Whirling Disease Investigations

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R. Barry Nehring
Principal Investigator

Thomas E. Remington, Director

Federal Aid in Fish and Wildlife Restoration

Job Progress Report

Colorado Division of Wildlife

Fish Research Section

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STATE OF COLORADO

Bill Ritter, Governor

COLORADO DEPARTMENT OF NATURAL RESOURCES

Sherman Harris, Executive Director

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Mark S. Jones, General Professional VI, Aquatic Wildlife Research Leader
Arturo Avalos, Technician III, Research Hatchery
Rosemary Black, Program Assistant I
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Harry Vermillion, Information Technology Professional II, F-239, Aquatic Data Analysis
Nicole Vieira, Physical Scientist III, Water Quality Studies

Paula Nichols, Federal Aid Coordinator
Kay Knudsen, Librarian

Prepared by: _____
R. Barry Nehring, GP V, Aquatic Wildlife Researcher

Approved by: _____
Mark S. Jones, Aquatic Wildlife Research Leader

Date: _____

The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Division of Wildlife policy by the Director or the Wildlife Commission.

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Job No. 1: *Myxobolus cerebralis* in Colorado's Cutthroat Trout Populations

Project Objective: To determine, and then document through professional publication, the impacts of the myxosporean parasite *Myxobolus cerebralis* on wild trout populations in selected stream ecosystems in Colorado with an overarching objective of developing risk assessment guidelines for the management of whirling disease.

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

Principal Investigator: R. Barry Nehring

Job Objective: Determine the extent of occurrence and severity of impact of *Myxobolus cerebralis* on populations of greenback *Oncorhynchus clarki stomias*, Rio Grande *O. c. virginalis*, and Colorado River cutthroat trout *O. c. pleuriticus* throughout Colorado.

INTRODUCTION

"Whirling disease" (WD) is a debilitating malady of trout and salmon that was first observed in cultured rainbow trout in Germany in 1893 (Hofer 1903). The name comes from the abnormal swimming behavior of fry or fingerling salmonids that can occur after becoming infected by the myxosporean parasite *Myxobolus cerebralis.* When frightened the fish appear to be chasing their tail like a run-away boomerang. It was considered a serious problem in aquaculture for much of the 20th century (Plehn 1905; Schäperclaus 1931; Uspenskaya 1957, 1982). However, the parasite life cycle was an enigma until described in the early 1980s (Markiw and Wolf 1983; Wolf and Markiw 1984). The complex 2-host life cycle alternates between a tubificid worm (*Tubifex tubifex*) and a salmonid fish. The parasite produces spores in each host that are infective to the alternate host. Myxospores produced in infected salmonids can be ingested by bottom-dwelling oligochaetes. Susceptible forms of *T. tubifex* that become infected produce a triactinomyxon (TAM) actinospore that is semi-buoyant, tumbles in moving water, and is infectious to susceptible salmonids.

Myxobolus cerebralis (Mc) was first detected in two public and two private trout rearing facilities in Colorado, late 1987 (Walker and Nehring 1995). Population level impacts among wild salmonid populations were unknown until the 1990s. However, severe losses of young rainbow trout first observed in major reaches of the upper Colorado, Cache la Poudre, Gunnison, Rio Grande, and South Platte rivers in Colorado in 1993 and 1994 were ultimately attributed to WD (Walker and Nehring 1995; Nehring and Walker 1996; Nehring et al. 1998; Nehring and Thompson 2001). The parasite became widely distributed in Colorado in the early 1990s through the stocking of millions of catchable size trout reared in waters enzootic for *M. cerebralis* (Schisler 2001). More than one million trout from *Mc*-infected hatcheries and rearing units were stocked into lakes, reservoirs and streams in the Cache la Poudre River drainage between 1990 and 2001. Moreover, this was not a highly unique scenario. Given such a management strategy, it is not surprising that *M. cerebralis* had been detected in feral salmonids at 118 different locations in lakes, reservoirs and major stream segments in Colorado by October 1997 and at 208

sites by spring 2000. It is estimated that *Mc* infections have negatively impacted recruitment of wild rainbow and brook trout fry in 560 – 600 km (350-400 miles) of stream in Colorado (Nehring and Thompson 2001). A special technical report, **Colorado's Cold Water Fisheries: Whirling Disease Case Histories and Insights for Risk Management,** summarized the effects of exposure to *M. cerebralis* upon Colorado's salmonid fisheries through 2005 (Nehring 2006).

Debilitating effects of the parasite were documented on wild rainbow trout in major reaches of the Madison River in Montana in the 1990s (Vincent 1996a,b). Research efforts between 1994 and 2004 revealed the parasite was enzootic in many coldwater habitats in Colorado (Nehring and Thompson 2003) and western Montana (Baldwin et al. 1998). It has been detected at one or more locations in almost all states west of the 100th meridian in the continental U.S. (Bartholomew and Reno 2002). Detected in Yellowstone cutthroat trout (*O. clarki bouvieri*) in 1998, *M. cerebralis* infections have had serious impacts on spawning runs in the Yellowstone River immediately downstream of Yellowstone Lake and in Pelican Creek and Clear Creek, major spawning tributaries that drain into the northeastern corner of the lake (Koel et al. 2005, Koel et al. 2006). Recent studies suggest that *M. cerebralis* may be enzootic in one or more streams in south central Alaska near Anchorage (Arsan 2006). There is increasing concern *M. cerebralis* infection may be affecting the mountain whitefish (*Prosopium williamsoni)* populations in some streams in the Rocky Mountain West.

