First introduced to the USA in 1958, *Myxobolus cerebralis*, the parasite responsible for whirling disease in salmonids, has since spread across the country causing severe declines in wild trout populations in the intermountain west. Recent development of risk assessment models used to assess the likelihood and consequences of exotic parasite introduction, have strengthened the process of science-based decision-making in aquatic animal health. In the case of *M. cerebralis*, it is necessary to use a risk assessment model with two unique segments that clearly address the distinct life stages and respective hosts of the parasite separately. The studies described examine the probability of *M. cerebralis* introduction and establishment for two regions: the state of Alaska, and the Willamette River basin, Oregon.

The Alaska risk assessment was based on the assumption that the parasite did not already occur in the state. However, in the process of validating this assumption, we documented the first polymerase chain reaction (PCR) detection of the parasite in the state. The pathogen was identified in hatchery rainbow trout (*Oncorhynchus mykiss*) from the Anchorage area. Although this is the first detection of the parasite in Alaska, clinical whirling disease has never been documented in the state.

To qualitatively assess the risk of further spread of *M. cerebralis* in Alaska, four potential routes of dissemination were examined: movement of fish by humans, natural dispersal (via migratory birds and stray anadromous salmon), recreational
activities, and commercial seafood processing. This research indicates the most likely pathway for M. cerebralis transport in Alaska is human movement of fish.

In the Willamette River basin, Oregon, introduction of M. cerebralis has already occurred, though establishment appears limited to a single private hatchery. Introduction in this region was considered the most likely to occur as a result of human movements of fish. Straying anadromous salmonids were also assessed and were present in higher numbers than predicted. However, they were not infected with the parasite, and thus the probability for introduction by this route is low. The probability of introduction of the parasite varies throughout the Willamette River basin. Areas with the highest probability for M. cerebralis introduction were identified as the Clackamas and Santiam River subbasins. The Clackamas River has already experienced an introduction of the parasite, has the largest concentration of hatcheries (state, federal, and private), has a popular sport fishery, and is the closest major tributary to the enormous piscivorous bird-populations in the Columbia River estuary. The Santiam subbasin has a popular sport fishery, received the highest number of stray fish in the Willamette River basin, and has the second largest concentration of hatcheries in the Willamette River basin.

Unique from introduction, establishment of the parasite is dependent upon several environmental and biological factors including: water temperatures, spatial/temporal overlap of hosts, and the distribution and genetic composition of the parasite’s invertebrate host, Tubifex tubifex. The distribution, genetic composition and susceptibility of T. tubifex, were considered the most important factor in the ability of M. cerebralis to establish in both systems. Surveys of oligochaete populations were conducted in both study regions.

In Alaska, T. tubifex was not detected from the southeast region and the apparent lack of appropriate tubificid hosts may prevent establishment in that part of the state. However, 4 lineages (I, III, IV, and VI) of the species were identified from
southcentral Alaska. Lineage IV has not been previously been described in North America and its susceptibility to *M. cerebralis* was unknown. When lineage IV *T. tubifex* and 3 mixed-lineage (I, III, IV and VI) groups were exposed to *M. cerebralis*, only lineage III became infected under our experimental conditions. Thus, if the parasite were dispersed, conditions are appropriate for establishment and propagation of the parasite life cycle in southcentral Alaska, although detrimental effects on fish populations may be reduced as a result of the presence of non-susceptible lineages of *T. tubifex*. The probability of further establishment in this area is greatest in Ship Creek, where the abundance of susceptible *T. tubifex*, the presence of susceptible rainbow trout (*Oncorhynchus mykiss*), and the proximity to the known area of infection make conditions particularly appropriate.

Similar to findings in Alaska, the Willamette River basin, Oregon also supports populations of susceptible *T. tubifex*. If the pathogen were introduced, probability of establishment is high in certain areas of the basin as all conditions are appropriate for propagation of the parasite life cycle. Tributaries to the mainstem Willamette River have the highest probability of establishment as these areas have the greatest numbers of susceptible *T. tubifex*. However, the abundance of resistant strains of *T. tubifex* could mitigate the effects of *M. cerebralis* if introduced.

Management recommendations to reduce the likelihood of parasite dissemination are similar for Oregon and Alaska since human movement of fish and angler activities were considered the most likely routes of introduction for both regions. Based on this research, steps should also be taken to limit human movement of fish, whether by restricting carcass planting for stream enrichment in Oregon, or by prohibiting use of fish heads as bait in southcentral Alaska. The states should also allot resources to angler education and awareness of the effects of angler activity and recreation on dispersal of *M. cerebralis*. This could be done using a combination of brochures and signage at boat ramps describing how to prevent spread of aquatic nuisance species.
Potential for Dispersal of the Non-native Parasite *Myxobolus cerebralis*:
Qualitative Risk Assessments for the State of Alaska and the Willamette River Basin, Oregon.

by
E. Leyla Arsan

A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented December 15, 2006
Commencement June 2007

APPROVED:

_______________________________________________________________
Major Professor, representing Fisheries Science

________________________________________________________________
Chair of the Department of Fisheries and Wildlife

________________________________________________________________
Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

_______________________________________________________________
E. Leyla Arsan, Author
ACKNOWLEDGEMENTS

I express my sincere gratitude to my major professor, Dr. Jerri L. Bartholomew, for the opportunity to pursue a graduate degree, and for her patience and guidance during my education. Special thanks to the ODFW pathology group and those at the Center for Fish Disease Research, Salmon Disease Lab for their assistance, guidance, and humor throughout my research. I would like to thank my friends and family for their encouragement and support. Lastly, I offer my tremendous appreciation to Matthew Giorgio for his unwavering belief in me.
CONTRIBUTION OF AUTHORS

Dr. Jerri L. Bartholomew was involved in the design, analysis, and writing of all phases of this project. Sascha L. Hallett and Stephen Atkinson assisted with experimental design and laboratory analysis and consultation. Dr. Theodore Meyers also provided consultation on experimental design and management recommendations for Alaska.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><em>Myxobolus cerebralis</em> description and lifecycle</td>
<td>1</td>
</tr>
<tr>
<td>Whirling Disease: distribution and impacts</td>
<td>2</td>
</tr>
<tr>
<td>Risk Assessment description and use in aquatic animal health</td>
<td>4</td>
</tr>
<tr>
<td>Research Goals</td>
<td>6</td>
</tr>
<tr>
<td><strong>TUBIFEX TUBIFEX FROM ALASKA AND THEIR SUSCEPTIBILITY</strong></td>
<td>7</td>
</tr>
<tr>
<td>Abstract</td>
<td>8</td>
</tr>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Methods</td>
<td>11</td>
</tr>
<tr>
<td>Results</td>
<td>23</td>
</tr>
<tr>
<td>Discussion</td>
<td>28</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>37</td>
</tr>
<tr>
<td>References</td>
<td>39</td>
</tr>
<tr>
<td>Tables</td>
<td>45</td>
</tr>
<tr>
<td>Figures</td>
<td>49</td>
</tr>
<tr>
<td><strong>EXPANDED GEOGRAPHICAL DISTRIBUTION OF MYXOBOLOS CEREBRALIS</strong></td>
<td>54</td>
</tr>
<tr>
<td>Abstract</td>
<td>55</td>
</tr>
<tr>
<td>Introduction</td>
<td>56</td>
</tr>
<tr>
<td>Methods</td>
<td>57</td>
</tr>
<tr>
<td>Results</td>
<td>61</td>
</tr>
<tr>
<td>Discussion</td>
<td>62</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>69</td>
</tr>
<tr>
<td>References</td>
<td>70</td>
</tr>
<tr>
<td>Tables</td>
<td>75</td>
</tr>
<tr>
<td>Figures</td>
<td>76</td>
</tr>
<tr>
<td><strong>A QUALITATIVE ANALYSIS OF RISK FOR THE INTRODUCTION AND ESTABLISHMENT OF MYXOBOLOS CEREBRALIS INTO THE STATE OF ALASKA, USA</strong></td>
<td>79</td>
</tr>
<tr>
<td>Abstract</td>
<td>80</td>
</tr>
<tr>
<td>Introduction</td>
<td>81</td>
</tr>
<tr>
<td>The Parasite Hazard</td>
<td>82</td>
</tr>
<tr>
<td>Risk Analysis</td>
<td>84</td>
</tr>
<tr>
<td>Release Assessment</td>
<td>86</td>
</tr>
<tr>
<td>Exposure Assessment</td>
<td>95</td>
</tr>
<tr>
<td>Conclusions and Risk Management</td>
<td>101</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th>104</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>106</td>
</tr>
<tr>
<td>Tables</td>
<td>116</td>
</tr>
<tr>
<td>Figures</td>
<td>118</td>
</tr>
</tbody>
</table>

POTENTIAL DISPERSAL OF THE NON-NATIVE PARASITE *MYXOBOLUS CEREBRALIS*: A QUALITATIVE ANALYSIS OF RISK FOR THE WILLAMETTE RIVER BASIN, OREGON

<table>
<thead>
<tr>
<th>Abstract</th>
<th>123</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>124</td>
</tr>
<tr>
<td>Methods</td>
<td>126</td>
</tr>
<tr>
<td>Release Assessment</td>
<td>128</td>
</tr>
<tr>
<td>Exposure Assessment</td>
<td>139</td>
</tr>
<tr>
<td>Conclusions and Risk Management</td>
<td>145</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>148</td>
</tr>
<tr>
<td>References</td>
<td>149</td>
</tr>
<tr>
<td>Tables</td>
<td>157</td>
</tr>
<tr>
<td>Figures</td>
<td>159</td>
</tr>
</tbody>
</table>

SUMMARY                                               | 164 |

BIBLIOGRAPHY                                          | 167 |
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Life cycle of <em>Myxobolus cerebralis</em></td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Map of the Columbia River basin with the <em>Myxobolus cerebralis</em> enzootic area circled and the Willamette River basin marked</td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Map of study locations, sampling sites for <em>Tubifex tubifex</em> (black dots), and rainbow trout (<em>Oncorhynchus mykiss</em>) hatcheries (triangles) in southcentral and southeast Alaska</td>
<td>50</td>
</tr>
<tr>
<td>2.2</td>
<td><em>Tubifex tubifex</em> lineages from Fort Richardson Hatchery, Alaska. Agarose gel electrophoresis of PCR-amplified genomic DNA using mt 16S rDNA lineage-specific primers. Two lineages were present: I (196 bp) and IV (2 bands at 320 bp and 147 bp). A negative control (water) and a positive control (lineage III, 147 bp) were included. A 50 bp DNA Ladder is shown on both sides of the gel (2% agarose)</td>
<td>51</td>
</tr>
<tr>
<td>2.3</td>
<td>First experimental exposures- Susceptibility of lineage IV <em>Tubifex tubifex</em> to <em>Myxobolus cerebralis</em>: triactinomyxon release per 100-worm replicate (3 Alaskan replicates are pooled as 1)</td>
<td>52</td>
</tr>
<tr>
<td>2.4a</td>
<td>Second experimental exposures- Comparative triactinomyxon release from Alaskan <em>Tubifex tubifex</em>. Number of triactinomyxons noted as absolute numbers 0-7000; greater values noted as &gt;7000 triactinomyxons and not as an absolute number</td>
<td>53</td>
</tr>
<tr>
<td>2.4b</td>
<td>Comparative triactinomyxon release from Alaskan <em>Tubifex tubifex</em> groups A, B, and C (enlargement of Figure 4a)</td>
<td>54</td>
</tr>
<tr>
<td>3.1</td>
<td>Agarose gel electrophoresis of PCR-amplified genomic DNA from 4 Alaska rainbow trout (sample numbers shown), using mt 18S rDNA specific primers. A 1 kb+ DNA Ladder is shown on both sides of the gel (1.5% agarose). A blank (B) well precedes each of the two positive (+) controls and a negative (-) control (water) is provided in the last well</td>
<td>77</td>
</tr>
<tr>
<td>3.2</td>
<td>QPCR cycle threshold values of Alaska hatchery rainbow trout plotted against reference samples spiked with known numbers of <em>Myxobolus cerebralis</em> myxospores (100, 1000).</td>
<td>78</td>
</tr>
<tr>
<td>4.1</td>
<td>Map areas of highest risk for <em>Myxobolus cerebralis</em> dissemination in southeast and southcentral Alaska. Rainbow trout hatcheries</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>depicted by triangles (Δ)</td>
<td>119</td>
</tr>
<tr>
<td>4.2 Model of potential <em>Myxobolus cerebralis</em> introduction in Alaska. Three main pathways of dissemination are shown with specific activities listed.</td>
<td>120</td>
</tr>
<tr>
<td>4.3 Scenario tree of risk of establishment of <em>Myxobolus cerebralis</em>, if disseminated in Alaska. Displays requirements for establishment and areas within the study sites that meet them.</td>
<td>121</td>
</tr>
<tr>
<td>5.1 Columbia River basin with Willamette River basin enlarged and <em>Myxobolus cerebralis</em> enzootic area depicted by dashed circle. Locations of fish hatcheries and collection sites of adult stray salmonids in the Willamette River basin are noted.</td>
<td>160</td>
</tr>
<tr>
<td>5.2 Pathways of potential parasite introduction in the release assessment</td>
<td>161</td>
</tr>
<tr>
<td>5.3 Scenario tree of risk of establishment in the exposure assessment</td>
<td>162</td>
</tr>
<tr>
<td>5.4 Locations of survey sites for <em>Tubifex tubifex</em> in the Willamette River basin</td>
<td>163</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Survey sites (excluding hatcheries) for <em>Tubifex tubifex</em> and their corresponding abundance and mitochondrial lineage</td>
</tr>
<tr>
<td>2.2</td>
<td><em>Tubifex tubifex</em> collected from Alaskan rainbow trout hatcheries</td>
</tr>
<tr>
<td>2.3</td>
<td>Prevalence and level of <em>Myxobolus cerebralis</em> infection in different mitochondrial lineages of <em>Tubifex tubifex</em> used in laboratory exposure experiments</td>
</tr>
<tr>
<td>2.4</td>
<td>Numbers of <em>Tubifex tubifex</em> exposed, infected and surviving experimental exposure to <em>Myxobolus cerebralis</em></td>
</tr>
<tr>
<td>3.1</td>
<td>Variable loci in the 18S SSU rRNA gene from different isolates of <em>Myxobolus cerebralis</em>. Loci are measured relative to 5’ position of universal primer ERIB1; heterogeneous bases: R=A+G, K=T+G, Y=C+T</td>
</tr>
<tr>
<td>4.1</td>
<td>Definitions of probability levels used in the risk analysis</td>
</tr>
<tr>
<td>4.2</td>
<td>Summary of the probability of introduction and within-state dissemination of <em>Myxobolus cerebralis</em> in Alaska</td>
</tr>
<tr>
<td>5.1</td>
<td>Summary of adult summer steelhead strays found at Oregon Department of Fish and Wildlife adult collection facilities in the Willamette River Watershed in 2004 and 2005</td>
</tr>
<tr>
<td>5.2</td>
<td>Survey sites and corresponding abundance and lineages of <em>Tubifex tubifex</em></td>
</tr>
</tbody>
</table>
DEDICATION

This thesis is dedicated to my aunt, Katherine Martin, who passed away during my research for this degree. She was always supportive of me and is deeply missed.

**CHAPTER 1: INTRODUCTION**

**Myxobolus cerebralis description and lifecycle**

*Myxobolus cerebralis*, a metazoan fish parasite exotic to North America, was first detected in the USA in 1958 (Hoffman 1962). There are some 1,350 known species of myxozoans, most of which are parasitic to fish at some point in their life cycle (Tops et al. 2004). The vast majority of myxozoans are relatively non-pathogenic. In contrast, *M. cerebralis* is one of the most pathogenic myxozoans known for fish (Hedrick et al., 1998).

![Figure 1.1. Life cycle of *Myxobolus cerebralis*.](image)

Figure 1.1. Life cycle of *Myxobolus cerebralis*. 
Life cycles are only known for 1% of the described myxosporeans (Kerans and Zale, 2002). The life cycle of *M. cerebralis* requires two obligate hosts: a salmonid and the aquatic oligochaete worm, *Tubifex tubifex* (Fig 1.1). There are also two distinct spore stages of the parasite (one particular to each of the hosts), the myxospore and the actinospore.

Myxospores develop in the fish host and are released upon death of the fish. These round and durable spores are characterized by two hardened valves surrounding two polar capsules with coiled filaments (Hedrick and El-Matbouli, 2002). Myxospores are ingested by *T. tubifex* as the worms consume sediment and bacteria.

Once inside the worm host, the parasite undergoes reproduction and structural transformation and is released in its actinospore stage as a triactinomyxon (TAM), morphologically different but genetically identical to a myxospore. The TAM attaches to its fish host in the water column, where the parasite sporoplasm is injected through the epidermis of the fish and migrates through the nervous system to the cartilage (El-Matbouli et al., 1995). The parasite consumes cartilage, causing lesions on the gills, cranium, and vertebral column of the fish (MacConnell and Vincent, 2002). Each spore stage requires approximately three months to develop within and be released from the respective host. Due to the myxospore’s durability (El-Matbouli and Hoffmann, 1991), this parasite stage is more likely to survive transport and be a risk for dissemination than the more fragile TAM stage.

**Whirling Disease: distribution and impacts**

Thought to have originated from a shipment of frozen infected brown trout (*Salmo trutta*) from Europe, *M. cerebralis* is now reported in a total of 24 US states in the contiguous U.S, (Bartholomew and Reno, 2002; Vermont Department of Fish and
Wildlife, 2002; Stromberg, 2006) and 26 different countries (Hoffman, 1962). The pathogen has been at the forefront of fish health research due to its potential impacts on rainbow trout culture and its implication in rapid dramatic declines of wild rainbow trout populations in Colorado and Montana (Walker and Nehring, 1995; Vincent, 1996; Nehring et al., 1998). The ecologic and economic impacts of *M. cerebralis*, in addition to its rapid spread and establishment across the globe, are indicative of the need for research to assist fishery managers in halting the spread or effects of the pathogen.

*Myxobolus cerebralis* was first detected in Oregon in 1986 (Lorz et al. 1989), and is now enzootic in certain tributaries of the upper Columbia River basin. Since the 1980’s, the parasite has been detected elsewhere in the basin from stray adult salmonids originating from these tributaries (Engelking 2002); however, there is no evidence of establishment of the parasite life cycle outside of the enzootic region. The parasite was detected further west in the state in 2001, at a private facility in the lower Willamette River Basin (Clear Creek). Infection at the facility was contained and there is no apparent establishment of the parasite in the wild (Bartholomew et al., 2007). Currently, *M. cerebralis* is not known to be established in the Willamette River basin outside of the private facility on Clear Creek. The closest enzootic area is the upper Columbia River basin (Figure 1.2).
Figure 1.2. Map of the Columbia River basin with the *Myxobolus cerebralis* enzootic area circled and the Willamette River basin marked.

**Risk Assessment description and use in aquatic animal health**

Risk analysis is a tool used to assess the likelihood and consequences of exotic parasite introduction. It addresses the following questions (MacDiarmid, 2000):

- What can go wrong?
- How likely is it to go wrong?
- What would be the consequences of its going wrong?
- What can be done to reduce either the likelihood or the consequences of its going wrong?

The International Aquatic Animal Health Code subdivides a risk analysis into: a) a release assessment, b) an exposure assessment and c) a consequence assessment (Office International des Epizooties [OIE], 2002). Risk assessments provide decision-makers with tools to assess management implications and to eliminate non-issues by using logical scientific arguments. Such assessments give managers a better understanding of where to allocate resources to help prevent introduction or spread of pathogens. In this way, risk analysis can be used as a type of map (MacDiarmid, 2000) to navigate through possible pathways or scenarios leading to an investigated risk. It is an essential first step in understanding potential outcomes and associated risks of management decisions.

Several risk assessment models have been created in the fields of animal health, biosecurity, and invasive species management (EPA 1992; Agriculture and Agri-Food Canada, 1994; Orr, 1995; OIE, 2002; Hayes, 2003). Typically, such qualitative risk assessments are initiated to either assess the risk of a newly proposed activity or pathway (i.e. importation) or to identify the route of introduction of a targeted species.
Most invasive species or biosecurity risk assessment models integrate the risk of introduction, the risk of survival during transport and risk of survival in the new environment, as the risk of establishment (EPA 1992; Agriculture and Agri-Food Canada, 1994; Orr, 1995). The establishment of *M. cerebralis* depends not only on the organism’s survival during transport and the environmental conditions of its new location, but also on spatial overlap with its definitive host, *T. tubifex*. The two distinct life stages and hosts of *M. cerebralis* necessitate the need for a risk assessment model with two distinct segments that clearly address each spore stage (and the respective host) separately. The model used in this research addresses the risk of introduction (myxospore stage) and the risk of establishment (actinospore stage) independently. The framework for this model (Bartholomew et al. 2005) was created in efforts to tailor such assessments to whirling disease. It is a combination of models employed by Orr (1993), OIE (2002) and MacDiarmid (2000), and is unique in that numerous potential pathways are assessed in one risk assessment model and that the targeted organism, *M. cerebralis*, has a complex two-host lifecycle that requires distinct assessments of each spore stage.

**Research Goals**

The goals of this research were to qualitatively assess the risk of introduction and establishment of *M. cerebralis* into the Willamette River basin, Oregon and the state of Alaska. Introduction in the Willamette River basin appears limited to a private hatchery (Batholomew et al. 2007) and the parasite was not known to be present in Alaska. The purpose of this study is to better understand the future risk of and routes of introduction, establishment, and further spread of the parasite into both areas. Specific study objectives are outlined below:

**Objective 1)** Assess the probability of introduction of *M. cerebralis* into the state of Alaska? **Approach:** Determine the validity of the current assumption that *M.*
cerebralis has not been established in Alaska. Determine and evaluate potential pathways for pathogen introduction and identify current critical gaps in data.

Objective 2) Assess the probability of establishment of M. cerebralis in the state of Alaska? Approach: Evaluate conditions that must be met to allow establishment in Alaska, including T. tubifex populations and lineages, and water temperatures.

Objective 3) Assess the probability of introduction of M. cerebralis into the Willamette River, Oregon? Approach: Determine the validity of the current assumption that M. cerebralis has not been established in the Willamette River basin. Determine and evaluate potential pathways for pathogen introduction and identify current critical gaps in data.

Objective 4) Assess the probability of establishment of M. cerebralis in the Willamette River, Oregon? Approach: Evaluate conditions that must be met to allow establishment in Willamette River basin, including T. tubifex populations and lineages, and water temperatures.
CHAPTER 2

TUBIFEX TUBIFEX FROM ALASKA AND THEIR SUSCEPTIBILITY TO MYXOBOLUS CEREBRALIS.

E. Leyla Arsan¹, Sascha L. Hallett², and Jerri L. Bartholomew²

¹Department of Fisheries and Wildlife, Center for Fish Disease Research, Oregon State University, 220 Nash Hall, Corvallis, Oregon 97331
²Department of Microbiology, Center for Fish Disease Research, Oregon State University, 220 Nash Hall, Corvallis, Oregon 97331
ABSTRACT

Although widespread throughout the continental United States, *Myxobolus cerebralis*, the myxozoan parasite that causes whirling disease in salmonids, has not been reported from the state of Alaska. As part of a risk assessment for the introduction and establishment of *M. cerebralis* into Alaska, the distribution of the invertebrate host, *Tubifex tubifex*, was surveyed and its genetic composition and susceptibility to the parasite determined. Many oligochaetes, but no *T. tubifex*, were collected from southeast Alaska; however, 4 lineages of *T. tubifex* (I, III, IV, and VI) were identified from southcentral Alaska. Lineage IV had not been previously described in North America and its susceptibility to *M. cerebralis* was unknown. When lineage IV *T. tubifex* and 3 mixed-lineage (I, III, IV and VI) groups were exposed to *M. cerebralis*, only lineage III became infected under our experimental conditions. Infection occurred in this lineage even when it comprised just 3% of the population. Implications of the presence of non-susceptible lineages of *T. tubifex* on Alaskan salmonids would be significant in areas where these lineages dominate *Tubifex* populations.
INTRODUCTION

*Myxobolus cerebralis*, the myxosporean parasite responsible for whirling disease in salmonids, has caused severe declines in wild trout populations in the intermountain west of North America (Walker and Nehring, 1995; Vincent, 1996; Nehring et al., 1998). The parasite’s definitive host, the oligochaete worm *Tubifex tubifex* (Wolf and Markiw, 1986), is a hearty and cosmopolitan species capable of withstanding extreme and variable environmental conditions. Such qualities allow the worm to inhabit areas where other species cannot compete, and thus to span across ecosystems as a widespread aquatic invertebrate.

*Tubifex tubifex* are commonly found in environments with abundant organic matter, fine sediments, and low flow (Brinkhurst and Gelder, 1991; Brinkhurst, 1996). The species is tolerant of low oxygen, desiccation, and variable temperature regimes (Reynoldson, 1987; Brinkhurst, 1996). Populations have consequently been found in environments extending from very unproductive high latitude lakes to eutrophic nutrient-rich lakes (Milbrink, 1994). *Tubifex tubifex* are important members of the aquatic ecosystem and play a vital role in breaking down organic matter and ingesting sediments (Brinkhurst and Gelder, 1991).

