THESIS

EVALUATION OF *MYXOBOLUS CEREBRALIS* DISTRIBUTION AND MEASURES OF INFECTION IN COLORADO RIVER CUTTHROAT TROUT IN LABARGE CREEK, WYOMING

Submitted by

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ABSTRACT

EVALUATION OF *MYXOBOLUS CEREBRALIS* DISTRIBUTION AND MEASURES OF INFECTION IN COLORADO RIVER CUTTHROAT TROUT IN LABARGE CREEK, WYOMING

Salmonid whirling disease caused by the invasive parasite *Myxobolus cerebralis* was first detected in the U.S. in 1956. It spread widely, causing declines of up to 90% in some wild salmonid populations. Colorado River Cutthroat Trout (CRCT), already threatened by habitat loss, introduced invasive species, and disease, now occupy only 13% of their historic Wyoming range. Despite extensive restoration efforts in LaBarge Creek, CRCT numbers remain critically low. My study examined the potential role of whirling disease in limiting CRCT recovery by assessing the spatial distribution and infection intensity of *M. cerebralis* in CRCT. I also compared two diagnostic approaches: the traditional pepsin-trypsin digestion (PTD) method and a quantitative polymerase chain reaction (qPCR) assay to evaluate whether raising fish for PTD analysis was necessary for estimating infection prevalence. Finally, I assessed if eDNA sampling could be used for detecting *M. cerebralis* to evaluate disease distribution and prevalence. Sampling of wild fish and a sentinel-caging experiment with naïve fish showed widespread but spatially structured infection risk, with site-level prevalence ranging from 0–100%. Infection was consistently detected in the mainstem and downstream portions of tributaries, while upstream headwater sites remained largely disease-free. Pepsin-trypsin digestion and qPCR showed high agreement (>95%) in detecting infection, and parasite maturation was not required for reliable

detection using qPCR. In contrast, environmental DNA sampling detected the parasite at only 5 of 24 sites, where sentinel fish became infected at 14 of 24 sites, and detection varied widely over the diel cycle. Taken together, these results indicate that whirling disease is established across much of the drainage and may be hindering CRCT recovery in the LaBarge Creek restoration area. They also demonstrate that qPCR applied to fish tissue is an efficient and reliable method for assessing infection prevalence, while eDNA may underestimate infection risk under typical sampling conditions.

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INTRODUCTION

Managers often attempt to reverse population declines through supplementing existing populations or by reintroducing species that have been extirpated. Reintroduction efforts are widespread, with numerous documented successes for mammals, birds, amphibians, and fishes (Bubac et al. 2019; Seddon et al. 2007). Despite these successes, reintroduction programs often face challenges that limit their effectiveness and can lead to failure (Bubac et al. 2019; Cochran-Biederman et al. 2015). Habitat degradation, invasive species, low juvenile survival, and disease are among the most common challenges to reintroduction programs (Bubac et al. 2019; Harding et al. 2025).

Disease can play a critical role in reintroduction failures. For instance, efforts to reintroduce the Black-footed Ferret (*Mustela nigripes*) have been constrained by the enzootic plague caused by *Yersinia pestis*, which affects both ferrets and their primary prey, prairie dogs (*Cynomys*; Matchett et al. 2010). Similarly, reintroduction of the Boreal Toad (*Anayyrus boreas*) has been hindered by the chytrid fungus (*Bactrachochytrium dendrobatidis*), which reduces survival in wild populations (Pilliod et al. 2010). When disease—particularly from nonnative pathogens—persists in the environment or is introduced during a reintroduction program, it may threaten long-term success. As a result, reintroduction and population supplementation programs must incorporate disease management and mitigation strategies.

Reintroduction management strategies are commonly used with declining native trout populations (Armstrong & Seddon, 2008; Penaluna et al. 2016). Of the 25 extant species and subspecies of trout native to the United States, 52% currently occupy less than 25% of their historic range (Williams et al. 2015). Colorado River cutthroat trout (CRCT, *Oncorhynchus clarkii pleuriticus*), which are native to the Green, Yampa, Colorado, and Gunnison river basins

on the west side of the southern Rocky Mountains (Bestgen et al. 2019; Metcalf et al. 2012), occupy only 3,304 km (~11%) of their historic range (Hirsch et al. 2013). The causes of population declines are complex and often interrelated. Anthropogenic and climate-induced habitat change, competition and hybridization with non-native salmonids, and disease caused by invasive parasites are all culprits in the declines of CRCT (Haak et al. 2010; Nehring & Walker, 1996; Vincent, 1996; Young, 1995).

State and federal agencies throughout the Southern Rockies have attempted to restore CRCT populations through both reinforcement and reintroduction. A particular large-scale reintroduction effort is the LaBarge Creek Restoration Project in western Wyoming. In the late 1990s, biologists with the Wyoming Game and Fish Department (WGFD) identified the LaBarge Creek drainage as a promising site for a reintroduction project due to its large size and the historic presence of CRCT in the drainage (Hirsch et al. 2013; LeCheminant, 2019). The goal was to create a genetically pure, self-sustaining population. Disease testing performed by WGFD in 1999 analyzed 72 fish from the drainage to confirm that it was whirling disease free before the reclamation process began. Rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and mountain whitefish (*Prosopium williamsoni*) were analyzed and all specimens tested negative for *M. cerebralis* infection (Wyoming Game and Fish Derpartment, 2000). By 2006, WGFD had constructed an upstream migration barrier and eradicated all fishes from the area, resulting in 58.5 miles of habitable stream. During the piscicide treatment 15,564 brook trout, 266 rainbow trout, 1,000 Colorado River cutthroat trout, 162 brown trout, 573 mountain white fish, 43 rainbow x cutthroat hybrids, 3,887 mottled sculpin (*Cottus bairdii*), and 7,231 mountain sucker (*Pantosteus platyrhynchus*) were removed from the drainage (Wyoming Game and Fish Department, Fish Division, 2021). Not all fish collected

during the treatment were counted; therefore, counts are reflective of relative species composition and not total population size. Between 2007 and 2026, routine stocking efforts released 481,414 CRCT into the drainage (Figure S1; Wyoming Game and Fish Department, n.d.). LaBarge's mainstem received 308,459 CRCT, with the rest being distributed between various tributaries. Of the stocked fish, 57,601 were stocked at less than 50 mm total length, 407,290 were stocked at a total length of 50–150 mm, and 16,523 were stocked at a total length greater than 150 mm. All stocked fish were from the North Piney Lake broodstock raised at the Daniel Fish Hatchery. Despite the stocking effort, populations remained low, and in 2023 WGFD estimated densities of only 0–86 fish per mile along LaBarge Creek (WGFD, unpublished data). A study to identify if *ex situ* rearing conditions or stocking locations were limiting establishment found that regardless of stocking strategy, survival remained low (LeCheminant et al. 2021). In 2021, as a part of screening for a potential translocation of CRCT into LaBarge, WGFD conducted pooled disease sampling on fish from upstream and downstream of the migration barrier. That sampling returned positive results for the presence of *Myxobolus cerebralis*, the causative agent of salmonid whirling disease (Wyoming Game and Fish Department Wildlife Forensic and Fish Health Laboratory, 2021).

My master's project was designed to characterize the distribution, prevalence, and infection intensity of *Myxobolus cerebralis* in the LaBarge Creek restoration area. In addition, I wanted to assess detection methods for the parasite to potentially streamline future studies. The specific objectives of my project were to 1) investigate the spatial distribution of *Myxobolus cerebralis* throughout the LaBarge Creek restoration area, and 2) understand how differing infection analysis techniques compare to each other. Objective 1 was addressed by using standard methods for wild fish collection alongside a sentinel caging study of naïve fish

deployed throughout the drainage. Objective 2 was divided into three components: first, comparing the traditional Pepsin-Trypsin Digestion (PTD) with modern quantitative polymerase chain reaction (qPCR) for infection analysis; second, assessing whether sentinel fish must be held beyond the field exposure period to allow for parasite maturation within host tissue; and third, evaluating the utility of environmental DNA (eDNA) as a non-lethal approach for assessing infection risk. Collectively, these techniques provide a comprehensive assessment of current disease status in the LaBarge Creek restoration area and establish a foundation for disease monitoring to support Colorado River Cutthroat Trout reintroduction efforts.

METHODS

Study Area

LaBarge Creek is located on the southeast side of the Wyoming Range mountains (Figure 1). Its headwaters arise in the Bridger-Teton National Forest, and it flows southeast ~70 miles. LaBarge Creek is a tributary to the Green River, entering the river ~7.2 river miles upstream of Fontenelle Reservoir. My study took place on the mainstem of LaBarge Creek and several of its tributaries upstream of the fish migration barrier that is ~2 miles upstream of the Bridger-Teton National Forest border (Figure 1). Tributaries that were assessed in this study included Indian Creek, Shafer Creek, South LaBarge Creek, Nameless Creek, Cabin Creek, Spring Creek, Clear Creek, Little Clear Creek, and Little Corral Creek. Elevation ranged from 7,730 ft to 8,618ft.

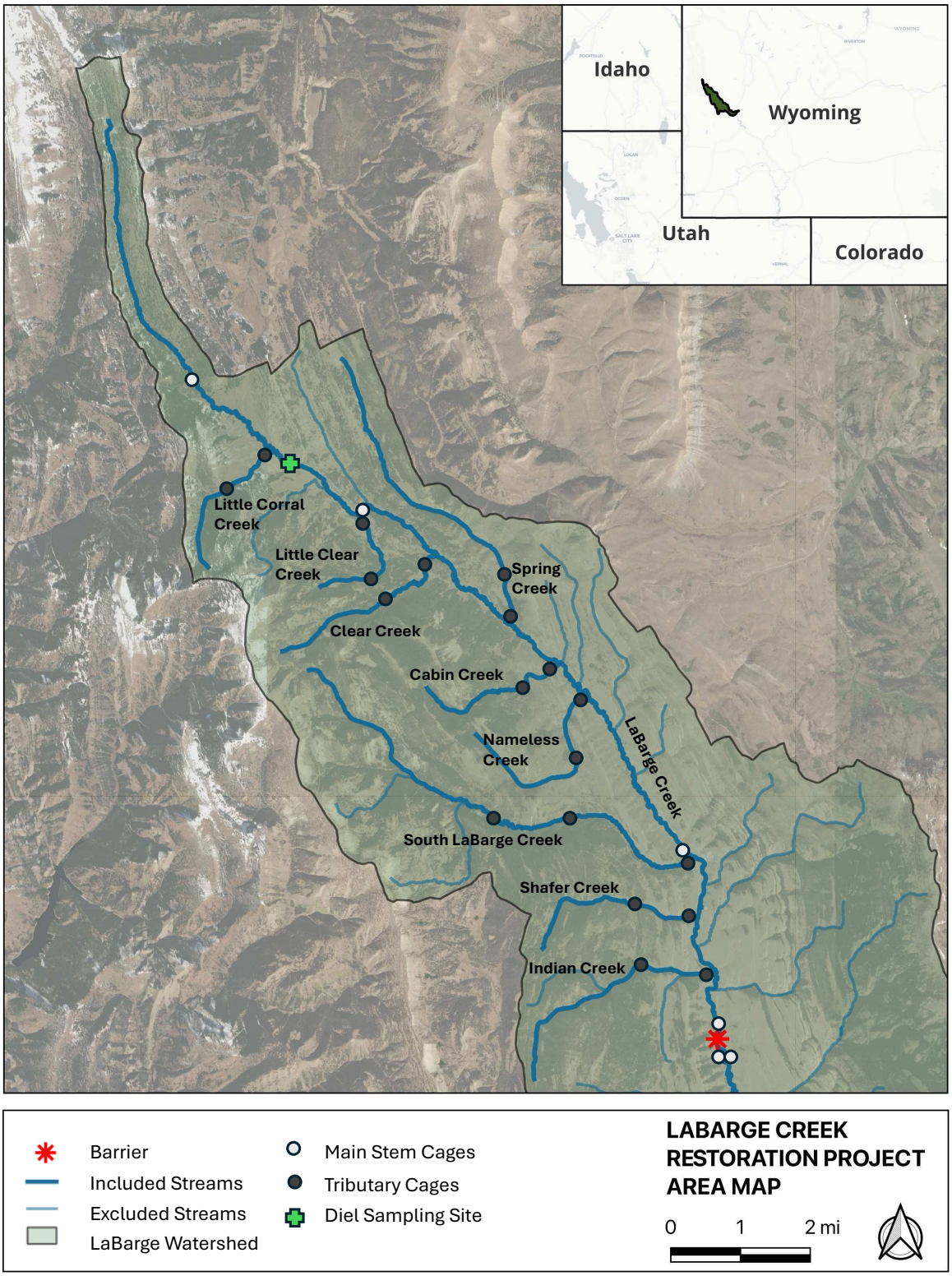

Figure 1. LaBarge Creek study area map showing streams that had sentinel fish cages or where wild fish were sampled, the upstream fish migration barrier, and the LaBarge watershed extent. The inset shows the location of the catchment within Wyoming.

Study Organisms

Colorado River cutthroat trout have two distinct lineages, coined the "blue" and "green" lineages, that are native to specific basins (Metcalf et al. 2012). My study specifically dealt with the blue lineage of CRCT, which are native to the Green, Yampa, and lower Colorado River basins in Wyoming, Colorado, and Utah (Bestgen et al. 2019; Metcalf et al. 2012). As of 2010, conservation populations of blue lineage CRCT, defined as those with at least 90% genetic purity, occupied only 13% of their historic range in Wyoming (Hirsch et al. 2013).

