MYXOSPORE DETECTION IN SOIL AND ANGLER MOVEMENT IN SOUTHWESTERN MONTANA: IMPLICATIONS FOR WHIRLING DISEASE TRANSPORT

by

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS -- CONTINUED

Page

LIST OF TABLES

LIST OF TABLES CONTINUED

Table Page

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

Figure Page

ABSTRACT

Movement of anglers among rivers in southwestern Montana presents a potential pathway for the spread of whirling disease and other aquatic nuisance species (ANS) on soil laden angling equipment. The objectives of this study were to 1) determine the effectiveness of a density extraction to isolate myxospores from soil and the effectiveness of polymerase chain reaction (PCR) at detecting *M. cerebralis* myxospores in soil; 2) quantify movement of resident and non-resident anglers in southwestern Montana and soil quantity carried on angling equipment; and 3) determine myxospore adherence to wading equipment materials. Myxospores were extracted from soil using a soil particle density separation technique. A single blind study was used for PCR experiments with varying quantities of myxospores and soil. Angler movement was assessed with a survey at fishing access sites on six southwestern Montana rivers. Soil samples were taken during the survey from boots and waders with a pressure sprayer to assess quantity of soil carried on angling equipment. Myxospore adherence to wading materials (lightweight, neoprene, rubber, and felt) was tested by exposing myxospores to material and rinsing with a pressure sprayer. Mean percent myxospore recovery with density extraction declined as soil quantity increased. Polymerase chain reaction experiments detected myxospores in treatments with ≤ 0.25 g of soil and ≥ 100 myxospores/0.25 g soil. Resident and non-resident anglers did not differ significantly in the number of fishing access sites used or drainages fished in during the previous 30 days. Non-residents fished in more states in the previous 30 days than residents and traveled greater distances to fish in the previous 7 and 30 days than residents. Mean quantity of sediment carried on one boot-wader leg was 8.39 g ($\pm$ 1.5, 95% CI). Lightweight waders and felt soled boots were the most prominent types of wading equipment materials used by anglers and myxospores adhered to felt more than rubber and the glass control. Integration of angler movement patterns and mean sediment quantities transported with angler numbers suggests that anglers in southwestern Montana are potentially moving tons of sediment among fishing access sites every year, thereby making transport of ANS likely.

CHAPTER 1

DETECTION AND EXTRACTION OF MYXOBOLUS CEREBRALIS MYXOSPORES IN SOIL

Introduction

Myxobolus cerebralis, the causative agent of whirling disease, is the most intensively studied member of the phylum Myxozoa (Hedrick 2002). *Myxobolus cerebralis* is a two-host metazoan parasite that alternates between an oligochaete worm host and a salmonid fish host (Hedrick et al. 1998). The parasite is responsible for major declines or near elimination of some salmonid year classes in Montana and throughout North America (Nehring and Walker 1996; Vincent 1996; Bergersen and Anderson 1997). Although speculation exists that the parasite is transported among drainages in the environmentally resistant spore stage (Bartholomew et al. 2005), little is known about its dissemination (Kent et al. 2001).

Myxobolus cerebralis exhibits a complex life cycle that was not clearly defined until the mid 1980s (Markiw and Wolf 1983). The environmentally resistant myxospore stage that develops in fish is ingested by the aquatic oligochaete host, *Tubifex tubifex* (Hedrick et al. 1998). The parasite undergoes a complex transformation into the triactinomyxon (TAM), which is released by *T. tubifex* (Hedrick et al. 1998). The TAM floats buoyantly in the water column and adheres to the epidermis of the salmonid host (Markiw 1989; El-Matbouli et al. 1995; Hedrick et al. 1998). The parasite feeds on the cartilage within the salmonid host, multiplies and eventually transforms into the myxospore (Hedrick et al. 1998). This developmental stage within the cartilage can

cause severe damage to juvenile fish and can ultimately lead to premature death. After the infected salmonid host dies and decomposes or is eaten, the myxospores are released into the environment (El- Matbouli et al. 1998). One infected salmonid host, upon decomposition, can release up to two million myxospores from its spinal column and cranium (Hedrick et al. 1998). After the myxospore is released into the environment, it is suspected to be deposited in low velocity areas with fine soil (Kerans and Zale 2002).

