FISHERY MANAGEMENT INTERVENTIONS TO ELIMINATE *MYXOBOLUS CERBRALIS* INFECTION IN LOWER SQUARE TOP LAKE, CLEAR CREEK COUNTY, COLORADO (1998 – 2014)

A PROGRESS REPORT

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INTRODUCTION

History and Background - Throughout most of the 20th century many studies purportedly demonstrated that myxospores of the *Mc* parasite were highly resistant to freezing (El-Matbouli and Hoffmann 1991) and drying (Plehn 1905, 1924; Hoffman and Putz 1969, 1971), required "aging" in mud before they became infective (Schäperclaus 1931; Uspenskaya 1957), and could possibly remain viable for two years (Hoffman et al. 1962), or even longer than a decade (Schäperclaus 1954; Bauer 1959). However, most of these studies were completed decades before the true life cycle of *Myxobolus cerebralis* was even understood. (Markiw and Wolf 1983; Wolf and Markiw 1984). Study results on the effects of freezing (Hoffman and Putz, 1969, 1971; El-Matbouli and Hoffmann 1991; Hedrick et al. 2008) and/or "aging" in mud on *Mc* myxospores (Schäperclaus 1931; Uspenskaya 1957; Hoffman and Putz 1971) were often equivocal or confusing. Differences in the thermal regime as well as the manner and/or duration of freezing could account for the inconsistent results.

However, the discovery that a tubificid worm was required in the life cycle of *M. cerebralis* (Markiw and Wolf 1983; Wolf and Markiw 1984) and the subsequent determination by many investigators that the lineage III *Tubifex tubifex* oligochaete is the only highly susceptible tubificid host (Arsan et al. 2007; Arsan and Bartholomew 2009; Beauchamp et al. 2001, 2005; DuBey and Caldwell 2004; DuBey et al. 2005; Steinbach-Elwell et al. 2006; Hallett et al. 2009; Zielinski et al. 2011; Nehring et al. 2014) dispelled the myth of the need for "aging" in the mud. Sediment contaminated with *Mc* myxospores requires the presence of a *Mc*-susceptible *T. tubifex* for elaboration of whirling disease. Those oligochaetes produce semi-bouyant waterborne triactinomyxon (TAM) actinospores that can infect many species of salmonid fishes (O'Grodnick 1979; Hedrick et al. 1998; Bartholomew and Wilson 2002). Salmonids that are infected with the *Mc* parasite produce myxospores that are infectious for susceptible oligochaetes.

Controlled exposures trials have repeatedly demonstrated that rainbow trout, brook trout, and many sub-species of cutthroat trout are highly susceptible to infection and can experience high mortality when exposed at a small size ($\leq$ 40 mm) or an early age ($<$ 9 weeks post hatch) (O'Grodnick 1979; Ryce et al. 2004, 2005; Schisler et al. 2000; Thompson et al. 1999; Vincent 2002). Exposure to ambient levels of *Mc* TAMs in the natural environment in the intermountain regions of the western U.S. has resulted in population level impacts for rainbow trout (Nehring and Walker 1996; Vincent 1996a,b; Nehring et al. 1998; Nehring 1996, 2006; Granath et al. 2007), brook trout (Nehring 2006) and cutthroat trout in spawning streams that are tributary to Yellowstone Lake in Yellowstone National Park (Koel et al. 2005, 2006; Gresswell 2011).

Spread of the *Mc* parasite in the natural environment can occur in many ways. Migrations of infected fish vector the myxospores throughout the aquatic environment. *Mc* TAMs are carried downstream from enzootic areas in streams. Piscivorous avian predators are capable of vectoring *M. cerebralis* from one place to another, as it has been demonstrated that *Mc* myxospores remain viable after passing through alimentary canal of avian predators such as the great blue heron (Meyers et al. 1970), kingfishers (Shaperclaus 1954), black crested night heron and mallard ducks (Taylor and Lott 1978; El-Matbouli and Hoffmann 1991). Similarly, myxospores also remain viable after passage through the digestive tract of piscine predators such as northern pike (El-Matbouli and Hoffmann 1991) and brown trout (Nehring et al. 2002). However, many investigators have repeatedly concluded that the primary mode of dissemination of the *Mc* parasite

has been through the transport and stocking of infected trout (Hoffmann 1970, 1990; Meyers et al. 1970; Modin 1998; Schisler 2000; Bartholomew and Reno 2002).

In Colorado it was determined that a total of 226 *Mc*- negative waters had been accidentally stocked with *Mc*-suspect fish in 1992 and 1996, from three state fish hatcheries that were considered to be free of the parasite at the time. Many of those stocking events were aerial plants of rainbow and cutthroat trout fry in high elevation lakes. As a result, a systematic study was undertaken to assess the extent of the spread and establishment of *M. cerebralis* in high elevation habitats resulting from these accidental stockings (Schisler 2000). During 1998 and 1999, Schisler applied the polymerase chain reaction (PCR) and pepsin-trypsin digest (PTD) tests using split-head analyses to detect *Mc* infections in trout collected from 69 high elevation (2,103 meters or 7,000 feet) lakes and streams. These waters were stratified into three classes, i.e., not stocked with infected or suspect fish prior to testing, stocked with fish from a hatchery the year prior to when the hatchery first tested positive for the *Mc* parasite, and stocked with fish from a hatchery after the hatchery was classified as positive for *M. cerebralis.* Those results are summarized in Table 1.