Myxobolus cerebralis is a myxozoan parasite and the causative agent of salmonid whirling disease (WD). Research in the 1980s demonstrated that *M. cerebralis* requires two hosts to complete its life cycle: salmonid fish and the aquatic oligochaete *Tubifex tubifex* (Fig 2; Markiw & Wolf, 1983). The parasite alternates between two sporogonic stages: triactinomyxon actinospores (TAMs), produced by *T. tubifex* and infectious to salmonids, and myxospores, produced in salmonids and infectious to *T. tubifex*. Infected *T. tubifex* release TAMs into the water column, where they can infect multiple fish upon contact. Myxospores are released into the environment by infected fish, both during life and following death and decomposition. Once settled into the substrate, myxospores are passively consumed by susceptible *T. tubifex*, completing the cycle (Nehring et al. 2018).

Clinical signs of whirling disease in salmonids include a blackened tail, skeletal and cranial deformities, and the characteristic "whirling" or circular swimming behavior when startled, caused by compression of the brain stem and spinal column (Hedrick et al. 1998; Rose et al. 2000). These behavioral and physical impairments reduce swimming and foraging efficiency in heavily infected fish, often leading to exhaustion and mortality (Hedrick et al. 1998).

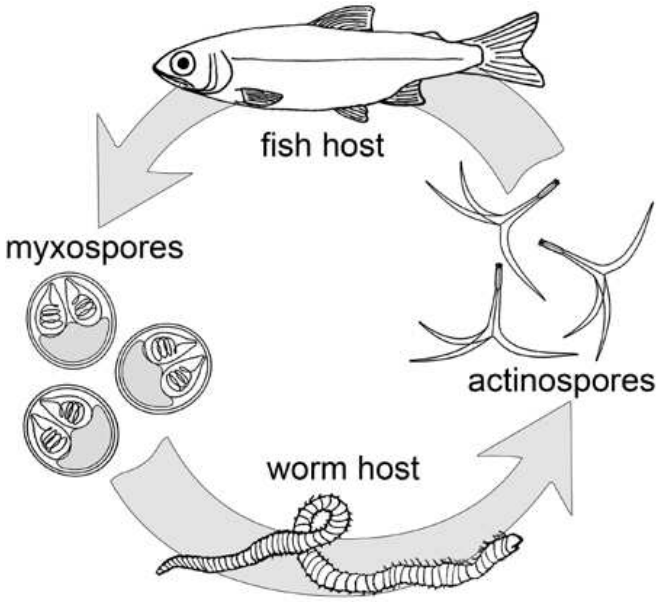

Figure 2. Life cycle of *Myxobolus cerebralis* showing salmonid and tubificid hosts (MacConnell & Bartholomew, 2012)

Wild Fish Collection

In July of 2023 we used one or two, depending on stream width, backpack electrofishing units to collect previously stocked or wild CRCT from the LaBarge study area. A total of 115 CRCT were collected. Thirty were collected from 5 different sites along the mainstem of LaBarge Creek (Figure S2). The remaining 85 CRCT were collected from tributaries. One hundred and eight of the 115 fish were both weighed (g) and measured (total length, mm). All fish were visually inspected for clinical signs of whirling disease. Eighty-seven fish were euthanized with an overdose of neutrally buffered tricaine methanesulfonate (MS-222). Twenty-four of the euthanized fish were collected from the mainstem and the remaining 63 were from tributaries. At the request of WGFD, when possible, we avoided euthanizing fish greater than 150 mm (spawners) and fish less than 100 mm. The former were avoided due to conservation value and the latter because they may not have been in the system long enough to fully develop

myxospores. All non-euthanized fish were returned to the approximate location from which they were collected. Following euthanasia, each fish was individually bagged, labeled, and stored on ice. Fish were transported to Colorado State University and stored at -18° C until they were analyzed using pepsin-trypsin digestion.

Sentinel Caging Experiment

In July of 2024, we performed a sentinel caging experiment to assess exposure risk throughout the drainage. We constructed 25 sentinel cages based on designs from previous research (Figure 3) (Winkelman & Gigliotti, 2018). Each cage was constructed of 24 in sections of 6 in SCH40 PVC pipe fitted with a threaded adapter and plug to allow access to the interior of the cage. Wire mesh with 2 mm openings was fitted to cover a horizontal opening on top of the tube and on the downstream end (Figure 3). The wire mesh allowed water to flow through the cage. Each cage was fitted with three eye bolts, one on the upstream capped end, and two on either side of the downstream end. Galvanized wire was tied from eyebolts to steel fence posts in the stream to anchor the cages in place.

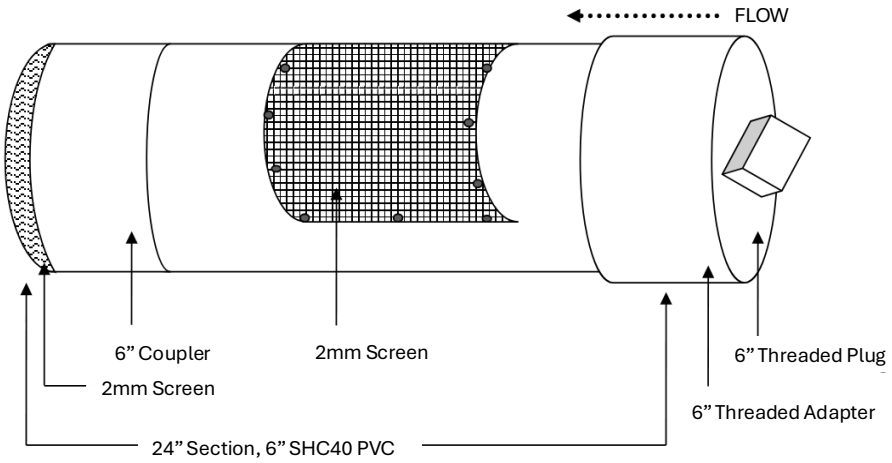

Figure 3. Sentinel cage design that housed naive fish in the wild. Adapted from Winkelman & Gigliotti (2018).

Six cages were placed in sites along the mainstem of LaBarge Creek: two below the fish migration barrier and the remaining four at locations spanning the mainstem study area. The remaining 19 cages were placed throughout tributaries to LaBarge Creek (Figure 1). All cages were placed at the edge of the stream near the bank when possible and in places that allowed the cages to be fully submerged.

Fish were held in sentinel cages from the July 9–30, 2024. Colorado River cutthroat trout were sourced from the WGFD Daniel Fish Hatchery (Daniel, WY) and were transported to LaBarge by hatchery staff. We attempted to put 30 fish in each cage; however, there was some variation due to counting errors during deployment and the range of CRCT per cage was 27–33 individuals. Rainbow Trout (RBT) from the WGFD Dan Speas Fish Hatchery (Casper, WY) were transported to LaBarge by WGFD staff, and 29 RBT were placed in the final cage below the fish migration barrier. Rainbow Trout were used as a comparative species, given that RBT are regarded as a highly susceptible species to *M. cerebralis* (Hedrick et al. 1999; Hedrick et al. 1998; O'Grodnick, 1979). Colorado River cutthroat trout averaged a total length of 33 mm, and rainbow trout averaged a total length of 51 mm at the beginning of the study. Fifteen fish, both CRCT and RBT, were euthanized and stored frozen (-18°C) to act as non-exposure controls. Each cage was visited every other day, when the mesh was cleaned and fish were fed a commercial diet at ~2% of their body weight. After 21 days of field exposure, all fish from individual cages were placed in 14 in x 36 in polyethylene bags that were filled with water from the same location as the cage. Bags were filled with compressed O_2, placed in coolers, and covered with ice for transportation to Colorado State University's Foothills Fishery Laboratory.

Environmental Data Collection

Local environmental data were collected at the caging sites to understand possible drivers of variation in exposure risk. At each mainstem site, and each of the confluence sites in the tributaries, a temperature logger (Hobo Pendant MX Water Temperature Data Logger, part no. MX2201) was attached to the cage, and a temperature reading was logged every 10 minutes over the caging period. Stream discharge (m^3/s) data was measured at the beginning (days 6 & 7) and the end (days 19 & 20) of the caging period at every location. Stream width (m) was measured directly upstream of the cage. Depth (m) and velocity (m/s) were measured at every 0.5 m of the stream cross section.

Gradient and drainage area at each site were measured with remote sensing data. Google Earth Pro (Version 7.3.6.10201) was used to collect stream gradients immediately upstream of each caging site. 500m of stream was traced using the "Add Path" tool. Elevations were recorded at the low end and high ends of the 500-meter path. The straight-line distance between elevation points was measured using the "Ruler" tool. The difference between elevations was divided by the straight-line distance to obtain the stream's gradient. The upstream drainage area (km^2) at each cage site was obtained using the USGS StreamStats online tool (U.S. Geological Survey, 2025). Drainage areas were delineated by selecting the cage site location within StreamStats and extracting the reported bankful basin characteristics.

Water Sample Collection

At the beginning, middle, and the end of the sentinel caging field exposure, four 1-L water samples were collected immediately upstream of each sentinel cage to quantify *M. cerebralis* DNA in the water column. This collection was designed to target the semi-buoyant TAM stage of *M. cerebralis*, but it is possible other sources of *M. cerebralis* DNA could be

collected in the water samples. Additionally, to investigate diel fluctuation of *M. cerebralis* DNA concentration in the environment, samples were collected every 2 hours for a 24-hour period starting at 1800 hours on July 24, 2024, and going till 1600 hours the following day. These samples were collected immediately downstream of the LaBarge meadow (Figure 1). I hypothesized that the LaBarge meadow had ample habitat for *T. tubifex* hosts and presumably high TAM production because of wild fish sampling documenting substantial infection in fish in the mainstem (Table 1), indicating an established parasite population. In addition, meandering morphology promotes fine sediment deposition and organic matter accumulation–conditions known to favor *T. tubifex* proliferation (Anlauf & Moffitt, 2010). Water samples were taken by submerging Nalgene bottles a third of the depth below the surface of the water and opening the lid. Samples were kept cool until filtering occurred. Vacuum filtering consisted of passing the one-liter sample through a 5 μm, 47 mm diameter membrane filter (MF-Millipore part no. SMWP04700). The filter was folded 3–4 times, placed in a 2.0 mL microfuge tube, and preserved with several drops of ethanol. Filters were kept cool until they were transferred to CSU, where they were stored at -18°C until they were analyzed.

Laboratory Rearing of Caged Fish

Ten fish per cage were euthanized after the field caging period upon arrival at CSU's Foothills Fishery Laboratory (FFL) to understand the immediate infection status after the field caging period. These fish are referred to as "T-0" fish (Figure 4). These T-0 fish were euthanized with a lethal dose of tricaine methanesulfonate (MS-222) and frozen at -18°C until analyzed. The remaining fish were reared until incurring 2000 degree-days of growth, and termed "T-2000" fish (Figure 4.).

At the CSU FFL, a flow-through aquarium system was constructed to house fish. The system consisted of 27 glass aquarium tanks, each with a capacity of 20 gallons. Tanks were gravity fed by two head tanks and flow rate to each tank was about 20 gal/hour. Water supply into the head tanks came from two sources, College Lake and on site well water. Each head tank was supplied with heated water, chilled water, and ambient college lake water. Solenoid valves and controllers were attached to inflows to regulate the water temperature in the head tanks. The temperatures in the tanks averaged 14.2°C throughout the entire raising period.

Upon arrival at the CSU FFL, all fish were acclimated to the lab's water temperature before being placed into their respective tanks. Each tank housed the fish from one sentinel cage, resulting in 25 tanks holding fish. Twice a day, in the morning and evening, the fish were fed, and tank temperatures were recorded. During the feeding, each tank was visually inspected to look for any mortalities. Tanks were cleaned 3 times per week. Mortalities were removed from the tank, weighed (g), measured (total length, mm) and placed into a Whirl-Pak. The Whirl-Pak was labeled with the date, the tank from which the fish came, any unusual physical characteristics of the fish, and was frozen for future disease analysis.

To assess the development of clinical signs of whirling disease in the fish, I did a standardized observation of each cage once during the raising period at roughly the mid-point of the raising period. At each cage, a 30-second video was taken and later analyzed. Any amount of blacktail, skeletal deformities, or whirling behavior was noted as evidence of the development of clinical signs of whirling disease.

Of fish that died during the study period, termed "Mortalities" (Figure 4), a subset was analyzed with histopathology (n = 13), and the others were analyzed with qPCR (n = 18). Two mortalities were caused during monitoring of clinical signs of disease when fish were caught

between the aquarium wall and a barrier used to concentrate fish. The remaining 26 mortalities were from unknown causes. Mortalities occurred across 10 cages. The first 13 fish to die were sent for histopathology and the following 18 were analyzed by qPCR. Three of the 13 fish that were sent for histopathology were not processed due to an overgrowth of Saprolegnia, a fungal growth that occurred post-mortem.

