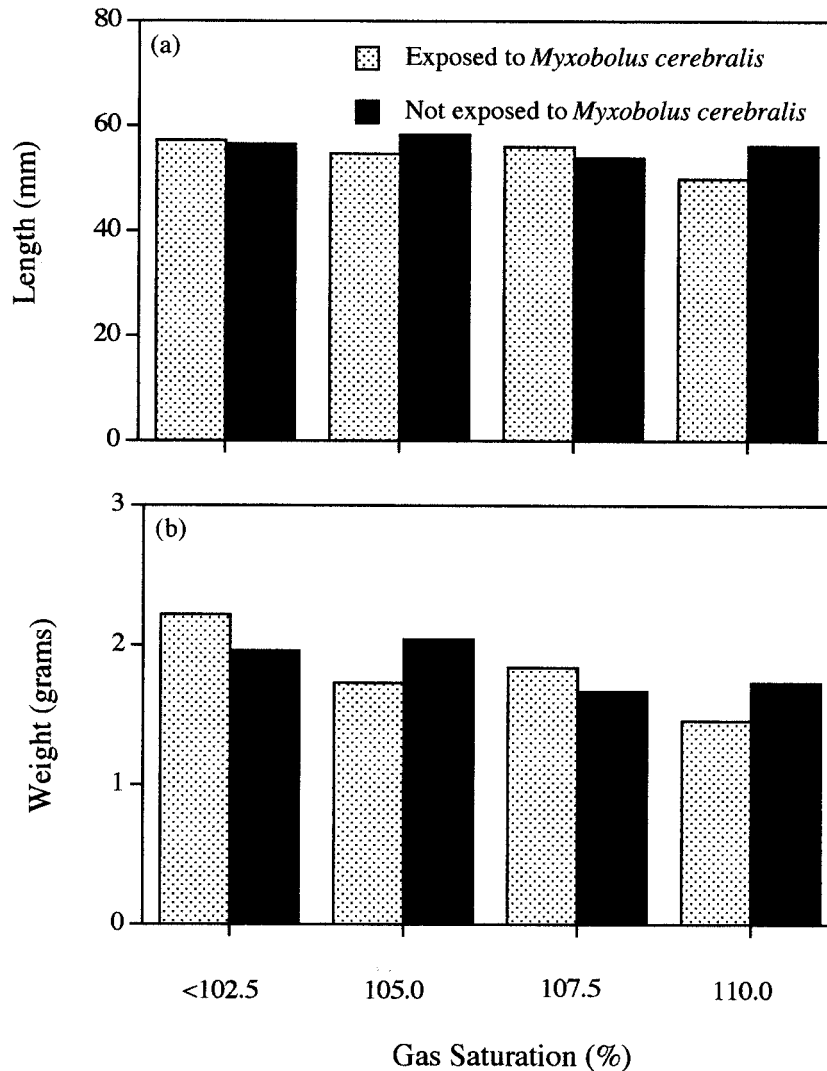
FIGURE 2.—(a) Mean lengths and (b) and weights of rainbow trout fingerlings exposed and not exposed to *M. cerebralis* and held at four different gas saturation levels for 10 weeks.

nificant. The most common signs of disease included blacktail, exophthalmia, and cranial deformities. No overt external signs of gas bubble trauma were observed among the fish held at any saturation level during the first evaluation.

Mean lengths and weights of fish were not noticeably affected by either *M. cerebralis* infection or by gas saturation level at 10 weeks postexposure (Figure 2). The ANOVA resulted in nonsignificant effects due to *M. cerebralis* exposure for both length ($F = 0.56$; df = 1, 16; $P = 0.4648$) and weight ($F = 0.03$; df = 1, 16; $P = 0.8654$). Saturation level also had a nonsignificant effect on length ($F = 0.52$; df = 3, 16; $P = 0.6727$) and

weight ($F = 0.97$; df = 3, 16; $P = 0.4309$). Interaction effects were nonsignificant for length ($F = 0.65$; df = 3, 16; $P = 0.5938$) and weight ($F = 0.50$; df = 3, 16; $P = 0.6904$).

Averages of 15.0–46.1% of examined gill filaments contained gas emboli after 10 weeks (Table 1). However, proportions of gill filaments with gas emboli were not significantly affected by *M. cerebralis* infection ($F = 2.14$; df = 1, 16; $P = 0.1630$) or gas saturation level ($F = 2.04$; df = 3, 16; $P = 0.1485$), and no significant interaction effect was observed ($F = 1.38$; df = 3, 16; $P = 0.2840$).