The inadvertent transport of viable myxospores by humans is possible, for example by anglers transporting soil on angling equipment among fishing access sites. The myxospore stage of *M. cerebralis* is resistant to environmental degradation unlike the more fragile TAM stage (Hedrick et al. 1998). The contents of the myxospore are sealed by a protective shell making the myxospore highly resistant to stresses such as smoking (Wolf and Markiw 1982), aging, freezing, chemical exposure, and digestion by fish-eating birds and fish (Hoffman and Putz 1969; El-Matbouli and Hoffmann 1991). The myxospore can withstand temperatures from -20° C to 60° C (Hoffman and Putz 1971; Hoffman and Markiw 1977) and can resist biodegradation for years while retaining its infectivity (Halliday 1976). These resilient features of the myxospore make it likely that it will persist in an environment until it is ingested by the oligochaete host.

Effective methods for extracting *M. cerebralis* myxospores from soil have not been developed. Attempts to extract *M. cerebralis* myxospores with the plankton centrifuge technique revealed that even a small percentage of silt in the sample reduced myxospore extraction efficiency (Lemmon and Kerans 2001). Increased silt and clay particles decrease interstitial spaces between soil particles causing aggregation and

reduced fluid movement between particles, which decreases particle extraction ability (Buffle and Leppard 1995). The use of sodium hexametaphosphate ($[NaPO_3]_6$), a mild detergent commonly used to prevent soil particle aggregation (Guillet and Rouiller 1982; Tan 1996), with the plankton centrifuge technique increased myxospore extraction but recovery percentages remained low (Lemmon and Kerans 2001).

Myxospore extraction from soil with silt particles may be possible using a process much like that used to determine soil texture by separating sub-fractions of sand, silt, and clay. *Myxobolus cerebralis* myxospores measure about 8.7 µm in length, 8.2 µm in width (Nehring et al. 2003), and if assumed to have similar properties to the myxospores of *Henneguya doori*, a relative density of 1.062 (McConnell and Cone 1992). This size and relative density make the myxospore similar in size and relative density to silt particles (2-50 µm in size and relative density of 1.00-1.60) (Tan 1996; McConnell and Cone 1992). Consequently, an optimal centrifuge procedure may be developed to separate the myxospores with silt.

Advances in deoxyribonucleic acid (DNA) analysis may provide another method for detecting myxospores in soil as opposed to extracting them. Nested and single-round polymerase chain reaction (PCR) analyses have been successfully used to identify the presence of *M. cerebralis* DNA in infected fish tissue (Baldwin and Mycklebust 2002; Kelley et al. 2004). Prior to the use of PCR analysis, the continuous plankton centrifuge (O'Grodnick 1975) and the pepsin trypsin digest method (PTD) (Markiw and Wolf 1974) were used to detect myxospores in fish. With the advent and refinement of PCR analysis in the mid 1990s, the detection of specific DNA segments was greatly improved (Welsh

and McClelland 1990; Williams et al. 1990). Polymerase chain reaction analysis is now considered a superior test to the previously used methods because it provides a more definitive diagnosis of the presence or absence of *M. cerebralis* DNA (Baldwin and Myklebust 2002). The ability of PCR to amplify small quantities of specific DNA segments make it a potentially useful tool for detecting the presence of *M. cerebralis* myxospores in soil.

The ability to extract and detect *M. cerebralis* myxospores in soil is of particular importance to understanding myxospore dispersal and parasite dissemination. Myxospore extraction and detection procedures would also provide a quantifiable method for determining infection severity in a waterbody. The objectives of this study were to determine the effectiveness of extracting *M. cerebralis* myxospores from soil and to assess the ability of PCR analysis to detect *M. cerebralis* DNA in soil.

Methods

Myxospore Preparation

Rainbow trout *Oncorhynchus mykiss* infected with *M. cerebralis* were supplied by the Montana State University Aquatic Sciences Laboratory. Myxospores were isolated from fish with the continuous plankton centrifuge technique (O'Grodnick 1975). After isolation, the myxospores were stained with aqueous methylene blue (Hoffman and Markiw 1977) to increase visibility of the myxospores in solution. After staining, myxospore abundances were estimated with a 0.4 mm Neubauer hemocytometer, compound microscope, and hand counter (Markiw and Wolf 1974). Counts were

replicated three times per grid on the hemocytometer (Lemmon and Kerans 2001). Additional counts were conducted if replicate counts exceeded 10% ± the mean.

Variability in the hemocytometer counting procedure produced variability in the abundance of myxospores used to inoculate treatments. I calculated 95% confidence intervals for the myxospore abundances added to treatments to measure the variability in counts. The confidence intervals are reported as mean 95% confidence intervals because the experiment was conducted over the course of several days and confidence intervals were calculated for each day.

