MYXOBOLUS CEREBRALIS IN NATIVE CUTTHROAT TROUT OF THREE SPAWNING TRIBUTARIES TO YELLOWSTONE LAKE: A QUALITATIVE ECOLOGICAL RISK ASSESSMENT

by

Silvia Murcia

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APPROVAL

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Silvia Murcia

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Dr. Billie L. Kerans and Dr. Todd M. Koel, Committee Chairs

Approved for the Department of Ecology

Dr. David Roberts, Department Head

Approved for the Division of Graduate Education

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ABSTRACT

Most environments impose periodic or stochastic stress on natural populations, which increase susceptibility to diseases. Infection by *Myxobolus cerebralis* (exotic parasite causing salmonid whirling disease) is strongly influenced by a stream's physicochemical attributes and stressors, which may also affect host pathology. Susceptibility to *M. cerebralis* varies greatly among different species and subspecies of the salmonid host, but little is known about lesion severity or location of infection among the native Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*). In 2002 and 2003 we performed a series of 10-day sentinel cutthroat fry exposures and habitat assessments in various sites of three *M. cerebralis*-positive tributaries to Yellowstone Lake: the Yellowstone River, Pelican Creek, and Clear Creek. At 90 and 150 days post-exposure, fry were examined by polymerase chain reaction and histology to determine prevalence, severity, and location of infection. The goal was to identify spatiotemporal patterns of infection, and physicochemical features of the streams influencing it, and potentially facilitating parasite invasion and establishment. Results on fish (young and adult) host infection data, environmental attributes, and tubificid host presence/absence data in the study streams were used to develop an ecological risk assessment for parasite establishment and whirling disease in this ecosystem. Results from our qualitative risk ranking systems suggest that the cutthroat trout of the Yellowstone Lake basin are highly susceptible to *M. cerebralis* infection, with the most severe lesions in cartilage of the cranium and jaws, especially in systems with high water temperatures and ionic content. Our results also suggest that such environmental features are most conducive to parasite establishment, especially in tributaries of the lake basin used by cutthroat trout as spawning and rearing habitats. Thus, this study has implications for both ecology and parasitology as it reveals that environmental components can affect when and where a pathogen resides within the host, and thereby affect manifestation of disease. Recognition of the specific environmental attributes most conducive to parasite establishment, and disease, can increase future diagnostics, detection, and management efforts, strengthening the likelihood of correctly predicting *M. cerebralis*' and similar pathogenic invasions and establishment in unsampled sites.

CHAPTER ONE

INTRODUCTION TO DISSERTATION

Biological invaders, such as parasitic and disease-causing organisms, have become one of the world's most pressing threats to native biodiversity, causing extensive ecological and economic costs (Mack et al. 2000, Pimentel et al. 2000, 2005). The exotic parasite causing salmonid whirling disease, *Myxobolus cerebralis*, was first detected in Europe in 1893 and introduced accidentally to US fish hatcheries in the 1950's (Hoffman 1990, Bergersen and Anderson 1997). Since its introduction to the U.S., the pathogen has been detected in numerous watersheds throughout the country and linked to severe declines of wild trout populations in the Intermountain West (Vincent 1996, Bartholomew and Reno 2002). Information about host and parasite responses to environmental conditions, and to each other is critical in order to assess the risk of whirling disease, and predict ecological and economic consequences of invasion and establishment in different systems (e.g., Leprieur et al. 2006). Given the unpredictable nature of invasions, the ability to identify abiotic and biotic factors influencing the pathology of infection by *M. cerebralis* will facilitate development of efficient detection, diagnostic, and management tools.

Assessing the risk of whirling disease in natural systems with native salmonids requires information on characteristics of the environment, the hosts, the pathogen, and factors influencing their interactions (Hedrick 1998). Since *M. cerebralis* was described as a detrimental fish parasite in the US, research has confirmed a two-host life cycle. Each host

produces a spore that is infective to the other host, with myxospores infecting the oligochaete *Tubifex tubifex* and the actinospores infecting the fish (Markiw and Wolf 1983, Wolf and Markiw 1984). *Tubifex tubifex* ingest the myxospores found in benthic sediment and once in the gut lining, myxospores undergo multiple divisions and mature into the actinosporean triactinomyxon spores, or TAMs (El-Matbouli and Hoffman 1998). The TAMs of *M. cerebralis* are released into the water column by egestion or following death of infected tubificids (Wolf and Markiw 1984, El-Matbouli and Hoffman 1998). Susceptible salmonids become infected through contact with waterborne TAMs or by ingestion of infected oligochaetes (Wolf and Markiw 1984, El-Matbouli et al. 1995). Once in the fish, *M. cerebralis* reproduce while destroying the salmonid's cartilage, causing skeletal deformities, erratic swimming behavior (whirling), and blackened tail (Hoffman 1990). Whirling disease can thus be lethal to hatchery and wild salmonids (El-Matbouli et al. 1992, Walker and Nehring 1995, Vincent 1996). When the fish die the myxospores are released into the stream and ingested by *T. tubifex*, completing the parasite's life cycle (El-Matbouli et al. 1995, El-Matbouli and Hoffman 1998).

Effects of parasite invasion, distribution, and host infection severity vary among sites on both large (within and across watersheds) and small (within streams) spatial and temporal scales (Downing et al. 2002, Krueger et al. 2006, Murcia et al. 2006). Factors contributing to the unpredictable incidence of *M. cerebralis* and risk of infection in both hosts among different systems remain understudied. Potential causes include features of the physical and chemical environment (Zendt and Bergersen 2000; Sandell et al. 2001; Hiner and Moffitt 2001); spatiotemporal overlap between hosts, spore stages, and environmental factors

(Downing et al. 2002, Krueger et al. 2006); factors influencing the parasite or pathology (susceptibility) of wild trout (Murcia et al. 2006, in review); as well as, the oligochaete host distribution, abundance, genetic lineage, and infection (Beauchamp et al. 2002, 2005, Blazer et al. 2003), or complex interactions among all of the above (Kerans and Zale 2002, Krueger et al. 2006).

Water temperature directly influences the biology and ecology of both, salmonids and *T. tubifex,* as well as spore development in each host, and thus spore production, release, survival, and abundance in aquatic systems (Markiw 1992, El-Matbouli et al. 1999b, Blazer et al. 2003, Kerans et al. 2005). High conductivity and percent organic content in the sediment are positively correlated with infection risk in both salmonid and oligochaete hosts (Hiner and Moffit 2001, 2002, Sandell et al. 2001, Krueger et al. 2006); and high water velocity, and large sediment size tend to negatively correlate with infection risk in rainbow trout, *T. tubifex* abundance, and TAM densities in the water column (Krueger et al. 2006, Hallett and Bartholomew 2007, Lukins et al. 2007).

Also, salmonid species and sub-species show a range of susceptibility to infection by *M. cerebralis* (O'Grodnick 1979, Hedrick et al. 1999a, Thompson et al. 1999) and rainbow trout seem, thus far, the most susceptible (O'Grodnick 1979, Hedrick et al. 1999a,b, Thompson et al. 1999). However, cutthroat trout coexist with rainbow trout in many watersheds of the Intermountain West, and are also highly vulnerable to *M. cerebralis* (Hiner and Moffit 2001, Koel et al. 2006, Murcia et al. 2006); but sub-species susceptibility remains uncertain (Hedrick et al. 1999a, Thompson et al. 1999, Hiner and Moffitt 2001, Wagner et al. 2002). Life history diversities among salmonids are also important to assess the risk of

parasite establishment in a system because in order to complete its life cycle, spores of *M. cerebralis* must come into contact with young fish. If timing and location of spawning, and fry emergence and rearing (most susceptible fish size and age), overlaps with TAM release by *T. tubifex* in stream systems, the risk of disease, and thus pathogen establishment, increases significantly (e.g., Downing et al. 2002, Hubert et al. 2002, Kerans and Zale 2002).

The Yellowstone cutthroat trout of Yellowstone Lake face significant threats posed by *M. cerebralis*, first detected in the lake in 1998 (Koel et al. 2006, Murcia et al. 2006, in review). Yellowstone Lake is one of the last refuges of this once widespread salmonid (Gresswell et al. 1994) but if populations continue to decline at the present rate (Koel et al. 2005) catastrophic economic and trophic consequences may cascade across the Yellowstone Ecosystem. Identifying susceptibility (microscopic pathology) and infection responses of the Yellowstone cutthroat trout (Murcia et al. 2006) is paramount to assessing the risk of population-level impacts (e.g., McCallum and Dobson 1995, Vincent 2002), and to develop practical diagnostic and management efforts across the Lake Basin. Microscopic pathology is examined by histology, typically at 90 days post-exposure to *M. cerebralis* (Markiw 1992, Vincent 2002), but recent studies suggest that infected fish should be examined 150 days post-exposure (e.g., Ryce et al. 2005). Hence, it was unclear when peak infection severity could be most effectively detected in Yellowstone cutthroat trout.

The present study examined Yellowstone cutthroat trout exposed in sentinel cages, and naturally (wild) reared, at three different times in three spawning tributaries to Yellowstone Lake, where incidence of *M. cerebralis* had been confirmed: Pelican Creek,

Clear Creek, and the Yellowstone River (Koel et al. 2006). In chapter two, the objectives were to determine whether the prevalence of clinical signs, prevalence of infection, and the severity and location of cartilage lesions within the head differed between 90 and 150 days post-exposure in Pelican Creek, where infection severity was highest. In chapter three, the objectives were to determine whether histopathology of infection in Yellowstone cutthroat trout was associated with prevalence of whirling behavior and several environmental variables in concert, and whether such associations differed among exposure sites and exposure times in Pelican Creek. The objectives in chapter four were to identify spatiotemporal variation in infection severity among sentinel cutthroat trout in the three aforementioned tributaries of Yellowstone Lake, and its potential relationships with physical and chemical attributes of each stream to identify contributing factors to *M. cerebralis* invasion and establishment in the basin. Finally, in chapter five, we used sentinel and wild fish-host infection data, environmental attributes, and tubificid-host presence/absence data in the three tributaries to develop an ecological risk assessment for additional establishment of whirling disease in the basin (chapter 5). Results from this study will assist fish biologists and regional fisheries managers in developing management strategies to reduce disease risk in wild trout populations.

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CHAPTER TWO

MYXOBOLUS CEREBRALIS INFECTION PATTERNS IN YELLOWSTONE CUTTHROAT TROUT AFTER NATURAL EXPOSURE

Abstract

Salmonid species and sub-species exhibit a range of susceptibility to *Myxobolus cerebralis* infection. Little is known about lesion severity and location, or time required for *M. cerebralis* myxospores to develop in Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*). In 2002 we performed three 10-day exposures of Yellowstone cutthroat trout fry in Pelican Creek, a *M. cerebralis* positive tributary to Yellowstone Lake. At 90 and 150 days post-exposure we examined the fish for clinical signs, for infection prevalence, and by histology to determine *M. cerebralis* infection location and severity of lesions. The most prevalent clinical signs in Yellowstone cutthroat were whirling behavior and skeletal deformities, especially at 90 days post-exposure. Prevalence of infection and severity of cartilage lesions were not statistically different between fish held for 90 or 150 days post-exposure. Histopathology was most severe in cartilage of the cranium and the lower jaw, whereas cartilage of the nares and gill arches was seldom damaged. This study suggests that Yellowstone cutthroat trout are highly vulnerable to *M. cerebralis* and that current population declines in the Yellowstone Lake basin may, in part, result from whirling disease. Our results answer important questions in fish health and will aid in the development of diagnostic tools and management efforts against this pathogen in native cutthroat trout and other vulnerable salmonids.

Introduction

The long-term survival of the Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*) is of significant conservation concern among fishery biologists and managers across the Intermountain West. Yellowstone Lake, located within Yellowstone National Park, has one of the largest remaining populations of Yellowstone cutthroat trout; however, recent invasion by non-native lake trout (*Salvelinus namaycush*) (Ruzycki et al. 2003, Koel et al. 2005) threatens this population, as potentially does the recently introduced New Zealand mud snail (*Potamopyrgus antipodarum*) (Kerans et al. 2005). The parasite causing whirling disease in salmonids, *Myxobolus cerebralis*, was first detected in Yellowstone Lake in 1998 and has since posed an additional threat to the status of the Yellowstone cutthroat trout population there (Koel et al. 2006). Information is needed on the susceptibility and pathology (e.g., location of lesions, clinical signs) in wild fish to guide future diagnostic and management efforts.

Salmonid species and sub-species exhibit a range of susceptibility to infection by *Myxobolus cerebralis* (O'Grodnick 1979, Hedrick et al. 1999a, MacConnell and Vincent 2002), and of the species studied to date, rainbow trout (*Oncorhynchus mykiss*) are the most susceptible (Halliday 1976, O'Grodnick 1979, Hoffman 1990, Markiw 1992, Hedrick et al. 1999a,b, Vincent 2002). Cutthroat trout coexist with rainbow trout in many watersheds of the Intermountain West and are also vulnerable to the parasite (Hedrick et al. 1998, Wagner et al. 2002). However, susceptibility of different cutthroat trout sub-species, including Yellowstone cutthroat, remains a subject of debate (e.g.,

Hedrick et al. 1999a, Thompson et al. 1999, Hiner and Moffitt 2001, MacConnell and Vincent 2002, Vincent 2002, Wagner et al. 2002).

The location of cartilage lesions caused by the pre-sporogonic stages of *Myxobolus cerebralis* differs among various salmonids (Hedrick 1999a, Vincent 2002). The parasite causes microscopic lesions in cartilage of the fin rays and gill arches of brown trout (*Salmo trutta*) when exposed to high parasite doses, but in rainbow trout, lesions are most common in cartilage of the cranium (Hedrick et al. 1999b, Baldwin et al. 2000, MacConnell and Vincent 2002). The regions of cartilage most severely damaged by the parasite have not been examined in Yellowstone cutthroat trout, although some studies have examined degree of microscopic pathology and prevalence of clinical signs (Hedrick et al. 1999a, Hiner and Moffitt 2001, Vincent 2002).

To determine microscopic pathology, histological examination is typically conducted at 90 days post-exposure when maximum cartilage inflammation and complete myxospores can be detected in rainbow trout (Halliday 1976, Markiw 1992, Vincent 2002). However, some research suggests that infected fish should be examined 150 days post-exposure (e.g., Ryce et al. 2005). Lesion severity in Yellowstone cutthroat trout has been examined 90, 123, and 150 days post-exposure (Hedrick et al. 1999a, Vincent 2002), but it remains unclear when peak infection severity can be most effectively detected in Yellowstone cutthroat trout.

To address these uncertainties, we examined Yellowstone cutthroat fry exposed at three different times in a *Myxobolus cerebralis* – positive tributary of Yellowstone Lake (Koel et al. in press) at 90 and 150 days post-exposure. Our objectives were to determine

whether (1) the prevalence of clinical signs; (2) the prevalence of infection; (3) the severity of cartilage lesions in sections taken from medial and lateral cuts of each fry head; and (4) the location of microscopic lesions within the head all differed between two post-exposure periods and among exposure times.

Methods

Study Area and Field Exposure

Pelican Creek Pelican Creek is the second largest tributary to Yellowstone Lake, Yellowstone National Park, Wyoming, USA (Figure 2.1). Most of Pelican Creek (2,400 meters above sea level) is low gradient, meandering through sub-alpine meadows (Parks 1998). Pelican Creek is highly infected by *Myxobolus cerebralis* (Koel et al. 2006).

We obtained parasite-free Yellowstone cutthroat fry (4 to 6 weeks old) to use in field exposures from wild Yellowstone River or Clear Creek brood-stocks that are collected annually by the Wyoming Game and Fish Department's Hatchery programs. Field exposures were conducted by placing fish in sentinel cages along the stream bank at four sites in the lower 14 km of Pelican Creek. The sentinel cages were screened, cylindrical enclosures about 1 m in height and 50 cm in diameter. Cages were replicated (two cages, 50 – 60 m apart) at all but one site. Sixty fry were exposed in each cage for 10 days in July (10^{th} – 20^{th}), August (7^{th} – 17^{th}), and September (August 28^{th} – September 7^{th}) 2002 (7 cages X 3 exposures = 21 cages used in total). Two cages were lost during the July exposure and fish had to be re-exposed between July 19 and 29. During each exposure, a control group of 60 Yellowstone cutthroat fry were held in the National Park

Service Lake Aquatic Resources Laboratory on well water for 10 days and fed a standard commercial trout feed. At the end of each exposure, fry were transported to the Wild Trout Research Laboratory (WTRL), Montana State University, Bozeman, and held in separate aquaria at 12-13^{o}C for either 90 or 150 days.

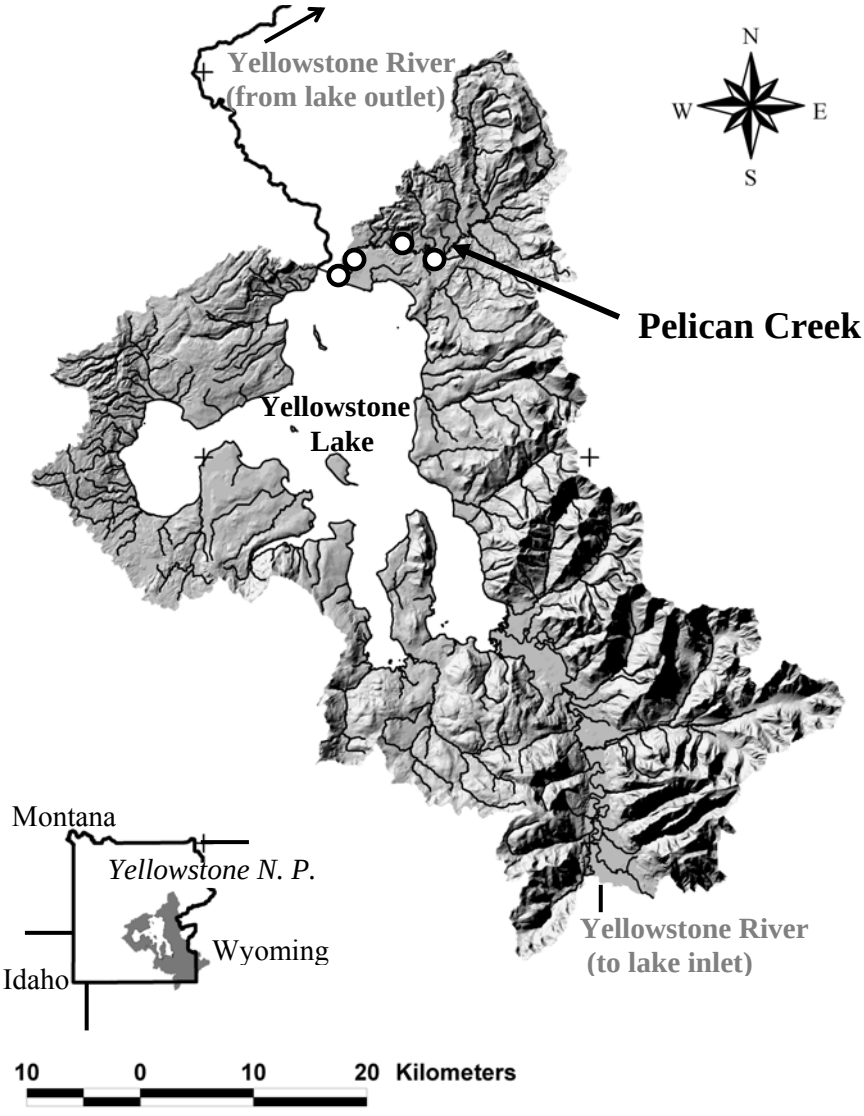

Figure 2.1 Map of Yellowstone Lake. Circles show study sites in Pelican Creek, where replicate sentinel cages were deployed three times during the summer (July-September) of 2002 (image adapted from Koel et al. 2006)

Water temperature was recorded hourly at each sentinel site using an Optic Stowaway Temperature Logger (Onset Computer Corporation, Bourne, MA). We measured specific conductivity and dissolved oxygen concentration at each site during each exposure using a Yellow Springs Instruments Inc. (Yellow Springs, Ohio; YSI model 85) multiparameter meter, and pH using an Oakton Instruments (Vernon Hills, IL) pH Tester.

Clinical Signs and Infection Prevalence

From each aquarium, random samples of 10 fish were collected on day 90 post-exposure and up to 20 fish on day 150 post-exposure. Fry were observed for one-half to one minute for clinical signs of whirling disease (whirling, black tail, skeletal deformities) prior to sacrificed in tricaine methane-sulfonate (MS-222). Heads were removed just behind the opercula and bisected along the sagittal line. One half was immediately preserved in Davidson's fixative (Humason 1979) for later histological examination and the corresponding half-head frozen at –70^{o}C for DNA analysis. Frozen half heads were tested by nested polymerase chain reaction (PCR) for *Myxobolus cerebralis* DNA (Andree et al. 1998). From each head we took a 5 mm cranial biopsy punch and pooled five punches in a 1.5 ml microcentrifuge tube with 180 ul of Qiagen-ATL lysis buffer (Qiagen Dneasy tissue kit # 69506).

Histology

Half-heads previously preserved in Davidson's fixative whose corresponding half-head tested positive for *Myxobolus cerebralis* DNA by PCR were processed for

microscopic examination using standard histological techniques (Humason 1979). Two 5 μm-thick tissue sections from each half head (a medial section near the brain, a lateral section near the skin) 150-200 μm apart, were stained with hematoxylin and eosin and examined for location and severity of lesions. In addition, a random sub-sample of 30 PCR-negative half heads was examined to verify lack of infection. Tissue sections were evaluated with no knowledge of sentinel cage site or PCR results. Microscopic lesions for six areas of head cartilage (nares, gill arches, lower jaw, upper jaw, vertebra, and cranium) were scored on a scale of zero (no infection) to five (severe infection) (Table 2.1) (Andree et al. 2002, Ryce et al. 2005).

Statistical Analyses

The non-parametric chi-square test of homogeneity was used to determine whether the frequency of clinical signs and the prevalence of infection differed between post-exposure periods (90 or 150 days) within each 10-day exposure (July, August, September) and among each 10-day exposure within each post-exposure period. Lesion severity scores for all areas of the head cartilage and inflammation were compared between post-exposure periods within medial and lateral sections and between sections within each post-exposure period using the same test. We pooled data from the three, 10-day exposures for lesion severity tests because we had insufficient data to examine them separately and our interest was in the patterns of pathology.

When chi-square *expected* values were < 1 we either combined cells to achieve expected values > 1 or we used the continuity correction for small sample sizes (Gotelli and Ellison 2004). All statistical analyses were carried out with the statistical software

program SAS 9.0 (SAS Institute 2004) and α-value 0.05 as the critical value in all tests of significance.

Table 2.1 Numerical scores, categories, and descriptions for cartilage lesion severity ratings used in this study. Adapted from: MacConnell-Baldwin scale for scoring lesions (Andree et al. 2002).

Numerical Score	**Category**	**Description**
0	No infection	No abnormalities noted, *M. cerebralis* not present.
1	Minimal	Small, discrete foci of cartilage degeneration, no inflammatory cells.
2	Mild	One locally extensive focus or several smaller foci of cartilage degeneration. No or minimal (inflammatory) host response causing no bone distortion or involvement of surrounding tissues, very localized response.
3	Moderate	Several foci of cartilage infected, cartilage degeneration and necrosis, inflammatory response has minimal or mild impact on surrounding tissues.
4	Moderate-severe	Several to coalescing areas of cartilage degeneration and necrosis, locally extensive areas of granulomatous inflammation, and moderate to severe involvement of surrounding tissues.
5	Severe	All areas of cartilage examined in the region are infected. Granulomatous inflammation is extensive with severe impact on surrounding tissues. This rating characterized by loss of normal architecture, such as bone displaced into the brain or spinal cord.

Results

Mean water temperature was highest during the July exposure, specific conductivity was highest during the September exposure, and dissolved oxygen and pH were highest during the August exposure (Table 2.2). Fish mortality in the field was 44% during the July exposure, 13.5% during the August exposure, and 16% during the September exposure. Laboratory mortality prior to the 90-day sampling was 12%, 12%, and 5% for the July, August, and September exposures, respectively. Laboratory mortality between 90 and 150-days was 5%, 2%, and 5%, for the July, August, and September exposures, respectively.

Table 2.2 Daily temperature, specific conductivity, dissolved oxygen, and pH measurements for each exposure in Pelican Creek were averaged across study sites. Mean (± 1 SE)

Exposure	Day temperature (^{o}C)	Specific conductivity (uS)	Dissolved oxygen (mg/L)	pH
July	19.8 (± 0.1)	313.5 (± 7.7)	8.7 (± 0.4)	7.6 (± 0.3)
August	16.9 (± 0.1)	382.3 (±10.5)	10.2 (± 0.4)	8.3 (± 0.2)
September	14.9 (± 0.1)	435.4 (± 8.8)	8.6 (± 0.4)	7.8 (± 0.1)

Clinical Signs and Infection Prevalence

Across all three exposures, whirling behavior was the most frequent sign of disease, occurring at an average rate of 30% of the fish at 90 days post-exposure (38/127, number of fry whirling divided by the total number examined, all ratios below are reported in a similar manner) and 16% (32/205) at 150 days post-exposure. The frequency of whirling did not differ between fish held for 90 or 150 days post-exposure

after the July and August exposures ($X^2_1 < 1.52$, $p > 0.2280$ for both tests) (Figure 2.2). After the September exposure, however, whirling was more frequent in fish held for 90 days than in fish held for 150 days ($X^2_1 = 17.39$, $p < 0.0001$) (Figure 2.2). Within the 90 day post-exposure period, frequency of whirling was lower after the August than after the July or September exposures ($X^2_2 = 16.42$, $p = 0.0003$) (Figure 2.2). Within the 150 day post exposure period frequency of whirling was lower after the September than after the July or August exposures ($X^2_2 = 13.26$, $p = 0.0013$) (Figure 2.2).