Widely distributed in the mountainous regions of Colorado, *M. cerebralis* has been detected in feral salmonid populations in close proximity to areas designated as cutthroat trout recovery streams. In 2003, at the initiation of this study there were no known cases where the parasite had negatively impacted fry recruitment for any of Colorado's three sub-species of cutthroat trout. At that time, the parasite was enzootic among Colorado River cutthroat trout in Trappers Lake in western Colorado and in greenback cutthroat trout in Zimmerman Lake in north central Colorado. Both trout populations have been managed for spawn-taking operations. Field exposure of young-of-the-year (YOY) of all three sub-species of Colorado's cutthroat trout to ambient levels of *M. cerebralis* in the Colorado River in the 1990s clearly demonstrated these fish are particularly vulnerable to developing a lethal infection after exposure (Thompson et al. (1999).

Total year class failure can occur among susceptible species of salmonids under the proper suite of environmental conditions once *M. cerebralis* becomes enzootic in an aquatic ecosystem,. Increasingly, it is being shown in this study and others (Koel et al. 2005, Koel et al. 2006) that the proper suite of environmental conditions is not very restrictive and does not necessarily involve environmental degradation.

In Colorado, the lack of a systematic effort to evaluate the distribution, establishment and spread of *M. cerebralis* into aquatic ecosystems capable of supporting native cutthroat trout was the primary impetus for the initiation of this research project.

STUDY DESIGN

The primary study objective is to determine whether or not the parasite has spread into habitats capable of supporting cutthroat trout populations. A multi-faceted approach is being used to determine whether or not significant exposure and spread of *M. cerebralis* has already occurred. In the event that there has been only minimal establishment in most regions of the state, an effort is being made to determine whether introduction actually took place or not. In the event introduction and exposure took place but the parasite did not become enzootic, the objective will be to determine what factor(s) might have prevented establishment of the life cycle. In those areas where the parasite is not enzootic and there is no record of initial exposure, the protocol will be to collect aquatic oligochaetes and genetically test them to determine whether or not they are the lineage of *T. tubifex* that is highly susceptible to infection by the parasite. A statewide systematic sampling process should provide insight(s) into the mechanisms and factors that facilitate the establishment and spread of *M. cerebralis*. The study should also provide significant insights into the potential risk for spread of the parasite through the development of statewide distribution maps for all lineages of *T. tubifex.* This is most important for the lineage III strain that produces very high numbers of TAMs even from a low dose of *M. cerebralis* myxospores.

For the first level of assessment, in most cases trout population estimations were conducted on one or more 91 meter (300 feet) segments of each study stream. When possible, two population estimates were conducted, one in the headwaters and another near the downstream end of the drainage. In general, the two-pass removal estimator was used to estimate population size and determine relative density, size and approximate age structure for all species of trout in the study reach (Seber and LeCren 1967). In most cases, study reaches were selected to include fry (YOY) and juvenile habitats in the population estimation process.

After the sampling process was completed, 10 young-of-the-year (YOY) trout and 10 age-1 juvenile trout were sacrificed to test for the *M. cerebralis* parasite. The YOY trout were tested by polymerase chain reaction (PCR) for genomic DNA unique to *M. cerebralis* (Cavender et al. 2004). Yearling, juvenile or adult trout were tested for myxospores of the *Mc* parasite using the pepsin-trypsin digest (PTD) method (Markiw and Wolf 1974). In most instances, cutthroat trout were euthanized for disease testing only when they occurred in allopatry. When cutthroat trout were sympatric with other salmonids, the other fish were sacrificed for disease screening to avoid unnecessary depletion of the cutthroat trout population.

In the event that the study reveals there is little evidence of spread, there are several plausible explanations for such an eventuality. First, in many instances the particular habitat being studied may have never been exposed to the parasite. Second, the habitat in question may have been exposed, but the parasite never completed its life cycle. If the parasite did not become enzootic there could be at least two plausible reasons. First, there may be very little stream habitat suitable for development of colonies of *T. tubifex* of sufficient density to sustain the life cycle in the aquatic oligochaete host. Second, aquatic oligochaetes may be present in the drainage but not the proper lineage of *T. tubifex* that is susceptible to *M. cerebralis.* Recent studies have shown that among the four different lineages of *T. tubifex* (I, III, V and VI) known to exist in Colorado, lineages I,V and VI are not susceptible to infection by *M. cerebralis*

(Beauchamp et al. 2001, 2002; Baxa and Hedrick 2008). Kerans et al. 2004 found that other tubificid oligochaetes such as *Limnodrilus hoffmeisteri and Ilyodrilus templetoni* do not become infected when exposed to *M. cerebralis* myxospores in a laboratory setting. Research efforts in New Mexico demonstrated that only lineage III *T. tubifex* become infected when exposed to *M. cerebralis* (DuBey and Caldwell 2004; DuBey et al. 2005). In the New Mexico studies lineages I, III and VI *T. tubifex* were tested.

