FINAL REPORT

Yellow Creek Whirling Disease Study: Investigating the Presence and Potential Severity of Whirling Disease in Yellow Creek

Duration of Contract
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Project Summary

Whirling disease (WD) is caused by *Myxobolus cerebralis*, a myxozoan parasite possessing a complex life cycle involving two hosts, a salmonid fish and the oligochaete *Tubifex tubifex*. Yellow Creek in Lassen and Plumas National Forests is a valuable trout fishery resource. The stream in the lower Humbug Valley has been designated as a wild trout fishery habitat by the California Department of Fish and Wildlife (CDFW). Concerns regarding proposed management and restoration efforts within the surrounding habitats and potential consequences to WD status in the drainages led to the inception of this study. Investigations on WD in 1997–1999 showed heavy spore loads and signs of WD among rainbow trout (RT) implicating carriers of the parasite and the long-term disease transmission in declining populations of wild RT populations. The goal of this study was to determine the current prevalence of WD in the drainage system by looking into spore loads of the *M. cerebralis* parasite in naïve rainbow trout exposed to ambient waters in the drainages (live cage study) and to assess the distribution and proportion of susceptible and resistant phenotypes of the *T. tubifex* oligochaete host (lineage typing).

A total of 16 study sites for collection of oligochaetes ("W") were designated in Humbug Valley on PG &E land (W1−W12) and Plumas National Forest (PNF) land (W13−W16). Field exposures of naïve rainbow trout ("F") via live cages were also deployed on PGE land in Humbug Valley: F1−F4 and PNF: F5−F6. Worms were collected from the sites 6 days prior to deployment of live cages; conventional PCR was used for lineage typing of oligochaetes with the specific genetic markers based on the mitochondrial 16S rDNA sequences. Groups of caged RT were exposed to ambient waters across the sites for 2 weeks. Fish recovered from the cages were transported and held in the UC Davis Fish Health Laboratory at the Center for Aquatic Biology and Aquaculture (CABA) for 4.5 months at 15°C. Fish were held in order to later assess WD development by spore counts using Pepsin-Trypsin Digest (PTD) and for determination of presence and quantification of *M. cerebralis* DNA by qPCR using specific primers and probes.

Results showed the presence of susceptible (lineages I & III) and non-susceptible (lineage V) *Tubifex tubifex* oligochaetes with their population proportion varying across the PGE and PNF study sites. Susceptible lineage III worms were moderately dominant at PGE sites (17.58%) compared to PNF sites (11.27%). Lineage I worms with both resistant and susceptible phenotypes were relatively greater in PNF (39.09%) and lower in Humbug Valley (PGE) (6.59%). Resistant lineage V worms were relatively dominant in both areas: PGE (45.49%) and PNF (30.07%). Field exposed RT from the various sites developed overt clinical signs of WD including deformed vertebral column, disfigured snout, and black tails. Fish exposed across sites in Humbug Valley (PGE) showed a relatively higher incidence and severity of WD compared to Plumas National Forest based on the prevalence of infected fish and abundance of spore loads and *M. cerebralis* DNA gene copies.

Our findings provide a baseline on the current status of WD in Yellow Creek that may be used for predicting WD following changes in habitat and water conditions deemed necessary for forest management and meadow restoration efforts for Yellow Creek and other drainages threatened by the devastating effects of WD. Based on these findings, annual surveillance for a period of 3-5 years post mitigation is highly recommended to determine effects on the restoration in reference to changes in prevalence and distribution of WD and changes in the prevalence of the susceptible oligochaete host.

Introduction

This study was conducted to evaluate the status of Whirling Disease (WD) within Yellow Creek and Humbug Valley. Plumas Corporation has proposed a Humbug Valley – Yellow Creek Meadow Restoration Project for Plumas County. The proposed project would restore 4,460 feet of the main channel of Yellow Creek and 1,936 feet of an unnamed tributary channel and the floodplain using a pond and plug technique. A portion of the main channel of Yellow Creek and the unnamed tributary would be rerouted into a historic channel on the surface of the meadow and approximately 14.4 acres of ponds would be created. The California Department of Fish and Wildlife, upon receiving the Meadow Restoration Project proposal, recommended that proposed impacts related to WD be evaluated within the Yellow Creek watershed (Pers. Comm., J. Drongesen, CDFW). Whirling disease was studied in Yellow Creek from 1997-2000 with spores being found in all three resident trout species from 1997 to 1999. Biologists noted physical deformities in rainbow trout (RT) indicative of heavy spore loads associated with WD. Today, RT numbers are very low in Yellow Creek, and while the cause is unknown, it is plausible that WD has contributed to the decline in the RT populations within this watershed. Currently, the status of WD in Yellow Creek in Humbug Valley is unknown, thus it is important to establish a new baseline study of WD within this drainage prior to the pond and plug restoration to better qualify and quantify WD.

Adequate knowledge is currently lacking for predicting the impacts on WD from any land management activity or restoration action within the Yellow Creek stream channel, meadow habitats, or adjacent forest uplands. Thus, the purpose of this study is to determine the current state of WD, including all aspects of its life cycle, within these watersheds to provide information that will support forest management decisions on evaluating proposed alternatives for stream restoration.