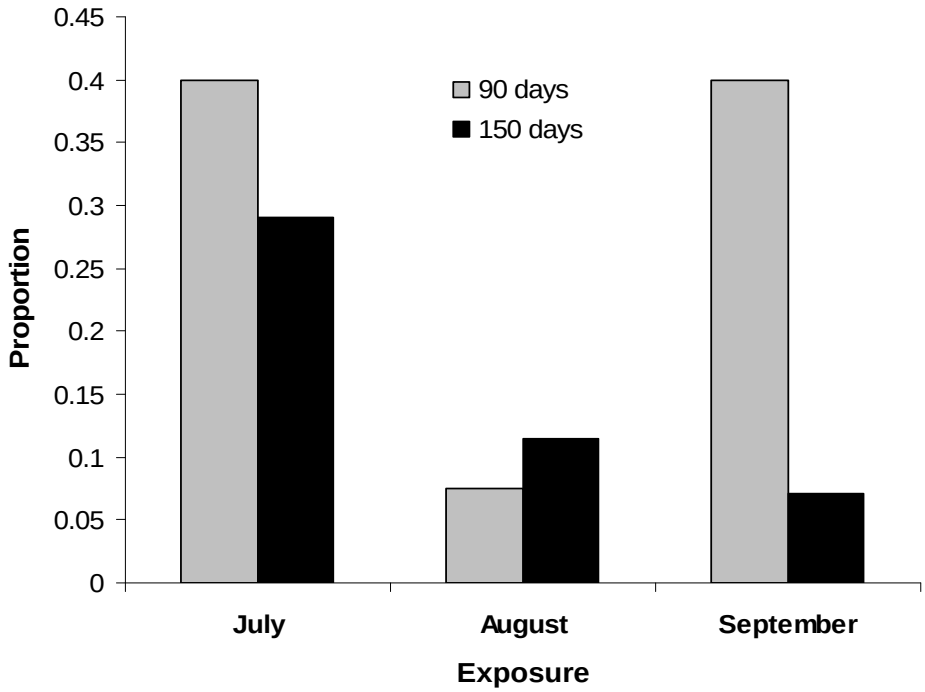

Figure 2.2 Proportion of sentinel Yellowstone cutthroat exhibiting whirling behavior 90 and 150 days post-exposure for the three exposures (July, August, September). Results shown were combined across study sites (Figure 2.1).

Across all three exposures, 8% (10/127) and 2% (5/205) of the fish exhibited skeletal deformities when examined at 90 and 150 days post-exposure, respectively. The frequency of skeletal deformities was higher in fish held for 90 days post-exposure than

in fish held 150 days post-exposure after the July (9%, 4/47; 0%, 0/65; respectively) and September exposures (15%, 6/40; 0%, 0/70 fish; respectively) ($X^2_1 > 7.15$, $p < 0.0075$ for both tests). However, after the August exposure, skeletal deformities were less frequent in fish held for 90 days (0%, 0/40) than in those held for 150 days post-exposure (7%, 5/70) ($X^2_1 = 4.66$, $p = 0.0310$). Within the 90 day post-exposure period, skeletal deformities were less frequent after the August exposure (0/40) than after the July (9%, 4/47) or September exposures (15%, 6/40) ($X^2_2 = 8.85$, $p = 0.012$). Within the 150 day post-exposure period, skeletal deformities were more frequent after the August (7%, 5/70) than after the July (0%, 0/65) or September (0%, 0/70) exposures ($X^2_2 = 10.99$, $p = 0.0041$).

Black tails were seldom observed. Only 6% (8/127) of fish exhibited black tails on day 90 post-exposure and this occurred only after the July exposure. No fish exhibited black tails 150 days post-exposure (n = 205). Thus, we did no statistical tests on prevalence of black tails.

Across all three exposures, the prevalence of infection was 68% in fish held for 90 days (17/25 five-fish samples) or 150 days (29/42 five-fish samples) post-exposure. We detected no difference in prevalence of infection between fish held for 90 or 150 days post exposure after the July (78%, 7/9 and 79%, 11/14, respectively), August (38%, 3/8 and 29%, 4/14 , respectively) and September (88%, 7/8 and 100%, 14/14 fish, respectively) exposures (all three $X^2_1 < 2.11$, $p > 0.1466$). The prevalence of infection was lower after the August (29%, 4/14 fish) than after the July (79%, 11/14) or September (100%, 14/14 fish) exposures in fish held for 150 days post-exposure ($X^2_2 =$

20.67, $p < 0.0001$). The same pattern occurred within the 90 day post-exposure period (July, 78%, 7/9 fish; August, 38%, 3/8 fish; September, 88%, 7/8 fish), but this was a marginally significant difference ($X^2_2 = 5.20$, $p = 0.0744$).

Microscopic Pathology

Infection was most frequent in cartilage of the cranium, lower jaw, and to a lesser degree, cartilage of the upper jaw (Figure 2.3). Cartilage of the nares and gill arches were seldom affected. Lesions were only observed twice in the vertebra and thus were not statistically analyzed (and not shown in Figure 2.3). We detected no differences in lesion severity scores in all regions of cartilage and overall inflammation between section depths in fish held for 90 or 150 days post-exposure (all $p > 0.0881$). Within section depth, the frequency of fish with microscopic lesions in the upper jaw was higher in fish held for 90 than 150 days in both the medial ($X^2_4 = 10.873$, $p = 0.0280$) and lateral sections ($X^2_4 = 17.432$, $p = 0.0016$) (Figure 2.3c). Severity of microscopic lesions did not differ between fish held for 90 or 150 days for all other regions of cartilage examined and for overall inflammation in both section depths (all $p > 0.1582$) (Figure 2.3).

Discussion

Whirling disease may be a significant stressor to an already declining population of Yellowstone cutthroat trout in Yellowstone Lake. We investigated the susceptibility of Yellowstone cutthroat trout to whirling disease and demonstrated that (1) they can be highly infected, especially in cartilage of the cranium and lower jaw; (2) it is sufficient to

examine them 90 days post-exposure to determine prevalence and severity of infection; and (3) lesion severity was similar between medial and lateral head sections.

Based on our results of infection prevalence (clinical signs and PCR) and disease severity (histology) in Yellowstone cutthroat trout, especially in the cranium, this salmonid's susceptibility to *Myxobolus cerebralis* infection may be high. Because damage in this area can significantly compromise long-term survival (Vincent 2002) our findings suggest that population level declines of infected Yellowstone cutthroat trout are likely. Damage to jaw cartilage and consequent deformities can hinder the fish's ability to feed normally (El-Matbouli et al. 1992, MacConnell and Vincent 2002) and further compromise survival in the wild. No prior studies have examined location of lesions in subspecies of cutthroat trout, information which is important in order to assess the risk of whirling disease among native trout populations.

Prompt detection and management for whirling disease in the wild are critical for the long term survival of the Yellowstone cutthroat trout. We suggest that prevalence and severity of infection in native cutthroat trout be examined 90 days after parasite exposure. Our results on infection prevalence and disease severity showed no significant difference between 90 and 150 days post-exposure. Likewise, whirling behavior was not significantly different between 90 and 150 days post-exposure, except in September when whirling was more prevalent at 90 than at 150 days after exposure. Skeletal deformities were also more prevalent 90 than 150 days post- exposure, except in August. Hence, it would be unnecessary to hold and maintain Yellowstone cutthroat trout in live aquaria for longer than 90 days after parasite exposure.

Furthermore, although histological examination of Yellowstone cutthroat fry at two different depths showed no statistical difference in severity of microscopic pathology, this could have resulted if our sections were not far enough apart. When we examined sections 500-600 μm apart, some fry with no apparent infection, showed moderate to high infections in the second (deeper) section (S. Murcia unpublished data). Therefore, we recommend additional testing for such infection differences by histologically examining fish heads at more than one depth, preferably with sections > 400-500 μm apart, or more for larger heads. Fry over 6 or 7 weeks of age can be large enough that histological analyses at a single cross-section of the head, 5 μm in thickness, may provide incomplete and inaccurate information on infection patterns. Fish heads are complex, three-dimensional structures where *Myxobolus cerebralis* is likely to reside, in variable amounts across given tissues, within different size or species of fish.

Susceptibility to *Myxobolus cerebralis* varies among different species and sub-species of salmonids (O'Grodnick 1979, Hedrick et al. 1999a,b, MacConnell and Vincent 2002), and manifestation of disease differs as well. Whirling behavior was the most frequent clinical sign we observed in Yellowstone cutthroat trout, whereas it was very seldom reported of brown trout (Hedrick et al. 1999b), and rainbow trout (Ryce et al. 2004). We observed marked skeletal deformities of the head (especially at the jaw) in 8% of sentinel Yellowstone cutthroat fry 90 days post-exposure. Black tail was the most prevalent manifestation of whirling disease in brown and rainbow trout (Hedrick et al. 1999b). However, this was the least prevalent manifestation of disease we observed in

Yellowstone cutthroat trout, with only a few fry from the July exposure showing black tails 90 days post-exposure.

These differences in external signs of disease among different trout species may reflect the different internal location of lesions caused by *Myxobolus cerebralis* in salmonids. When the parasite invades cartilage of the posterior spinal column, increasing pressure on the caudal nerves controlling pigmentation, it causes the darkened caudal regions (or black tails) of infected salmonids (Halliday 1976, Hedrick et al. 1999b, MacConnell and Vincent 2002). However, cartilage lesions and consequent inflammation restricting the lower brain stem and spinal cord were proposed causes of whirling behavior among infected trout and salmon (Hedrick et al. 1999b, Rose et al. 2000). The high prevalence and severity of lesions in cranial cartilage and inflammation in Yellowstone cutthroat trout may partially explain why whirling was a frequent symptom of disease in this salmonid, but seldom reported of brown trout (Hedrick et al. 1999b) or bull trout (Hedrick 1999b, Vincent 2002). Though we detected lesions throughout cartilage of the cranium in Yellowstone cutthroat comparable to those reported for rainbow trout (Hedrick et al. 1999b, Baldwin et al. 2000, MacConnell and Vincent 2002), the prevalence of black tail was negligible in Yellowstone cutthroat but usually high in rainbow trout (and brown trout) (Hedrick 1999a, Vincent 2002, MacConnell and Vincent 2002). This may suggest that infection in vertebral cartilage of Yellowstone cutthroat trout is rare or if vertebrae do get infected lesions are not as significant as in axial skeleton of rainbow trout (Markiw and Wolf 1974, Baldwin et al. 2000, Rose et al. 2000). For example, 50% of our fry head sections (265 slides) included

vertebrae, 99.2% of which showed no infection in this region. Alternatively, the lack of black tails among Yellowstone cutthroat trout fry may be due to impenetrability of the skin, or peripheral nerve location, in caudal regions in comparison to that of other salmonids (e.g., rainbow trout; El-Matbouli et al. 1995, 1999).

Several investigations describe rainbow trout as the most susceptible salmonid to *Myxobolus cerebralis* infection (O'Grodnick 1979, Hoffman 1990, Hedrick et al. 1998), but Yellowstone cutthroat trout showed infection severity similar to that reported of rainbow trout when exposed to *M. cerebralis*-infected waters in the field (Hiner and Moffitt 2001, Vincent 2002). Genetic similarities among the two salmonid species may exist but this remains a controversial topic. Recent evidence suggests a lower genetic divergence from rainbow trout by Yellowstone cutthroat trout than by other subspecies of cutthroat (Smith et al. 2002), while others report smaller genetic distances between rainbow trout and Westslope cutthroat or Coastal cutthroat trout (*Oncorhynchus clarki clarki)* than between rainbow and Yellowstone cutthroat trout (Allendorf and Leary 1988, Behnke 1992). We found infection levels similar to, or higher than, those reported for younger (10-15 days, Hiner and Moffitt 2001) and older (3 months, Hedrick et al. 1999a) Yellowstone cutthroat trout than ours. Genetic stock differences may, in part, explain such differences in response to infection among Yellowstone cutthroat trout. The genetic make up of cutthroat trout used in previous investigations (e.g., Hedrick et al. 1999a, Hiner and Moffitt 2001) was probably different from that of pure Yellowstone cutthroat trout of Yellowstone Lake tributaries. The native cutthroat trout of the Yellowstone Lake basin were isolated from the downstream watershed during deglaciation about 12,000

years B. P. (Behnke 1992). This isolation combined with the highly stable, nutrient-rich hydrothermal environment of Yellowstone Lake (Kilham et al. 1996) may render the native cutthroat ill-adapted against recently introduced pathogens.

All our study sites tested positive for *Myxobolus cerebralis*. Infection severity and development of clinical signs are directly related to parasite spore concentration (Markiw 1992, Ryce et al. 2004), thus, our results suggest that *M. cerebralis* abounds in Pelican Creek. If instead, parasite concentrations are low in Pelican Creek our results suggest that Yellowstone cutthroat trout are vulnerable to whirling disease even at low spore doses. Spore concentrations of *M. cerebralis* in this tributary were not measured directly (e.g., Thompson and Nehring 2000, Lukins 2004), but we found close similarities in infection response between our Yellowstone cutthroat trout and reports of rainbow trout exposed to controlled, high parasite doses (Hedrick et al. 1999a,b, Ryce et al. 2004). The severity of infection we observed in cranial and lower jaw cartilage could significantly reduce the young fry's ability to survive in the wild.

A number of environmental factors may also influence fish-host susceptibility to whirling disease in natural settings (de la Hoz Franco and Budy 2004, Krueger et al. 2006). In Pelican Creek, the August exposure showed the least prevalence and severity of infection, when mean water temperature was 16^{o}C. *Myxobolus cerebralis* infection among rainbow and cutthroat trout was positively correlated to mean water temperature in Montana (Baldwin et al. 2000) and Idaho (Hiner and Moffitt 2002), but may decline significantly in rainbow trout above a threshold temperature of about 16^{o}C (Hedrick et al. 1998). During our August exposure, mean dissolved oxygen and pH were higher than in

the July and September exposures. This possibly reduced environmental stress and thereby the fry's disposition to infection, or increased their ability to cope with infection. Cutthroat trout require waters with high dissolved oxygen content and a slightly basic pH ranging 6.5 – 8.0 (Hickman and Raleigh 1982). In contrast, the most prevalent and severe infection generally occurred during the July exposure, when mean water temperature was nearly 20^{o}C and dissolved oxygen was low. The stress of warm, widely fluctuating temperatures may have weakened the physiological condition of sentinel fry (e.g., Schaperclaus 1992, Hedrick 1998). Despite mean water temperature being favorable for cutthroat trout during the September exposure, specific conductivity was higher than the July and August exposures. Conductivity was positively correlated with salmonid infection risk (Hiner and Moffit 2001, 2002, Sandell et al. 2001) and infection was high during our September exposure (especially 90 days post-exposure).

The native Yellowstone cutthroat trout is currently listed as a species of special concern by state and federal agencies and the Pelican Creek population has already shown a dramatic decline since the mid 1980's (Koel et al. 2005, Koel et al. in press). Establishment of *Myxobolus cerebralis* in this and other tributaries may contribute to further reductions in the Yellowstone Lake population of native cutthroat trout. It is therefore important that we continue to investigate this system as it offers a great opportunity to study the dynamics of whirling disease in the natural environment.

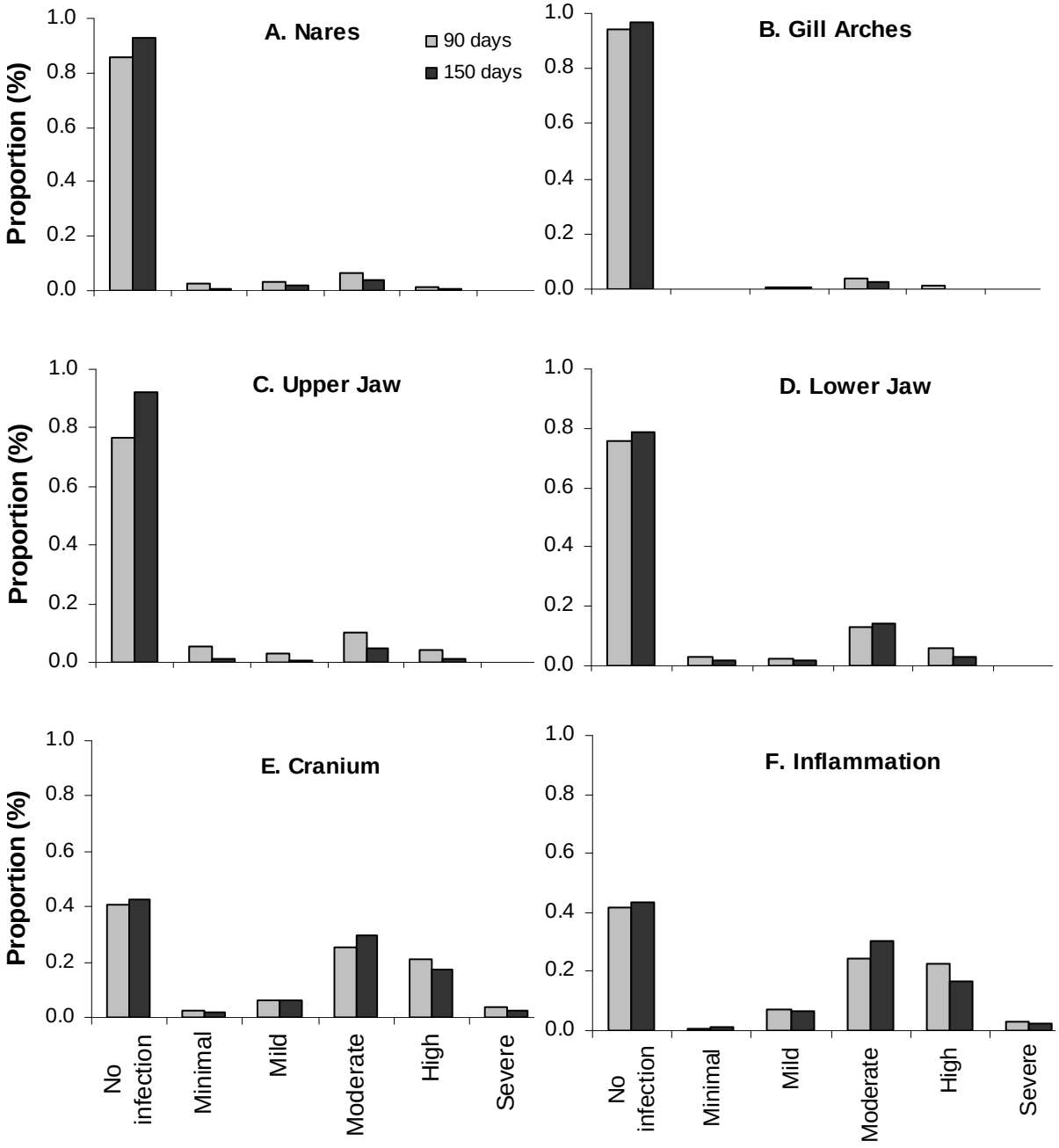

Figure 2.3 Severity and location of microscopic pathology in PCR-positive Yellowstone cutthroat fry; A-E) five regions of head cartilage, and F) inflammation, were histologically examined and individually rated 90 and 150 days post-exposure (lesion severity categories described in Table 2.1). Results were combined for the three exposures, study sites, and histological section depths.

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CHAPTER THREE

CORRELATING ENVIRONMENTAL ATTRIBUTES WITH HISTOPATHOLOGY OF NATIVE YELLOWSTONE CUTTHROAT TROUT NATURALLY INFECTED WITH *MYXOBOLUS CEREBRALIS*

Abstract

Most environments impose periodic or stochastic stress on natural populations, which increase susceptibility to diseases, often accelerating host population declines. Infection by *Myxobolus cerebralis* (exotic parasite causing salmonid whirling disease) is strongly influenced by a stream's physico-chemical characteristics, which may also affect host pathology. We examined whether environmental characteristics of a *M. cerebralis*-positive tributary to Yellowstone Lake, Yellowstone National Park, correlated with histopathological anomalies of naturally infected native cutthroat trout *Oncorhynchus clarkii bouvieri*. Host inflammatory response and cranial cartilage lesions were the main correlates with whirling behavior. Results from canonical correlation analyses showed that the prevalence of fish with moderate and higher lesion severity in lower jaw and cranial cartilage was highest in areas with a combination of high day-time temperatures and low specific conductivities. We conclude that *M. cerebralis* infection in salmonids cannot be examined the same in every system and every species. Instead, it should be context-dependent and biologically relevant (to the fish) by including histological examination of various cartilage regions in tandem, as well as including the synergistic effects of potential environmental drivers of disease. Our results have implications for both ecology and parasitology as they reveal that environmental components can affect

when and where a pathogen resides within the host, and thereby affect manifestation of disease. Recognition of the specific environmental attributes most conducive to parasite establishment, and disease, can increase future detection abilities, strengthening the likelihood of correctly predicting *M. cerebralis*' and similar pathogenic invasions and establishment in unsampled sites. The success of eradication and control measures, or management efforts, may rest on our ability to predict new invasions in marginal parasite ranges or new regions.

Introduction

Natural populations are often exposed to seasonal or stochastic environmental stress. Environmental stressors can increase susceptibility to parasitism and disease among host populations, or decrease their ability to survive infection (Sousa and Gleason 1989), by directly affecting the physiology, reproduction, and survival of parasites and their hosts (Schaperclaus 1992; Harvell et al. 2002). A variety of stressor inputs are known to increase stress hormone release (corticosteroids), which reduce natural and acquired resistance to infections (Pickering 1993). A fish's tolerance to pathogens, for instance, will tend to be lower when other stresses operate at the same time (Myers 1995; Lafferty and Kuris 1999). The occurrence and severity of disease thus depends on a range of factors including characteristics of the environment, the host, the pathogen, and their interactions (Hedrick 1998). Understanding how interactions affect pathology of diseased organisms is critical for early detection, and diagnostic and management purposes, but this remains understudied.

Histopathology is important to managers because infection and disease diagnostics change with environmental conditions. Histopathological anomalies in fish are frequently used as indicators of chemical pollution in marine and fresh water environments (Schwaiger 2001; Wester et al. 2002) but, a histopathological approach to assess the effects of environmental stressors on development of disease from parasitic infection in the wild is seldom used. Numerous laboratory studies with terrestrial (e.g., Brown et al. 2003; Greer et al. 2005) and aquatic host organisms (e.g., Sousa and Gleason 1989; Jokela et al. 2005) show that environmental characteristics, such as warming temperatures and water chemistry, can significantly influence parasitic infection and diseases (Schaperclaus 1992; Pickering 1993; Harvell et al. 2002). Hydrothermal and high elevation streams are examples of systems where the physical and chemical environment is variable and can inflict additional stresses on the native biota beyond seasonal or other natural fluctuations. In Lake Wabamun, for example, thermal effluents facilitated parasite transmission between hosts throughout the year and increased prevalence of certain parasites (Sankurathri and Holmes 1976). Yet, we found no examples in the scientific literature of studies examining potential relationships between the environment and microscopic pathology of parasitic infection in the wild.

Given the unpredictable nature of invasions, the ability to identify local abiotic conditions influencing the pathology of infection by *Myxobolus cerebralis* (exotic parasite causing salmonid whirling disease) among native trout will facilitate development of efficient management tools. Effects of parasite invasion may differ across sites and the ability to characterize it and predict its impact requires information

about host and parasite responses to local conditions, and to each other (e.g., Leprieur et al. 2006). This information can then lead to increased vigilance at the sites of most likely establishment, which raises detection rates and, consequently, can expedite response to invasion and early stages of establishment (Hulme 2006).

A stream's physico-chemical characteristics influence infection by *M. cerebralis* in rainbow trout *Oncorhynchus mykiss*, the species most frequently examined. For example, infection prevalence in this salmonid, non-native to the intermountain west of the US, was positively correlated to mean water temperature in Montana (Baldwin et al. 2000; Krueger et al. 2006), Idaho (Hiner and Moffit 2002), Utah (Franco and Budy 2004), and in laboratory studies (Halliday 1976; Markiw 1992). A decreasing severity of whirling disease was noted with decreasing stream flow (Hallett and Bartholomew 2007); and conductivity was positively correlated to increased prevalence of infection and clinical signs (whirling swimming, black tails, skeletal deformities) in sentinel rainbow trout (Sandell et al. 2001). Environmental components may affect when and where the parasite resides within the fish, and thereby manifestation of disease. The parasite spore stages infective to the fish have a chemotactic specificity for salmonids and selectively bind to host living tissue (El-Matbouli et al. 1995; El-Matbouli et al. 1999). Physico-chemical constituents of a stream, such as conductivity, dissolved solids, or water velocity, may play a role in *M. cerebralis*' host recognition, attachment, and burrowing rate into skin (El-Matbouli et al. 1999, Hallett and Bartholomew 2007)

Once inside the fish, the pathogen's affinity for cartilaginous tissue causes variable lesion severity depending on characteristics of the host. In rainbow trout, severe

and lethal pathology occurs in cranial cartilage, but other salmonids survive *M. cerebralis* infection when lesions occur in cartilage of the caudal peduncle area (e.g., *Prosopium williamsoni*; MacConnell and Vincent 2002) or the fin rays and gill arches (e.g., *Salmo trutta*; Hedrick et al. 1999a,b, Baldwin et al. 2000). Little is known, however, about factors influencing the pathology of native Yellowstone cutthroat trout *O. clarkii bouvieri*, or its potential to survive *M. cerebralis* infection, especially in high-country streams of the intermountain west.

Disease can accelerate host population declines or species extinctions (Harvell et al. 2002). If the affected host population is a keystone species its removal by parasitism or disease can have catastrophic ecological consequences (Ernest and Brown 2001). In the Greater Yellowstone Ecosystem, numerous species of birds and mammals depend on the native Yellowstone cutthroat trout for nutrition (Gresswell 1995; Varley and Schullery 1998). In addition, economically, the native salmonid supports an important recreational fishery throughout the region (Varley and Schullery 1998; Koel et al. 2005). Over the last decade, however, *M. cerebralis* (first detected in Yellowstone Lake in 1998), has posed a growing threat on the declining cutthroat trout population there (Koel et al. 2005; 2006; Murcia et al. 2006).

Scientific research predicts that global environmental change, and its consequent increase in pathogen development, survival, disease transmission, and host susceptibility, will increase frequency and severity of disease impacts in most host-parasite systems (Harvell et al. 2002). Therefore, our ability to predict declines and epidemics in wild populations may require that we distinguish individual from interactive effects of

multiple environmental drivers (Harvell et al. 2002) on infection pathology and disease impact.