Though *T. tubifex* is thought to be cosmopolitan, it is not usually a common species (Brinkhurst, 1996) and its identification can be complicated. Positive identification is based on morphology of the reproductive structures such as the penis sheath, vas deferens, and atrium (Brinkhurst, 1996). Not only must worms be sexually mature to be confidently identified, but reproductive organs are
reabsorbed after breeding (Poddubnaya, 1984), a further hindrance to identification. Additionally, number of chaete, another important diagnostic trait, may vary depending on environmental conditions (Chapman and Brinkhurst, 1987). Non-mature *T. tubifex* can be difficult to distinguish phenotypically from other oligochaetes with similar physical characteristics, such as *Ilyodrilus templetoni* and *Rhyacodrilus* spp. Genetic assays have been developed for such differentiation among species (Beauchamp et al., 2001; Hallett et al., 2005).

In addition to complexities with identification of the species, recent studies have revealed the presence of 6 cryptic mitochondrial (mt) lineages of *T. tubifex* (lineages I - VI) (Sturmbauer et al., 1999; Beauchamp et al., 2001). Lineages cannot be distinguished morphologically, and therefore must be differentiated genetically. Four lineages (I, III, V, and VI) have been reported from North America (Beauchamp et al., 2001) and 5 are known from Europe (I-V) (Sturmbauer et al., 1999). Lineages II and IV have been described only from Europe, and lineage VI only from North America. Lineages commonly cohabitate, with more than a single given lineage found at a site, though populations can be mono-lineal (Beauchamp et al., 2006). Lineages of *T. tubifex* differ in susceptibility to *M. cerebralis* from highly susceptible to completely resistant. Populations of lineages I and III have been shown to propagate the parasite whereas lineages V and VI do not (Beauchamp et al. 2002, 2005, & 2006; DuBey et al., 2005). Although mt 16S rDNA does not directly confer resistance to *M. cerebralis*, the relationship can be useful in assessing general susceptibility.
This study was conducted as part of a risk assessment for the introduction and establishment of *M. cerebralis* into the state of Alaska. *Myxobolus cerebralis* has never been reported in Alaska, though monitoring for the parasite in the state is limited. The nearest known enzootic area is the Snake River basin in northeast Oregon and Idaho. The risk assessment included a *T. tubifex* survey to determine the presence, relative abundance, and lineages of *T. tubifex* from sites sampled in southeast and southcentral Alaska. Subsequent laboratory parasite-exposure experiments were conducted to determine the susceptibility of these lineages to *M. cerebralis*, with particular focus on a lineage previously unknown in North America. This paper describes results from both the *Tubifex* survey and lineage susceptibility experiments.

**MATERIALS AND METHODS**

**Sampling design**

A qualitative survey was used to determine presence/absence and relative abundance by order of magnitude of *T. tubifex*. Areas most likely to contain the organisms were targeted for sampling, i.e., those with low flow, fine sediments, and accumulations of organic material. Due to the magnitude in geographic size of Alaska [the state is approximately the size of the continent of Europe (Pagano 2000; Atlas A-Z 2001)], this study focused on the areas of the state where we considered the probability for introduction and establishment of *M. cerebralis* to
be highest: southeast and southcentral Alaska (the Cook Inlet basin) (Figure 2.1). These sites have physical attributes that are well-suited for parasite introduction and proliferation, such as, temperatures appropriate for parasite propagation, populations of salmonids susceptible to the parasite, a large commercial or sport fishery, and accessibility by boat or car.

**Site descriptions**

Oligochaetes were sampled from waterways in southeast and southcentral Alaska during August and September 2005 (Figure 2.1). Additionally, 2 fish hatcheries were sampled, representing the only locations in the state where rainbow trout [the species most susceptible to *M. cerebralis*, (MacConnell and Vincent, 2002; Sollid et al., 2002)] are reared. These samples were collected in November 2004 and May 2005 at Fort Richardson (FTR) and Elmendorf hatcheries in southcentral Alaska.

In southeast Alaska, 2 creeks near Juneau were sampled: Peterson Creek and Montana Creek. Southeast Alaska has a maritime climate and is within the Pacific Northwest temperate rainforest ecosystem. The area has cool winters and wet summers with stream temperatures generally warmer than those in the interior of the state. Peterson Creek annual water temperatures ranged from 0-16 C during 2002-2004 [R. Harding, Alaska Department of Fish and Game (ADFG), personal communication]. The natural hydrographs of these creeks are influenced by spring snowmelt and autumn rainfall (Chaloner et al., 2004; Montgomery et al., 1996;
Milner et al., 1997). Peterson and Montana Creeks have small basins, typical of coastal southeast Alaska, where mountains and icefields rise sharply from sea level and create relatively short watersheds that flow into the Pacific Ocean. Both creeks originate in the Taku Mountain Range and flow less than 15 km (Alaska Atlas and Gazetteer, 1998) to the protected waters of the inside passage off the Gulf of Alaska. The creeks support various salmonid populations including, steelhead trout (*Oncorhynchus mykiss*), pink (*O. gorbuscha*), chum (*O. keta*), and coho (*O. kisutch*) salmon, Dolly Vardon (*Salvelinus malma*), and coastal cutthroat trout (*O. clarkii*) (Harding and Jones, 1992; Chaloner et al., 2004). There is a barrier to fish migration approximately 2 km from the Peterson creek mouth; sampling was conducted below this barrier and before the short tidal area at the creek mouth.

In southcentral Alaska, three river systems within the Cook Inlet basin were sampled: Ship Creek, Campbell Creek, and the Kenai River. The Cook Inlet basin is home to over half the state’s human population. The area has a transitional climate (National Climate Center, 1982) and is the ecotone between the Pacific Northwest rainforest and the northern boreal forest. Annual water temperatures in these streams range from 0-16.5 C [U.S. Geological Survey (USGS), 2006a and 2006b]. Hydrographs in the basin are highly predictable and influenced by snow and glacier melt in the summer; typical freshwater inflow into Cook Inlet is 15 times higher in July than in February (Dorava and Milner, 2000).
Ship and Campbell Creeks, which originate in the Chugach Mountains, run west through the city of Anchorage and are impacted by urban development. The Kenai River flows approximately 132 unregulated km across the width of the Kenai Peninsula. The river has several distinct sections. The upper section (Kenai Lake to Skilak Lake) is included in the Kenai National Wildlife Refuge and has a more pristine character; the middle and lower sections of the river (Skilak Lake to Cook Inlet) contain more private property, industry, urbanization, and motorized vessels.

The Kenai supports populations of rainbow trout, Chinook, coho, pink, and sockeye (*O. nerka*) salmon, and Dolly Vardon and is the largest freshwater sport fishery in Alaska (Hammarstrom, 1988). Ship and Campbell Creeks support populations of these species as well as chum salmon (Miller and Bosch, 2004). Ship Creek is the most popular sport fishery in the Anchorage area and sustains the only 2 hatcheries, FTR and Elmendorf, in the state that rear rainbow trout. Ship and Campbell Creeks and the lower Kenai River have numerous sources of potential organic loading due to their urban proximity, commercial and industrial activity, streambank degradation due to recreational traffic, and large pulses of organic material from spawning salmon runs. Sampling sites in the Cook Inlet were chosen to represent water bodies with and without fish hatcheries, urban development, and glacial sources.
Tubifex tubifex collection and isolation

Oligochaete samples from hatchery settling ponds at FTR and Elmendorf (both on Ship Creek) were obtained as initial pilot samples in autumn 2004 before the complete T. tubifex survey was conducted. Sediments were collected with a shovel and shipped in fresh water and on ice to the Salmon Disease Laboratory (SDL), Corvallis, Oregon.

All other sediment samples were obtained via 5-gal bucket and 500 µm sieve or by 500 µm mesh kicknet. A bucket was used to scrape the substrate of the stream and collect sediment. Sediment was swirled with water in the bucket to dislodge worms and allow heavy particulates to sink to the bottom. Water with suspended material was poured through a sieve and washed with water to remove fines. In areas too deep or too rocky for a bucket, a kicknet was used. The remaining sample was deposited in a bag with clean water, placed on ice and sent to the SDL.

Once in the laboratory, the water in which samples were shipped was passed through a 20 µm mesh filter and inspected microscopically to determine if myxozoan actinospores were present. The sediment was then rinsed through a 246 µm screen to flush out fines. Bulk organic matter was retained and placed in a 24.8 x 14 cm shallow container and covered with water. The sample was swirled to evenly distribute sediments and organisms and a 24-cell grid was placed over the top of the container. Three subsamples were taken by simultaneously inserting
plexiglass squares (roughly 4 x 3.6 cm) into 3 randomly assigned cells in the grid. The material within each square was removed (approximately 30 ml) and sorted on a white tray. Oligochaetes were collected and inspected under a microscope to determine presence of hair chaete. In subsamples with greater than 500 worms, only 7.5 – 15 ml was sorted and numbers extrapolated to total 30 ml subsample volume. This was only necessary in subsamples from 3 sites: Centennial and Eagle Rock Boat Landings on the lower Kenai River, and from above Amalga Lake on Peterson Creek in Southeast Alaska.

Tubifex tubifex characterization

A subsample of 10 worms with both hair and pectinate chaete was mounted on slides for positive identification (Brinkhurst, 1986). Another 10 worms with both hair and pectinate chaete were assayed using a species-specific PCR (Hallett et al., 2005) to determine percentage of worms with morphological T. tubifex characteristics that were actually T. tubifex. All 10 worms suspected to be T. tubifex morphologically were determined to be so by genetic tests, thus hair and pectinate chaete were used as the definitive identification method for the remainder of the sorting process. Rinsed samples were maintained in culture at 12.8 C in an incubator with an air stone or on flow-through well water.
Oligochaete genetic analyses

In addition to the 10 worms subsampled for species characterization, 20 more *T. tubifex* per sample site were genetically assayed to determine lineage. In samples with fewer than 20 total *T. tubifex*, all *T. tubifex* found were assayed. Worms were digested with 95 µL ATL buffer (QIAGEN, Valencia, California) and 5 µL Proteinase K at 55°C, boiled for 5 minutes, and diluted 1:101 with buffer AE (QIAGEN) then stored frozen. A *T. tubifex* species-specific PCR assay was conducted following Hallett et al. (2005). Lineages were determined based on the PCR assays described by Beauchamp et al. (2001) and Sturmbauer et al. (1999). PCR products were visualized by gel electrophoresis (2% agarose) and UV illumination (Figure 2.2).

Worms morphologically identified as *T. tubifex* but which did not visibly amplify in the lineage assay had their mt 16S rDNA sequenced to determine whether they were a novel lineage or a different species. Two other morphologically similar tubifids, *Rhyacodrilus sodalis* and *Ilyodrilus templetoni*, whose sequences were not available on GenBank, were also sequenced. The gene was amplified with either the primers Tub16SF and Tub16SR (Beauchamp et al., 2001) or 16sar and 16sbr (Sturmbauer et al., 1999). Products were purified using a QIAquick PCR purification kit (QIAGEN). DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Samples were sequenced in 1 direction with Tub16SF or 16sar and ABI Big Dye Terminator chemistry on an Applied Biosystems Capillary 3100 Genetic Analyzer (Foster City, California) at the
OSU sequencing facility (Center for Gene Research and Biotechnology, Central Service Laboratory). Sequences were aligned in BioEdit (Hall, 1999) with other *T. tubifex* and selected oligochaetes from GenBank. A standard nucleotide-nucleotide BLAST (blastn) search was also conducted (Altschul et al., 1997).

**Myxobolus cerebralis susceptibility experiments**

Two exposure experiments were conducted: the first concentrated on lineage IV *T. tubifex* whereas the second included *T. tubifex* of all identified lineages from Alaska. The initial focus of these experiments was on lineage IV, as the susceptibility of this novel group was unknown. However, after the start of this study, other studies have changed our understanding of what lineages are susceptible, finding that only lineage III is susceptible to the parasite (authors unpublished data; B. Nehring, Colorado Division of Wildlife, personal communication). Thus, a second exposure experiment was conducted to compare susceptibility of all lineages found in Alaska with those from elsewhere in North America.

*First exposure- Susceptibility of lineage IV T. tubifex to M. cerebralis:*

FTR (Fort Richardson) *T. tubifex* were chosen for this study as the population was shown to have a high concentration of lineage IV worms. The FTR *T. tubifex* collected in 2004 were 81% lineage IV, and the remainder lineage I. Samples (in original sediment) from FTR were allowed to acclimatize to 12.8 C water for approximately 30 days before the start of the experiment. A known *M. cerebralis*-susceptible strain of *T. tubifex* (lineage III) was collected for a reference
triplicate groups of 100 worms from both FTR and WF and a single unexposed FTR control group were used for the parasite challenge (total of 7 groups). Worms were held in 38.5 cm² plastic containers at a density of 2597 worms/m² with 1-2 cm of coarse and fine sand and slow-flowing 12.8 C well water.  

*Myxobolus cerebralis* myxospores were collected from rainbow trout infected and held at the SDL. Heads of infected fish were removed and processed in a blender. Spores from the resulting suspension were enumerated using a hemocytometer. Worms were exposed to 500 myxospores each. Running water was diverted for the first 48 hours to allow myxospores to settle to the bottom of containers. Spores were left in the worm containers after exposure to imitate a natural source of infection that would likely not be removed after 24-48 hr. Worms were fed once a week (after screening) with ALGAMAC-2000 (Biomarine Inc., Hawthorne, CA).  

Samples were filtered for triactinomyxon (TAM) spores through a 20 µm mesh screen twice a week beginning at 60 days post-exposure (PE). This time period was chosen as the earliest date of initial TAM release from *T. tubifex* has occurred at 74 days PE at 15 C (Gilbert and Granath, 2001), the latest at 182 days PE (Kerans et al., 2005). Water inflow was diverted for 24 hr prior to screening. Site replicates were pooled for screening until TAMs were detected. Two 50 µl aliquots of the retained screened material were placed on a glass microscope slide, air-dried and examined with a compound microscope at 100x magnification with
phase contrast. TAMs were extrapolated to number/ml.

At peak TAM release in the WF reference group, subsamples of 20 worms per replicate were separated into 24-well plates and monitored for 48 hr for individual worm TAM release. After 48 hr, the worms were frozen for infection verification via QPCR. Since no TAMs had been detected in the FTR group, 20 worms per replicate were separated at the same as the WF group, with individual worms directly frozen for genetic analysis. A *M. cerebralis* QPCR assay (Kelley et al., 2004, see below) was performed to ascertain if worms were infected but not releasing TAMs. The lineage of all worms was also determined.

Biweekly filtering was continued until TAM numbers significantly declined or were near zero, 191 days PE. Worms were held until 280 days to assess post infection survival and reproduction. At this point, remaining individual worms were counted and assessed as adults or juvenile progeny from original worms placed in containers at day 0. Juveniles were frozen for lineage analysis to assess changes in lineage structure of the population.

*Second exposures- Comparative susceptibility of Alaskan T. tubifex to M. cerebralis:*

A second round of experimental exposures to *M. cerebralis* was conducted to test the susceptibility of all the lineages found in Alaska: I, III, IV, and VI. Six groups of *T. tubifex* were used: 3 mixed lineage groups, an FTR group from the first experimental exposure, a WF reference group, and a WF unexposed control group. Group A (roughly 71% I, 21% III, and 10% IV) was chosen to represent
lineage III as it had the largest proportion of that lineage. Group B, representing lineage VI, was roughly 90% VI and 10% III, and group C, representing lineage I, was approximately 84% I, 8% III, 3% IV, and 5% VI. These lineage proportions are representative of samples collected in Alaska.

Lineage IV worms from the first experimental exposures were re-exposed to assess if their susceptibility is dependant on maturity or size. At the time of previous exposure they were small in size in comparison with other worms we have sampled from the Pacific Northwest. As these are the first data on Alaskan *T. tubifex*, it was unknown if these specimens were simply smaller due to the climatic and environmental pressures of their high latitude environment, and if given the opportunity, they would grow larger. Indeed, after being held at 12.8 C, being fed every week, and having an absence of predators, the worms grew much larger, matured and reproduced and thus were re-exposed as known adults.

In the second exposure challenge, the first round methods were modified to ensure similar methods, and thus comparison, with research in other laboratories. Two hundred worms per container were used (density 5194 worms/m²), with 1 cm clean coarse and fine sand. Filtering was conducted every 7-10 days starting at 100 days PE, a date by which other studies have documented TAM release to begin (Gilbert and Granath, 2001; Blazer et al., 2003; Kerans et al., 2004; Beauchamp et al., 2006). Numbers of TAMs released from the WF reference group were counted until numbers exceeded 7000 TAMs per 100 µl of filtrate.
Numbers above this value were noted as “greater than 7000”. Filtering ended at 160 days PE, as TAM release in the Alaskan groups had begun to decline.

At 120 days, 50 worms from each group that was releasing TAMs, were separated into 24-well plates and monitored for 48 hours for individual worm TAM release. This number was selected to detect an infection prevalence of at least 5% (with 95% confidence) (USFWS and AFS-FHS 2003). At the end of the experiment, all worms were counted and adults frozen for genetic analyses described in the first experimental exposures.

**Myxobolus cerebralis QPCR**

To detect *Myxobolus cerebralis* infected worms, the QPCR assay described by Kelley et al. (2004) was followed except that 4 µl of extracted DNA was used in a final reaction volume of 20 µl. Reactions were performed in an ABI PRISM 7000 Sequence Detection System in either ABI PRISM™ optical tubes or MicroAmp® optical 96-well reaction plates. A negative (water) and positive (spores or infected fish) control were included in each run. In the second experimental exposures, individual extracted samples were pooled into groups of 4 worms per reaction, with 1 µL from each worm used. This method was tested with worms with known levels of infection to ensure that pooling samples would be sensitive enough to detect 1 worm with a low infection (authors unpublished data). If infection was detected, worms in that pool were assayed individually. The pooled samples were run singly and rerun if the amplification plot was
dubious. An individual sample presenting a cycle threshold (Ct) value of 38 or less was considered infected (Hallett and Bartholomew, 2006).

RESULTS

*Tubifex tubifex survey*

Over 2,700 oligochaetes were collected from southeast Alaskan sample sites (Table 2.1); however, none of the worms assayed were *T. tubifex*. Many of the non-mature worms from this area were identified as *T. tubifex* morphologically, until contrary genetic identification; sequencing indicated these specimens to be most similar to *Rhyacodrilus sodalis*, an oligochaete with comparable morphological traits to *T. tubifex*, such as hair chaete. Because worms from other sample sites in southcentral Alaska that had hair chaete were in fact *T. tubifex*, this trait was used as a determining factor while processing samples. This method was also successful in other studies (Zendt and Bergerson, 2000; Allen and Bergerson, 2002) but proved unsuccessful for the samples from southeast Alaska.

*Tubifex tubifex* were abundant at certain locations in southcentral Alaska, with the highest numbers on the lower Kenai River, particularly at Centennial (~RM 20.4) and Eagle Rock (~RM 11.3) Boat Landings (Table 2.1). *Tubifex tubifex* were also identified from Ship and Campbell Creeks in the Anchorage subbasin, though not in the Upper or Middle Kenai River on the Kenai Peninsula. Furthermore, settling ponds at the 2 fish hatcheries provided many *T. tubifex*
Other oligochaetes morphologically identified in the survey include: *Lumbriculus variegatus*, *Spirosperma nikolski*, *Kincaidiana hexatheca*, and members from the Naididae.

The *T. tubifex* populations in southcentral Alaska were a mixture of 2 or 3 mt lineages (Table 2.1). Four mt lineages were identified in the state: I, III, IV, and VI. Lineage I dominated (71-86%) sites on Ship Creek and Campbell Creek, whereas Lineage VI dominated (69%) sites on the Lower Kenai River. Lineage III was present in low numbers (7-21%) throughout the Cook Inlet basin at 7 out of the 9 sites where *T. tubifex* was found. However, it was not identified from any of the hatchery sites.

The FTR hatchery settling pond on Ship Creek had the greatest abundance of lineage IV worms. This lineage was also found in Ship Creek near both FTR and Elmendorf hatcheries, but not elsewhere in Alaska. Four of the lineage IV worms were sequenced to confirm the results of the lineage assay; a BLAST search revealed them to be most similar to a lineage IV *T. tubifex* from Europe (Sturmbauer et al., 1999; AJ225910). There were 4 base differences (1.2%) over the aligned 325 bases, which is consistent with the intra-lineage variation determined by Sturmbauer et al. (1999).

Mt 16S rDNA from a subset of worms that did not amplify in the lineage PCR but was amplified in the *T. tubifex* species-specific PCR was sequenced. Sequences were genetically identical to each other and to a lineage I *T. tubifex* from Europe (Sturmbauer et al., 1999; AJ225904). However, they differed (in 2-4
base positions over 300bp) to 2 other European lineage I worms (AJ225903 and AJ225906).

**Actinospore screening from survey sites**

No *M. cerebralis* TAMs were observed in filtrates from any of the sites sampled in the *T. tubifex* survey. However, actinospores of several other myxozoans were found in both southeast and southcentral Alaska; these were genetically identified as *Sphaerospora oncorhynchi, Myxobilatus gasterostei*, and the CKX organism triactinomyxon [unknown coho kidney parasite (Jones et al., 2004)] (S. Atkinson, Center for Fish Disease Research, personal communication). Other unidentified triactinomyxon and raabeia type actinospores were also found. This validates that our methodology was sufficient for detecting *M. cerebralis* TAMs.

**Susceptibility of lineage IV *T. tubifex* to *M. cerebralis***

No TAMs were detected from any of the 3 replicates of lineage IV worms from FTR (Table 2.3, Figure 2.3). Typical signs of infection (decreased growth, slowing or absence of reproduction) were not observed; to the contrary, the FTR worms continued to reproduce and grow in size. QPCR verified no *M. cerebralis* infection.

TAM release in the susceptible WF reference group of *T. tubifex* began at 98 days PE (Table 2.3, Figure 2.3). This group exhibited peak TAM release (>270
spores per worm per day) between 112 and 143 days PE. The experiment ran for 191 days, at which time no WF replicates were releasing TAMs. When individual worms were monitored for TAM release at peak infection, 90% were releasing TAMs; at least 2151 TAMs per worm were detected in a 48-hr period. Infection was verified by QPCR in 85% of WF worms.

All WF worms assayed at peak TAM release (n = 60) were lineage III. FTR worms assayed at the same time were comprised of 68% lineage IV, 30% lineage I, and 2% unknown lineage. For the purposes of this study, a lineage is considered unknown when the specimen amplifies as *T. tubifex* in a species-specific PCR but does not amplify in a lineage PCR, or vice versa. This will be explained further in the discussion of this paper.

Worms were held after filtering ended at 191 days PE to assess post-infection survival and reproduction. At 280 days PE, 38-50% of adult Alaskan worms remained among replicates, with 42–131 additional juvenile worms (Table 2.4). In contrast, only 7% of the WF worms survived in 1 lone replicate. All other WF worms did not survive to 280 days PE. Juvenile Alaskan worms from the end of the experiment (progeny of original worms placed in experiment) were approximately 74% lineage IV, 23% lineage I, and 3% unknown lineage.

**Comparative susceptibility of Alaskan *T. tubifex* to *M. cerebralis***

Table 2.3 and Figure 2.4 show TAM release from all groups in our second experimental exposures. TAMs were first detected in Alaskan worms at 108 days
PE, with numbers peaking at only 188-280 TAMs per 7 days. In contrast, the reference WF group exhibited heavy infection with detection of TAMs by 100 days PE (day of first filter) and numbers peaking at >7000 TAMs per 7 days. Once again, the FTR group did not become infected. Only group C worms were individually separated into 24-well plates and monitored for TAM release. Results from this separation indicated this method is ineffective in detecting TAM release from groups with low infection prevalence.

Group A began releasing TAMs at 120 days PE. Of the 90 total surviving worms at 160 days PE, 3 (3%) were lineage III. These were the only worms from group A that were infected as determined by QPCR.

In group B, a single TAM was detected at 132 days PE and then another again at 153 days. Extrapolated to total volume of filtrate, this is the equivalent of 11 TAMs on day 132 and 9 TAMs at 153 days. The number of TAMs detected per Alaskan group was not proportional to infection prevalence as determined by QPCR. Group B had the highest infection prevalence (10%) and the lowest peak number of TAMs detected (11) (Table 2.3) whereas group C had the highest peak number of TAMs detected (280) and the lowest infection prevalence (0.5%).

No TAMs were detected in worms from group C that were sampled at 120 days PE, and QPCR confirmed none of these worms were infected. This indicates that infection prevalence in this group was less than 5%. Indeed, QPCR analysis at the end of the experiment confirmed that only 1 worm out of the 158 surviving
(0.6% of the group) was infected. The single infected worm was lineage III. The 6 other surviving lineage III in this group were not infected.

Worm survival was similar to that of the first experimental exposure (Table 2.4). The WF reference group had poor adult survival of only 17.5%, with only 9.5% of the original population replenished by juvenile worms. In contrast, the population of the FTR group increased with an adult survival rate of 100% and an additional 98% of the original population reproduced as juvenile worms. Groups A-C had adult survival rates similar or better than that of the unexposed WF control group.

DISCUSSION

*Myxobolus cerebralis* has not been described from Alaska, although it is recognized as an economically and ecologically important pathogen of salmonid fishes elsewhere in North America. To assess the potential for the parasite to become established in that state, we investigated the distribution of the invertebrate host, *T. tubifex*, which *M. cerebralis* requires to propagate. Prior to our study, there was no record of the distribution of *T. tubifex* in Alaskan freshwaters and no data on whether lineages present in the region were susceptible to *M. cerebralis* should the pathogen be inadvertently introduced.