Background – Whirling Disease

Whirling disease is caused by a non-native parasite myxosporean, *Myxobolus cerebralis* and can have devastating effects on native salmonids. All salmonids in the genus *Oncorhynchus* are susceptible at varying degrees. Brown trout have been shown to carry a higher level of resistance as opposed to rainbow trout which are highly susceptible to the disease. Whirling disease mainly affects juvenile salmonid fish (fingerlings and fry) and causes skeletal deformation and neurological damage. Fish "whirl" forward in an awkward corkscrew-like pattern instead of swimming normally, find feeding difficult, and are more vulnerable to predators. The severity of the disease is inversely related to the age of the fish upon exposure; 100% mortality has been reported in fry and 90% mortality in infected populations (Hedrick et al., 2003). Fish that survive are deformed by the parasites residing in their cartilage and bone that act as a reservoirs for the parasite (i.e. carriers). Spores are released into the water following the fish's death. Even fish that are several years old can become infected via gill cartilage and remain potential carriers of the parasite causing WD.

Myxobolus cerebralis has a two-host life cycle requiring a susceptible salmonid and the tubificid oligochaete *Tubifex tubifex* (Figure 1). Briefly, myxospores are released upon death of fish and ingested by tubificid worms and attach to their gut lumen. Around 60–90 days post-infection, sexual cell stages of the parasite undergo sporogenesis, and develop into pansporocysts that contain eight triactinomyxon-stage spores (TAM). TAMs are released from the oligochaete

anus into the water and infect the salmonids host. The triactinomyxon spores swim through the water to infect a salmonid through the skin. The spores penetrate the fish in only a few seconds. Within five minutes, a sac of germ cells called a sporoplasm enters the fish epidermis, and within a few hours, the sporoplasm splits into individual cells that will spread through the fish (Figure 1). TAMs can be released by an infected tubificid for over a year. Fish can also be infected by eating an infected oligochaete.

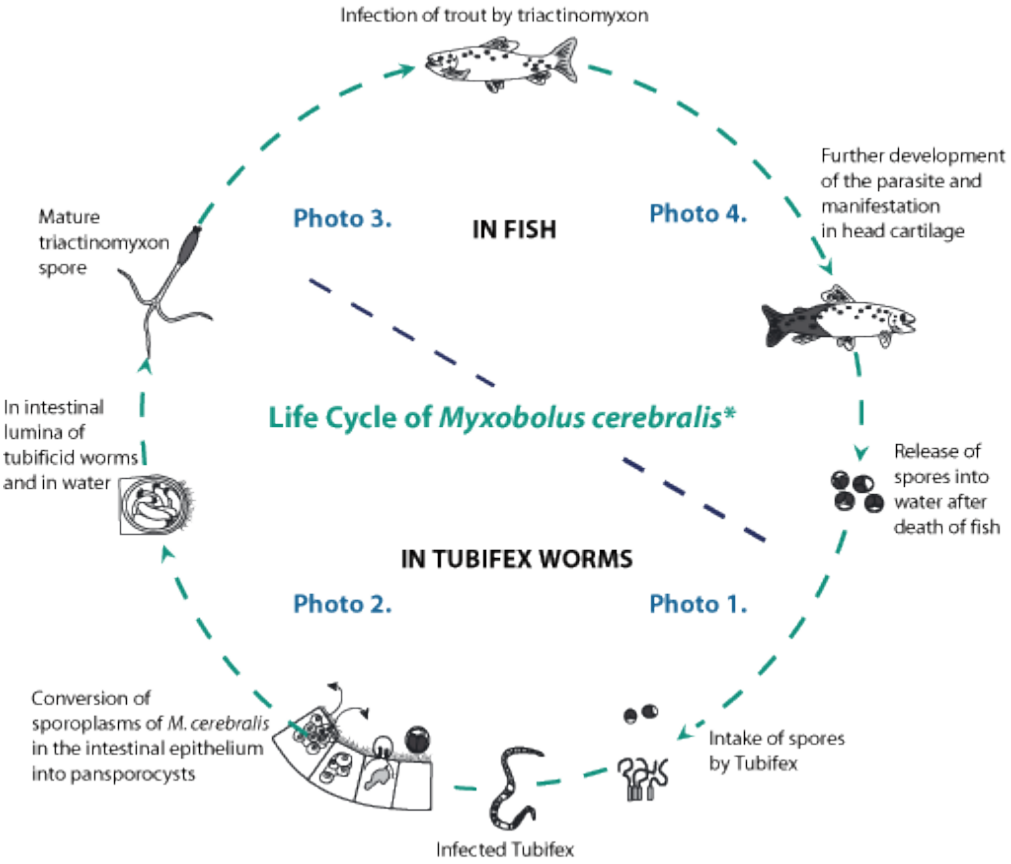

Figure 1. Life cycle of *Myxobolus cerebralis.* http://whirlingdisease.montana.edu/about/lifecycle.htm *Adapted from M. El Matbouli, T. Fischer Scherl, and R.W. Hoffmann. 1992. Annual Review of Fish Diseases, p. 392.