Substantial effort has been expended to collect substrate samples containing aquatic oligochaetes in as many habitats as possible to determine which possibility might be the most plausible explanation. Collections were made concurrent with the trout population estimation surveys. The samples were sorted to determine the relative abundance of "haired" and "non-haired" oligochaetes. The standard protocol was to separate and sort oligochaetes until two aliquots of 50 "haired" worms per collection site were identified and preserved in 70% reagent grade ethanol for quantitative PCR testing (hereafter qPCR) to determine which lineages of *T. tubifex* (if any) were present in the study reach. Recent advances in testing and development of DNA-based genetic markers specific to at least six different lineages of *T. tubifex* make this possible (Beauchamp et al. 2001, 2002). Each 50-worm composite sample was quantitatively screened for DNA markers specific to each lineage within the mitochondrial DNA 16S *T. tubifex* oligochaete group. A private laboratory (Pisces Molecular) developed a 4-probe-multiplex qPCR test that screens a sample of up to 50 aquatic oligochaetes for the percentage of DNA for each of the four lineages of *T. tubifex* contained in the sample. The test also provides a relative indication of the total amount of DNA from *T. tubifex* in the sample. This test has facilitated development of spatial distribution maps for the various lineages of *T. tubifex* by drainage basin and on a statewide basis (Nehring 2008).

In addition, each worm sample can be screened by qPCR using the Hsp70 test (Cavender et al. 2004) to determine if DNA of *M. cerebralis* is present in the worm sample. The Hsp70 test targets a highly conserved region of the heat shock protein gene 70 that is found in a wide array of living organisms and also occurs in the genome for *M. cerebralis.*

METHODS

Trout Population Assessment - In most study streams, the objective was to estimate the salmonid species composition, density and size structure of the trout population at two or more sampling sites using the two-pass removal estimator as described by Seber and Le Cren (1967). Data collected during this effort were run through the Colorado Division of Wildlife's GOLDMEDL or JAKOMATIC computer software programs to develop the population estimates (N), 95% confidence limits, density (n/ha), biomass (kg/ha) and develop a relative estimate of year class abundance for the first 3 year classes based primarily on length-frequency distribution. All sampling sites were identified by GPS to facilitate mapping the collection locations using the mapping software package ARC VIEW 9.

Parasite Screening in Fish – In streams where adequate numbers of salmonids were present, we collected 10 YOY and 10 juvenile (≥age 1) trout for screening for *M. cerebralis* infection. YOY trout were screened for parasite DNA using the Hsp 70 (Heat shock protein gene

70) test (Cavender et al. 2004). Juvenile trout were tested for *M. cerebralis* using the PTD methodology (Markiw and Wolf 1974).

Aquatic Oligochaete Studies –Sediment-laden microhabitats from multiple locations at each study lake or stream were sampled for aquatic oligochaetes. All samples were thoroughly screened for aquatic oligochaetes. During the 2008 field season, most oligochaetes were examined by hand-held, 25-power, 4-ounce monocular microscope(s) in the field. Haired worms were preserved in 70% reagent grade ethanol and distilled water for qPCR testing. Haired oligochaetes have a high probability of being *T. tubifex* (Kathman and Brinkhurst 1998). The study protocol was to preserve at least one 50-worm sample of haired oligochaetes from each collection to determine whether or not the sample contained DNA specific any of the four lineages (I, III, V or VI) for *T. tubifex.* Each sample was prepped for total DNA extraction to preserve all of the genetic material in the sample. When large numbers of worms were encountered, two aliquots of 50 haired worms were preserved. Each 50-worm aliquot was screened using the 4-probe-multiplex qPCR test to quantify the percentage of DNA specific for the different lineages of *T. tubifex.* Total DNA extraction preserved any *M. cerebralis* DNA in the sample, affording subsequent testing of the worms for *M. cerebralis* infection (Cavender et al. 2004).

Beauchamp et al. (2001, 2002) developed a PCR-based genetic screening method that allowed differentiation and assignment of an individual oligochaete to one of four lineages of *T. tubifex* from a mixture of morphologically indistinguishable worms or genotypes. Drawing upon this pioneering work, a 4-probe multiplex qPCR protocol was developed in 2003 that allowed simultaneous testing and quantification of mitochondrial DNA specific for four different lineages of *T. tubifex* in a single sample (John Wood, Pisces Molecular; personal communication). A large amount of testing was done to develop and refine the 4-probe multiplex qPCR test for quantifying the relative amount of DNA for the various lineages (I, III, V and VI) of *T. tubifex* in aquatic oligochaetes. A detailed summary of the development of the 4-probe multiplex qPCR protocol has been presented previously (Nehring 2008) and will not be reiterated here.

RESULTS AND DISCUSSION

Historically, nine major river basins in Colorado have supported native cutthroat populations. These include the Arkansas, Colorado, Dolores, Gunnison, Rio Grande, San Juan, South Platte, White and Yampa river systems. Greenback cutthroat trout are native to the Arkansas and South Platte river basins. Rio Grande cutthroat trout are thought to be native to the Rio Grande basin. Colorado River cutthroat trout are native to the Colorado, Dolores, Gunnison, San Juan and White and Yampa river systems. No cutthroat trout were native to the North Platte drainage in Colorado. An overview of the number of streams and sites sampled each year for each of the three sub-species of native cutthroat trout is summarized in Table 1 below.

Table 1. Number of streams and sampling sites (stratified by year and cutthroat trout sub-species) between 2003 and 2007.