*Tubifex tubifex survey*
Tubifex tubifex were identified in southcentral Alaska, but not from the sites sampled in southeast Alaska. Sites sampled in southeast Alaska were approximately 900 km from sites sampled in southcentral Alaska. The sites with highest abundance of T. tubifex were Centennial and Eagle Rock Boat Landings on the lower Kenai River, areas with high sedimentation and organic loading (primarily decaying salmon carcasses) and heavy recreational use. The second highest abundance was at FTR hatchery settling pond. Excluding hatcheries, the area with the second highest T. tubifex abundance was near the inflow for Elmendorf fish hatchery on Ship Creek.

Tubifex tubifex are commonly associated with fish hatcheries, likely due to the large amount of organic matter available and thus the provisions of both a seemingly endless food source and a habitat of accumulated sediment. In reference to the dense populations of worms frequently found where organic enrichment is high, the term sludge worms is often used to describe T. tubifex. Researchers have found the worms in hatchery settling ponds, but not in the surrounding stream sediments (Allen and Bergersen, 2002; Bartholomew et al., 2007). Fish hatchery worms were tallied separately in this study (Table 2.2) because settling ponds are independent from the main creek channel and are not representative of T. tubifex abundance in the wild.

Our data support Brinkhurst’s (1996) statement that, contrary to expectation, T. tubifex is not a common species. The taxon was present at only 10 of the 37 sites (27%) that we sampled (outside of hatcheries) in Alaska. Where it
was present, it was usually also abundant, comprising at least half the total worms
found at 5 out of the 10 sites.

Greater numbers of *T. tubifex* in the lower Kenai River could be due to the
influence of Skilak Lake (9945 ha, from approximately RM 50-65) on both
biological processes and the attenuation of glacial runoff. Lakes have been shown
to dampen peak flows in rivers, sustain high flows in summer, supplement low
flows in winter, increase river temperatures, and provide settling for suspended
sediment (Oswood et al., 1995). Macroinvertebrate densities and diversity are
greater below Skilak Lake than above, and higher in the Kenai River in general
than in other glacial-fed rivers in the Cook Inlet basin (Dorava and Milner, 2000).
Milner and Petts (1994) suggest this is because of increased temperature and
channel stability due to lake regulation. The USGS’s NAWQA (National Water
Quality Assessment) data shows the lower Kenai River as one of the sites with the
highest numbers of worms (species not specified) in the Cook Inlet basin (Glass et
al., 2004). The area was one of only two sites in the basin where aquatic worms
made up nearly one-third of all macroinvertebrates sampled.

While water temperatures affect *M. cerebralis* proliferation within *T.
tubifex* (Hedrick and El-Matbouli, 2002; Blazer et al., 2003), they are not known to
affect worm presence or absence. *Tubifex tubifex* is tolerant of variable
temperature and oxygen regimes (Reynoldson, 1987; Brinkhurst, 1996), and
populations have been found in environments extending from unproductive high
latitude lakes to eutrophic nutrient-rich lakes (Milbrink, 1994).
Additionally, the lower Kenai River may provide more suitable substrates for *T. tubifex*. The lower river is approximately 43% silt/sand as opposed to the upper and middle sections of the river that are only 2% silt/sand and 32-41% cobbles (12.7-25.4 cm) (Bendock and Bingham, 1988).

The discovery of non- *M. cerebralis* actinospores in our water filtrates confirms that other myxozoan lifecycles have established in Alaska. Some of the Alaskan myxozoans were found in the oligochaetes *Ilyodrilus templetoni* and *Limnodrilus hoffmeisteri* (S. Atkinson, Center for Fish Disease Research, personal communication), species commonly associated with *T. tubifex*. Several of these specimens were collected from southeast Alaska.

While *T. tubifex* were not found in southeast Alaska, it is difficult to determine whether the absence is true or simply due to a lack of detection or small sample size. As our methods of detection have proven effective in other parts of Alaska (southcentral) as well as in other studies (Bartholomew et al., 2007; authors unpublished data), we believe this study accurately represents the relative abundance of *T. tubifex* in the areas sampled. It is possible that more extensive surveys may detect *T. tubifex* in southeast Alaska. This study did not sample glacial rivers in these areas as they did not meet our sampling criteria of being at highest risk for *M. cerebralis* introduction (i.e., supporting populations of rainbow or steelhead trout and being close to a road system). Glacial Rivers in southeast Alaska are generally longer than non-glacial streams, as they follow a path that has gouged through mountains by a receding glacier. In contrast, non-glacial streams
in this area of the state are typically short, low order, and steep, a reflection of the topography of the Alaskan Coast Range that rises sharply from the Pacific Ocean. This may limit appropriate habitat for *T. tubifex*. However, the large numbers of other oligochaetes found in southeast Alaska, shows that there is habitat to support some species of this class of annelids commonly associated with *T. tubifex*. Additionally, the more pristine character of some southeast Alaskan streams may allow proliferation of oligochaete species that could out-compete *T. tubifex*.

Three of the four North American *T. tubifex* lineages were found in Alaska (I, III, and VI). These lineages are documented from other states such as Colorado, Montana, and Utah (Beauchamp et al., 2002), as well as New Mexico (Dubey and Caldwell, 2004), Pennsylvania (Kaeser et al., 2006), and Oregon (authors’ unpublished data). In addition, we discovered a fifth mt lineage, lineage IV, which has previously been documented only in Europe (Sturmbauer et al., 1999).

While determining presence and lineages of *T. tubifex*, we encountered some discrepancies in our genetic analyses. Several worms did not amplify in the lineage PCR but were confirmed as *T. tubifex* in the species-specific PCR. The mt 16S rDNA of these worms was sequenced to investigate the possibility of the presence of a lineage not targeted by the original lineage assay. The worms were verified as lineage I as they were identical to a lineage I worm in GenBank (from Europe; Sturmbauer et al., 1999), but they had a single base difference in the lineage I primer annealing location. Two of the 3 lineage I worms in GenBank also
had this base difference (GenBank accession numbers AJ225904, AJ225906); the third worm (AJ225903) did not. Thus, if only a lineage PCR is performed in lieu of a species-specific PCR, false negatives could be reported.

Another problem encountered in the *T. tubifex* molecular assays was inhibition in samples taken directly from the field. Inhibition was eliminated by dilution of extracted DNA to 1:100. Although the exact source of inhibition is unknown, humic substances have been known to cause inhibition in PCR by forming complexes with metal ions (De Boer et al., 1995). Peterson Creek in southeast Alaska has dark humic-stained water and a high level of dissolved organic carbon (Chaloner et al., 2004). We observed similar dark-colored water at other sampling sites with non-glacial sources in both southeast and southcentral Alaska. Samples held in the laboratory in clean sediment and water did not exhibit inhibition of the PCR assays.

**Susceptibility of Alaskan *T. tubifex* to *M. cerebralis***

Of the 4 mt lineages (I, III, IV, and VI) of *T. tubifex* that were exposed to *M. cerebralis* myxospores, only lineage III worms became infected and released actinospores. Even when comprising a low proportion of the population (Table 2.3), the lineage became infected and, in 2 of the 3 Alaskan groups, released a steady if meager supply of TAMs. This lineage has been consistently susceptible to *M. cerebralis*; all exposed populations from North America propagate the parasite, including California and Colorado (Beauchamp et al., 2002, 2006),
Montana (Stevens et al., 2001), New Mexico (DuBey et al., 2005), and Oregon (authors’ unpublished data).

We found the severity of infection (as determined by QPCR) and numbers of TAMs produced in Alaskan lineage III *T. tubifex* to differ from lineage III from other geographical locations. A 10-100 fold greater number of TAMs have been documented from lineage III from: Mt. Whitney Fish Hatchery, California (Beauchamp et al., 2002, 2006), the Deschutes and Metolius Rivers, Oregon (authors’ unpublished data) and the Madison River, Montana (Stevens et al., 2001). Upwards of 23,000 TAMs were produced in 3 days in lineage III *T. tubifex* from Mt. Whitney (Beauchamp et al., 2006) and Metolius River lineage III (WF reference group for this study) produced over 11,000 TAMs in a 4 day period (Figure 2.3). In contrast, TAM production in Alaskan lineage III *T. tubifex* peaked at a meager 188-280 TAMs in 7 days (Figure 2.4). Given the low abundance of lineage III *T. tubifex* found in Alaska and the small number of TAMs released from the group, salmonids in Alaskan streams would likely experience a very low exposure to *M. cerebralis* (if the parasite were introduced).

Our study is the first to examine the susceptibility of lineage IV to *M. cerebralis*. We found the lineage not to be susceptible to the parasite when exposed to 500 myxospores per worm. This dosage and exposure appears to be appropriate to cause infection, as our reference control group (WF) became heavily infected and released TAMs. Water temperatures during the experiment were similar to those tubifex populations would encounter during an Alaskan
summer. However temperatures would be lower in the spring and fall causing delayed parasite propagation and prolonged TAM release (El-Matbouli et al., 1999; Blazer et al., 2003).

Worm populations collected from Alaska and used in our experiments were a mixture of lineage IV (65%) and lineage I (31%). Lineage I *T. tubifex* have been shown elsewhere to be susceptible to the parasite in mixed lineage populations, though virulence can vary among different geographical strains of *T. tubifex* (Beauchamp et al., 2002; Kerans et al., 2004).

Myxospores are typically ingested as worms burrow through sediment to consume particles and bacteria (Brinkhurst, 1996). Kerans and Lemmon (in Kerans and Zale, 2002) found that the negative effects of myxospores on the biomass of *T. tubifex* were reduced if the species was grown in culture with *L. hoffmeisteri*, presumably because myxospores were consumed by *L. hoffmeisteri* and were thus unavailable to *T. tubifex*. Other studies have shown a 70% reduction in parasite production in mixed (susceptible and resistant) cultures of *T. tubifex* compared to pure cultures of susceptible worms (Beauchamp et al., 2006). It is possible that because lineage IV worms were not susceptible and were also dominant in the population used in the first experimental exposures, that they ingested spores, inactivating or making them unavailable to lineage I worms. However, 30% lineage I seems to be a sufficient presence in a population to become infected if susceptible; populations of approximately the same proportion of susceptible to resistant *T. tubifex* have been used in similar parasite exposure
studies (Beauchamp et al., 2006) and became infected with *M. cerebralis*. Similarly, in the second exposures, Group A and C were 80-89% lineage I, yet only lineage III worms became infected (Table 2.3).

Other studies have shown that even resistant worms have adverse effects from the parasite, such as impaired reproduction, even though spores were not released (Steinbach Elwell et al., 2006). Our data show the control unexposed FTR worms had 29-69% higher reproductive success than their exposed counterparts. Thus it appears that both lineage IV and lineage I *T. tubifex* experience some adverse effects from parasite exposure, though infection and TAM release were never detected. However, considering the relatively large proportion of Alaskan worms alive at 280 days PE (compared to the reference group), it is clear that adult survival and reproduction in the Alaskan lineages exposed to the parasite is much greater than that of the susceptible WF worms. The 4 lone worms that remained in the WF group after the same amount of time is an indication of poor adult survival and reproductive success in infected lineage III worms.

**Conclusions**

Positive implications of the resistance of lineages I, IV, and VI *T. tubifex* from Alaska toward the potential for establishment of *M. cerebralis* could be significant in areas where the lineages dominate *Tubifex* populations. Other researchers have found lineage susceptibility to correspond to severity of infection
in fish (Beauchamp et al., 2006; B. Nehring, Colorado Division of Wildlife, personal communication). This suggests that when resistant lineages are in high abundance, the effects of *M. cerebralis* may be diminished. Lineage III represents the highest risk to Alaskan salmonids. However, given the low abundance of lineage III *T. tubifex* found in Alaska and the low level of parasite production from the group, negative impacts on Alaskan salmonids are likely to be reduced if the parasite were introduced.

**ACKNOWLEDGMENTS**

This work was supported in part by the National Partnership for the Management of Wild and Coldwater Species, the U.S. Fish and Wildlife Service, and the U.S. Geological Survey. We are grateful to: Tammy Burton of the Alaska Department of Fish and Game (ADFG) for providing live worm samples and field support; Stephen Atkinson, Harriet Lorz, and Don Stevens at the Center for Fish Disease Research, for assistance with worm identification and for efforts in maintaining oligochaete cultures; Jenny Dubanoski and Genny Cobarrubias at Oregon State University for assistance with genetic assays; and Paul Reno and Carl Schreck at Oregon State University, Department of Fisheries and Wildlife for providing critical comments on this manuscript. We acknowledge Ted Meyers and Tim McKinley of the ADFG for their help in the field; Ted Meyers also provided essential consultation on study design and background. We are thankful to Alaska Wildland Adventures for providing equipment and field support on the
upper Kenai River. We appreciate the identified non-*T. tubifex* DNA provided by Charlotte Rasmussen of the USGS Western Fisheries Research Center and Billie Kerans of Montana State University.
REFERENCES


Beuchamp, K. A., M. El-Matbouli, M. Gay, M. P. Georgiadis, R. B. Nehring, and


Hallett, S. L. and J. L. Bartholomew 2006. Effects of water flow on the infection dynamics of *Myxobolus cerebralis*. Page 71 in Abstracts from the Fifth International Symposium on Aquatic Animal Health, San Francisco, California, USA.

Hammarstrom, S. L. 1988. Angler effort and harvest of Chinook salmon *Oncorhynchus tshawytscha* and coho salmon *O. kisutch* by the recreational fisheries in the lower Kenai River, 1987. Alaska Department of Fish and Game, Division of Sport Fish, Fisheries Data Series No. 50, Juneau, Alaska.


Jones, S., G. Preoperi-Porta, S. Dawe, J. Blackbourn, K. Taylor, G. Lowe, and A.


National Climate Center. 1982. Climate of Alaska. NOAA Environmental Data Service, Asheville, NC.


Steinbach Elwell, L. C., B. L. Kerans, C. Rasmussen, and J. R. Winton. 2006. Interactions among two strains of Tubifex tubifex (Oligochaeta: Tubificidae) and Myxobolus cerebralis (Myxozoa). Diseases of Aquatic Organisms 68: 131-139.


Table 2.1. Abundance and mitochondrial lineage of *Tubifex tubifex* from survey sites (excluding hatcheries).

<table>
<thead>
<tr>
<th>River</th>
<th>Sites sampled</th>
<th>Sites with <em>Tubifex tubifex</em></th>
<th>Total worms*</th>
<th>*Tubifex tubifex†</th>
<th>Worms assayed ‡</th>
<th>Worms sequenced</th>
<th>Lineages (% of sampled population)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Southeast Alaska</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peterson Creek</td>
<td>4</td>
<td>0</td>
<td>2,741</td>
<td>0</td>
<td>61</td>
<td>2</td>
<td>n/a</td>
</tr>
<tr>
<td>Montana Creek</td>
<td>4</td>
<td>0</td>
<td>54</td>
<td>0</td>
<td>42</td>
<td>1</td>
<td>n/a</td>
</tr>
<tr>
<td>Total southeast</td>
<td>8</td>
<td>0</td>
<td>2,795</td>
<td>0</td>
<td>103</td>
<td>3</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Southcentral Alaska</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ship Creek</td>
<td>6</td>
<td>5</td>
<td>1,545</td>
<td>251</td>
<td>71</td>
<td>3</td>
<td>I (71%), III (21%), IV (7%)</td>
</tr>
<tr>
<td>Campbell Creek</td>
<td>6</td>
<td>2</td>
<td>415</td>
<td>135</td>
<td>49</td>
<td>2</td>
<td>I (86%), III (14%)</td>
</tr>
<tr>
<td>Upper Kenai River</td>
<td>6</td>
<td>0</td>
<td>117</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Mid Kenai River</td>
<td>6</td>
<td>0</td>
<td>55</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Lower Kenai River (before brackish area)</td>
<td>4</td>
<td>3</td>
<td>6,578</td>
<td>4,805</td>
<td>61</td>
<td>2</td>
<td>I (24%), III (7%), VI (69%)</td>
</tr>
<tr>
<td>Total southcentral</td>
<td>28</td>
<td>10</td>
<td>8,710</td>
<td>5,191</td>
<td>199</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>36</td>
<td>10</td>
<td>11,505</td>
<td>5,191</td>
<td>302</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Total worms; 3 subsamples of 30 ml each per sample processed.
† Total *Tubifex tubifex* in 3 subsamples of 30 ml each from each site. Total is adjusted: total worms morphologically identified as *T. tubifex* minus percent identified as false positive (determined to be another species by PCR).
‡ Assayed by both *T. tubifex* species-specific and lineage PCRs.
Table 2.2. *Tubifex tubifex* collected from Alaskan rainbow trout hatcheries

<table>
<thead>
<tr>
<th>Hatchery settling pond</th>
<th>Fall</th>
<th>Spring</th>
<th>Worms assayed</th>
<th>Worms sequenced</th>
<th>Lineages (% of sampled population)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total worms</td>
<td><em>Tubifex tubifex</em> (%)</td>
<td>Total worms</td>
<td><em>Tubifex tubifex</em> (%)</td>
<td></td>
</tr>
<tr>
<td>Elmendorf</td>
<td>248</td>
<td>47 (19%)</td>
<td>106</td>
<td>9 (9%)</td>
<td>10</td>
</tr>
<tr>
<td>Ft. Richardson 1</td>
<td>1048</td>
<td>140 (13%)</td>
<td>1535</td>
<td>138 (9%)</td>
<td>187</td>
</tr>
<tr>
<td>Ft. Richardson 2</td>
<td>116</td>
<td>14 (12%)</td>
<td>98</td>
<td>5 (5%)</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>1412</td>
<td>201 (14%)</td>
<td>1739</td>
<td>152 (9%)</td>
<td>207</td>
</tr>
</tbody>
</table>

* 3 subsamples of 30 ml each per sample processed.
† Assayed by both *T. tubifex* species-specific and lineage PCRs. Includes worms from exposure experiments.
‡ Unk: Unknown, sample amplified in *T. tubifex* species-specific PCR, but not in lineage PCR.
‖ Primary settling pond
§ Secondary settling pond
Table 2.3. Prevalence and level of *Myxobolus cerebralis* infection in different mitochondrial lineages of *Tubifex tubifex* used in laboratory exposure experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Lineage (% of sampled population)*</th>
<th>TAM Release period (days PE)†</th>
<th>Maximum TAMs detected per week</th>
<th>Infection prevalence (by QPCR)</th>
<th>Lineages infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ft. Richardson hatchery‡</td>
<td>I (30%), IV (68%), Unknown (2%)</td>
<td>n/a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>Wizard Falls (reference)‡</td>
<td>III (100%)</td>
<td>98-182</td>
<td>14,967</td>
<td>85%</td>
<td>III</td>
</tr>
<tr>
<td>Second Exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>I (89%), III (3%), IV (5%), VI (2%), Unknown (1%)</td>
<td>120-160</td>
<td>188</td>
<td>3%</td>
<td>III</td>
</tr>
<tr>
<td>B</td>
<td>III (12%), VI (88%)</td>
<td>132-153</td>
<td>11</td>
<td>10%</td>
<td>III</td>
</tr>
<tr>
<td>C</td>
<td>I (80%), III (4%), VI (14%), Unknown (2%)</td>
<td>108-160</td>
<td>280</td>
<td>0.6%</td>
<td>III</td>
</tr>
<tr>
<td>Ft. Richardson hatchery</td>
<td>I (65%), IV (35%)</td>
<td>n/a</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>Wizard Falls (reference)</td>
<td>III (100%)</td>
<td>100-160</td>
<td>≥7000</td>
<td>100%</td>
<td>III</td>
</tr>
</tbody>
</table>

* Unknown: sample amplified in *Tubifex tubifex* species-specific PCR, but not in lineage PCR.
† Second experiment ended at 160 days post-exposure (PE), triactinomyxon (TAM) release not monitored after this date.
‡ Total of three replicates.
Table 2.4. Numbers of *Tubifex tubifex* exposed, infected and surviving experimental exposure to *Myxobolus cerebralis*

| Groups: | First Exposure | | | Second Exposure | | | | | |
|--------|----------------|-----------------|-----------------|----------------|-----------------|----------------|
|        | Ft. Richardson hatchery | Wizard Falls (reference) | Control (Ft. Richardson hatchery) | A | B | C | Ft. Richardson hatchery | Wizard Falls (reference) | Control (Wizard Falls) |
| Exposed | 300* | 300* | 0 (100 unexposed) | 200 | 200 | 200 | 100 | 200 | 0 (200 unexposed) |
| Monitored at peak infection (number infected) | 60 (0) | 60 (54) | 20 (0) | 0† | 0† | 50 (0) | 0† | 20 (20) | 0† |
| Surviving Adults‡ (number infected) | 127 | 4 | 29 | 90 (3) | 137 (13) | 108 (1) | 100 (0/20) | 15 | 97 |
| Progeny | 205 | 0 | 131 | 28 | 41 | 50 | 98 | 19 | 57 |

* In three replicates of 100.
† Not individually monitored as no TAMs were detected.
‡ Total worms remaining at end of experiment; does not include worms removed for monitoring during experiment.
Figure Captions

Figure 2.1. Map of study locations, sampling sites for *Tubifex tubifex* (black dots), and rainbow trout (*Oncorhynchus mykiss*) hatcheries (triangles) in southcentral and southeast Alaska.

Figure 2.2. *Tubifex tubifex* lineages from Fort Richardson Hatchery, Alaska. Agarose gel electrophoresis of PCR-amplified genomic DNA using mt 16S rDNA lineage-specific primers. Two lineages were present: I (196 bp) and IV (2 bands at 320 bp and 147 bp). A negative control (water) and a positive control (lineage III, 147 bp) were included. A 50 bp DNA Ladder is shown on both sides of the gel (2% agarose).

Figure 2.3. First experimental exposures- Susceptibility of lineage IV *Tubifex tubifex* to *Myxobolus cerebralis*: triactinomyxon release per 100-worm replicate (3 Alaskan replicates are pooled as 1).

Figure 2.4a. Second experimental exposures- Comparative triactinomyxon release from Alaskan *Tubifex tubifex*.

Figure 2.4b. Comparative triactinomyxon release from Alaskan *Tubifex tubifex* groups A, B, and C (enlargement of Figure 2.4a).
Figure 2.1

[Map of Alaska showing key locations such as Anchorage, Ship Creek, Campbell Creek, Cook Inlet, Kenai River, Skilak Lake, Kenai Lake, Montana Creek, Peterson Creek, Mendenhall River, and Juneau.]
Figure 2.2
Figure 2.3
Figure 2.4a

Figure 2.4b
CHAPTER 3

EXPANDED GEOGRAPHICAL DISTRIBUTION OF MYXOBOLUS CEREBRALIS: FIRST DETECTIONS FROM ALASKA.

E. Leyla Arsan1,2, Stephen D. Atkinson2,3, Sascha L. Hallett2, Theodore Meyers4, and Jerri L. Bartholomew2*

1 Department of Fisheries and Wildlife, Center for Fish Disease Research, Oregon State University, Corvallis, Oregon, USA
2 Department of Microbiology, Center for Fish Disease Research, Oregon State University, Corvallis, Oregon, USA
3 School of Molecular and Microbial Sciences, University of Queensland, Brisbane Queensland, Australia
4 Alaska Department of Fish and Game, Juneau Fish Pathology Laboratory, Juneau, Alaska, USA
Abstract

Introduced to the USA in 1958, Myxobolus cerebralis, the parasite responsible for salmonid whirling disease, has since spread across the country causing severe declines in wild trout populations. While assessing the risk of introduction of this pathogen into the state of Alaska, an area where clinical whirling disease has never been documented, we detected the parasite using a species-specific polymerase chain reaction (PCR). This is the first report of M. cerebralis in Alaska. Testing of 180 hatchery rainbow trout (Oncorhynchus mykiss) by pepsin-trypsin digest and quantitative PCR (QPCR) revealed fourteen positive samples. Infection in two of these was confirmed by sequencing the parasite 18S rRNA gene and by a nested PCR assay based on the same gene. We were unable to visually identify myxospores, indicating that either infection was light or mature spores had not formed. Sequence comparison of M. cerebralis from several locations demonstrated the Alaska isolates were genetically distinct and therefore not false positives arising from contamination during processing. This paper presents DNA sequence data from the Alaska M. cerebralis isolates, provides a brief history of the fish and facility of origin, and discusses implications of different testing methods on asymptomatic fish populations.
Introduction

Myxobolus cerebralis, a freshwater metazoan parasite exotic to North America, was first detected in the USA in 1958 (Hoffman, 1962). Thought to have originated from a shipment of infected brown trout (Salmo trutta) from Europe, it is now reported in a total of 24 states in the USA and 26 countries worldwide (Bartholomew and Reno, 2002; Vermont Department of Fish and Wildlife, 2002; Stromberg, 2006). The pathogen has been at the forefront of fish health research in the USA due to its implication in dramatic rapid declines of wild rainbow trout (Oncorhynchus mykiss) populations in the North American intermountain west, primarily in Colorado and Montana (Vincent, 1996; Nehring et al., 1998).

Myxobolus cerebralis is not known to occur in Alaska, though little monitoring for the pathogen has been conducted. In response to concerns regarding allocation of resources for monitoring programs, research was initiated to assess the risk of introduction and establishment of the parasite. In the process of validating the initial assumption that the parasite did not occur in Alaska, we detected the pathogen by PCR in samples from a state hatchery.

The closest M. cerebralis enzootic area to Alaska is the Snake River basin in northeast Oregon, southeast Washington, and Idaho. The parasite has also been reported in wild and cultured salmonids from the Sakhalin Islands off the east coast of Russia (Bogdanova, 1960, 1968). However, the distance from the Sakhalin Islands to southcentral Alaska is approximately double that from the Snake River basin.