Here, we investigate the potential relationship between a suite of environmental attributes and histopathology of *M. cerebralis* infection in native Yellowstone cutthroat trout exposed in the wild. This information is paramount to guiding predictions of potential population-level effects (e.g., McCallum and Dobson 1995) and future diagnostic and management efforts. We examined Yellowstone cutthroat fry exposed at four different times in three sites of a *M. cerebralis* – positive tributary to Yellowstone Lake (Koel et al. 2006; Murcia et al. 2006). Our objectives were to determine whether histopathology of infection in Yellowstone cutthroat trout was associated with (1) prevalence of whirling behavior (fish spin until exhaustion) and (2) environmental variables; and (3) whether such associations differed among exposure sites and exposure times.

Methods

Study Area and Field Exposures

Pelican Creek, at the north end of Yellowstone Lake (Figure 3.1), is the lake's second largest tributary. It flows south and west for 53 km from its headwaters to the lake. The stream is low gradient, meandering through a valley of sub-alpine meadows and grass-covered banks. Many of Pelican Creek's over 100 tributaries are hydrothermal springs. The lack of canopy cover throughout most of the stream and warm, hydrothermal influences, lead to high temperature and primary production.

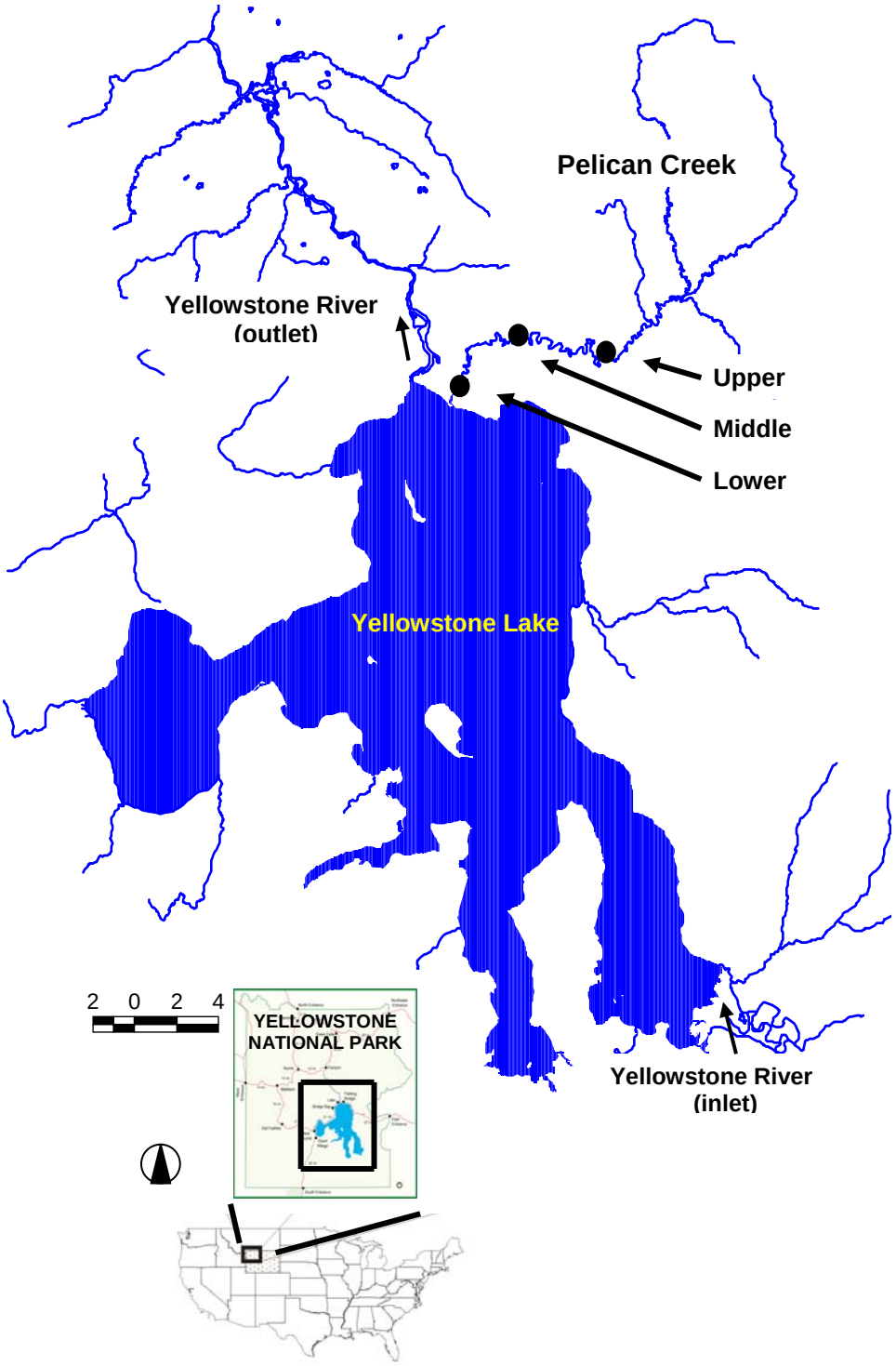

Figure 3.1 Map of Yellowstone Lake, Yellowstone National Park. Circles show study sites in Pelican Creek (*M. cerebralis*-positive tributary) where four 10-day exposures of cutthroat trout were performed using duplicate sentinel cages in July, August, and September 2002, and July 2003.

We examined *M. cerebralis* infection in Yellowstone cutthroat trout using four histopathology response variables and whirling behavior. Variables were measured in fry exposed to the parasite in sentinel cages in their natural environment. Cages were screened, cylindrical enclosures 1 m in height and 50 cm in diameter. Parasite-free Yellowstone cutthroat fry (4 to 6 weeks old) were obtained from the Wyoming Game and Fish Department's broodstock, originating from the Yellowstone River and Clear Creek, a large tributary on the lakes' eastern shore.

Field exposures were conducted as in Murcia et al. (2006) at three sites (upper, middle, lower, Figure 3.1) in the lower 13 km of Pelican Creek. Locations for sentinel cages were selected to represent a wide range of environmental characteristics (e.g., water temperature, velocity) and typical native fry habitat, while considering logistic constraints and minimizing the probability of cages being discovered, or tampered with, by park visitors or wildlife. Cages were replicated (two cages, 50 – 60 m apart) at each site. Sixty fry were exposed in each cage for 10 days in July (10 – 20), August (7 – 17), and September (August 28 – September 7) 2002. In 2003, fry were only exposed in July (8 – 19) because high temperatures and several wild fires in August and September led to high fry mortality or precluded access to the sites entirely. During each exposure, a control group of 60 Yellowstone cutthroat fry were held in the park's Lake Aquatic Resources Laboratory on well water for 10 days and fed a standard commercial trout feed. At the end of each exposure, fry were transported to the Wild Trout Research Laboratory, Montana State University, Bozeman, and held in separate aquaria at 12-13 ^{o}C for 90 days. Holding fish for 90 days allows for parasite development such that

examinations can be undertaken to determine prevalence and severity of infection (Murcia et al. 2006).

Histopathology and Whirling Behavior

On day 90 post-exposure, 10 fry were randomly selected from each aquarium and observed for 30 to 60 s for whirling behavior prior to being sacrificed in tricaine methane-sulfonate. We did not record prevalence of skeletal deformities and black tails because these clinical signs were seldom present (Murcia et al. 2006). Fry heads were removed and half heads prepared for testing by nested polymerase chain reaction (PCR) for *M. cerebralis* DNA (Andree et al. 1998) in pools of five as in Murcia et al. (2006). Nested PCR was used to detect infection presence/absence and thereby identify which fry heads required histological analysis to determine location and severity of pathology. Fry heads that tested positive for *M. cerebralis* DNA by PCR were processed for microscopic examination using standard histological techniques (Humason 1979) as in Murcia et al. (2006). A random sub-sample of 30 PCR-negative half heads was examined to verify lack of infection. We examined lesions in cartilage of the cranium, lower jaw, upper jaw, and inflammation to measure pathology because lesions were rarely observed in cartilage of the nares, vertebrae, and gill arches (Murcia et al. 2006). Microscopic lesions and inflammation were scored on a six category scale of no infection/inflammation, minimal, mild, moderate, moderately severe, and severe (Andree et al. 2002). Fish in the moderate and above categories had several to all cartilage regions with mild to severe degeneration and necrosis, and granulomatous inflammation with small to extensive impact on surrounding tissues, including bone displacement in severe cases (for detailed category

description see Andree et al. 2002). Tissue sections were evaluated blindly – with no knowledge of sentinel cage site or PCR results.

The proportions of fry with moderate or higher inflammation and lesions in cranial and jaw cartilage were used for statistical analysis. Infection severities of moderate and above were considered to be biologically meaningful because below that there is little to no impact on surrounding tissue (Murcia et al. 2006), nor population-level impacts (Vincent 2002).

Environmental Characteristics

In order to identify potential factors influencing pathology of *M. cerebralis* in the wild, we measured several environmental attributes of the study streams as predictor variables. Day-time (8a.m. – 8p.m.) water temperature (^{o}C), specific conductivity (µS), and dissolved oxygen concentration (mg/L) were measured at each cage location during each exposure using a YSI model 85 (Yellow Springs Instruments Inc., Ohio, USA) multiparameter meter. Water velocity (m/s) was measured by setting random transects across the stream channel, perpendicular to the flow direction and adjacent to each cage, and channel depth (m) and current velocity were measured with a Marsh-McBirney Flo-Mate 2000 (Marsh-McBirney Inc., Maryland, USA) flow meter at 10-15 equidistant intervals. Measurements were averaged into one mean depth and velocity measurement per cage location. Due to a malfunctioning flow meter, we could not measure water velocity during the September exposure at the Pelican upper site. Instead, the average of cage 1 and the average of cage 2 from the other three exposures at this site were used as a surrogate of a velocity measure at cages 1 and 2, respectively, for September.

Statistical Analyses

Correlations (Pearson correlation coefficients) were used to determine which of the histopathology measures was best associated with prevalence of whirling behavior. Residuals were examined for homogeneity of variance and no transformation was needed. Canonical correlations were used to examine potential relationships between the set of histopathology (response) variables and the set of environmental (predictor) variables simultaneously (James and McMulloch 1990; Manly 2005). In canonical correlation analysis, linear combinations of the attributes (canonical variables) are created for each data set such that the correlations between canonical variables of the two data sets are maximized. These combinations are analogous to the eigenvectors of principal component analysis (Manly 2005). Eight new canonical variables were created, four from the environmental attributes (ENV_1, ENV_2, ENV_3, ENV_4) and four from the histopathology response measures (PAT_1, PAT_2, PAT_3, PAT_4). The canonical correlations (between ENV_1 and PAT_1, between ENV_2 and PAT_2, etc.) give an overall feel for the degree of association between the environmental and histopathology variables (James and McMulloch 1990; Manly 2005). Only canonical correlations with p-values < 0.05 were considered to be significant, and were identified. All statistical analyses were carried out with the statistical software program SAS 9.1 (SAS Institute 2007).

Results

Histopathology and Whirling Behavior

Across all four exposures, whirling behavior occurred at an average rate of 13% of the fish (50/157, number of fry whirling divided by the total number examined), at the upper site, 30% at the middle, and 54% at the lower. The highest prevalence of fry whirling was observed at the lower site during the July 2002 and September exposures (Table 3.1). Moderate and higher inflammation and cranial lesions were most frequent at the lower site during all exposures (Table 3.1). But, cartilage of the jaws was also highly infected during the 2002 and 2003 July exposures at all sites, except the middle site in July 2002 (Table 3.1). The strongest correlation between histopathology measures and prevalence of whirling behavior was with inflammation ($r = 0.56$, $P = 0.005$), and with lesions in cranial ($r = 0.50$, $P = 0.01$) and lower jaw cartilage ($r = 0.44$, $P = 0.03$) (Figure 3.2).

Histopathology and Environment

Day-time temperature was generally high and specific conductivity low during the July exposures in 2002 and 2003 at all sites, except the upper site in July 2003 and the middle site in July 2002 (Table 3.2). Velocity was lowest at the lower site during the 2002 and 2003 July exposures, and at the middle site in August, when depth was also the lowest (Table 3.2).

Table 3.1 Proportion of Yellowstone cutthroat fry with moderate or higher infection severity (histology score ≥ 3) in each cartilage and inflammation, and proportion of fry exhibiting whirling behavior, after four 10-day natural exposures to *Myxobolus cerebralis* in Pelican Creek, Yellowstone National Park. Shown are the mean proportions for duplicate cages (N = 20 fry) at each site. SE = ± 1 standard error.

Exposure	**Exposure**	**Whirling**	**Histopathology**			
Site	**Period**	**Behavior**	**Cranium**	**Upper Jaw**	**Lower Jaw**	**Inflammation**
UPPER	July - 02	0.32 (± 0.18)	0.45 (± 0.45)	0.25 (± 0.25)	0.25 (± 0.25)	0.45 (± 0.45)
	August	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
	September	0.20 (± 0.2)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
	July - 03	0.0 (± 0.0)	1.00 (± 0.0)	0.59 (± 0.09)	0.50 (± 0.20)	1.00 (± 0.0)
MIDDLE	July - 02	0.50 (± 0.1)	0.40 (± 0.0)	0.10 (± 0.10)	0.10 (± 0.10)	0.85 (± 0.05)
	August	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
	September	0.30 (± 0.3)	0.30 (± 0.10)	0.0 (± 0.0)	0.0 (± 0.0)	0.30 (± 0.10)
	July - 03	0.40 (± 0.0)	1.00 (± 0.0)	0.89 (± 0.11)	0.95 (± 0.05)	0.95 (± 0.05)
LOWER	July - 02	0.80 (± 0.0)	1.00 (± 0.0)	0.45 (± 0.05)	0.90 (± 0.10)	0.40 (± 0.0)
	August	0.10 (± 0.1)	0.30 (± 0.30)	0.0 (± 0.0)	0.0 (± 0.0)	0.30 (± 0.30)
	September	0.80 (± 0.0)	0.70 (± 0.30)	0.40 (± 0.20)	0.20 (± 0.0)	0.90 (± 0.10)
	July - 03	0.45 (± 0.05)	0.85 (± 0.05)	0.79 (± 0.09)	0.90 (± 0.10)	0.85 (± 0.05)

Table 3.2 Environmental attributes measured at three study sites in Pelican Creek, Yellowstone National Park, on two or more days (8a.m.– 7p.m.) during each of four 10-day exposures of Yellowstone cutthroat fry to *Myxobolus cerebralis*. Shown are the means for duplicate cage locations at each site. SE = ± 1 standard error.

Exposure Site	Exposure Period	Temperature (^{o}C)	Spfc. conductivity (µS)	Dissolved oxygen (mg/L)	Velocity (m/s)	Depth (m)
UPPER	July - 02	18.5 (± 0)	278.1 (± 0.05)	9.2 (± 0.01)	0.20 (± 0.06)	0.34 (± 0.03)
	August	11.7 (± 0.05)	329.9 (± 2.00)	10.8 (± 0.26)	0.12 (± 0.04)	0.27 (± 0.05)
	September	11.3 (± 0.05)	280.8 (± 0.05)	6.82 (± 0.14)	0.16 (± 0.03)	0.27 (± 0.05)
	July - 03	14.7 (± 0.15)	286.3 (± 1.00)	8.47 (± 0.06)	0.16 (± 0.02)	0.31 (± 0.02)
MIDDLE	July - 02	16.1 (± 0.10)	236.3 (± 1.30)	10.8 (± 0.05)	0.18 (± 0)	0.32 (± 0.05)
	August	18.5 (± 0.15)	361.8 (± 1.60)	12.0 (± 0.20)	0.04 (± 0.02)	0.18 (± 0.06)
	September	11.0 (± 0.05)	313.1 (± 0.50)	7.39 (± 0.07)	0.13 (± 0.01)	0.28 (± 0.02)
	July - 03	21.0 (± 0)	303.7 (± 2.40)	12.9 (± 0.40)	0.19 (± 0.02)	0.27 (± 0.01)
LOWER	July - 02	22.4 (± 0.05)	300.9 (± 0.20)	7.2 (± 0)	0.16 (± 0.01)	0.27 (± 0.04)
	August	13.7 (± 0.10)	308.4 (± 0.75)	8.94 (± 0.01)	0.23 (± 0.02)	0.23 (± 0.04)
	September	12.4 (± 0.05)	318.7 (± 0.10)	8.15 (± 0.05)	0.24 (± 0.11)	0.25 (± 0.02)
	July - 03	20.0 (± 0)	285.1 (± 1.05)	7.4 (± 0.01)	0.09 (± 0.05)	0.47 (± 0.28)

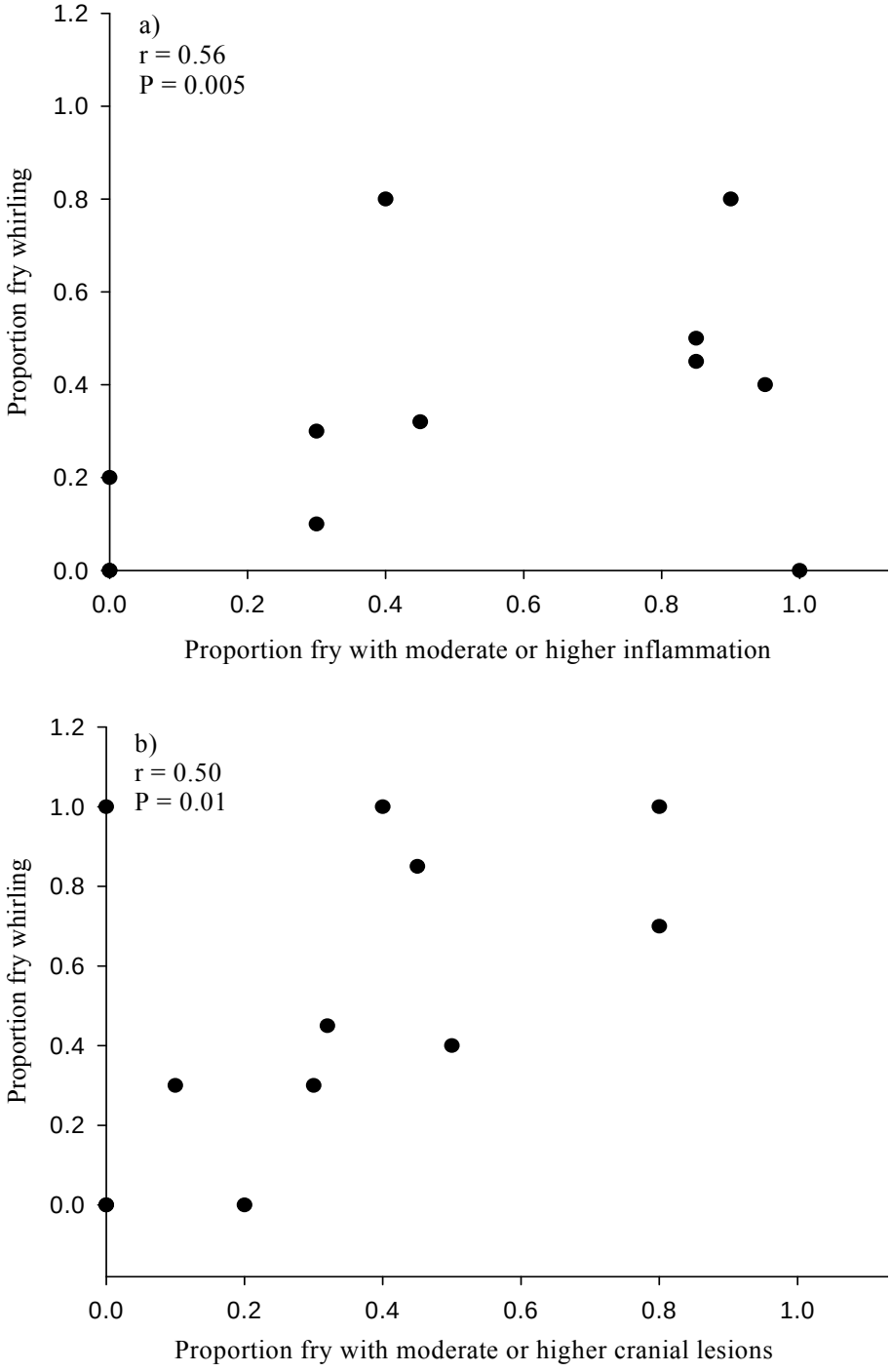

Figure 3.2 Correlation between the proportion of Yellowstone cutthroat trout with a) moderate and higher (score ≥ 3) inflammation and proportion of fry whirling, and b) moderate and higher cranial cartilage lesions and fry whirling proportions. Shown are the means of two cages per site per exposure in Pelican Creek (*Myxobolus cerebralis*-positive tributary to Yellowstone Lake).

We found a high correlation between the first pair of canonical variables ($r = 0.819$; $F_{20, 50.7} = 1.87$; $p = 0.038$), and low correlations between the second, third, and fourth pairs of canonical variables (all $p > 0.376$). ENV_1 was a measure of high temperature and low specific conductivity, and PAT_1 indicated high infection in cartilage of the lower jaw and cranium (and to a lesser extent in the upper jaw; Table 3.3). Thus, only the first pair of canonical variables was identified and interpreted because it explained the strongest relationship between pathology and environmental characteristics.

Table 3.3 Correlations between the environmental attributes measured at Pelican Creek, Yellowstone National Park, and their canonical variables (ENV_1); and the correlations between pathology response measures for *M. cerebralis*-infected Yellowstone cutthroat trout and their canonical variables (PAT_1).

	Correlations with canonical variables		
	ENV1		PAT1
Temperature	**0.915**		
Specific Conductivity	**-0.811**	Cranium	**0.840**
Dissolved Oxygen	-0.035	Upper Jaw	**0.817**
Velocity	0.138	Lower Jaw	**0.982**
Depth	0.263	Inflammation	0.786

Interpretation of the first pair of canonical variables suggested that the frequency of moderate and higher lesion severity in lower jaw and cranial cartilage was high in areas with high temperatures and low specific conductivity. Such patterns were observed at the lower site in July 2002 and at all sites during the July 2003 exposures. These sites had the highest values of the first canonical variable for infection, PAT_1, and the environment, ENV_1 (Figure 3.3).

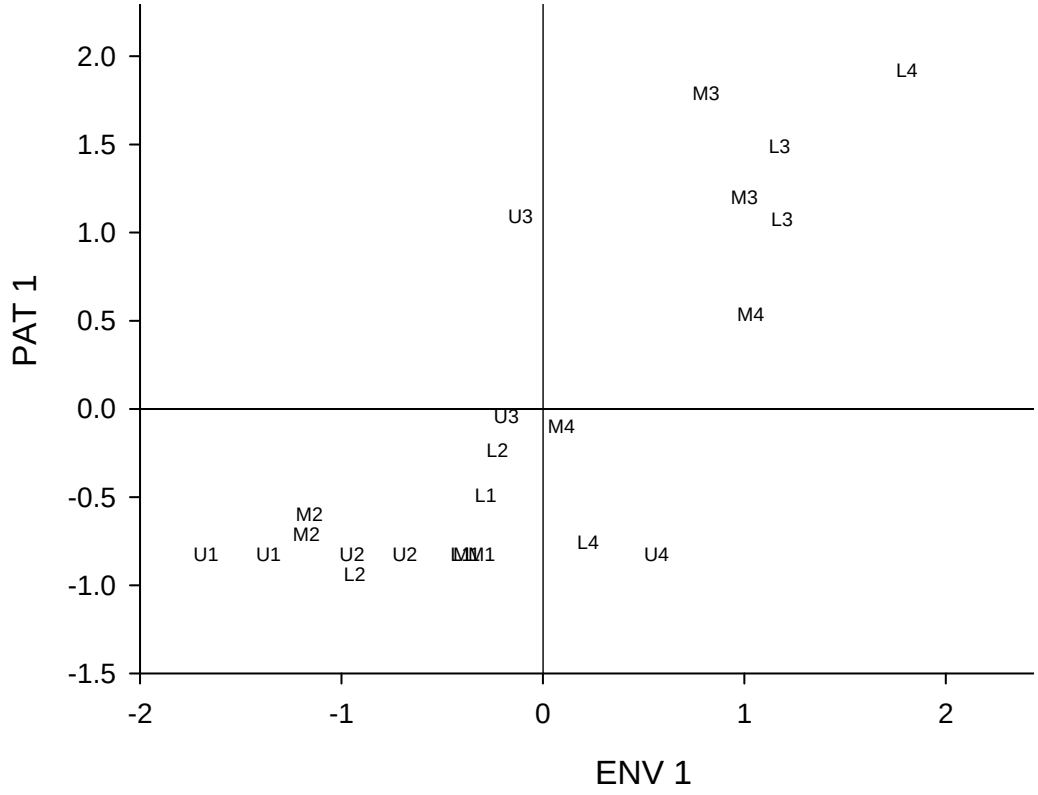

Figure 3.3 Canonical correlations between canonical variables ENV_1 and PAT_1, representing linear combinations of five environmental predictor variables and four pathology response variables. Variables were measured at three sites during four 10-day sentinel fry exposures in Pelican Creek, Yellowstone National Park. Symbols indicate site (L=lower, M=middle, U=upper) and exposure (1=July 2002, 2=August 2002, 3=September 2002, 4=July 2003).

Discussion

Parasitic infection may render hosts more susceptible to adverse environmental conditions than otherwise healthy individuals (Schaperclaus 1992; Jokela et al. 2005). In turn, very small environmental changes may dramatically modify host responses to parasitism (Lenihan et al. 1999; Thomas and Blanford 2003; Jokela et al. 2005). Comprehensively understanding the abiotic mechanisms facilitating pathogenic invasion

and establishment success is paramount to advance our prediction and detection abilities (Leprieur et al. 2006). Our study was designed to test whether exposure of Yellowstone cutthroat trout to *M. cerebralis* under naturally stressful environmental conditions influenced infection pathology (location and severity of lesions) and whirling behavior over multiple sites and exposure times.