Year Greenback cutthroat			Rio Grande cutthroat		Colorado River cutthroat	
	Streams sites		Streams	Sites	streams	Sites
2003	9	12	9	13	22	29
2004	3	5	18	26	24	36
2005	9	12	18	24	10	12
2006	---	---	10	18	49	73
2007	1	2	3	5	48	61
Total 22 31			**58**	**86**	**153**	**201**

Detailed information regarding streams and sites sampled from 2003 through 2007 can be seen in Nehring 2004, 2005, 2006, 2007, 2008. Results from five years of field studies indicated that *M. cerebralis* was enzootic in numerous stream trout populations throughout Colorado. The primary reason for this seemed to be that the *M. cerebralis*-susceptible lineage III *T. tubifex* is the most cosmopolitan of the four lineages of this tubificid worm in Colorado. Moreover, there was no apparent thermal or elevation barrier that appeared limiting to the occurrence or establishment of *M. cerebralis* in aquatic habitats up to 11,000 feet or 3,354 meters. Stratification of the occurrence, relative abundance and distribution of the various lineages (I, III, V and VI) of *T. tubifex* into 1,000 foot elevation zones for the five-year data set revealed that the *M. cerebralis*-susceptible lineage III strain of *T. tubifex* was far more abundant and widely distributed in all elevation strata between 6,000 and 11,000 feet than the three non-susceptible *T. tubifex* lineages (I, V and VI) combined (Nehring 2008).

However, mitochondrial 16s rDNA for lineage III *T. tubifex* was detected in only one of eight oligochaete samples collected at sites > 11,000 feet between 2003 and 2007. This is an inadequate sample size to determine whether or not harsh environmental conditions at elevations > 11,000 feet might be a factor limiting the distribution of *T. tubifex*. For these reasons the field study was extended an additional year to concentrate on collecting trout and aquatic oligochaete samples from aquatic habitats at sampling locations > 11,000 feet elevation. During 2008, most of the sampling was concentrated in or near lakes at or above timberline. The results of six years of aquatic oligochaete sampling are summarized in Table 2.

Table 2. Number and frequency of detection and percent occurrence (in parentheses) of mt16s rDNA specific for lineage I, III, V and VI *T. tubifex* and tubificid oligochaetes with "haired" chaetae stratified within 1,000 foot elevation zones, and within and among strains or lineages across all elevation strata (2003 – 2008).

Elevation (ft.)	**Number of Sites where each Lineage of *Tubifex tubifex* was present**				
	Lineage I	**Lineage III**	**Lineage V**	**Lineage VI**	**No Lineage**
5,000 – 6,000	0	3 (43)	0	4 (57)	0
6,001 – 7,000	6 (11)	28 (54)	3 (6)	15 (29)	3
7,001 – 8,000	1 (3)	29 (74)	2 (5)	7 (18)	6
8,001 – 9,000	8 (11)	46 (61)	5 (7)	16 (31)	19
9,001- 10,000	3 (7)	33 (73)	2 (4)	7 (16)	25
10,001- 11,000	1	17 (81)	1 (5)	2 (9)	14
11,001 – 12,000	0	4 (57)	0	3 (43)	24
> 12,001	0	2 (50)	0	2 (50)	13
Total	**19 (5)**	**162 (46)**	**13 (4)**	**56 (16)**	**104 (29)**

These data suggest that the prevalence of non-*T. tubifex* aquatic oligochaetes appears to be greater at high elevations. However, at those sites where mt16s rDNA specific for *T. tubifex* was detected, the lineage III DNA was detected in 50% or more of the samples within all 1,000 foot elevation strata > 6,000 feet (Table 2).

Aquatic Oligochaete Sampling – Since the late 1990s, substantial research efforts have been directed at developing a better understanding the factors that affect the population dynamics and distribution of aquatic oligochaetes in the natural environment. In addition, much has been learned about the relative differences in susceptibility to *M. cerebralis* among the different lineages of *T. tubifex* (Beauchamp et al. 2001, 2002, 2005, 2006; DuBey and Caldwell 2004; DuBey et al. 2005; Kaesar and Sharpe 2006; Kerans et al. 2004: Winkelman and Nehring 2007).

As more and more research investigations have been directed at the aquatic oligochaete side of the life cycle of *M. cerebralis* it has become increasing clear that the presence of the lineage III *T. tubifex* in an aquatic environment is often the primary determining factor governing whether or not *M. cerebralis* becomes established after the initial introduction occurs. In the San Juan River below Navajo Dam in New Mexico, DuBey and Caldwell (2004) found that only lineage III *T. tubifex* were infected with *M. cerebralis*, even though *T. tubifex* belonging to lineages I and VI were also present in the stream. Moreover, in a follow-up laboratory study where worms from lineages I, III and VI were exposed to myxospores of *M. cerebralis*, evidence of infection was only detected in lineage III worms (DuBey et al. 2005). Similar outcomes have emerged from laboratory tests where lineage I, III, IV, V and VI *T. tubifex* have been exposed to varying concentrations of *M. cerebralis* myxospores in Colorado (Nehring, unpublished data), Oregon (Dr. Jerri Bartholomew, personal communication), California (Baxa and Hedrick 2008) and in states in the eastern U.S. (Dr. Vicki Blazer, personal communication). Although variations in sediment type or quality, (i.e., sand, mud or organically rich muck) can enhance the severity of infection among *T. tubifex* worms that are susceptible to *M. cerebralis*, parasite development and infectivity is not altered in lineages of worms that are refractory (V and VI) or highly resistant (I) to infection, regardless of sediment type (Baxa and Hedrick 2008). For these reasons, ascertaining the distribution and relative abundance of the various lineages of *T. tubifex* in

Colorado's cutthroat trout streams is a critically important component in assessing the risk of establishment and spread of *M. cerebralis* in Colorado.