Table 1. Summary of the PCR and PTD split-head analyses test results to detect *Myxobolus cerebralis* infection in trout from high elevation (2,103 meters or 7,000 feet) habitats. The lake and stream populations tested were stratified into three classes (0, 1 and 2). Class 0 waters were not stocked with infected or suspect fish prior to testing. Class 1 waters had been stocked with fish from a hatchery the year prior to when the hatchery tested positive for the *Mc* parasite. Class 2 waters were stocked with fish from a hatchery after the hatchery was classified as positive for *M. cerebralis.*

Class	Number of Waters	PCR Positive	PTD Positive
Waters ≥ 2,103 meters or 7,000 feet			
0	24	16	6
1	35	18	9
2	10	8	8
Total	69	42	23
Waters ≥ 3,036 meters or 9,958 feet			
0	13	6	0
1	26	9	4
2	4	2	2
Total	43	17	6

Over the period of two field seasons in 1998-1999, Schisler (2000) detected evidence of *Mc* infection in salmonids from 21 high elevation (≥ 3,036 m) lakes and streams (Table 1). In that study however, it was unknown whether or not the parasite was actually enzootic, due to the short period of time between the accidental stocking and subsequent collection of fish for disease testing. At the end of the 1990s, evidence of infection had been detected in 145 trout populations in Colorado.

In a six-year (2003-2008) follow-up study, it was determined that the parasite was enzootic in 18 lakes and streams in seven wilderness areas in Colorado (Nehring 2010). Moreover, the highly-susceptible lineage III *T. tubifex* worm was the most widely distributed aquatic oligochaete, detected almost twice as often as the resistant (lineage I) and refractory (lineages V and VI) tubificids combined, and occurring at all elevation zones from 1,524 m (5,000 feet) to 3,678 (12,064 feet). In 2008, 14 of the high elevation lakes sampled in the 1998-1999 study where evidence of infection was found were re-sampled (Nehring 2010). In this study it was determined that *Mc* was still enzootic in four of the 14 lakes, Upper and Lower Chicago lakes, Kite Lake and Lower Square Top Lake, ranging in elevations from 3,481 to 3,678 meters. Lower Square Top Lake is the subject of this study.

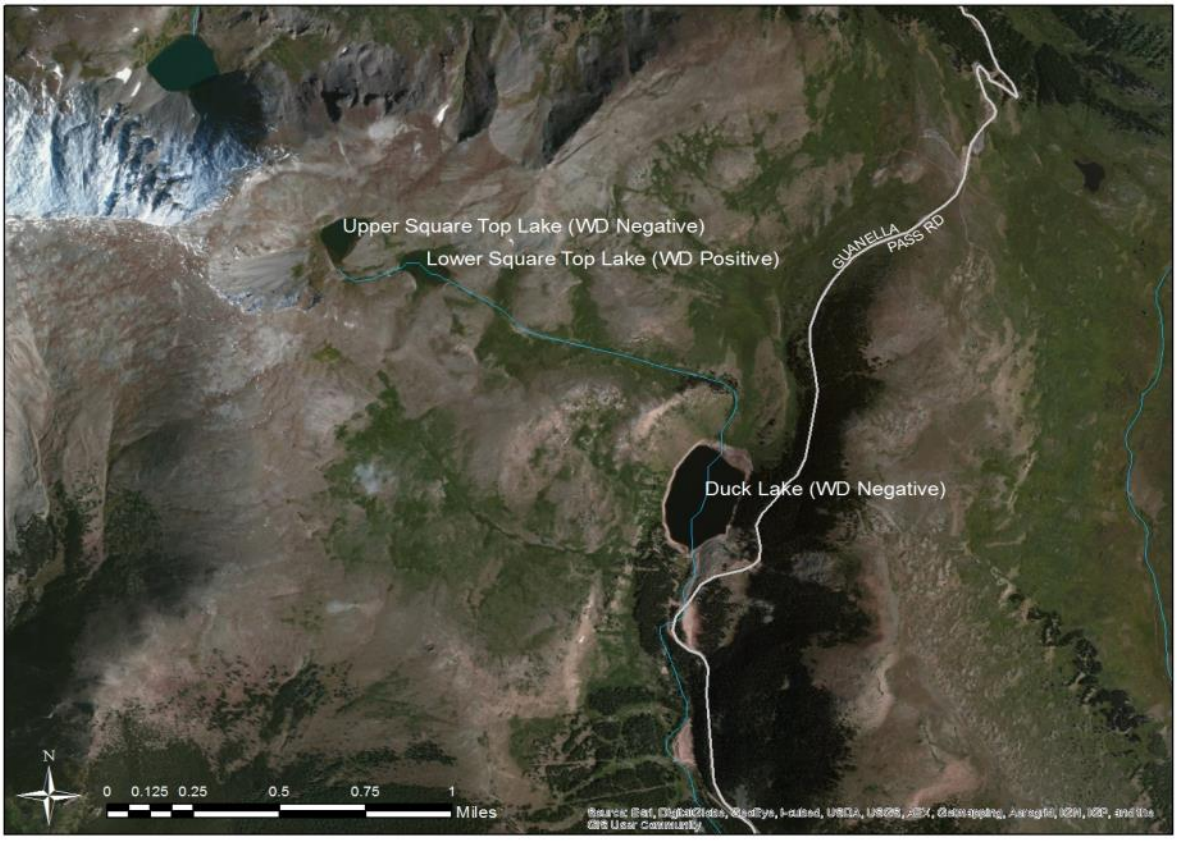
Lower Square Top Lake Study Area – Situated in a cirque basin at an elevation of 3,673 m (12,047 feet) and 2.82 ha (6.99 acres) in area, Lower Square Top Lake (LSTL) is located in Clear Creek County, approximately 3 km west of the Guanella Pass (Figure 1). Upper Square Top Lake (USTL), with a surface area of 3.05 Ha (7.55 acres) at an elevation of 3,738 m (12,257 feet), is located approximately 0.4 km directly west of LSTL. Water from both lakes flows out of LSTL into Duck Creek, which runs into Duck Lake at an elevation of 3,354 m (11,000 feet). According to Colorado Parks and Wildlife hatchery records, USTL and LSTL were stocked annually in mid- to late-summer with aerial plants of various strains of cutthroat trout fry from 1973 through 1995. No stocking occurred in 1996-1997. Between 1998 and 2008, aerial stocking of 800 to 1,000 cutthroat fry occurred in even years. No stocking has occurred in either lake since September 2008.