Results of correlation analyses suggested a strong relationship between the host's moderate or higher inflammatory response and cranial lesions and whirling behavior. These results were expected because when a pathogen invades cranial cartilage the host's inflammatory response may restrict nerves along the lower brain stem and spinal cord causing the whirling motion observed amongst infected trout and salmon (Hedrick et al. 1999b; Rose et al. 2000). Though this may be a reasonable hypothesis, lesions in cartilage of the lower jaw also correlated with prevalence of whirling behavior. Exceptions occurred in some sites and exposures, however, where fry showed whirling signs though neither inflammation or cranial and jaw lesions were severe. This suggests that factors other than *M. cerebralis* infection, such as other parasites, environmental stressors, or physiological deficiencies may have also caused whirling and abnormal swimming behavior (Schaperclaus 1992; Margolis et al. 1996) among those fry. Alternatively, fry could have severely damaged cartilage and inflammation of surrounding tissue throughout the head and exhibit no overt clinical signs of disease (e.g., Thompson et al. 1999; Sipher and Bergersen 2005). Thus, although clinical signs are an important gauge of overall fish health, they may not be a main indicator of *M. cerebralis*

pathology or population survival potential, as they may reflect internal damage to less vital cartilage regions (Vincent 2002) than the cranium or jaw.

Based on our canonical correlation results, the proportion of Yellowstone cutthroat fry with moderate or higher lesion severity in cartilage of the lower jaw and cranium (and to a lesser extent the upper jaw) was strongly correlated with high water temperature and low specific conductivity. This was most notable at the middle and lower sites in July, when day-time water temperature ranged between 20 and 22.4^{o}C and specific conductivity was 285 to 328 µS. The influence of temperature on *M. cerebralis* infection in rainbow and cutthroat trout has been well recognized (Markiw 1992; Baldwin et al. 2000; Vincent 2002; Franco and Budy 2004). But, few studies have examined the relationship with conductivity (but see Sandell et al. 2001), or the potentially synergistic effects of temperature and conductivity, or additional stressors, and salmonid vulnerability to whirling disease. In Sandell et al. (2001) only conductivity correlated with infection prevalence among sentinel rainbow trout, but not temperature, pH, or total dissolved solids; perhaps because these were not analyzed concurrently but individually against presence/absence of infection.

Although prior research shows an optimal temperature range of 15-17^{o}C for *M. cerebralis* spore production, and infection and development in the fish host (Halliday 1976; MacConnell and Vincent 2002), we did not find a high proportion of moderately to highly infected fish in that temperature range (e.g., middle site in July 2002, 16.1^{o}C). The fact that we were not correlating infection severity with temperature alone but in concert with several other factors might explain these results. Although our

measurements could not capture diel temperature variation, the high day-time temperatures we recorded at severely infected sites are well above the metabolically optimal range requirements of trout (Dickerson and Vinyard 1999; Leprieur et al. 2006). This probably worsened the physiological condition of our sentinel trout and increased their vulnerability to parasitic infection. Then again, at these sites where temperature was highest, specific conductivity remained under 328 µS. Thus, we cannot argue for the importance of one factor over the other, but perhaps for the overlap of the two, compounded with the presence of the parasite in the system, as the likely source for the onset of disease (e.g., Hedrick 1998). Synergisms may arise in the wild when two or more environmental factors interact in such a way that the outcome is not additive but multiplicative (Myers 1995).

Our most severely infected sites were the lower sites of Pelican Creek, which probably resulted from the cumulative effects of spores, warm water (e.g., from extended sun exposure), and similar biotic or physico-chemical stressors carried throughout the drainage from upstream. This is a common pattern of *M. cerebralis* infection in the wild whereby infection prevalence and severity increase in a downstream progression (Hiner and Moffitt 2001; Sandell et al. 2001; Hubert et al. 2002). For example, we observed severe jaw and cranial infection in fry from the lower site in September even under low temperature (12.4^{o}C) and high specific conductivity (319 µS). At this time in the season increased water conductivity probably results from increased summer productivity (Holmes 1990; Edwards and Helvey 1991), or the spring snow melt effects now vanishing, increasing the hydrothermal discharge influences, or a combination of all.

Hence, we suggest that pathology may correlate differently with single environmental attributes or with several factors in unison. We further propose that conclusions on fish host pathology should not be drawn based on site or exposure time alone because pathology in the cutthroat trout host differed between sites and with exposure times. This may imply important spatio-temporal factors in host infection pathology that could otherwise be missed if evaluated on a seasonal or spatial basis alone.

Unlike findings in Oregon (Sandell et al. 2001) and Montana (Krueger et al. 2006), *M. cerebralis* infection in our study system was negatively correlated to specific conductivity – except at the lower site in September. But, a low conductivity range in Pelican Creek (285 – 328 µS) is probably comparable to the high ends of the range in other systems (e.g., Sandell et al. 2001; Krueger et al. 2006). This may indicate a conductivity threshold range of 160 (or 200) µS to approximately 330 µS below and above which prevalence and severity of *M. cerebralis* declines considerably in some systems (e.g., Sandell et al. 2001; Krueger et al. 2006). Those prior studies, however, were conducted with rainbow trout, and did not examine the collective effects of conductivity and other environmental factors against the collective host pathology responses.

Under variable environmental conditions, host pathology will vary depending on many physico-chemical and biotic attributes and their synergism (Myers 1995; Lenihan et al. 1999). We propose that *M. cerebralis* infection in salmonids cannot be examined in the same manner in every system and every species (e.g., Hedrick et al. 1999a,b), but should be context-dependent and biologically relevant (to the fish) by including

histological examination of various cartilage regions in tandem. For whirling disease diagnostic and sampling purposes, examination of cranial cartilage should be an effective means of assessing pathology in most salmonids because this organ is most consistently and intensely damaged. Biologically, however, lesions in the jaw are extremely important for survival and feeding purposes, and should also be assessed.

We know of no prior investigation linking *M. cerebralis*-host pathology in detail to natural environmental stressors, but can identify some plausible explanations for our results. Though current knowledge of the immune response to *M. cerebralis* in the salmonid host is still limited, initial response is reportedly stimulated early in infection with macrophage defense just below skin surface and during the active feeding phase of the parasite: during cartilage destruction (El-Matbouli et al. 1999; MacConnell and Vincent 2002). Presumably, *M. cerebralis* can then escape the host immune response by migrating to cartilage through the nervous system (Halliday 1976; El-Matbouli et al. 1999). The cellular immune response, however, varies considerably among host salmonid species (Baldwin et al. 2000; Hedrick et al. 1999a,b; MacConnell and Vincent 2002). In Yellowstone cutthroat trout moderate to severe lesions were consistently most prevalent in lower jaw cartilage. It is possible that the parasite enters its host through the gills (operculum) and preferentially resides and consumes the closest cartilage, that of the lower jaw, instead of taking the longer migration to reach rib, vertebral, or some further cartilaginous tissue. Alternatively, in traveling to those areas in cutthroat trout *M. cerebralis* might encounter greater resistance from the host immune response, such as dense eosinophilic granular leukocytic (EGL) response in nerve ganglia (El-Matbouli et

al. 1995; Hedrick et al. 1999a; MacConnell and Vincent 2002), and thus greater difficulty reaching upper jaw, cranial, and further cartilage. A distinct EGL response was observed in the nerves of various *M. cerebralis*-exposed salmonids (Hedrick et al. 1999a), suggesting that in these fish those cells play a role in eliminating or preventing the parasite from reaching cartilage (Hedrick et al. 1999a; MacConnell and Vincent 2002). The diverse location of parasite lesions observed among salmonid species and subspecies (Hedrick et al. 1999a) may consequently reflect the different host immune responses of fish, under variable ambient conditions, and diverse geographic areas.

The interactions between the native cutthroat trout, *M. cerebralis*, and the naturally harsh environment of Pelican Creek, are an example of how the effects of an invasive parasitic species relate habitat, disease, and fisheries management (e.g., Lenihan et al. 1999). In Yellowstone cutthroat trout cranial and lower jaw cartilages were the worst afflicted by this pathogen, especially in fish under the combined stresses of high temperature and conductivity in the 200 to 330 μS range. The native cutthroat trout of the Yellowstone Lake basin are liable to become more susceptible to whirling disease and other parasitic or infectious diseases in the wake of high-temperature and productivity (elevated atmospheric CO_2) stresses that are likely to accompany global warming (Holmes 1990; Dukes and Mooney 1999; Lenihan et al. 1999; Marcogliese 2001).

Most often, invasive pathogens and other invaders are opportunists taking advantage of environmental mismanagement and degradation (Manchester and Bullock 2000; Hulme 2006). Even a relatively-pristine and protected ecosystem such as that of Yellowstone Lake and its tributaries has shown the effects of changing climate over the

last decade, with stream temperatures rising and stream flows declining daily, and remaining above and below long term averages, respectively (U.S.G.S. stream gauging stations). As global climate continues to change so will the environmental conditions within these stream systems (Cook et al. 2004). Our results suggest that rising temperatures will be beneficial for the proliferation of *M. cerebralis* and (potentially) other, similar pathogenic invaders. Additional components of global climate change we are yet to examine, such as increased nitrogen deposition and atmospheric CO_2 concentration (Dukes and Mooney 1999) may also influence success of invasive species. We suggest that the occurrence of *M. cerebralis* may be indicative of future incursion by similar or other species in the same or similar sites.

This has implications for management because alteration of habitat, or similar management actions to mitigate for an invasion are seldom, if ever, an option within protected areas such as national parks. Only through the prevention of introduction or establishment will the integrity of these areas be maintained. Possible tactics against further parasite establishment and spread across the Yellowstone Lake ecosystem and beyond should include increased public education and heightened vigilance against the transfer of potentially parasite spore-laden (e.g., muddy and/or wet) camping and fishing equipment among streams (e.g., Leprieur et al. 2006; Gates 2007). Although construction is typically minimal in protected areas, an important preventive measure should also include close monitoring of ongoing construction (e.g., roads) or similar projects near streams which have the potential to cause environmental degradation. Degradation through changes to the physical and chemical environment of streams (e.g.,

substrates, sinuosity, sediment deposition, surface and groundwater chemistry, water table stage; Hulme 2006) may likely facilitate parasite establishment and onset of disease. Continued research and monitoring are necessary in order to discover new biotic and abiotic stressors (singly or in tandem) that might facilitate pathogenic invasions, and to improve our alertness and predictions.

This salmonid is of significant conservation concern throughout the Greater Yellowstone Ecosystem and the Pelican Creek population has already declined notoriously since the mid 1980's (Koel et al. 2005; 2006). It is therefore important that we continue to study the role of biotic and environmental synergisms on the dynamics of whirling disease in the natural environment. Defining areas of high risk is important not only for systems within Yellowstone National Park but outside park boundaries as well, and thus implications are beyond the local or stream scale.

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CHAPTER FOUR

SPATIOTEMPORAL VARIATION IN WHIRLING DISEASE AMONG NATIVE CUTTHROAT TROUT IN THREE, ENVIRONMENTALLY DISTINCT TRIBUTARIES TO YELLOWSTONE LAKE, YELLOWSTONE NATIONAL PARK

Abstract

Ecological invasions by non-native species are now extremely common components of every environment on Earth, altering natural community structures and ecosystem dynamics. In the Greater Yellowstone Ecosystem, invasion by the parasite causing salmonid whirling disease (*Myxobolus cerebralis*) has already reduced native cutthroat trout population numbers in the Yellowstone Lake basin, and may soon damage natural ecosystem dynamics of the lake and tributary streams. In summer 2002 and 2003 we performed a series of sentinel cutthroat trout exposures and habitat assessments in various reaches of three spawning tributaries to the lake to identify spatiotemporal patterns of infection severity, and physicochemical features of the streams potentially facilitating parasite invasion and establishment. Results of principal component analysis (PCA) of physical features correlated with infection in lower jaw cartilage of cutthroat trout and the PCA of chemical features correlated with infection in cranial cartilage. Infection in the lower jaw increased as day-time water temperature increased and velocity decreased. Cranial infection tended to be high in naturally enriched stream waters with high ionic levels (sulfate, total phosphorus, chloride). Percent organic material in the sediments and percent of fine sediments did not predict cranial or lower jaw infection severity. We suggest that the extent to which environmental features vary (range sizes)

across short spatiotemporal scales within a stream or season, rather than the specific features alone, is perhaps what predisposes salmonids to infection and facilitates parasitic invasion. The native cutthroat trout of the Yellowstone Lake Basin face increasing threats from other non-native invasions in these and nearby systems, and their long-term survival is crucial to maintaining the Yellowstone Lake Ecosystem function.

Introduction

Invasive non-indigenous species, such as parasitic and disease-causing organisms, have become one of the world's most costly threats to native biodiversity and natural ecosystem function. Introductions of animal or plant pathogens often lead to native species' population declines, extinctions, or range restrictions, altered community composition, and immeasurable economic costs (OTA 1993, Mack et al. 2000). For example, introduction of the parasitic sea lamprey (*Petromyzon marinus*,) in the Great Lakes of North America devastated the stocks of native lake trout (*Salvelinus namaycush*), burbot (*Lota lota*), whitefish (*Coregonus clupeaformis*), and lake herring (*Alosa* sp.) (Lawrie 1970, Christie 1974, Leach 1976). This added to economic losses of nearly $ 1 billion per year due to the negative effects of non-native fish on native aquatic biota (Pimentel et al. 2000).

Myxobolus cerebralis (myxozoan parasite causing salmonid whirling disease; Hoffman 1990, Hedrick et al. 1998), has enormous potential to similarly affect the native trout of the Intermountain West of North America. Endemic to Eurasia, this pathogen was introduced to the United States in the 1950's and currently exists in the wild in 25 states

(Hoffman 1990, Bergersen and Anderson 1997, Bartholomew and Reno 2002, Arsan et al. 2007). The parasite has been linked to severe declines in some populations of non-native, wild rainbow trout (*Oncorhynchus mykiss*; Walker and Nehring 1995, Vincent 1996, Schisler et al. 2000). Cutthroat trout are native to the Intermountain West, coexist in many watersheds with non-native salmonids, and are also highly vulnerable to *M. cerebralis* infection (Wagner et al. 2002a, Murcia et al. 2006). Given their ecological and economic value, information is needed on the factors influencing whirling disease in the wild to assess the risk posed to native salmonid populations and to guide management efforts.

Since *M. cerebralis* was first described as a detrimental wild fish parasite in the United States in the 1990's, research has confirmed a two-host life cycle. Each host produces a spore that is infective to the other host, with myxospores infecting the oligochaete *Tubifex tubifex* and the actinospores infecting the fish (Markiw and Wolf 1983, Wolf and Markiw 1984). Whirling disease can significantly reduce the fish's ability to avoid predators and feed normally (El-Matbouli et al. 1992), increasing mortality among hatchery and wild salmonids (Walker and Nehring 1995, Vincent 1996). When fish die myxospores are released into the water and sediments where they can be ingested by *T. tubifex*, completing the parasite's life cycle (El-Matbouli et al. 1995, El-Matbouli and Hoffman 1998).

In the wild, prevalence and severity of whirling disease among salmonids vary on large (within and across watersheds) and small (within streams) spatial scales (Hiner and Moffit 2001, Cavender et al. 2002, Downing et al. 2002, Krueger et al. 2006, McGinnis 2007). Rainbow trout populations have declined in some drainages in Colorado (Walker

and Nehring 1995) and Montana (Vincent 1996, Baldwin et al. 2000), but not in California (Modin 1998) and Oregon (Sandell et al. 2001). Factors contributing to variable parasite incidence and severity among different aquatic systems and geographic areas remain unclear. Potential causes include features of the physical and chemical environment, the ecologies of the oligochaete and fish hosts, or complex interactions among these attributes (Kerans and Zale 2002). The life histories of both, salmonids and *T. tubifex* are directly influenced by water temperature, as is spore development in each host, and thus spore production, release, survival, and abundance in aquatic systems (Markiw 1992, El-Matbouli et al. 1999, Blazer et al. 2003, Kerans et al. 2005). Water velocity can be inversely correlated to severity of salmonid whirling disease (Hallett and Bartholomew 2007), *T. tubifex* abundance, and triactinomyxon densities (Krueger et al. 2006, Lukins et al. 2007). At the stream scale, conductivity and percent organic content in the sediment have been positively correlated with infection risk in both salmonid and oligochaete hosts (Hiner and Moffit 2001, 2002, Sandell et al. 2001, Krueger et al. 2006).

The Yellowstone Lake Ecosystem, with its subpopulations of cutthroat trout, provides an excellent system to identify mechanisms responsible for the variable effects of *M. cerebralis* in the wild. Both, *M. cerebralis* and the oligochaete host, occur in the system (Koel et al. 2006a) and the highly diverse physicochemical features of the streams provide great potential for assessing possible factors contributing to parasite invasion and establishment success. This information is key to help balance management priorities among this and other invasive species in the system. Besides *M. cerebralis* (first detected in the lake in 1998), other non-native species (e.g., lake trout; Ruzycki et al. 2003), have

increasingly threatened the long term survival of this valuable fishery over the last decade.

Invasions of all types are often more successful on island habitats, such as oceanic islands, parks, and reserves (Loope and Mueller-Dombois 1989, Marvier et al. 2004). Also, environments predisposed to invasion can be those under frequent disturbance or most affected by climatic changes (Hulme 2006). Even a protected and relatively-pristine ecosystem such as that of Yellowstone National Park has shown the effects of changing climate over the last decade, with stream temperatures rising and stream flows declining (U.S.G.S. stream gauging stations, unpublished data). As global climate continues to change across the Intermountain West (Cook et al. 2004, Arnold and Koel 2004) so will the environmental conditions within the Yellowstone Lake basin and its stream systems, further threatening the long-term survival of native cutthroat trout populations.

The goal of this study was to identify spatiotemporal variation in infection severity among Yellowstone cutthroat trout in three spawning streams where incidence of *M. cerebralis* had been confirmed (Koel et al. 2006a). The objectives were to detect potential relationships between infection severity in sentinel cutthroat fry and the streams' environmental characteristics. This was to identify potential factors influencing parasite incidence and disease severity in streams, and thereby, assist in guiding the park's fisheries management efforts. This study was one of three precursors to our ecological risk assessment for whirling disease in the Yellowstone Lake Basin.

Methods

Study Area

Yellowstone Lake is 2,360 m above sea level in southeastern Yellowstone National Park of northwest Wyoming (Figure 4.1). The lake has 124 tributaries and approximately 60 of these were, at one point at least, used by cutthroat trout for spawning (Gresswell et al. 1994, Keading et al. 1996). Testing near the mouth of lake tributaries between 1999 and 2001 confirmed the presence of *M. cerebralis* in Clear Creek, Pelican Creek, and the Yellowstone River outlet (Koel et al. 2006a). Infection gradients across these tributaries ranged from mild in Clear Creek, to moderate in the Yellowstone River outlet, to severe in Pelican Creek (Koel et al. 2006a).

The Yellowstone River outlet flows north and west as it leaves the lake at its northern shore (Figure 4.1). Before reaching the Canyon and Upper Falls, 26 river Km (Rkm) downstream, the river is low-gradient and 120 – 150 m in width, generally meandering through meadows of grass-covered banks. Approximately 7 Rkm below the lake, past LeHardy Rapids (Figure 4.1), the Yellowstone River flows through the old lake-bed deposits of Hayden Valley for another 9 – 10 Rkm. Closed to fishing, this broad valley dominated by sagebrush and grasses, is heavily used by wildlife such as bison (*Bison bison*), elk (*Cervus elaphus*), Canada geese (*Branta canadensis*), and pelicans (*Pelecanus erythrorhynchos*). Two tributaries to this section of the Yellowstone River are Trout Creek and Alum Creek, which enter the river from the west side almost 15 Rkm and 20 Rkm below the lake, respectively (Figure 4.1). These creeks transport large quantities of sediments into the river, forming large deltaic deposits at their mouth

(Skinner 1977 in Kelly 1993). Nez Perce Ford is a side channel to the main stem of the Yellowstone River, approximately 12 Rkm below Yellostone Lake. This section represented important cutthroat spawning and fry rearing habitat (Kelly 1993). These sections in Hayden Valley of the Yellowstone River have potentially high organic content in the sediments (likely *T. tubifex* habitats), or comprise important cutthroat trout spawning and rearing habitat (Kelly 1993), or both.

Pelican Creek, the lake's second largest tributary, flows south and west for 53 Rkm from its headwaters to the lake (Figure 4.1). Most of the stream is low gradient, meandering through a wide valley of sub-alpine meadows and grass-covered banks (0.5 – 1 m above the water). Many of Pelican Creek's >100 tributaries are hydrothermal springs (Gresswell et al. 1994). The lack of canopy cover throughout most of the stream and warm, hydrothermal influences, lead to high temperatures and primary production. Flocks of geese, and herds of bison and elk also frequent most of Pelican Creek possibly exacerbating stream bank erosion, and organic content in the sediments, probably increasing stream temperature and productivity.

Clear Creek flows west for nearly 20 Rkm from the Absaroka Mountains before entering Yellowstone Lake on the northeast shore (Figure 4.1). This is a first-order, freestone stream that flows through forests of lodgepole pine (*Pinus contorta*), spruce (*Picea* spp), and sub-alpine fir (*Abies lasiocarpa*), and a few small, open meadows. The high elevation and its heavy canopy cover make this creek the coldest of the three study streams (mean daily 9^{o}C; field data) with little primary production.

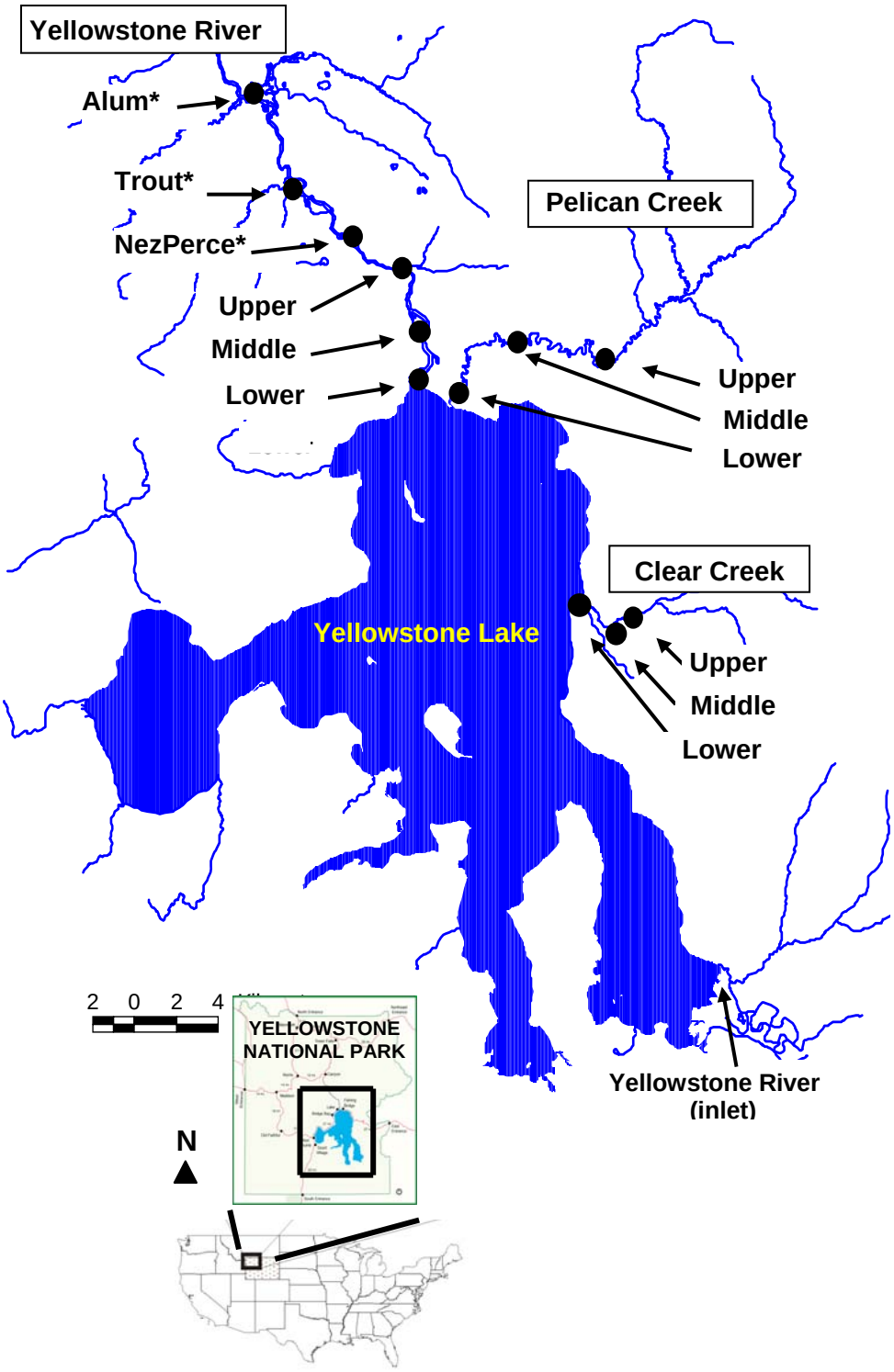

Figure 4.1 Map of Yellowstone Lake. Circles show study sites where 10-day exposures of cutthroat trout were performed using duplicate sentinel cages in July, August, and September 2002; July and August 2003 *Three new sites in the Yellowstone River were tested in July and August 2003 using a single, non-replicated cage per site.

Experimental Design

Field exposures were conducted as in Murcia et al. (2006, in review)), but for this study, we deployed sentinel cages at three sites (upper, middle, lower, Figure 4.1) along the upper 13 Rkm of the Yellowstone River outlet, below Yellowstone Lake, the lower 13 Rkm of Pelican Creek, the lower 2 Rkm of Clear Creek, and at three additional sites (NezPerce, Trout, Alum, Figure 4.1) in 2003 along 10 Rkm of Hayden Valley in the Yellowstone River. Locations for sentinel cages were selected to represent a wide range of environmental characteristics and typical native fry habitat (the age when trout are most susceptible to the parasite (Ryce et al. 2005), while considering logistic constraints. We placed two cages (60 – 70 m apart) along the stream bank at each study site, but due to logistics and time constraints, in 2003 we placed a single sentinel cage at three of the six sites in the Yellowstone River (i.e., in addition to replicated cages at the other three sites) and at two of the three sites in Clear Creek. Four study sites in the Yellowstone River (2002 – 2003) were on side channels to the main stem. Sentinel cages were screened cylindrical enclosures about 1 m in height and 50 cm in diameter.