Cutthroat Trout Population Status - More often than not, Colorado's self-sustaining populations of cutthroat trout persist at high elevations in stream reaches where the water temperatures are cold and the growing season can be quite short. In some cases these populations exist in habitats near the upper thermal limits of the species (Coleman and Fausch 2006). At lower elevations where the thermal regime may be more conducive to successful reproduction, growth and survival, cutthroat trout face extirpation due to competition from nonnative brown and brook trout (Peterson and Fausch 2003a; Peterson and Fausch 2003b; Peterson et al. 2004). Hybridization with nonnative rainbow trout can dilute the genetic purity of native cutthroat trout. These factors, coupled with the high sensitivity of Colorado's cutthroat trout to infection by *M. cerebralis* (Thompson et al. 1999), present daunting obstacles for resource managers charged with recovery efforts.

During the 2008 field season, salmonid samples were collected from 40 lakes, reservoirs, and beaver ponds, and five streams. The fish were euthanized and submitted for PTD testing for evidence of infection by *M. cerebralis.* Results of the PTD testing are summarized in Table 3. There were 14 additional lakes and one stream visited where no fish samples were preserved for PTD testing. Seven of the 14 lakes were either known to be devoid of fish, no fish were observed, or we were unsuccessful in collecting trout for testing. In seven other lakes fish were observed and/or caught but not sacrificed for various reasons. When fish were caught and not sacrificed, they were too large, there were too few fish in the lake, or fish caught from the inlet or outlet streams were sacrificed for testing, making the lake sample redundant. Except for the collections from the Rio Grande near Creede and Idaho Springs Reservoir all collection sites were at elevations ≥ 11,000 feet (3,354 m). Thirty-five collection sites were at elevations between 11,000 and 12,000 feet, and 22 were located at elevations > 12,000 feet (3,659 m).

Results of the collections and testing of salmonids in wilderness areas of Colorado are summarized in Table 4. A review of the fish sampling and aquatic oligochaete collections from 2008, together with similar results from the previous studies (Nehring 2004, 2005, 2006b, 2007, 2008; Schisler 2000, 2001) indicate that *M. cerebralis* has spread into high elevation lakes and streams in Colorado that either already support cutthroat trout, or are capable of supporting cutthroat trout. The Wilderness Institute website (Wilderness.net) lists 41 wilderness areas in Colorado. Samples of fish and/or aquatic oligochaetes have been collected from at least 50 sites in 14 of the 41 wilderness areas in the state. *Myxobolus cerebralis* infections have been detected in salmonids at 18 sites in one or more aquatic habitats in 6 of the 14 wilderness areas visited. Detections occurred in 11 streams and six lakes. The *Mc* parasite was detected in 41% (18 of 44) of the fish samples collected in wilderness areas.

Table 3. *Myxobolus cerebralis* cranial myxospore concentrations in salmonids collected from various sites in high elevation lakes and streams in Colorado during the 2008 field season.

Date Mmddyy	Collection Site (Water Name/Water Code)	Species	Sample Size		Myxospores	
			№	№ +	Mean	Range in Positive Fish
08/14/08	Ptarmigan Lake/ 91861	GBN	16	0	0	0
08/21/08	Lower Copper Lake/ 89121	CRN	34	0	0	0
07/14/08	Lower Hancock Lake/80527	GBN	10	0	0	0
07/15/08	Upper Pomeroy Lake/81074	GBN	10	0	0	0
07/18/08	North Fork Reservoir/79891	Rainbow	10	6	23,833	1,389 – 196,389
10/13/08	Rio Grande near Creede/42539	Brown	10	2	2,434	2,989 – 21,350
09/24/08	Emma Lake/80414	GBN	10	0	0	0
09/24/98	Linkins Lake/67783	CRN/BRK	11	0	0	0
09/24/08	Independence Lake/67416	CRN/BRK	17	0	0	0
07/16/08	Hartenstein Lake/80565	GBN/BRK	13	0	0	0
07/17/08	Ptarmigan Lake/81086	GBN	13	0	0	0
07/08/08	Henry Lake/90439	CRN	30	0	0	0
07/12/08	Upper Lamphier Lake/90871	CRN	10	0	0	0
07/12/08	Lower Lamphier Lake//90869	CRN	20	0	0	0
07/30/08	Middle Fork Saguache Creek/42806	RGN	17	1	187	2,811
08/20/08	Big Verde Lake/92926	Brook	20	0	0	0
08/07/08	Poage Lake/91760	SRN/BRK	17	0	0	0
08/13/08	Lower Deadman Lake/89397	RGN	10	0	0	0
08/21/08	Little Highland Mary Lake/90491	CRN	1	0	0	0
08/31/08	Big Highland Mary Lake/90489	Brook	5	0	0	0
07/23/08	Mill Lake/91265	CRN	10	0	0	0
07/25/08	Upper Lottis Lake/91087	CRN	13	0	0	0
07/27/08	Lake Fork Cochetopa Creek/39215	CRN	12	0	0	0
07/29/08	Wallace Lake	CRN	7	0	0	0
07/02/08	Texas Creek (Rio Grande)/43620	Brook	10	0	0	0
06/30/08	East Willow Creek/44076	Brook	10	0	0	0
08/27/08	Murray Lake Beaver ponds/55788	GBN	10	0	0	0
07/24/08	Lower Powderhorn Lake/91823	Brook	20	0	0	0
09/08/08	Silver Dollar Lake/56487	GBN	6	0	0	0
08/28/08	Naylor Lake	RBT/BRN	10	0	0	0
08/28/08	W. Summit Lake (Mt. Evans)/56641	GBN	8	0	0	0
09/19/08	Murray Lake/55788	GBN	12	0	0	0
09/10/08	Chinns Lake/54368	BRK/SPL	6	0	0	0
09/10/08	Sherwin Lake/56451	BRK/GBN	8	0	0	0
09/09/08	Slater Lake/56514	GBN	10	0	0	0
09/05/08	Idaho Springs Reservoir/54320	RBT/GBN	11	0	0	0
09/11/08	Lost Man Lake/68064	Brook	10	0	0	0
08/19/09	Cunningham Creek/39506	Brook	9	0	0	0
07/24/08	Powderhorn L. Beaver ponds/91823	Brook	6	0	0	0
09/03/08	Lower Chicago Lake/54332	GBN/BRK	28	2	469	11,244
09/16/08	Upper Chicago Lake/54344	GBN	23	21	63,879	2,811 – 368,256
09/08/08	Kite Lake (S. Platte Basin)/80717	GBN	20	4	8,293	14,056 – 70,278
09/17/08	Lower Square Top Lake/56576	GBN	4	2	22,256	22,256 – 66,767
09/17/08	Upper Square Top Lake/56588	GBN	11	0	0	0