The assumption that M. cerebralis was not present in Alaska was based on a lack of observed clinical disease and on data from previous limited monitoring for the parasite in wild fish stocks by the US Fish and Wildlife Service (USFWS) as part of the National Wild Fish Health Survey (NWFHS). The USFWS uses pepsin trypsin digest (PTD) for parasite detection and PCR as confirmation of infection (USFWS and AFS-FHS, 2003). Examination of 2,049 fish from 26
locations did not result in detection of the parasite [USFWS, 2006; T. Burton, Alaska Department of Fish and Game (ADFG), personal communication]. Thus, PCR has never been conducted. However, the NWFHS tests for a number of fish pathogens and does not target areas at high-risk for *M. cerebralis*. As a result, only 18% of fish tested by USFWS (361 fish from seven locations) were from southcentral Alaska. Hatchery fish in Alaska are not monitored for the parasite. To provide further information on the status of infection in fish in Alaska, we tested rainbow trout from state hatcheries, using PCR as the initial screening method.

**Methods**

*Origin of fish tested*

Rainbow trout, the salmonid most susceptible to the parasite (MacConnell and Vincent, 2002; Sollid et al., 2002), were obtained from the only two hatcheries in Alaska that routinely rear the species: Fort Richardson (FTR) and Elmendorf (ELM). Both hatcheries are located on Ship Creek in the Cook Inlet basin in southcentral Alaska. In 2005, 60 fish each from FTR and ELM were tested for the parasite. In 2006, another group of 60 fish from ELM (of the same broodyear as those tested in 2005) was assayed. Rainbow trout at ELM are hatched at FTR and transferred to ELM after 4-8 months.

*Detection of Myxobolus cerebralis*

In 2005, the 60 fish sampled from each hatchery were randomly segregated into five-fish pools and cartilage from whole heads was digested by pepsin trypsin digest (PTD) following USFWS and AFS-FHS (2003) procedures with minor modifications noted below. After trypsin was added, the pH was adjusted to 8.0 [not 8.5, as stated in USFWS and AFS-FHS (2003)] with 1 N NaOH. The PTD pellet was suspended in 1 mL phosphate buffered saline (PBS) and frozen for later analysis. DNA was extracted from the entire thawed sample using a QIAGEN
DNeasy Tissue Kit and eluted with 50 µL buffer AE. This was then tested for *M. cerebralis* by QPCR (Kelley et al., 2004) with 4 µL of extracted DNA used in a 20 µL reaction volume. Duplicate reactions were performed in an ABI PRISM 7300 or 7500 Sequence Detection System. In 2006, fish were tested individually (not pooled). Testing methodology was the same as 2005, except that only 200 µL of fully homogenized (vortexed) PTD product suspended in PBS was used for DNA extraction. The remainder of the PTD product was retained for visual inspection for myxospores. As the blue book procedures do not specify the volume of sample to use for DNA extraction, we consulted with the Oregon Department of Fish and Wildlife (ODFW), Fish Pathology Division (J. Kaufman, pers. comm.) who have found 200 µL of PTD product to be sufficient volume for parasite confirmation in Oregon.

Samples positive for *M. cerebralis* by QPCR in 2006 were visually inspected for myxospores under 200 and 400X magnification (USFWS and AFS-FHS, 2003). However, due to the difficulty of visual confirmation of low-grade infections, a nested PCR assay that targeted the 18S rRNA gene (Andree et al., 1998) was also used on selected samples to provide further evidence that the organism amplified in QPCR was actually *M. cerebralis*. As entire 2005 PTD samples were processed for DNA extraction (by microwaving), any spores would have been destroyed and therefore were not discernible by a visual inspection.

A positive control and negative control (water) were used in each molecular assay. The positive control was an adult Chinook salmon cranial core (Lostine River, Oregon *M. cerebralis* enzootic area) that had been previously digested and determined positive for *M. cerebralis* by PTD detection and PCR confirmation. Based on our reference samples, a fish sample presenting any cycle threshold (Ct) value was considered infected. Samples presenting a Ct in one of their two duplicate wells were rerun. Further discussion on Ct values and their reflection of parasite load appears below.
Verification of testing methods and determination of QPCR threshold

To verify QPCR and PTD results and determine the relationship between Ct value and myxospore number, we created a reference set of samples spiked with known numbers of *M. cerebralis* myxospores. Six half-heads of uninfected juvenile rainbow trout originally obtained as eggs from Trout Lodge, WA and held at the Salmon Disease Laboratory, Corvallis, Oregon on well water were digested by PTD then spiked with either 100 or 1000 myxospores (three half-heads each per dose). Myxospores used to spike samples were obtained from three fish exposed as fry to *M. cerebralis* actinospores at the Salmon Disease Laboratory and were added after the PTD to maximize recovery. Each half-head sample was processed for DNA extraction and QPCR exactly as described for the 2006 samples. Samples and serial 10-fold dilutions were run in triplicate to determine the limit of detection in the QPCR assay. We deemed (in consultation with ABI QPCR representatives) that if one of three triplicates of a sample fluoresced within our limit of detection, then the sample was positive. The limit of detection was defined by the sample set in which fewer than all (three) wells tested, fluoresced. Amplification plots (curves) and delta Rn values were evaluated to confirm the efficiency and threshold of the QPCR reaction.

The remainder of the PTD product was also visually inspected using both methods (hemocytometer and wet mount preparation) in the Blue Book (USFWS and AFS-FHS, 2003). In wet mount preparation, 380 fields at 200x magnification were examined per 20 and 40 µl of sample for the 1000-myxospore references and 20 µl were examined for the 100-myxospore samples.

**DNA sequencing**

For two samples (one from each year) positive by the QPCR assay, we amplified parasite 18S rDNA to confirm the presence of *M. cerebralis*. We also sequenced the QPCR positive control (Lostine River adult fish) and our *M.
cerebralis laboratory isolate, to examine the possibility of positive assay results due to DNA contamination.

The *M. cerebralis* SSU 18S rRNA gene was amplified by two-round PCR: amplification of the entire gene by universal primers ERIB1 and ERIB10 (Barta et al., 1997) followed by semi-nested reactions using primer pairs ERIB1-ACT1r and MYXGEN4f-ERIB10 (Hallett & Diamant, 2001, Kent et al., 2000). Reactions were carried out in 20 µL volumes, which comprised: 2-4 µL template DNA, 0.5 µL each primer (10 µM stock), 0.4 µL dNTPs (10 mM), 0.5 µL BSA (Bovine Serum Albumin, 10 mg/mL), 1 µL Rediload loading dye (Invitrogen), 1.2 µL MgCl₂ (25 mM), 4 µL Colorless GoTaq Flexi buffer (5X) (Promega), 0.25 µL GoTaq Flexi (5 U/µL) (Promega) and water. This was scaled up to 50 µL to generate products for sequencing. PCRs were run on a MJ Research PT100 thermacycler, using the following program: block preheated to 95°C, then initial denaturation @ 95 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, terminal extension of 72 °C for 10 min, then rest at 4 °C. DNA was either purified directly from PCR products, or electrophoresed on an agarose gel and the appropriate band excised and purified using Qiagen kits and standard protocols. DNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Fragments were sequenced using the same primers as in the amplifications, with ABI Big Dye Terminator chemistry on an Applied Biosystems Capillary 3100 Genetic Analyzer (Foster City, California) at the Oregon State University sequencing facility (Center for Gene Research and Biotechnology, Central Service Laboratory). Sequences were aligned and edited in BioEdit (Hall, 1999) with other *M. cerebralis* isolates from Genbank (see Table 3.1 for accession numbers).
Results

PCR detection of Myxobolus cerebralis

In 2005, assays of 12 five-fish pools from ELM revealed two positive pools. QPCR indicated low target DNA quantities (mean Ct values 33.6 and 38.7). Potentially positive samples as well as several negative samples were blind-tested three different times by two different researchers and results were consistent. None of the fish from FTR were positive.

Testing in 2006 of individual fish of the same cohort but reared at ELM for an additional year, revealed 12 out of 60 fish to be positive (mean Ct range 32.2–39.1). Two more fish fluoresced in the QPCR assay, but presented Ct values which were above the limit of detection of our reference samples and thus were not included in the total. The nested PCR assay amplified DNA from 2 of 4 fish that were determined to have the greatest quantity of parasite DNA by QPCR (Figure 3.1).

Comparison of QPCR Ct values of ELM samples with the reference samples containing known numbers of spores indicated that in 2005 one pool (5 fish) had approximately 1000 myxospores and the other 100 myxospores (Figure 3.2). In 2006, two samples (1 fish each) had 1000 or more myxospores, three had approximately 100 myxospores and the remainder had less than 100 myxospores (approximately 10) (Figure 3.2).

Visual detection of Myxobolus cerebralis

Myxospores were not detected by visual inspection of the PTD product of QPCR-positive samples in either 2005 or 2006.
Verification of testing methods

*Myxobolus cerebralis* was not visually detected in samples of uninfected rainbow trout tissue spiked with 100 myxospores, and only one disrupted spore was observed in samples spiked with 1000 myxospores, when recommended procedures were followed (USFWS and AFS-FHS, 2003). In contrast, all of the spiked samples were positive by QPCR. Samples spiked with 1000 myxospores produced Ct values ranging from 33.3-35.4, with a mean Ct value of 34.4 (Figure 3.2). Fish spiked with 100 myxospores had QPCR Ct values ranging from 35.3-39.4, with a mean Ct value of 37.3. The 10-fold dilution of a 1000 myxospore sample had a Ct of 37.1-38.5 (mean 37.6), consistent with a 100 myxospore sample. The limit of detection was the equivalent of 10 myxospores (either a 10-fold dilution of a 100 myxospore sample or a 100-fold dilution of a 1000 myxospore sample) since not all triplicate wells of these samples fluoresced (0 to 2 wells per sample).

18S rDNA sequence analysis

*Myxobolus cerebralis* DNA was amplified and sequenced from two Alaskan fish that tested positive by QPCR, as well as our laboratory isolate and the positive control (Lostine River). The SSU 18S rRNA gene was determined to be 2120 bp in length (including terminal primers ERIB1 and ERIB10). Twenty variable loci, including seven sites with heterogeneous bases, were identified from alignment of our novel *M. cerebralis* isolates with sequences in GenBank (Table 3.1). The two Alaska isolates differed from both the laboratory isolate (6-8 loci) and the positive control (3-5 loci), and from each other (6 loci).

Discussion

The molecular detection of *M. cerebralis* from hatchery rainbow trout is the first record of the parasite in Alaska. The rainbow trout that tested positive for
M. cerebralis were hatched at FTR and transferred to ELM after 4-8 months. FTR is supplied by well water and no fish examined from that facility were positive for the parasite. ELM is supplied by surface water from Ship Creek and the fish tested were reared at this facility for 11 months (2005 samples) and 23 months (2006 samples).

Because M. cerebralis had not previously been detected in Alaska and the fish tested had no clinical signs of disease, we used the most sensitive methods (PCR, QPCR) for diagnostic screening. However, we were unable to visually detect myxospores from samples that tested positive by these assays. Given the low DNA quantity indicated by QPCR, we suspected that visual detection of a low number of spores in a sample would be highly improbable and designed reference samples to test this. In our reference samples that had been spiked with 1000 myxospores after PTD (then frozen before examination) we were able to identify only one presumptive spore. This observation is in accordance with Markiw and Wolf (1974), who documented that in fish spiked with 1000 myxospores, less than 0.2% of spores were recovered by the PTD process. Thus, visual confirmation in asymptomatic fish with low or early infections may be unattainable.

For regulatory inspection purposes, PCR testing is offered along with histology as a confirmatory test after presumptive identification of M. cerebralis by visual inspection of myxospores in enzymatic tissue digests (USFWS and AFS-FHS, 2003). Molecular tests in general are not approved diagnostic screening techniques because of the difficulties in verifying positive results using a second, often less sensitive, test. For bacterial and viral pathogens that can be amplified by culture techniques, there is generally not a significant difference in sensitivity between culture and molecular detection. However, detection of pathogens that cannot be amplified by culture is more problematic and likely to result in false negatives when pathogen numbers are low, even when using concentration procedures like PTD. This is compounded when identification relies on detection of a single life stage, like the myxospore. In studies that have compared these
testing methods (Schisler et al., 2001; Kelley et al., 2004), PCR was found to be an effective diagnostic tool and is approximately 10-fold more sensitive than PTD (Andree et al. 2002). Kelley et al. (2004) determined that any of the five diagnostic approaches they evaluated was effective in identifying moderately to heavily infected fish. Schisler et al. (2001) screened free-ranging and hatchery-reared fish with various levels of infection and documented that PCR identified *M. cerebralis* more often than PTD, and that the heightened sensitivity of PCR resulted in detection of the parasite in areas previously thought to be free of the pathogen.

Another advantage of genetic tests as detection tools is their capability to detect not only the different stages of parasite development, but also mature spores that have lost their morphological integrity under harsh extraction conditions. In contrast, PTD is capable of detecting only mature final stages of parasite development that are visually distinguishable.

Estimates of infection levels in the Alaskan hatchery fish were inferred by comparison of their QPCR Ct values with those of the reference samples that contained known numbers of myxospores and were processed identically. All six reference samples spiked with either 100 or 1000 myxospores each were positive by QPCR as were 10-fold dilutions of a 1000 spore sample. Dilutions equivalent to 10 myxospores of the 100 and 1000 spore samples fluoresced in zero to two of their three replicate wells, thus defining the limit of detection as a Ct value less than 40 (Figure 3.2). Infections in hatchery fish ranged from 10 myxospores to over 1000 (mean Ct range 32.2–39.1).

For infection levels in the range of our estimates for the Alaska fish (1000 myxospores or less), we found that neither of the two visual detection methods described in the Blue Book is a valid method for enumeration of spores following PTD digest (USFWS and AFS-FHS, 2003). Firstly, for hemocytometer data to be accurate, 15-50 spores per 1 mm² grid (with four grids per hemocytometer) must be observed (Cascade Biologics, Inc., 2002). This suggests that a minimum of
150,000 spores per mL is required for an accurate enumeration by hemocytometer assessment. In samples with low infections such as 1000 myxospores, an average of only 1 spore per 10 grids would be present. Secondly, if the other visualization method stipulated in the Blue Book (examination of all myxospores in 150 fields under 200X magnification) is used, we should have seen 20 myxospores in 20 µL of the 1000-spore sample. We saw only one disrupted spore in a single sample, which suggests the PTD method jeopardized the integrity of the spore.

As we were unable to visually detect *M. cerebralis* spores, we used a second PCR assay and DNA sequencing of the second round product as an alternate method of confirmation of parasite presence. The two fish from Alaska from which *M. cerebralis* DNA was sequenced contained approximately 1000 myxospores. Another two samples that were positive by QPCR, but could not be amplified in the nested PCR, likely had infections on the order of 100 myxospores. Schisler et al. (2001) observed an exponential increase in myxospore numbers with increasing PCR band strength, and that “weak positives” had a mean spore count of 958 myxospores. These researchers also found that only 2 out of 40 “weak positive” samples produced enough DNA to be sequenced. Thus, it is not surprising that only two of the positive samples from Alaska produced sufficient DNA for sequencing.

The Alaska isolates differed in at least five loci from the positive control and our lab isolates, which indicates cross-contamination was unlikely. Overall variation between *M. cerebralis* isolates was very low: 20 loci in 2120 (<1%), and the maximum variation between isolates was only 12 loci (0.56%). This low level of intraspecific variation in the 18S is consistent with data from ITS sequences (Whipps et al., 2004). Some variations were only present in the older GenBank sequences and may be sequence noise or misreads, especially the insertion at base position 1387, which was not present in any of our isolates. The presence of heterogeneous bases at loci in several isolates indicates that source fish may either be infected with multiple ‘genotypes’ of *M. cerebralis*, or that some isolates may
have multiple alleles of the 18S rRNA gene present – a feature we have observed in another myxozoan parasite of salmonids, Parvicapsula minibicornis (Atkinson et al., 2006). Interestingly, the Alaska isolates differed between themselves at 6 loci, which suggests different sources of the parasite may be involved. Although, given the degree of anthropogenic mixing and translocation of both host and parasite populations in North America, it is difficult to draw conclusions on finescale phylogeny of M. cerebralis.

The source of infection for the hatchery fish remains unknown. ELM is supplied by surface water from Ship Creek, with incoming surface water screened for debris but not treated for potential biota. Thus, M. cerebralis myxospores, actinospores, or even Tubifex tubifex [the alternate oligochaete host of the parasite (Wolf and Markiw, 1984)] could potentially enter the hatchery if the parasite were present in the Creek. Also, until 2004, water sources for both hatcheries were heated to ambient temperatures as high as 14°C, either by wastewater (ELM) or by heat exchanger from power plants nearby. Heated water temperatures could have contributed to parasite propagation, as temperatures from 10-15°C accelerate the parasite life cycle and are optimal for parasite development (El-Matbouli et al., 1999). Effluent from both ELM and FTR is routed through earthen settling ponds [that contain populations of T. tubifex (Arsan et al., chapter 2 of this volume)] and flows back into Ship Creek untreated. Thus, fish in Ship Creek could also be exposed to parasites in hatchery effluent.

While the origin of M. cerebralis in Alaska remains unknown, the state of Alaska has made significant efforts to limit movements of fish and hence sources of pathogen introduction, and the origin of M. cerebralis in the state remains unknown. Anecdotal evidence suggests that rainbow trout were introduced as eggs in southcentral Alaska in the 1950’s from broodstock at Winthrop National Fish Hatchery, Washington. Interior Alaska received transplants (eggs) from Idaho and Montana in 1952 (Marvich and McRea, 1953; Morrow, 1980). As these imports were eggs and also were made before M. cerebralis was known to have
been introduced into the USA (Hoffman, 1962; Bartholomew and Reno, 2002), it is unlikely that they resulted in parasite transfer. Additionally, since the mid 1970s, Alaska has adopted strict regulations that prohibit the import of live non-ornamental fish into the state (Alaska Administrative Code [AAC] 5: 41.070).

Though it is unknown how long the parasite has been present in Alaska, it is likely that previous monitoring would have missed a low prevalence or low level of infection due to the limited sensitivity of testing methods. The use of PTD as the sole screening method could miss low infections that would be identified by molecular tests such as PCR, which are approximately 10-fold more sensitive (Andree et al., 2002). However, for management purposes, it may only be necessary to detect infections that cause negative impacts on fish populations or are considered “significant” infections. Each state has its own stipulations as to what constitutes such an infection and what the implications of detection are. For example, the state of Colorado transfers only fish that are PCR negative for *M. cerebralis* to areas where the parasite is not known to occur. The 2004 broodyear rainbow trout from ELM were transplanted throughout southcentral and interior Alaska prior to parasite detection. Many of these stocked locations were land-locked lakes with no outlets, which would limit the spread of the parasite, but some fish were also stocked in open stream systems where susceptible lineages of *T. tubifex* have been documented (Arsan et al., chapter 2 of this volume). Thus, some degree of spread may already have occurred. However, the low prevalence and severity of infection in the fish populations tested and the low abundance of suitable oligochaete hosts (Arsan et al., chapter 2 of this volume) may limit further spread of the parasite.

The first detection of *M. cerebralis* in Alaska is not without consequence. Though the risk of parasite dissemination by ELM fish may be low, it is nevertheless unacceptable by current ADFG fish disease policies. An interim management strategy will include restricting all fish transplants from ELM to land-locked lakes having no native susceptible resident salmonid species or to seawater
netpens for terminal commercial and sport fisheries. Release of Chinook salmon smolts into Ship Creek will continue in order to maintain returning broodstock. This species develops resistance to *M. cerebralis* more rapidly than rainbow trout (Sollied *et al.* 2003) and thus presents a lower risk of parasite dissemination. Future plans include replacement of the current Elmendorf Hatchery with a new facility constructed on a nearby site with water supplied from wells rather than Ship Creek. The FTR will be discontinued entirely once the new ELM facility is completed. The use of well water should facilitate breaking the parasite life cycle, and will be corroborated by additional QPCR testing of hatchery fish stocks in the new facility. Negative results may allow future unrestricted fish transplants. Placement of juvenile sentinel rainbow trout in cages within Ship Creek would also determine if the parasite has become established within the basin, an approach that has been used in similar situations after isolated parasite introduction (Bartholomew *et al.*, 2007).

While this is the first detection of *M. cerebralis* in Alaska, clinical whirling disease has never been reported. Because hatchery rainbow trout in Alaska originated from the contiguous USA (Marvich and McRea, 1953; Morrow, 1980), their susceptibility to the pathogen is likely similar to other resident rainbow trout strains. We found rainbow trout from ELM to have very low infections (as determined by QPCR), which probably reflects exposure to a low number of actinospores or exposure when fish are older and have already gained some degree of resistance to the parasite. In laboratory susceptibility challenges with *T. tubifex* from different locations in Alaska, infection prevalence in populations fed 500 myxospores/worm ranged from 0.5 – 10 % depending on the lineage composition of the population, and each group of 200 exposed worms produced up to several hundred actinospores (Arsan *et al.*, chapter 2 of this volume). While this number is relatively low, each triactinomyxon that reaches its fish host is capable of proliferating 1000-fold (Kerans and Zale, 2002), though environmental conditions and water temperatures may slow development. Thus, even introduction of fish
with low infections of *M. cerebralis* can be significant in areas where physical or environmental conditions are optimal for parasite proliferation.

Current physical and environmental conditions in Alaska likely contribute to keeping infection rates low and disease from becoming apparent. Abundance of resistant lineages of *T. tubifex* (Arsan et al., chapter 2 of this volume), as well as cooler water temperatures, may slow the proliferation and life cycle of *M. cerebralis* and cumulatively prevent the parasite from severely impacting salmonid populations in this region. In such cases, disease may never become apparent.

A complete analysis of risk of further spread and of potential routes of introduction of *M. cerebralis* into Alaska is forthcoming.

**Acknowledgements**

This work was supported in part by the National Partnership for the Management of Wild and Coldwater Species, the USFWS, the U.S. Geological Survey, and the ADFG. We are grateful to: Tammy Burton of the ADFG and to FTR and ELM hatchery personnel for providing fish samples; John Kaufman and Nadine Hurtado of the ODFW for providing positive control samples as well as assistance and advice regarding parasite assays; Harriet Lorz at the Center for Fish Disease Research for her tremendous help with the pepsin trypsin digest and with reference samples; and Jenny Dubanoski and Kyle Thames at Oregon State University for preparing reference samples and for assistance in the laboratory.
References


Hallett, S. L. and A. Diamant. 2001. Ultrastructure and small-subunit ribosomal DNA sequence of *Henneguya lesteri* n.sp. (Myxosporea), a parasite of sand whiting *Sillago analis* (Sillaginidae) from the coast of Queensland, Australia. Diseases of Aquatic Organisms 46: 197-212.


Table 3.1 Variable loci in the 18S SSU rRNA gene from different isolates of *Myxobolus cerebralis*. Loci are measured relative to 5’ position of universal primer ERIB1; heterogeneous bases: R=A+G, K=T+G, Y=C+T.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>156</td>
</tr>
<tr>
<td>Munich DE WV, USA</td>
<td>T</td>
</tr>
<tr>
<td>CA, USA OR, USA</td>
<td>G</td>
</tr>
<tr>
<td>OR, USA Lab OR, Lostine + AK, Fish 1</td>
<td>G</td>
</tr>
<tr>
<td>AK, Fish 2</td>
<td>G</td>
</tr>
</tbody>
</table>
Figure Legend

**Figure 3.1** Agarose gel of PCR-amplified genomic DNA from four rainbow trout from Elmendorf Hatchery, Alaska (sample numbers shown), using mt 18S rDNA specific primers. A 1 kb+ DNA Ladder is shown on both sides of the gel (1.5% agarose). A blank (B) well precedes each of the two positive (+) controls and a negative (-) control (water) is provided in the last well.

**Figure 3.2** QPCR cycle threshold values of Alaska hatchery rainbow trout plotted against reference samples spiked with known numbers of *Myxobolus cerebralis* myxospores (100, 1000). Data are represented in log scale. Values for fish samples are an average of duplicates, all other values are individual.
Figure 3.1
Figure 3.2
CHAPTER 4

A QUALITATIVE ANALYSIS OF RISK FOR THE DISSEMINATION OF *MYXOBOLUS CEREBRALIS* IN THE STATE OF ALASKA.