Parasite-free Yellowstone cutthroat fry (4 to 6 weeks old) to use in field exposures were progeny of the Yellowstone River or Clear Creek that were collected annually as embryos by the Wyoming Game and Fish Department's Hatchery programs. Sixty fry were exposed in each cage for 10 days in July (10 – 20), August (7 – 17), and September (August 28 – September 7) 2002. In 2003, fry were only exposed in July (8 – 19) and August (5 – 15) because high temperatures and several wild fires in late August and September led to high fry mortality or precluded access to the sites entirely. During

each exposure, a control group of 60 Yellowstone cutthroat fry were held in the park's Lake Aquatic Resources Laboratory on well water for 10 days and fed a standard commercial trout feed. At the end of each exposure, fry were transported to the Wild Trout Research Laboratory, Montana State University, Bozeman, and held in separate aquaria at 12-13 ^{o}C for 150 days. Holding fish for 90 – 150 days allowed for development of *M. cerebralis* myxospores such that prevalence and severity of infection could be examined (e.g., Hedrick et al. 1999b, Vincent 2002, Ryce et al. 2005, Murcia et al. 2006).

Infection Severity

On day 150 post-exposure, 10 fry were randomly selected from each aquarium and sacrificed in tricaine methane-sulfonate. Fry heads were removed and half heads prepared for testing in pools of five by nested polymerase chain reaction (PCR) for *M. cerebralis* DNA (Andree et al. 1998). Nested PCR was used to detect the presence of infection and thereby identify which fry heads required histological analysis to determine location and severity of pathology. Fish from (pooled) samples that tested positive for *M. cerebralis* DNA by PCR were individually processed for microscopic examination, to determine lesion severity, using standard histological techniques (Humason 1979) as in Murcia et al. (2006). Disease severity was measured by the proportions of fry with moderate or higher severity of infection (Krueger et al. 2006) in cranial and lower jaw cartilage because these are the regions most consistently damaged by the parasite in Yellowstone cutthroat trout (Murcia et al. 2006). Infection severities of moderate and above (Andree et al. 2002) were considered to be biologically meaningful because below

that there is little to no impact on surrounding tissue (Murcia et al. 2006), nor population-level impacts (Vincent 2002).

Environmental Characteristics

We quantified physical and chemical attributes of the study sites as predictor variables to identify potential factors influencing *M. cerebralis* infection in the wild. The physical attributes measured were stream day-time temperature, depth, current velocity, percent fines and percent organic content in the sediments. The chemical attributes measured were water conductivity, dissolved oxygen concentration, sulfate, total phosphorus, ammonium, and chloride concentrations a triple space above and a double space (1 blank line in-between text) below second level headings; align with the left margin. Capitalize, punctuate and underscore the same as first level headings. If a second level heading is longer than half the width of the page, single-space.

Physical Variables: Water temperature (^{o}C) was measured manually using a Yellow Springs Instruments Inc. (Yellow Springs, Ohio; YSI model 85) multiparameter meter. We measured day-time (8a.m. – 8p.m.) temperature two or more times during each 10-day exposure at each cage location because access to sites was restricted after 7p.m. The mean (± SE) temperature per site was calculated for a total of three measurements per stream per exposure for statistical analysis. Water velocity (m/s) and depth (m) were measured with a Marsh-McBirney Flo-Mate flow meter at 8-12 equidistant intervals along transects set across the stream channel, perpendicular to the flow direction, and adjacently to each cage (two transects per site). If we could walk

across the stream (e.g., when in side channels) width (m) was also measured. If we could not walk across (e.g., Yellowstone River), we measured velocity and depth up to 2-3 m to each side of the cage. We averaged measurements into one mean depth and velocity value per site per exposure for statistical analysis. In 2003 we measured velocity with one more replicate per site by randomly setting three transects across the stream, up to 25-30 m up- or downstream of each sentinel cage. Along these transects we measured channel depth and current velocity with the same flow meter at 10 equidistant intervals for a total of 30 measurements per cage. These measurements were then averaged into one mean (± SE) depth and velocity measurement for each cage. For each variable (depth and current velocity measured by each of the two cages) two mean values were then averaged into a mean (± SE) for each site and exposure period.

Chemical Variables: Conductivity (μS) and dissolved oxygen concentration (mg/L) were measured with the same YSI multiparameter meter once or twice during each exposure, at each cage, and averaged by study site for a total of three measurements per stream per exposure. Also, from each study site one water sample was collected during each exposure in 2002. Each sample was chemically analyzed for nutrients and major chemical constituents in the laboratory at the Great Lakes Water Center, University of Wisconsin, Milwaukee. A total of three measurements of sulfate (SO_4, μM), total phosphorus (TP, μM), ammonium (NH_4, μM), chloride (Cl^-, μM), conductivity, and dissolved oxygen concentrations per stream per exposure were used for statistical analysis.

Substrate Characteristics: At random locations (between the two cages) along transects in 2003, three wet sediment samples were collected with a small trowel to quantify percent organic material following standard laboratory protocols (Wetzel and Likens 1991). We calculated the mean (± SE) from a total of three percent values of organic content in the sediment for each site. Sediment size composition at each site was assessed in 2003 using two techniques: the surface-fines grid, which differentiates sediment into fine (< 2 mm diameter) and non-fine (> 2 mm diameter) categories, and pebble counts as described by Wolman (1954) and Marcus et al. (1995). These techniques partition sediments into five categories: gravel (3-15 mm), pebble (16-64 mm), cobble (65-255 mm), and boulder (> 256 mm diameter) (Cummins 1962, Overton et al. 1997). The proportion of 30 surface-fines grid measurements of fines and non-fines were averaged into a mean (± SE) proportion of fines for each site. Through the pebble counts (Wolman 1954, Marcus et al. 1995) we determined the composition of sediments larger than 2 mm in diameter using the same ten random transects used for surface-fines grid measurements, as described in Krueger et al. (2006). The sediment particles were organized into the modified Wentworth classification size categories as the percent (mean ± SE) gravel, pebble, cobble, and boulder (Cummins 1962) per site.

Statistical Analyses

We examined the means of infection response variables, and the coefficient of variation (CV) of environmental predictor variables to examine variability. A principal component analysis (PCA, correlation matrix) was conducted on the set of physical variables and a separate PCA (correlation matrix) on the set of chemical variables to

identify the features that best described differences among the study sites and the streams. Two separate PCAs were done because physical and chemical variables were measured a different number of times in different ways. We used PCA because many environmental variables covary in streams (e.g., conductivity and chloride) and the PCA generated independent axes that could be correlated with fish infection severity (e.g., Krueger et al. 2006). The physical and chemical variables with eigenvectors > |0.45| were considered the most influential features of the PCA axes (Table 4.5).

Linear regression analyses (SAS 2007) were used to investigate relationships among the results of the PCA of both, physical and chemical variables and infection severity in cartilage of both the cranium and lower jaw. We used the mean proportions of July 2002 and July 2003, and the mean proportions of August 2002 and August 2003, of fry with moderate or higher infection severity when only one year of measurements for predictor variables were available (e.g., chemical and sediment variables). Regressions were done with the first two PCA axes because they explained most of the variation among exposure sites and exposure times. Data were examined for normality and no transformations were necessary.

Regression analyses were also used to examine whether percent organic content and fine sediments correlated with infection severity in cartilage of the cranium and lower jaw. The residuals were normally distributed and no transformations were necessary. All statistical analyses were carried out with the statistical software program SAS 9.1.3 (SAS 2007).

Results

Infection Severity

Infection in cranial and lower jaw cartilage was highest in all sites in Pelican Creek, especially in July 2002 and 2003 (Table 4.1). In the Yellowstone River, 10 to 20% of sentinel cutthroat trout showed moderate and higher infection severity in two of the six sites in July 2003. No fry exposed in Clear Creek tested positive for *M. cerebralis* in 2002 or 2003 (Table 4.1).

Environmental Characteristics

Average day-time water temperature per exposure (July, August, or September) over the three sites was generally higher in the Yellowstone River and Pelican Creek than in Clear Creek in 2002 and 2003 (Table 4.2). The CV for temperature within streams was highest in Pelican Creek in August 2002 (23%) and July 2003 (18%). Water velocity was generally highest in Pelican Creek when averaged over the three sites tested in 2002, except for August. In 2003, however, mean velocity was generally highest in the Yellowstone River (both, in the outlet and Hayden Valley sites). Clear Creek often had the lowest mean water temperature and velocity, especially in 2002 (Table 4.2). The CV for velocity within streams was highest in the Yellowstone River (138%).

Table 4.1 Proportion of sentinel Yellowstone cutthroat fry with *M. cerebralis* infection severity ≥ moderate in cartilage of the cranium and lower jaw, after five (two in the Yellowstone River, Hayden Valley in 2003, Figure 4.1) 10-day exposures at three sites (upper, middle, lower) in the Yellowstone River and Pelican Creek, Yellowstone National Park. Clear Creek is not shown because all fry had zero infection in 2002 and 2003.

			2002			2003	
Study Stream	Site	Infection	JULY	AUGUST	SEPTEMBER	JULY	AUGUST
Yellowstone River	*Upper*	Cranium	0.00	0.00	0.00	0.20	0.00
		Lower Jaw	0.00	0.00	0.00	0.10	0.00
	Middle	Cranium	0.00	0.00	0.00	0.00	0.00
		Lower Jaw	0.00	0.00	0.00	0.00	0.00
	Lower	Cranium	0.00	0.00	0.00	0.00	0.00
		Lower Jaw	0.00	0.00	0.00	0.00	0.00
Pelican Creek	*Upper*	Cranium	0.95	0.00	0.05	0.75	0.00
		Lower Jaw	0.40	0.00	0.00	0.50	0.00
	Middle	Cranium	0.45	0.00	0.20	1.00	0.00
		Lower Jaw	0.10	0.00	0.00	0.90	0.00
	Lower	Cranium	0.78	0.22	0.74	1.00	0.00
		Lower Jaw	0.50	0.00	0.15	0.86	0.00
Yellowstone-Hayden	*Alum*	Cranium	--	--	--	0.00	0.00
		Lower Jaw	--	--	--	0.00	0.00
	NezPerce	Cranium	--	--	--	0.00	0.00
		Lower Jaw	--	--	--	0.00	0.00
	Trout	Cranium	--	--	--	0.20	0.00
		Lower Jaw	--	--	--	0.00	0.00

Table 4.2 Physical variables measured during five 10-day sentinel cutthroat fry exposures to *M. cerebralis* in three tributaries to Yellowstone Lake, Yellowstone National Park, in 2002 – 2003. Shown are the mean (± SE) over the three sites tested each exposure in the upper 13 Rkm of the Yellowstone River outlet, the lower 13 Rkm of Pelican Creek, lower 2 Rkm of Clear Creek, and 10 Rkm further downstream in the Yellowstone River, Hayden Valley. SE = ± 1 standard error.

Exposure		Study Stream	Temperature (^{o}C) N = 6	Velocity (m/s) N = 6	Depth (m) N = 6
2002	**JULY**	Yellowstone River	18.3 (± 0.58)	0.13 (± 0.02)	0.49 (± 0.04)
		Pelican Creek	19.0 (± 1.82)	0.18 (± 0.01)	0.31 (± 0.02)
		Clear Creek	11.9 (± 1.1)	0.08 (± 0.04)	0.27 (± 0.03)
	AUGUST	Yellowstone River	17.3 (± 1.35)	0.09 (± 0.06)	0.36 (± 0.07)
		Pelican Creek	14.8 (± 1.98)	0.10 (± 0.03)	0.24 (± 0.03)
		Clear Creek	8.8 (± 0.14)	0.16 (± 0.03)	0.30 (± 0.01)
	SEPTEMBER	Yellowstone River	15.0 (± 0.08)	0.03 (± 0.02)	0.29 (± 0.01)
		Pelican Creek	11.6 (± 0.41)	0.23 (± 0.09)	0.27 (± 0.01)
		Clear Creek	8.8 (± 0.19)	0.12 (± 0.02)	0.32 (± 0.02)
2003	**JULY**	Yellowstone River	13.3 (± 0.33)	0.30 (± 0.13)	0.86 (± 0.09)
		Pelican Creek	18.6 (± 1.93)	0.13 (± 0.04)	0.41 (± 0.12)
		Clear Creek	9.5 (± 0.26)	0.31 (± 0.04)	0.24 (± 0.02)
		Yellowstone-Hayden*	14.1 (± 0.21)	1.11 (± 0.37)	0.59 (± 0.15)
	AUGUST	Yellowstone River	18.1 (± 0.87)	0.10 (± 0.02)	0.64 (± 0.06)
		Pelican Creek	19.0 (± 1.38)	0.12 (± 0.01)	0.24 (± 0.03)
		Clear Creek	12.3 (± 0.46)	0.16 (± 0.01)	0.27 (± 0.02)
		Yellowstone-Hayden*	18.9 (± 0.40)	0.19 (± 0.07)	0.42 (± 0.14)

*N = 3. A single, non-replicated cage was used in each of the three sites during the two 10-day exposures carried out in 2003

All the water chemistry measurements, but especially conductivity, sulfate, and ammonium, were higher in Pelican Creek than in the Yellowstone River (except Chloride in July) or Clear Creek (Table 4.3). The Yellowstone River showed the next higher levels of mean conductivity, sulfate, ammonium, and chloride; whereas Clear Creek was low in all but dissolved oxygen (Table 4.3). The CV for sulfate (77%), total phosphorus (86%), and ammonia (130%) within streams were highest in Pelican Creek in July, September, and August, respectively.

The percent organic content in the sediment was generally highest in the Yellowstone River outlet sites (closest to the lake) and Pelican Creek, followed closely by percent levels in the Hayden Valley sites of the Yellowstone River (Table 4.4). The least percent fine sediments were in Clear Creek and those sites of the Yellowstone River and Pelican Creek farthest from the lake outlet (Table 4.4; Figure 4.1).

Infection Severity and Environment

The first two PCA axes described 80% of the variation in physical characteristics among study sites and exposure times (Table 4.5). PC 1 differentiated among sites based on mean water temperature and mean velocity, whereas PC2 differentiated among sites based on water velocities (Table 4.5, Figure 4.2). Severity of infection in cartilage of the lower jaw was significantly related to the first PC of physical variables ($P = 0.042$; Figure 4.3), but not with PC 2; thus, prevalence of moderate and higher infection in the lower jaw increased as water temperature increased and velocity decreased at the exposure sites, or over exposure times, or both. Severity of infection in the cranium was not related to either of the first two PCA axes of physical predictor variables (both $P > 0.13$).

The first two major PCA axes described 85% of the variation in chemical characteristics among study sites and exposure times (Table 4.5). PC 1 differentiated among sites based on sulphate, phosphorus, chloride, ammonium, and conductivity (Figure 4.4). PC 2 was reflective mostly of dissolved oxygen (Table 4.5; Figure 4.4). Severity of infection in cranial cartilage was significantly related to PC 1of chemical variables ($P = 0.046$; Figure 4.5), but not with PC 2; thus, prevalence of moderate and higher cranial infection increased as ionic concentrations in the water increased at the exposure sites, or exposure periods, or both. Severity of infection in the lower jaw was not related to either of the first two PCA axes of chemical predictor variables (both $P > 0.25$).

Neither percent organic content in the sediment nor percent fine sediments correlated with severity of infection in cartilage of the cranium or the lower jaw (all $P > 0.48$).

Table 4.3 Chemical variables measured during three 10-day sentinel cutthroat fry exposures to *M. cerebralis* in three tributaries to Yellowstone Lake, Yellowstone National Park, in 2002. Shown are the mean (± SE) over the three sites tested each exposure in the upper 13 Rkm of the Yellowstone, the lower 13 Rkm of Pelican Creek, and the lower 2 Rkm of Clear Creek. SE = ± 1 standard error.

Exposure	Study Stream	Conductivity (uS) N = 3	Diss. oxygen (mg/L) N = 3	Sulfate (µM) N = 3	T. phosphorus (µM) N = 3	Ammonium (µM) N = 3	Chloride (µM) N = 3
JULY	Yellowstone R	77.7 (± 1.26)	7.09 (± 0.21)	84.0 (± 7.5)	0.77 (± 0.05)	0.90 (± 0.12)	127.6 (± 3.0)
	Pelican Creek	281.2 (± 6.25)	9.28 (± 1.37)	242.7(± 107.6)	1.78 (± 0.31)	6.87 (± 4.65)	117.5 (± 49.4)
	Clear Creek	48.7 (± 0.19)	7.64 (± 0.16)	27.2 (± 1.2)	0.44 (± 0.02)	0.37 (± 0.09)	8.6 (± 3.0)
AUGUST	Yellowstone R	83.8 (± 1.60)	8.35 (± 0.30)	53.2 (± 4.2)	0.71 (± 0.19)	0.93 (± 0.38)	85.6 (± 6.2)
	Pelican Creek	364.8 (± 8.69)	10.22 (± 0.79)	350.7 (± 97.2)	1.19 (± 0.32)	2.80 (± 2.10)	318.5 (± 96.3)
	Clear Creek	59.1 (± 0.42)	8.37 (± 0.16)	17.2 (± 3.0)	0.29 (± 0.04)	0.10 (± 0.0)	3.2 (± 0.7)
SEPTEMBER	Yellowstone R	76.8 (± 0.05)	9.09 (± 0.20)	52.1 (± 10.0)	0.66 (± 0.09)	0.53 (± 0.12)	83.1 (± 16.5)
	Pelican Creek	304.1(± 11.79)	7.44 (± 0.37)	772.1 (± 34.5)	2.95 (± 1.46)	11.35(± 7.90)	578.6 (± 46.0)
	Clear Creek	62.0 (± 0.04)	8.42 (± 0.05)	38.6 (± 7.1)	0.50 (± 0.04)	0.47 (± 0.03)	12.5 (± 3.4)

Table 4.4 Percent organic content and percent fines in stream substrate at the three study sites (upper, middle, lower) of the Yellowstone River Outlet, Pelican Creek, Clear Creek, and the three study sites (Alum, NezPerce, Trout) of the Yellowstone River at Hayden Valley, Yellowstone National Park, as determined by Wolman (1954) pebble counts and weight-after-burn (percent organic content in sediment) in July 2003. Shown are the mean (± SE) for three random replicate samples/measurements per site.

Study Stream	Study Site	% Organic Content N = 3	% Fine Sediments N = 3
Yellowstone River	Upper	3.38 (± 0.42)	70.35 (± 3.93)
	Middle	1.93 (± 0.38)	69.38 (± 3.54)
	Lower	3.35 (± 0.30)	12.18 (± 1.95)
Pelican Creek	Upper	2.45 (± 0.61)	1.97 (± 0.95)
	Middle	2.86 (± 0.32)	11.65 (± 1.38)
	Lower	2.84 (± 0.64)	78.11 (± 9.46)
Clear Creek	Upper	2.71 (± 0.20)	0.07 (± 0.06)
	Middle	1.42 (± 0.15)	9.48 (± 3.78)
	Lower	1.28 (± 0.52)	6.07 (± 3.96)
Yellowstone -Hayden	Alum	2.54 (± 0.59)	39.90 (± 6.61)
	NezPerce	3.56 (± 1.43)	18.61 (± 8.46)
	Trout	2.14 (± 0.31)	4.52 (± 1.49)

Table 4.5 The three PCA axes of physical variables, and two PCA axes of chemical variables that best describe (i.e., >15% of the variation) the environmental features of the sentinel cutthroat trout exposure sites and exposure times in three tributaries to Yellowstone Lake, Yellowstone National Park, during the summers of 2002 and 2003.

	EIGENVECTORS	
	PC1 (42%)	PC2 (37%)
Physical Variables		
Temperature	**0.75**	0.18
Velocity	**-0.62**	**0.51**
Depth	0.22	**0.84**
	PC1 (63%)	PC2 (22%)
Chemical Variables		
Conductivity	0.41	0.44
Dissolved oxygen	-0.02	**0.80**
Sulfate	**0.48**	0.04
Total phosphorus	**0.45**	-0.26
Ammonium	0.42	-0.28
Chloride	**0.47**	0.10

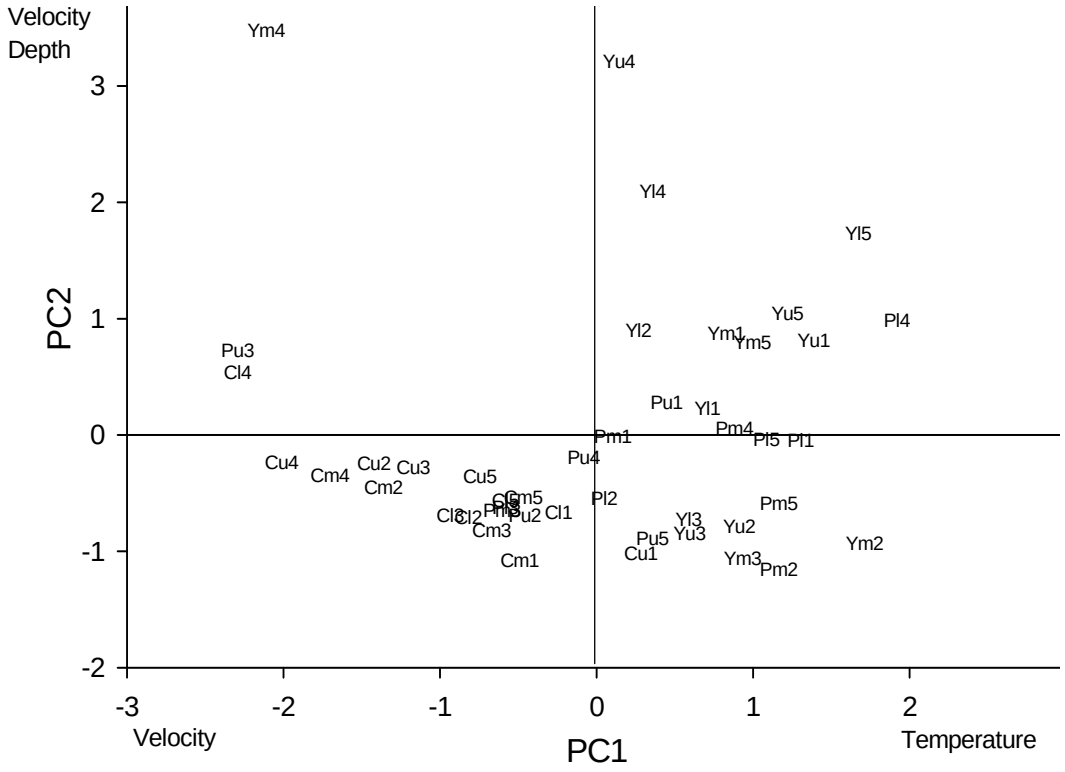

Figure 4.2 Features of the physical environment in three tributaries [Pelican Creek (P), Clear Creek (C), and the Yellowstone River Outlet (Y)] to Yellowstone Lake, Yellowstone National Park. Three sites per tributary [upper (u), middle (m), lower (l)] were tested three times each in July (1), August (2), and September (3) of 2002, and July (4) and August (5) of 2003. Principal component one and principal component two from a principal component analysis differentiated sites according to the variation among physical features of the environment. High values of the features that were most influential in the principal components are indicated on the axes (Table 4.5).