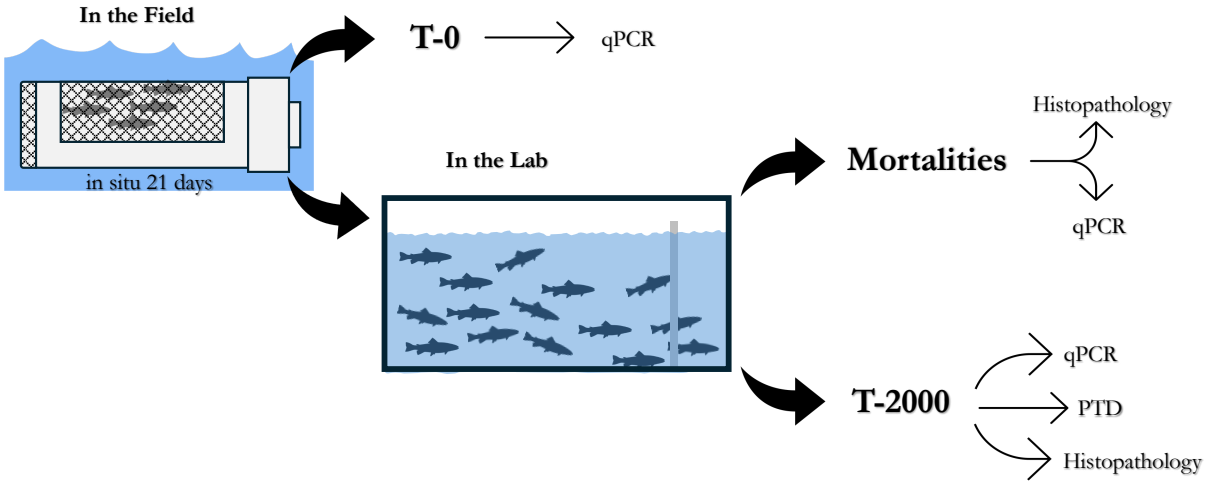

Figure 4. Fate diagram of fish included in the sentinel caging study. Bold text refers to the name given to each subset of fish, and the text following the arrow away from the group names refers to how these fish were analyzed. Sample numbers are reported in tables 2, 3, and 4.

Pepsin-Trypsin Digestion Analysis

Pepsin-trypsin digestion (PTD) was performed on heads of fish that were collected from the wild in 2023 (n=87), and on heads of fish that either died during the laboratory raising period (n=16) or survived to the end of the 2000 degree-days of growth (n=444). Pepsin-trypsin digestion is a highly effective approach for the detection and quantification of parasitic myxospores in yearling and older salmonids (Markiw & Wolf, 1974). This procedure involves the digestion of cranial skeletal tissues pepsin and trypsin. The resulting digestion yields a pellet containing myxospores, which can be subsequently identified and counted using a microscope.

Fish heads designated for PTD analysis were removed immediately posterior to the opercular plate using a sterile surgical scalpel. For wild fish, whole heads were processed using PTD. For T-2000 fish, heads were split along the midsagittal plane and only one half was analyzed with the other half being analyzed with qPCR; resulting spore counts were multiplied by a factor of two to account for the subsampling.

We followed the PTD assay defined by Colorado Parks and Wildlife: Aquatic Animal Health Laboratory in their SOP AAHL-PTD-2. Specifically, we performed this assay on frozen samples and obtained quantified results. The assay consists of five different steps. First, the heads of the fish were removed from the body using a sterile surgical scalpel. The heads are then cooked in 45º C water until pupils become opaque and flesh is tender. Second, all bones and cartilage are removed from the head and placed in a 50mL tube. Third, the bones are digested for the first time in a pepsin solution until a granular pellet is formed and myxospores are released. Fourth, the material is digested for a second time in a trypsin solution resulting in a pellet in which myxospores are fully free from bone and cartilage material. Fifth, the remaining pellet is suspended in a buffer solution, and the spores are visually enumerated using a microscope. The final step will result in a number of myxospores visually counted across 18 grids of a hemacytometer. Total myxospores per head are estimated using a calculation developed by CPW that considers the total volume of material digested as well as number of spores counted and grids examined (Figure S3) and is a proxy for infection intensity.

Quantitative Polymerase Chain Reaction

Quantitative PCR (qPCR) analysis was run on DNA extracted from fish tissue, and on environmental DNA extracted from filtered water samples. The qPCR assay, outlined in Kelley et al. 2004, was used to detect *Myxobolus cerebralis*. Amplifying the 18S rDNA gene of *M.*

cerebralis allows the identification of the presence of myxospores or triactinomyxon spores (TAMs) in the samples. The DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and included the following modifications: a 5 mm tissue punch was used to collect cranial tissue from near the midline ventral calvarium prior to extraction and purification, and extracted DNA was eluted to a final volume of 120 µL. Tissue punches were taken from two different head preparations: first, punches were taken from whole heads of T-0 fish and mortalities; and second, tissue from T-2000 fish came from punches from half of a head allowing for PTD to be run on the remaining half head.

The DNA was extracted from environmental water samples using the protocol described by Hallett et al. (2012), which included dissolving the filter membrane in an acetone wash, followed by the extraction and purification of DNA according to the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) with an additional wash with buffer AW2, and finished with a final elution volume of 120 µL.

All qPCR reactions were run on an Applied Biosystems QuantStudio 3 Real-Time PCR System. All samples were run in triplicate and when quantitation was reported in results, it was the average of the replicates. Fish tissue samples were considered positive if 2 of 3 wells fluoresced at a cq score of less than 37, a threshold chosen to limit unreliable detection of low template concentrations. Environmental DNA samples were considered positive if 2 of 3 wells fluoresced at a cq score of less than that of 1.1 TAMs, which was the smallest positive control on each plate run. Delta Rn values were manually adjusted to 0.1 for every reaction to allow for cross-plate comparisons. Quantification of TAMs was performed using Applied Biosystems Design & Analysis Software v3.1.0, based on a standard curve generated from a dilution series starting from a known sample of 10 TAMs.

Histopathology

One fish from each cage (n = 25, T-2000 fish), as well as mortalities during laboratory rearing (n = 13), were analyzed by histopathology. Fish were immersion-fixed whole in 10% neutral buffered formalin for >48 hours. Fish heads were then bisected with a scalpel on the midsagittal plane, and both halves were prepared for histopathology. For small heads, each half was processed in its own cassette. Larger heads required splitting the tissue into 4 or more total cassettes. An additional cassette contained spleen, liver, kidney, and sections of gastrointestinal tract. Heart was often included in the head sections or were processed with the viscera. Head sections were decalcified for 6-8 hours in Nitrical prior to further processing. Cassettes were routinely dehydrated through graded alcohol, xylene, and paraffin, embedded in paraffin blocks, and processed to 5-micron thick sections on positively charged microscope slides. Sections were stained routinely with hematoxylin and eosin stain for review by an anatomic veterinary pathologist with experience in salmonid pathology.

Heads were scored for the presence or absence of *Myxobolus* lesions as positive, negative, or suspect. Suspect cases included cases with small sites (usually <100 microns) of cartilage degeneration, necrosis, and/or granulomatous inflammation without identification of *Myxobolus* organisms. The number of discrete sites of chondritis in the most severely affected half of the head was recorded. Samples scored as positive or suspect were also graded according to Baldwin et al. (2000) and Hedrick et al. (1999). Separate Hedrick scores were given for gill and for cranium according to this grading scheme. Scores were given according to the most severe half of the head for each. Where the sample did not fit well into the proposed grading scheme based on number or size of lesional foci a “best fit” approach was applied according to pathologist discretion.

Data Analysis

We analyzed the relationship between the length of wild fish and their estimated total cranial spore load using a simple linear regression model, with the spore load $\log_{10}$-transformed to meet assumptions of normality. A simple linear regression model was also used to evaluate the relationship between Cq values, derived from qPCR, and estimated total cranial spore load, $\log_{10}$-transformed and derived from PTD analysis of sentinel caged fish. To analyze differences in infection prevalence, or infection load (Cq or Spores per Head), between sentinel cage sites or raising period groups (T-0 vs T-2000) we used the Kruskal-Wallis rank-sum test to allow for non-normally distributed data. When significant differences were indicated, pairwise comparisons between groups were conducted with Wilcoxon rank-sum tests with Bonferroni corrections. The relationship between *M. cerebralis* DNA in water samples collected and stream temperature during the diel sampling period was examined using Pearson's correlation coefficient.

To evaluate patterns of environmental variables between sentinel caging sites, we conducted a principal component analysis (PCA). Variables included in the PCA were mean stream temperature, width, average depth, discharge, elevation, drainage area and upstream gradient. All variables were standardized (centered to a mean of 0 and scaled to a standard deviation of 1) prior to analysis to ensure comparability among variables measured on different scales. Infection prevalence based on qPCR analysis was paired to each site ID to explore if environmental variables may play a role in limiting parasite establishment.

All statistical analyses and visualizations were conducted in R version 4.3.1 (Core Team, 2023). Data were manipulated using R packages *dplyr*, *tidyr,* and *hms*; multivariate analyses were performed with *vegan*; and figures were created using *ggplot2* and *ggrepel* in R.

RESULTS

Infection in Wild Fish

Overall, 46% of wild fish tested positive for *M. cerebralis* infection based on pepsin-trypsin digestion testing (Table 1). Infected fish were found at each collection site along the mainstem, and five of eight collection sites along tributaries (Table 1). The percentage of fish infected ranged from 0–83% per site (Table 1). The infection intensity varied among and within sites, with myxospore counts in positive fish averaging 74,749 spores/head and ranged from 411 to 528,267 spores per head (Table 1). The number of myxospores per head was positively related to fish length ($R^2 = 0.239$, $p = 0.001$; Figure 5). Euthanized fish averaged a length of 122 mm (range: 64–290 mm) and a weight of 27.6 g (range 2.4–308.9 g; Table S1). No fish exhibited any visual clinical signs of whirling disease.

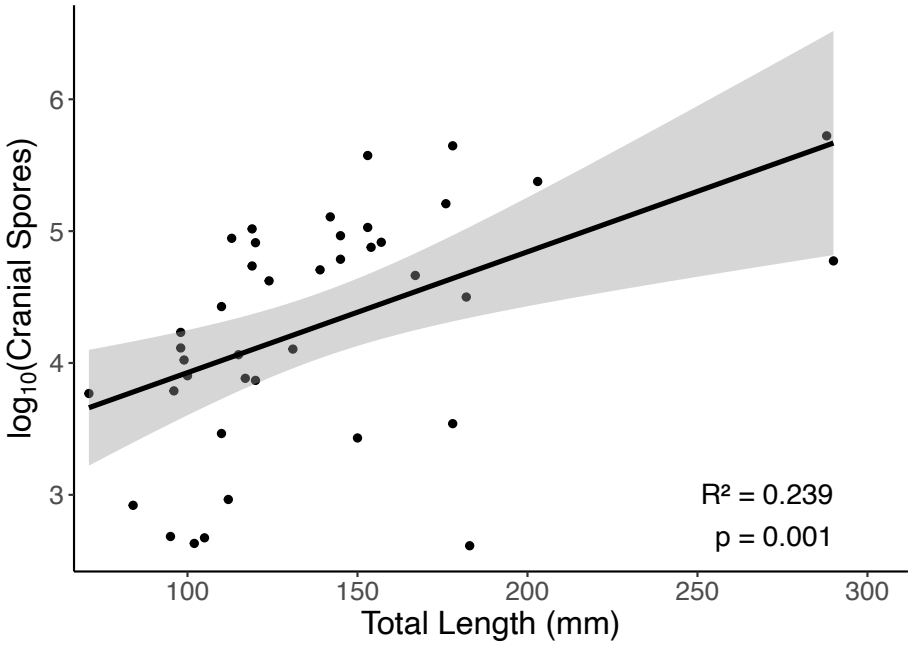

Figure 5. Relationship between total length (mm) of wild captured Colorado River cutthroat trout and total cranial myxospores (log_{10} transformed). Solid line shows the linear regression fit with 95% confidence interval.

Table 1. Measurements and infection status of Colorado River cutthroat trout (CRCT) euthanized (n = 87) from the LaBarge Creek restoration project area in 2023, based on pepsin-trypsin digestion analysis.

Stream Name	Sample Size	n Positive	Mean Spores per Head and Range	Mean Length and Range (mm)	Mean Weight and Range (g)	Mean Relative Weight and Range
LaBarge Creek	24	20 (83%)	106,041 (922-528,267)	144 (81-290)	46 (5.18-309)	96.4 (86-114)
Indian Creek	6	1 (16%)	411	110 (90-183)	17.3 (6.3-63.4)	93.7 (86-97)
Shafer Creek	10	8 (80%)	8,183 (428-17,061)	104 (96-120)	11.6 (8.77-17)	98.4 (89-107)
South LaBarge Creek	11	6 (55%)	128,135 (3,467-443,856)	174 (151-191)	53.4 (31.4-71.3)	91.7 (82-99)
Nameless Creek	2	0	N/A	70 (64-76)	3.62 (2.43-4.82)	103 (95-111)
Spring Creek	10	3 (30%)	9,242 (472-26,772)	106 (95-116)	11.2 (8-15)	92.6 (77-103)
Clear Creek	1	0	N/A	128	20.5	93
Little Clear Creek	10	0	N/A	81.4 (71-101)	5.07 (2.6-10)	90.7 (65-125)
Little Corral Creek	13	2 (15%)	3,350 (833-5,867)	111 (71-201)	22.9 (4.1-100)	93 (77-116)

Sentinel Caged Fish

Of the 252 T-0 fish, 129 (51%) were found to be infected by *M. cerebralis* based on qPCR (Table 2). The proportion of fish infected by *M. cerebralis* per site ranged from 0 to 100% prevalence (Table 2). Ten of the sites had no infection, with the 15 infected sites ranging from 40 to 100% prevalence (Table 2). Mean Cq values among positive samples ranged from 28.3–35.7, indicating varied infection intensities (Table 2). The mean length and weight of T-0 RBT were 61.5 mm and 1.41 g. The mean length and weight of T-0 CRCT varied by cage and ranged from 37.1 to 45 mm and 0.24 to 0.49 g, respectively (Table S2). Histopathology of fish from each site confirmed infection trends at most sites that were noted with qPCR and PTD analysis (Table S3).

Four fish from sites that were found to have no infection by either qPCR or PTD were deemed 'suspect' by histopathology, though the parasite was not positively identified in them (Table S3). All non-exposure control fish tested negative for *M. cerebralis* infection based on qPCR.