Table 4. Summary of *Myxobolus cerebralis* testing in wilderness areas of Colorado (2003-2008).

Wilderness Area	Location (Lake or Stream Name)	Elevation (feet)	Species Infec tion	Severity
Collegiate Peaks Wilderness	Hartenstein Lake	11,451	GBN	None
Collegiate Peaks Wilderness	Ptarmigan Lake	11,758	GBN	None
Eagles Nest Wilderness	Piney River ↓ Piney Lake	9,315	BKT/LOC	Moderate-Severe
Eagles Nest Wilderness	Piney River ↑ Piney Lake	9,405	BKT/LOC	Moderate-Severe
Flat Tops Wilderness	Cabin Creek ↑ Trappers Lake	9,642	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Fraser Creek ↑ Trappers Lake	9,650	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Heberton Creek ↑ Trappers Lake	9,650	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Upper Marvine Lake	9,324	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Lower Marvine Lake	9,314	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Slide Lake	8,654	BKT/RBT	Moderate-Severe
Flat Tops Wilderness	Marvine Creek	9,299	BKT/RBT	Moderate-Severe
Flat Tops Wilderness	Trappers Lake	9,635	BKT/CRN	Moderate-Severe
Fossil Ridge Wilderness	Henry Lake	11,757	CRN	None
Fossil Ridge Wilderness	Upper Lamphier Lake	11,703	CRN	None
Fossil Ridge Wilderness	Lower Lamphier Lake	11,227	CRN	None
Fossil Ridge Wilderness	Mill Lake	11,457	CRN	None
Gunnison Gorge Wilderness	Gunnison River	5,300	LOC/RBT	Moderate-Severe
Hunter-Fryingpan Wilderness	Lost Man Lake	12,450	BKT	None
Hunter-Fryingpan Wilderness	Independence Lake	12,490	BKT/CRN	None
Hunter-Fryingpan Wilderness	Linkins Lake	12,008	BKT/CRN	None
La Garita Wilderness	Middle Fork Saguache Creek	11,956	RGN	Very light
La Garita Wilderness	Cochetopa Creek↑Stewart Creek	10,289	BKT/BNT	Moderate-Severe
La Garita Wilderness	Cochetopa Creek↑Canon Diablo	10,921	CRN	None
La Garita Wilderness	Lake Fork Cochetopa Creek	11,231	CRN	None
La Garita Wilderness	Machin Lake	12,480	RGN	No Sample
La Garita Wilderness	Wallace Lake	12,621	CRN	None
Mt. Evans Wilderness	Upper Chicago Lake	11,755	GBN	Severe
Mt. Evans Wilderness	Lower Chicago Lake	11,420	GBN	Very light
Mt. Evans Wilderness	West Summit Lake	12,841	GBN	None
Powderhorn Wilderness	Upper Powderhorn Lake	11,877	No fish	No Sample
Powderhorn Wilderness	Lower Powderhorn Lake	11,663	BKT/CRN	None
Powderhorn Wilderness	Beaver ponds ↓Powderhorn Lakes	11,600	BKT	None
Sangre de Cristo Wilderness	West Deadman Lake	11,772	No Fish	No Sample
Sangre de Cristo Wilderness	Upper Deadman Lake	11,709	RGN	None
Sangre de Cristo Wilderness	Lower Deadman Lake	11,661	RGN	None
Sangre de Cristo Wilderness	Deadman Creek	11,740	RGN	None
Maroon Bells-Snowmass	Lower Copper Lake	11,321	CRN	None
South San Juan Wilderness	East Fork Piedra River	7,992	BKT/BNT	Mild
South San Juan Wilderness	North Fork Conejos River	10,286	BKT	None
South San Juan Wilderness	Middle Fork Conejos River	10,249	BKT	None
South San Juan Wilderness	Rio de los Pinos	10,365	RGN	None
Uncompahgre Wilderness	Fall Creek	11,344	CRN	None
Weminuche Wilderness	Lower Flint Lake	11,620	CRN	None
Weminuche Wilderness	Highland Mary Lakes	12,096	BKT/CRN	None
Weminuche Wilderness	Big Verde Lake	12,186	BKT	None
Weminuche Wilderness	Lost Lake	12,190	CRN	No Sample
Weminuche Wilderness	Weminuche Crk.↑ Rio Grande Rsvr.	10,356	BKT	Very Light
Weminuche Wilderness	Ute Creek ↑ Rio Grande Reservoir	9,473	BKT/RBT	Moderate
West Elk Wilderness	North Golden Lake	11,028	No Fish	No Sample
West Elk Wilderness	South Golden Lake	11,066	GOLDEN	No Sample

