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Response of rainbow trout *Oncorhynchus mykiss* to exposure to *Myxobolus cerebralis* above and below a point source of infectivity in the upper Colorado River

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ABSTRACT: We exposed 9 wk old rainbow trout *Oncorhynchus mykiss* to ambient levels of *Myxobolus cerebralis* infectious stages at 4 sites of suspected differing infectivity in the Colorado River. Exposure was estimated by periodic filtration of river water at each exposure location. After a 32 d exposure, the fish were held in the Colorado River at a common site for over a year. Resulting infection was evaluated by the presence of clinical signs (whirling behavior, cranial deformity/exophthalmia, and black tail), severity of microscopic lesions, and myxospore counts (8, 10, 12, and 14 mo post-exposure). Two exposure sites that were immediately downstream of Windy Gap Reservoir were much higher in infectivity than the site above the reservoir or the site 26 km downstream of the reservoir. Rainbow trout exposed at those locations showed higher prevalence of clinical signs of whirling disease, more severe histological evidence of infection and higher average myxospore concentrations than those exposed above the reservoir or 26 km below the reservoir. Many more *M. cerebralis* actinospores were observed from water filtration at the 2 sites immediately below the reservoir compared to the other sites.

KEY WORDS: *Myxobolus cerebralis* · Whirling disease · Rainbow trout

INTRODUCTION

The upper Colorado River in Middle Park, Colorado, supported a self-sustaining rainbow trout *Oncorhynchus mykiss* fishery throughout the middle and latter decades of the twentieth century. Failures in rainbow trout recruitment during the early 1990s resulted in several consecutive missing year classes, and led to extensive field studies to determine the causative factors. Whirling disease, caused by the myxosporean parasite *Myxobolus cerebralis*, has been documented as the primary factor in the recruitment failures and subsequent rainbow trout population decline (Walker & Nehring 1995, Nehring & Walker 1996, Nehring 1998, Nehring et al. 1998, Nehring & Thompson 2001). Other potential factors, such as gas bubble disease, bacterial coldwater disease, or ectoparasites, have proven to be inadequate explanations for recruitment failures (Schisler & Bergersen 1999, Schisler et al. 1999, 2000). Indeed, in a 4-stressor laboratory experiment, *M. cerebralis* was the most important contributor to mortality among rainbow trout (Schisler et al. 2000).

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Schisler et al. concluded that their 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.'

By early 1997, more prevalent and severe clinical signs of whirling disease among young-of-the-year brown trout *Salmo trutta* and rainbow trout from the Colorado River in the proximity of Windy Gap Reservoir indicated that infection rates were higher below the reservoir than in areas further downstream (Nehring 1998). This suggested that the area near the reservoir was a point source of infectivity, but whether the source was the reservoir or the spill basin was unclear. Trout migrating upstream would be stopped in the spill basin below the dam, and we hypothesized that many might remain there only to be stranded and perhaps die in the spill basin when releases ceased for the winter (winter water releases occur through a separate channel). These fish, if infected, could then be a source of *Myxobolus cerebralis* infection in the *Tubifex tubifex* population.

These observations were the impetus for an experiment conducted in 1997 to 1998. We conducted a sentinel fish test designed to expose the fish to differing levels of *Myxobolus cerebralis* infectious units, and determine if the spill basin or Windy Gap Reservoir was the primary source of infectivity in this area.