Table 2. Infection status of T-0 fish (n=10 from each cage, n=12 from MS4) based on qPCR analysis. * Indicates values calculated from only infected fish.

Site ID	Stream	Species	Sample Size	n Positive	% Positive	Average Cq Value and Range *
MS1	LaBarge	RBT	10	10	100	33.8 (32.5-36.4)
MS2	LaBarge	CRCT	10	10	100	31 (29.4-35.3)
MS3	LaBarge	CRCT	10	10	100	31.1 (29.9-33.1)
MS4	LaBarge	CRCT	12	12	100	33.1 (31.9-35)
MS5	LaBarge	CRCT	10	10	100	29.6 (28.7-30.7)
MS6	LaBarge	CRCT	10	0	0	N/A
IC7	Indian	CRCT	10	4	40	34.8 (32.8-36.2)
IC8	Indian	CRCT	10	0	0	N/A
SHC9	Shafer	CRCT	10	7	70	35.7 (33.6-36.7)
SHC10	Shafer	CRCT	10	0	0	N/A
SLBC11	South LaBarge	CRCT	10	10	100	33 (32-34.2)
SLBC12	South LaBarge	CRCT	10	10	100	33.1 (32.1-34)
SLBC13	South LaBarge	CRCT	10	0	0	N/A
NLC14	Nameless	CRCT	10	10	100	32.3 (31.1-33.5)
NLC15	Nameless	CRCT	10	0	0	N/A
CAC16	Cabin	CRCT	10	0	0	N/A
CAC17	Cabin	CRCT	10	0	0	N/A
SPC18	Spring	CRCT	10	10	100	34.8 (33.1-36.1)
SPC19	Spring	CRCT	10	0	0	N/A
CLC20	Clear	CRCT	10	9	90	35.3 (34.3-36.5)
CLC21	Clear	CRCT	10	0	0	N/A
LCLC22	Little Clear	CRCT	10	10	100	28.3 (27.4-29.4)
LCLC23	Little Clear	CRCT	10	0	0	N/A
LCOC24	Little Corral	CRCT	10	7	70	35.4 (34.8-36.5)
LCOC25	Little Corral	CRCT	10	0	0	N/A

Histopathology results of the analyzed mortalities confirmed infection for five fish (MS2, n =3; MS5, n=1; SLBC13, n=1), suspected infection for three fish (SLBC13, n=3), and were inconclusive for two fish (MS4, n=1; SLBC13, n=1) analyzed due to an overgrowth of saprolegnia (Table S4). *M. cerebralis* infection was identified by qPCR analysis in 16 of 18 mortalities during the laboratory raising period (Table 3).

Table 3. Infection status of mortalities during the raising period in the laboratory and analyzed with qPCR. $^{+}$indicates the fish was killed during the monitoring of clinical sign development.

Site ID	Sample Size	n Positive	% Positive	Average Cq Value and Range
MS2	4	4	100	23.4 (21.1-26.8)
MS4	1	1	100	32.6
MS5	2	2	100	20.9 (19.2-22.6)
SLBC12	1	1	100	31.9
SLBC13^{+}	1	0	0	N/A
NLC14	4	4	100	20 (17.7-21.7)
CAC16	1	0	0	N/A
SPC19^{+}	1	1	100	24.8
LCLC22	2	2	100	20.1 (19.9-20.3)
LCOC24	1	1	100	31.7

Of the T-2000 fish, *M. cerebralis* infection was detected in 15 of the 20 cages from both qPCR and PTD analysis (Table 4). Infection was found in five of the six mainstem sites, and 10 of the tributary sites (Table 4). Prevalence in cages with infection ranged from 6 to 100% (Table 4). All mainstem sites had high infection prevalence except for the highest site in the drainage (MS6, Figure 6). Tributaries usually had high infection prevalence at the confluence site with the exceptions of one site having moderate infection prevalence (IC7, Figure 6) and another having no infection (CAC16, Figure 6). Tributary upstream sites were generally uninfected except for one site having moderate infection prevalence (SPC19, Figure 6). Additionally, the middle site

on South LaBarge Creek (SLBC12) had high infection prevalence (Figure 6). Clinical signs of whirling disease were observed in 8 of the 25 tanks (Table S5). Clinical signs observed included the pigmentation of the caudal peduncle and caudal fin, erratic ‘whirling’ or ‘tail-chasing’ swimming behavior, and skeletal deformations.

Cq values and estimates of total spores per head varied substantially within and between sites (Table 4). Histopathology positively identified *M. cerebralis* in 11 of the 25 fish, a suspect infection was found in 7 of the 25 fish, and no infection was found in the remaining 7 fish (Table S3). RBT averaged a length of 158 mm, and a weight of 40.8 g. CRCT average lengths ranged from 87.8 mm at LCLC22 to 120 mm at SLBC13 and average weights ranged from 6.59 g at LCLC22 to 16.4 g at SLBC13 (Supplemental Table S2).

Table 4. Summary of *Myxobolus cerebralis* detection results and measures of infection intensity of T-2000 fish at each sampling site. The table includes sample size, histopathology results, number of positive detections from qPCR and PTD, as well as average Cq values (with ranges) from qPCR and estimated total spore counts (and ranges) for positive samples. Site MS1 contained RBT and all other sites contained CRCT.

Site	Sample Size	Histology Result (n=1)	n Positive qPCR	Average Cq and Range	n Positive PTD	Average Total Spores and Range
MS1	17	Y	17 (100%)	26.7 (20.8–36)	16 (94%)	227,981 (16,622-280,178)
MS2	14	Y	14 (100%)	29.5 (21.8–35.9)	14 (100%)	88,060 (20,400-290,644)
MS3	22	Y	21 (95%)	27.8 (22.7–34.3)	22 (100%)	65,728 (7,556-195,000)
MS4	17	Y	14 (82%)	32.9 (26.8–36.3)	14 (82%)	48,425 (6,933-101,444)
MS5	14	Y	13 (93%)	28.8 (24.3–33.9)	13 (93%)	120,926 (19,311-221,333)
MS6	19	N	0	N/A	0	N/A
IC7	19	Y	8 (42%)	33.6 (28.7-36.9)	7 (37%)	36,214 (967-108,611)
IC8	19	Y	0	N/A	0	N/A
SHC9	21	Y	17 (81%)	29 (23.9-34.3)	17 (100%)	38,741 (922-167,844)
SHC10	18	S	0	N/A	0	N/A
SLBC11	19	Y	19 (100%)	30.4 (24.4-34.3)	19 (100%)	112,414 (1,844-260,067)
SLBC12	15	N	14 (93%)	32.9 (28.1-35.4)	15 (100%)	124,755 (10,000-252,933)
SLBC13	12	N	0	N/A	0	N/A
NLC14	16	Y	16 (100%)	30.8 (26.9-35.6)	16 (100%	92,653 (18,911-175,833)
NLC15	18	S	0	N/A	0	N/A
CAC16	19	N	0	N/A	0	N/A
CAC17	19	N	0	N/A	0	N/A
SPC18	19	S	15 (79%)	33.5 (29.8-36.7)	19 (100%)	61,167 (3,333-202,267)
SPC19	18	S	2 (11%)	36.6 (36.3-37)	1 (6%)	800
CLC20	19	Y	18 (95%)	27.8 (24-32.9)	18 (95%)	47,823 (12,911-144,400)
CLC21	18	N	0	N/A	0	N/A
LCLC22	17	Y	17 (100%)	25.5 (19.9-30.8)	17 (100%)	257,473 (84,000-441,700)
LCLC23	18	N	0	N/A	0	N/A
LCOC24	18	S	11 (61%)	32.9 (28.4-36)	15 (83%)	27,176 (2,167-108,656)
LCOC25	19	S	0	N/A	0	N/A

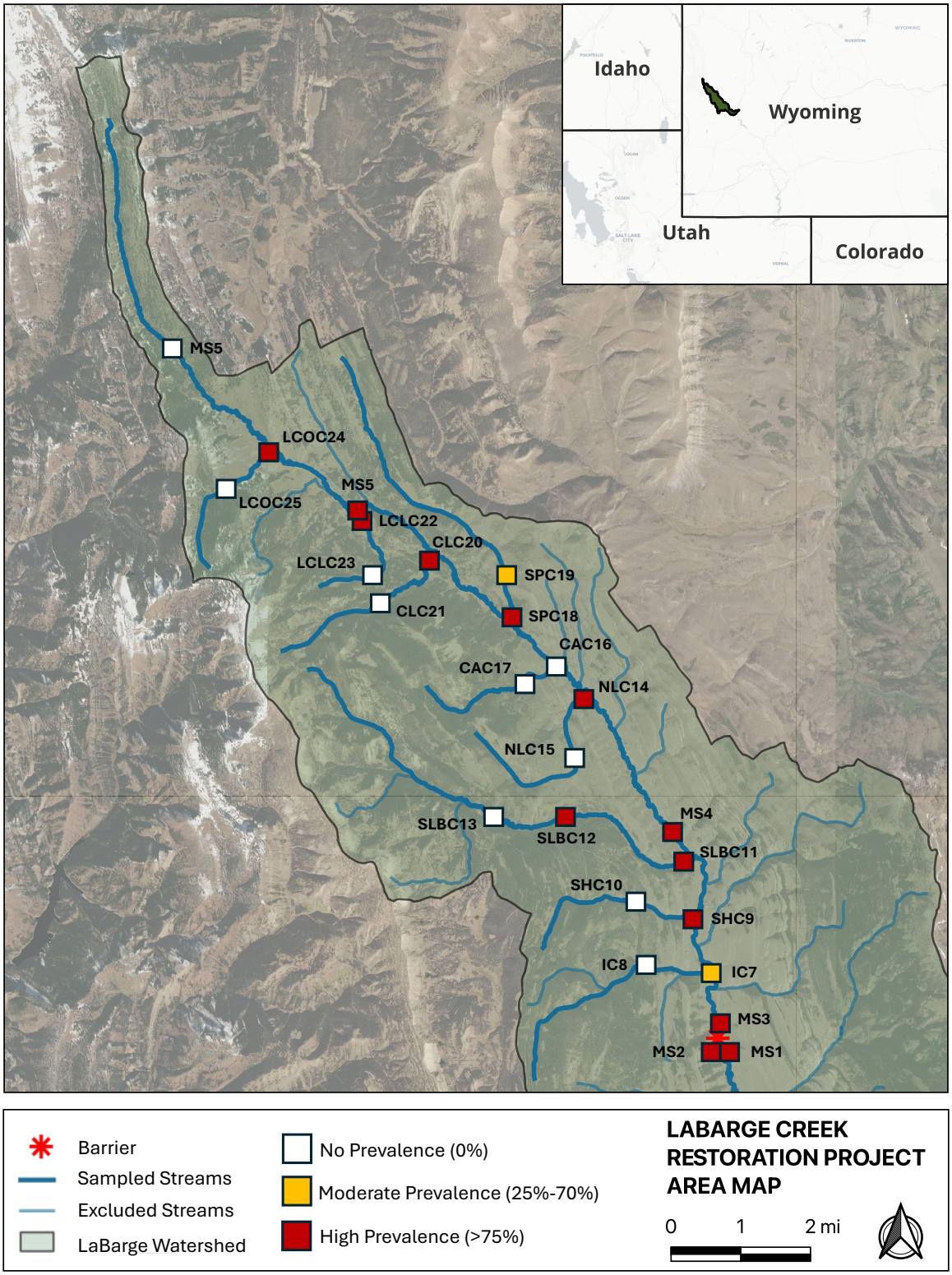

Figure 6. Infection prevalence of *Myxobolus cerebralis* in Colorado River Cutthroat Trout and Rainbow Trout (MS1) at LaBarge Creek caging sites as shown by qPCR analysis.

Table 5. Comparison of qPCR and PTD detection of *Myxobolus cerebralis*. A total of 444 fish were analyzed.

		PTD	
		Positive	**Negative**
qPCR	**Positive**	209 (94%)	7 (3%)
	Negative	14 (6%)	214 (97%)
	Total	223	221

PTD and qPCR produced highly concordant results, with molecular estimates of parasite load closely reflecting cranial spore counts and consistent infection prevalence across mortality periods. Comparing PTD to qPCR showed a high level of agreement between the two tests with 94% agreement for positive fish and 97% for negative fish (Table 5). There was a significant negative relationship between average qPCR Cq and $\log_{10}$-transformed total cranial spore count among fish that tested positive for *M. cerebralis* infection by both qPCR and PTD analyses (Figure 7) as indicated by the linear regression model ($p<0.001$). As Cq values increased (indicating a lower concentration of parasite DNA), the total spore count estimation decreased. Fish from each mortality period were analyzed by qPCR, and Fishers exact test indicated that there were no significant differences in prevalence between T-0 fish and fish T-2000 fish at each caging site (Figure 8).