E. Leyla Arsan¹,² and Jerri L. Bartholomew²*

¹Department of Fisheries and Wildlife, Center for Fish Disease Research, Oregon State University, 220 Nash Hall, Corvallis, OR 97331, USA
²Department of Microbiology, Center for Fish Disease Research, Oregon State University, 220 Nash Hall, Corvallis, OR 97331, USA
Abstract

*Myxobolus cerebralis*, the myxosporean parasite responsible for whirling disease in salmonids, was first introduced to the United States in 1958. It has since spread across the country causing severe declines in wild trout populations in the intermountain west. The recent detection of the parasite in Alaska is further evidence of the species’ capability to invade and colonize new habitat, though it is unknown when colonization occurred. The goal of this study was to qualitatively assess the risk of further spread and establishment in the state by examining potential routes of dissemination. This paper explores potential routes of introduction of *M. cerebralis* in Alaska by assessing four main elements of dispersal: movement of fish by humans, natural dispersal (via migratory birds and stray anadromous salmon), recreational activities, and commercial seafood processing. Establishment of the parasite is dependent upon several environmental and biological factors including: water temperatures, spatial/temporal overlap of hosts, and the distribution and genetic composition of the oligochaete host, *Tubifex tubifex*. The most likely pathway of *M. cerebralis* transport in Alaska is human movement of fish by stocking. While the extent of *M. cerebralis* infection in Alaskan salmonid populations is unknown, if the parasite were dispersed, conditions are appropriate for establishment and propagation of the parasite life cycle in some areas of southcentral Alaska. The probability of further establishment is greatest in Ship Creek where the abundance of susceptible *T. tubifex*, the presence of susceptible rainbow trout (*Oncorhynchus mykiss*), and the proximity to the known area of infection, make conditions particularly suitable. The apparent lack of appropriate tubificid hosts in southeast Alaska may prevent establishment in that part of the state.
INTRODUCTION

*Myxobolus cerebralis*, a metazoan parasite that causes salmonid whirling disease, is exotic to North America and was first detected in the USA in 1958 (Hoffman, 1962). Thought to have originated from a shipment of frozen infected brown trout (*Salmo trutta*) from Europe, the parasite is now reported in 25 US states and 26 different countries (Bartholomew and Reno, 2002; Vermont Department of Fish and Wildlife, 2002; Stromberg, 2006; Arsan et al., chapter 3 of this volume), and its range continues to expand. Though the pathogen appears to have little impact on fish populations in the eastern and coastal western states (Modin, 1998), it has caused dramatic rapid declines of wild rainbow trout (*Oncorhynchus mykiss*) populations in the intermountain west, primarily in Colorado and Montana (Walker and Nehring, 1995; Vincent, 1996; Nehring et al., 1998). The ecologic and economic impacts of *M. cerebralis*, in addition to its rapid spread and establishment across the globe, are indicative of the need for specific recommendations to assist fishery managers in halting the spread or effects of the pathogen.

*Myxobolus cerebralis* was first detected in Alaska in 2006 (Arsan et al., chapter 3 of this volume) in rainbow trout from a hatchery on Ship Creek in Anchorage in southcentral Alaska. Prior to this detection, the closest enzootic area was the upper Columbia River basin (CRB) in northeast Oregon, southeast Washington, and Idaho. The parasite has also been reported in wild and cultured salmonids from the Sakhalin Islands off the east coast of Russia (Bogdanova, 1960, 1968). However, the distance from the Sakhalin Islands to southcentral Alaska is approximately double that from the upper CRB.

The parasite was detected and confirmed in Alaska using molecular techniques as the low level of infection precluded detection by less sensitive methods (Arsan et al., chapter 3 of this volume). The prevalence of infection was low and clinical whirling disease has never been reported from the hatchery or
other locations in the state. Cultured salmonids in Alaska are not monitored for
the parasite, and limited monitoring occurs in wild salmonids [US Fish and
Wildlife Service (USFWS), 2006]. As salmonids are inextricably linked to the
culture and economy of Alaska [Kenai River salmon runs alone generate annual
revenues of $70 million (Glass et al., 2004)], the potential impacts of *M. cerebralis*
could be catastrophic both ecologically and economically.

This paper uses risk analysis as a tool to assess the likelihood and
consequences of exotic parasite introduction. The framework for this type of risk
assessment (Bartholomew et al., 2005) was created in efforts to tailor such
assessments to whirling disease. We use risk analysis as a type of map
(MacDiarmid, 2000) to navigate through possible pathways or scenarios leading to
parasite introduction and establishment, and to assess where to allocate resources
to help prevent such an occurrence. This is an essential first step in understanding
potential outcomes and associated risks of management decisions. Our aim is to
provide decision-makers with tools to assess management implications and to
eliminate non-issues by using logical scientific arguments. This paper assesses the
risk for future spread of *M. cerebralis* within southcentral Alaska and to southeast
Alaska, and explores the potential for new introductions to the state.

THE PARASITE HAZARD

Tracking the epidemiology of parasites requires investigation of an
organism’s life cycle, biophysical properties, and hosts. The life cycle of *M.
cerebralis* requires two obligate hosts: a salmonid and the aquatic oligochaete
worm, *Tubifex tubifex*. The parasite maintains two distinct spore stages, one
unique to each of the hosts, the myxospore and the actinospore. Each spore stage
requires approximately three months to develop within and be released from the
respective host. Myxospores develop in the fish host and are released upon death
of the fish. These round and durable spores are ingested by *T. tubifex* as they
burrow through sediment (Brinkhurst, 1996). The parasite then undergoes
reproduction and structural transformation and is released as a triactinomyxon (TAM). When the waterborne TAM attaches to its fish host, the parasite sporoplasm is injected through the epidermis of the fish and migrates through the nervous system to the cartilage (El-Matbouli et al., 1995). The parasite consumes cartilage, causing lesions in the gills, cranium, and vertebral column of the fish (MacConnell and Vincent, 2002).

The biophysical properties of *M. cerebralis* are also a factor in its potential dissemination, with introduction most likely occurring by the myxospore stage. Myxospores are far more resilient than TAMs and are capable of withstanding severe environmental variables (El-Matbouli & Hoffmann, 1991) that might occur during transport and dissemination. Of the two obligate hosts of *M. cerebralis*, the fish host is more mobile; thus, myxospores are more likely to be distributed over a broader area than TAMs. Indeed other researchers have speculated that myxozoan colonization on a landscape would most likely occur via myxospores (Cone et al., 2006).

Susceptibility of hosts is another contributing factor to parasite dispersal and establishment. Salmonid hosts of *M. cerebralis* differ in their susceptibility to infection and subsequent disease caused by the parasite. Rainbow trout have repeatedly shown the highest susceptibility in laboratory and field experiments (O’Grodnick, 1979; Hedrick et al., 1999a, 1999b; MacConnell and Vincent, 2002). Most other salmonids, except lake trout (*Salvelinus namaycush*) and arctic grayling (*Thymallus arcticus*), are also susceptible to the parasite (MacConnell and Vincent, 2002), but infections result in varying degrees of clinical disease.

Whereas many species of salmonids are susceptible to the parasite, only one species of oligochaete, *T. tubifex*, is capable of propagating the pathogen. *Tubifex tubifex* is a hearty and cosmopolitan species that proliferates in areas with high organic content. The worm’s ability to withstand extreme and variable environmental conditions allow it to inhabit areas where other species cannot compete, and thus to span across ecosystems as a widespread aquatic invertebrate. These qualities also have made control efforts of this host unpractical or
unsuccessful (Wagner, 2002). However, susceptibility of *T. tubifex* to *M. cerebralis* varies greatly, and has been linked to the worm’s lineage. There are six cryptic mitochondrial lineages of *T. tubifex*. Five lineages (I, III, IV, V, and VI) have been reported from North America (Beauchamp et al., 2001; Arsan et al., chapter 2 of this volume) and five are known from Europe (I-V) (Sturmbauer et al., 1999). Susceptibility of these different lineages varies from highly susceptible to the parasite to completely resistant. While lineage does not confer susceptibility, researchers have found the genetic composition of tubifex populations to correspond to severity of infection in fish (Beauchamp et al., 2006).

**RISK ANALYSIS**

To begin the risk assessment process, we narrowed the magnitude of geographic size of Alaska [the state is approximately the size of the continent of Europe (Pagano, 2000; Atlas A-Z, 2001)], by focusing on areas of the state where we considered the probability for new introduction, or further within-state spread, of *M. cerebralis* to be highest: southeast and southcentral Alaska (the Cook Inlet basin) (Figure 4.1). These areas have high concentrations of susceptible fish hosts, high angler traffic and/or a large commercial fishery, the highest concentration of human populations in the state, and high potential organic loading. The areas are also close to the road system and ports, and in the migration path of fish from enzootic areas. Definitions of risk levels used in this analysis are displayed in Table 4.1.

**Study Sites**

*Southeast Alaska*

Southeast Alaska has a maritime climate and is within the Pacific Northwest temperate rainforest ecosystem. The area has cool winters and wet
summers with stream temperatures generally warmer than those in the interior of the state. Typical hydrographs for Southeast Alaska creeks are influenced by spring snowmelt and autumn rainfall (Chaloner et al., 2004; Montgomery et al., 1996; Milner et al., 1997). River basins of coastal southeast Alaska are generally small, due to mountains and icefields that rise sharply from sea level and create relatively short watersheds that flow into the Pacific Ocean. Two streams were selected based on the criteria described above: Peterson and Montana Creeks near Juneau.

Southcentral Alaska

The Cook Inlet, in southcentral Alaska, is home to over half the state’s human population. The area has a transitional climate (National Climate Center, 1982) and is the ecotone between the Pacific Northwest rainforest and the northern boreal forest. Hydrographs in the basin are highly predictable and influenced by snow and glacier melt in the summer; typical freshwater inflow into Cook Inlet is 15 times higher in July than in February (Dorava and Milner, 2000). This study focuses on Ship and Campbell Creeks in the Anchorage area and on the Kenai River on the Kenai Peninsula. All three streams are popular sport fisheries due to abundant fish runs, proximity to major population centers, and accessibility by roads. They also have numerous sources of potential organic loading due to their urban proximity, commercial and industrial activity, streambank degradation due to recreational traffic, and large pulses of organic material from spawning salmon runs.

The Kenai River supports populations of rainbow trout, Chinook, coho, pink, and sockeye (O. nerka) salmon, and Dolly Varden and is the largest freshwater sport fishery in Alaska (Hammarstrom, 1988). Ship and Campbell Creeks support populations of these species as well as chum salmon (Miller and Bosch, 2004). Ship Creek is the most popular sport fishery in the Anchorage area and sustains the only two hatcheries in the state that rear rainbow trout: Ft. Richardson (FTR) and Elmendorf (ELM). Detections of M. cerebralis in Alaska were in rainbow trout from ELM (Arsan et al., chapter 3 of this volume). This
hatchery uses surface water that, until 2004, was heated to 14°C [an optimal temperature for parasite development (El-Matbouli et al., 1999)] by wastewater from a power plant near the facility. The incoming surface water at ELM is screened for debris but is not treated for potential biota. Thus, *M. cerebralis* spores or even *T. tubifex* could potentially enter the hatchery through Ship Creek water. Effluent from both ELM and FTR is routed through earthen settling ponds and flows back into Ship Creek untreated. Thus, fish in Ship Creek could also be exposed to parasites in hatchery effluent. Furthermore, *T. tubifex* were identified from settling-ponds at both hatcheries (Arsan et al., chapter 2 of this volume).

**RELEASE ASSESSMENT**

The release assessment explores potential pathways of pathogen introduction and is focused on the myxospore stage of the parasite. As the parasite has been detected in southcentral Alaska, the release assessment provides insight into how introduction may have occurred and by what route further dissemination is most likely. Four main pathways of introduction of *M. cerebralis* were identified: movement of fish by humans, natural dispersal, recreational activities, and commercial seafood processing (Figure 4.2). These potential introduction routes could include: legal and illegal movement of fish by humans, importation of frozen food fish, dispersal of parasite via migratory birds, angler activities, dissemination by stray anadromous fish from enzootic areas, and effluent discharge from seafood processing plants.

**Human Movement of Fish**

The primary spread of many invasive species has been by human activity. In the case of *M. cerebralis*, the pathogen is thought to have spread throughout the USA as a result of shipments of infected fish, both commercially and through state and federal stocking programs (Hoffman, 1990; Bartholomew and Reno, 2002). Since the mid 1970’s, the state of Alaska has adopted strict laws prohibiting the
import of live non-ornamental fish (Alaska Administrative Code [AAC] 5: 41.070). However, frozen food fish are regularly imported in the state. Imported frozen rainbow trout (with heads still on) are common supermarket items in Alaska [T. Meyers, Alaska Department of Fish and Game (ADFG), personal communication]. Given that the majority of rainbow trout produced in the USA originate in the Hagerman Valley, Idaho (in the *M. cerebralis* enzootic area), it is likely these fish are sold in Alaska. Because *M. cerebralis* myxospores survive freezing (El-Matbouli and Hoffmann, 1991), frozen fish still pose a risk for parasite dissemination and are thought to be the original pathway of introduction of *M. cerebralis* to the USA (Hoffman, 1962). However, the resulting whirling disease epizootics that ensued from the parasite’s initial introduction to the USA likely stemmed from the use of the frozen imports as food for hatchery fish.

Hatchery practices have changed considerably since the 1950’s, and it is unlikely that frozen fish imported for human consumption would end up in Alaskan waters. Though the potential for pathogen dissemination by frozen fish is low, the risk can be further reduced by requiring that imported fish have heads removed.

Little information is known regarding the number and distribution of private fish-ponds in the state or possible illegal importation of fish. However, instances of illegal importation from out-of-state are thought to be low considering general lack of accessibility and proximity to ports in addition to legal complications and climatic logistics of private fishpond operation in Alaska.

State regulations (AAC 5: 41.005) also prohibit within-state transport, possession, export, or release of any live fish or fish eggs without a Fish Transport Permit. However, illegal transfer (stocking) of fish has occurred and is thought to be the primary reason for the presence of northern pike (*Esox lucius*) in the upper Cook Inlet basin and Kenai Peninsula (ADFG, Southcentral Alaska northern pike brochure).

Another factor influencing potential within-state dissemination is the use of game fish (fresh or frozen) as bait. Heads, tails, fins and viscera of legally caught game fish may be used as bait (AAC 5: 75.026), though live fish may not. Fish
heads, where parasite concentrations would be highest, may thus be dispersed in rivers throughout the state. To add to this potential pathway of dissemination, the parasite survives passage through the alimentary canal of predatory fish (El-Matbouli and Hoffmann, 1991), and thus may be further distributed beyond an initial introduction as bait.

Assessment of risk

Although human movement of fish presents a low risk for new introductions of *M. cerebralis* to Alaska, it is the most likely pathway for parasite dissemination within the state. Prior to parasite detection, potentially infected fish were transplanted throughout southcentral and interior Alaska. Many of these stocked locations were land-locked lakes with no outlets, which would limit the spread of the parasite, but some fish were also stocked in open stream systems. Thus, some degree of spread may already have occurred. As the estimated prevalence of infection in production rainbow trout from ELM is 23% (Arsan et al., chapter 3 of this volume), if 10,000 fish are stocked, 2,300 potentially have some degree of infection. Typically, 2,000-10,000 fish are transplanted per site in southcentral Alaska (ADFG, 2006b). This would readily disperse the parasite throughout the stocking area. Thus, without management action, the probability for *M. cerebralis* spread by human movement of fish is high. In contrast, if stocking of ELM fish is limited to no-outlet lakes where susceptible native resident fish are absent or to seawater netpens for terminal commercial and sport fisheries, and anglers are prohibited from using heads of fish as bait, the probability of parasite dissemination by human movement of fish decreases (Table 4.2) to a low level. Detailed management recommendations are discussed in the conclusions of this paper.

Commercial Seafood Processing

Fish from *M. cerebralis* enzootic areas, such as the upper Columbia River basin, are regularly caught in commercial fisheries in Alaska [ADFG, 2006a; Regional Marking Information Systems (RMIS), 2006]. If effluent from seafood
processors were discharged into freshwater where *T. tubifex* were present, *M. cerebralis* could potentially be introduced. Fish solids released into marine waters utilized by migratory birds or scavengers, such as gulls, could also result in dispersal from marine environments inland to riverine systems via excrement and egested pellets. (See Birds Pathway.)

Authorized seafood processors in Alaska individually discharge 30,000 to over 10 million pounds of waste solids annually (USEPA, Fact Sheet a). Shore-based fish processors in Alaska are required (Clean Water Act, section 301(a), 33 U.S.C.) to grind solid effluent waste to 0.5 inch (1.27 cm) or less prior to discharge. These smaller pieces, including cartilage where myxospores would be concentrated, would be less attractive to scavengers that may transport the parasite in freshwater ecosystems. Processors are allowed to discharge into freshwater rivers with a waiver. However, very few of these waivers are granted. Seafood processing solid wastes (i.e.: fish heads where myxospores would be concentrated) are taken to by-product reduction facilities for reduction to fish meal or other secondary products (USEPA, Fact Sheet b). Though the majority of Alaska fish meal is comprised of whitefish (Alaska Office of Economic Development, By-Product Fact Sheet), all fish meal is brought to 100-600°C during processing (USEPA, 1995), temperatures that would destroy *M. cerebralis* myxospores.

There are 218 fish processing plants with National Pollutant Discharge Elimination System (NPDES) permits in the state of Alaska (USEPA, 2006). None of the 28 permits in the Cook Inlet Basin or the 22 permits in southeast Alaska have a waiver to discharge effluent into freshwater. As the majority of processors are near the coast, discharges into freshwater would likely be near a river mouth and hence would be tidal. Tidal areas are not suitable habitat for *T. tubifex* (Seys et al., 1999; Rundle et al., 1998), thus discharging into these areas would present little risk for introduction of *M. cerebralis*.

Seafood processors discharging less than 1,000 pounds of seafood waste per day and less than 15 tons per calendar year are not required to have a permit. This limit was imposed to allow subsistence processing and direct market
processors (processors receiving mostly processed seafood requiring minimal further processing) to discharge without a permit. These processors are not included in this risk assessment, as information regarding their effluent discharges is unavailable.

Assessment of risk

We consider the probability that commercial seafood processing practices would result in introducing *M. cerebralis* into Alaskan fresh waters to be negligible. None of the permitted processing plants discharge into freshwater, and discharges into marine waters are made less attractive to scavengers by grinding effluent to a small size. Scavengers are also more likely to be attracted to tissue than cartilage where parasite loads would be greatest.

Dispersal via Recreation

Alaska has a world-class sport fishery that attracts anglers from across the country and the globe; many of these anglers use equipment in Alaska that has been used in other river systems. River systems most likely to experience introduction of the parasite via recreational activity would be those with popular sport fisheries and accessibility by road. The Kenai River is the largest freshwater sport fishery in Alaska (Hammarstrom, 1988). Ship Creek is the most popular sport fishery in the Anchorage area (accounting for 69% of the 2002 Anchorage angling effort) (Miller and Bosch, 2004). Peterson Creek is the only recreational steelhead (*Oncorhynchus mykiss*) fishery on the Juneau road system (Harding and Jones, 1992).

Anglers in these popular areas could introduce *M. cerebralis* by inadvertently transporting the parasite on the soles of their waders. Myxospores trapped in the moisture of a felt-sole would likely remain wet and thus viable far longer than in a less absorbent material. Anecdotal data collected prior to the determination of the life cycle of *M. cerebralis* (Schäuperclaus, 1931; Hoffman and O’Grodnick, 1977) suggest myxospores remain viable after drying. Yet, more current studies indicate myxospores do not remain viable after drying for 24 hr (R.
Hedrick, University of California, Davis, personal communication). A recent laboratory study demonstrated removable felt soles were able to transport myxospores and TAMs (P. Reno, Oregon State University, personal communication). However, spores became less viable after drying the soles for 8-24 h, and infectious parasites were no longer transmitted after drying for 7 d. Because of their fragility, individual TAM stages are less likely to be transported by this route. However, infected *T. tubifex* adhered to a felt sole provide a suitable environment for TAMs to remain viable. More research is needed to determine the duration of drying required to disinfect entire waders. However, the length of time required to dry and thus disinfect waders will vary by environmental conditions.

While the probability of transporting *M. cerebralis* in a single event (i.e.: a single angler or single angling day) by recreational activities may be low, when all angler days in a year are considered, the probability increases. In 2001, the state saw 421,000 anglers (US Department of Interior, 2001); 239,000 were not Alaska residents and may have used gear that was used outside of Alaska. The sheer number of anglers from within and outside the state indicates the potential for parasite transport is not only high, but continues to grow, with a 36% increase in number of anglers from 1991 to 2001 (U.S. Department of Interior, 2001).

Recreational activities can also influence the risk of potential *M. cerebralis* introduction in indirect ways by enhancing *T. tubifex* habitat. *Tubifex tubifex* are commonly found in environments with abundant organic matter, fine sediments, and low flow (Brinkhurst and Gelder, 1991; Brinkhurst, 1996). Recreational foot traffic (such as angling from the bank) can damage vegetation and increase streambank erosion causing more sediment to enter surrounding waters. Motorized boats also create wakes that can increase streambank erosion. Previous studies have documented these activities and their effects on the Kenai River (Liepitz, 1994; Weiner, 1998).

*Assessment of risk*
The risk of recreational activities as a pathway of parasite introduction is difficult to assess, as the lack of information regarding potential spore transfer via felt-soled waders represents a significant data gap. A conservative estimate of the probability of new introductions of *M. cerebralis* by recreational activity is moderate, but the probability for within-state transfer of the parasite is low, if prevalence and severity of infection remain low (Table 4.2), and if management actions are taken to limit stocking and thus dissemination of infected sportfish. However, the cumulative and long-term effects of angler and recreational activities in heavy-use areas could be much greater than the likelihood of introduction (or further spread) in the short term or by a single event.

**Natural dispersal: birds**

The role of piscivorous birds in fish pathogen dissemination has been examined in several studies (Taylor and Lott, 1978; El-Matbouli and Hoffmann, 1991). Because *M. cerebralis* survives passage through the guts of birds, long food retention times would lengthen the distance the pathogen could be dispersed. If all food is voided within a short period, the parasite could be contained locally. Other omnivorous fish-consumers such as bears (*Ursus americanus*, *Ursus arctos*) and river otters (*Lutra canadensis*) do not present a risk for *M. cerebralis* dispersal, as the parasite does not survive passage through the guts of mammals (El-Matbouli et al., 2005).

Alaska is a migratory destination for thousands of birds worldwide. Although the probability of a bird releasing viable myxospores over a waterbody remains unknown, deposition near (and not in) water may be sufficient for transport of the parasite under certain environmental conditions. It is unknown how long myxospores deposited in bird feces would remain moist, and thus viable. Time to inactivation of spores is likely to vary by environmental conditions. Thus, if spores are deposited near a waterbody, and are rapidly washed into the river by high water or precipitation, dissemination of the parasite could occur.
Since *M. cerebralis* manifests in cartilage of fish, it is likely that the parasite would be egested (regurgitated) in pellets of birds. Piscivores that swallow their prey whole (e.g. non- raptors) present the biggest risk of *M. cerebralis* dispersal. Double-crested cormorants (*Phalacrocorax auritus*) have a simple gut structure and egest bones, pieces of fish, and solid markers 1-2 days following ingestion (Brugger, 1993). In contrast, the passage time of rainbow trout through bald eagles (*Haliaeetus leucocephalus*), which have a more complex gut morphology, is approximately 62 hours (F. Barrows, USFWS, personal communication). Thus spores could be excreted 2-3 d after eating an infected fish.

Raptors and large waterbirds (waders, seabirds, etc) have some of the fastest known migration speeds among birds; bald eagles travel 201 km/day in migration (Kerlinger, 1995), and ospreys (*Pandion haliaetus*) 108-431 km/day (Alerstam, 2003; Hake et al., 2001). The nearest *M. cerebralis* enzootic area outside Alaska [upper Columbia River Basin (CRB)] is approximately 1800 km from southeast Alaska and 2750 km from southcentral Alaska. An osprey would have to retain food material for 4.2 to 16.7 days to transport spores to Juneau and for 6.4 to 25.5 days to transport spores to Anchorage.

*Assessment of risk*

Whereas the probability of new parasite introduction via bird transport from the CRB is negligible, the probability of within-state transfer by bird transport is greater, but still low. Because of the low prevalence and severity of infection in the state, and the fact that numerous events must align in order for dissemination to occur, the probability of parasite transport by birds is likely to remain low unless prevalence or severity of infection increases.

**Natural Dispersal: Stray anadromous salmon**

Anadromous salmonids may stray into non-natal streams during their return migration to spawning grounds. Pathogen dissemination is an often-overlooked effect of straying. For example, introduction of *M. cerebralis* as a result of straying salmonids has been documented in the Deschutes River, Oregon
Some anadromous salmonids from the CRB migrate north to waters off of Alaska to feed for 3-5 years before typically returning south to spawn (Groot and Margolis, 2003; RMIS, 2006).

Fish that have strayed into non-natal waters are usually identified by examination of fin clips or by evidence of a coded-wire tag (CWT). As any single fin clip pattern is commonly used by more than one hatchery in the Pacific Northwest in a given year, fin clips reveal only the “stray” status of a fish and a rough list of potential origins. A CWT or fin clip unique to a certain hatchery would be required for identification of exact hatchery of origin.

Though CRB fish are commonly harvested in commercial marine fisheries off the coast of Alaska, little data are available on salmon straying into the fresh waters of Alaska. There is only one such record: in 2001, a Chinook salmon from Marion Forks Hatchery, North Santiam River, Oregon (lower CRB) was recovered in the Copper River, Southcentral Alaska (RMIS, 2006; ADFG, 2006a). Because stray wild fish cannot be visually identified, no data (current or historical) are available on the stray rates of wild CRB salmon in Alaska, yet these fish are potential carriers of *M. cerebralis*. This represents a data gap in this risk analysis.