Extraction of Myxospores from Soil

Two experiments were designed to determine the effectiveness and sensitivity of myxospore extraction from soil. Sand and potting soil were used to create treatments for both experiments with various quantities of sand or soil and various quantities of myxospores. Potting soil was selected over natural benthic or terrestrial soil to eliminate the possibility of wild myxospores being present and contaminating the treatments. I assumed sand had fewer silt particles than potting soil and therefore presented fewer challenges. Thus, extraction experiment one treatments contained sand and potting soil alone and experiment two treatments contained potting soil alone.

Treatments consisted of 10.5 g of dry sand with 30 ml aqueous $[NaPO_3]_6$ and 1.5E+05 stained myxospores combined in a 50 ml centrifuge tube in the first extraction experiment. Treatments were replicated three times. Aqueous $[NaPO_3]_6$ was prepared with 6200 mg of $[NaPO_3]_6$/L of de-chlorinated water (Lemmon and Kerans 2001). I used 10.5 g of sand because that was the average quantity of soil carried by anglers on one leg

boot-wader combination in southwestern Montana (see Chapter 2). Treatments were agitated for 4 minutes to distribute myxospores throughout and de-aggregate particles. Treatments were separated into four particle categories including sand (50-2000 μm size and 1.2-1.8 g/cm^3 density), coarse silt (20-50 μm), course to medium silt (15-25 μm), and medium to fine silt (5-15 μm) (Tan 1996). Sand was the coarsest material and settled out within 40 seconds (Tan 1996). Subsequently, the supernatant was decanted, placed in another 50 ml centrifuge tube, agitated for an additional 4 min, and settled for 7.5 min to allow the coarse silt to separate from the solution (Tan 1996). The supernatant was carefully decanted again, placed in another 50 ml centrifuge tube, and centrifuged for 3 min at 250 rpm to separate the course to medium silt from the solution (Tan 1996). The final supernatant containing medium to fine silt was decanted, transferred to another 50 ml tube, and centrifuged at 3500 rpm for 20 min to form a pellet at the bottom of the tube (Lemmon and Kerans 2001). The supernatant was decanted, discarded, and the remaining pellet was re-suspended in 5 ml of aqueous $[NaPO_3]_6$ by agitating for 2 min. Myxospore abundance retrieved in the re-suspended pellet was enumerated with a hemocytometer as previously described. The above process was repeated with 10.5 g of dry potting soil. A two-sample t-test with an alpha value of 0.05 was used to determine if any significant differences existed between myxospore recovery from sand and potting soil (SAS Institute 2005).

A two-way factorial experimental design was used to determine the sensitivity of myxospore extraction (quantity of potting soil that began to interfere with extraction) in experiment two (SAS Institute 2005). Treatments contained one of two quantities of

stained myxospores (1.E+04 and 1.E+05), one of three quantities of potting soil (0.01, 0.1, and 1.0 g), and a control with no potting soil. Treatments were combined with 30 ml of aqueous $[NaPO_3]_6$ in a 50 ml centrifuge tube. The same extraction technique described above was used with three replicates of each treatment. A two-way ANOVA and a least squares means multiple comparison procedure were used to identify significant differences among treatments (myxospore count and soil quantity).

Detection of Myxospores in Soil

The designs of the PCR experiments were an iterative process that evolved based on the results from each experiment. Treatments consisted of various soil quantities (myxospore count held constant) and various myxospore quantities (soil quantity held constant) (Table 1.1). This design allowed me to assess the effects of soil on PCR analysis and the number of myxospores necessary in a soil sample to receive a positive score (sensitivity of the analysis). Samples for PCR analysis were sent to Pisces Molecular of Boulder (CO). All PCR experimental designs were single-blind with the PCR laboratory unaware of sample contents. Pisces Molecular recommended the use of a single-round PCR technique to detect *M. cerebralis* DNA (Baldwin and Myklebust 2002).

In the first PCR experiment, myxospore quantity was held constant (1.E+03) and potting soil quantity was varied (Table 1.1). Treatments contained various quantities of myxospores and potting soil combined in 2 ml centrifuge tubes (Table 1.1). Each tube was agitated for 2 min by repeated inversion to distribute the myxospores throughout. Tubes were immediately frozen and shipped overnight to Pisces Molecular. The first

PCR experiment treatments contained dry soil measured in ml^3 as stipulated by Pisces to help prepare the samples for PCR analysis. The second and third PCR experiments contained dry soil treatments measured in grams to standardize with the myxospore extraction experiments.

Table 1.1– Study design for three PCR experiments. Soil treatment columns indicate quantity of soil added to sample. Experiment one and two controls contain 0 g of soil and experiment three controls contain no myxospores. Number of replicates for each treatment is indicated as N. Cubic ml soil volumes correspond to weight in g as follows: 0 ml^3 = 0 g, 0.25 ml^3 = 0.21 g, 0.5 ml^3 = 0.43 g, 1.0 ml^3 = 0.85 g.