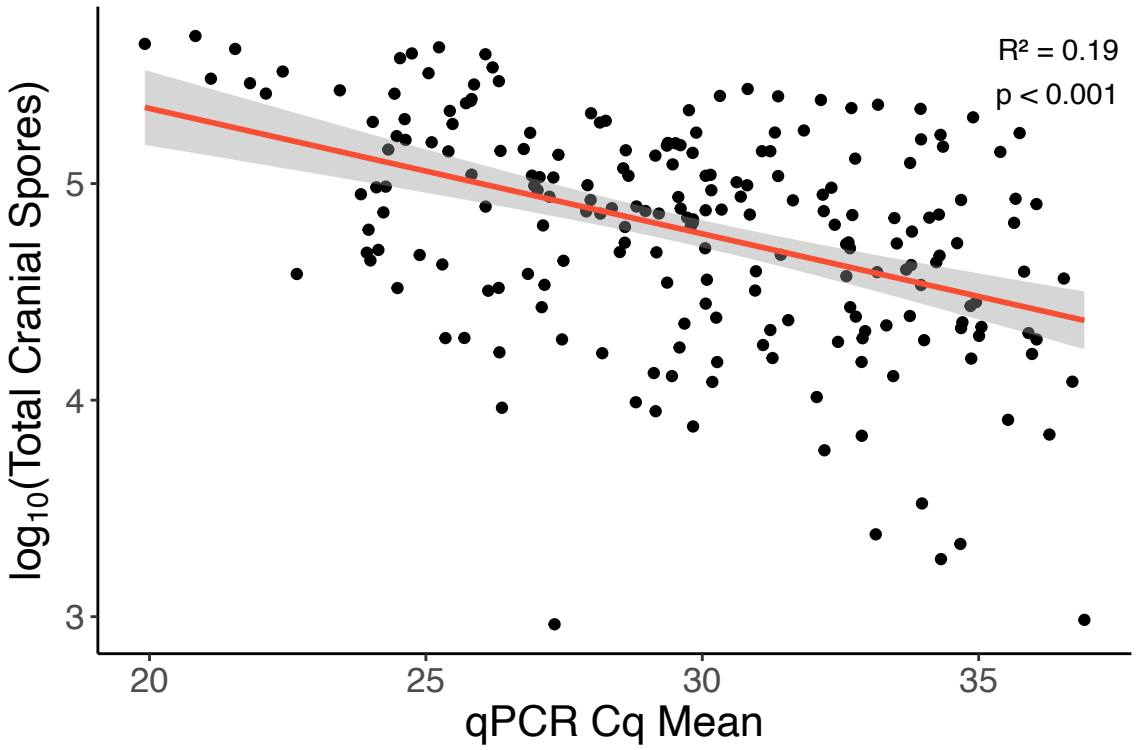

Figure 7. Relationship between qPCR and PTD results for fish that tested positive by both analyses. Each point represents an individual fish. The red line shows the fitted linear regression with the 95% confidence interval.

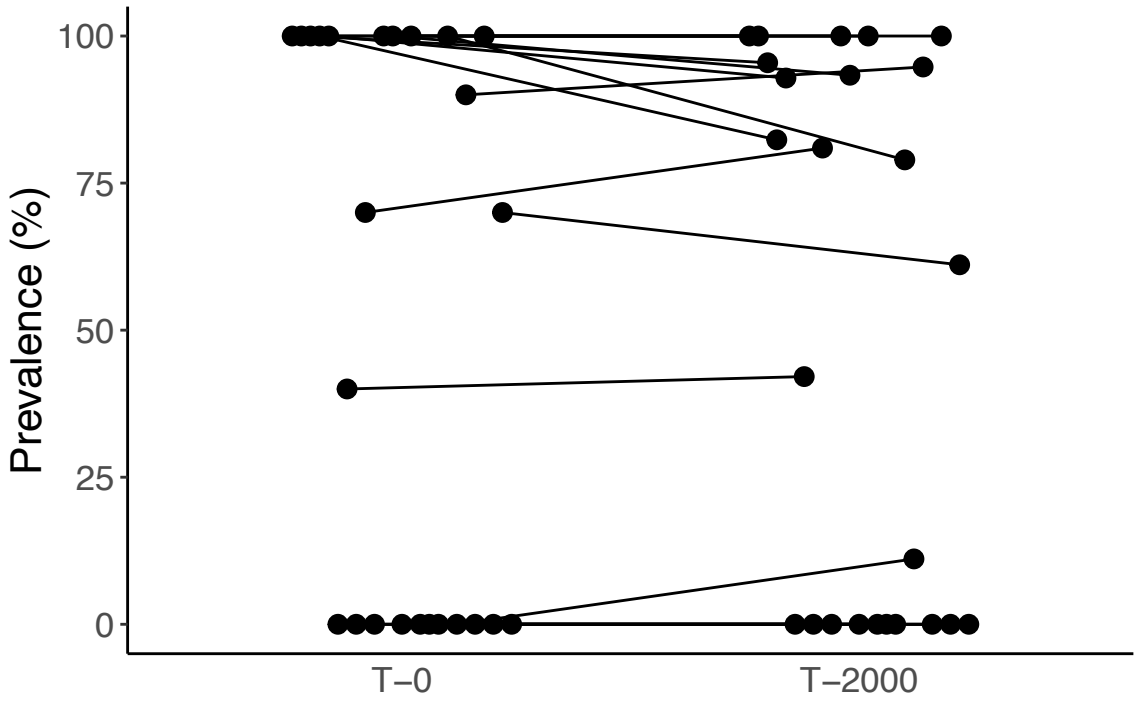

Figure 8. Disease prevalence based on qPCR analysis of fish that were euthanized post-caging (T-0) and fish that survived to 2000 degree-days of growth (T-2000). Points represent prevalence per cage, with lines connecting cages between euthanasia periods.

Sites with high infection prevalence correlated with larger stream width, depth, and discharge, as well as larger upstream drainage area in the Principal Component Analysis (PCA). These sites grouped together in the middle and left of the PCA biplot (Figure 9). Most mainstem sites had higher depth, larger width, higher discharge, larger drainage area, and are grouped on the left side of the biplot (Figure 9). Tributary sites were grouped together, and were associated with shallower depths, smaller widths, low discharge, and smaller drainage areas (Figure 9). Disease-free sites generally grouped together on the right side of the biplot, and were associated with shallow depths, smaller widths, low discharge, low temperatures, and higher gradients (Figure 9). Axis 1 explained 68.2% of the variation among sites and axis 2 explained 11.9% of

the variance in the PCA. A summary of environmental variables collected is displayed in Supplemental Table 6.

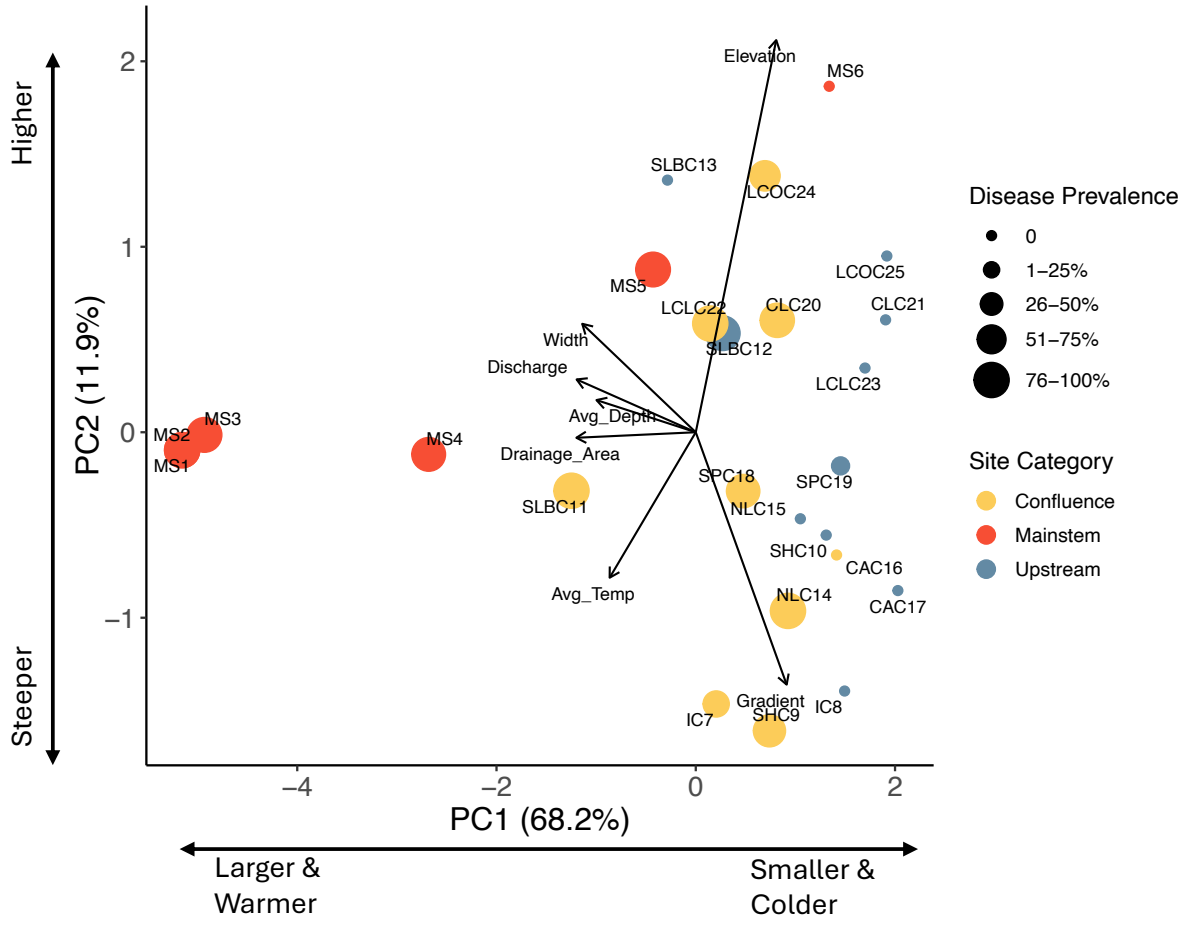

Figure 9. Principal component analysis biplot. Each circle represents a sentinel caging site, and its size represents the disease prevalence found at that site from qPCR. Sites are colored by mainstem sites, tributary confluence sites, and tributary upstream sites. Vectors show environmental variables included in the PCA.

A summary of the recorded temperature data is displayed in Supplemental Table 7. Mainstem sites experienced mean stream temperatures between 7.5°C (MS6) and 14.2°C (MS1/2). The highest recorded temperature, 21.5°C, was recorded at the most downstream site (MS1/2), and the lowest recorded temperature, 4.1°C, was recorded at the most upstream site. Mean temperatures at tributary confluence sites varied between 7.6°C (CLC20) and 14.7°C

(LCLC22). The single upstream site with a temperature logger, SHC10, had a mean stream temperature of 7.5°C (Figure S4).

Environmental DNA

Environmental DNA of *Myxobolus cerebralis* was not detected in any water samples collected during the 2023 field season (n = 105). Of the 215 samples analyzed from the 2024 season, 15 were positive for *M. cerebralis* DNA. These 15 samples came from 5 unique sites (Figure 10) and comparatively, fish were infected during the sentinel caging study at 15 of the 24 unique sites (Figure 10). At ten sites with positive fish from cages, we did not detect *M. cerebralis* DNA in the water samples. LCLC22 was the only site where *M. cerebralis* DNA was detected in each replicate, and during each sampling event (Figure 10). Other positive detections were from a single replicate and single sampling event, besides at site MS5 where the parasite was detected during two sampling events, and in two replicates from the first sampling event (Figure 10).

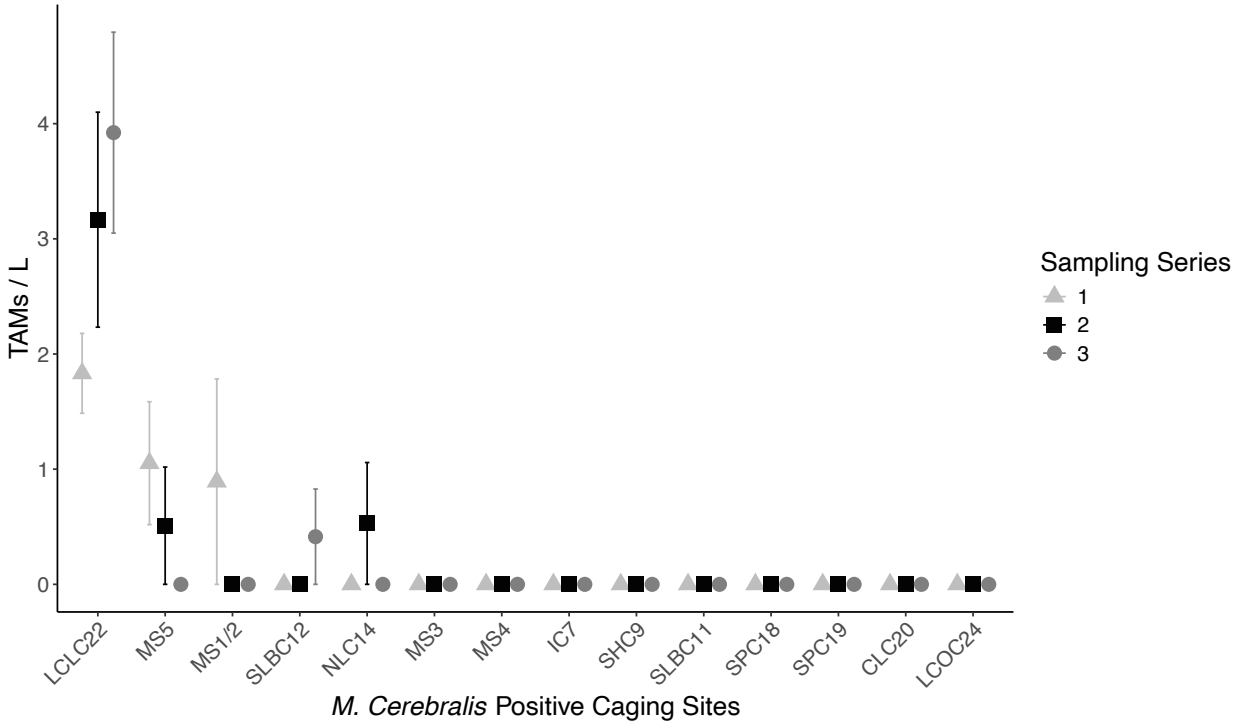

Figure 10. Average quantities of TAMs collected during eDNA sampling per liter of filtrate. The x-axis is comprised of all sites that infection was detected at during sentinel caging experiment. Sites with positive eDNA detections are grouped on the left. Shapes represent different sampling time periods, with the triangle representing the first, the square representing the second, and the circle representing the third.