Preliminary Studies - In 2008, 2009 and 2010, repeated gillnet sampling and PTD testing of the fish in USTL and LSTL indicated that very few of the cutthroat trout fry being stocked in LSTL were surviving through the first year after stocking (see Tables 2 and 3). Despite many more hours of gill net sampling in LSTL over the 3-year period, only 4 cutthroat trout were caught that ranged from 115 to 162 mm in length, the proper size range for survivors of the September 2008 aerial plant. In contrast, 12 of 20 cutthroat trout captured in one gill net that was set in USTL for just 18 hours in August 2009 were smaller than 199 mm in length (Table 3), suggesting that the September 2008 aerial plants into the upper lake had survived and grown very well. No fish collected from the upper lake have ever tested positive for *Mc* myxospores, compared to the heavy infection levels in the fish in the lower lake (Table 4). Sampling of Duck Creek (between LSTL and Duck Lake) in late August 2013 revealed that the cutthroat trout ≥ age 2 were infected with the parasite. In contrast, cutthroat trout collected by gill netting in Duck Lake (DL) in September 2011 and tested by PTD were negative for evidence of *Mc* infection.

At the outset, the absence of any evidence of *Mc* infection in cutthroat trout in DL seems highly unlikely. However, several lines of evidence and sets of data gathered from the three lakes in the Duck Creek basin and other areas during the 6-year study from 2003 through 2008 reveal that this is quite plausible (Nehring 2009, 2010). First, field studies conducted in 2008 at Upper and Lower Chicago lakes in the Mt. Evan Wilderness indicated that a vertical drop of 120 – 150 m for TAMs cascading down water falls or a talus side was not quite adequate to physically destroy TAMs. Lineage III worms were present in Upper Chicago Lake (UCL) and the cutthroat trout were heavily infected with the *Mc* parasite. Moreover, fish from UCL were heavily infected in 1998 (Schisler 2000). Extensive sampling efforts and lineage-testing of oligochaetes in Lower Chicago Lake (LCL) in 2008 indicated there was no lineage III DNA present in the worms. However, a low percentage of the fish from LCL were lightly infected, suggesting that a few

TAMs survive the 120 – 150 m vertical fall from the outlet of UCL. A low prevalence of lightly infected fish was also the case for LCL in 1998 (Schisler, *ibid).* Second, collection and lineage-typing of aquatic oligochaetes from USTL and DL using the qPCR techniques outlined in Nehring et al. 2014, revealed that only mitochondrial 16S rDNA belonging to lineage I and V *Tt* worms was present in DL, while only DNA belonging to lineage VI oligochaetes was detected in worms

Figure 1. Satellite image of Duck Lake, Upper and Lower Square Top lakes and the surrounding area west of Guanella Pass, Clear Creek County, Colorado.



collected from USTL. In contrast, qPCR testing of *Tt* worms samples from LSTL in 2008, 2009 and 2012, revealed only DNA belonging to lineage III oligochaetes was detected. The vertical drop from LSTL to DL is > 305 m (1,000 feet), over many cascades and waterfalls and no evidence of infection has been detected among the cutthroat trout sampled by PTD and qPCR from DL. This is *prima facie* evidence that a vertical drop > 305 m is more than sufficient to destroy TAMs flowing out of LSTL, given that *Mc* has been enzootic in the lake since 1998 or longer (Schisler 2000). Moreover, the detection of *Mc* myxospores in cutthroat trout collected from Duck Creek below LSTL in 2013 is also empirical evidence supporting the hypothesis that some TAMs have come out of the lake and infected some of the cutthroat trout in Duck Creek (Table 5). But, without the presence of a lineage III worm in DL, the life cycle of the parasite cannot be sustained.

Study Design – Given all of the foregoing information and data, the decision was made in 2010 to net all of the cutthroat trout out of LSTL and keep the lake fish-free for a period of 2-3

years. The working hypothesis was that by keeping the lake free of any *Mc*-susceptible fish host, the parasite could be eliminated from LSTL. This hypothesis is based on the results of a number of studies. First, it has been demonstrated in a laboratory study that the viability of *Mc* myxospores maintained in a medium of fine sand and water and a thermal regime of 5 – 15 ˚C decreases at an exponential rate with less than 0.1% remaining viable at six months post-inoculation (PI) and none remained viable after one year (Nehring 2010). In this study, 250 lineage III tubificid worms were sequentially introduced into 0.9 L replicate containers of sand and water that had been inoculated with 10,000 myxospores/replicate (dose=50 myxospores/worm). Groups of 250 worms per container were introduced into two replicates at each time-delayed interval of 0, 15, 30, 60, 90, 135,180 and 365 days PI. Second, laboratory studies conducted on lineage III tubificids infected with the *Mc* parasite revealed individual worms remained infected for life, stochastically releasing bursts of TAMs for a few days at 6-9 month intervals, but no worms survived longer than 24 months post-exposure (Gilbert and Granath 2001). Therefore, the working hypothesis is that if LSTL can be kept fish-free for at least 3 years and that all lineage III worms infected with the *Mc* parasite do not live longer than 3 years, the life cycle of the parasite should be broken in the lake by the end of 2014.

To test this hypothesis, a floating live cage was placed in LSTL during the open-water period each year beginning in 2011, and stocked with fry or fingerling rainbow and/or cutthroat trout. Exposed trout fry/fingerlings were to be cropped at approximately 7-day intervals and tested by qPCR techniques for evidence of infection by the parasite. Fish were to be maintained in the floating live car for minimum of approximately two months during the late summer and early fall. Surviving fry or fingerlings were to be removed from the lake just prior to ice up and onset of winter conditions and taken to the state Fish Research Laboratory at Parvin Lake and maintained for 9 months, then euthanized and tested for the prevalence and severity of infection using the PTD methodology (Markiw and Wolf 1974). It is anticipated that with each passing year the prevalence and severity of infection in the exposed fingerling trout should decrease, and hopefully disappear from the lake in 2014.