At 10 weeks postexposure, mortality of fish ex-

TABLE 1.—Percentages of rainbow trout fingerling gill filaments containing gas bubbles after 10 and 22 weeks of exposure to *Myxobalus cerebralis* at four different levels of gas supersaturation.

| Saturation (%) | Exposed | | Not exposed | |
|---|---|---|---|---|
| | Mean | SD | Mean | SD |
| **10 weeks exposure** | | | | |
| <102.5 | 36.7 | 19.7 | 40.1 | 16.6 |
| 105.0 | 21.4 | 8.5 | 30.0 | 16.9 |
| 107.5 | 16.9 | 17.7 | 46.1 | 12.8 |
| 110.0 | 20.6 | 14.3 | 15.0 | 12.4 |
| **22 weeks exposure** | | | | |
| <102.5 | 10.6 | 5.3 | 20.0 | 20.0 |
| 105.0 | 8.1 | 6.0 | 8.3 | 5.2 |
| 107.5 | 8.9 | 5.0 | 15.3 | 13.2 |
| 110.0 | 26.7 | 14.0 | 3.5 | 1.3 |

posed to *M. cerebralis* was higher than unexposed fish at all saturation levels, except at 100–102.5% saturation (Figure 3). Average mortality among fish exposed to *M. cerebralis* was 19.7%, while those not exposed averaged 14.7%. A significant effect due to *M. cerebralis* infection was found ($F = 4.89$; df = 1, 16; $P = 0.042$), while neither saturation level ($F = 2.70$; df = 1, 16; $P = 0.0804$) nor interaction effects were significant ($F = 2.04$; df = 3, 16; $P = 0.1485$).

Frequency of signs of disease at the end of the experiment were much higher among fish exposed to *M. cerebralis* than those not exposed (Figure 4). Virtually all fish in the groups exposed to *M. cerebralis* exhibited at least one sign of disease at the end of the experiment. An average of 1.78 (SD = 0.62) signs per fish were found among fish exposed to *M. cerebralis* and 0.11 (SD = 0.22) signs per fish among fish not exposed to *M. cerebralis.* Data were transformed (square-root) because of non-uniform variances. The raw data and ANOVA results indicate signs of disease were affected by *M. cerebralis* infection ($F = 99.85$; df = 1, 16; $P = 0.001$) but not by saturation level ($F = 0.60$; df = 3, 16; $P = 0.6239$). Interaction effects were also nonsignificant ($F = 0.96$; df = 3, 16; $P = 0.4352$). Reactions to the gas saturations used in this experiment varied from fish to fish. External signs of gas bubble trauma such as severe exophthalmia and obvious bubble formation in the orbit of the eyes occurred only in a few individual fish at the highest saturation level.

The percentage of gill filaments containing gas emboli averaged from 3.5 to 26.7% at the end of the experiment (Table 1). The interaction effect of saturation level and *M. cerebralis* exposure on emboli was not significant ($F = 3.02$; df = 3, 16; $P$
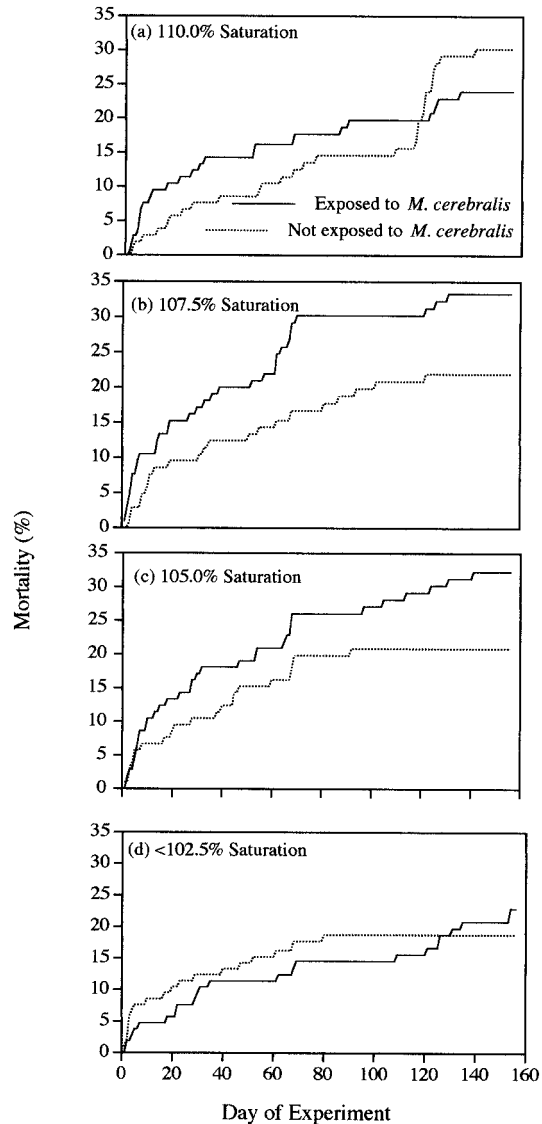


FIGURE 3.—Cumulative mortalities of fingerling rainbow trout exposed and not exposed to *M. cerebralis* and held in four levels of saturated water at 12.5°C for 160 d.