Whirling Disease: Problems and Solutions (Excerpted from *Weber ES. 2012)*

Though speculative, the myxozoan parasite *Myxobolus cerebralis* likely reached North America in the 1950s with frozen rainbow trout or imported brown trout (*Salmo truffa*) from Europe (Hoffman 1990; Bartholomew and Reno 2002). The first recorded epizootic in North America was among brook trout at a hatchery in Pennsylvania. Subsequent episodes were reported in numerous other Eastern state trout hatcheries, frequently among rainbow trout, one of the more susceptible species of salmonids (Hoffman 1990). Under conditions of high infectivity, whirling disease may induce severe cranial and spinal deformations and death as parasite stages feed upon and destroy skeletal cartilage prior to bone formation in young fish (Halliday 1973).

The myxospore stages of *M. cerebralis* in the skeletal elements of chronically infected trout may not result in the blackened tail and erratic swimming characteristic of acute disease. If the spores are not detected, the parasite will spread to new geographic regions with the transport of hatchery fish for stocking in rivers and streams for the sport fishery. This mode of parasite movement has facilitated the spread of the parasite to over 25 of the United States, affecting most profoundly wild trout in the intermountain West (e.g., Colorado, Montana) where large-scale population declines, particularly among rainbow trout, have destroyed once highly prized sport fisheries (Nehring and Walker 1996; Vincent 1996). The two-host life cycle of *M. cerebralis* has added to the complexity associated with the control and management of whirling disease. However, a combination of improved diagnostic procedures for parasite detection, in both fish and oligochaetes, and new management procedures in aquaculture are improving the ability to control whirling disease. The advent and now widespread application of PCR can detect the pathogen among fish with subclinical disease, thus preventing the inadvertent movement of infected fish (Andree et al. 1998, 2002). Improved sanitation in trout hatcheries using both treatments of the water supply with ultraviolet irradiation and several common disinfectants for ponds and equipment, including transport vehicles, has further reduced the spread of the parasite (Hedrick et al. 2007, 2008). Finally, the discovery and exploitation of rainbow trout strains resistant to whirling disease has provided a powerful management approach to significantly reducing and perhaps eliminating whirling disease from certain hatchery environments and ideally from selected wild trout sport fisheries (Hedrick et al. 2003b; Schisler et al. 2006). The most broadly exploited whirling disease-resistant rainbow trout strain is the product of 120 years of selection in a commercial hatchery in Europe. This strain is now being reared in state and commercial fish hatcheries in the United States. It is also being bred with wild trout with the aim of restoring selected wild populations of rainbow trout in Colorado.

Lineage Typing of *Tubifex tubifex*

Tubifex tubifex is an oligochaete species that commonly occurs in mixed assemblages with other related oligochaete species. Previous phylogenetic studies using mitochondrial 16S ribosomal DNA has shown that there are 6 genetically distinct lineages of *T. tubifex* (Sturmbauer et al. 1999, Beauchamp et al. 2001). Four of these lineages (I, III, V, and VI) are found in North America and were shown to demonstrate variable responses to infections with *M. cerebralis* (Beauchamp et al. 2005). Oligochaetes from lineage III are highly susceptible to *M. cerebralis* myxospore infection. Worms in lineage I are both susceptible and resistant to the parasite with resistant phenotypes more predominant. To date, lineages V and VI contain only resistant phenotypes. While morphologically indistinguishable, *T. tubifex* are genetically diverse. Genetic markers such as the 18S rRNA gene, mitochondrial (mt) 16S rDNA sequences, and the ITS1 region throughout the oligochaete genome have provided methods for grouping *T. tubifex* (Sturmbauer et al. 1999, Beauchamp et al. 2001, Kerans et al. 2004, Rasmussen et al. 2008). In this study, the mt 16S rDNA sequences developed at the Fish Disease Laboratory at UC Davis by Beauchamp et al. (2001) were used to distinguish I, III, and V *T. tubifex* specific lineages of the worms sampled from the different study sites. The goal is to determine the relative proportion of susceptible vs. non-susceptible worms within the Yellow Creek study region.

Detection of *Myxobolus cerebralis* in Infected Fish

There are various methods for detecting *Myxobolus cerebralis* myxospores among infected fish, one of the most common being the Pepsin-Trypsin Digest (PTD). In this method, potentially infected fish are euthanized, heads removed and halved. Half the head is digested (see "methods" below) for spore enumeration and the remaining half is fixed in 10% neutral buffered formalin and archived for confirmatory diagnosis. Spores can then be enumerated under a microscope or simply reviewed for presence or absence within the fish head sample. While this method is reliable and common, it is tedious and prone to cross-contamination leading to false positives (Kelley et al, 2004).