**Assessment of risk**

Based on the available data, the probability of *M. cerebralis* dispersal by straying anadromous hatchery salmon from the CRB is negligible. However, presence of the parasite at ELM on Ship Creek provides a local source of dissemination. While it is unknown if *M. cerebralis* is established in Ship Creek, if naturally reproducing fish were infected, this would increase the potential for parasite dispersal. As rainbow trout are typically resident species and do not make long migrations (Morrow, 1980; Behnke, 1992), spread of the parasite by natural migration of this species is likely to be local. Parasite dispersal by straying of anadromous fish from Ship Creek is more likely, though *M. cerebralis* has not been documented in anadromous fish species in Alaska.
Summary of the release assessment

The probability of re-introduction and within state dissemination of *M. cerebralis* in Alaska is summarized in Table 4.2. While the overall probability of new introductions from out-of-state is low; the likelihood of further transport within the state is high if no management actions are taken. The pathway with the greatest likelihood for within-state parasite transfer is stocking of infected rainbow trout, since this would repeatedly introduce large numbers of potentially infected fish. However, if stocking is limited to no-outlet lakes where native susceptible resident salmonid species are absent, or to seawater netpens for terminal commercial and sport fisheries, the probability of further spread of *M. cerebralis* is low. Illegal stocking of fish remains a risk for dissemination of the pathogen. The importation of whole frozen food fish also presents a risk for parasite introduction. The risk of parasite transfer posed by the use of fish heads as bait could also be reduced if this practice were prohibited with fish from ELM-stocked areas. Because of the low prevalence of infection in the state, the likelihood of parasite transport by pathways other than human movement of fish is likely to remain low unless prevalence or severity of infection increases.

EXPOSURE ASSESSMENT

The exposure assessment, which explores the risk of parasite establishment, is focused on the TAM stage of *M. cerebralis* and its oligochaete host. Establishment of *M. cerebralis* has already occurred in southcentral Alaska at ELM on Ship Creek. Whether establishment has also occurred outside the hatchery, remains unknown.

Establishment of *M. cerebralis* is dependent upon several environmental and biological factors including: the distribution and genetic composition of *T. tubifex* populations, water temperatures, and spatial temporal overlap of hosts. Each of these factors determines the outcome of the exposure assessment, as depicted by the scenario tree in Figure 4.3.
**Tubifex tubifex** habitat and populations

Habitat for *T. tubifex* is defined as areas with fine sediment, low flow, and organic matter (Brinkhurst and Gelder, 1991). These areas were found throughout the study area.

A *T. tubifex* survey was conducted to ascertain relative abundance of *T. tubifex* in selected streams in the study area (Arsan et al., chapter 2 of this volume). Over 2,700 oligochaetes were collected from southeast Alaskan sample sites; however, morphological and genetic analysis demonstrated that none of the worms examined were *T. tubifex*. In contrast, *T. tubifex* were identified from southcentral Alaska, with the highest numbers on the lower Kenai River, particularly at Centennial and Eagle Rock Boat Landings, areas with high sedimentation and organic loading (primarily decaying salmon carcasses) and heavy recreational use. As many as 1,768 *T. tubifex* out of 3,024 total worms were detected in 30ml of sediment at Centennial Boat Landing. The species was also identified from Ship and Campbell Creeks, though not from the upper or middle Kenai River.

Oligochaetes gathered in the *T. tubifex* survey were held in water that was screened for *M. cerebralis* actinospores. No *M. cerebralis* TAMs were observed from any of the sites sampled in the *T. tubifex* survey. However, actinospores of several other myxozoans were detected (Arsan et al., chapter 2 of this volume), confirming that other myxozoan life cycles have established in both southeast and southcentral Alaska.

**Tubifex tubifex** lineages

Though *T. tubifex* is the only species of oligochaete capable of harboring *M. cerebralis*, susceptibility to the parasite varies dramatically within the species and has been correlated to the taxa’s mitochondrial lineage. Populations of lineages I and III have been shown to propagate the parasite whereas lineages IV, V, and VI do not (Arsan et al., chapter 2 of this volume; Beauchamp et al., 2002,
Four lineages were identified in the state: I, III, IV, and VI (Arsan et al., chapter 2 of this volume). Lineage IV had previously been reported only from Europe.

Parasite exposure experiments using *T. tubifex* collected from our study sites demonstrated that lineages I, IV, and VI from Alaska do not support the parasite life cycle (Arsan et al., chapter 2 of this volume). Lineage III was the only lineage from the state to propagate the parasite. This lineage, though susceptible to *M. cerebralis*, was a low parasite producer, releasing a maximum of 280 TAMs in 7 d, as compared to the control lineage III population from Oregon that released over 7000 TAMs during the same time period when exposed to the same number of myxospores.

*Tubifex tubifex* populations in southcentral Alaska were a mixture of two or three lineages. Lineage I dominated (71-86%) at sites on Ship Creek and Campbell Creek, whereas Lineage VI dominated (69%) at sites on the Lower Kenai River. Lineage III was present in low numbers (7-21%) throughout the Cook Inlet basin at 7 out of the 9 sites where *T. tubifex* were collected, however, it was not identified from any of the hatchery sites.

The presence of resistant lineages I, IV, and VI *T. tubifex* could have significant beneficial implications for Alaskan salmonids in areas where these lineages dominate *Tubifex* populations. Other researchers have found lineage susceptibility to correspond to severity of infection in fish (Beauchamp et al., 2006). This suggests that when lineages I, IV, and VI are in high abundance, the effects of *M. cerebralis* may be less severe.

Populations of lineage III *T. tubifex* from other geographic regions are highly susceptible to *M. cerebralis* (DuBey et al., 2005; Beauchamp et al., 2006; Hallett and Bartholomew, 2005) and detection of the lineage in Alaska is cause for concern. Though this lineage composed approximately 14-21% of worm populations in Ship and Campbell Creeks and only 7% on the lower Kenai River, it can become infected with *M. cerebralis* and release TAMs when in proportions as low 0.6% of the total population (Arsan et al., chapter 2 of this volume). Hallett
et al. (2007) documented similar lineage III infection dynamics when the lineage was in proportions as low as 5% of the total *T. tubifex* population. Researchers have suggested that only a few *T. tubifex* and TAMs are required to produce infection and population loss in fish in cold, oligotrophic streams (Allen and Bergersen, 2002).

**Water temperature seasonally reaching 10-15 °C**

Water temperature influences *M. cerebralis* development in each of its hosts (Hedrick and El-Matbouli, 2002; Blazer et al., 2003). Both severity of infection in fish and development and release of TAMs from oligochaetes are highest when average daily water temperatures are between 11-15°C (El-Matbouli et al., 1999; Baldwin et al., 2000; Granath and Gilbert, 2002). Temperatures below 10°C delay the development of TAMs and those above 20°C hinder parasite development and can be lethal to the TAM stage of *M. cerebralis*.

Lower water temperatures retard the spore formation process in both fish and oligochaetes, and could delay TAM development and release at high latitudes. However, *M. cerebralis* has been found to persist and cause reduced juvenile trout recruitment even in cold, oligotrophic, sediment poor, high-gradient streams (Allen and Bergersen, 2002) and at elevations as high as 3,300m (10,800 ft) in Colorado (Nehring and Thompson, 2002). Temperatures in such areas are comparable, if slightly warmer, to those in our study sites in Alaska. Since snow covers the ground for more than 6 months of the year, streams in southcentral Alaska are generally cooler than those in the contiguous US, typically with a total of 1780 annual degree days (Oswood, 1997). In comparison, the Madison River in Montana (enzootic for *M. cerebralis*) has roughly 2650 annual degree days (USGS, 2005).

Mean summer (June- August) water temperatures in the lower Kenai River ranged from 10.7- 11.2°C between 1999-2001 (USGS, 2005), with an average of 79 days/year continuously greater than 10°C, and 1801 degree days annually. Temperatures in Campbell Creek ranged from 9.5-10.5°C, with an average of only
50 days above 10°C each year from 2000-2001 (USGS, 2002). Lineage III *T. tubifex* from Alaska required 1382-1536 degree days for *M. cerebralis* to develop. Therefore, water temperatures in southcentral Alaska are sufficient for parasite development and propagation, though complete life cycle duration may be longer than in warmer climates. The lower Kenai River has water temperatures most conducive to propagation of the *M. cerebralis* life cycle.

Future climate trends could also influence parasite development; USGS reports water temperatures within the Cook Inlet basin are likely to increase 3°C in the next 10 years (Kyle and Brabets, 2001). If such an increase occurs, water temperatures in southcentral Alaska would be similar to that of the Madison River, Montana, an area where *M. cerebralis* has caused population loss in rainbow trout (Krueger et al., 2006).

**Spatial/ temporal overlap of hosts**

For the parasite to establish after introduction of myxospores, obligatory spatial overlap must occur between parasite and host. The complex life cycle of *M. cerebralis* requires spatial overlap to occur twice: once between myxospores and *T. tubifex*, and subsequently between salmonids and TAMs.

Researchers have documented varied patterns of seasonality associated with TAM release in different geographic locations. In Montana, TAM numbers peak in the spring warming and fall cooling periods (Gilbert and Granath, 2003); in high altitude waters in Colorado, TAM release peaks in the fall through the winter (Nehring and Thompson, 2002). Yet, other areas of Colorado tend to exhibit peak TAM numbers in the summer and early fall, with the lowest number of TAMs occurring in the spring (Thompson and Nehring, 2000; Allen and Bergersen, 2002). Seasonality of TAMs is believed to relate to water temperature and the availability of myxospores as influenced by fish stocking schedules. We speculate that high latitude Alaskan water temperatures would be similar to high altitude water temperatures in Colorado and that seasonal TAM release in Alaska would be comparable to these areas.
Worm infection and TAM release are dependant on myxospore availability. Myxospores are released upon the death of the fish host and subsequent degradation of the cartilage. As breakdown of this tissue is likely to occur gradually in slow moving or cold waters, release may occur over a long period (Hallett and Bartholomew, 2006). Once infected, worms can remain persistently infected throughout their lifespan (Gilbert and Granath, 2001) with TAM release occurring when water temperatures are appropriate. The majority of young salmonids (both wild and hatchery) in southcentral Alaska hatch December through August (ADFG, 2003; Quinn, 2005) and would be most susceptible to infection during the first weeks post-hatch. Rainbow trout (and to a lesser extent steelhead) are most susceptible to the parasite from 0-9 weeks post-hatch (up to 756 degree days at 12°C) (Ryce et al., 2004). However, rainbow trout hatched in June would exhibit resistance to the parasite by September. On this schedule, young rainbow trout may avoid peak TAM release from oligochaetes if peak release begins in September. Thus, it may be possible for the parasite to proliferate at cold temperatures and yet have little impact on rainbow trout populations (Kerans et al., 2005).

Salmonid species composition will also affect the outcome of introduction. Generally, areas with the highest *T. tubifex* abundance, like the lower Kenai River, would have a higher likelihood of spatial-temporal overlap of hosts and parasite and thus of *M. cerebralis* establishment. However, the juvenile salmonid assemblage in this area is 88% Chinook salmon, with rainbow trout making up only 0.1% of the total assemblage (Bendock and Bingham, 1988). While Chinook salmon are susceptible to *M. cerebralis*, they acquire resistance to the parasite more quickly and are less susceptible than rainbow trout (MacConnell and Vincent, 2002; Sollid et al., 2003). Thus, the likelihood of spatial overlap of susceptible hosts in the lower Kenai River is low. Juvenile salmonid assemblages in Ship Creek are speculated to be greater than 80% Chinook and coho salmon (D. Bosch, ADFG Sport Fish Division, personal communication), with proportions of rainbow trout unknown.
Summary of the exposure assessment

The probability of further establishment of *M. cerebralis* in southcentral Alaska is variable between locations (Figure 4.3). Distribution of susceptible *T. tubifex*, water temperatures, and the juvenile salmonid species composition would be determining factors for establishment. Thus the probability of establishment in the upper Kenai River is low, due to a lack of oligochaete hosts. While the lower Kenai River maintains a high abundance of *T. tubifex*, the lack of juvenile rainbow trout lowers the risk of *M. cerebralis* establishment. Ship and Campbell Creeks have environmental conditions representative of non-glacial streams in southcentral Alaska, and remain the most likely areas to experience parasite establishment (Figure 4.3). Yet while these creeks support populations of *T. tubifex*, abundance and susceptibility of the worms appear to be low. Not only is susceptibility limited to lineage III *T. tubifex*, but Alaska strains of this lineage are low parasite producers (Arsan et al., chapter 2 of this volume). Thus, although conditions are permissive in southcentral Alaska for establishment of *M. cerebralis*, they are not ideal and may keep infection rates low and disease from becoming apparent. Changes to physical or environmental conditions, such as climate change, may alter the probability of parasite establishment in the state.

The probability of establishment in southeast Alaska is negligible, as no *T. tubifex* were detected. However, oligochaete surveys have been limited (Arsan et al., chapter 2 of this volume).

CONCLUSIONS AND RISK MANAGEMENT

Risk of new introductions

The probability of new introductions of *M. cerebralis* into the state of Alaska is low, with the most likely pathway for introduction being recreation and angler activity. Thus, areas most likely to first experience introduction are high-use sport fisheries such as the Kenai River and Ship Creek. If introduction occurs,
the probability of parasite establishment is moderate, particularly in rivers like Ship Creek where permissive temperatures, susceptible *T. tubifex*, and potential rainbow trout populations overlap.

Although conditions (temperatures, *T. tubifex* populations, and spatial/temporal overlap of hosts) in Ship Creek are permissive for parasite development, they are not optimal, and may slow parasite development and lower the risk for establishment. For example, water temperatures are acceptable for parasite development, but are low enough to abate rapid proliferation. Susceptible *T. tubifex* are present in the creek, but populations are dominated by resistant strains, again lowering the risk of rapid parasite proliferation. Lastly, fish species composition in southcentral Alaskan creeks may also help to lower the risk of parasite establishment, as Chinook salmon (having lower susceptibility to the parasite) tend to dominate areas where susceptible *T. tubifex* were collected. This study focused on the first two steps of a risk analysis (release and exposure assessments), but did not complete a consequence assessment to evaluate the effects of parasite introduction and establishment in Alaska.

Policies that prevent importation of live salmonids into Alaska have been the most effective tool for limiting introduction. Existing regulations discourage establishment of private ponds, which are believed to contribute to spread of the parasite in areas of the contiguous USA (B. Nehring, Colorado Division of Wildlife, personal communication). We also recommend the state maintain the policy requiring all seafood processor effluent be disposed in marine waters, and to restrict effluent waivers in areas considered high risk (Cook Inlet Basin).

Although the potential for introduction by anglers is moderate, the risks could be further reduced, especially in light of recent data on the vulnerability of myxospores to desiccation. To further reduce the risk of introduction by anglers, we urge the state to allot resources to angler education and further research on effects of angler activity on dispersal. This could be accomplished with signage at boat ramps, parking areas, or other access points, brochures distributed upon purchase of fishing licenses, and by an informational web page.
Risk of further dissemination within Alaska

The probability of further transport of *M. cerebralis* within the state is high due to the presence of the parasite at a state hatchery. The pathway presenting the greatest risk for within-state parasite transfer is human movement of fish (e.g. transplanting of infected trout and use of fish heads as bait) since large numbers of infected fish could be repeatedly introduced. Because of the low prevalence of infection in the state, the likelihood of parasite transport by other pathways is likely to remain low unless prevalence or severity of infection increases. Although the probability of establishment of *M. cerebralis* in southeast Alaska is considered negligible due to the lack of suitable invertebrate hosts, only a few sites were surveyed. To gain more confidence in this assessment, additional sites should be surveyed for susceptible lineages of *T. tubifex*.

While it is unknown how long the parasite has occurred in Alaska, it is likely that previous monitoring using the pepsin-trypsin digest as an initial screening test would have missed a low infection prevalence. Not only did all prior monitoring efforts in Alaska use the pepsin-trypsin digest, but testing was non-targeted and did not focus on areas at risk for *M. cerebralis* introduction. Molecular tests such as PCR are approximately 10-fold more sensitive (Andree et al., 2002) and may have detected the parasite. For management purposes it may only be necessary to detect infections that cause negative impacts on fish populations or are considered “significant” infections, and both pepsin-trypsin digest and histology are adequate for this purpose. However, if the criterion for a “significant” infection is presence of the parasite, then use of more sensitive assays is necessary. Each state has its own stipulations as to what constitutes such an infection and what the implications of detection are. For example, the state of Colorado transfers only fish that are PCR negative for *M. cerebralis* to areas where the parasite is not known to occur.

The first detection of *M. cerebralis* in Alaska is not without consequence. Clearly the risk of parasite dissemination is low (if stocking fish from ELM is
limited), but nevertheless unacceptable by current ADFG fish disease policies. Future plans include replacement of ELM with a new facility constructed on a nearby site with water supplied from wells rather than Ship Creek. The FTR will be discontinued entirely once the new ELM facility is completed. The use of well water should facilitate breaking the parasite life cycle, and will be corroborated by additional QPCR testing of hatchery fish stocks in the new facility. Negative findings would suggest the possibility of future unrestricted fish transplants.

Exposure of sentinel rainbow trout fry in Ship Creek would also determine if the parasite has become established outside the hatchery, and has been used in similar situations after isolated parasite introduction (Bartholomew et al., 2007). Given the low prevalence and severity of infection in ELM fish, examination of other salmonid species or monitoring of Ship Creek water for TAMs would likely prove ineffective. Regular monitoring for *M. cerebralis* in cultured salmonids would provide baseline data if prevalence or severity of infection were to change. Monitoring of wild salmonids should focus on areas of highest risk for parasite introduction and establishment.

In addition to Alaska’s *M. cerebralis* interim management plan, we recommend the state prohibit the use of fish heads from ELM-stocked areas as bait. Allotment of resources toward angler education would further benefit this action.

The risk of parasite dissemination in Alaska is an evolving process and will vary with changes to environmental or physical conditions that affect parasite proliferation and development, such as climate change or land use modifications. This risk assessment provides a framework for future re-evaluation of the risk of *M. cerebralis* dispersal in Alaska.

ACKNOWLEDGEMENTS

This work was supported in part by the National Partnership for the Management of Wild and Coldwater Species, the Whirling Disease Foundation, the United States Fish and Wildlife Service, and the United States Geological
Survey. We are grateful to the following members of the Oregon State University, Center for Fish Disease Research: Sascha Hallett and Stephen Atkinson for providing critical consultation on oligochaete sampling, identification and genetic assays, Don Stevens also assisted with worm identification, and Harriet Lorz for her efforts in maintaining oligochaete cultures. We also thank a number of ADFG personnel for their assistance: Tammy Burton for providing live worm samples and field support, Ted Meyers and Tim McKinley for their help in the field, Ted Meyers also provided essential consultation on study design and background. We are thankful to Alaska Wildland Adventures for providing equipment and field support on the upper Kenai River.
REFERENCES

ADFG (Alaska Department of Fish and Game).  Southcentral Alaska northern pike brochure, Matanuska-Susitna Valley / Kenai Peninsula / Anchorage Bowl. Recreational Fishing Series, Division of Sport Fish, Southcentral Region, Anchorage, Alaska. [http://www.sf.adfg.state.ak.us/region2/pdfpubs/02pike.pdf]


National Climate Center. 1982. Climate of Alaska. NOAA Environmental Data
Service, Ashville, NC.


State of Alaska. Department of Fish and Game. Recreational Fishing Series, Southcentral Region, Division of Sport Fish.


Table 4.1. Definitions of probability levels used in the risk analysis

<table>
<thead>
<tr>
<th>Probability</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>The event would be expected to occur</td>
</tr>
<tr>
<td>Medium</td>
<td>There is less than an even chance of the event occurring</td>
</tr>
<tr>
<td>Low</td>
<td>The event is unlikely to occur</td>
</tr>
<tr>
<td>Negligible</td>
<td>The chance of the event occurring is so small that in practical terms it can be ignored</td>
</tr>
</tbody>
</table>
Table 4.2. Summary of the probability of introduction and within-state dissemination of *Myxobolus cerebralis* in Alaska.

<table>
<thead>
<tr>
<th>Pathway of parasite dissemination</th>
<th>Probability of introduction from outside Alaska</th>
<th>Probability of dissemination within Alaska</th>
<th>Probability of dissemination with limited stocking*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human movement of fish</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Commercial fisheries</td>
<td>Negligible</td>
<td>Negligible</td>
<td>Negligible</td>
</tr>
<tr>
<td>Natural dispersal: birds</td>
<td>Negligible</td>
<td>Low†</td>
<td>Low†</td>
</tr>
<tr>
<td>Natural dispersal: stray salmonids</td>
<td>Negligible</td>
<td>Low*</td>
<td>Negligible‡</td>
</tr>
<tr>
<td>Recreation</td>
<td>Moderate</td>
<td>Low‡</td>
<td>Low‡</td>
</tr>
<tr>
<td>Overall Probability</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

* Limited to no-outlet lakes where native susceptible resident salmonid species are absent, or to seawater netpens for terminal commercial and sport fisheries.
† If infection prevalence and severity remain low
‡ If parasite not established outside of hatchery
Figure Legend

Figure 4.1. Map of areas of highest risk for *Myxobolus cerebralis* dissemination in southeast and southcentral Alaska. Rainbow trout hatcheries depicted by triangles (Δ).

Figure 4.2. Model of potential *Myxobolus cerebralis* introduction in Alaska. Three main pathways of dissemination are shown with specific activities listed.

Figure 4.3. Scenario tree of risk of establishment of *Myxobolus cerebralis*, if disseminated in Alaska. Requirements for establishment and areas within the study sites that meet them are displayed.
Figure 4.1.
Figure 4.2.

- Hatchery fish transfers and stocking
- Illegal importation of fish

**Human Movement of Fish**

**Commercial Activities**
- Infected fish in commercial harvests

**Introduction**

**Natural Dispersal**
- Migration of piscivorous birds and mammals
- Stray fish from enzootic areas

**Recreational Activities**
- Angler/boating activity
Figure 4.3.

Establishment does not occur

No

Southeast Alaska

Habitat for *T. tubifex*

Yes

Population of susceptible *T. tubifex*

Yes

Water temperature seasonally reaching 10-15 °C

Yes

Spatial/temporal overlap of hosts

Yes

Establishment occurs

Throughout Alaska

Southcentral Alaska: lower Kenai River, Ship and Campbell Creeks

Southcentral Alaska: lower Kenai River, Ship and Campbell Creeks

Where susceptible salmonid assemblages overlap with susceptible lineages of *T. tubifex*: Ship and Campbell Creeks

In Ship and Campbell Creeks
CHAPTER 5

A QUALITATIVE ANALYSIS OF RISK FOR DISPERSAL OF THE NON-NATIVE PARASITE *MYXOBOLUS CEREBRALIS* IN THE WILLAMETTE RIVER BASIN, OREGON

E. Leyla Arsan\(^1\) and Jerri L. Bartholomew\(^2\)

\(^1\)Department of Fisheries and Wildlife, Center for Fish Disease Research, Oregon State University, 220 Nash Hall, Corvallis, OR 97331, USA
\(^2\)Department of Microbiology, Center for Fish Disease Research, Oregon State University, 220 Nash Hall, Corvallis, OR 97331, USA
Abstract

*M. cerebralis*, the myxosporean parasite responsible for whirling disease in salmonids, was first detected in the United States in 1958. It has since spread across the country causing severe declines in wild trout populations in the intermountain west. This study qualitatively assesses the risk of introduction and establishment of the pathogen into the Willamette River basin, Oregon by examining potential routes of dissemination and relationships among obligate hosts, the parasite, and the environment. The risk of *M. cerebralis* introduction in the Willamette River basin is addressed as a function of three main elements of dispersal: 1) movement of infected fish by humans, 2) natural dispersal (via migratory birds and stray anadromous salmonids), 3) and recreational activities. Establishment of the parasite is dependent upon several environmental and biological factors including: water temperatures, density and spatial/temporal overlap of hosts, and the distribution and genetic composition of the oligochaete host, *Tubifex tubifex*. This study finds the probability of introduction of the parasite to vary throughout the Willamette River basin. Areas with greater probability have been identified as the Clackamas and Santiam River subbasins. If the pathogen were introduced, probability of establishment is high in certain areas of the basin as conditions are appropriate for propagation of the parasite life cycle.
INTRODUCTION

*Myxobolus cerebralis*, a metazoan fish parasite exotic to North America, was first detected in the USA in 1958 (Hoffman, 1962). Thought to have originated from a shipment of frozen infected brown trout (*Salmo trutta*) from Europe, it is now reported from 25 US states and 26 different countries (Bartholomew and Reno, 2002; Vermont Department of Fish and Game, 2002; Stromberg, 2006; Arsan et al., chapter 3 of this volume). The pathogen has been at the forefront of fish health research due to its potential impacts on rainbow trout culture and its implication in rapid dramatic declines of wild trout populations in Colorado and Montana (Vincent, 1996; Nehring et al., 1998). The ecologic and economic impacts of *M. cerebralis*, in addition to its rapid spread and establishment across the globe, are indicative of the need for specific recommendations to assist fishery managers in halting the spread or effects of the pathogen. These recommendations can be developed through the process of risk analysis (MacDiarmid, 2000), and be tailored for a specific area. This study assesses the risk of introduction and establishment of *M. cerebralis* into the Willamette River basin, Oregon, a major tributary of the lower Columbia River.

Preventing pathogen introduction is a primary objective in fish health management. However, once introduction occurs, parasitic infestations are often managed by disrupting the parasite life cycle, generally by altering accessibility to one or more of the organism’s hosts. The life cycle of *M. cerebralis* requires two obligate hosts: a salmonid and the aquatic oligochaete worm, *Tubifex tubifex*. There are also two distinct spore stages of the parasite (one particular to each of the hosts). The myxospore stage develops in the fish host and is released upon death and decomposition of the fish. These round and durable spores are ingested by *T. tubifex* as the worms burrow through sediment consuming bacteria (Brinkhurst, 1996). The parasite then undergoes reproduction and structural transformation and is released in its actinospore stage as a triactinomyxon (TAM). The TAM attaches to its fish host in the water column, where the parasite sporoplasm is injected.
through the epidermis of the fish and migrates via the nervous system to the cartilage (El-Matbouli et al., 1995). Each spore stage requires approximately three months to develop and be released from the respective host. The two distinct life stages and hosts of *M. cerebralis* also necessitate the need for a risk assessment model with two distinct segments that address each spore stage (and the respective host) separately.