= 0.0602), although a high proportion of filaments contained emboli among exposed fish at 110% saturation. Neither saturation level ($F = 0.57$; df = 3, 16; $P = 0.6403$) nor *M. cerebralis* exposure ($F = 0.19$; df = 1, 16; $P = 0.6692$) had significant effects on proportions of gill filaments containing emboli.

Average weights and lengths of fish at the end of the experiment were lower among fish exposed to *M. cerebralis* at every level of gas saturation tested (Figure 5). Average weight was 4.7 g (SD
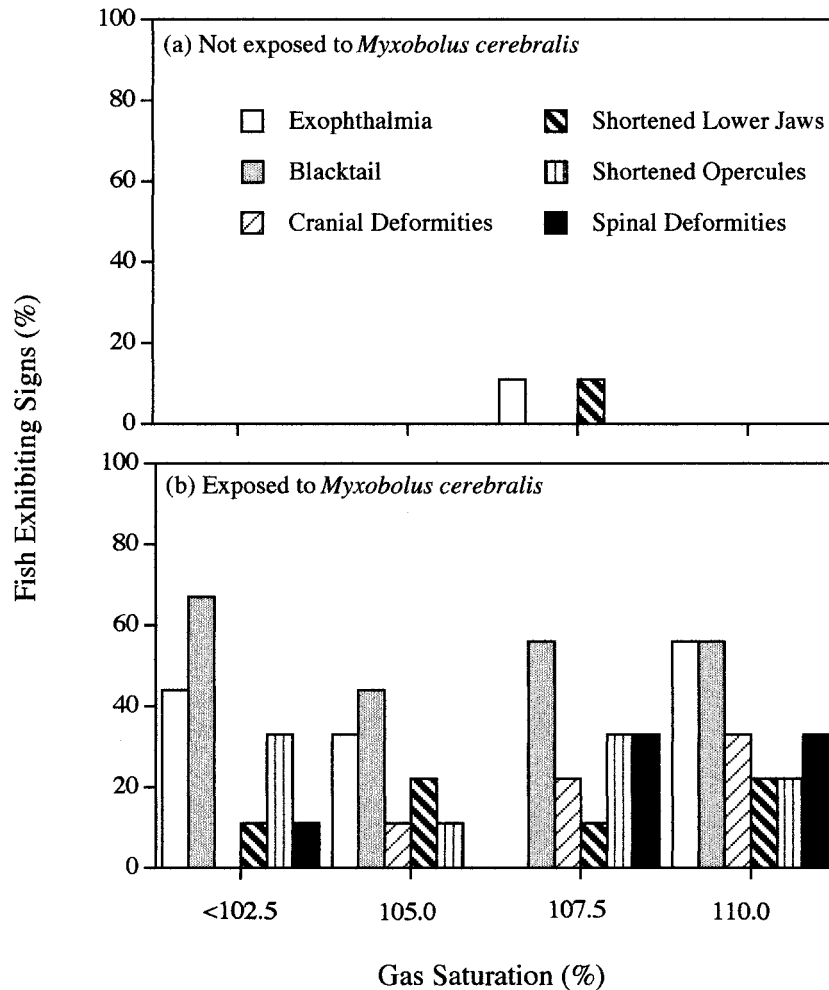
FIGURE 4.—Percentage of fingerling rainbow trout (a) not exposed and (b) exposed to *M. cerebralis* exhibiting signs of disease after 22 weeks at four gas supersaturation levels.