			Soil Treatment			
Experiment	N	Myxospores	1	2	3	4
Experiment 1						
Potting Soil	5	1.E+03	0 ml^3	0.25 ml^3	0.5 ml^3	1.0 ml^3
Experiment 2						
Potting Soil	3	1.E+05	0 g	0.01 g	0.1 g	1.0 g
Potting Soil	3	1.E+04	0 g	0.01 g	0.1 g	1.0 g
Experiment 3						
Natural Soil	3	1.E+03	0.25 g	0.75 g	1.0 g	-
Natural Soil	3	1.E+02	0.25 g	0.75 g	1.0 g	-
Natural Soil	3	0	0.25 g	0.75 g	1.0 g	-

Based on results from the first PCR experiment, a second experiment with different treatments was designed. The new treatments contained greater numbers of myxospores and the same potting soil quantities that were used in the density extraction (Table 1.1). Treatments were placed in 2 ml centrifuge tubes. Each tube was agitated for 2 min to distribute the myxospores throughout and was replicated three times. Tubes were immediately frozen and shipped overnight to Pisces Molecular.

Based on the results from the second PCR experiment, a third experiment was designed containing treatments with smaller quantities of myxospores and natural soil in lower magnitudes of difference among treatments (Table 1.1). These quantities were selected to determine what level of natural soil above 0.1 g and below 1.0 g would not interfere with PCR analysis. Natural soil used in these samples was obtained from below the surface topsoil outside the Montana State University Aquatic Science Laboratory. I assumed the probability of myxospore presence in the natural terrestrial soil was low because of the distance from an infected waterbody. Treatments with natural soil and myxospores were placed in 2 ml centrifuge tubes, agitated for 2 min, and replicated three times. Tubes were immediately frozen and shipped overnight to Pisces Molecular.

The following protocol was used by Pisces Molecular to assay for the presence of the *M. cerebralis* Hsp70 gene segment with single-round PCR analysis. Deoxyribonucleic acid was extracted from soil samples with DNeasy columns and the rodent tail protocol (Qiagen, Inc.). All extracted DNA samples were treated to remove potential PCR inhibitors (frequently present in environmental samples) with Gene Releaser© tissue-lysis buffer (BioVentures, Inc.). For the first PCR experiment, 1 ml of lysis buffer was added to all samples. For the second and third PCR experiments, the amount of lysis buffer added differed among soil treatments (0-0.1 g received one times the sample volume, 0.1 g received two times the sample volume, and 1 g received 10 times the sample volume). The third PCR experiment was also pre-treated to remove inhibitors. After incubation at 55º C for one hour, total DNA was extracted using a spin-column DNA purification procedure. Results are

reported as positive or negative for the presence of *M. cerebralis* or as inhibited. Inhibited results imply that DNA could not be extracted from the sample.

Variability in the hemocytometer counting procedure produced variability in the myxospore abundances used to inoculate treatments. To measure the variability in counts I calculated 95% confidence intervals for the myxospore abundances added to the treatments in each experiment. The confidence intervals are reported as 95% confidence intervals for each experiment.

Results

Extraction of Myxospores from Soil

Treatments in extraction experiment one were inoculated with 150,000 ± 16,590 myxospores. Mean recovery of myxospores from the sand treatment (23% ± 5%, 95% CI) was significantly greater than from the potting soil treatment (12% ± 2%) (t_4 = -5.38, P = 0.006).

Treatments in extraction experiment two were inoculated with either 10,000 ± 2,041 or 100,000 ± 14,696 myxospores. Percent recovery differed significantly among soil quantity treatments ($F_{7, 16}$ = 11.15, P = <0.0001) (Figure 1.1). Mean percent myxospore recovery was highest in the controls (Table 1.2). Percent myxospore recovery did not differ significantly between myxospore quantities ($F_{1, 16}$ = 3.3, P = 0.09). There was no interaction between myxospore quantity and soil quantity ($F_{3, 16}$ = 0.98, P = 0.43). Mean percent myxospore recovery declined as potting soil quantity increased (Figure 1.1).

Table 1.2– Mean percent recovery of myxospores from each treatment (myxospore count and potting soil quantity). Controls contained no soil.