A second method of detection is qPCR analysis using specific primers and a probe developed at the Fish Disease Laboratory at UC Davis by Kelley et al. (2004). In this method, half heads are sampled with a biopsy punch and the tissue core gDNA extracted (see "methods below). The gDNA is analyzed by qPCR that provides a qualitative (presence/absence of parasite DNA) and a quantitative (18S gene copy numbers/10^6 host cells) assessment of the parasite abundance and WD severity.

Experimental Design and Methods

Studies previously done in our laboratory as well as by others have shown that knowledge on current infection levels of fish and the genotype and abundance of oligochaetes present in a particular watershed provide critical insight into the potential negative effects of whirling disease on wild trout populations. In order to establish baseline data on the presence and severity of WD in Yellow Creek in Humbug Valley, it was necessary to determine the genotype and abundance of *T. tubifex* present in selected representative habitats. Genetic typing of oligochaetes was conducted along with spore enumeration from fish in the live-cage studies, across the designated study sites, using conventional and molecular diagnostic protocols.

Oligochaete Examinations

Sixteen sites were chosen for determining the presence and genotype of *T. tubifex*. Of the sixteen sites, 12 were located on PG & E territory with the remaining 4 in Plumas National Forest territory. Oligochaete (worm) samples were collected on October 20, 2011, 6 days prior to the live-cage study. At each site, 2 gallon buckets were half filled with sediment. The sites for worm collection are listed as "W" on the sampling map (Figure 2) and were chosen by various collaborators of the project based on strategic locations within the watershed and type of sediment present. Sediment-worm samples were taken below the water surface at the shallow sides of the creek beds. Buckets with sediment were transported back to the UC Davis Fish Health Laboratory within CABA. All buckets were aerated and maintained in 15^{o}C flow through well-water until sediment and worms were collected from all the designated sites. The sediments from each site were visually examined for the potential presence of *T. tubifex* worms. The sediment samples were filtered through a USA Standard Testing Sieve by Fisher Scientific with a mesh opening of 53 micrometers to remove fine clay, leaving any worms for collection. Next,

small portions of the sediment samples were visually examined and following morphological examination, possible *T. tubifex* worms were collected and individually placed in 1.5µl centrifuge tubes containing lysis buffer (Qiagen DNeasy Blood & Tissue kit) and later used for genomic DNA (gDNA) extraction. Up to 100 worms were collected per site. At sites where 100 worms were not available, all possible *T. tubifex* worms collected from the entire bucket of sediment were analyzed. Worms that did not meet morphological characteristics of *T. tubifex* were placed back in the bucket with remaining sediment.

For genetic screening and lineage typing, up to 50 worms per site were gDNA extracted using the Qiagen DNeasy Blood and Tissue Kit. All extracted worms were then PCR analyzed using the lineage-specific primers, excluding primers for lineage VI*. The PCR cocktail contained 3 lineage-specific primers (I, III, & V) and the *Tubifex*-specific reverse primer. Negative controls were used for each PCR reaction. PCR products were visualized on 2% agarose gel and lineage-specific bands recorded, or the absence of a band, for each oligochaete tested.

*Lineage VI was excluded as we were mostly interested in susceptible lineages present (I & III) and it is difficult to distinguish lineage VI positive bands from from lineage III upon agarose gel visualization.

Live−Cage Study

The presence of *M. cerebralis* and the severity of whirling disease were examined among rainbow trout held in cages that were deployed across the selected sites. For this study, 6 live cage sites were chosen, 4 on PGE lands and 2 on PNF. Representative sites were chosen at strategic locations within the watershed (Figure 2) and listed as "F" sites on the sampling map. Groups of 100+ triploid rainbow trout, <1g in weight, were placed in stream locations on October 26th, 2011. Sentinel cages were held for approximately 2 weeks within the study locations. On November 10th, 2011 the cages and surviving fish were removed from study sites and transported back to the UC Davis Fish Health Laboratory within CABA. Following exposure from the study sites, fish recovered from each cage were held for approximately 4.5 months in 130L aquaria with 15^{o}C flow through well water and fed a commercial diet (1.5% body weight) daily until termination of the study. Fish were observed daily for evidence of the onset of whirling disease (e.g. erratic swimming and black tail) and for the presence of other potential pathogens. All mortalities were recorded and examined for evidence of other pathogens by wet mounts and bacterial cultures using general isolation media such as Blood Agar and Tryptone Yeast Extract Salts (TYES) for fastidious organisms such as flexibacteria.

On April 16, 2012, five months post initial exposure, all remaining fish were euthanized. Fish were processed at the UC Davis Fish Health Laboratory within the Veterinary Medicine and Medical Epidemiology (VMME) Department. Heads were removed and then halved. One half head was examined for the presence and abundance of *M. cerebralis* spores by quantitative PCR (qPCR) and the other half was tested per PTD. All fish in each group were qPCR tested. For qPCR testing, half heads were sampled with a 4mm biopsy punch inserted just posterior and dorsal to the eye to remove the head cartilage. Samples (N=308 total) were then gDNA extracted using the Qiagen DNeasy Blood and Tissue Kit. For qPCR runs, each sample was run in triplicate using the *Myxobolus*18S specific primers and probe. Each run contained positive (*M. cerebralis* plasmid DNA) and negative controls (molecular grade water). The qPCR method

was conducted according to Kelley et al. (2004). To enumerate the number of host cells present per sample, trout IGF-1 specific primers and probe were also run in triplicate. Triplicate results were then averaged and the copy numbers of 18S rDNA per 10^6 host cells was calculated.