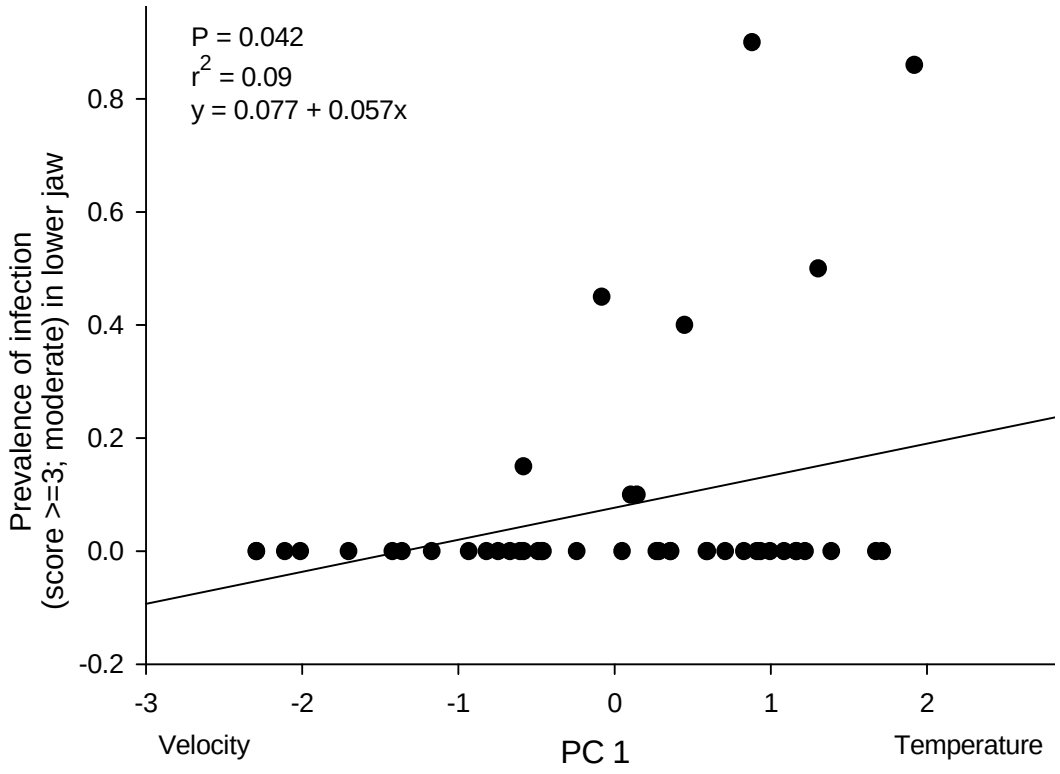

Figure 4.3 The relationship between prevalence of sentinel Yellowstone cutthroat fry with *M. cerebralis* infection severity ≥ moderate in lower jaw cartilage and PC1 of physical features of the water at three sites (upper, middle, lower) in three tributaries (Pelican Creek, Clear Creek, and the Yellowstone River Outlet) of Yellowstone Lake, Yellowstone National Park, in summer of 2002. On the PC 1 axes are the physical characteristics most correlated (eigenvalues >|0.45|)

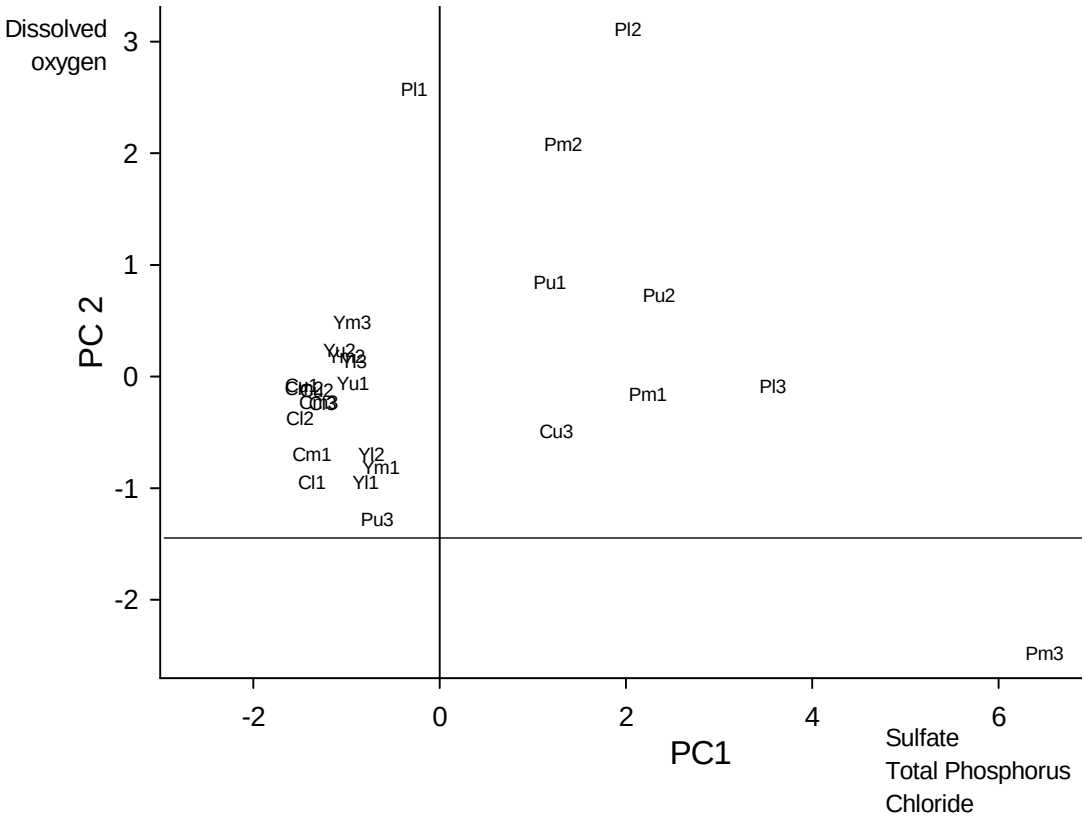

Figure 4.4 Features of the chemical environment in three tributaries [Pelican Creek (P), Clear Creek (C), and the Yellowstone River Outlet (Y)] to Yellowstone Lake, Yellowstone National Park. Three sites per tributary [upper (u), middle (m), lower (l)] were tested three times each in July (1), August (2), and September (3) of 2002. Principal component one and principal component two from a principal component analysis differentiated sites according to the variation among chemical features of the environment. High values of the features that were most influential in the principal components are indicated on the axes (Table 4.5).

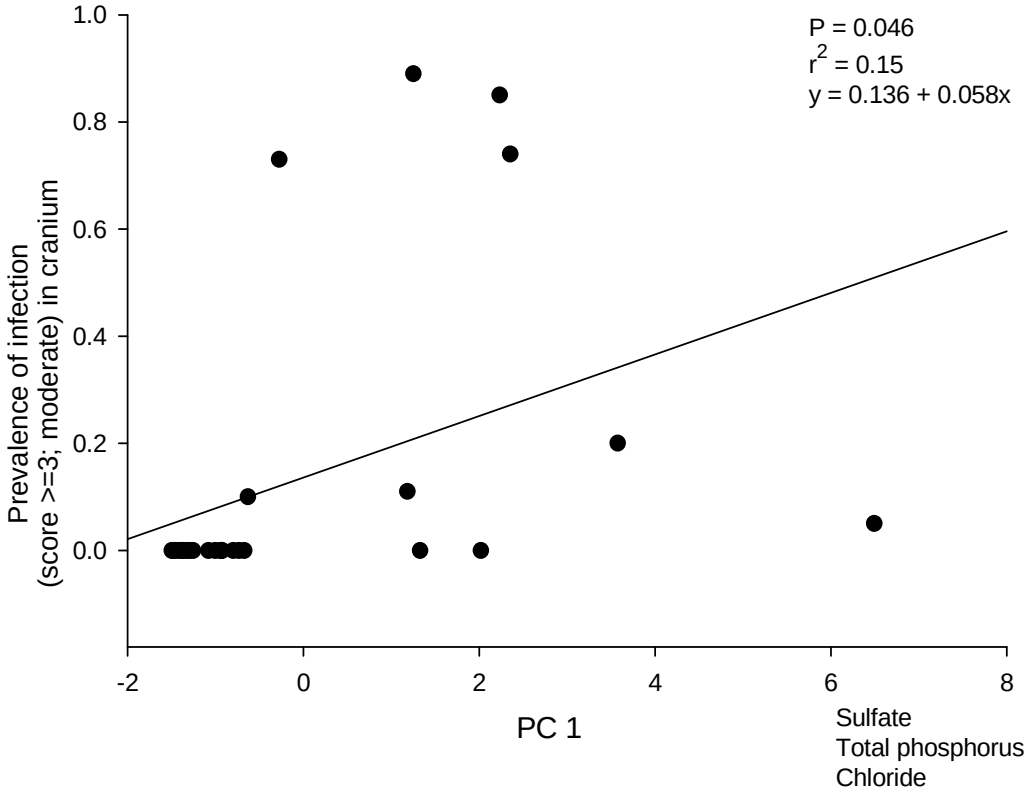

Figure 4.5 The relationship between prevalence of sentinel Yellowstone cutthroat fry with *M. cerebralis* infection severity ≥ moderate in cranial cartilage and PC1 of chemical features of the water at three sites (upper, middle, lower) in three tributaries (Pelican Creek, Clear Creek, and the Yellowstone River Outlet) to Yellowstone Lake, Yellowstone National Park, in summer of 2002. On the PC 1 axes are the chemical characteristics most correlated (eigenvalues >|0.45|).

Discussion

Myxobolus cerebralis was widespread in Pelican Creek, especially in July 2002 and 2003. Moderate and higher infection severity among Yellowstone cutthroat trout in the Yellowstone River was also notable by July 2003, especially in two sites of the 23 Rkm tested. Some sites would have been more difficult for cutthroat trout to avoid the parasite than others, and most importantly, some exposure times (e.g., July) were clearly most conducive to high infection. Spatiotemporal variation in the distribution of *M.*

cerebralis and salmonid infection severity among different aquatic systems and geographic areas is often reported (Cavender et al. 2002, Downing et al. 2002, McGinnis 2007), but factors contributing to such variation remain a subject of debate. Our results suggest that certain physical and chemical characteristics of stream waters of the Yellowstone Lake basin can explain the dynamics of infection incidence and severity among trout. Furthermore, it appears that the extent to which these parameters vary (ranges) across space and time within a given system, rather than the specific values or the parameters alone, is what may predispose salmonids to infection and facilitate parasitic invasion.

Based on our results of correlation analyses, high stream temperatures and low water velocities were the best predictors of infection in lower jaw cartilage in Yellowstone cutthroat trout, especially in the Yellowstone River and Pelican Creek. This supports our findings from prior research on this salmonid's susceptibility to *M. cerebralis* in a single tributary to Yellowstone Lake (Murcia et al. 2006). Also, despite fish being examined at later stages of infection in the present study than previous investigations (e.g., Vincent 2002, Murcia et al. 2006), and including observations from more than one stream, our results agree with prior findings relating temperature and velocity to incidence of *M. cerebralis* in the Intermountain West (Baldwin et al. 2000, Vincent 2002, Franco and Budy 2004, Krueger et al. 2006).

Temperatures of 12–15^{o}C are considered most optimal for parasite spore production and development in both hosts, and infection prevalence in salmonids (Markiw 1992, El-Matbouli et al. 1999, Hiner and Moffitt 2002), decreasing or ceasing

above such threshold. However, peak infections in rainbow trout occurred at temperatures ranging from 6 to 12°C in spring creeks in Montana (Anderson 2004). In Pelican Creek and the Yellowstone River, mean day-time water temperatures, especially during the July and August exposures, were generally well above 15°C. In these tributaries and exposure periods, sentinel fry infection was most severe. This suggests seasonal patterns of *M. cerebralis* infection risk in the Yellowstone Lake basin, as well as possibly a tolerance for higher mean water temperatures than have been documented for other systems (Hiner and Moffitt 2002, Franco and Budy 2004) and laboratory trials (Markiw 1992, El-Matbouli et al. 1999, Blazer et al. 2003). On the contrary, it is possible that we detected unusual temperature effects on infection severity because our measurements could not capture diel temperature variations, which may have changed overall mean temperature values and thus the strength of its relationship to infection.

The importance of low water velocities on increased incidence of *M. cerebralis* and infection severity in both hosts has been observed in the wild (Zendt and Bergersen 2000, Franco and Budy 2004, Krueger et al. 2006) and the laboratory (Lemmon and Kerans 2001, Hallett and Bartholomew 2007). Low water velocity areas often relate to increased incidence of *M. cerebralis* and salmonid infection (Balzer et al. 2003, Franco and Budy 2004, Hallett and Bartholomew 2007) because fine, silty organic material and diverse tubificids communities can abound in those areas (Lazim and Learner 1987, Sauter and Gude 1996); and *M. cerebralis* myxospores may persist amongst fine sediments longer than in coarser sediments (Lemmon and Kerans 2001). In contrast, high water flows may reduce *M. cerebralis* infection rates by diluting the concentration

of actinospores infective to fish, destroying them, or scouring fine-sediment habitat of tubificids and myxospores (Zendt and Bergersen 2000, MacConnell & Vincent 2002).

We found a strong correlation with both, high water temperature and low velocity (PC1; physical features), and lesion severity in the lower jaw, but not the cranium. Unlike other *Oncorhyncus* species, Yellowstone cutthroat trout appear highly prone to severe jaw infections (Murcia et al. 2006). This is significant because lesions caused by *M. cerebralis* in this cartilage, and its consequent host-inflammatory response, lead to severe jaw deformities that preclude fry from feeding normally and increase mortality (El-Matbouli et al. 1992, 1999, Vincent 1996). Population declines of native cutthroat in the Yellowstone Lake basin have accelerated over the last four years, especially in our three study streams (Koel et al. 2005, 2006b, 2007). Although studies have suggested some resilience of this subspecies to whirling disease (Hedrick et al. 1999b, Thompson et al. 1999, Koel et al. 2006a), their propensity to severe jaw (and cranial; i.e., lethal) lesions may be one more explanation for the accelerated losses in this system.

It is also possible that the observed relationships between PC1 and lower jaw infection reflect the range size effects of these variables on infection. For example, severe lesions in lower jaw were frequent in July 2003 in the Yellowstone River even though mean velocity appears high (Table 4.1, 4.2). But, the highest degree of variation (CV >74%) in velocity among sites was observed during this exposure in this stream. Similarly, temperatures in Pelican Creek were high across sites during this exposure, when jaw infection was also highest in this stream. This may represent a variety of

stressor inputs, which are known to reduce natural and acquired resistance to infections (increasing stress hormone release; Pickering 1993).

Our results from correlation analyses with chemical constituents and ionic content (e.g., PC 1: sulfate, chloride, conductivity) of the water suggest these features to be important predictors of cranial infection in Yellowstone cutthroat trout, especially in Pelican Creek. This tributary showed the widest ranging levels of ions in the water, with sulfate, for instance, ranging from 40 μM in the middle site in July to 832 μM in the upper site in September. At these same sites, chloride levels were also consistently lowest in July and highest in September, ranging between 26 and 650 μM. Because this tributary continually shows the highest incidence and severity of *M. cerebralis* over the last 5-6 years (Koel et al. 2006a, Murcia et al. 2006), it is possible that cutthroat trout in this system have suffered the novel stresses of parasite invasion worse than other, less environmentally stressful streams in the basin. In Pelican Creek, geothermal inputs are numerous, more than in Clear Creek or the Yellowstone River, as the majority of its tributaries are thermal springs (Fournier 1989, Gresswell et al. 1994, Varley and Schullery 1998). This probably influences greatly factors such as temperature, nutrients, and ionic content of the stream, which vary monthly and over short distances, and also increase conductivity (Boylen and Brock 1973, Fournier 1989). Widely fluctuating environments, especially over short spatiotemporal scales, tend to be physiologically stressful to species, and in our study streams it may exacerbate the cutthroat trout host-responses to a novel pathogen (Schaperclaus 1992, Hedrick 1998, Dickerson and Vinyard 1999; Thomas and Blanford 2003; Jokela et al. 2005, Leprieur et al. 2006).

Sources of ionic inputs such as chloride, sulfate, ammonium, and total phosphorus in streams include atmospheric gases (precipitation), particles in the air, weathering of sedimentary rock (carbonate mineral input; Allan 1995), or as in Yellowstone National Park, local seepage of sulphurous springs (Boylen and Brock 1973, Fournier 1989, Allan 1995, Astrom 2001). Also, acid sulfate soils often leach sulfate and other elements (e.g., calcium, sodium, aluminum; Astrom 2001) into stream waters further altering pH and conductivity levels. Though we could not use water pH in our statistical analyses, we visually examined our measurements (where available) against infection data. These pH measurements were also consistent with the park's long-term water quality monitoring program (Koel et al. 2007). All sites and exposures in the Yellowstone River (e.g., upper, July 2003) and Pelican Creek that showed infection consistently revealed the highest pH levels (8.2 – 9). The mildly basic waters may constitute an advantage for *M. cerebralis* to successfully invade and establish, and become infective to trout, in a system. The parasite's naturally resilient spore stages are known to withstand a variety of stresses, including passage through the digestive tract of predatory animals (El-Matbouli and Hoffman 1991, Wagner et al. 2003), and these spores may thus, be unaffected by high or widely fluctuating pH, conductivity, ionic or other physical and chemical features of the system.

Prior studies examining potential relationships between the stream's pH and *M. cerebralis* infection in rainbow trout and tubificids did not detect a correlation (Zendt and Bergersen 2000, Krueger et al. 2006). But pH in those study systems never fluctuated beyond the 8.3 – 8.6 range, whereas in Pelican Creek it varied widely (6.6 – 9.0) across

sites and exposures, as did all other physical and chemical attributes we measured there. Salmonids prefer pH-neutral and cool waters because outside the 6.5 to 8.0 pH and 12 to14^{o}C temperature ranges immature stages can be severely affected, and deformities and disruption of ion exchange across their gills may occur, leading to metabolic dysfunctions (Hickman and Raleigh 1982, Trzaskos 2003, Johnstone and Rahel 2003).

Differences on the role of physical and chemical features on *M. cerebralis* distribution and severity among systems or geographic areas might include differences in *T. tubifex* strain and salmonid species susceptibility (O'Grodnick 1979, Hedrick et al. 1999a,b, Thompson et al. 1999, MacConnell and Vincent 2002), the study's methodology (e.g., timing of histology; Murcia et al. 2006), and spatiotemporal scale of the study. Other environmental features of a stream, besides those measured in this study, may be better predictors of *M. cerebralis* infection in some salmonid species (Sandell et al. 2001, MacConnell and Vincent 2002, Krueger et al. 2006) than in Yellowstone cutthroat trout; or when examined at temporal and spatial scales in the basin (and other drainages; Franco and Budy 2004, Krueger et al. 2006) other than the scale used here.

The methodology used to measure predictor and response variables may affect the degree of correlations between them, or degree to which relationships are observable. For example, one potential limitation of the study is that field measurements cannot be logistically replicated enough over space and time to capture the entire stream conditions. Instead, replicate measurements of local conditions across study sites and exposure periods were assumed to represent the entire system conditions (e.g., Madison River; Krueger et al. 2006). Also, histological analysis is generally performed 90 days post-

infection when peak host response and maximum cartilage inflammation in salmonids can be observed (Hedrick et al. 1999b, Vincent 2002). The fact that we examined Yellowstone cutthroat trout 150 days post-exposure instead, may partially explain our infection results (e.g., Murcia et al. 2006). Moderate to severe lesions were consistently most prevalent in the cranium and the lower jaw, and only water chemistry correlated with cranial lesions, and temperature and velocity with lesions in the jaw. It is possible that correlations existed between infection in the lower jaw and water chemistry, but that infection severity in those cartilage sections had weakened after 150 days (Hedrick et al. 1999b, Vincent 2002, Murcia et al. 2006), making potential relationships undetectable by the time we tested them. Alternatively, certain characteristics of chondrocytes making up the matrix of cranial cartilage, and lacking in other cartilage regions, may have driven key reactions with the water's ionic content and chemical make-up (e.g., intracellular messenger signals through $[Ca^{2+}](i)$; Erickson et al. 2001).

The amount of cartilage in the lower jaw is far less than that of the cranium in young fish, which constitutes less of a resource for pre-sporogonic (feeding) stages of the parasite (e.g., Baldwin et al. 2000, MacConnell and Vincent 2002). Infections in cartilage of the lower jaw would thus tend to be lighter (low parasite numbers) or smaller, or both, and hence potentially closer to healing five months after infection than cranial lesions (e.g., Hedrick et al. 1999b, Baldwin et al. 2000, MacConnell and Vincent 2002, Vincent 2002). Conversely, larger (cranial) or heavier infections (e.g., high parasite numbers) may have worsened by day 150 post-exposure, strengthening extant relationships with chemical attributes of the water that would otherwise be undetected

had lesions been, possibly, already healing. Current knowledge of the immune response to *M. cerebralis* in the fish host is still limited (MacConnell and Vincent 2002), but clearly, timing of histology may explain some of the differences between our observed relationships among infection and PCA axes of chemical and physical features of the streams and those of similar studies (Hiner and Moffitt 2001, Downing et al. 2002, Krueger et al. 2006), and studies with other subspecies of cutthroat trout (Hedrick et al. 1999b, Vincent 2002, Franco and Budy 2004).

Infection by *M. cerebralis* in this study was not related to percent organic content and fine sediments, but such physical features of streams have been shown to be important to parasite invasion and establishment success in other systems. Perhaps the fact that we could not include substrate characteristics in forming the PCA axes of physical features may have affected our correlations with infection, as that may not be an ecologically realistic approach to identify environmental influences on the invasiveness of *M. cerebralis* in the wild. Species invasiveness cannot be predicted from a limited number of criteria because they often results from a combination of several (abiotic and biotic) characteristics (Devin and Beisel 2007). We suggest that a biologically realistic approach to investigate *M. cerebralis* invasion potential in the wild should comprise data on both hosts (biotic factors) in addition to environmental data (abiotic factors). Biotic factors that were not included in our analyses, such as tubificid infection and density, or biotic stresses common at elevated temperatures (e.g., bacterial, fungal, or viral pathogens; Schisler et al. 2000, Trzaskos 2003), may be important factors predisposing infection to *M. cerebralis* in cutthroat trout of the Yellowstone Lake basin, and possibly

as important as abiotic features. We collected data on tubificid density and infection during the summer of 2002, but excluded them from this study because they were used in our risk assessment of whirling disease in three tributaries to Yellowstone Lake (Murcia et al. in review,b).

The relationship between environmental attributes and invasion success may differ among habitats to the extent that traits important in the invasion of one habitat are unimportant in another (Pysek et al. 1995). In addition, environmental attributes of streams are probably in continuous flux with the current, rapidly changing environments. Presently, organisms face a growing number and variety of stresses, singly or in tandem (Myers 1995; Lafferty and Kuris 1999), ranging from introduced pathogens to large scale climatic changes. Rising temperatures, land-use alterations, habitat fragmentation, and countless other natural and anthropogenic disturbances affecting aquatic systems are widely recognized catalysts to biological invasions and diseases (D'Antonio and Vitousek 1992, Cohen and Carlton 1998, Marvier et al. 2004). Invasive pathogens and other invaders tend to be opportunists taking advantage of environmental degradation, mismanagement, and disturbance (Manchester and Bullock 2000; Hulme 2006).

Disturbances such as high road densities and a low proportion of forested riparian area within drainages in Montana were the most significant correlates with high prevalence and severity of *M. cerebralis* (McGinnis 2007). In Clear Creek during our studies, for instance, no fish tested positive for the parasite. This apparent lack of invasion, or establishment, by the parasite in Clear Creek may relate to the lack of roads and greater riparian forest cover at this site than in Pelican Creek or the Yellowstone

River. Alternately, the parasite may have invaded the system but environmental conditions are not conducive to establishment, or *M. cerebralis* does not exist yet in sufficiently large numbers to have noticeable effects on the cutthroat trout population there. *Tubifex tubifex* have been found in Clear Creek and were susceptible to infection (J. Alexander, Montana State University, personal communication); and infected, adult cutthroat trout presumably spawn throughout Clear Creek (Koel et al. 2006b, 2007), releasing myxospores into the system if they die after spawning. The parasite is easily and unknowingly transported by animals, birds, and humans (El-Matbouli and Hoffman 1991, Wagner et al. 2003, Gates et al. in press, Koel et al. in review) and thus very likely to continue to spread to drainages within the park currently uninfected and with the environmental characteristics likely to support establishment.

We conclude that continued protection of relatively undisturbed habitats within the Yellowstone Lake basin, and Yellowstone National Park, should be a key strategy against further spread and establishment of *M. crebralis* and other invasive species in the region. In the wake of environmental changes, occurrence of this parasite in tributaries of Yellowstone Lake may be indicative of future incursion by similar or other species in the same or similar sites. This invasion represents a major threat to the native salmonid and the ecosystem, and thus, continued research is paramount to learn more about it and the dynamics of whirling disease in the wild.

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CHAPTER FIVE

AN ECOLOGICAL RISK ASSESSMENT FOR WHIRLING DISEASE IN YELLOWSTONE CUTTHROAT TROUT FROM THE YELLOWSTONE LAKE BASIN

Abstract

Biological invasions, including parasitic and disease-causing organisms, have caused substantial ecological and economic costs. *Myxobolus cerebralis,* a parasite causing salmonid whirling disease, was introduced to US fish hatcheries in the 1950's. It now exists in numerous watersheds and has caused severe declines of wild trout populations in the Intermountain West of the US. Ecological risk assessment models have the potential to improve our understanding of the effects of non-indigenous species, and provide useful prediction and decision-making tools for how best to manage them. We used fish-host infection data, environmental attributes, and tubificid-host presence/absence data in three tributaries to Yellowstone Lake (Yellowstone National Park) to develop a qualitative risk ranking system for parasite establishment and whirling disease in native Yellowstone cutthroat trout *Oncorhynchus clarkii bouvieri*. Our results suggest that cutthroat trout of the Yellowstone Lake basin are at high risk of whirling disease, especially in systems with environmental attributes such as high water temperatures and ionic content, and used by cutthroat trout as spawning and rearing habitat. We identified information gaps and uncertainties that will require further investigation for future, comprehensive ecological risk analyses of *M. cerebralis* establishment in similar systems. Results from this study will assist the park's fish

biologists, regional fisheries managers, and researchers in developing management strategies to reduce disease risk in native cutthroat trout populations and the probabilities of the pathogen reaching other systems.

Introduction

The introduction of animal or plant pathogens to new hosts or geographic areas has caused substantial ecological and economic costs world wide. Such costs include extinctions or range restrictions of native species with subsequent changes in community composition (OTA 1993, Mack et al. 2000), and an estimated cost of $137 billion per year in the USA (Pimentel et al. 2000). There are numerous examples of harmful introductions of pathogenic agents and parasites to aquatic systems (parasitic sea lamprey in the Great Lakes; Lawrie 1970, Christie 1974, Leach 1976; parasitic trematode of salmon in Norway; Appelby and Mo 1997; Paisley 2001), resulting in significant changes to the system's natural processes and components.

Endemic to Eurasia, the parasite causing whirling disease in several species of trout and salmon, *Myxobolus cerebralis* (Hoffman 1990, Hedrick et al. 1998), was presumably introduced in Pennsylvania's fish hatcheries via frozen fish imported from Europe during the late 1950's. It has since spread throughout the USA and currently exists in the wild in 25 states (Hoffman 1990, Bergersen and Anderson 1997, Bartholomew and Reno 2002, Arsan et al. 2007). Since the early 1990's, *M. cerebralis* has been implicated in the catastrophic declines of wild trout populations throughout the western states (Walker and Nehring 1995, Vincent 1996, Schisler et al. 2000), causing >

80% reductions in some populations of non-native, wild rainbow trout (*Oncorhynchus mykiss*; Walker and Nehring 1995, Vincent 1996, Schisler et al. 2000). Cutthroat trout are native to the Intermountain West of the US, coexist in many watersheds with non-native salmonids, and are also highly vulnerable to *M. cerebralis* infection (Hedrick et al. 1998, Wagner et al. 2002, Murcia et al. 2006). Information is needed on ecological factors influencing whirling disease in the wild, native salmonid populations to assess the risk of establishment of this introduced parasite and help guide management efforts.

Ecological risk analysis is increasingly being applied to aquatic animal diseases and non-native species introductions (e.g., USOSTP 1999, Paisley 2001, Simberloff 2005, Colnar and Landis 2007, Schleier III et al. 2008). Risk assessment, a primary component of risk analysis, is the process of evaluating the likelihood that adverse ecological effects are occurring, or may result, from exposure to one or more stressors (U.S. EPA 1998, Lackey 1997), such as chemical pollutants or invasive species. Risk assessment methodologies provide information to decision-makers about the potential effects of various management strategies to reduce and control the risk of spread and establishment of invasive pathogens.