Gill nets were to be continuously set in LSTL each summer for several weeks each year to insure that no cutthroat trout migrate down the cascading waterfalls from USTL to LSTL or upstream from DL or Duck Creek into LSTL during the spring run-off, becoming exposed to TAMs in LSTL and thereby keeping the life cycle of *Mc* going in the lake.

Monthly water filtration efforts were conducted during the summer of 2012 in an attempt to see if *Mc* TAMs could be detected in the water during the time when the fingerling trout were being exposed in the sentinel live cage.

Tubificid worms from LSTL were collected in 2008, 2009 and 2012 and tested for the presence of DNA of the *Mc* parasite. These worms were also exposed to *Mc* myxospores in a laboratory trial during 2009-2010 to assess their vulnerability to the parasite in relation to other populations of lineage III *Tt* worms from 10 major drainage basins all across Colorado (Nehring et al. 2014).

Table 2. Length (mm) and weight (g) for GBN cutthroat trout captured by gill net sampling of Lower Square Top Lake for various periods of time from 2008 through 2013.

Date	Fish		Fish		Fish		Fish	
Mm/dd/yyyy	Length	Weight	Length	Weight	Length	Weight	length	Weight
09/16/2008	429	1,000	325	690	430	1,050	430	905
08/27/2009[b]	495	---	262	---	347	---	281	---
	240	---	253	---	400*	---	420*	---
	281	---	335	---	435	---	450	---
	451	---	123	---	---	---	---	---
08/28/2009[b]	162	---	115	---	---	---	---	---
08/18/2010[c]	500	2,000	310	450	420	1,250	215	150
	215	125	228	150	415	1,175	296	395
	205	50	405	750	328	435	---	---
08/19/2010[c]	225	185	---	---	---	---	---	---
08/20/2010[c]	223	115	---	---	---	---	---	---
08/24/2010[c]	160	50	---	---	---	---	---	---
08/25-28/2010[c]	Zero catch on all nights in all seven nets fished.							
07/15/2011[d]	No data	No data	No data	No data	No data	No data	No data	No data
07/20/2011 – 08/15/2011[d]	Gill nets were fished continuously, and checked once each week or oftener over this period, and no additional fish were caught.							
07/20/2012 –	0	0	0	0	0	0	0	0
08/20/2012[e]	0	0	0	0	0	0	0	0
06/11/2103 –	0	0	0	0	0	0	0	0
08/15/2013[f]	0	0	0	0	0	0	0	0

Note([a]): 2 -150 foot gill nets set each night during the 2008 gill net surveys. Three GBN trout caught on nite of 9/16-17, and 1 caught on nite of 9/17-18.

Note([b]): 3 -150 foot gill nets set each night during the 2009 gill net surveys.

Note([c]): 7 -150 foot gill nets set each night during the 2010 gill net surveys.

Note([d]): 3 -150 foot gill nets set each night during the 2011 gill net surveys. 6 larger GBN cutthroat were caught during the first week after ice out. Four heads were saved for PTD analysis. All were PTD negative. The 3 – 150 foot gill nets were fish 24 hours a day for the next 3-4 weeks and no other fish were caught. There was no length-weight data taken on these 6 fish.

Note([e]): 3 -150 foot gill nets set each night during the 2012 gill net surveys. The 3 – 150 foot gill nets were fish 24 hours a day for 30 days and no fish were caught.

Note([f]): 3 -150 foot gill nets set each night during the 2013 gill net surveys. The 3 – 150 foot gill nets were fish 24 hours a day for 66 days and no fish were caught.

Table 3. Length (mm) and weight (g) for GBN cutthroat trout captured by gill net sampling of Upper Square Top Lake for various periods of time from 2008 through 2013.

Date	Fish		Fish		Fish		Fish	
Mm/dd/yyyy	Length	Weight	Length	weight	Length	Weight	Length	Weight
09/17/*2008* [a]	490	1,030	409	995	305	380	295	298
	260	203	271	280	495	1,400	395	770
	366	735	384	850	282	280	---	---
08/28/2009 [b]	158	---	113	---	161	---	182	---
	132	---	198	---	152	---	160	---
	168	---	175	---	131	---	137	---
	241	---	282	---	297	---	310	---
	356	---	310	---	390	---	450	---
08/20/2010 [c]	278	250	260	200	270	290	340	620
	295	340	320	455	235	170	217	100
	318	395	263	240	165	20	410	960
	295	285	304	395	336	520	286	280
	285	295	233	140	222	125	244	155
	262	200	253	190	274	245	355	590
	310	380	254	190	210	95	233	150
	231	140	226	140	203	85	211	110
	228	135	196	95	298	315	183	65
	190	70	---	---	---	---	---	---
08/20/2013	435	---	490	---	445	---	383	---
	465	---	530	---	470	---	530	---
	495	---	465	---	550	---	495	---
	490	---	465	---	500	---	---	---
08/27/2013	528	---	570	---	441	---	---	---
09/05/2013	480	---	500	---	---	---	---	---
09/10/2013	525	---	500	---	---	---	---	---
09/16/2013	457	1,400	---	---	---	---	---	---
09/19/2013	All nets were empty							
09/24/2013	557	---	---	---	---	---	---	---

Note([a]): 1- 125 foot gill net set for a single 24-hour overnite set in 2008.
Note([b]): 1- 150 foot gill net set for a single 21-hour overnite set in 2009.
Note([c]): 1- 150 foot gill net set for a single 21-hour overnite set in 2010.

RESULTS and DISCUSSION

Water Filtration Studies – Filtration and concentration of 1890 L of water from LSTL was completed once a month between July and September 2012. No TAMs were ever detected in any of the filtrates, indicating that the raw densities of TAMs in the lake were < 1 TAM/100 L of lake water.