MATERIALS AND METHODS

We chose 4 exposure locations in the Colorado River that we believed represented differing levels of infectivity (Fig. 1). The uppermost location was 0.5 km upstream of Windy Gap Reservoir, labeled 'Above Windy Gap'. Two locations were immediately downstream of Windy Gap Reservoir in the 'Spill Basin' and in the alternate outlet channel that we termed the 'North Outlet'. These were necessary choices to deter-

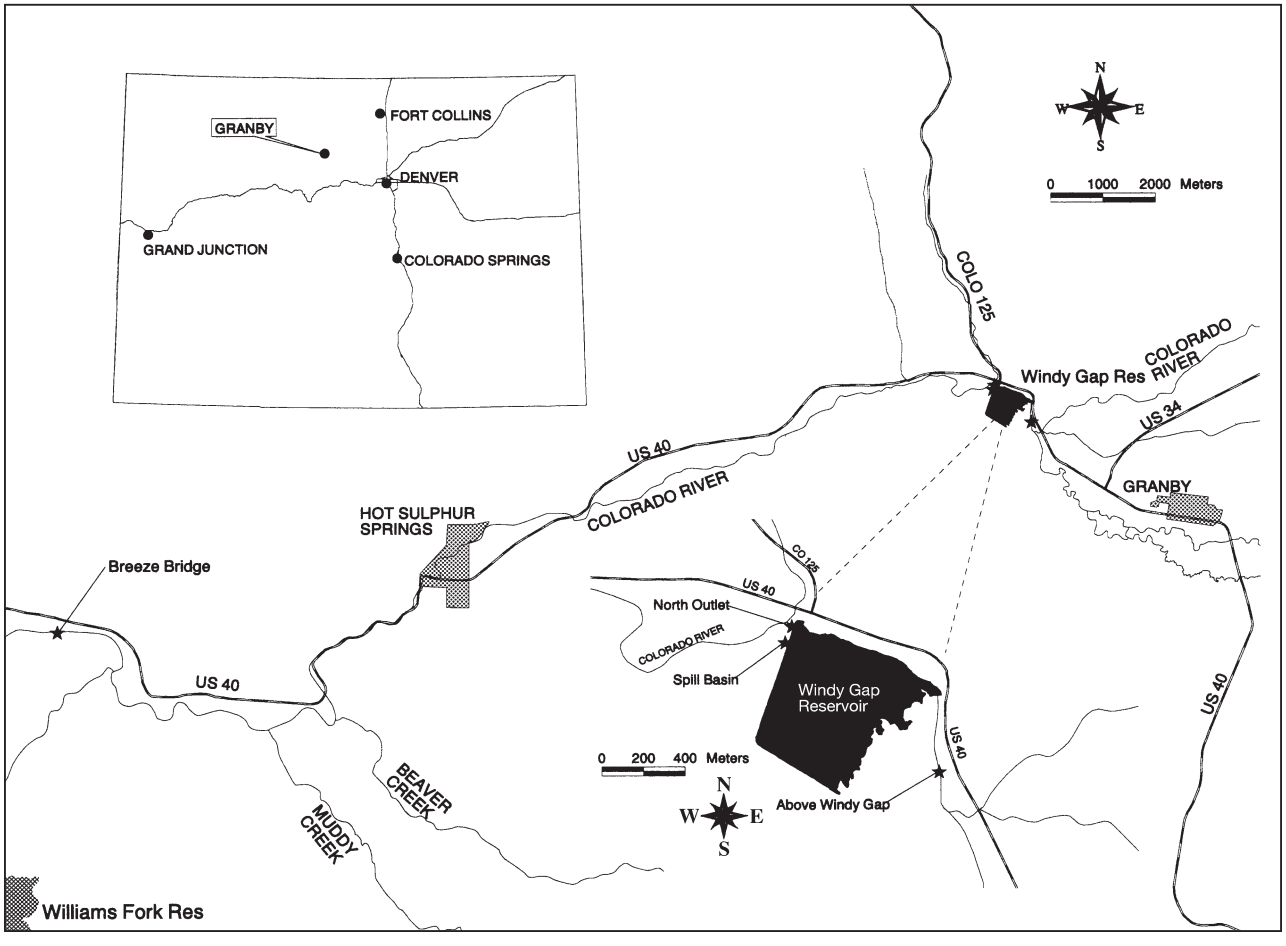

Fig. 1. The upper Colorado River study area. Stars indicate the locations where initial exposures of *Oncorhynchus mykiss* occurred. All fish were held at the Breeze Bridge site after the initial exposure period. CO 125 and COLO 125: Colorado State Highway 125; US 40: US Route 40; US 34: US Route 34

mine whether the reservoir or the Spill Basin was the source of infectivity. The lower location was 26 km downstream of Windy Gap Reservoir at 'Breeze Bridge', the site of previous sentinel fish studies (Thompson et al. 1999). This was also where all fish were held after the initial exposure period at different locations, since it remains ice-free during the winter months.