Myxobolus cerebralis DNA was detected during each collection event of the diel sampling effort and showed a clear diurnal pattern, increasing at night and decreasing during the day (Figure 11). The sample with the fewest number of TAMs was collected at 6:00 am (2.52 TAMs) and the highest counts were collected at 12:00 am (29.5 TAMs). Stream temperature varied throughout the sampling effort (range: 9.2–17.3°C). Pearson's correlation coefficient between average TAMs/L and stream temperature was 0.67.

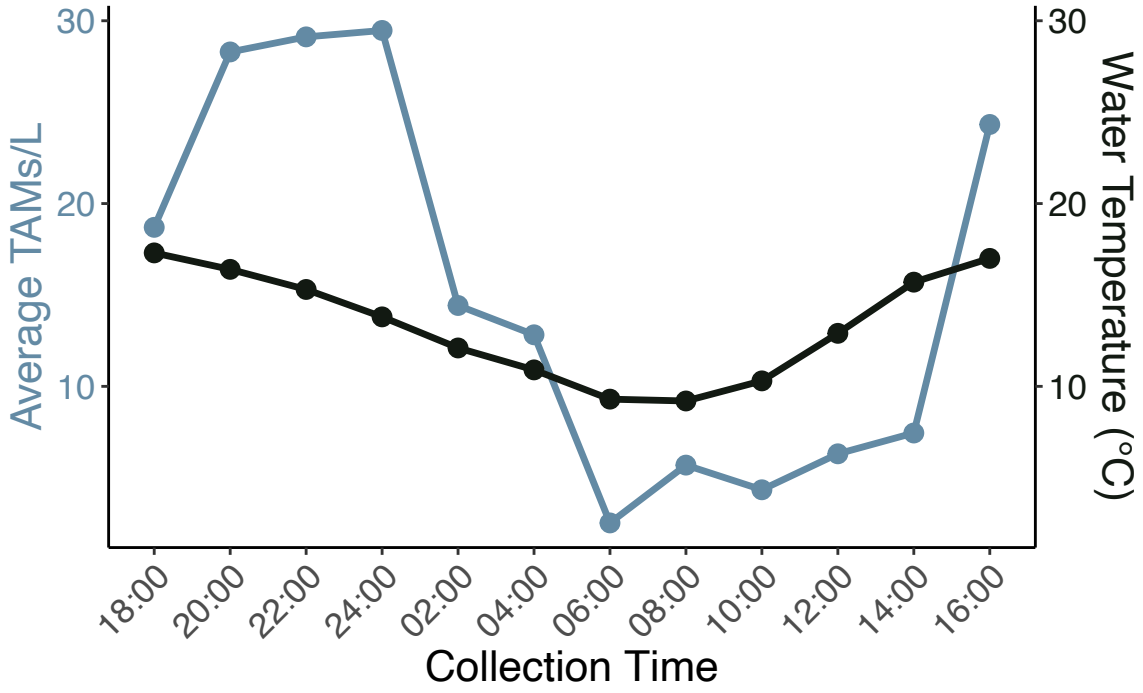

Figure 11. Diel variation in average triactinomyxon spore (TAM) concentrations (TAMs/L, left axis) and water temperature (°C, right axis) over a 24-hour period. Points represent measurements at each collection time, and lines connect sequential samples to show temporal trends. Pearson's correlation coefficient between TAMs/L and stream temperature is 0.67.

DISCUSSION

Evaluation of *Myxobolus cerebralis* distribution within the LaBarge Creek Restoration Area demonstrated that infection risk is spatially structured across the drainage, with many upstream sections having lower exposure risk than downstream reaches. Diagnostic methods were consistent in determining infection status: qPCR provided a time-efficient alternative to PTD when infection prevalence was the primary objective, while PTD remained the most effective approach for assessing infection intensity. Allowing parasite maturation in fish tissue did not alter detectability using molecular methods, indicating that immediate post-exposure

processing is sufficient when estimating infection prevalence. In contrast, molecular analysis of environmental DNA proved to be a sub-optimal indicator of infection risk, likely due in part to pronounced diel fluctuations in TAM concentrations that may have reduced the effectiveness of the sampling approach.

Based on the caging study, infection risk within the LaBarge Creek Restoration Area was higher on the mainstem of LaBarge Creek and the lower reaches of most tributaries, compared to upper stream reaches where caged fish remained disease-free. The continued presence of the parasite throughout much of the drainage may be partially explained by strong hydrologic connectivity and the absence of major barriers to fish movement. Consequently, nearly all mainstem locations—except for the most upstream segment—appear to represent areas where infection can occur. Apart from three outliers, tributaries containing paired caging sites consistently showed infection at lower sites and no infection at upstream sites. South LaBarge Creek, the largest tributary, followed this pattern: infection was detected at the lower and middle sites, while the upper site remained free of infection. Similarly, the upper site on Spring Creek exhibited low but detectable infection prevalence (~10%). In contrast, Cabin Creek showed no infection at either the lower or upper site. These large-scale spatial patterns echo findings from investigations of other systems following parasite invasion, where *M. cerebralis* becomes widespread throughout the watershed and, when absent from portions of the drainage, upstream reaches can remain disease-free (Allen & Bergersen, 2002; James et al., 2021).

The absence of infection at certain sites suggests that the *M. cerebralis* life cycle may be interrupted by the absence of one or both obligate hosts. In Cabin Creek and at the most upstream LaBarge Creek mainstem site, large beaver dam complexes separate these reaches from the rest of the LaBarge Creek mainstem. Although beaver dams are generally not considered

effective barriers to fish movement (Lokteff et al., 2013), they may still reduce dispersal (Kemp et al., 2012) and thereby limit parasite spread by restricting fish movement into these areas. Upstream sites that remained free of infection exhibited similar conditions to one another that may disrupt the parasite life cycle. These sites were typically smaller in depth, width, and discharge, colder, and steeper in gradient. It is probable that many upstream segments of tributaries experience mean summer stream temperatures associated with low or no growth and recruitment of juvenile CRCT (Roberts et al., 2013), which may be a factor in low to no densities of fish in these areas. Importantly, stream temperatures associated with most upstream tributary sites included in the PCA were averaged from spot measurements taken during the day while checking cages. These measurements likely reflect a higher temperature than the true mean stream temperature averaged over 24 hours. If the true mean temperatures were available, it is possible that temperature could have explained more site level variation between upstream and downstream confluence sites. In fact, a study of the effect of *ex situ* rearing strategies on survival probabilities of CRCT in LaBarge Creek showed that six of nine sites analyzed had temperatures associated with low to no growth and recruitment of CRCT (LeCheminant, 2019; Roberts et al., 2013). If fish are present in these upstream reaches, population densities are likely lower than at downstream sites closer to the mainstem due to reduced habitat size (Harig & Fausch, 2002) and sub-optimal temperature regimes (Roberts et al., 2013). Additionally, these upstream reaches are less associated with habitats that increase fine sediment deposition, a key factor influencing the abundance of *Tubifex tubifex*, the obligate host of *M. cerebralis* (Lazim & Learner, 1987). This combination of low salmonid densities, cold temperatures, and limited oligochaete habitat may explain the lower infection risk to trout in upstream tributary sites.

PTD and qPCR produced highly consistent results for determining infection presence or absence but differed in their ability to characterize infection intensity. Agreement between the two assays exceeded 95% when determining infection status, with most non-conforming results arising from samples that tested positive by PTD but negative by qPCR. This disparity likely reflects methodological differences between the assays. PTD involves homogenization of an entire half-head, capturing infections occurring anywhere within that tissue, including localized or focal infections. In contrast, the qPCR assay analyzed a standardized 5-mm tissue punch collected from a standardized cranial location on each fish. Histological analyses indicate that *M. cerebralis* infections can occur throughout multiple regions of cartilage and bone within the cranium; therefore, localized tissue sampling may fail to capture infections present in areas such as the gill arches or rostrum, resulting in discordant outcomes between qPCR and PTD. This incomplete spatial sampling of infection within each fish head may also contribute to the low R^2 value observed in Figure 5. Additionally, qPCR detects DNA from all parasite life stages, whereas PTD identifies only mature, intact myxospores. As a result, qPCR may overestimate myxospore infection intensity when DNA from immature or degraded spores is detected, while PTD provides a more conservative estimate of myxospore infection. Collectively, these findings support previous work demonstrating that qPCR is a reliable and efficient method for detecting *M. cerebralis* in fish tissue (Cavender et al., 2024; James et al., 2021; Kelley et al., 2004), while reaffirming that the most accurate estimates of relative infection intensity of myxospores in the cranium are obtained using the Blue Book standard PTD assay, though at a costly time investment.

Infection prevalence did not differ between T-0 fish and T-2000 fish, indicating that delayed processing to allow parasite maturation does not influence detection by qPCR. Because

qPCR detects all parasite life stages regardless of maturation state or form, fish analyzed immediately following field exposure yield infection status results equivalent to those obtained from fish reared to meet PTD requirements. When infection intensity is not required to address the research objective, this molecular approach can substantially reduce time, cost, and labor by eliminating the need for a post-exposure rearing period. A similar sentinel caging study showed that 100% of fish from sites low in the drainage tested positive for *M. cerebralis* infection by qPCR in just 7 to 14 days post-exposure (James et al., 2021). An evaluation of rapid assessments of sentinel exposed fish analyzed at 10 days, 67 days, and 5 months post-exposure showed that there was no correlation between the quantitative values (*M. cerebralis* genome copies) obtained at 10 days and 67 days to the values obtained after maturation of the parasite took place; however, evaluation of fish 10 days post-exposure was a means to predict the proportion of fish that would be positive at 5 months post-exposure (Kelley et al., 2006). This disparity between the estimated *M. cerebralis* genome copies is likely due to the developmental process of *M. cerebralis*, where the parasite undergoes a series of replications in the host's cartilage (Hendrick & El-Matbouli, 2002).

Environmental DNA was a poor predictor of infection risk in this study, though limitations in sample collection methodology likely reduced its effectiveness. eDNA sampling detected the parasite's triactinomyxon (TAM) stage at only five sites, substantially underrepresenting infection risk compared to the sentinel caging study, which documented infections at 14 of the 24 sites. TAMs were detected in all sample replicates at only a single location, situated immediately downstream of a large beaver complex. Beaver ponds may create favorable habitat for *Tubifex* worms (Fritz, 2020), potentially resulting in elevated TAM production and release upstream of this site. This may explain why it was the only site with

consistent TAM detection across sampling time points. A statewide investigation of the impacts of *M. cerebralis* on fish populations in Colorado concluded that a single detection of a TAM over 12 separate sampling events was indicative of potential population level effects on wild rainbow trout populations (Nehring & Thompson, 2003), further providing evidence that *M. cerebralis* may be significantly impacting the population of CRCT in the LaBarge Creek project area, despite very low detection of TAMs via eDNA.

Diel sampling revealed that TAM concentrations experienced pronounced fluctuations throughout the 24-h period. TAM concentrations were highest during evening and nighttime hours and declined sharply by morning. This pronounced temporal fluctuation may explain the mismatch between eDNA results and sentinel caging outcomes, as eDNA samples at caging sites were collected in the morning through early afternoon, when TAM concentrations were lowest. The mechanisms driving this diel pattern remain unclear, though TAM concentrations were strongly correlated with stream temperature. Ambient light does not influence the number of TAMs released from infected worms under laboratory conditions (Wagner et al., 2002) and worms held at 13°C in mud substrate produce more TAMs than worms at other temperatures and in other substrates (Baxa & Nehring, 2022; Blazer et al., 2003). Regardless of the underlying mechanism, these results suggest that single-time-point eDNA sampling may inadequately capture infection risk in systems with strong temporal variability. An investigation of whirling disease in the Cache le Poudre River near Fort Collins, CO showed that a diel fluctuation in TAM concentration may exist, with the highest concentration at 0:400 h (Allen & Bergersen, 2002). More representative estimates of infection potential may be obtained using automated samplers that collect water at regular intervals over a 24-hour period, producing a temporally integrated sample for analysis (Hallett et al. 2012).

While histopathology findings largely mirrored molecular and pepsin-trypsin digest results, several upstream sites exhibited unexpected inconsistencies among diagnostic methods. Interestingly, mortality samples from the most upstream site on South LaBarge Creek were incongruent to the results obtained by qPCR and PTD analysis. qPCR and PTD showed no amount of infection occurred in any fish from this site, but histopathology showed three fish with suspected infection, one fish with positively identified infection, and one fish inconclusive. It is unclear why these mortalities showed signs of infection while the remaining fish remained unaffected. One possible explanation for this pattern is the presence of a small, localized population of infected *Tubifex* worms upstream of the site. These worms may have released a limited number of triactinomyxon spores (TAMs) into the environment, resulting in infection of only a few caged fish. If this exposure occurred late in the experimental period, only those fish initially infected would have had sufficient time for infection to be detected. Four T-2000 fish had histopathology results that were misaligned with the trends seen in other infection analysis techniques. Four sites which are all upstream sites along tributaries, were deemed to have 'suspected' infection, though *M. cerebralis* was not visually identified. It is important to note that samples classified as 'suspect' did not have *M. cerebralis* visually identified, though damage consistent with the parasite was found. Fish with the most severe infections based on the number of discrete infection sites and severity scores (Table S3) were from the lowest mainstem sites, consistent with other literature showing severe infections in downstream reaches (James et al. 2021; Murcia et al. 2011).