Aquatic Oligochaete Studies – Fifty-worm aliquots of haired *Tt* oligochaetes from USTL, LSTL and DL were assayed to determine lineage and tested for the presence or absence of *Mc* DNA using the four-probe multiplex qPCR technique described by Nehring et al. 2014. These assays were conducted on worms from USTL and LSTL in 2008, 2009 and 2012, and from DL in 2011. Tubificid worms were very abundant in the shallow water (depth < 1m) areas of all three lakes where substrates of fine sediment and muck were found. Analysis of two-50 worm aliquots collected from LSTL in August 2008, proved to contain only DNA belonging to lineage III *Tt* oligochaetes, but they did not test positive for *Mc* DNA. QPCR assays of worms from LSTL in

2009 were shown to be pure lineage III oligochaetes that were producing TAMs at the time of collection, and tested positive for *Mc* DNA. Eleven 50-worm aliquots of *Tt* collected from LSTL in August 2012 were screened by qPCR for determination of lineage and evidence of *Mc* infection. Once again, all 11 aliquots were shown to be pure lineage III worms, but no *Mc* DNA was detected, indicating that the prevalence of infection in this collection was < 0.18%.

Sediment samples collected at six separate sites in DL in the fall of 2011 all contained tubificid worms. The sediment from all six sites was pooled and four aliquots of 50 haired worms were screened by qPCR for determination of lineage composition and presence or absence of *Mc* DNA**.** These assays indicated the *Tt* oligochaete population was comprised of *Tt* worms belonging to lineages I and V. No lineage III DNA or *Mc* DNA was detected in any of the samples. All qPCR assays of haired oligochaetes from USTL collected in 2008, 2009 and 2012 tested negative for Mc DNA and were shown to be comprised of lineage VI *Tt* worms.

Worms collected in August 2009 were exposed to 50 *Mc myxospores/*worm to evaluate the total TAM production of lineage III worms from LSTL compared with TAM production among more than 30 separate populations of lineage III oligochaetes from 10 major drainage basins across Colorado that were similarly exposed to the same myxospore densities. Approximately 95% of the water in each 1-L replicate container was filtered once each week and assayed to determine the amount of TAM production (Nehring et al. 2014). All replicate treatments contained 250 *Tt* worms that were held and exposed under identical conditions. Total TAM production in each replicate was determined over a period of 210 days. Total TAM production among the two replicates of lineage III worms from LSTL was 2.751 and 5.918 million. No TAMs were produced among two exposed replicates of lineage VI worms from USTL in the same exposure experiment.

Gillnet surveys – Results of the gill net surveys for USTL and LSTL from 2008 through 2013 are summarized in Tables 2 and 3. The data for the years 2008 and 2009 indicate that the survival of the cutthroat trout fry stocked from the air by fixed wing aircraft in late summer 2006 and 2008 are dramatically different for the two lakes. The 2008 overnight gill netting of USTL resulted in a catch of 11 trout of various sizes, four less than 300 mm in length, most likely survivors of the August 2006 aerial stocking. Similarly, the 2009 gillnet results for a single overnight gillnet set (with just one net) resulted in a catch of 20 cutthroat trout, including 12 (60% of the catch) that were under 200 mm in length, suggesting that they were survivors of the September 2008 aerial plant. In August 2010, 37 cutthroat trout were caught in a single net overnight gill net set of 18 hours (Table 3).

In the late - summer of 2013, the management decision was made to eliminate all cutthroat trout from USTL, in order to establish a pure population of pure GBN cutthroat trout as part of the management effort to re-establish a free-ranging population of this sub-species. The most recent genetic analyses from museum specimens and all other known sources seems to indicate that there is currently only one extant population of these fish left on the planet, that being found in the Bear Creek drainage arising from the east slope of Pikes Peak, near Colorado Springs, Colorado (Kevin Rogers, personal communication). Gill nets set in USTL from September 17 through September 24 resulted in a catch of only 24 cutthroat trout. However, it is not known whether all of the fish have been netted out. Nets will have to be set in the lake again in 2014 after ice out to insure that all of the fish have been removed.

Table 4. Results of PTD disease testing for cranial myxospore concentrations in greenback native (GBN) cutthroat trout from Upper and Lower Square Top Lakes, Clear Creek County, Colorado 1998 – 2013.

Collection Date Mo/Da/Yr	Species	Age (Yrs)	Sample Size		Mean Myxospore Burden	Myxospores in Positive Fish	
			No.	No.+		Mean	Range
Upper Square Top Lake							
09/15/1998	Cutthroat	≥ 1+	7	0	0	0	-------
09/17/2008	GBN	≥ 1+	11	0	0	0	-------
08/28/2009	GBN	≥ 1+	19	0	0	0	-------
08/20/2010	GBN[a]	≥ 1+	37	0	0	0	-------
Lower Square Top Lake							
09/15/1998	Cutthroat[b]	≥ 1+	20	12	23,318	38,863	934 – 252,688
09/15/1998	Cutthroat[c]	3+	3	3	31,948	31,948	14,156 – 57,688
09/15/1998	Cutthroat[d]	4+	10	7	28,340	40,486	934 – 252,688
09/15/1998	Cutthroat[e]	5+	3	0	0	0	-------
09/15/1998	Cutthroat[f]	6+	1	1	4,444	4,444	4,444
09/15/1998	Cutthroat[g]	7+	2	1	41,333	82,666	0 – 82,666
09/15/1998	Cutthroat[h]	8+	1	0	0	0	--------
09/23/2008	GBN	≥ 4+	4	2	22,256	44,512	22,256 – 66,767
08/28/2009	GBN	1+	3	3	17,302	17,302	8,233 – 28,622
08/28/2009	GBN	≥ 4+	11	6	179,407	328,913	8,322 – 1,851,111
08/17/2010	GBN	≥ 2+	9	6	37,052	55,579	9,367 – 86,333
08/17/2010	GBN	≥ 4+	4	1	2,539	10,156	10,156
07/15/2011	GBN[k]	≥ 4+	4	0	0	0	-------

Note([a]): Based on length differences of trout in the collection, it was estimated that there were 4 separate year classes of fish represented in the 2010 sample: 22 from the 2008 aerial plant, 11 from the 2006 plant, 3 from the 2004 plant and 1 from the 2002 aerial plant.