Water temperatures were monitored in the Colorado River at each location during the exposure period and at Breeze Bridge throughout the study. We used electronic monitors attached to an experimental tank at each location to record the water temperature every half-hour during the exposure period. During the remainder of the study at Breeze Bridge, a monitor recorded the temperature every hour. These temperature data were used to generate mean daily water temperatures for each location.

Rainbow trout, progeny of wild Colorado River brood fish, were hatched and reared in pathogen-free water prior to exposure in the Colorado River at 9 wk post-hatch. Mean weight for the fish at exposure was 1.81 g. Approximate mean length (estimated from a length-weight table for fish with a condition factor C of 4000×10^{-7}: Piper et al. 1982) at the time of distribution to exposure sites was 55 mm. Ten fish were sampled prior to exposure and individually tested for the presence of nucleic acid of the *Myxobolus cerebralis* parasite by a polymerase chain reaction (PCR) test (Andree et al. 1998), modified as a single round procedure (Schisler et al. 2001).

We estimated infectivity by filtering 1900 l samples of water through a 20 µm Pecap® (TETKO) screen on several occasions at each exposure location during the exposure period and microscopically examining 20 subsamples of each filtrate for the presence of *Myxobolus cerebralis* actinospores (Thompson & Nehring 2000). Actinospore density estimates from the water samples were averaged over the exposure period and used to model the potential exposure of a 60 mm fish over a range of water velocities similar to that experienced by the experimental fish. We also filtered water and estimated actinospore densities at Breeze Bridge after the exposure period at the 4 different sites.

The experiment was designed as a randomized complete block, with each block exposed at a different location. A 'block' consisted of two 3.7 m livestock feed troughs, each modified into a flow-through floating tank with 4 quadrant cells (see Thompson et al. 1999), and tethered adjacently in the river. One cell of each tank was randomly chosen to hold the test fish to obtain 2 replicates at each exposure location. Fish were assigned to cells randomly in 15 groups of 5 and 1 group of 2, resulting in 77 fish per replicate at the beginning of the exposure. The other 3 cells of each tank held groups of fish that are not reported on in this paper.

The fish were placed at exposure locations directly from the transport truck on August 14, 1997, and moved to Breeze Bridge on September 16, 1997, resulting in a 32 d exposure at separate locations. The fish continued to be exposed to actinospores at Breeze Bridge during the remainder of the experiment. Mortalities were collected daily from each of the 8 cells, beginning August 15. The fish were fed ad libitum with a commercial trout diet 4 to 6 times daily, depending on fish size and water temperature. The fish tanks were cleaned daily.

Once each month, beginning in September, a complete inventory of all cells was performed. The fish in each cell were counted and individually characterized for the presence of overt clinical signs of whirling disease including black tail, whirling behavior, cranial and skeletal deformities, and exophthalmia.

In November, 5 fish from each cell were sampled for histological examination. Sagittal sections from the head of each fish were examined and scored from '0' to '4' in 4 categories: number of lesions, severity of lesions, number of organisms, and severity of inflammation (Table 1). Point totals were then used to assign each fish on a rating scale from '0' (no evidence of infection) to '4' (marked evidence of severe infection) to describe the severity of infection.