Management Implications

The results highlight several important considerations for the conservation of Colorado River Cutthroat Trout in the LaBarge Creek Restoration Area and similar systems. Upstream

reaches that remain free of *Myxobolus cerebralis* may represent critical habitats for protection. While balancing the trade-offs between habitat connectivity and protective isolation is challenging (Fausch et al. 2009), limiting the invasion of infected fish into disease-free reaches may be essential for conserving native salmonid populations. The installation of barriers could protect these areas, though such structures may also be problematic. For example, structures may restrict access to spawning habitats for migratory adults, the amount of upstream habitat above barriers may be too small for conservation populations, and temperature regimes in these areas may be outside of the zone allowing for successful reproduction and growth. Although another large-scale reclamation of LaBarge Creek is unlikely, eradication of *M. cerebralis* has been demonstrated (Nehring et al. 2018), suggesting that targeted, small-scale eradication of *M. cerebralis* from select tributaries with viable temperature regimes could be a feasible strategy for creating disease-free habitat within the system. To be effective, targeted reaches would need to remain fishless for ~14 months (Nehring et al. 2018) which likely would require the construction of additional barriers and does not seem to be a realistic option for LaBarge Creek.

Stocking strategies may also benefit from consideration of spatial variation in infection risk. If young, highly susceptible fish (Ryce et al. 2004) continue to be stocked, prioritizing release of fish at upstream locations with viable temperature regimes with low or no infection potential could reduce early exposure. Allowing juveniles to grow in these areas may enable them to develop partial resistance or tolerance prior to encountering the parasite in downstream reaches (Ryce et al. 2005). Another stocking strategy that could be utilized to bolster the 'catchable' fishery in LaBarge Creek would be to increase the stocking of larger and older fish. Ryce et al. (2005) showed that rainbow trout develop increased resistance to whirling disease when exposed to TAMs at nine weeks post-hatch and at a total length of 40 mm. It is unknown

whether these age and size thresholds are similar for CRCT, but if a similar trend holds true, these older and larger fish may help support the population in LaBarge Creek. If either approach is taken, we believe it would be beneficial to mark the stocked fish to track their survival.

Finally, the evolution of genetic tolerance or resistance to *M. cerebralis* represents a potential long-term management strategy. A Rainbow Trout strain chronically exposed to the parasite in a German hatchery evolved resistance (R. Hedrick et al. 2003) and was subsequently crossed with locally adapted strains to restore rainbow trout populations in the Colorado River while retaining disease resistance (Fetherman et al. 2014; Nehring & Walker, 1996). This precedent suggests that identifying and monitoring extant Colorado River Cutthroat Trout populations that persist in the presence of *M. cerebralis* could provide insight into the potential for natural resistance or tolerance to develop on the landscape. Greenback Cutthroat Trout, O*ncorhynchus clarkii stomias*, in Colorado are currently the subject of a large-scale conservation effort. Due to the low numbers of wild greenbacks to repopulate conservation areas, discussion around genetic manipulation to increase genetic diversity into the population has taken place. In this context, a strategy of 'genetic rescue'—introducing alleles that enhance disease tolerance—could be a feasible approach for promoting resistance in Colorado River Cutthroat Trout populations. Importantly, the concept of "genetic purity" would need to be considered when attempting to manipulate these genomes to ensure conservation goals and native lineages are preserved, while attempting to create and place high fitness individuals onto the landscape.

CONCLUSION

This study investigated the distribution and detection of the parasite *Myxobolus cerebralis*, the causative agent of whirling disease, throughout the LaBarge Creek Restoration Area and evaluated the effectiveness of multiple diagnostic approaches. Infection potential was

not uniform across the drainage, with upstream reaches of many tributaries and the mainstem having lower infection risk. Molecular assays proved to be a reliable alternative for determining infection status when compared to the traditional “Blue Book” standard of pepsin-trypsin digestion (PTD), though PTD remains the most effective method for quantifying relative myxospore infection intensity. Additionally, molecular analysis of exposed fish with unmatured parasite loads produced reliable prevalence estimates, while environmental DNA analysis was less informative than the use of sentinel fish under the conditions of this study.

In a broader context, these findings demonstrate that low disease risk habitats persist within the restoration area and that fish can be stocked into these sites without immediate exposure to *M. cerebralis*. Although raising fish to allow parasite maturation facilitates the detection of clinical disease, this step is unnecessary when infection prevalence is the primary objective, offering substantial savings in time, labor, and cost. Finally, pronounced diel fluctuations in triactinomyxon (TAM) density highlight important considerations for future eDNA-based monitoring efforts and emphasize the need for carefully designed temporal sampling strategies. Collectively, this work provides practical guidance for whirling disease monitoring and management and supports more efficient approaches to conserving native salmonids in restored stream systems.

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SUPPLEMENTAL INFORMATION

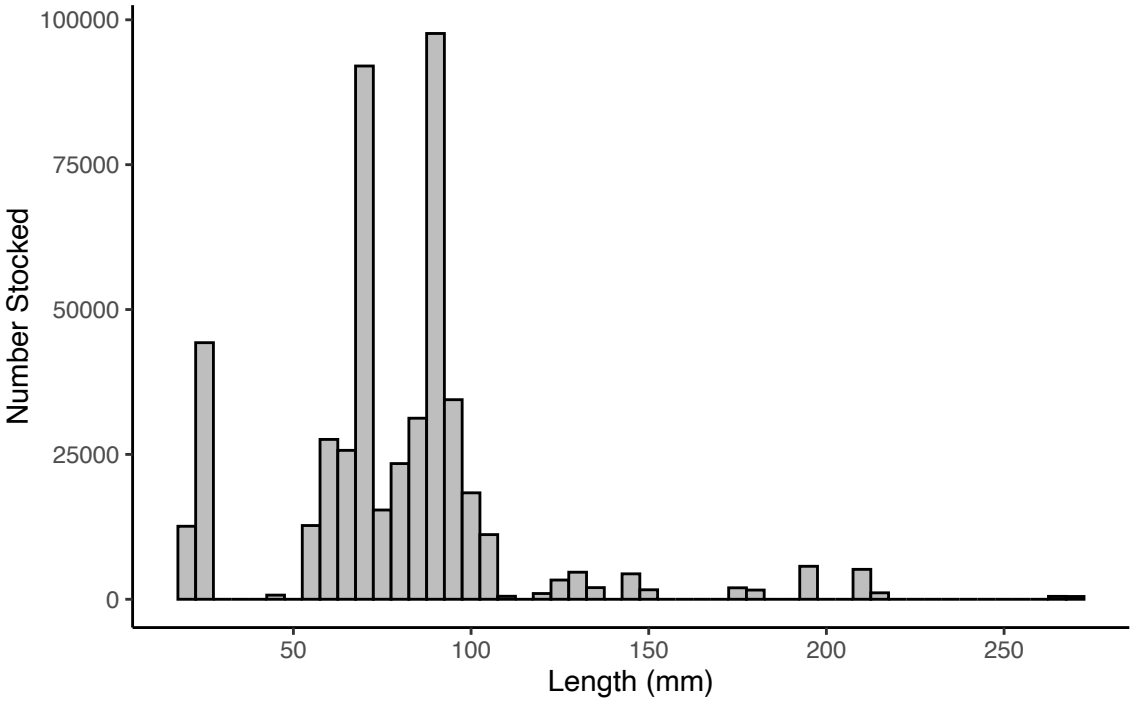

Figure S1: Number of fish stocked into the LaBarge restoration project area from 2007-2026 by size (Total length, mm; Wyoming Game and Fish Department, n.d.).

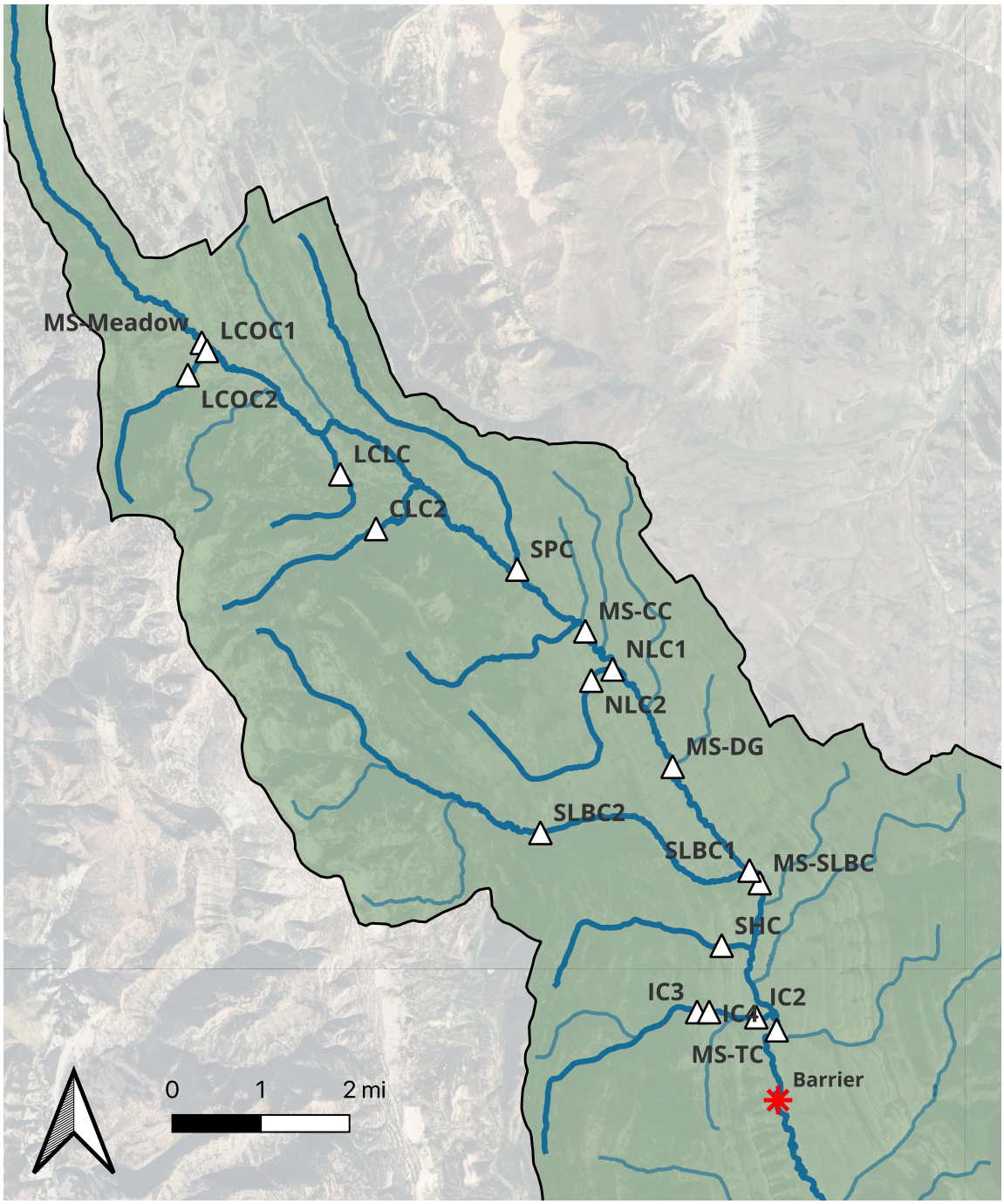

Figure S2. Sampling locations of wild fish collected in 2023.

$$Total\ Spores\ per\ Head = \frac{Number\ of\ Spores\ Counted}{Number\ of\ Grids\ Counted} * Final\ Volume\ * 10^4$$

$$Final\ Volume = Volume\ of\ Buffer\ in\ Tube + 0.03$$

Figure S3. Calculation of total myxospores per head. Value was multiplied by 2 when PTD was run on a half-head.

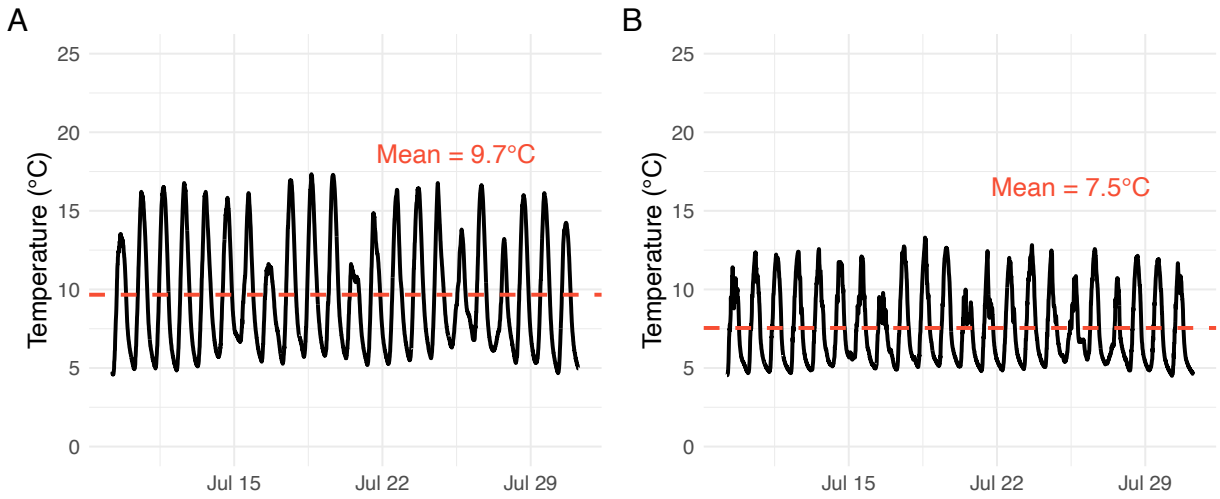

Figure S4: Temperature data recorded at Shafer Creek caging sites during the sentinel caging experiment. Panel A is the confluence site, and panel B is the upstream site. The black line represents the recorded temperature, and the red dashed line represents the mean temperature from the entire deployment.