The data collected during this study over the past six field seasons demonstrate that there is no elevation or thermal barrier prohibiting the establishment of the parasite in Colorado. *Myxobolus cerebralis* infections were documented in cutthroat trout in four lakes at elevations > 11,400 feet, including two lakes over 12,000 feet. The infections in two of the four lakes (Upper Chicago and Lower Square Top lakes) would be considered moderate to severe (≥ 50% prevalence). The presence of lineage III *T. tubifex* oligochaetes and a salmonid fish appear to be all that is required for establishment of the life cycle of the *Mc* parasite. Lineage III *T. tubifex* oligochaetes were collected in all four lakes with infected cutthroat trout located at elevations ≥ 11,400 feet.

All the foregoing does not provide much hope for slowing the spread of *M. cerebralis* or better yet breaking the life cycle of the parasite once it becomes enzootic in a specific aquatic habitat. However, recently published results of laboratory tests are tantalizingly suggestive that myxospores of the *Mc* parasite might not be near as resistant to degradation in the natural environment as previously thought (Uspenskaya 1957). In one study (Hedrick et al. 2008), TAM production among lineage III *T. tubifex* inoculated with *Mc* myxospores held in water suspension for seven days at 5 °C and 22 °C was reduced 64% in the replicates held at the higher temperature prior to the exposure to the oligochaetes. In a second study (Hedrick et al. 2008), replicate groups of *Mc* myxospores were held in water suspension at 4 °C, 10 °C and 20 °C for 60 days prior to inoculating lineage III *T. tubifex.* Mean TAM production among the 4, 10 and 20 °C treatments was 20,550, 17,450 and 1.5, respectively. Similarly, mean TAM production among lineage III *T. tubifex* exposed to myxospores held in 5 °C water for seven days and 4 °C water for 60 days prior to inoculation was 71,735 and 20,550 for the seven-day and 60-day holding periods, respectively. Mean TAM production was reduced 71% in the 60-day pre-inoculation treatment group. In all cases, the worms were held in 15 °C water for the 200-230 day post-inoculation/TAM-enumeration test period. These findings are strongly suggestive that *Mc* myxospores degrade and become non-viable in a much shorter period of time under environmental conditions that would support salmonids than previously believed (Uspenskaya 1957).

Freezing, exposure to sunlight, and simple desiccation for less than 24 hours were also shown to render *Mc* myxospores non-viable. Hedrick et al. (2008) found *Mc* myxospores were rendered non-viable by freezing at -20 °C and -80 °C for 7 days. *Mc* myxospores in a 1-mL suspension held in an uncovered petri dish were rendered non-viable by exposure to direct sunlight for 105 minutes. Temperature at the petri dish was 18 °C at the start and 42 °C at the end of the time period. Similarly, 1- mL suspensions of *Mc* myxospores were rendered non-viable when exposed to the air for 18.5 hours at a temperature of 22 °C. In all of these experiments, the myxospores were used to inoculate lineage III *T. tubifex* that were subsequently screened for TAM production for 200-230 days post-exposure.

All of the foregoing in the Hedrick et al. (2008) study suggest that it may be possible to break the life cycle of the *Mc* parasite in smaller high altitude lakes without a source of Mc-contaminated water by removing fish and suspending fish stocking for only a year or two.

CONCLUSIONS

Recent developments in the DNA typing and testing of the various lineages of *T. tubifex* for susceptibility or resistance to *M. cerebralis* infection are very encouraging. Studies conducted in Colorado, California , Oregon, New Mexico, and West Virginia have repeatedly shown that only lineage III *T. tubifex* are capable of producing TAMs of *M. cerebralis*. Oligochaetes belonging to lineages I, IV, V and VI are either refractory or highly resistant to infection by the parasite and do not produce fish-infective TAMs. These results offer hope that non-susceptible *T. tubifex* lineages can act as "biofilters" to consume and deactivate *M. cerebralis* myxospores in habitats where the parasite is already enzootic, and dramatically reduce ambient levels of infection. Indeed, this appears to have been occurring at Windy Gap Reservoir in Colorado for the past 5-6 years (Winkelman and Nehring 2007).

Aquatic oligochaete sampling and testing over the past six field seasons reveal that the *Mc*-susceptible lineage III *T. tubifex* is the most widely distributed of the four lineages *T. tubifex* occurring in Colorado. Mitochondrial DNA specific to the lineage III oligochaete has been detected in far more worm samples at all elevation zones between 6,000 and 12,000 feet in Colorado than for worms belonging to lineages I, V and VI. These findings indicate that the risk of establishment of *M. cerebralis* is quite high, once introduction into a previously unexposed aquatic ecosystem occurs. Moreover, there is no indication that high elevation and/or cold thermal conditions will prohibit establishment of *M. cerebralis* even above 12,000 feet.

After six field seasons, it is evident that *M. cerebralis* has become established in numerous aquatic habitats that support native cutthroat trout populations. The degree of spread of the parasite into high elevation habitats in the White and Yampa River basins is especially disconcerting. Many locations where the parasite is enzootic have direct connectivity to streams and lakes that support excellent populations of Colorado River cutthroat trout. It is critical that efforts be increased to construct barriers to isolate these populations and prevent invasion by non-native salmonids carrying the parasite from other areas where it is already enzootic.