Assessing the risk of whirling disease in natural systems and native salmonids requires information on the biotic and abiotic factors influencing host ecology and susceptibility (morbidity), parasite prevalence and infection severity, and knowledge of the environmental attributes of the system. Since *M. cerebralis* was first described as a detrimental fish parasite in the US, intensive research has confirmed a two-host life cycle. Each host produces a spore that is infective to the other host, with myxospores infecting the aquatic oligochaete *Tubifex tubifex* and the actinospore, triactinomyxon (TAM) infecting the fish (Markiw and Wolf 1983,

Wolf and Markiw 1984). Much progress has also been made in examining ecological factors influencing risk of infection in both hosts, such as physico-chemical attributes of the system (Zendt and Bergersen 2000; Sandell et al. 2001; Hiner and Moffitt 2001), spatio-temporal overlap between hosts, spore stages, and environmental factors (Krueger et al. 2006), and complex interactions among several attributes and host ecologies (Kerans and Zale 2002, Krueger et al. 2006).

We used data and results from our own (and others') previous investigations (e.g., Bartholomew et al. 2005, Koel et al. 2006, Krueger et al. 2006, Murcia et al. 2006, in review) and a ranking system to develop a qualitative risk assessment for whirling disease in Yellowstone cutthroat trout. Ecological risk assessments often utilize risk rankings, which assign values to data that correspond and represent increments in risk as the value increases (Landis 2003, Schleier III et al. 2008). Such qualitative risk assessments are commonly performed when quantitative risk assessment methodologies are not feasible (Schleier III et al. 2008).

Our approach was similar to that of Landis and Wiegers (1997), Wiegers et al. (1998), and Bartholomew et al. (2005), but used the modifications of Schleier III et al. (2008) for the ranking system. This was a "retrospective" ecological risk assessment because we evaluated the likelihood of establishment and effects caused by past and present exposure to the parasite (stressor) (e.g., U.S.EPA 1998, Wolt and Peterson 2000). But, this could be combined later with a "prospective" ecological risk assessment approach to predict effects of such pathogen introduction and establishment in unsampled sites. Our goal was to determine the degree to which various, previously identified

ecological, host, and environmental factors influence risk of whirling disease in the native salmonid. Through the risk assessment, we also identify information gaps that require further investigation to improve future ecological risk analyses and management decisions regarding this introduced pathogen.

Methods

Risk assessment is defined in quantitative terms as a function of effects and exposure. In our assessment to estimate ecological risk from *M. cerebralis* establishment, we used a step-wise procedure with four distinct steps. First, *problem formulation*, describing what needs protection and is potentially at risk. Second, *hazard identification*, which in this case is the pathogenic agent that could potentially be introduced. Third, the *analysis phase* of the assessment models the likelihood of a given pathogen (e.g., *M. cerebralis*) being introduced and established in a given watershed (exposure assessment) and potential pathways of introduction (Bartholomew et al. 2005); as well as predicting ecological and economic consequences of such occurrence (effects assessment; Landis 2003, Peterson and Shama 2005, Schleier III et al. 2008). Fourth, *risk characterization*, which combines the elements of risk analysis, exposure and effects assessments, to estimate overall risks associated with the identified hazard(s), such as potential distribution, abundance, spread, impacts, costs (Stohlgren and Schnase 2006, Schleier III et al. 2008).

Problem Formulation

The rapidly declining population of Yellowstone cutthroat trout in the Yellowstone Lake ecosystem is of significant conservation concern because it places the

entire system potentially at risk of collapse (Koel et al. 2005). Throughout the Yellowstone Lake basin, numerous species of resident and migratory birds and mammals depend on the native cutthroat trout for nutrition (Gresswell 1995, Varley and Schullery 1998). In addition, the native salmonid supports an economically important recreational fishery (Gresswell and Liss 1995, Varley and Schullery 1998).

Several of the major spawning tributaries to Yellowstone Lake have already shown moderate to severe reductions in cutthroat trout populations (Koel et al. 2005, 2006). We used environmental, fish-host infection data, and oligochaete-host population data from Clear Creek, Pelican Creek, and the Yellowstone River (outlet) to develop our ecological risk assessment (Figure 5.1). We chose these three streams because they represent a wide range of physico-chemical characteristics (e.g., substrates, temperature, water chemistry, velocity), they have tested positive for *M. cerebralis* over the last seven years (Koel et al. 2006, Murcia et al. 2006), and the oligochaete host is known to inhabit these systems (Koel et al. 2006). Information is also available from sentinel caged trout experiments in the three streams – on pathogen prevalence, cutthroat trout infection pathology, severity, and the systems' environmental attributes (Murcia et al. 2006, in review). In addition, Clear Creek, Pelican Creek, and the Yellowstone River represent important spawning and rearing grounds (presently and/or historically) for the native Yellowstone cutthroat trout of the lake basin (Gresswell et al. 1994).

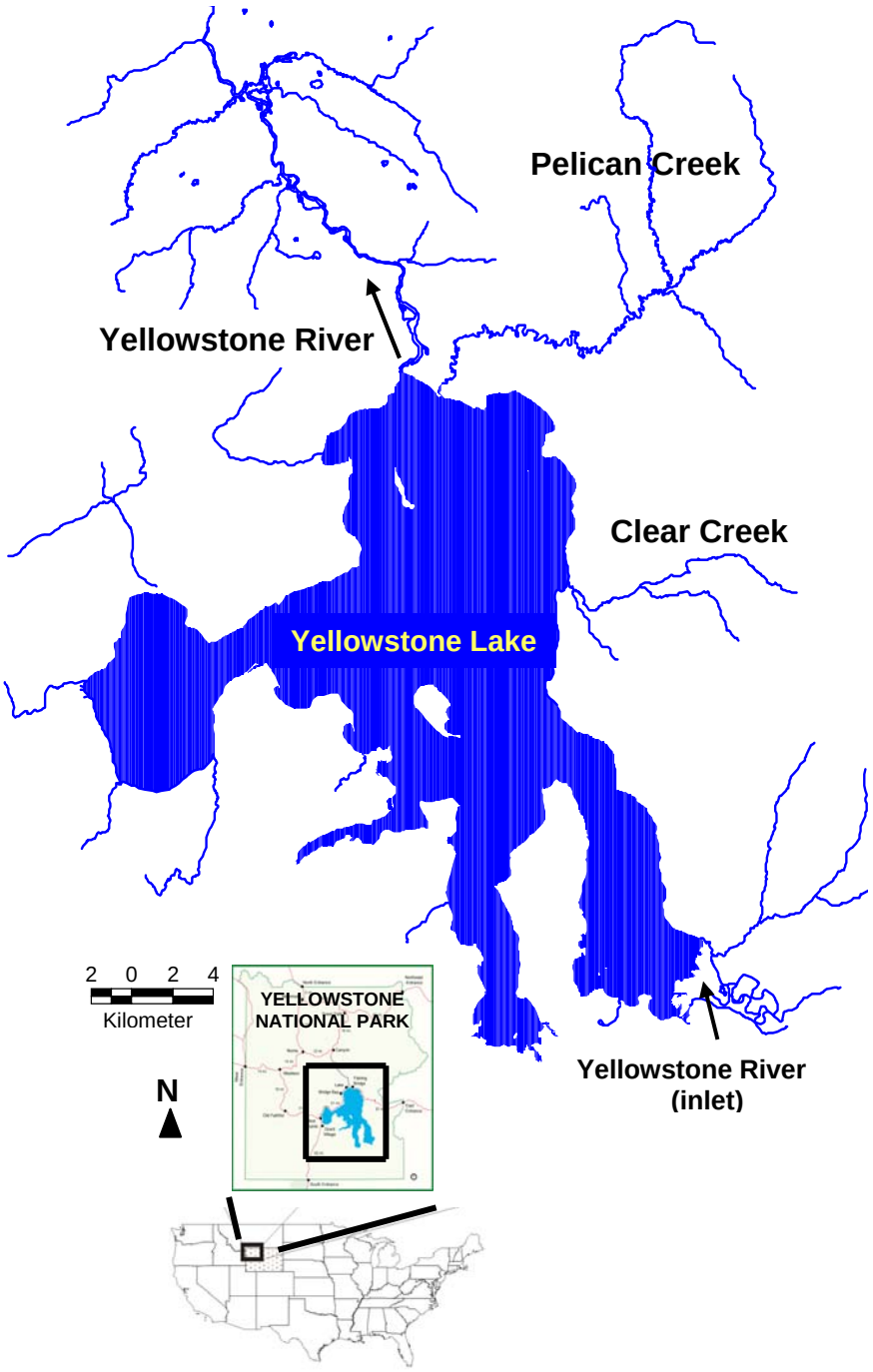


Figure 5.1 Map of the northern section of Yellowstone Lake showing the study streams. Data elements from these systems, including fish infection severity and pathology (from cage exposures) and environmental characteristics (e.g., temperature, water velocity, water chemistry, substrate composition), were used for this study's ecological risk assessment of whirling disease in Yellowstone cutthroat trout.

Hazard Identification

For the present study, we are concerned with a single hazard, the parasite *M. cerebralis*, which is the primary stressor (e.g., MacDiarmid 2003, Landis 2003, Bartholomew et al. 2005). However, not all aquatic systems respond equally to this stressor. For instance, a system may have low parasite prevalence but its environmental attributes induce whirling disease among the resident cutthroat trout by weakening tolerance to infection through physiological stress. Alternatively, a salmonid can be infected with *M. cerebralis* but not have whirling disease if the system's environmental conditions are physiologically favorable for the fish (Hedrick 1998).

Analysis

Exposure Assessment: We examined the factors potentially conducive to pathogen establishment in each system. These included environmental factors and oligochaete host population density. The occurrence and severity of disease depends on a range of factors including characteristics of the environment, the host/s, the pathogen, and their interactions (Hedrick 1998).

We included environmental factors in the exposure assessment because they are key determinants for the onset of disease (Schaperclaus 1992, Hedrick 1998, Reno 1998), and thus likely facilitators of pathogen establishment. Because environmental stressors can directly affect the physiology, reproduction, and survival of parasites and their hosts (Schaperclaus 1992; Harvell et al. 2002) and susceptibility to parasitic infection and disease (Sousa and Gleason 1989), we considered disease risk to be high when other

stresses would operate at the same time (e.g., Myers 1995; Lafferty and Kuris 1999). Thus, environmental characteristics previously identified as potentially good predictors of prevalence and severity of infection were ranked based on known physiological stressor effects on either host (e.g., Schaperclaus 1992, Hedrick 1998, Trzaskos 2003, Blazer et al. 2003), and published effects on *M. cerebralis* infection and disease severity (Hiner and Moffit 2001, 2002, Krueger et al. 2006, Koel et al. 2006, Murcia et al. 2006, Hallett and Bartholomew 2007, Murcia et al. in review) (Table 5.2).

Numerous experiments with *M. cerebralis* show on optimal threshold temperature of 15-16 ^{o}C for parasite spore production and development in both hosts, infection and disease severity in sentinel-caged and wild trout, and infection prevalence in *T. tubifex* (Markiw 1992, El-Matbouli et al. 1999, Hiner and Moffitt 2002, Blazer et al. 2003, Franco and Budy 2004). In spring creeks in Montana, peak infections in rainbow trout occurred at temperatures ranging from 6 to 12^{o}C (Anderson 2004). But, research with Yellowstone cutthroat trout in the wild suggests the most prevalent and severe infection occur when mean water temperature is nearly 20^{o}C or fluctuating by more than 10^{o}C within a month in a given system (Koel et al. 2006, Murcia et al. 2006, in review). Such high, widely fluctuating temperatures in those systems could be well above the metabolically optimal requirements of trout (Dickerson and Vinyard 1999; Leprieur et al. 2006), and may have weakened the physiological condition of cutthroat fry (e.g., Schaperclaus 1992, Hedrick 1998), increasing their vulnerability to parasitic infection. The ecology of both, salmonids and *T. tubifex,* is directly influenced by water

temperature, as is spore development in each host, and thus spore production, release, survival, and abundance in aquatic systems (Markiw 1992, El-Matbouli et al. 1999).

We ranked stream temperature in July, August, and September based on stressor and infection effects. These months are commonly used for fisheries research in the Yellowstone Lake basin because the snow, high water flows, or legal restrictions by the National Park Service limit access to study sites at other times. If average daily water temperature was $<16^{o}C$, or it varied by $< 5^{o}C$, that stream received a temperature stress score of 1 (minimal disease risk; Table 5.1). At the opposite extreme, if average daily water temperature was $\geq 16^{o}C$ and it ranged by $> 10^{o}C$ from one study site to the next within a stream or a month (e.g., ranged $11^{o}C$ to $23^{o}C$ within Pelican Creek; Table 5.2) the stream received a temperature score of 3 (high disease risk; Table 5.1).

Conductivity has also been positively correlated with *M. cerebralis* infection risk in both salmonid and tubificid hosts (Hiner and Moffit 2001, 2002, Sandell et al. 2001, Krueger et al. 2006). *Tubifex tubifex* can grow and reproduce faster, possibly releasing more parasite spores, where conductivity and thus productivity, are high (Kaster 1980). Sulfate, chloride, and total phosphorus may also influence *T. tubifex* and fish physiology and spore production, and indirectly affect disease risk in trout. These three chemical constituents were important predictors of severe cranial infection in caged Yellowstone cutthroat trout (Koel et al. 2006, Murcia et al. in review). We ranked the streams' water chemistry characteristics based on infection and potentially stressor effects to the hosts (e.g., Schaperclaus 1992, Sibley et al. 1997) in a manner similar to temperature. For

example, if conductivity ranges were narrow, varying by < 20 µS, that stream received a conductivity score of 1 (minimal risk; Table 5.1).

Two other risk factors we included were stream water velocity and tubificid presence/absence. High water velocities have been associated with low incidence of *M. cerebralis* infection rates as they may dilute the concentration of actinospores infective to fish, destroying them, or scour fine-sediment habitat occupied by *T. tubifex* and myxospores (Zendt and Bergersen 2000, MacConnell and Vincent 2002, Vincent 2002). Low water velocity areas such as side channels, backwaters, and pools, may hold *M. cerebralis* myxospores amongst fine sediments (Lemmon and Kerans 2001). These areas can also hold fine organic matter with diverse tubificid communities (Kerans and Zale 2002) and are often associated with high incidence of *M. cerebralis* and salmonid infection (Balzer et al. 2003, Franco and Budy 2004, Hallett and Bartholomew 2007). We ranked water velocity and tubificid data into two risk categories (Table 5.1). Because the mean of the highest and lowest water velocities combined was 0.148 m/s, if velocity was > 0.148 m/s the stream received a score of 1, if it was ≤ 0.148 the stream received a score of 2. Tubificids were collected at various sites in 2002 as in Krueger et al. (2006), and ranked by presence/absence (Table 5.1).

Table 5.1 Risk ranking scores of the exposure assessment portion of a whirling disease risk assessment for Yellowstone cutthroat trout in tributaries of Yellowstone Lake, Yellowstone National Park. The exposure assessment included environmental variables and tubificid presence/absence data, which are all factors previously identified as potentially stressful for cutthroat trout, or conducive to *M. cerebralis* establishment in a system, or both (e.g., El-Matbouli et al. 1999, Sandell et al. 2001, Blazer et al. 2003, Franco and Budy 2004, Murcia et al. in review).

Risk Score	**Temperature (^oC)**	**range (^oC)**	**Velocity range (m/s)**	**Conductivity range (µS)**	**Sulfate range (µM)**	**Chloride range (µM)**	**T Phosphorus range (µM)**	**Tubificids**
1 LOW	< 16	< 5	> 0.148	< 20	< 40	< 40	< 0.50	absent
2 MEDIUM	≥ 16	5 - 8	≤ 0.148	20 - 100	40 - 200	40 - 200	0.50 - 1.0	present
3 HIGH	≥ 16	> 8		> 100	> 200	> 200	> 1.0	

Table 5.2 Results of risk estimation. Shown are the risk ranking scores of the exposure (environmental and tubificid data) and effects (wild Yellowstone cutthroat trout; YCT) assessments. Effects assessment scores include *M. cerebralis* infection among adult YCT, and score (N) for number of YCT fry collected (< 10 fry score = 1; > 10 fry score = 2) multiplied by the score (INF) for presence/absence of infection in fry (no infection = 1; infection = 2). Risk characterization scores (RCS) resulted from multiplying the sum of exposure scores *by* the sum of effects scores for each stream.

Study Stream	**Temperature score**	**Velocity score**	**Conductivity score**	**Sulfate score**	**Chloride score**	**Total Phosphorus score**	**Tubificid score**	**Wild YCT-adult score (N*INF)**	**Wild YCT-fry score (N*INF)**	**RCS**
Clear Creek	1	1	1	1	1	1	1	1	2	**21**
Yellowstone R	2	2	2	2	2	2	2	2	4	**84**
Pelican Creek	3	1	3	3	3	3	2	2	2	**72**

Effects Assessment: This examines potential ecological consequences of parasite invasion and establishment. The *effects*, as defined in the present risk assessment, occur when the environmental and host conditions in the system lead to *M. cerebralis* establishment and the onset of whirling disease.

Fish host susceptibility to *M. cerebralis* was presumed high throughout the present risk assessment based on published (and our own) histopathology analyses of sentinel-caged Yellowstone cutthroat trout in these systems (Koel et al. 2006, Murcia et al. 2006, Murcia et al. in review). Sentinel fish cages placed at appropriate sites within a stream are a standardized methodology for assessing *M. cerebralis* infection risk to salmonids in the wild (Hoffman 1990, Baldwin et al. 1998, 2000). Caged fish provided an indirect measure of parasite TAM abundance in our three study streams (Koel et al. 2006, Murcia et al 2006, in review) because they eliminate confounding factors associated with studying *M. cerebralis* infection risk in the wild (e.g., fish migration, diseased fish mortality; Hoffman 1990, Baldwin et al. 1998, 2000, Downing et al. 2002). But, where and when cages are deployed in a given system cannot be (logistically) replicated sufficiently to account for the entire system and accurately determine the spatio-temporal distribution of patchy organisms like *M. cerebralis*. Therefore, it was important that, in the effects assessment, we also examined the wild fish population of native cutthroat trout (and tubificid; exposure assessment) to complement the sentinel fry infection data.

Fish host susceptibility to infection, and lesion severity and location (pathology) are known to vary among different species of trout and salmon (O'Grodnick 1979, Hedrick et al. 1999a, MacConnell and Vincent 2002). Cutthroat trout are highly vulnerable to the parasite

(Hedrick et al. 1998, Wagner et al. 2002), and Yellowstone cutthroat, especially, exhibit severe and lethal pathology in cranial and jaw cartilage (Murcia et al. 2006). Such lesions lead to severe host inflammatory response, bone displacement, and thus increased pressure on caudal nerves, lower brain stem and spinal cord (Halliday 1976, Hedrick et al. 1999b, MacConnell and Vincent 2002). This results in overt clinical signs of disease: erratic swimming (whirling), skeletal deformities (e.g., jaws, skull), and darkened tails (from damaged nerves controlling pigmentation; Halliday 1976, Hedrick et al. 1999b). Whirling disease can thereby reduce the fish's ability to avoid predators and feed normally, which in turn increases mortality among hatchery and wild salmonids (El-Matbouli et al. 1992, Walker and Nehring 1995, Vincent 1996).

Life history stage is an important risk factor because small, young trout are most susceptible to *M. cerebrlis* infection and death, as they have more cartilage relative to body size than adult fish (Hoffman and Byrne 1974, Vincent 1996; Ryce et al. 2005). The parasite feeds on, and destroys, cartilaginous tissue of its salmonid host (Markiw 1991, 1992). Larger fish may have skin with heavier mucous and more complete scale cover that is more difficult to penetrate by the TAM stage of *M. cerebrlis* than young, developing fish (Markiw 1992). The importance of life history strategies of susceptible salmonids has also been widely recognized (Sandell et al. 2001, Downing et al. 2002, Hubert et al. 2002). Risk of infection and establishment increases where the spatiotemporal overlap between parasite spore production and fry emerge from the gravel is greatest (Downing et al. 2002, Hubert et al. 2002, Kerans and Zale 2002).

Likewise, where and when fish spawn and rear may influence the parasite's distribution and infection success (Sandell et al. 2001, Downing et al. 2002). Death of salmonids after spawning and subsequent decomposition of carcasses could significantly contribute to the myxospore loading of a given stream (Hedrick et al. 1999a,b, Kerans and Zale 2002). Once released into the sediment or passively dispersed by water currents, myxospores may be deposited in backwater or side channel areas where water velocities are low (Kerans and Zale 2002) and where fry typically remain for long periods after emergence (e.g., Sandell et al. 2001, Downing et al. 2002, Hubert et al. 2002), during their most susceptible age to infection. These are also the areas where tubificid communities are most common (Lazim and Learner 1987, Sauter and Gude 1996, Blazer et al. 2003, Kerans and Zale 2002), which possibly contributes to an increased risk of whirling disease and parasite establishment in those regions.

We used infection prevalence in wild-caught spawning (adult) Yellowstone cutthroat trout and presence of, and infection in, wild-caught young (fry) Yellowstone cutthroat trout in the effects assessment to rank wild fish risk factors. If infected spawning cutthroat trout were collected from a stream that system received a score of 2, if no infection was detected in adult spawning cutthroat trout the stream received a score of 1. Also, if > 10 young, susceptible-age fry per season were found in a stream, we assigned a score of 2 to that system, if < 10 fry were found in the stream, the system received a score of 1. Infection among the fry was ranked in a similar manner whereby no infection corresponded with a score of 1 and infection with a score of 2. We multiplied the scores corresponding to number (N) of wild fry collected by the score

corresponding to infection (INF) for a total wild fry score. For example, if > 10 fry per season were found in a stream (N score 2) but no infection was detected (INF score 1) that stream received a wild fry score of 2 (N*INF = 2*1 = 2; Table 5.2).

Although an effects assessment for *M. cerebralis* may consider such outcomes as production losses, hatchery facility closures, or other costs in some regions, we focused on effects to Yellowstone cutthroat trout and potential ecosystem–level effects. Such effects would involve native species above and below the cutthroat trout's trophic level. Negative impacts can include reduced fish host fitness (if surviving infection), sharp declines, or complete loss, of native cutthroat trout sub-populations (within a stream system), and consequent effects on lower and higher trophic levels (e.g., minnow prey, or pelicans, otters, respectively) and fish host competitors. We did not predict economic consequences of native cutthroat trout population declines or losses (e.g., angler dissatisfaction, park visitors, wildlife viewing) because this was beyond the scope of our assessment.

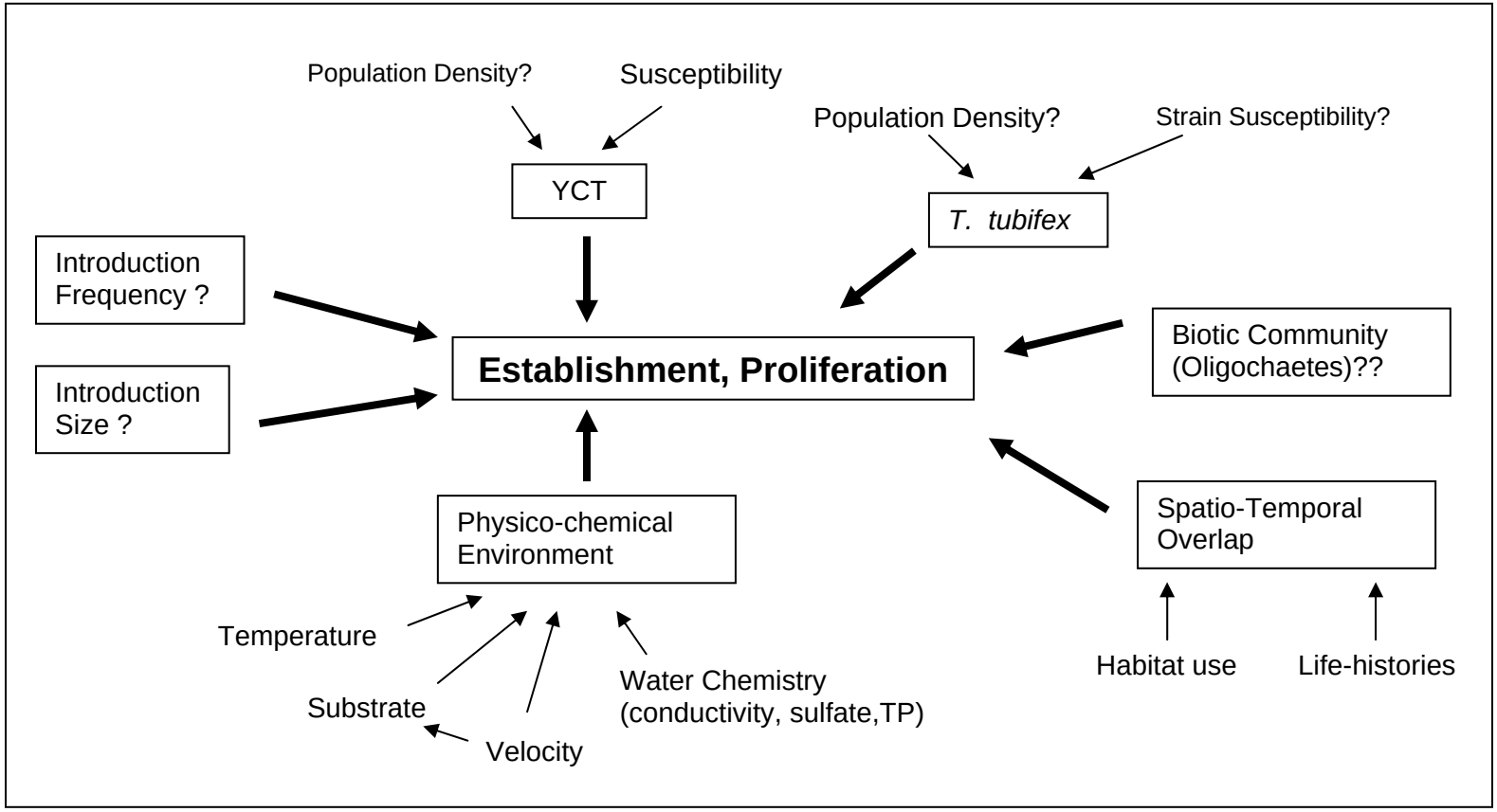
The effects of 'successful' invasion and establishment of the pathogen in a system (e.g., disease) depend on complex interactions among many variables (Figure 5.2) involving, not only the parasite, but characteristics of the two hosts and the system's environmental conditions (Travis and Hueston 2001). Dramatically different outcomes have been documented in watersheds where the pathogen and vulnerable hosts coexist. In some locales, whirling disease has caused little or no adverse effects on wild salmonid populations (Modin 1998, Sandell et al. 2001), but evidence from other regions suggests severe population declines as a consequence of disease (Walker and Nehring 1995,

Vincent 1996, Schisler et al. 2000). If severely diseased to the point of meta-population crash, *M. cerebralis* could significantly alter Yellowstone Lake ecosystem dynamics, park tourism activities, and the sport fishery of Yellowstone National Park, having serious economic consequences.