Note ([b]): Based on length differences of trout in the collection, it is estimated that there were 6 separate year classes represented in the group of 20 trout. Examination of aerial planting records for Lower Squaretop Lake indicates the lake was stocked every year between 1990 and 1995 with 400 to 900 cutthroat trout fry per year. ([c]):1995 year class; ([d]):1994 year class; ([e]):1993 year class; ([f]):1992 year class; ([g]):1991 year class; ([h]):1990 year class. ([k]):These fish were believed to be downstream migrants from Upper Squaretop Lake that left the lake right after ice-out. The flows were extremely high due to the near 600% of normal snowpack.

Only four large cutthroat trout were caught in LSTL during the 2008 gillnetting operation. All were larger than 300 mm and three were ≥ 429 mm in length. Only three of 16 (18.8%)

cutthroat trout caught during the 2009 gill net surveys in LSTL were < 200 mm, suggesting a poor 1-year survival rate in LSTL compared with that of USTL. In August 2010, when the management decision had been made to net all of the trout out of LSTL, a total of 14 cutthroat trout were captured during an 11-day period of netting with a total of seven nets fishing the lake continuously. Eleven of the 14 fish were caught during the first 24 hours of netting. None were caught during the last 3 nights of netting (Table 2).

Evidence of M. cerebralis infection - Results of PTD testing of cranial tissues to determine the prevalence and severity of infection by the *Mc* parasite in USTL, LSTL, DL and Duck Creek are summarized in Tables 4 and 5. These results compiled over a period of 15 years demonstrate the high prevalence and severity of infection among the cutthroat trout in LSTL, as well as the lack of infection in USTL and DL. It is noteworthy that there was no evidence of infection (by PTD testing) among 13 fingerling cutthroat trout that were a year old, the result of natural reproduction in 2012. These yearling trout were collected by electrofishing in Duck Creek during late-August 2013 (Table 5). This suggests a number of possibilities. First, the level of TAM production escaping from LSTL from late August 2012 through August 2013 was so low or non-existent that none of these fish became infected. Second, if they were indeed infected, the exposure occurred too late in time for any infection to proceed to the elaboration of myxospores in the cranial tissues at the time of collection. In this case, infection could have been detected if these 13 fish had been tested by qPCR for *Mc* DNA, which was not done. Third, any TAMs that had escaped from LSTL during or after August 2012 were rendered non-viable by the hydraulic turbulence as they tumbled down the dozens of rapids and cascading vertical drops below the outlet of LSTL.

Table 5. Estimated concentrations of *Myxobolus cerebralis* myxospores in cranial tissues of cutthroat trout from Duck Lake and Duck Creek between the outlet of Lower Square Top Lake and the inlet to Duck Lake (2011 – 2013).

Collection Date Mo/Da/Yr	Species	Age (Yrs)	Sample Size		Overall Mean Myxospore Burden	Myxospores in Positive Fish	
			No.	No.+		Mean	Range
Duck Lake							
09/02/2011	Cutthroat	≥ 3+	17	0	0	0	-------
Duck Creek from Duck Lake to the lower fish barriers (1st impassable waterfall)							
08/27/2013	Cutthroat	1	11	0	0	0	-------
08/27/2013	Cutthroat	≥ 2	19	9	21,750	45,916	2,267 – 118,833
Duck Creek between lower and upper fish barriers (1st and 2nd impassable waterfalls)							
08/27/2013	Cutthroat	1	2	0	0	0	-------
08/27/2013	Cutthroat	≥ 2	4	1	16,043	64,167	0 – 64,167

Sentinel Cage Exposure Tests – In 2011, 2012 and 2013, fingerling rainbow trout (RBT) and greenback (GBN) native cutthroat trout fry were placed in a double-walled floating live cage in early August and continuously exposed to the water and TAMs in LSTL. Each year between 50

and 100 fingerlings of each species were placed in the cage. RBT fingerlings were introduced during August, and GBN fry were placed in the cage in early September. Five to ten or more of these fish were withdrawn from the cage at approximately 7-day intervals, euthanized, and preserved in 70% ETOH in a 15 mL test tube, and submitted for qPCR testing to determine the prevalence and severity of infection by *Mc.* The sentinel cage and all surviving GBN fry were taken from the lake during the first two weeks of October, just prior to the lake surface freezing over for the winter. The results of those qPCR tests are summarized in Figures 2 and 3. Taken together the test results strongly support the hypothesis that the ambient level of TAM densities has declined significantly over the past three years. The prevalence of infection (% testing positive) for a given time period of the first 14-21 days has declined each year. The time required for the weekly samples to reach 100% prevalence increased from 2011 to 2012 and then never reached 100% for either test species during the 2013 exposures. For the RBT fingerlings, the maximum prevalence of infection only reached 70% in 2013 after 63 days PE, compared to 100% prevalence after 28 days PE in 2011. Among the GBN fry, the maximum observed prevalence never exceeded 20% at any sampling interval over the 35-day exposure period in 2013.

The prevalence and severity of infection, as determined by cranial myxospore concentrations among the 2011 and 2012 treatment groups of GBN fry/fingerlings held over for nine months at the Parvin Lake Research Lab are summarized in Table 6. The prevalence of infection (% PTD positive) was essentially 100% for all exposure groups in 2011 and 2012, congruent with the qPCR test results for those two years as shown in Figures 2 and 3. However, it

Table 6. Estimated concentrations of *Myxobolus cerebralis* myxospores in cranial tissues of GBN cutthroat trout exposed in a floating live car in Lower Square Top Lake for approximately 30-35 days in September and early October 2011 and 2012, and then transferred to the Colorado Parks and Wildlife Research Lab at Parvin Lake and held for 8 months, and then euthanized at evaluated for prevalence and severity if *M. cerebralis* infection in 2012 and 2013.