Trappers Lake and Upper and Lower Marvine Lakes lie within the Flattops Wilderness Area in the White River National Forest. The headwaters of Piney Creek (above Piney Lake) north of Vail, Colorado lie within the Eagles Nest Wilderness Area. Upper and Lower Chicago Lakes lie at elevations over 11,400 feet in the Mt. Evans Wilderness. Lower Square Top Lake (west of Guanella Pass) and Kite Lake in the Mosquito Mountain Range west of Alma are located at elevations above 12,000 feet. Like Yellowstone Lake in Yellowstone National Park, these aquatic habitats are in pristine areas, located at relatively high elevation, and have no habitat degradation problems. Yet the cutthroat trout populations in all of these aquatic ecosystems are heavily infected with *M. cerebralis.* These cases belie the conventional "wisdom" that this parasite can only thrive in degraded or organically polluted environments. Rather, there are two important factors common to all of these ecosystems. Those factors are 1) the presence of a highly susceptible salmonid host, and 2) the presence of lineage III *T. tubifex.* It has been a proven fact for a decade that brook trout and Colorado's three sub-species of native cutthroat trout are more prone to develop a lethal infection after exposure to *M. cerebralis* than either brown trout or rainbow trout exposed under identical conditions (Thompson et al. 1999).

After six years of sampling and testing, we know that lineage III *T. tubifex* are highly abundant and the most widely distributed of the four lineages of *T. tubifex* known to occur in Colorado. Moreover, lineage III worms have been readily collected at all elevations in the state up to 3,354 meters (11,000 feet). The majority of core conservation populations of Colorado's native cutthroat trout occur in lakes and streams at elevations between 2,439 and 3,354 meters (8,000 – 11,000 feet). The empirical evidence collected over the past six years reveal there is a **very high degree of congruence** between aquatic habitats that 1) either sustain or are capable of supporting core conservation populations of native cutthroat trout, and 2) support dense populations of lineage III *T. tubifex*. Given these realities, it would be foolhardy for fisheries resource managers to assume that threat or risk of exposure of Colorado's native cutthroat trout to *M. cerebralis* is minimal. On the contrary, the risk is high once the parasite is introduced.

RECOMMENDATIONS

It is recommended that this research project be continued for an additional year to further explore two areas of research. First, the findings of Hedrick et al. (2008) suggesting that myxospores of *M. cerebralis* degrade and become non-viable in a matter of months need to be more thoroughly investigated. Uspenskaya (1957) and others concluded that *Mc* myxospores were extremely durable and remained viable for decades even in desiccated fish ponds. To determine whether or not myxospores of the *Mc* parasite degrade in a matter of months, 12,500 freshly harvested myxospores will be seeded into replicate 1-L containers of sterilized sand and aerated water and held at temperatures of 10 – 15 °C. At time intervals of 1, 15, 30, 60, 90, 120 and 180 days, 250 lineage III *T. tubifex* oligochaetes will be placed into two replicate 1-L containers and held for up to 270 days. Screening for TAM production will be conducted weekly on each replicate beginning at 80 days post exposure (PE) and continued for a minimum of 120 days after TAM production commences. At the end of all screening for TAM production, 100 worms from each replicate will be tested by the Hsp 70 PCR test for evidence of infection by *M. cerebralis*. If the viability of the myxospores attenuates in a relatively short period of time total TAM production should decrease as the time between myxospore inoculation (at day 0 in all replicates) and the seeding of the lineage III *T. tubifex* oligochaetes at each subsequent time period. Alternatively, if there is no decrease in total TAM production among the replicates at time intervals 90, 120 and 180 days compared to the time intervals 0, 15, 30 and 60 days, that would support the conclusions of the early investigations of Uspenskaya (1957) and others.

The second area of investigation is to determine whether or not non-susceptible lineage *T. tubifex* actually consume and de-activate *M. cerebralis* myxospores or do they pass through the gut lumen of these oligochaetes unaffected and remain viable for subsequent ingestion and infection of susceptible lineage III worms. This experiment would proceed along a similar design with 1-L replicates of sterilized sand being hydrated and held at 10-15°C and seeded with 12,500 myxospores. There will be two phases in the experimental design. In Phase One, treatment groups of 250 *T. tubifex* belonging to lineage I, III, V and VI would be seeded into the 1-L containers of sand, water and myxospores. There will be four replicate containers for each lineage treatment group. At time intervals of 15, 45, 90 and 120 days PE, all worms will be removed from one replicate per lineage treatment group and reseeded into clean sterilized sand without myxospores. Each replicate with the original sand (that had the worms removed during

Phase One) will be reseeded with unexposed lineage III *T. tubifex*. This is the Phase Two portion of the experiment. TAM production in all replicates for both phases will be screened and tabulated weekly for 200 - 270 days PE and compared among all replicates and treatments. If TAM production among all Phase Two replicates of lineage III worms are equivalent this would indicate that the non-susceptible lineages I, V and VI *T. tubifex* did not deactivate the myxospores in their respective replicates during Phase One. Alternatively, if TAM production was reduced among the Phase Two lineage III worms that were seeded into sand from Phase One that had contained lineage I, V and VI worms, this would be strong evidence that the non-susceptible lineages do consume and deactivate myxospores of *M. cerebralis* and therefore function as true "biological filters". This would be particularly true if TAM production was dramatically lower among the 15 and 45 day time interval replicates.

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