The definitive host, *T. tubifex*, is a hearty and cosmopolitan species capable of withstanding extreme and variable environmental conditions. Such qualities allow the worm to inhabit areas where other species cannot compete, and thus to span across ecosystems as a widespread aquatic invertebrate. These qualities also have made control of this host impractical or unsuccessful (Wagner, 2002). Although *T. tubifex* is the only species of oligochaete capable of acting as a host for *M. cerebralis* (Wolf et al., 1986), its susceptibility to the parasite varies greatly and has been correlated to the specimen’s mitochondrial lineage (Beauchamp et al., 2006). Thus, the potential for *M. cerebralis* establishment depends not only on the presence and density of *T. tubifex*, but also on the genetic composition of the population.

In contrast to the single species of oligochaete capable of acting as a definitive host, numerous salmonid species have varying degrees of susceptibility to *M. cerebralis*. Rainbow trout (*Oncorhynchus mykiss*) have repeatedly shown the highest susceptibility in laboratory and field experiments (O’Grodnick, 1979; Hedrick et al., 1999a, 1999b; MacConnell and Vincent, 2002). Other salmonids in Oregon, except lake trout (*Salvelinus namaycush*), are also susceptible to the parasite (MacConnell and Vincent, 2002).

This study evaluates the probability of introduction and establishment of *M. cerebralis* into the Willamette River basin (WRB), Oregon. The parasite is enzootic in portions of the upper Columbia River basin (CRB), and although it has not been demonstrated to cause loss to fish populations in other parts of Oregon, its detection in the WRB would effectively stop commercial transport of fish from the area and is thus of economic as well as ecologic importance. The framework for
this risk assessment (Bartholomew et al., 2005) was created in efforts to tailor such assessments to whirling disease. This paper includes a synthesis of historical data as well as new research to fill data gaps. Our aim is to provide decision-makers with tools to assess management implications, to eliminate non-issues by using logical scientific arguments, and to provide guidance on where to allocate resources to help prevent introduction of the pathogen.

METHODS

Study Area

*Myxobolus cerebralis* was first detected in Oregon in 1986 (Lorz et al., 1989), and is now enzootic in certain tributaries of the upper CRB (Figure 5.1). Since the 1980’s, the parasite has been detected elsewhere in the basin in stray adult salmonids originating from these tributaries (Engelking, 2002). However, there is no evidence of establishment of the parasite life cycle outside of the enzootic region. The parasite was detected further west in the state in 2001, at a private facility in the lower WRB (Clear Creek). Infection at the facility was contained and discharges are no longer made into creek (Bartholomew et al., 2007). Currently, *M. cerebralis* is not known to be established in the WRB outside of the private facility on Clear Creek.

The Willamette River originates in the Cascade Mountains (up to 10,000 ft) and flows approximately 300 km to the lower Columbia River (10 ft). The WRB houses 70% of the population of Oregon and is the fastest growing and most economically and agriculturally developed region in the state (USEPA, 2006a, 2006b). The river, which is the 13th largest in terms of discharge in the contiguous US (Urish and Wentz, 1999), flows through Oregon’s three largest cities, and accounts for 15% of the total flow in the Columbia River (Payne and Baker, 2002). Stream flow in the basin reflects precipitation, with the majority (60-85%) of the runoff occurring from October to March (Urish and Wentz, 1999). The basin
contains the richest native fish fauna in the state (Rathert et al., 1999) and provides a migratory corridor and spawning grounds for a variety of anadromous salmonids.

This study concentrated on four areas of the WRB that we believed to have the highest risk for introduction and establishment of *M. cerebralis*: the Willamette River mainstem, Clackamas River subbasin, McKenzie River, and North and South Santiam Rivers (Figure 5.1). These areas have high concentrations of susceptible fish hosts, high angler traffic, hatcheries rearing susceptible fish species, private fish rearing ponds, and high potential organic loading leading to increased *T. tubifex* habitat.

**Validation of preliminary assumption**

To verify the preliminary assumption that *M. cerebralis* is not present in wild or hatchery salmonids in the WRB, we used data from 20 years of Oregon Department of Fish and Wildlife (ODFW) parasite testing, representing over 12,000 wild and cultured fish (N. Hurtado, ODFW, unpublished data).

From 1986-1987, immediately following the first detection of the parasite in Oregon, 82 fish from 6 locations in the basin were presumed positive for the parasite. All fish were transfers from a positive private facility in the upper CRB to other private facilities in the WRB, and the ODFW subsequently halted transfers of fish from the enzootic area. The parasite was not detected in further monitoring of these 6 locations.

Though more presumptive positive fish were detected in the WRB from 1988-2001, infection was never confirmed. Presumptive identification of *M. cerebralis* is based on myxospore morphology, a trait that is similar in many *Myxobolus* species. Until 2001, confirmation of infection was based on histopathology, a method with high specificity but low sensitivity. Since 2001 and the incorporation of sensitive genetic tests such as polymerase chain reaction (PCR) into monitoring regimes, the only locations in the WRB where *M. cerebralis* has been detected are in Clear Creek. Because of the low sensitivity of parasite
detection and confirmation methods used before 2001, previous detection of the parasite was likely underestimated. The infected private facility on Clear Creek was shut down in March 2003 (Bartholomew et al., 2007) and the pathogen has not been detected in the creek since, thus confirming it was the point source of infection.

The United States Fish and Wildlife Service’s (USFWS) National Wild Fish Health Survey (NWFHS) also conducts limited testing of wild salmonids in the WRB. The NWFHS reports results of *M. cerebralis* tests for 468 wild fish from 18 locations in the basin (USFWS, 2006); *M. cerebralis* has never been detected.

Test results from both the ODFW and NWFHS support the assumption that the *M. cerebralis* lifecycle is not established in the WRB.

**RELEASE ASSESSMENT**

The release assessment explores potential pathways of pathogen introduction and is focused on the myxospore stage of the parasite. Myxospores are far more resilient than TAMs and are capable of withstanding severe environmental conditions (El-Matbouli & Hoffmann, 1991) that might occur during transport and dissemination. Of the two obligate hosts of *M. cerebralis*, the fish host is more mobile; thus, myxospores are more likely to be distributed over a broader area than TAMs. Indeed, other researchers have speculated that myxozoan colonization on a landscape would most likely occur via myxospores (Cone et al., 2006).

Three main pathways for introduction of *M. cerebralis* into the WRB were identified: movement of fish by humans, natural dispersal, and recreational activities (Figure 5.2).

**Human Movement of Fish**

Historically, the spread of *M. cerebralis* in the USA is thought to be a consequence of shipments of infected fish, both commercially and as a result of
state and federal stocking programs (Bartholomew and Reno, 2002). All import, export, or transfers of live fish in Oregon require a Fish Transport Permit (Oregon Administrative Rules [OAR] 635-007-0600 and 635-007-0615) that entails an annual health examination of both the broodstock and of the lot of fish that are to be imported (OAR 635-007-0585 and 635-007-0990). Currently, permits are not granted if the exporting facility has a history of *M. cerebralis* infection, even if the importing area is enzootic for the parasite. All state (6), federal (1), and private (6) trout rearing facilities in the WRB are monitored annually for the parasite (Figure 5.1).

When buying live fish in Oregon, it is the buyer’s responsibility to know the rules and regulations surrounding fish transfers and required permits. Buyers are often unaware of required permits (T. Amandi, ODFW, personal communication), which may result in illegal (even if inadvertent) movements of fish that cannot be traced. Because rules and regulations can be lengthy and ambiguous, and because businesses want to ensure sales, most licensed fish sellers in Oregon acquire ODFW fish transport permits for prospective buyers and often explain regulations and procedures. While there are six facilities licensed to sell fish in the WRB, the number of private ponds that contain fish is more difficult to estimate. Only 30 permits are on record to legally impound water and hold fish in the WRB (Oregon Water Resources, 2006), yet transport records from a single private facility show that greater than 400 sites in the WRB received fish, demonstrating that numbers of private ponds stocked with salmonids is far higher. Hobby ponds and U-Catch facilities are examples of private ponds where fish may be reared and resold or transferred by owners who may be unaware of the need for fish transport permits.

Even legal transports of fish present a risk. For example, although establishment of *M. cerebralis* at the private facility on Clear Creek was contained, the facility legally transferred potentially infected fish throughout the state of Oregon before detection of the parasite occurred. Over 760 shipments to 430 different locations in the WRB were received between 1999-2000 alone (N. Hurtado, ODFW, personal communication). Three of the facilities in the WRB that
received the largest number of fish have been tested for establishment of *M. cerebralis* and were negative (authors’ unpublished data). Additionally, some of the buyers that received shipments had recirculating facilities or no-outflow ponds that would limit the spread of the parasite into surrounding water bodies.

There are no laws governing the movement of frozen or fresh-killed fish (for bait, food, or fish meal markets) in Oregon. As myxospores can survive freezing (El-Matbouli and Hoffmann, 1991), this is one of the ways in which *M. cerebralis* is thought to have been introduced into the USA (Hoffman, 1962). However, disease resulting from this initial shipment of frozen fish was likely a consequence of the use of the imports as food for hatchery fish. Hatchery feeding practices have changed considerably since the 1950’s and the likelihood that infected fish would be used for this purpose is low. Similarly, the likelihood of introduction as a result of release of infected baitfish is also low as salmonids are not a legal baitfish species.

Movement of fish carcasses for stream enrichment is another potential route for introducing myxospores. Although this practice is “restricted to the originating river basin” (OAR 635-007-1000), fish of the same broodstock, but of different rearing basins can be transferred among basins.

*Assessment of risk*

Human movement of fish represents a high risk for parasite introduction for the following reasons: 1) the regulations pertaining to live fish transport are ambiguous and are not easily enforced or widely known; 2) introduction (though locally contained) has already occurred; 3) shipments of infected fish have already been made in the WRB; 4) shipments of infected fish could introduce a large number of myxospores; and 5) historically, many fish pathogens and non-native species introductions have been a result of human movement of organisms.

*Dispersal via Recreation*

Willamette River tributaries have sport fisheries that attract anglers from across the state and region; many of these anglers use equipment that has been used
in other river systems. In a laboratory study, felt-soled waders were demonstrated to transport myxospores and TAMs (P. Reno, Oregon State University, personal communication). However, after drying waders for 8-24 h, spores became less viable, and after drying for 7 d, spores were no longer able to cause infection in appropriate hosts. These findings support other studies that suggest that myxospores do not remain viable after drying for 24 hr (R. Hedrick, University of California at Davis, personal communication). Myxospores trapped in the moisture of a felt-sole would likely remain wet and thus viable for longer than in a less absorbent material. Prior data on myxospore viability after drying (Schäuperclaus, 1931; Hoffman and O’Grodnick, 1977) were collected before the determination of the *M. cerebralis* life cycle and are anecdotal at best. Time required to dry and thus disinfect waders will vary with environmental conditions. Because of their fragility, it is unlikely that TAM stages could be transported large distances by this route. However, infected *T. tubifex* adhered to a felt sole provide a suitable environment for TAMs to remain viable.

While the probability of transporting *M. cerebralis* in a single event (i.e.: a single angler or single angling day) by recreational activities may be low, when all angler days in a year are considered, the probability increases. The Middle Fork of the Willamette River and South Santiam River saw 22,501 and 17,937 anglers respectively in 1975 (Moring, 1985). These areas, along with the Clackamas River (the closest major tributary and sport fishery to Portland) would be most likely to experience introduction of the parasite via recreational activity. While the number of anglers traveling between the enzootic area and the WRB is unknown, anglers from Portland [the area with the greatest number of anglers (US Department of Interior, 2001)] could recreate in NE Oregon (a renowned recreational destination) and return home to use gear in local waterways.

Boat bilge and engine cooling water represent another route by which recreational activities could introduce *M. cerebralis*. TAMs would be more likely than myxospores to be suspended in the water column and be retained in bilge and engine cooling water. Survival of TAM stages is temperature dependent, with
spores surviving for 5-7 d at 7°C and only 2 d at 20°C (Markiw, 1992b). Thus, TAMS are unlikely to survive multiple days of transport or high summer temperatures that would alter the condition of small amounts of bilge water. However, water caught in the covered intake of the engine cooling system does not experience elevated temperatures and has been shown to transport live larval zebra mussels (Johnson et al., 2001). While the volume of spores transported per boat is likely to be low, when considering all boats recreating in a year, the potential for *M. cerebralis* transfer increases. In 2005, 3,380 boats registered in the WRB were used in the enzootic area (Oregon State Marine Board, 2005). Likewise, 330 boats registered in the enzootic area were used in the WRB in 2002 (Oregon State Marine Board, 2002).

**Assessment of risk**

A conservative estimate of recreational activities as a pathway for transfer of *M. cerebralis* to the WRB is that it represents a moderate risk. Numbers of spores introduced in a single angling or boating event are expected to be low, but the cumulative and long-term effects of angler and recreational activities in heavy-use areas would be much greater. Recreational activity is also one of the few pathways where management steps can be taken to reduce the risk of introduction. Management considerations are discussed in the conclusions of this paper.

**Natural dispersal: birds**

The possibility of *M. cerebralis* dissemination by birds has been considered in several studies (Taylor and Lott, 1978; El-Matbouli & Hoffmann, 1991). However, none of these studies have aligned the numerous events that must occur for *M. cerebralis* to be spread long distances via bird transport. Below, we outline the chain of events that must occur to result in introduction.

1) **Bird eats infected fish.**

The CRB estuary houses the largest colony of Caspian terns (*Sterna caspia*) in North America and the second largest colony of double-crested cormorants (*Phalacrocorax auritus*) - 11,223 and 7,501 birds respectively in 1998 (Collis et al.,
A smaller Caspian tern colony (990 birds in 1997) is located at Crescent Island, just below the confluence of the Snake and Columbia Rivers and near the enzootic area. Birds from these colonies eat juvenile salmonids as their primary food source, with steelhead smolts being the most vulnerable to predation (Collis et al., 2001). This species is also among the most susceptible to *M. cerebralis* (MacConnell and Vincent, 2002).

While prevalence of *M. cerebralis* infection in juvenile salmonids in the CRB is unknown, the proportion of infected fish is higher in the enzootic area than in the estuary. However, the estuary houses larger piscivorous bird colonies and timing of seasonal dispersal from these colonies coincides with peak migration of highly susceptible juvenile salmonids. Thus, birds from both the enzootic area and the estuary represent a risk of *M. cerebralis* dispersal. In addition to the Caspian tern and double-crested cormorant colonies, numerous other piscivorous bird species inhabit the CRB, and may also present risks for *M. cerebralis* dispersal.

2) **Myxospores survive passage through gut of piscivorous bird.**

*M. cerebralis* myxospores have been demonstrated to survive passage through the guts of piscivorous birds and fish (El-Matbouli & Hoffmann, 1991).

3) **Bird enters WRB.**

Banding data on the dispersal of birds from particular breeding colonies are limited, thus flight paths mentioned here are speculative based on expert opinion (D. Roby, USGS-Oregon Cooperative Fish and Wildlife Research Unit, personal communication). Caspian terns at Crescent Island have been banded and resighted 1-2 days later in the Columbia River estuary. Although these birds do not nest in the WRB, they do use foraging sites in the WRB during spring and fall migration and terns from breeding colonies in eastern Washington may use this route post-nesting. Immature and sub-adult eagles undertake seasonal movements in search of foraging areas and could disperse from NE Oregon to the WRB. Migratory paths of ospreys after the breeding season could take the birds west following the Columbia River and south through the WRB.
4) Bird retains infected fish in gut for flight to WRB.

As *M. cerebralis* manifests in cartilage of fish, it is likely that birds would egest, or regurgitate, the parasite in pellets. Double-crested cormorants have a simple gut structure and egest bones, pieces of fish, and solid markers 1-2 days following ingestion (Brugger, 1993). In contrast, the passage time of rainbow trout marked with trace minerals through the guts of bald eagles (which have a more complex gut morphology) is approximately 62 hours (F. Barrows, USFWS, personal communication). Thus spores could be excreted 2-3 d after eating an infected fish.

Raptors and large waterbirds have the fastest known migration speeds among birds (Kerlinger, 1995). Migrating bald eagles travel 201 km/day (Kerlinger, 1995), and ospreys 108-431 km/day (Alerstam, 2003; Hake et al., 2001). As the nearest enzootic area is approximately 450-500 km from the WRB, an osprey would have to retain food material for 1-4.2 days and bald eagles for 2.2-2.5 days to transport spores to the WRB. Thus transport of spores from the enzootic area to the WRB is possible.

5) Bird releases viable spores over water body.

The probability of such an occurrence is unknown, and is likely to vary by habits and behaviors of species. For example, waterbirds (waders, waterfowl, gulls, etc.) characteristically spend more time over water than raptors. Although the probability of a bird releasing viable myxospores over a waterbody remains unknown, deposition near (and not in) water may be sufficient for transport of the parasite to a new location under certain environmental conditions. Anecdotal evidence suggests that myxospores can withstand drying for long periods of time (Schäuperclaus, 1931), but more current studies indicate myxospores do not remain viable after drying for 24 hr (R. Hedrick, University of California at Davis, personal communication). It is unknown how long myxospores deposited in bird feces would remain moist, and thus viable. Time required for inactivation of spores is likely to vary by environmental conditions. Thus, if spores are deposited near a
waterbody, and are rapidly washed into the river by high water or precipitation, dissemination of the parasite could occur.

Assessment of risk

Although the likelihood of parasite introduction by a single bird may be low, if the event were to occur, a considerable number of spores could be released because many piscivores swallow their prey whole. As many as $1.7 \times 10^6$ myxospores have been reported from experimentally challenged rainbow trout, though such numbers are variable (Markiw, 1992b; Sollid et al., 2002). Therefore, even a single introduction could have large consequences. Still, numerous events must align in order for birds to transport *M. cerebralis* long distances, thus the probability of birds transporting the pathogen to the WRB is low. The cumulative risks when considering the large numbers of birds traveling through the WRB in a year would be much higher.

Data gaps identified in this part of the release assessment are the infection rate of salmonids migrating from both the Snake River basin and the Columbia River estuary, and time required to disinfect myxospores in guano or egested pellets.

Natural dispersal: stray anadromous salmonids

Anadromous salmonids may stray into non-natal streams during their return migration to spawning grounds. An often-overlooked effect of straying is the potential for pathogen dissemination. Introduction of *M. cerebralis* as a result of straying salmonids has been documented in the Deschutes River, Oregon (mid CRB) (Engelking, 2002). Between 1983 and 2005, 32-92% of adult hatchery steelhead collected annually during peak migration were strays (J. Seals, ODFW, unpublished data). Examination of over 450 stray adult steelhead between 1997 and 2000 showed that at least 5% were infected with *M. cerebralis* (Engelking, 2002), with unconfirmed but suspected detections in an additional 15% of the fish. Infection prevalence in stray Chinook salmon was lower (1% confirmed; 7% presumptive).
Stray fish are typically documented by examination of fin clips or evidence of a coded wire tag (CWT). As any single fin-clip pattern is commonly used by more than one hatchery in the Pacific Northwest in a given year, fin clips reveal only the “stray” status of a fish and a rough list of potential origins. A CWT or fin clip unique to a certain hatchery would be required for identification of exact hatchery of origin.

Very little data (current or historical) are available on the stray rates of cultured or wild salmonids in the WRB. A search of recovery data for the WRB through the Regional Mark Information System (RMIS) database, shows only 16 recoveries of stray Chinook salmon between 1977-2003 (RMIS, 2006). Four of the hatcheries of origin of these fish, representing 81% of the historical strays, are within the enzootic area of the Snake River basin.

As the frequency of adult salmonids straying into the WRB was identified as a significant data gap for this risk assessment, we conducted a study to investigate the frequency and prevalence of infection of straying salmonids in the WRB.

*Methods for investigation of frequency and infection of stray fish in the WRB*

From 2004 to 2005, ODFW personnel at all adult salmonid collection facilities in the WRB (Figure 5.1) monitored returning adults for marks or tags which would indicate they were strays from other river basins. Traps were located on the McKenzie, Clackamas, Middle Fork Willamette, and North and South Santiam Rivers.

Collected fish were killed, checked for coded-wire tags, the fins or maxillary marks recorded, and the heads removed and frozen for *M. cerebralis* testing. The RMIS and ODFW databases were queried for the origin of salmonids with marks corresponding to those of the stray fish collected. Additional efforts were made by the National Marine Fisheries Service (NMFS, Conservation Biology Division, DNA Laboratory, Seattle, WA) to genetically identify these fish using microsatellite markers.
To determine \textit{M. cerebralis} infection in strays, core samples were taken from the cranium using an electric hand drill with a 2.5 cm diameter coring-bit. Cores were maintained individually and processed by pepsin-trypsin digest (PTD) (USFWS and AFS-FHS, 2003). The defleshed cores were blended with a commercial Waring blender for 30 seconds to accelerate digestion in pepsin solution. Because the samples were stored frozen until processing, 0.05% trypsin in Rinaldini’s saline solution was used during the trypsin digestion phase. The final pellet of digest was suspended in 1 ml of phosphate buffered saline or 70% ethanol and observed microscopically for myxospores by examining wet mounts of undiluted and diluted (1:5 and 1:10) digest at 250X and 400X magnification. In 2004, the PTD product was tested by the PCR assay using protocols developed by Andree et al. (1998). In 2005, a quantitative version of this assay (Kelly et al., 2004) was used to increase sensitivity.

\textit{Results and discussion of stray fish studies in the WRB}

In contrast to the low number of reported stray salmonids from 1977-2003, 129 adult summer steelhead with fin clips identifying them as strays were recovered at traps in the WRB during 2004-2005. Table 5.1 lists the locations, number and percent strays found and the different marks observed on these fish. Most of the strays were collected in the Santiam River subbasin (97%), possibly because the area releases more steelhead than other WRB tributaries (ODFW, unpublished data). If strays follow the masses of fish migrating upriver, they would be more likely arrive in the Santiam River subbasin. Also, mis-marked fish (i.e. not strays) would be more probable in an area where more fish are clipped. No stray fish were obtained from traps on either the Clackamas or Middle Fork Willamette Rivers.

Fin marks recorded from WRB strays were used by a number of hatcheries releasing summer steelhead in the Pacific Northwest during 1999-2002 (RMIS, 2006; ODFW, Fish Liberation Database, unpublished data). Of the 19 facilities, seven (37\%) are in the \textit{M. cerebralis} enzootic area (Table 5.1). Interestingly, ADLVRV (adipose, left ventral, right ventral), one of the most prominent markings
(31%), was not recorded in RMIS or any of the federal, state, or regional databases from Oregon or surrounding states. Efforts to genetically identify the WRB strays did not reveal a hatchery of origin (Melanie Paquin, NMFS, personal communication); however, the high level of genetic differentiation between the WRB strays and Snake River basin hatchery summer steelhead indicate that these fish likely originated outside the enzootic region.

Stray Chinook salmon were not encountered in this study, which could be explained by a decreased rate of straying compared with steelhead, or by the lack of distinctive marks. Chinook salmon rarely receive marks other than an adipose fin clip and determination of hatchery of origin would require a CWT. However, Chinook salmon also represent a lower risk for introduction because of their decreased susceptibility to the parasite (Sollid et al., 2003).

Overall, numbers of strays collected in the WRB in 2004-2005, while lower than that of mid CRB tributaries (ODFW, unpublished data), are far higher than indicated by database records. It is likely that this increase in number can, at least in part, be attributed to a concerted effort by ODFW to train personnel to recognize stray fish. If similar efforts had been made in previous years a different pattern of straying may have emerged. It is also possible that the years 2004 and 2005 were abnormal. As there are few historical data, we cannot determine a “normal” stray rate per year. However, stray rates of 0-20% are not uncommon for hatchery salmonids (Quinn, 1997). Actual numbers of strays in the WRB are likely to be higher than seen in this study as all stray wild fish as well as unmarked or single-marked (only adipose clip) hatchery fish were not recorded as out of basin fish. Another source of error in estimating straying frequency is that collections occurred only at fish traps; fish spawning in the river and tributaries were not counted. It is also possible that some stray fish were actually mis-marked fish from WRB hatcheries and were indeed not strays.

Spores of a *Myxobolus* spp. were detected in 3 fish. All samples were negative by PCR, suggesting that the spores detected were of another *Myxobolus* species.
Assessment of risk

Although the straying frequency for steelhead was higher than expected, none of these fish had detectable *M. cerebralis* infection and therefore are not likely to have originated from the enzootic area. If we assume this stray rate and origin is representative of strays in other years, the likelihood of introducing *M. cerebralis* as a result of stray adult salmonids is low. However, if it were to occur, its probability is higher in the Santiam River subbasin as the majority of stray fish were recovered in this area.

Summary of the release assessment

The probabilities described in this assessment are defined as: high – the event would be expected to occur, moderate – less than an even chance of the event occurring, low - the event is unlikely to occur, and negligible – the chance of the event occurring is so small that it can be ignored. The probability of introduction of *M. cerebralis* into the WRB is summarized as:

- Dispersal via human movement of fish: high
- Dispersal via recreation: moderate
- Dispersal via natural dispersal (birds and stray anadromous salmonids): low

Overall probability of introduction: moderate (though long-term cumulative probability is higher.)