Collection Date Mo/Da/Yr	Species	Age (Yrs)	Sample Size		Overall Mean Myxospore Burden	Myxospores in Positive Fish	
			No.	No.+		Mean	Range
Lower Square Top Lake September 2011 exposures held at Parvin Lake through July 5, 2012							
07/05/2012	GBN lot 1	1+	14	14	77,272	77,282	14,767 – 242,667
07/05/2012	GBN lot 2	1+	15	14	85,949	92,088	26,667 – 200,978
07/05/2012	GBN lot 3[a]	1+	20	3	1,755	11,698	4,817 – 21,356
Lower Square Top Lake September 2012 exposures held at Parvin Lake through July 9, 2013							
07/09/2013	GBN	1+	30	30	33,565	34,307	1,600 – 130,667

Note ([a]): The fish were negative controls from Duck Lake that were captured by electrofishing seven days after being stocked into Duck Lake from the Shavano Hatchery. These fish were tested by qPCR for DNA of the *M. cerebralis* parasite 3 times, first at the Mt. Shavano Hatchery BEFORE transport for being stocked into the live car at Lower Square Top Lake in September 2013, also BEFORE being stocked into the live car at Lower Square Top Lake, and AFTER being stocked into Duck Lake for seven days prior to transfer to the Parvin Lake Lab. Therefore, it is almost a certainty that these "negative controls" became lightly positive in the aquaria at Parvin Lake since the water supply comes from Parvin Lake and is known to periodically be positive for *M. cerebralis.*

Figure 2. Summary of the results of the floating sentinel cage exposures of Greenback native (GBN) cutthroat trout fry in Lower Squaretop Lake during September and early October, 2011, 2012, and 2013. The tests were to determine whether or not the *Myxobolus cerebralis* DNA in the fry. Five GBN fry were sacrificed for qPCR testing at each 7-day sampling interval during the 2011 exposure period. During the 2012 and 2013 exposure periods, 10 GBN fry were sacrificed for qPCR testing at each 7-day sampling interval.

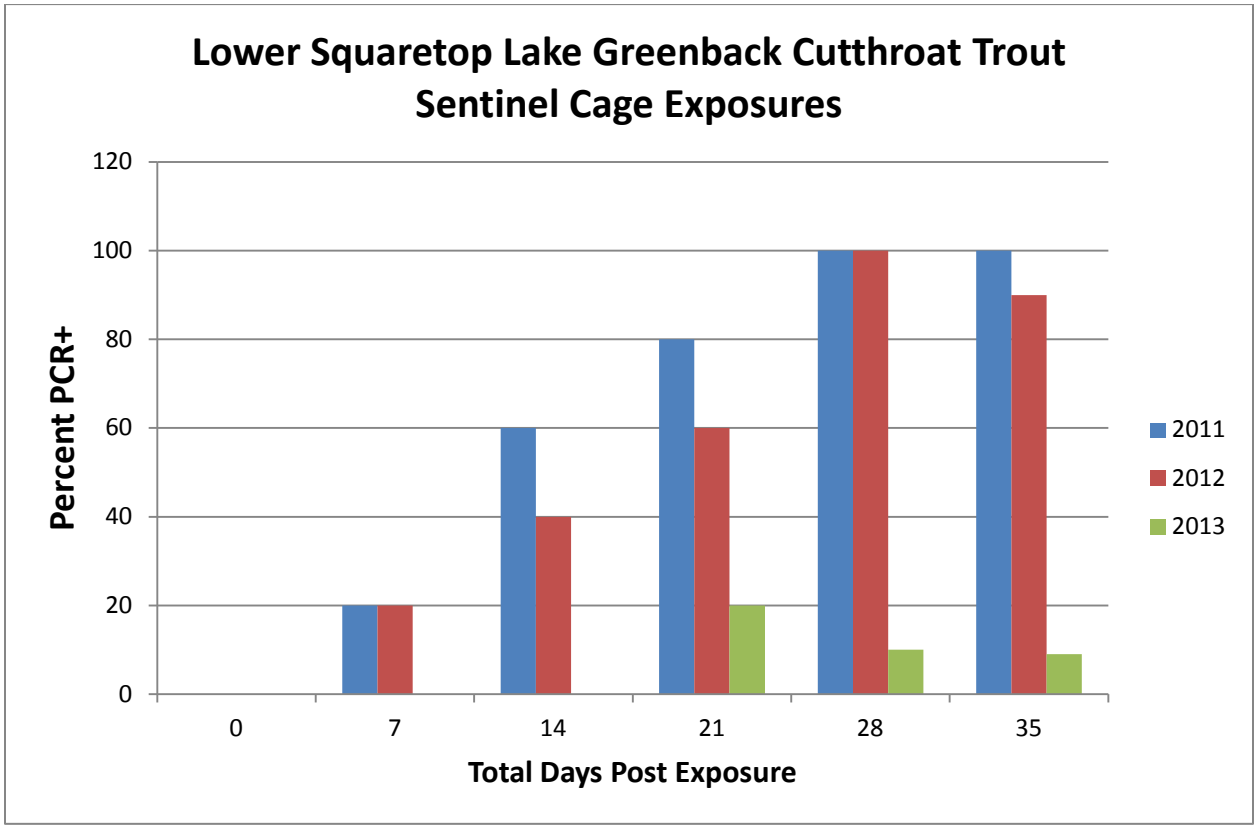
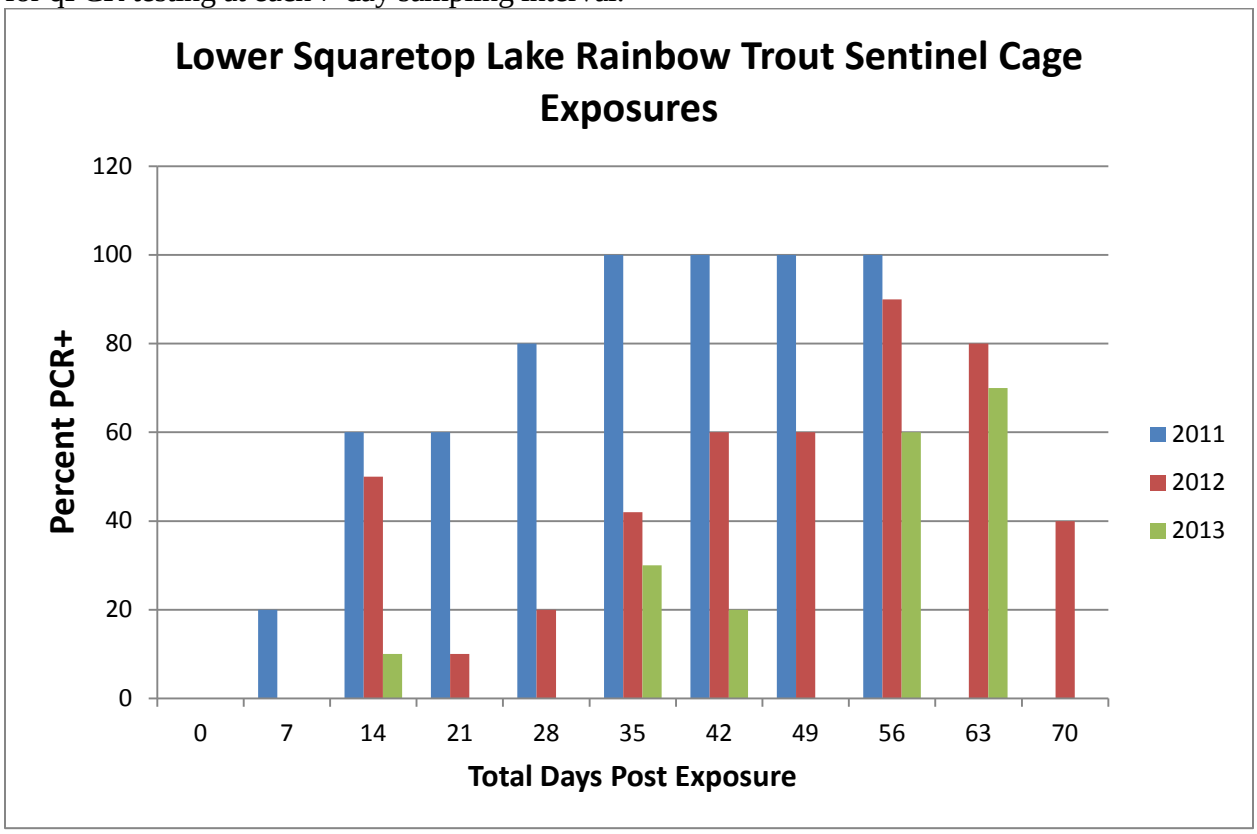