= 1.11) for fish exposed to *M. cerebralis,* and 6.73 g (SD = 1.02) for fish not exposed to *M. cerebralis.* Average length was 76.1 mm (SD = 6.2) for fish exposed to *M. cerebralis* and 85.3 mm (SD = 4.8) for fish not exposed to *M. cerebralis.* No obvious trend in lengths and weights occurred among saturation levels. The ANOVA resulted in significant effects due to *M. cerebralis* exposure on both length ($F$ = 15.51; df = 1, 16; $P$ = 0.0012) and weight ($F$ = 17.25; df = 1, 16; $P$ = 0.0007) of the fish. Nonsignificant effects for gas saturation level were observed for length ($F$ = 0.43; df = 3, 16; $P$ = 0.7357) and weight ($F$ = 0.30; df = 3, 16; $P$ = 0.8263). Interaction effects were nonsignificant for length ($F$ = 0.97; df = 3, 16; $P$ = 0.4328), and weight ($F$ = 0.39; df = 3, 16; $P$ = 0.7622).

Estimated numbers of mature spores varied widely among the fish evaluated. Spore counts averaged 578,578 (SD = 544,675) among all fish evaluated and ranged from 6,667–2,826,667 (Table 2). Because of the nonuniform variation in spore counts, a log-transformation was used before analysis. A one-factor ANOVA resulted in nonsignificant differences in spore counts among saturation levels ($F$ = 1.11 df = 3, 8 $P$ = 0.3266). A subsample of one fish from each aquarium containing fish not exposed to *M. cerebralis* was submitted for spore counts as a negative control. No spores were found in any of the fish not exposed to *M. cerebralis.*

At the end of the experiment, mortalities were higher in the groups exposed to *M. cerebralis* at every saturation except 110%. This contributed to
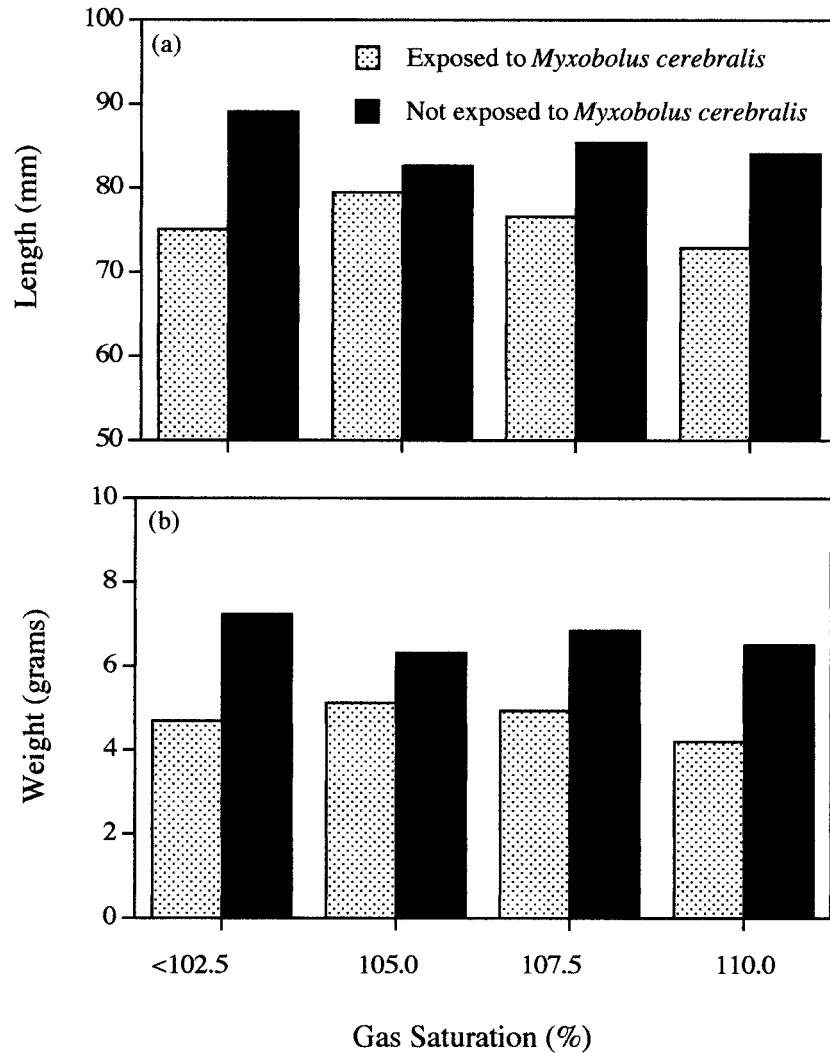
FIGURE 5.—(a) Mean lengths and (b) weights for rainbow trout fingerlings exposed and not exposed to *M. cerebralis* and held at four different gas saturation levels for 22 weeks.

the interaction effect between saturation level and *M. cerebralis* infection on mortality ($F = 2.78$; df $= 3$, $16$; $P = 0.0750$). Saturation level was not found to be a significant contributor to mortality when tested with the ANOVA ($F = 1.58$; df $= 3$, $8$; $P = 0.2326$). Average mortality at the end of the experiment was 28.1% for fish exposed to *M. cerebralis* and 22.9% for fish not exposed to *M. cerebralis*; this difference was significant ($F = 4.27$; df $= 1$, $16$; $P = 0.0553$).