For the PTD analysis, 20 fish from each group, except F5 (see results below), were digested per standard methods. Full myxospore counts per half head was conducted on 10/20 fish per site and presence/absence of myxospores was determined from the remaining 10 fish. Both methods were used to assess the severity of whirling disease across the designated sites within Yellow Creek, Humbug Valley.

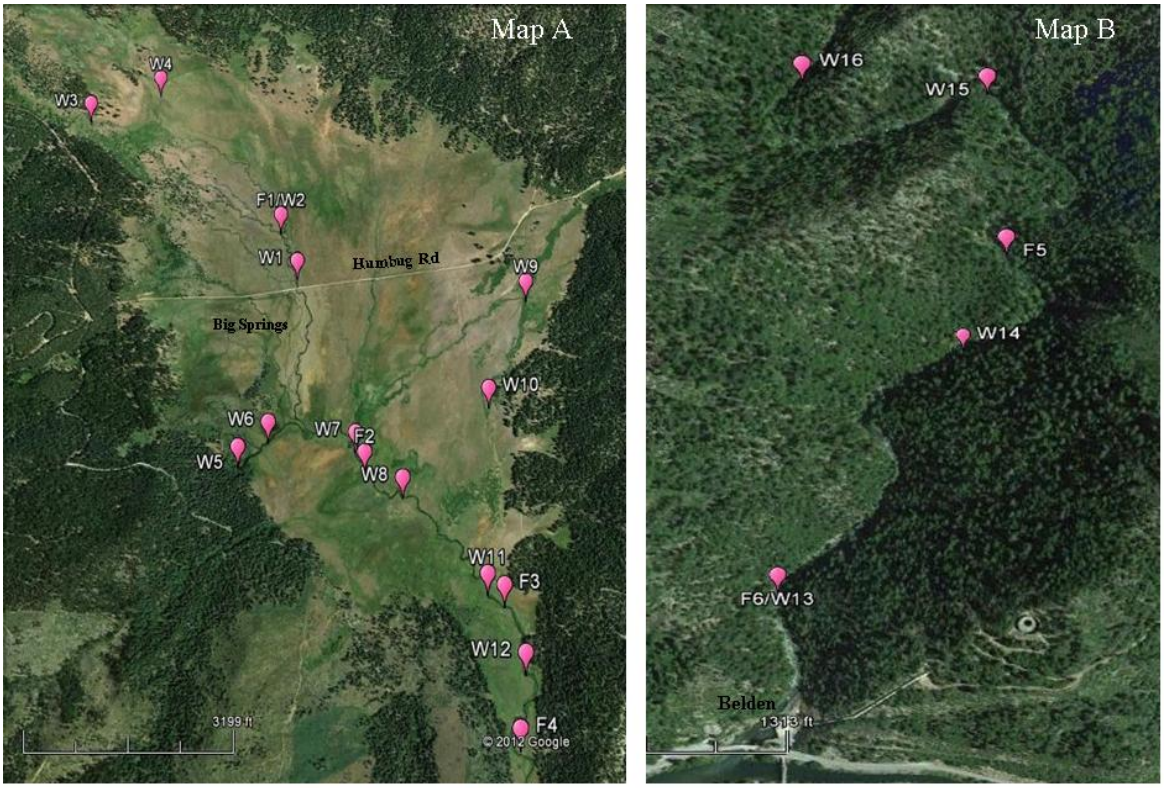

Figure 2: Study sites at Yellow Creek: (A) Humbug Valley – PG & E sites and (B) Belden Plumas National Forest. Sampling locations for oligochaetes are designated as "W" and "F" for sentinel cages containing rainbow trout. Oligochaetes were sampled 6 days prior to deployment of live cages that were exposed to the water column for 2 weeks. Fish recovered from the cages were held in the laboratory for 4.5 months, post-exposure, at 15°C and then examined for presence and severity of whirling disease.

Results

Oligochaete Examinations

Lineage-specific typing using conventional PCR showed that *T. tubifex* lineages I and III (phenotypes susceptible to *M. cerebralis*) including non-susceptible lineage V were present throughout Yellow Creek in Humbug Valley and Plumas National Forest (Belden) (Table 1). The highest concentration of susceptible worms (lineage III) were most abundant in the following sites: W3-above Big Springs with 29/50 lineage III worms; W7-just below Big Springs with 36/50 lineage III worms. W13-closest to Belden Dam showed abundant lineage I worms 44/50 that may be susceptible or resistant. At sites W6 & W9, no worms were found

within the entire sample collected. Sites W4, W12, W15 and W16 had less than 100 worms from the entire site, thus all worms collected were lineage typed.