All replicates were sampled in April, June, August and October 1998 for determination of cranial myxospore concentrations by the pepsin-trypsin digest (PTD) technique (Markiw & Wolf 1974). Analysis of variance (ANOVA) was used to examine differences in log-transformed myxospore concentrations from *Myxobolus cerebralis*-positive fish among all exposure locations and sample dates.

Survival was analyzed at inventory dates using arcsine square-root transformations of the data to stabilize variances. Mean survival is reported in this paper. No survival comparisons were performed beyond March 1998.

RESULTS

Water temperatures in the Colorado River during the period of this study were suitable for trout (Figs. 2 & 3). Summertime mean daily water temperatures at Breeze Bridge ranged from 11 to 16°C during August and September 1997, and from 8 to 19°C from June through September 1998.

Experimental fish placed at sites below Windy Gap Reservoir experienced much higher exposure to actinospores than those exposed Above Windy Gap or at Breeze Bridge (Table 2). Modeled exposure at Spill

Basin and North Outlet was 84 to 96 times higher than at Above Windy Gap, and 33 to 38 times higher than at Breeze Bridge.

None of the 10 fish sampled for the presence of parasite DNA prior to exposure tested positive by the PCR test. This was expected, since the fish came from a *Myxobolus cerebralis*-free hatchery.

Cranial deformities and exophthalmia were so highly correlated among the test fish that we report them as a single sign of disease. The experimental fish showed dramatically more prevalent whirling behavior and cranial deformity/exophthalmia in the replicates exposed below Windy Gap Reservoir in the North Outlet and the Spill Basin than in the replicates exposed Above Windy Gap or at Breeze Bridge (Fig. 4). Black tail and spinal deformities were rarely observed, with average prevalence for each site always less than 1%. Black tail was observed on at least 1 inventory occasion among fish from each exposure site except Above Windy Gap. Spinal deformity was only observed in a single fish exposed at Breeze Bridge.

Table 1. *Oncorhynchus mykiss*. Rating scheme used to describe *Myxobolus cerebralis* infection. Scores from all 4 categories were summed. A total score of 0 = no significant microscopic lesions; 1–4 = Grade 1 (minimal infection evident); 5–8 = Grade 2 (mild *M. cerebralis* infection evident); 9–12 = Grade 3 (moderate infection evident); 13–16 = Grade 4 (marked evidence of severe infection)

Category	Score	Description
Number of lesions	0	No lesions observed
	1	A single lesion
	2	Multifocal; 2 lesions
	3	Multifocal; 3 lesions
	4	Multifocal, extensive; >3 lesions
Severity of lesions	0	No chondrolysis
	1	Minimal chondrolysis
	2	Mild chondrolysis
	3	Moderate chondrolysis
	4	Severe chondrolysis
Number of organisms	0	No organisms noted
	1	Rare intralesional *M. cerebralis* organisms
	2	Few intralesional *M. cerebralis* organisms
	3	Moderate intralesional *M. cerebralis* organisms
	4	Abundant intralesional *M. cerebralis* organisms
Degree of inflammation	0	No inflammation
	1	Minimal inflammation
	2	Mild inflammation
	3	Moderate inflammation
	4	Severe inflammation

Histological sectioning and analysis of samples taken on November 19 (97 d post-exposure) revealed that lesions and/or organisms consistent with *Myxobolus cerebralis* infection were present in 73% of fish overall. However, there were clear patterns in the prevalence and severity of infection among exposure sites (Table 3). Lesions and/or organisms were seen in 60% of fish from Above Windy Gap, 100% of fish from North Outlet and Spill Basin, and 30% of fish from Breeze Bridge. Higher relative numbers of *M. cerebralis* organisms seen within lesions were observed among fish that were exposed at the 2 sites below Windy Gap Reservoir compared to those exposed Above Windy Gap or at Breeze Bridge (Table 3). The average grade of infection was also higher in the fish exposed below Windy Gap Reservoir.