Table S1. Length (mm), weight (g), relative weight (Wr), and euthanasia status of wild fish collected in 2023 (n=115).

Fish Number	Length (mm)	Weight (g)	Wr	Euthanized (Y/N)
1	256	194	103	N
2	212	115	110	N
3	164	52	110	N
4	140	30	104	N
5	159	31	72	N
6	144	32	101	N
7	142	30	99	Y
8	115	15	95	Y
9	131	24	102	Y
10	150	32	89	Y
11	145	31.5	98	Y
12	120	19	106	Y
13	145	32	99	Y
14	119	18.5	106	Y
15	154	39.5	102	Y
16	153	36	94	Y
17	113	13	87	Y
18	117	16.38	99	Y
19	124	18.68	94	Y
20	153	37.17	97	Y
21	139	25.35	89	Y
22	103	11.01	98	Y
23	96	8.79	98	Y
24	146	28.92	88	Y
25	112	13.94	96	Y
26	81	5.18	97	Y
27	203	84.43	92	Y
28	119	15.6	89	Y
29	290	239.12	86	Y
30	288	308.85	114	Y
31	77	4	88	N
32	90	6.2	84	N
33	75	3.5	84	N
34	80	4.4	86	N
35	81	4.7	88	N

36	82	4.9	89	N
37	74	3	75	N
38	81	5.8	109	N
39	85	6.7	109	N
40	81	4.1	77	Y
41	71	4.1	116	Y
42	106	12	98	Y
43	96	7.8	87	Y
44	88	5.75	84	Y
45	82	5.5	100	Y
46	84	5	84	Y
47	81	4.25	80	Y
48	88	7	102	Y
49	82	4.4	80	Y
50	201	100	113	Y
51	195	75	93	Y
52	183	63	95	Y
53	128	20.5	93	Y
54	64	2.9	113	N
55	76	3.5	80	N
56	101	10	95	Y
57	89	6.6	93	Y
58	81	5.5	104	Y
59	79	4.8	98	Y
60	79	3.3	67	Y
61	71	4.4	125	Y
62	84	5.7	96	Y
63	74	2.6	65	Y
64	76	3.5	80	Y
65	80	4.3	84	Y
66	108	11	85	N
67	110	14.5	106	N
68	103	9.6	86	N
69	116	13.5	83	Y
70	110	11.5	84	Y
71	112	15	103	Y
72	110	10.5	77	Y
73	105	12	101	Y
74	96	8	89	Y
75	107	13	103	Y

76	100	10.5	103	Y
77	95	8.8	101	Y
78	104	9.5	82	Y
79	64	2.43	95	Y
80	76	4.82	111	Y
81	N/A	N/A	N/A	N
82	178	58.3	96	Y
83	90	N/A	N/A	N
84	179	N/A	N/A	N
85	182	N/A	N/A	N
86	182	N/A	N/A	N
87	215	N/A	N/A	N
88	221	N/A	N/A	N
89	182	64.27	98	Y
90	185	56.85	83	Y
91	167	46.00	92	Y
92	191	71.26	94	Y
93	180	51.57	82	Y
94	174	54.15	95	Y
95	151	31.43	86	Y
96	157	35.67	86	Y
97	176	58.27	99	Y
98	178	59.70	98	Y
99	224	138.89	112	N
100	183	63.42	95	Y
101	98	9.25	96	Y
102	90	7.11	97	Y
103	101	9.75	93	Y
104	90	6.3	86	Y
105	95	8.26	95	Y
106	105	10.53	89	Y
107	98	9.55	100	Y
108	102	10.98	101	Y
109	114	16.42	107	Y
110	99	9.16	93	Y
111	96	8.77	97	Y
112	120	17.05	95	Y
113	100	10.58	104	Y
114	98	9.58	100	Y
115	110	13.38	98	Y

Table S2. Size metrics of sentinel caged fish raised in euthanasia groups T-0 and T-2000.

Site	Group	n	Weight (g) ± SD	Length (mm) ± SD
MS1	T-0	10	1.41 ± 0.65	61.5 ± 7.6
	T-2000	18	40.76 ± 15.57	157.72 ± 18.80
MS2	T-0	10	0.43 ± 0.28	42.8 ± 7.8
	T-2000	15	10.11 ± 7.17	96.27 ± 19.99
MS3	T-0	10	0.28 ± 0.12	39.5 ± 5.3
	T-2000	23	10.49 ± 4.44	100.74 ± 17.31
MS4	T-0	12	0.24 ± 0.07	37.1 ± 4.6
	T-2000	18	12.54 ± 5.36	106.33 ± 14.62
MS5	T-0	10	7.34 ± 4.73	91.27 ± 16.62
	T-2000	15	0.29 ± 0.10	39.7 ± 4.6
MS6	T-0	10	0.24 ± 0.10	38.7 ± 4.1
	T-2000	20	11.18 ± 3.37	105.55 ± 9.63
IC7	T-0	10	0.36 ± 0.15	41.8 ± 4.0
	T-2000	20	12.73 ± 3.80	110.80 ± 8.15
IC8	T-0	10	0.28 ± 0.15	38.2 ± 6.5
	T-2000	20	13.25 ± 3.86	112.45 ± 10.57
SHC9	T-0	10	0.42 ± 0.14	45.0 ± 4.8
	T-2000	22	10.97 ± 2.82	106.73 ± 9.01
SHC10	T-0	10	0.28 ± 0.09	39.2 ± 4.9
	T-2000	19	13.98 ± 3.66	114.32 ± 8.62
SLBC11	T-0	10	0.27 ± 0.09	40.8 ± 4.0
	T-2000	20	12.12 ± 4.24	108.05 ± 11.90
SLBC12	T-0	10	0.27 ± 0.12	42.0 ± 4.3
	T-2000	16	12.78 ± 3.74	110.06 ± 10.27
SLBC13	T-0	10	0.28 ± 0.06	40.3 ± 4.4
	T-2000	13	16.37 ± 3.45	120.15 ± 7.82
NLC14	T-0	10	0.31 ± 0.11	41.8 ± 5.3
	T-2000	17	12.88 ± 4.98	108.24 ± 19.03
NLC15	T-0	10	0.28 ± 0.09	40.9 ± 3.9
	T-2000	19	14.03 ± 3.76	115.11 ± 9.46
CAC16	T-0	10	0.36 ± 0.10	42.9 ± 4.0
	T-2000	20	11.77 ± 4.82	108.65 ± 13.06
CAC17	T-0	10	0.28 ± 0.12	39.9 ± 5.7
	T-2000	20	13.69 ± 3.39	114.70 ± 9.83
SPC18	T-0	10	0.49 ± 0.20	43.2 ± 5.4
	T-2000	20	13.37 ± 4.59	111.65 ± 12.76
SPC19	T-0	10	0.44 ± 0.10	42.8 ± 4.3

	T-2000	19	13.91 ± 4.63	112.42 ± 13.93
CLC20	T-0	10	0.31 ± 0.11	38.1 ± 5.0
	T-2000	20	13.00 ± 3.31	112.45 ± 8.99
CLC21	T-0	10	0.48 ± 0.10	43.4 ± 4.1
	T-2000	19	13.74 ± 2.93	116.00 ± 8.02
LCLC22	T-0	10	0.43 ± 0.19	39.4 ± 6.3
	T-2000	18	6.59 ± 4.01	87.78 ± 16.47
LCLC23	T-0	10	0.35 ± 0.13	39.1 ± 4.0
	T-2000	19	13.71 ± 3.31	113.32 ± 8.05
LCOC24	T-0	10	0.42 ± 0.12	41.1 ± 4.0
	T-2000	19	13.43 ± 3.14	114.37 ± 8.43
LCOC25	T-0	10	0.35 ± 0.16	38.1 ± 3.8
	T-2000	20	13.36 ± 3.82	113.75 ± 11.76

Table S3. Summary of histopathology analysis from T-2000 fish. (Y-Yes, N-No, S-Suspect)

Site	MC ID	# discrete sites	Baldwin grade	Hedrick score (gill)	Hedrick score (cranium)	Hedrick score (jaw)
MS1	Y	6	4	4	4	0
MS2	Y	6	4	2	4	0
MS3	Y	2	2	0	3	0
MS4	Y	4	4	0	4	0
MS5	Y	5	4	2	4	0
MS6	N	0	0	0	0	0
IC7	Y	3	3	0	3	0
IC8	S	2	2	0	2	0
SHC9	Y	3	4	1	4	0
SHC10	S	1	1	0	1	0
SLBC11	Y	4	4	0	4	0
SLBC12	N	0	0	0	0	0
SLBC13	N	0	0	0	0	0
NLC14	Y	1	2	0	4	0
NLC15	S	1	1	0	1	0
CAC16	N	0	0	0	0	0
CAC17	N	0	0	0	0	0
SPC18	S	3	3	0	3	0
SPC19	S	1	1	0	2	0
CLC20	Y	3	4	0	4	0
CLC21	N	0	0	0	0	0
LCLC22	Y	4	4	3	4	0
LCLC23	N	0	0	0	0	0
LCOC24	S	1	1	0	1	0
LCOC25	S	1	1	0	1	0

Table S4. Summary of histopathology analysis from fish mortalities. (Y-Yes, N-No, S-Suspect, I-Inconclusive)

Site	MC ID
MS4	I
SLBC13	I
MS2	Y
MS2	Y
MS2	Y
SLBC13	S
SLBC13	S
MS5	Y
SLBC13	Y
SLBC13	S

Table S5. Summary of clinical sign development of caged fish.

Site	Sample Size	n Clinical Sign Development
MS1	18	0
MS2	19	17
MS3	23	4
MS4	18	3
MS5	17	17
MS6	20	0
IC7	20	0
IC8	20	0
SHC9	22	0
SHC10	19	0
SLBC11	20	4
SLBC12	17	1
SLBC13	14	0
NLC14	21	4
NLC15	19	0
CAC16	21	0
CAC17	20	0
SPC18	20	0
SPC19	18	0
CLC20	20	0
CLC21	19	0
LCLC22	20	19
LCLC23	19	0
LCOC24	20	0
LCOC25	20	0

Table S6. Summary table of environmental variables collected and included in the principal component analysis. Temperature measurements marked with an * are based on spot measurements instead of hobo logger measurements.

Site ID	Mean Temp (c)	Width (m)	Mean Depth (m)	Elevation (m)	Upstream Drainage Area(km^2)	Upstream Gradient (%)	Discharge (cms)
MS1	14.23	8.5	0.56	2356	146.59	0.79	0.041646467
MS2	14.23	8.5	0.56	2356	146.59	0.79	0.041646467
MS3	13.75	10.6	0.36	2359	145.81	1.14	0.04577475
MS4	13.96	6.7	0.29	2400	78.47	1.02	0.023096441
MS5	12.54	5.3	0.16	2545	25.90	1.97	0.012259503
MS6	7.49	2.1	0.16	2627	6.55	1.83	0.003694811
IC7	10.2	2	0.34	2373	11.34	4.64	0.001025365
IC8	9.45*	0.9	0.16	2437	6.92	6.27	0.001516298
SHC9	9.66	1.3	0.27	2382	5.41	5.41	0.002539908
SHC10	7.54	1	0.11	2427	4.09	3.40	0.001285735
SLBC11	10.78	4.6	0.39	2397	25.28	2.11	0.012031407
SLBC12	5.33*	3.9	0.28	2475	21.13	3.32	0.012724974
SLBC13	5.83*	3.7	0.42	2514	17.48	1.62	0.013273691
NLC14	11.66	1.2	0.13	2440	5.02	3.96	0.000832721
NLC15	12.41*	0.8	0.12	2491	3.11	3.45	0.000548012
CAC16	8.99	1.3	0.11	2460	4.04	4.54	0.001696514
CAC17	8.123*	1.3	0.12	2494	3.32	6.76	0.000988511
SPC18	10.23	1.4	0.36	2478	7.51	3.89	0.002207643
SPC19	8.663*	1.7	0.13	2503	6.81	4.51	0.001765864
CLC20	7.62	1.4	0.27	2520	8.18	2.85	0.006839624
CLC21	5.69*	3.8	0.1	2571	6.73	5.70	0.004448705
LCLC22	14.68	2	0.19	2547	3.55	1.15	0.002537952
LCLC23	7.71*	3.1	0.22	2582	1.76	6.28	0.003059568
LCOC24	8.92	3.1	0.27	2585	4.07	2.09	0.002210059
LCOC25	6.85*	1.5	0.22	2611	2.85	4.82	0.001592423

Table S7. Summary table of stream temperatures recorded by hobo loggers during the sentinel caging study. All temperatures are Celsius.

Site	Stream	Min	Max	Mean
MS1/2	LaBarge	8.3	21.5	14.2
MS3	LaBarge	7.8	21	13.8
MS4	LaBarge	8.2	20.4	14
MS5	LaBarge	7.4	18.8	12.5
MS6	LaBarge	4.1	13.7	7.5
IC7	Indian	5.5	16.2	10.2
SHC9	Shafer	4.6	17.3	9.7
SHC10	Shafer	4.5	13.3	7.5
SLBC11	South LaBarge	5.3	18.2	10.8
NLC14	Nameless	6.9	16.3	11.7
CAC16	Cabin	4.8	15.1	9
SPC18	Spring	5.4	18.1	10.2
CLC20	Clear	3.8	15.9	7.6
LCLC22	Little Clear	10.3	20.8	14.7
LCOC24	Little Corral	4.8	15.4	8.9