Comparing the 2 study sites, susceptible lineage III worms were moderately dominant (17.58%) in PGE (Humbug Valley) compared to Plumas National Forest (11.27%) (Table 1). A higher incidence of lineage I worms (39.09%) was found in PNF and while at PGE sites in Humbug Valley, lineage I worms were prevalent at 6.59%. Resistant lineage V worms were relatively dominant in both sites: PGE (45.49%) and PNF (30.07%). A percentage of worms in both sites (PGE – 30.32%, Plumas – 18.79%) did not type as mitochondrial lineages (I, III, or V). These worms may either be: 1) non *Tubifex tubifex* or, 2) the PCR lineage typing did not work due to potential inhibitor present in the worm samples.

Table 1. Lineage typing of oligochaetes from sampling locations at PG & E sites (Humbug Valley) and Plumas National Forest.

Sampling Site	Lineage I	Lineage III	Lineage V	*Negative for Lineages I, III, V
PG & E Sites (Humbug Valley)				
W1	0/50	0/50	0/50	50/50 (Not *T. tubifex*)
W2	4/50	3/50	39/50	4/50
W3	1/50	28/50	18/50	3/50
W4	1/19	0/19	6/19	12/19
W5	12/50	0/50	30/50	8/50
W6	No worms found at this site			
W7	0/50	36/50	12/50	2/50
W8	1/50	3/50	45/50	1/50
W9	No worms found at this site			
W10	1/50	4/50	44/50	1/50
W11	6/50	5/50	4/50	35/50
W12	4/36	1/36	9/36	22/36
Lineage prevalence	30/455 6.59%	80/455 17.58%	207/455 45.49%	138/455 30.32%
Plumas National Forest Sites				
W13	44/50	2/50	0/50	4/50
W14	1/50	11/50	26/50	12/50
W15	1/19	3/19	14/19	1/19
W16	6/14	0/14	0/14	8/14
Lineage prevalence	52/133 39.09%	16/133 11.27%	40/133 30.07%	25/133 18.79%

Note: Lineage I – *T. tubifex* are resistant and susceptible but mostly resistant
Lineage III – *T. tubifex* are susceptible and resistant but mostly susceptible
Lineage V – *T. tubifex* are all resistant
*The absence of amplification products suggests the oligochaete is not *T. tubifex* or the PCR assay did not work due to a potential inhibitor present in the worm sample.

Live-Cage Study

Mean water temperatures across the study sites were 8.9°C, with little variation between sites, during deployment of the live cages. At each site, 100+ fish were retrieved and transported except for site F5 where only 8 fish were recovered. Fish (N=60) were euthanized from each holding tank, at 4.5 months post initial exposure and analyzed per qPCR and PTD. The results shown in Table 2 represent the average of 60 fish per sampling site (group) except for site F5 where all 8 fish were analyzed. During the course of the holding period, mortalities were low however, from daily observations, signs of whirling disease were seen in various fish including erratic swimming (whirling), deformation of the spine, and shortening of the snout (Figure 3).

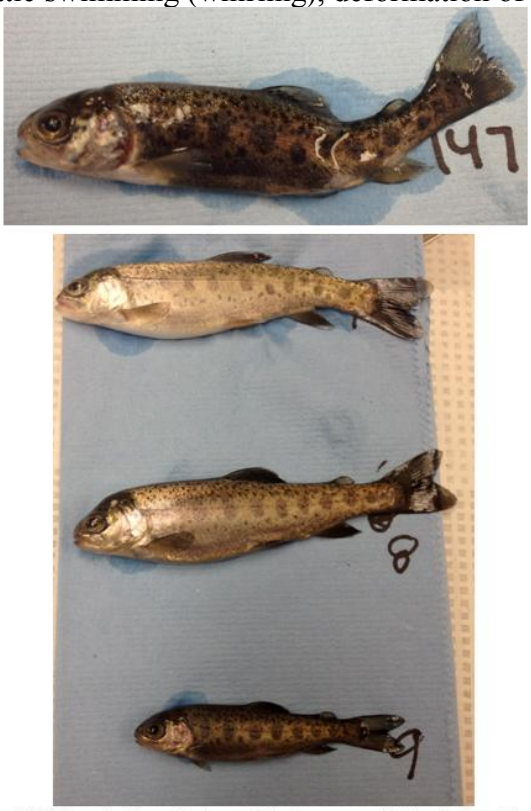

Figure 3: Rainbow trout in sentinel cages following exposure for 2 weeks to ambient water at study sites in Humbug Valley and Belden Plumas National Forest. Fish showed typical signs of whirling disease such as deformed vertebral column and dark tail (top) and shortened snout (bottom) following rearing in the laboratory for 4.5 months post-exposure at 15°C.

Results of qPCR analysis showed that all fish were positive for *M. cerebralis* at sites F1-F4. Site F5 had 7/8 fish positive and site F6 had 43/60 fish positive. Fish from sites F1-F4 were all highly positive with results in the hundreds of thousands or higher of 18S gene copies per 10^6 host cells. Only one fish from site F1 (out of 180 fish) had a result under 100,000, showing a result of 76,297 per 10^6 host cells. The qPCR results showed that the fish from sites F1-F4 had moderate to severe infections with WD. Sites F5 and F6 had 18 fish negative for *M. cerebralis*, however the majority of positive fish in these groups were still highly positive. Severity of infections with *M. cerebralis* as determined by qPCR and PTD are summarized in Table 2. The entire data set of qPCR for each individual fish tested may be provided upon request.