Risk Characterization

We used a qualitative, three-category visual scale (low, medium, and high risk) to rank the likelihood and ecological effects of parasite establishment in a stream, and disease in fish (i.e., outcome of successful invasion; MacDiarmid 2003, Bartholomew et al. 2005). For this, we summed the physical (temperature, velocity), chemical (conductivity, sulfate, chloride, total phosphorus), and tubificid risk scores for each stream to generate an exposure assessment score. We summed the risk score for wild adult cutthroat trout infection and the total wild fry (N*INF) infection score for each stream to generate an effects assessment score. Effects and exposure risk scores were multiplied to obtain a final risk characterization score (RCS; modified from Schleier et al. 2008) for each study stream (Table 5.2). No specific procedure was necessary to partition the final RCS into visual risk categories (e.g., low, medium, and high risk).

Figure 5.2. Conceptual model depicting examples of data elements necessary for an effects assessment (modified from Bartholomew et al. 2005) of parasite establishment and proliferation (i.e., whirling disease) in Yellowstone cutthroat trout (YCT) from three *M. cerebralis*-infected tributaries to Yellowstone Lake (Figure 5.1).



Results

Water temperature ranges were narrow in Clear Creek (varied by 4.6°C) across July, August, and September, but Pelican Creek temperatures varied widely (Table 5.3). The Yellowstone River had the lowest mean water velocity across sites and seasons (July, August, September 2002-2003) and thus received a high risk score for this variable (score = 2; Table 5.2). The widest ranges in conductivity, sulfate, chloride, and total phosphorus concentrations occurred in Pelican Creek (Table 5.3), which thus received a score of 3 for each of these chemical features (Table 5.1, 5.2). Based on the final RCS, undoubtedly, Clear Creek falls in a relatively low risk category, while in Pelican Creek and the Yellowstone River the risk of disease and establishment of *M. cerebralis* is more than four times higher than that of Clear Creek (Table 5.2).

Table 5.3 Environmental variables measured during a study of whirling disease in Yellowstone cutthroat trout from three tributaries to Yellowstone Lake, Yellowstone National Park, during the summers of 2002-2003. Shown are the mean ranges of each variable potentially affecting host physiology and risk of *M. cerebralis* establishment and disease severity among native cutthroat trout in the Yellowstone Lake Basin.

Study Stream	Temperature range (°C)	Velocity mean(m/s)	Conductivity range (µS)	Sulfate range(µM)	Chloride range(µM)	TPhosphorus range(µM)
Clear Creek	8.4 - 13	0.166	45 - 63	13 - 50	2 - 18	0.24 - 0.57
Yellowstone R	13 - 19	0.129	74 - 99	37 - 97	59 - 131	0.40 - 1.11
Pelican Creek	11 - 23	0.152	238 - 400	39 - 832	26 - 650	0.69 - 5.70

Tubificids were present in highest densities in Pelican Creek and the Yellowstone River, but were not found in Clear Creek in 2002 (Table 5.4). Only three of the tubificids collected released actinospores, which DNA tests failed to classify as *M. cerebralis*.

About 150 tubificids that were not producing actinospores were also tested and two of these, one from Pelican Creek and one from the Yellowstone River, were positive for *M. cerebralis* (Table 5.4). Thus, estimates of infection prevalence among the parasite's tubificid host were low in our study systems, but presence of this host may constitute a potentially high risk of parasite invasion, and whirling disease. Infection prevalence in tubificid is typically low even in systems with high disease risk (Gilbert and Granath 2001, Beauchamp et al. 2005, Krueger et al. 2006).

Table 5.4 Mean density estimates (+/- 1 SE) of oligochaetes (indiv/m^2) from three tributaries to Yellowstone Lake (Figure 4.1). Six core samples were taken at various sites during the summer (July-Sept) 2002 for a total of 18 samples per stream. Oligochaetes were identified to family whenever possible (Kathman and Brinkhurst 1998) and tubificid presence/absence used in the exposure assessment portion of the risk assessment. Oligochaetes were tested for *M. cerebralis* DNA and two were positive for the parasite.

Collection	Site	density (# /m^2)	tested	Mc-positive	releasing TAMs
JULY	Clear Creek	0	0	0	0
	Yellowstone River	96	0	0	0
	Pelican Creek	1699	17	1	2
AUGUST	Clear Creek	0	0	0	0
	Yellowstone River	160	1	0	0
	Pelican Creek	578	3	0	0
SEPTEMBER	Clear Creek	0	11[a]	0	0
	Yellowstone River	577	4	1	1
	Pelican Creek	545	0	0	0

[a] these 11 oligochaetes tested for *M. cerebralis* had been collected by kick net samples, instead of core samples as with those for which density was calculated

Another factor we included in the present assessment (effects) of whirling disease risk was infection prevalence in wild-caught Yellowstone cutthroat trout. Our digital fish-counting station at the mouth of Clear Creek counted 6,613 upstream-migrating adult Yellowstone cutthroat trout in 2002 and 3,432 in 2003, of which, only 46% returned to the lake after spawning (in 2003). No adult Yellowstone cutthroat trout trapped at Clear Creek during spawning migrations tested positive for *M. cerebralis* by the Pepsin-Trypsin Digest (PTD) method for spore counts. However, *M. cerebralis* spores have been detected in relatively large numbers among adult Yellowstone cutthroat trout accidentally killed in gillnets (used to remove non-native lake trout from Yellowstone Lake), especially from nets set in the northern sections of the lake, near Clear Creek (Koel et al. 2006). Although there is no spawning counter at either the Yellowstone River or Pelican Creek, we tested a small sample of adult, spawning cutthroat trout from both streams (3 fish from the Yellowstone River, 5 from Pelican Creek) in May-June 2002. Infection among these fish ranged between nearly 9,000 to more than 19,000 parasite spores per cutthroat in the Yellowstone River (Fishing Bridge, 3 fish tested), and between 5,000 to 251,000 spores per cutthroat trout in Pelican Creek (5 fish tested). We tested more than 100 wild fry collected from sites in Clear Creek throughout the summers of 2002 and 2003, and none tested positive for *M. cerebralis* (Table 5.5). Of the 141 wild fry from the Yellowstone River, 7.2% were positive for the parasite. We found no wild fry throughout our study sites in Pelican Creek in 2002 and only 9 fry total in 2003, of which, 77.8% were positive for *M. cerebralis* (Table 5.5).

Table 5.5 Wild Yellowstone cutthroat fry tested for *M. cerebralis* infection during the summers of 2002-2003. Fry were collected at the three stream sites in each study stream where sentinel-caged fry exposures were conducted (Murcia et al. 2006; in review) and during, or shortly after, each 10-day exposures. Fry were tested by PCR 150 days post-collection to allow for parasite development within fish cartilage and to correspond with sentinel fry testing. Shown are the total number of fry tested per stream (three sites).

Collection	Stream	Fry tested	*M. cerebralis*--positive*
JULY 2002	Clear Creek	30	0
	Yellowstone River	17	0
	Pelican Creek	n/a	n/a
AUGUST 2002	Clear Creek	30	0
	Yellowstone River	10	0
	Pelican Creek	n/a	n/a
SEPTEMBER 2002	Clear Creek	29	0
	Yellowstone River	14	1*
	Pelican Creek	n/a	n/a
JULY 2003	Clear Creek	30	0
	Yellowstone Outlet	30	0
	Yellowstone Hayden	51	2*
	Pelican Creek	7	2**
AUGUST 2003	Clear Creek	0	n/a
	Yellowstone River	22	0
	Yellowstone Hayden	0	n/a
	Pelican Creek	2	0

* Fry were tested in pools of five per sample.
** One of these two positive samples from Pelican Creek in July 2003 had 3 fry, the other had 4 fry

Based on the Yellowstone cutthroat trout infection susceptibility (Hiner and Moffitt 2001, Koel et al. 2006, Murcia et al. 2006), its relationship to the systems' environmental attributes (Schaperclaus 1992, Hedrick 1998), the tubificid presence/absence data (exposure), and the fish infection results (effects) we identified two of our three study systems, the Yellowstone River and Pelican Creek (Figure 5.1) at high risk of *M. cerebralis* establishment and whirling disease (Table 5.2). Yellowstone cutthroat trout (young and adult) from these tributaries were the most severely infected

(and diseased; sentinel fish data; Koel et al. 2006, Murcia et al. 2006, in review) by the parasite, and tubificids were present in highest densities in this tributaries as well. Based on these findings, the risk of trout population declines due to parasite establishment in the Yellowstone River and Pelican Creek is high.

Clear Creek showed the lowest risk of *M. cerebralis* establishment and whirling disease (Table 5.2). This tributary's environmental attributes were the least conducive to physiological stresses or disease among the Yellowstone cutthroat trout, and parasite establishment and proliferation. Cutthroat trout (young and adult) from Clear Creek showed no infection and we found no tubificids there in 2002. However, we cannot classify this population of cutthroat trout or the system to be at no risk because *T. tubifex* have been found in Clear Creek and were susceptible to infection (J. Alexander, Montana State University, personal communication); adult, infected cutthroat trout from the northeastern region of the lake migrate upstream to spawn in this system (Koel et al. 2006), and may release large numbers of parasite spores if they die after spawning; one Yellowstone cutthroat fry tested positive for *M. cerebralis* during caged exposures in Clear Creek in 2000 (Koel et al. 2006).

Discussion

By ranking various, previously and presently identified ecological, host, and environmental factors influencing disease risk in Yellowstone cutthroat trout we conclude that the Yellowstone River and Pelican Creek are at high risk of *M. cerebralis* establishment, and proliferation (e.g., disease outbreaks) and Clear Creek is at low risk.

Data from this and prior investigations on the relationships between environmental factors and the native cutthroat susceptibility to *M. cerebralis* (Hedrick et al. 1999b, Koel et al. 2006, Murcia et al. 2006; in review) show the native salmonid is at high risk of whirling disease and death, especially in the Yellowstone Lake ecosystem. Severe lesions in cranial and jaw cartilage in this salmonid were most frequent in areas with high water temperatures, conductivity, sulfate, chloride, total phosphorus, and low velocity (Koel et al. 2006, Murcia et al. 2006; in review). Such patterns were observed in our prior studies at almost all our sentinel cage sites in Pelican Creek and three of our six cage sites in the Yellowstone River, especially during the July exposures, but never in our sites at Clear Creek (except the one infected fish in 2000; Koel et al. 2006). This tributary was characterized by low water temperatures, low velocity, conductivity and nutrient concentrations, and little to no percent organic content in the sediment.

Ecologically, deleterious cascading effects of many keystone extinctions world wide have resulted from introductions of parasitic and disease-causing organisms like *M. cerebralis* (Simberloff and Boecklen 1991, Simberloff 2005). If invasive species and parasite-driven declines of the Yellowstne cutthroat trout continue at the present rate the entire Yellowstone Lake ecosystem may face considerable ecological and economic consequences.

We excluded anthropogenic facilitators of parasite establishment such as restoration, stocking, or commercial activities from the exposure assessment portion of the present risk assessment because they are not applicable to watersheds under National

Park Service management, but should be considered for systems under state or private management allowing these practices.

In the course of working through this assessment, we also recognized several information gaps that require further investigation or more detailed analyses. As research fills in the gaps identified, the quality of future ecological risk assessments can improve, supporting or modifying our results, and management actions can be designed accordingly. For example, data elements desirable for the exposure assessment of an ecological risk assessment on whirling disease in salmonid environments, which were not available to us, include information on the variable susceptibility of different genetic lineages for the oligochaete host (Beauchamp et al. 2002, 2005), natural pathogen dispersal (fish migration, avian or other fish predators' movement), and recreational activities by park visitors (anglers, hikers, campers; e.g., Gates et al. in press).

Our exposure assessment of *M. cerebralis* in Clear Creek, Pelican Creek, and the Yellowstone River did not include data on the tubificid host genetic lineages and habitat preferences (e.g., confined systems; J. Alexander personal communication). Genetically distinct strains of *T. tubifex* exhibiting varying degrees of susceptibility to *M. cerebralis* and ability of TAM production have been described (Stevens et al. 2001, Kerans et al. 2004, Beauchamp et al. 2005) and often coexist in the wild (Beauchamp et al. 2002). If our study streams contain large populations of susceptible strains (lineages) of *T. tubifex*, the likelihood that *M. cerebralis* will become established and proliferate in these systems is high. The higher the abundance of susceptible tubificid and fish hosts in a given system, the more likely that *M. cerebralis* will encounter a permissive host and

proliferate there (Bartholomew et al. 2005). Also, the biotic community is important because tubificid assemblages may consist of not only multiple strains or genetic lineages, but also oligochaete species (e.g., *Limnodrilus hoffmeisteri*) in which *M. cerebralis* may have differential proliferation and thereby influence infection in its definitive host (Steinbach-Elwell et al. 2006).

Tubifex tubifex is a cosmopolitan species, but little is known about distribution and abundance in cold, high altitude headwater systems of the Yellowstone Lake Basin such as Clear Creek where it may co-occur with native cutthroat trout. *Tubifex tubifex* are typically associated with sediments characterized by fine substrates and feed on the bacteria associated with organic matter (McMurtry et al. 1983). This tubificid can abound in habitats degraded by siltation, nutrient enrichment, or low oxygen levels, however, populations can also be found in oligotrophic conditions typical of high altitude headwater streams in the western United States (Zendt and Bergersen 2000) and Yellowstone National Park.

An additional data element that would be needed for a comprehensive risk assessment of whirling disease in trout environments is natural dispersal factors (e.g., pathways; in exposure assessment). Fish spawning migrations and spreading by piscivore predators (birds, wildlife, fish), for instance, can significantly facilitate parasite introduction, establishment, and spread. For example, 40 to 60% of Yellowstone cutthroat trout die after spawning in some of the Lake tributaries (Gresswell et al. 1994) and if these adults are infected the possibility exists for thousands to millions of *M. cerebralis* myxospores to be released into the stream environment following

decomposition of carcasses (Hedrick et al. 1999a,b, Kerans and Zale 2002). At the myxospore stage *M. cerebralis* can survive for long periods of time outside the fish (El-Matbouli and Hoffman 1991, Kerans and Zale 2002). Therefore, the likelihood that infectious myxospores will find a vulnerable tubificid host will increase with increasing numbers being introduced.

At this stage, the parasite (myxospore) reportedly survives passage through the guts of predators (El-Matbouli and Hoffmann 1991, Koel and Kerans 2007), such as piscivorous birds, mammals (e.g., otters, bears; Koel et al. 2005), and fish (e.g., lake trout), which are thus very likely vectors of parasite transmission across systems. If the pathogen is introduced through wildlife dispersal of myxospores between or within systems, there is great potential for the parasite to survive long enough to encounter its tubificid host. We could not measure the extent of movement and subsequent death of adult, spawning cutthroat in our study streams, nor that of avian and other wildlife predators frequenting these systems. But, these data, as well as data on time and place of trout emergence (e.g., fry traps) would be important to future exposure assessments.

Prior investigations in Colorado and Montana have measured parasite spore concentrations in the water column (e.g., TAM-ometer; Hubert et al. 2002, Lukins et al. 2007). Although we had no information on actual parasite spore doses in our study streams, infection severity among sentinel-caged trout provides an indirect measure of parasite spore concentration in a stream (Baldwin et al. 1998, 2000). We used histopathology data from sentinel exposures of trout under their susceptibility threshold size and age (45mm TL, < 9 weeks old; Ryce et al 2005) for this risk assessment, as well

as environmental, tubificid presence/absence, and wild fish infection data. But, establishment and proliferation of *M. cerebralis* are also dependent on factors maintaining continuity of parasite life cycle and biotic assemblage in which both, host and parasite reside (Bartholomew et al. 2005). These data elements would strengthen the risk assessment because risk is a function of both exposure and effect. Spatiotemporal overlap between vulnerable fish and susceptible tubificid host (life-stage, genetic strain, habitat use) with viable spores is critical (Kerans and Zale 2002), as is the frequency and size of introduction and spreading beyond the point of introduction. Actual measurements of parasite spore concentrations in the water, and sediments, could strengthen the applicability and predictive power of future risk assessments in these systems and other regions.

Causes of uncertainty in an ecological risk assessment are generally attributed to lack of knowledge (published data), stochastic properties (natural variability), and investigator bias (Wiegers et al. 1998). In the present risk assessment there are several areas of uncertainty related to physical and chemical characteristics of the streams. Whereas several environmental factors (e.g., temperature, substrate type) and their effect on the life cycle of *M. cerebralis* have been investigated (Hiner and Moffitt 2002, Blazer et al. 2003, Kerans et al. 2004, Krueger et al. 2006) the importance of water velocity, for instance, and water chemistry, are difficult to separate from other factors (e.g., temperature) in field studies and remain to be thoroughly investigated (MacConnell and Vincent 2002, Hallett and Bartholomew 2007).

Unlike temperature, no data currently exist explicitly reporting a threshold level of water velocity above or below which *M. cerebralis* infection intensities in either host significantly increase or decrease in a given system. Part of the reason may be that some studies use discharge (m^3/s; Franco and Budy 2004) while others, in the laboratory use velocity (cm/s; Hallett and Bartholomew 2007). Based on the available literature, we had no reference values to use in creating our risk categories for water velocity. Instead, we mathematically partitioned velocity into two risk levels based on field observations specific to the Yellowstone Lake Basin. But, number and range-size of risk categories for velocity may vary and require modifiable system-dependent criteria in different systems and regions. Water velocity is an important variable to include as a risk factor influencing establishment and propagation of the parasite.

There are also no robust data for specific ranges of nutrients (sulfate, total phosphorus) or other chemical constituents of streams influencing risk of and prevalence of *M. cerebralis* in either host. For example, no relationships were identified in Utah between the parasite's prevalence and nutrients (nitrogen, phosphorus), periphyton, substrate composition, and tubificids (Franco and Budy 2004). But, in the Yellowstone Lake basin, Pelican Creek (where cutthroat trout were most severely infected) showed sulfate, chloride, and total phosphorus concentrations far above most other streams tested (Koel et al. 2006, Murcia et al. in review). Based on these findings we chose three sulfate and total phosphorus concentration levels (low, medium, high) likely to influence risk of parasite establishment, but different concentration ranges may facilitate establishment and proliferation to variable degrees in different systems.

Part of our goal with the present exercise was to provide a risk assessment framework that, perhaps with slight context-dependent modifications, could be widely applied to various aquatic systems and geographic areas where introduced pathogens, and invasive species, threaten native populations. A logical next step would be to validate the present risk assessment by examining other systems for which data on incidence of *M. cerebralis* is available and seeing what their exposure and effects assessment scores are (risk ranking scores; followed by risk characterization). For example, verification of our model could be done for other tributaries of Yellowstone Lake by assigning risk scores to streams based on their range of stream temperatures, conductivity and chemical constituent concentrations, velocity and tubificids presence/absence data, according to our proposed ranking system. The final RCS would then be compared to the available *M. cerebralis* data for the systems as preliminary means of verifying the predictive power of our risk assessment. This was a "retrospective" ecological risk assessment that could thus be combined with a "prospective" ecological risk assessment approach to predict the chances of future adverse effects in infected watersheds within and outside national park boundaries, for instance, and evaluate the consequences of future trout population declines and management actions there.

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CHAPTER SIX

CONCLUSIONS

The growing frequency of species invasions, including those of animal or plant pathogens to new hosts or geographic areas, has made non-native species common components of every ecosystem on Earth (Marvier et al. 2004, Pimentel et al. 2005). Such invasions are a major threat to the native composition and community structure, biodiversity, and ecosystem function, which often result in immeasurable ecological and economic costs. Over the last decade, non-native species in the Yellowstone Lake ecosystem have increasingly threatened the long term survival of the Yellowstone cutthroat trout (Ruzycki et al. 2003, Koel et al. 2005, Murcia et al. 2006). The present study was designed to identify biotic and abiotic factors facilitating invasion and establishment by *M. cerebralis*, including native cutthroat trout host-response to parasite exposure (pathology, disease severity), spatiotemporal patterns of parasite incidence in three tributaries to the lake, and presence of, and infection prevalence in, the wild cutthroat trout and oligochaete hosts. The mechanisms identified as conducive to pathogen invasion and establishment were used, with risk assessment methodologies, to predict and rank the risk of population-level impacts associated with whirling disease in the Yellowstone Lake basin.

The first half of this investigation utilized data on infection response and environmental predictor variables from Pelican Creek only, where incidence of *M. cerebralis* was known to be highest (Koel et al. 2006, Murcia et al. 2006). The first

portion of the study focused on the patterns of infection pathology (location and severity of lesions) in cutthroat trout and the relationships between the environment and histopathology of parasitic infection in the wild. The second half of the study, however, incorporated data on additional response and predictor variables collected in the Yellowstone River and Clear Creek (Figure 4.1; chapter 4, 5).

Results from this investigation suggested that the native Yellowstone cutthroat trout from Yellowstone Lake are highly vulnerable to infection by *M. cerebralis*, especially in cartilage of the cranium and lower jaw. For whirling disease diagnostic and sampling purposes, examination of cranial cartilage should be an effective means of assessing pathology in susceptible salmonids (i.e., *Oncorhynchus* species) because this organ was most consistently and intensely damaged. Biologically, however, lesions in the jaw are extremely important for survival and feeding ability, and should also be assessed. This information was critical in order to assess the risk of population-level impacts among Yellowstone cutthroat trout because no prior studies had examined location of lesions in subspecies of cutthroat trout.

Whirling behavior was the most frequent clinical sign of disease in Yellowstone cutthroat trout, even though it is seldom reported of other salmonids (e.g., brown trout, rainbow trout; Hedrick et al. 1999, Ryce et al. 2004). Whirling behavior in native cutthroat trout was strongly correlated to moderate or higher inflammatory response and cranial lesions, which were expected results. When a pathogen invades cranial cartilage the host's inflammatory response may restrict nerves along the lower brain stem and spinal cord causing the whirling motion observed amongst infected trout and salmon

(Hedrick et al. 1999; Rose et al. 2000). Also, the proportion of cutthroat trout with moderate or higher lesion severity in cartilage of the jaws and cranium in Pelican Creek was strongly correlated with the combined effects of this system's water temperatures and specific conductivity. This suggested that pathology may correlate differently with single environmental attributes (e.g., Hiner and Moffitt 2001, Sandell et al. 2001) or with several factors in unison, which was also important information to assess the risk of *M. cerebralis* establishment in the lake basin. No prior investigations have linked salmonid-host pathology in detail to natural environmental stressors, or have examined the potentially synergistic effects of various environmental stressors on salmonid vulnerability to infection.

The most severely infected sites were the lower ones tested in the Yellowstone River and Pelican Creek, which is a common pattern of *M. cerebralis* infection in the wild, whereby parasite prevalence increases in a downstream progression (Sandell et al. 2001; Hubert et al. 2002). In addition, moderate and higher infection severity among the native salmonid was most prevalent in the July exposures, suggesting important spatiotemporal patterns in parasite incidence in the Yellowstone Lake Basin. Some sites were more difficult for cutthroat trout to avoid the parasite than others, and some exposure times (e.g., July) were most conducive to high infection, especially in cranial and jaw cartilage. Such spatiotemporal patterns in prevalence and infection seemed best explained by physical (e.g., temperature, velocity) and chemical features (e.g., sulfate, chloride), and by the extent to which these varied (coefficient of variation), across space and time within a system. Such fluctuations can be physiologically stressful to fish,

potentially predisposing trout to infection, and thereby, predisposing the system to this and further pathogenic invasions.

This second portion of the study examined the histopathology of cutthroat trout from the three streams 150 days instead of 90 days post-exposure. By examining Yellowstone cutthroat trout 150 days post exposure it is possible to miss the acute infection period, detecting chronic infection stages instead, and thus the degree of extant relationships with environmental attributes. Clearly, timing of histology is critical, and results of this research suggest that Yellowstone cutthroat trout should be examined 90 days post-exposure, as demonstrated in the first portion of the study. Besides timing of histology, water chemistry (ionic content) and the physical features of the system identified above (e.g., temperature) were also critical factors influencing risk of disease among the native cutthroat. The physicochemical features of tributaries to Yellowstone Lake are probably unusual compared to those of systems used in similar studies in other regions (e.g., Downing et al. 2002; Franco and Budy 2004, Krueger et al. 2006) due to geothermal or other (e.g., elevation, wild life) influences; or lack thereof (e.g., anthropogenic).

In conclusion, the findings from this study suggested a considerable risk of population-level impacts of infected cutthroat trout in the Yellowstone Lake Ecosystem. By ranking the identified ecological, host, and environmental risk factors influencing disease risk in Yellowstone cutthroat trout this study concludes that the Yellowstone River and Pelican Creek are at high risk of *M. cerebralis* invasion, establishment, and proliferation (e.g., disease outbreaks) and Clear Creek is at low risk. Based on the native

cutthroat susceptibility to *M. cerebralis* (Murcia et al. 2006; in review) this salmonid is at high risk of whirling disease and death, especially given the environmental characteristics of the Yellowstone Lake ecosystem.

The native cutthroat trout of the Yellowstone Lake basin are likely to become more susceptible to whirling disease and other parasitic or infectious diseases in the wake of global climate changes. The occurrence of *M. cerebralis* may be indicative of future incursion by similar or other species in the same or similar sites, which already show high (and widely variable) temperatures, ionic levels (e.g., sulfate, ammonium), and other stressful environmental features. Increased prevention of future biological invasions may be the key to maintaining the integrity of this ecosystem. The present study showed how risk assessment methodologies can provide important predictive tools for decision-makers about the potential effects of various management strategies to reduce and control the risk of spread and establishment of invasive species. The applicability and predictive power of this risk assessment can also be increased through context-dependent modifications for a diversity of aquatic systems and geographic areas where introduced pathogens and invasive species threaten wild trout populations and native species.

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