Areas with the highest probability for *M. cerebralis* introduction are the Clackamas and Santiam River subbasins. The Clackamas River has already experienced an introduction of the parasite, has the largest concentration of hatcheries (state, federal, and private), has a popular sport fishery, and is the closest major tributary to the enormous piscivorous bird populations in the Columbia River estuary. The Santiam subbasin has a popular sport fishery, received the highest number of stray fish in the WRB, and has the second largest concentration of hatcheries in the WRB.
EXPOSURE ASSESSMENT

The exposure assessment, which explores the risk of parasite establishment, is focused on the TAM stage of *M. cerebralis* and its oligochaete host. Establishment of *M. cerebralis* is dependent upon several environmental and biological factors including: water temperatures, spatial temporal overlap of hosts, and the distribution and genetic composition of *T. tubifex* populations. Each of these factors determines the outcome of the exposure assessment, as depicted by the scenario tree in Figure 5.3.

**Habitat for *T. tubifex***

Habitat for *T. tubifex* is defined as areas with fine sediment, low flow, and organic matter (Brinkhurst and Gelder, 1991). These areas were found throughout the WRB.

**Sustainable populations of susceptible *T. tubifex***

Bartholomew et al. (2007) documented only a single specimen of *T. tubifex* in a survey of Clear Creek, Clackamas River subbasin. In contrast, a private hatchery adjoining the creek had dense *T. tubifex* populations. A literature review revealed little other data regarding *T. tubifex* populations in the WRB. The prevalence and susceptibility of *T. tubifex* was identified as a significant data gap in the risk analysis and a qualitative survey for *T. tubifex* was conducted with subsequent characterization of specimens to determine mitochondrial lineage and susceptibility.

**Tubifex tubifex survey methods**

A qualitative survey was used to determine relative abundance of *T. tubifex* in selected streams in the study area. Areas most likely to contain the organisms were targeted for sampling, i.e. those with: low flow, fine sediments, and accumulations of organic material. Thirty-three sample sites with these attributes as well as with temperatures appropriate for parasite propagation, populations of
salmonids susceptible to the parasite, a large sport fishery, documented stray fish, and accessibility by road were chosen (Figure 5.4). Ten sites were located in the mainstem Willamette River, 12 in the South Santiam River, four in the North Santiam River and seven in the McKenzie River. The Clackamas River has previously been surveyed (Bartholomew et al., 2007) and no further collections were made in that system.

Oligochaetes in sediment were collected by 5 gal bucket and 500 µm sieve or by 500 µm kicknet, maintained, sorted, and presumptively characterized as *T. tubifex* following methods described in Arsan et al. (chapter 2 of this volume). A subsample of 20 worms presumed to be *T. tubifex* were then genetically analyzed to confirm identity as *T. tubifex* (Hallett et al., 2005) and to determine lineage (Sturmbauer et al., 1999; Beauchamp et al., 2001). In preparation for genetic analysis, worms were by digested with 95 µL ATL buffer (QIAGEN) and 5 µL Proteinase K at 55°C, boiled for 5 minutes, and diluted 1:101 with buffer AE (QIAGEN) then stored frozen.

To determine whether worms genetically identified as *T. tubifex* (in a species-specific PCR) but which did not visibly amplify in the lineage assay (lineage-specific PCR) were a novel lineage or a different species, a portion of their mitochondrial 16S rDNA was sequenced. Mitochondrial 16S DNA was amplified with either the primers Tub16SF and Tub16SR (Beauchamp et al., 2001) or 16sar and 16sbr (Sturmbauer et al., 1999) using reaction conditions described by Arsan et al. (chapter 2 of this volume).

Water in which samples were transported was retained and passed through a 20 µm mesh filter and inspected microscopically to determine if actinospores were present. Actinospores were identified to group and species, when possible.

Results of *T. tubifex* survey

Over 5,300 oligochaetes were collected from 33 sites, with *Tubifex tubifex* identified from 19 sites (Figure 5.4) and accounting for approximately 10% of the total oligochaetes collected (Table 5.2). Other oligochaetes morphologically identified from the WRB include: *Tubifex ignotus, Limnodrilus hoffmeisteri,*
Lumbriculus varigatus, Kincaidianha hexatheca, Aulodrilus pluriseta, Dero digitata, Spirosperma nikolski, Ophidonais serpentina, Chaetogaster limnaei, and Pristonella jenkinae.

The largest numbers of *T. tubifex* were observed from the South Santiam and McKenzie Rivers, particularly at sites near Foster (RM 36.5) and Leaburg (RM 29.4) Dams, and at Hendrick Boat Landing on the McKenzie River. These sites receive heavy recreational use and had areas with deep sediments and organic enrichment (due to both decaying salmonid carcasses and overhanging vegetation).

While this study presents some of the few data on WRB *T. tubifex* populations, our surveys were qualitative and limited by access. Accessibility to sample sites on the North Santiam River was restricted to areas accessible by road. As only four of these sites had appropriate *T. tubifex* habitat, data from that river are limited.

Susceptibility of *T. tubifex* to *M. cerebralis* was inferred from results of the lineage analysis. Different lineages vary in susceptibility. Populations from lineages I and III have been shown to be susceptible to the parasite while lineages IV, V and VI are considered resistant (Beauchamp et al., 2002, 2005, & 2006; DuBey et al., 2005; Arsan et al., chapter 2 of this volume). All five *T. tubifex* lineages reported from North America were found in the WRB (Table 5.2), including lineage IV, which had previously been described only from Europe and Alaska (Sturmbauer et al., 1999; Beauchamp et al., 2001; Arsan et al., chapter 2 of this volume).

The presence of resistant lineages IV, V, and VI *T. tubifex* could have significant beneficial implications for WRB salmonids in areas where these lineages dominate *Tubifex* populations. Together, these lineages comprised approximately 88% of the *T. tubifex* population in the mainstem Willamette River and 24-54% in river tributaries. Other researchers have found lineage susceptibility to correspond to severity of infection in fish (Beauchamp et al., 2006). This suggests that when lineages IV, V, and VI are in high abundance, the effects of *M.*
cerebralis may be diminished if the parasite were introduced and established in the WRB.

Lineage III is the most susceptible to M. cerebralis (Beachamp et al., 2002; Dubey et al., 2005; Hallett and Bartholomew, 2007; Arsan et al., chapter 2 of this volume) and its detection in the WRB is cause for concern. This lineage comprised approximately 12-56% of worm populations, with the greatest numbers in the North Santiam River and fewest in the mainstem of the Willamette River (Table 5.2). Even in proportions as low as 2% of the total population, lineage III T. tubifex become infected with M. cerebralis and release TAMs (Beachamp et al., 2006; Hallett and Bartholomew, 2007; Arsan et al., chapter 2 of this volume). Populations of T. tubifex from the North Santiam, McKenzie and Middle Fork Willamette Rivers that have been experimentally exposed to the parasite (Hallett and Bartholomew, 2007) were moderate TAM producers, releasing up to 48,960 in 4 d when exposed to 500 myxospores and held at 12.8°C.

No M. cerebralis TAMs were observed in filtrates from any of the sites sampled. However, actinospores of other myxozoans were observed and were genetically identified as Myxobilatus gasterostei and other Myxobolus spp. (Stephen Atkinson, Center for Fish Disease Research, unpublished data). Other unidentified triactinomyxon, aurantiactinomyxon, siedleckiella, echinactinomyxon and raabeia type actinospores were also found. Several of these unidentified actinospores were released from T. tubifex hosts. The detection of other myxozoans validates our methodology was sufficient for detecting M. cerebralis TAMs. This also confirms that other myxozoan life cycles, some with documented T. tubifex hosts, are established in the WRB.

**Water temperature seasonally reaching 10-15 °C**

Water temperature influences parasite development in both hosts (Hedrick and El-Matbouli, 2002; Blazer et al., 2003). In the oligochaete, 10 to 15°C has been demonstrated to be optimal for development and release of the TAM stage of the parasite (El-Matbouli et al., 1999). Temperatures below 10°C delay the
development of TAMs and those above 20°C hinder parasite development and can be lethal to the TAM stage of *M. cerebralis*. Highest prevalence of infection in fish occur when average daily water temperatures are between 11-14°C (Baldwin et al., 2000; Granath and Gilbert, 2002).

Because continuous water quality monitoring is a relatively new phenomenon, temperature data for certain rivers are limited to only a few years. For WRB sites where temperature data were available, average summer (May-September) water temperatures ranged from 11.5-16.5°C between 2001-2005 (USGS, 2006), with an average of 142-204 d/yr continuously >10°C.

In major tributaries, temperatures are within the optimum range for parasite development from approximately May to October. The mainstem Willamette River has higher water temperatures and could temporally support parasite development for over half the year. However, there are locations where temperatures may be too warm for TAM development and survival during some parts of the summer.

**Spatial/ temporal overlap of hosts**

For the parasite to establish after introduction of myxospores, obligatory spatial overlap of myxospores and *T. tubifex*, with subsequent spatial overlap of salmonids and TAMs would be required.

If an introduction of myxospores occurred, the spores could remain viable in river sediments for up to five months (El-Matbouli and Hoffmann, 1991), with anecdotal evidence suggesting they may survive up to 30 years (Halliday, 1976). Furthermore, worms can remain persistently infected throughout their lifespan, which can be at least 3 yr (Gilbert and Granath, 2001, 2003) with TAM release occurring when water temperatures are appropriate. Depending on the temperature of the water when myxospores are introduced, this cycle could be prolonged in the winter months or require the minimal 80-90 days in the summer or early fall months. However, the number of TAMs to which fish would be exposed would be diluted during the winter by seasonal high water flows typical of western Oregon
streams. Thus peak infections (in both fish and oligochaetes) would likely occur seasonally in response to temperature and water flows.

The majority of young salmonids in the WRB emerge April to October, coinciding with the period when temperatures are conducive for complete parasite life cycle proliferation. Younger fish are more susceptible to the parasite, with rainbow trout obtaining some resistance at 9 weeks post-hatch (Ryce et al., 2004). Chinook salmon require less time to become resistant to the pathogen (0-3 weeks post-hatch) (Sollid et al., 2003). Thus, the progeny of late fall spawners that emerge once water temperatures have cooled and flows have increased could have a lower risk of infection of *M. cerebralis* by avoiding peak TAM release periods earlier in the season.

**Summary of the exposure assessment**

The probability of establishment of *M. cerebralis* in certain locations in the WRB is high, as all of the following exist:

- Habitat for *T. tubifex*
- Populations of susceptible *T. tubifex*
- Seasonal water temperatures of 10-15°C
- Spatial temporal overlap of hosts

If introduced, conditions are appropriate for the parasite life cycle to proliferate. Tributaries to the mainstem Willamette River have the highest probability of establishment as these areas also have the greatest numbers of susceptible *T. tubifex*. However, the large numbers of resistant strains of *T. tubifex* could mitigate the effects of *M. cerebralis* if introduced.

**CONCLUSIONS AND RISK MANAGEMENT**

Human movement of fish is the most likely route for introduction of *M. cerebralis* into the WRB. The ODFW has taken steps to decrease this risk by
increasing the frequency of inspections of fish transferred from public and private facilities and by working cooperatively with fish sellers regarding fish transfer regulations and permit acquisition. However, although rules, regulations and policies exist, they are not well known to the general public in regards to transfers of live salmonids. These could be articulated more clearly and made more prominent by emphasizing policies to the buyers of fish (mostly private pond owners) in the form of a brochure or website. Additionally, efforts should be made to restrict carcass planting for stream enrichment to streams where fish were reared, regardless of broodstock. Alternatively, carcasses could be de-headed and frozen individually, with outplanting occurring after fish health testing has been conducted. This method is already in place at several hatcheries in the Pacific Northwest (S. Gutenberger, USFWS, personal communication).

Recreation and angler activity was evaluated as the second most likely route for introduction of *M. cerebralis* into the WRB. This potential pathway of *M. cerebralis* introduction is another point where management steps can be taken to reduce the risk of introduction. The state should allot resources to angler education and awareness of the effects of recreation and angler activity on dispersal of *M. cerebralis* and other invasive species. This could be done using a combination of brochures and signage at boat ramps and other river access points, describing how to prevent spread of aquatic nuisance species.

The role of infected anadromous fish in disseminating *M. cerebralis* was difficult to evaluate because stray fish have not always been identified at fish traps, marks are commonly used by multiple hatcheries in the CRB and databases within the CRB are not coordinated. While a designated clip used uniquely by enzootic-area hatcheries would allow more thorough investigations of stray fish and their potential as pathogen dispersal agents, the practice may not be feasible. Marks are usually used to differentiate lots within a hatchery, not a geographic area. The use of a CWT would alleviate the need for a distinctive clip, but would require more time and resources. Additionally, results of this study suggest that stray fish are not routinely identified and thus historic stray rates in the WRB have been
underestimated. To improve detection of strays, fish trap handlers should be instructed on how to identify fish with markings indicating they are from out of basin, and to remove these fish from the population. Lastly, efforts should be made to coordinate databases within the CRB, so fish release and recovery data are compiled in one easily accessible database.

In contrast to introduction, few steps can be taken to reduce the probability of establishment of *M. cerebralis*, as measures for eliminating its oligochaete host are impractical or have proven unsuccessful (Wagner, 2002). Yet the presence of resistant lineages of *T. tubifex* may help to diminish negative effects of *M. cerebralis* in areas where these strains dominate, such as the mainstem of the Willamette River. However, data on the distribution of *T. tubifex* is limited, and qualitative oligochaete surveys in this study were narrow in scope.

Similarly, little can be done once parasite establishment has occurred; there are no vaccines or effective treatments against *M. cerebralis*. This makes the management of potential parasite introduction more imperative. Steps to control the pathogen should start by focusing resources on areas where probability of introduction and establishment are greatest: the Santiam River subbasin. This subbasin has the second largest concentration of hatcheries (state and private) in the WRB, a popular sport fishery, close proximity to the enormous piscivorous bird populations in the Columbia River estuary, the highest number of documented stray fish in the WRB, and the greatest numbers of susceptible *T. tubifex*. The Clackamas River subbasin also has a high probability of introduction of the parasite (already experienced an introduction, has the largest concentration of hatcheries in the WRB and the closest proximity to piscivorous bird populations in the Columbia River estuary). Yet, the probability of parasite establishment in the area appears low (limited oligochaete surveys in the subbasin indicate few *T. tubifex* inhabit the tributary where introduction previously occurred) (Bartholomew et al., 2007).

Probabilities of parasite introduction and establishment discussed in this paper may vary with changes to physical or environmental conditions, such as climate change or land-use modifications. Land-use practices that enhance *T.
tubifex habitat by increasing sedimentation or organic enrichment or by decreasing flow may increase the probability of *M. cerebralis* establishment.

**ACKNOWLEDGEMENTS**

This work was supported in part by the National Partnership for the Management of Wild and Coldwater Species, the United States Fish and Wildlife Service, the United States Geological Survey, and the Oregon Department of Fish and Wildlife (ODFW). We are grateful to the following members of the Oregon State University - Center for Fish Disease Research: Sascha Hallett and Stephen Atkinson for critical consultation on oligochaete sampling and genetic assays, Rich Holt for assays of stray fish, Don Stevens for assistance with worm identification, Harriet Lorz for her efforts in sampling and maintaining oligochaete cultures, Lindsay Osborne and Richard Stocking for help in the field. We also thank a number of ODFW personnel for their assistance: hatchery personnel for collecting stray fish, Ken Kenaston and crew for assistance in the field, Mark Lewis and Nadine Hurtado for help using agency databases, and Tony Amandi for providing essential consultation on study design and background. Genetic work on stray steelhead was done by Paul Moran and Melanie Paquin of NMFS. We acknowledge Paul Reno and Carl Schreck of Oregon State University, Department of Fisheries and Wildlife for providing critical comments on this manuscript.
REFERENCES


Colonial waterbird predation on juvenile salmonids tagged with integrated transponders in the Columbia River estuary: vulnerability of different salmonid species, stocks, and rearing types. Transactions of the American Fisheries Society 130: 385-396.


Fish Passage Center. 2006. Database of adult salmonid returns.


Whirling disease: reviews and current topics. American Fisheries Society Symposium 29, Bethesda, Maryland.


RMIS (Regional Mark Information System). 2006. Pacific States Marine Fisheries


Table 5.1. Summary of adult summer steelhead strays found at Oregon Department of Fish and Wildlife adult collection facilities in the Willamette River basin in 2004 and 2005.

<table>
<thead>
<tr>
<th>Observed marks* (number of fish)</th>
<th>Recovery river (number of fish)</th>
<th>Total collected</th>
<th>Percent of total strays collected</th>
<th>Potential Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADLM (2)</td>
<td>Mckenzie (2)</td>
<td>2</td>
<td>1.5%</td>
<td>Seymour R, BC; Chehalis R, BC; Deschutes R; Umpqua R N Fk</td>
</tr>
<tr>
<td>ADRM (2)</td>
<td>Mckenzie (2)</td>
<td>2</td>
<td>1.5%</td>
<td>Chehalis R, BC; Capilano R, BC; Hood R E Fk; Deschutes R</td>
</tr>
<tr>
<td>ADLV (45)</td>
<td>S Santiam (36), N Santiam† (6), N Santiam M‡ (3)</td>
<td>45</td>
<td>35%</td>
<td>Wallowa R; Umatilla R; Clearwater R; Grande Ronde R; Salmon R; Pahsimeroi R; Cowlitz R; Lewis R; Big Sheep Cr; Big Canyon Hatchery</td>
</tr>
<tr>
<td>ADRV (27)</td>
<td>S Santiam (23), N Santiam† (4)</td>
<td>27</td>
<td>21%</td>
<td>Columbia R; Wilson R; Cedar Cr; Deschutes R</td>
</tr>
<tr>
<td>ADLVVRV (45)</td>
<td>S Santiam (26), N Santiam† (1), N Santiam M‡ (18)</td>
<td>45</td>
<td>35%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Other marks/tags (8)</td>
<td>S Santiam (8)</td>
<td>8</td>
<td>6%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Total</td>
<td>n/a</td>
<td>129</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

* ADLVVRV=adipose left and right ventral clip; ADLV=adipose and left ventral clip; ADRV=adipose and right ventral clip; ADLM=adipose and left maxillary clips; ADRM=adipose and right maxillary clips.
† NS = North Santiam River at Lower Bennett Trap
‡ NSM = North Santiam River at Minto Pond.

Bold letters connote facilities in the *Myxobolus cerebralis* enzootic area.
Table 5.2. *Tubifex tubifex* survey sites and corresponding abundance and mitochondrial lineages.

<table>
<thead>
<tr>
<th>River</th>
<th>Sites sampled</th>
<th>Sites with <em>Tubifex tubifex</em></th>
<th>No. total worms*</th>
<th>No. <em>Tubifex tubifex</em>†</th>
<th>Worms assayed</th>
<th>Worms sequenced</th>
<th>Lineages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mainstem Willamette</td>
<td>10</td>
<td>3</td>
<td>414</td>
<td>8</td>
<td>27</td>
<td>4</td>
<td>III (12%), VI (88%)</td>
</tr>
<tr>
<td>North Santiam</td>
<td>4</td>
<td>2</td>
<td>462</td>
<td>53</td>
<td>45</td>
<td>0</td>
<td>I (20%), III (56%), IV (12%), V (8%), VI (4%)</td>
</tr>
<tr>
<td>South Santiam</td>
<td>12</td>
<td>8</td>
<td>2532</td>
<td>235</td>
<td>132</td>
<td>2</td>
<td>I (13%), III (45%), V (1%), VI (41%)</td>
</tr>
<tr>
<td>McKenzie</td>
<td>7</td>
<td>6</td>
<td>1939</td>
<td>242</td>
<td>126</td>
<td>1</td>
<td>I (3%), III (43%), IV (2%), VI (52%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>33</strong></td>
<td><strong>19</strong></td>
<td><strong>5347</strong></td>
<td><strong>538</strong></td>
<td><strong>330</strong></td>
<td><strong>7</strong></td>
<td><strong>I, III, IV, V, VI</strong></td>
</tr>
</tbody>
</table>

* Total worms; 3 subsamples of 30 ml each per sample processed.
† Total *Tubifex tubifex*; 3 subsamples of 30 m each per sample processed. Number reflects total number morphologically identified as *T. tubifex* subtracted by the proportion of false positives (worms genetically identified as another species).
Figure Legend

**Figure 5.1.** Columbia River basin with Willamette River basin enlarged and *Myxobolus cerebralis* enzootic area depicted by dashed circle. Locations of fish hatcheries and collection sites of adult stray salmonids in the Willamette River basin are noted.

**Figure 5.2.** Pathways of potential introduction of *Myxobolus cerebralis* in the Willamette River basin.

**Figure 5.3.** Scenario tree of the risk of establishment of *Myxobolus cerebralis* in the Willamette River basin.

**Figure 5.4.** Locations of survey sites for *Tubifex tubifex* in the Willamette River basin.
Figure 5.1.

- Federal hatchery
- State hatchery
- Private hatchery

Fish trap with stray fish
Fish trap without stray fish
Figure 5.2.

- Hatchery fish transfers and stocking
- Movement of frozen fish
- Illegal importation of fish
- Movement of fish carcasses for stream enrichment
- Migration of piscivorous birds
- Stray fish from enzootic areas
- Angler/boating activity

Introduction

Natural Dispersal

Recreational Activities
Figure 5.3.

If introduction occurs:

- Habitat for *T. tubifex*
  - Yes

- Sustainable populations of susceptible *T. tubifex*
  - Yes

- Water temperature seasonally reaching 10-15 °C
  - Yes

- Spatial/temporal overlap of hosts
  - Yes

**Establishment occurs**

**Establishment does not occur**
Figure 5.4.

- **Tubifex tubifex** detected
- **Tubifex tubifex** not detected
The risk of *M. cerebralis* introduction and establishment in both the Willamette River basin, Oregon and the state of Alaska was investigated to provide information to managers concerned with resource allocation for parasite monitoring. Several potential pathways of introduction were identified: human movement of fish, natural dispersal by migratory piscivorous birds, natural dispersal by stray anadromous salmonids, recreation and angler activities, and commercial seafood processing. In addition, factors necessary for establishment were examined: populations and lineages of *T. tubifex*, water temperatures, and spatial-temporal overlap of hosts.

**Summary of research conclusions**

*Willamette River Basin*

Introduction risks:

1. The most likely pathway for parasite dissemination is human movement of fish.
2. Recreation (boats and angler activities) is the second most likely route of parasite introduction to the Willamette River basin, though the number of spores transported would be fewer than by human movement of fish.
3. Stray salmonids in the Willamette River basin present little probability of parasite introduction; while we documented a greater number of strays than previously recorded, none of the strays encountered were infected with *M. cerebralis*.
4. Piscivorous birds also present little probability of parasite introduction as numerous events must align in order for parasite dissemination to occur.
5. Areas with greatest probability of introduction are the Clackamas and Santiam River subbasins.

Establishment potential:

1. As susceptible *T. tubifex* were detected throughout the Willamette River basin, and temperatures in the basin are conducive to parasite propagation, the probability of parasite establishment is high.

2. Areas with greatest probability of establishment are the tributaries of the mainstem Willamette River.

Management recommendations:

1. Steps should also be taken to limit human movement of fish by clearly and prominently articulating fish transport policies to the buyers of fish in the form of a brochure or website.

2. Human movement of fish can also be limited by restricting carcass planting for stream enrichment to streams where fish were reared, regardless of broodstock.

3. The state should allot resources to angler education and awareness of the effects of angler activity and recreation on dispersal of *M. cerebralis* and other invasive species. This could be done using a combination of brochures and signage at boat ramps describing how to prevent spread of aquatic nuisance species.

4. To reduce the likelihood of dissemination by stray fish, fish trap handlers should be instructed on how to identify fish with markings indicating they are from out of basin, and to remove these fish from the population. Efforts should also be made to coordinate databases within the Columbia River basin, so fish release and recovery data are compiled in one easily accessible database.

*Alaska*
Introduction risks:

1. The most likely pathway for parasite dissemination is human movement of fish and recreational activities.
2. Recreation and angler activities present a low probability of parasite transfer, if the prevalence and severity of *M. cerebralis* infection in Alaska remain low.
3. Natural dispersal by stray salmonids and piscivorous birds present little probability of parasite dissemination if the parasite is not established outside of Elmendorf Hatchery.
4. Seafood processing has a negligible probability of parasite introduction.

Establishment potential:

1. As susceptible *T. tubifex* were detected in southcentral Alaska, and temperatures in the region are permissible (though not ideal) for parasite propagation, the probability of parasite establishment is moderate.
2. Areas most likely for establishment: Ship and Campbell Creeks.

Management recommendations:

1. Steps should also be taken to limit human movement of fish by limiting transplants of fish from Elmendorf Hatchery and by prohibiting use of fish heads as bait in southcentral Alaska.
2. The state should allot resources to angler education and awareness of the effects of angler activity and recreation on dispersal of *M. cerebralis* and other invasive species. This could be done using a combination of brochures and signage at boat ramps describing how to prevent spread of aquatic nuisance species.
3. Maintain the policy requiring all seafood processor effluent be disposed in marine waters, and restrict effluent waivers in areas with high probability of introduction and establishment (Cook Inlet Basin).


Tops, S. and B. Okamura. 2003. Infection of bryozoans by Tetracapsuloides