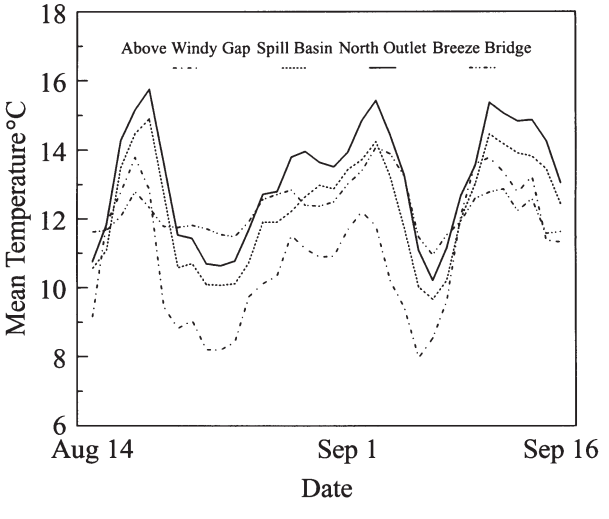

Fig. 2. Mean daily temperature (°C) at the 4 exposure locations during the exposure period (August 14 to September 16, 1997)

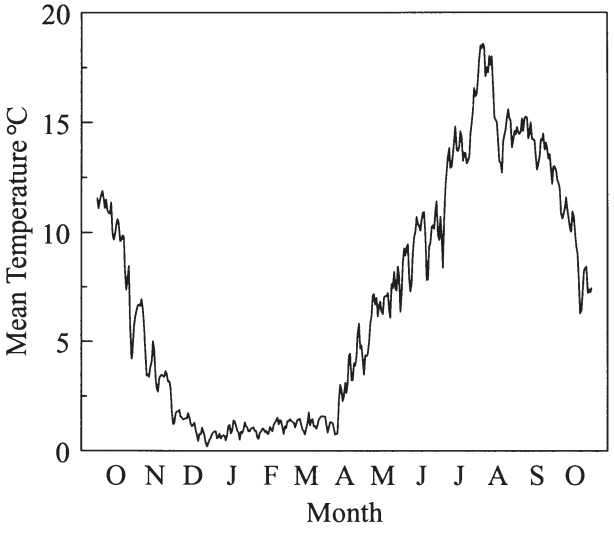

Fig. 3. Mean daily temperature (°C) at the Breeze Bridge holding site (September 16, 1997, to October 14, 1998)

Table 2. *Oncorhynchus mykiss*. Estimates of actinospore (Act) density and potential daily fish exposure at the 4 exposure locations during the period August 14 to September 16, 1997, and at Breeze Bridge after the exposure period until November 5, 1997. Daily exposure was modeled on the estimated mean actinospores l^{-1} (Act l^{-1}) of river water at a given water velocity (cm s^{-1}), and a fish length of 60 mm. n: number of water samples from which mean actinospores l^{-1} was estimated

Location	Duration (d.mo)	n	Mean Act l^{-1}	CI	Exposure at velocity of 9	15	21
Above Windy Gap	14.8 – 16.9	8	0.047	0.008, 0.086	24	40	56
North Outlet	14.8 – 16.9	10	4.517	2.585, 6.449	2305	3842	5378
Spill Basin	14.8 – 16.9	13	3.970	2.011, 5.929	2026	3376	4727
Breeze Bridge	14.8 – 16.9	4	0.119	0, 0.266	61	101	142
	23.9 – 5.11	7	0.083	0, 0.190	42	71	99

Survival through the March inventory was 99% for fish exposed Above Windy Gap and at Breeze Bridge, and 97% for fish exposed in the North Outlet and Spill Basin (Table 4), adjusted for the intentional removals that occurred in November for histology analysis. Means comparisons by month revealed that survival among the fish exposed in the North Outlet was significantly lower than that in Above Windy Gap ($p = 0.0443$) and Breeze Bridge ($p = 0.0130$). However, the differences were obviously trivial from a biological perspective. No other statistically significant differences in survival were noted. After the March inventory, 1 replicate from the North Outlet was depleted by about 18 fish by mink or raccoon depredation. Additional fish were sampled from each replicate at the April inventory. These circumstances made survival analyses difficult beyond the March inventory, and they were not attempted.