Results from PTD closely matched that of the qPCR results (Table 3). The first 20 fish from each site were PTD analyzed, except for site F5 where all 8 fish were processed for PTD analysis. Twenty of twenty fish from sites F1-F4 were positive for myxospores and WD. At site F5, 7/8 samples were positive and the single fish that was negative corresponds to the same fish that was qPCR negative. For site F6, 13/20 fish were found to be WD positive. The seven fish that were negative by PTD were also negative by qPCR. Average results per positive fish for each site are shown in Table 2. PTD results indicate severe infection in sites F1-F4 and moderate to severe infection in sites F5-F6.

Table 2. Summary of qPCR and PTD analyses of rainbow trout from the live cage studies. Numbers of gene copies and myxospores refer to averages of positive fish.

Site of fish cages	qPCR No. fish WD+ No. fish tested	Mean copy no. of 18S rDNA/10^6 host cells (qPCR)	PTD No. fish WD+ No. fish tested	Mean myxospore cells/ml (PTD)
F1	60/60	4,011,596	20/20	8.2×10^5
F2	60/60	2,415,920	20/20	2.5×10^5
F3	60/60	3,759,030	20/20	5.4×10^5
F4	60/60	4,227,112	20/20	7.3×10^5
F5	7/8	391,873	7/8	4.3×10^5
F6	43/60	382,450	13/20	2.8×10^5

F1 – F4: PG & E (Humbug Valley)
F5 – F6: Plumas National forest

Discussion

Prevalence and severity of Whirling Disease is influenced by many factors. Factors influencing disease are as follows: Environmental stress – environmental stressors such as pollution, crowding, or abnormal temperatures generally make fish more susceptible to disease; Infective dose – as fish are exposed to increasing doses of triactinomyxons, the severity of disease increases; Fish age – older fish are less susceptible to disease than younger fish, since the cartilage that is affected by the parasite ossifies to bone as fish age; and Fish species – different species of fish differ in their susceptibility to whirling disease; generally speaking, rainbow trout are considered among the most susceptible species while brown trout are highly resistant.

In addition to the factors that affect the susceptibility to disease of individual fish, it appears that ecological factors may play a significant role in determining the severity of effects on wild populations. Whirling disease infectivity appears to be greater in high-productivity steams. Water with more sediment and organic matter may also have greater disease problems as it provides more favorable habitat for tubifex worms. Water temperature can have pronounced effects on the release of triactinomyxons (TAMs); it appears that 15°C may be optimal for production of TAMs, while releases are slower at lower temperatures and may be eliminated at temperatures of 20°C or more. There also appear to be infection "point sources," locations where production of TAMs is especially high and disease is especially severe.

The results of this study show that *Myxobolus cerebralis* and whirling disease are present in Yellow Creek. Naïve juvenile rainbow trout showed the presence of the parasite and the

disease following exposure for 2 weeks in the water column in cages deployed across the study sites and then held in the laboratory for 4.5 months. Despite the suboptimal water temperatures (8.9°C) at the time of field exposures, infective triactinomyxon spores (TAMs) were presumably released from susceptible oligochaetes rendering infections of rainbow trout with *M. cerebralis* spores and severe signs of WD at the end of the study. Numerous myxospores (as determined by PTD) and *M. cerebralis* DNA gene copies (qPCR) were observed among infected fish. Furthermore, the results demonstrate a higher prevalence and severity of WD at PGE sites based on a 100% prevalence of infected fish in all of the 4 sites examined in terms of fish frequency with positive spore loads (PTD) and abundance of parasite DNA (qPCR) (Table 2). The mean 18S gene copies of fish examined in PGE is 9.3 times greater than fish examined from Plumas National Forest (PNF). In addition, the methods employed for evaluating the parasite and the disease among exposed fish (i.e. PTD and qPCR) showed closely matched results suggesting an effective diagnostic molecular approach of determining WD prevalence in the watershed.