## Discussion

The results of this study indicate that chronic gas saturation up to 110% does not have a signif-icant effect on morbidity or mortality of fingerling rainbow trout infected with *M. cerebralis*. The lim-ited effect of the increased gas supersaturations supports the notion that 110% saturation is a safe upper limit for salmonids under the conditions of this study.

The results indicate that growth of fingerling rainbow trout is significantly affected by *M. cer-ebralis* infection, but not significantly affected by changes in gas saturation levels from 100 to 110%. Fish infected with *M. cerebralis* in this study ap-peared to be less efficient feeders. Noticeably more waste products and uneaten food were observed in the bottoms of the aquaria containing fish in-

TABLE 2.—Myxospore loadings (in thousands) for fingerling rainbow trout exposed to *Myxobalus cerebralis* and held at four different levels of gas supersaturation for 22 weeks.

| Saturation (%) | Fish | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|
| <102.5 | 4 | 1,001 | 1,268 | 67 | 2,826 |
|  | 4 | 813 | 432 | 488 | 1,412 |
|  | 4 | 695 | 286 | 270 | 880 |
| 105.0 | 4 | 324 | 246 | 129 | 668 |
|  | 4 | 1,335 | 768 | 351 | 2,088 |
|  | 4 | 199 | 338 | 7 | 705 |
| 107.5 | 4 | 512 | 481 | 174 | 1,223 |
|  | 4 | 287 | 220 | 55 | 586 |
|  | 4 | 328 | 205 | 91 | 592 |
| 110.0 | 4 | 465 | 287 | 197 | 844 |
|  | 4 | 480 | 368 | 97 | 985 |
|  | 4 | 498 | 174 | 327 | 716 |

fected with *M. cerebralis.* Exposed fish would often exhibit whirling behavior during feeding, resulting in fewer food particles being consumed. Exposed fish searched less actively for uneaten food particles after the initial feeding than fish not exposed to *M. cerebralis.* Energy expended during the whirling episodes may also result in a higher metabolic rate, resulting in larger amounts of waste products.

Signs of disease were strongly affected by *M. cerebralis* infection, but not by gas supersaturation. Overt signs of gas bubble trauma did not appear until after 14 weeks of exposure to 110% saturated water. Certain individual fish developed overt signs of gas bubble trauma, while other fish residing in the same aquarium appeared to be normal. Individual fish resistance seems to play a large part in their tendency to develop gas bubble disease.

The presence of bubbles in the gill filaments of fingerling rainbow trout was not a good indicator of gas bubble trauma in this experiment. Other authors (Walker and Nehring 1995; Maule et al. 1997) have reported highly variable association between presence of bubbles in gill filaments and the occurrence of gas bubble trauma. An observation that may explain this variation is the tendency for air to be drawn into the severed end of the gill filaments by capillary action. This would also explain the higher number of gill filaments containing gas bubbles in the first examination than in the second. The fish were much smaller during the first examination, so relatively more damage occurred to the filaments during excision.

Myxospore loadings were not affected by gas saturation level. Although all fish were exposed to the same number of triactinomyxons at the same time, spore counts were quite variable from fish to fish within the same treatment groups. Variability of myxospore load could be affected by numbers of triactinomyxons successfully attacking individual fish. However, as with the onset of signs of gas bubble trauma, total myxospore load appeared to be largely determined by individual fish resistance.

Lower growth rates, higher occurrences of deformities, and higher mortality rates among fish infected with *M. cerebralis* are a concern for fish culture operations. Although gas saturation did not significantly affect these variables in this experiment, *M. cerebralis* has the potential to reduce productivity of a fish culture operation in the absence of other exacerbating factors. The results of this experiment indicate that gas saturations found in the upper Colorado River are not severe enough to explain the total loss of rainbow trout year-classes in the river. Factors such as reduced fitness of fish infected with *M. cerebralis* are probably affecting the survival of fingerling rainbow trout to a greater degree than gas supersaturation. Altered feeding and predator avoidance behaviors could greatly affect the survival of fish infected with *M. cerebralis* in natural systems. Additional experiments should be conducted to determine how these factors affect survival of infected fish. Clarification of the role of bacterial pathogens such as *Flavobacterium psychrophilum* on the virulence of *M. cerebralis* should also be addressed in future studies.

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