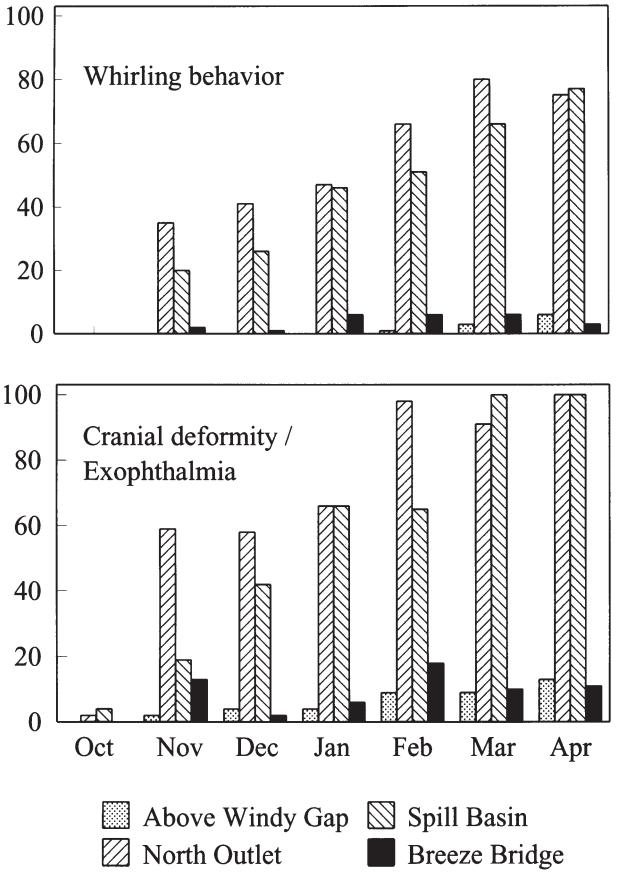

Fig. 4. *Oncorhynchus mykiss*. Average prevalence (percent, n = 2 replicates per site) of whirling behavior and cranial deformity/exophthalmia among rainbow trout exposed at 4 different locations in the upper Colorado River

Table 3. *Oncorhynchus mykiss*. Results of histological examination of fish collected from each replicate (n = 5) on November 19, 1997. Relative numbers of organisms observed within lesions were judged as none (N), rare (R), few (F), moderate (M), or abundant (A); asterisk indicates that relative number of organisms was not determined for that fish

Location	Fish with lesions	Relative no. of organisms	Mean grade
Above Windy Gap	3	R, *, *	0.6
	3	R, R, M	1.2
North Outlet	5	F, M, M, M, A	3.0
	5	M, M, M, A, A	3.4
Spill Basin	5	M, M, M, A, A	3.2
	5	F, F, M, M, A	2.6
Breeze Bridge	2	N, R	0.4
	1	R	0.2

Table 4. *Oncorhynchus mykiss*. Mean percent of fish from each exposure location (n = 2 replicates) surviving at monthly inventories from September 1997 through April 1998

Exposure location	Percent surviving at inventory in: Sep	Oct	Nov	Dec	Jan	Feb	Mar
Above Windy Gap	99.4	98.7	98.7	98.7	98.7	98.7	98.7
North Outlet	99.4	99.4	98.7	98.0	98.0	97.3	97.3
Spill Basin	99.2	98.6	98.6	98.6	98.6	98.6	97.2
Breeze Bridge	99.4	99.4	99.4	99.4	99.4	99.4	99.4