Susceptible and non-susceptible *Tubifex tubifex* oligochaetes were found throughout the study area. Worm sub-samples that were examined showed that the proportion of susceptible and resistant worms varied across the study sites in PGE and PNF. Susceptible Lineage III worms were relatively more prevalent in PGE sites (17.58%) when compared to PNF sites (11.27%). The abundance of susceptible lineage III worms at these sites may have contributed to more severe infections (greater prevalence of fish with myxospores and higher 18S gene copies, Table 2) observed at PGE in contrast to PNF. The overlapping occurrence of susceptible *T. tubifex* and salmonid hosts in the aquatic environment is one factor affecting the successful establishment of *M. cerebralis* and the development of whirling disease. Several research investigations conducted to date in our laboratory and by other investigators have shown that lineage III contains the most number of *T. tubifex* susceptible to *M. cerebralis* while lineage I, although both resistant and susceptible phenotypes are present, contain more worms that are resistant to the parasite. In the current study, Lineage I worms were more dominant in PNF sites (39.09%) than PGE sites (6.59%). The proportion of resistant and susceptible worms in the lineage I worm assemblages in these sites is unknown, which can only be verified by infectivity trials where worms are exposed to myxospores and then evaluated for parasite production. Resistant lineage V worms were moderately abundant in both sites (PGE: 45.49% and PNF: 30.07%). Although some of the worms from the study sites may not be *T. tubifex*, they may help in regulating WD severity by acting as resistant phenotypes based on their inability to host the parasite. Resistant worms may represent one avenue by which parasite abundance can be down regulated in a given habitat as shown in other studies (M. El-Matbouli unpublished data, Beauchamp et al. 2006). These previous investigations demonstrate that resistant worms could alter the course of *M. cerebralis* infections by not releasing TAM stages that are infectious to susceptible young fish. Even certain populations of mt 16S lineage III *T. tubifex* that showed arrested development (i.e. presence of parasite but absence of TAM release) were also considered resistant phenotypes (Baxa et al. 2008). In all of these studies, resistant worms can diminish parasite propagation by providing biological filtration through ingestion of myxospores that hatch but never develop fully to mature stages for release. The presence of resistant phenotypes effectively removes myxospores of *M. cerebralis* from the sediment with a presumed corresponding decrease in overall infectivity (Beauchamp et al. 2006).

Variations on the production of triactinomyxon spores of *M. cerebralis* from the *T. tubifex* host are attributed to several factors such as genetic markers and sediment types. Genetic markers such as the mt 16S rDNA sequences used in this study may provide a method for

grouping *T. tubifex*. While useful for determining the role of the oligochaete host on whirling disease severity, genetic markers are not based directly upon DNA regions likely to target genes that may influence resistance or susceptibility to infections with *M. cerebralis*. Thus, assessing responses to *M. cerebralis* ultimately relies on infectivity trials where worms are exposed to myxospores and then evaluated for parasite production.

Sediment type in the current study may have contributed in sustaining oligochaete assemblages and severity of infections associated with whirling disease. Sediment comprising fine particles (e.g. sand and mud) may enhance oligochaete reproduction and production of the infectious stages (i.e. TAMs) of *M. cerebralis* in *T. tubifex* host (Arndt et al. 2002, Blazer et al. 2003). Fine sediment is associated with increased risk of *M. cerebralis* infection and infection severity in fish as fine particles are likely to collect in the same areas as the small myxospores (Lemmon and Kerans 2001). Furthermore, *T. tubifex* distribution is strongly influenced by the composition and organic content of the substrate, as they prefer silt and clay substrates (Gilbert and Granath 2003, Krueger et al. 2006). Worms also reproduce faster in silt than in coarse substrate types (Arndt et al. 2002), and produce more triactinomyxons (Arndt et al. 2002, Blazer et al. 2003). Other studies have also shown that sediment affects the severity of infections with *M. cerebralis* among the *T. tubifex* host lineages containing worms that vary in susceptibility to the parasite (i.e. I, III) while parasite development and infectivity cannot be altered in lineages containing only parasite resistant worms (V, VI) regardless of substratum type (Baxa et al. 2006).

In conclusion, this study provides baseline data on the current prevalence and severity of WD in Yellow Creek. The presence and abundance of *M. cerebralis* spores were demonstrated in naïve cohorts of a susceptible salmonid host (i.e. rainbow trout) that were deployed, via live cages, along potentially WD-affected drainages across the Yellow Creek region. The development of WD among field exposed fish was observed following certain periods of captivity in the laboratory using conventional (PTD) and state of the art molecular techniques (qPCR). We also demonstrated the presence and estimated the relative abundance of the *T. tubifex* host lineages across the designated study sites. Variable proportions of susceptible (lineage I, III) and resistant (lineage V) lineages present among the drainages examined may contribute to varying risk levels of WD along Yellow Creek as shown in previous studies (Beauchamp et al. 2005).

Studying both fish and oligochaete hosts of *M. cerebralis* has provided critical knowledge on the current extent and severity of WD in Yellow Creek. This information may be used to predict potential scenarios for WD following changes in habitat and water conditions deemed necessary for forest management and meadow restoration efforts.

Although our understanding of WD in fish has progressed over the last 15 years, little is known about what and how environmental factors affect the interaction between the parasite and its oligochaete host in the wild. The worm host is now viewed as a key to the severity and management of WD in wild trout populations. Future efforts may be directed towards establishing long-term baseline data on WD status for Yellow Creek and other drainages impacted by WD that may provide valuable insights for activities and projects relevant for protection, conservation, and restoration of key aquatic resources and habitats. Based on these findings annual surveillance for a period of 3-5 years post mitigation is highly recommended to determine positive and or negative effects on restoration in reference to changes in prevalence and distribution of WD and host oligochaetes in the subsequent drainage basin.

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