Table 7. Length and weight data for greenback native (GBN) cutthroat trout from a gill net catch in Duck Lake, September 2, 2011.

Date	Fish		Fish		Fish		Fish	
Mm/dd/yyyy	length	Weight	length	weight	Length	weight	length	Weight
09/02/2011	330	331	345	386	360	437	380	504
	363	436	343	373	351	432	335	351
	346	402	370	470	370	455	352	387
	385	468	402	675	365	432	340	377
	310	281	---	---	---	---	---	---

Figure 3. Summary of the results of the floating sentinel cage exposures of rainbow trout fry in Lower Squaretop Lake from early August through early October, 2011, 2012, and 2013. The tests were to determine whether or not the *Myxobolus cerebralis* DNA was present in the fry. Five rainbow fry were sacrificed for qPCR testing at each 7-day sampling interval during the 2011 exposure period. During the 2012 and 2013 exposure periods, 10 rainbow trout fry were sacrificed for qPCR testing at each 7-day sampling interval.



is noteworthy that the average cranial myxospore concentrations was reduced by more than 50% among the GBN groups exposed in 2012 compared with the 2011 exposure groups (Table 6). It was unfortunate that the 60-70 GBN cutthroat fry that were exposed in 2013 all died very suddenly in the laboratory in just one day approximately 25 days after they were removed from the sentinel cage in LSTL on October 1, 2013, making it impossible to obtain comparative PTD results from the 2013 exposure group in mid-summer 2014.

The percent prevalence of infection for GBN and RBT fry/fingerlings) as they were cropped at 7-day intervals for qPCR analyses for all three years are shown graphically in Figures 2 and 3. It is evident that the prevalence and severity of infection has been definitely declining each year. Those declines were much greater between 2012-2013 than between 2011 and 2012. Therefore it seems reasonable top hope that the *Mc* parasite may be eradicated from LSTL in 2014; however, sentinel cage exposures will need to be carried out again in 2014 to ascertain whether or not that will be the outcome.

Finally, the length-weight data for the 17 GBN cutthroat trout captured in the gill net survey of DL in September 2011 are summarized in Table 7. The results of the PTD screening of those fish are summarized in Table 5. The PTD tests indicated all of the fish were negative for evidence of *Mc* infection.

RECOMMENDATIONS AND CONCLUSIONS

It appears from all of the results and data presented above that in is not improbable that the life cycle of *M. cerebralis* may be broken in LSTL in 2014. However, it is imperative that sentinel cage exposures be carried out again in the lake during the summer of 2014. It is recommended that the protocol of the exposure efforts in 2014 be changed from that used for the exposures carried out in 2011, 2012 and 2013. It is recommended that approximately 30 trout fry be exposed in the floating live cage for 30 days beginning on or about June 15, 2014, if the ice has gone off the lake by that time. After 30 days of exposure those fish should be removed, preserved in 70% ETOH and submitted for qPCR analysis for evidence of infection by the *Mc* parasite. This 30-day cycle of exposure and qPCR analysis should be repeated in July-August, August-September and September-October. In the event that all qPCR analyses indicate no detection of *Mc* DNA, it is safe to conclude that the life cycle of the parasite in all likelihood has been eliminated from LSTL.

Gillnet sets should be made in USTL and LSTL until it is almost certain that no trout are left in either lake. This is critically important for LSTL, to insure that no fish host gets back into this lake and survives long enough to jump-start the infection cycle again. In the event that all exposed trout from the LSTL sentinel cage exposures test negative for *Mc* DNA in 2014, it should be safe to restock both USTL and LSTL in 2015 with GBN cutthroat trout fry.

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