Table 5. *Oncorhynchus mykiss*. Mean *Myxobolus cerebralis* myxospore concentration in samples taken from each replicate on 4 separate occasions. Degree-days were calculated from August 14, 1997, when fish were distributed to exposure locations. Spore concentrations represent only fish in which spores were detected; lower and upper 95% CLs, back-transformed from the natural logarithm, are in parentheses. –: second replicate exposed at Breeze Bridge was not sampled on August 19 or October 15 because no fish remained (51 remaining fish lost to raccoon or mink depredation about June 30, 1998)

Exposure location	Degree-days	No. examined	No. positive	Spore concentrations		
				Mean	Low (lower CL)	High (upper CL)
April 14, 1998						
Above Windy Gap	1116	5	5	14800	9200 (9800)	22600 (22400)
		5	5	42100	8800 (11700)	106000 (151500)
North Outlet	1192	5	5	344700	119000 (133200)	841800 (892300)
		5	5	308800	102300 (133700)	587900 (712900)
Spill Basin	1163	5	5	355400	218600 (226300)	510800 (558200)
		5	5	166200	31400 (51100)	423600 (540600)
Breeze Bridge	1163	5	5	91200	13500 (26200)	172500 (317400)
		5	5	475400	75700 (131100)	812000 (1724700)
June 22, 1998						
Above Windy Gap	1689	15	15	82400	23700 (56900)	213200 (119200)
		15	15	98600	30300 (76100)	160700 (127700)
North Outlet	1765	15	15	225800	25700 (144000)	681100 (354200)
		15	15	92300	48300 (76300)	140800 (111700)
Spill Basin	1736	15	15	89300	28600 (62500)	236900 (127700)
		15	15	169900	229300 (109300)	529300 (264100)
Breeze Bridge	1736	15	15	23100	1100 (10800)	114800 (49400)
		15	10	15800	2400 (8300)	34700 (30200)
August 19, 1998						
Above Windy Gap	2551	15	15	269800	3900 (125700)	794800 (578800)
		15	15	216900	10200 (109500)	737300 (429400)
North Outlet	2627	15	15	659800	5400 (294400)	1378100 (1479100)
		15	15	959700	115500 (620800)	2506700 (1483400)
Spill Basin	2598	15	15	881200	133300 (579100)	1953600 (1326900)
		15	15	796200	182800 (539000)	2161600 (1176300)
Breeze Bridge	2598	14	14	144800	12200 (78900)	353000 (266000)
	–	–	–	–	–	–
October 15, 1998						
Above Windy Gap	3233	34	32	85600	8000 (59400)	338900 (123200)
		33	32	160300	3700 (102200)	362500 (251600)
North Outlet	3309	10	10	693800	87600 (337000)	1870000 (1428600)
		21	21	393700	52500 (286500)	882800 (541100)
Spill Basin	3208	6	6	528000	199400 (300200)	1074500 (928800)
		23	23	701700	80100 (466900)	2088400 (1054700)
Breeze Bridge	3208	10	8	88200	18500 (42000)	228000 (185200)
	–	–	–	–	–	–

Of the 401 fish analyzed by PTD, myxospores were detected in 386 (Table 5). All 40 fish sampled in April contained myxospores, at less than 1200 degree-days post-exposure. However, the myxospore values in Table 5 and a significant date effect in the ANOVA model (df = 3, $F = 26.17$, p = 0.0001) indicate that myxospore formation was not complete in April.

On each sample occasion except June, fish from the North Outlet and Spill Basin sites exhibited significantly more average spores than fish from Above Windy Gap (all $p < 0.004$), as tested by least-squares means. Myxospore concentrations in fish from Breeze Bridge were not significantly different from those at North Outlet and Spill Basin in April (p = 0.399 and 0.669 respectively), but they were significantly lower in June (p = 0.0001 and 0.0002). Breeze Bridge myxospore concentrations were not tested against the other sites in August or October because one of the Breeze Bridge replicates was missing after a depredation incident at the end of June. However, it appears from the data for the 1 remaining replicate (Table 5) that myxospore concentrations in fish from that site continued to be substantially lower than those seen at the North Outlet and Spill Basin sites. Myxospore con-

centrations in fish from the North Outlet and Spill Basin did not test significantly different from each other on any occasion.

DISCUSSION

The similar responses of trout from the North Outlet and Spill Basin sites indicate that the reservoir, rather than the spill basin, is the primary source of *Myxobolus cerebralis* infectivity. This study represents additional evidence that Windy Gap Reservoir is a site of heightened infectivity compared to the sites Above Windy Gap and at Breeze Bridge (e.g. see Thompson & Nehring 2000). Actinospore filtering and the response of the experimental fish as measured by clinical signs, histology and myxospore concentrations all support this conclusion. The actinospore evidence is plain from Table 2: actinospores were many times more abundant immediately below Windy Gap Reservoir than they are just above the reservoir or at the Breeze Bridge 26 km downstream. The overt clinical signs of whirling behavior, cranial deformity, and exophthalmia were more prevalent in fish exposed at the 2 locations below Windy Gap Reservoir than at the other sites. Fish exposed at the North Outlet and Spill Basin sites just below Windy Gap Reservoir exhibited more severe lesions, greater abundance of lesions, and greater numbers of *Myxobolus cerebralis* organisms within those lesions than did fish that were exposed Above Windy Gap or 26 km below the reservoir at the Breeze Bridge. Myxospore concentrations were much higher in fish exposed below Windy Gap Reservoir than at the other sites, especially on the last 2 sample occasions when myxospore formation from initial infection should have been completed. This result indicates a higher exposure for the fish below Windy Gap Reservoir compared to the other sites, and is in agreement with laboratory challenges that showed rainbow trout produce more myxospores as the challenge dose increases (Hedrick et al. 1999).

The results of the myxospore concentration analysis for the June samples were troubling. Although considerable variability is inherent in myxospore counts, it seems improbable to observe the apparent large decrease in mean concentration from April to June, followed by large increases in August. We are not aware of any published study designed to determine the point at which myxospores begin to develop, or at what point the process is largely complete. Halliday (1973, 1976) found acid-fast myxospores that he called mature in as little as 840 to 884 degree-days (120 d at 7°C or 52 d at 17°C). We previously observed myxospores by PTD at 1255 degree-days in rainbow trout *Oncorhynchus mykiss*, and in several subspecies of the cutthroat trout *O. clarki* (Thompson et al. 1999, July 9, 1996 to April 8, 1997; however, degree-days are not detailed in the paper). El-Matbouli et al. (1992) found that myxospores could be demonstrated at 1440–1530 degree-days (90 d at 16 to 17°C, or 120 d at 12 to 13°C). However, myxospore development does not appear to be complete until much later, as evidenced by the higher values observed in August and October in this study. Markiw (1992) suggested that myxospore concentrations plateaued between 5 and 6 mo post-exposure (at 12.5°C, 1875 to 2250 degree-days). Samples collected in April were 1116 to 1192 degree-days post-exposure, a point at which myxospore formation should be well underway, but not complete. Our June samples were collected at 1689 to 1765 degree-days, and August samples were obtained at 2551 to 2598 degree-days. The June samples should have shown higher average myxospore concentrations than the April samples, but perhaps a bit lower or about the same as the August samples. We believe that the June myxospore samples were probably compromised during analysis. The nature of the error is unknown, but possibilities would include a trypsin concentration stronger than intended, or too long a time spent in the trypsin solution.

This study conclusively demonstrates that foci of *Myxobolus cerebralis* infectivity exist in wild habitats, and that Windy Gap Reservoir is one such site. This naturally leads to the hypothesis that if such sites are discrete enough to be identified and remedied, natural populations of rainbow trout might respond positively to the abatement of *M. cerebralis* infectivity.

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