Treatment		Mean percent recovery
1.E+04	Control	113
	0.01 g	100
	0.1 g	53
	1.0 g	42
1.E+05	Control	97
	0.01 g	90
	0.1 g	24
	1.0 g	44

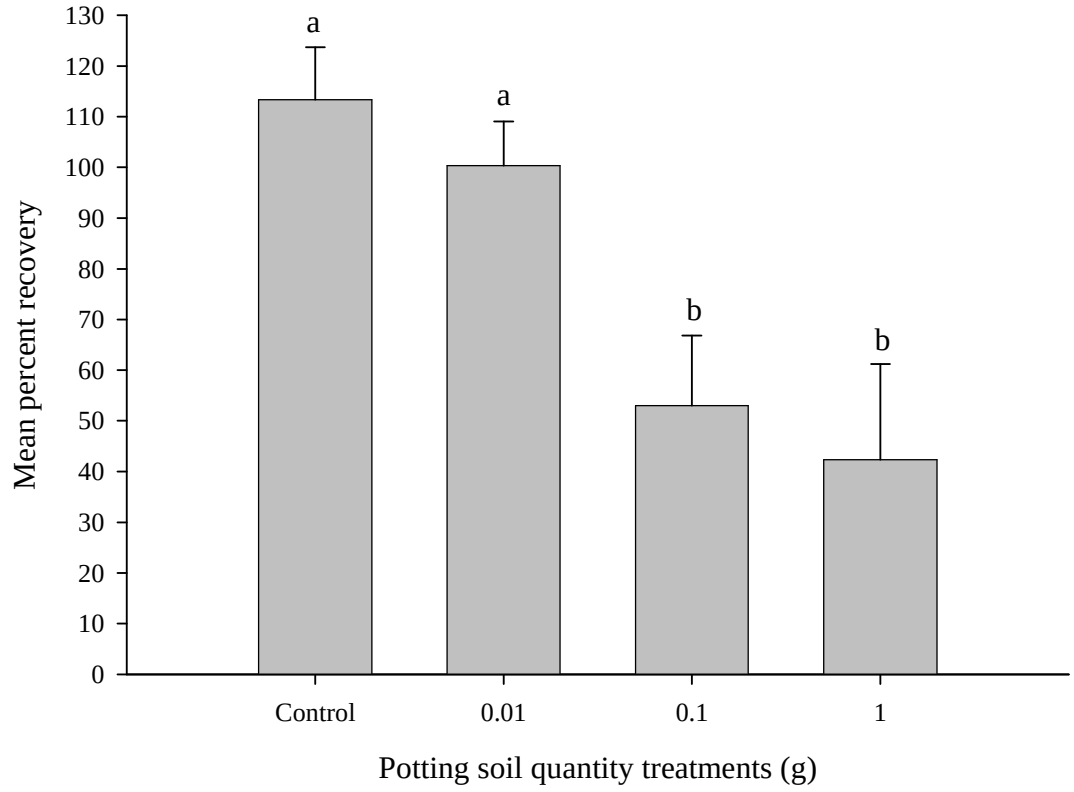

Figure 1.1-- Mean percent myxospore recovery using a density extraction by varying quantities of potting soil. Treatments labeled with the same letter do not differ significantly. Error bars delineate one standard error.

Detection of Myxospores in Soil

Treatments in the first PCR experiment were inoculated with 1,004 ± 144 myxospores. All five replicates of the positive control tested negative for *M. cerebralis* DNA (Table 1.3). Four of the five replicate treatments containing 0.25 g of potting soil and 1.E+03 myxospores tested positive. As the quantity of potting soil added to each treatment increased, the number of positive PCR tests for *M. cerebralis* decreased except with the controls (Table 1.3).

Table 1.3—Results of the first PCR experiment. All samples contained potting soil. Polymerase Chain Reaction (PCR) scores indicate how many of the replicates tested positive or negative for the presence of *Myxobolus cerebralis* DNA.

Sample Number	Treatment		PCR Scores
	Myxospore Count	Soil Quantity (ml^3)	
1-5	1.E+03	Control	5 negative
6-10	1.E+03	0.25	4 positive, 1 negative
11-15	1.E+03	0.5	3 positive, 2 negative
16-20	1.E+03	1.0	2 positive, 3 negative

Treatments in the second PCR experiment were inoculated with either 10,000 ± 1,071 or 100,000 ± 10,714 myxospores. The controls from the second PCR experiment all tested positive for *M. cerebralis* DNA (Table 1.4). Two of the replicates from the treatment containing 1.0 g of potting soil and 1.E+05 myxospores were inhibited (Table 1.4). Similarly, one of the replicates for the treatment containing 1.0 g of potting soil and 1.E+04 myxospores was also inhibited.

Table 1.4—Results of the second PCR experiment. All samples contained potting soil. Myxospores included in samples were extracted from infected rainbow trout hours prior to sample mixing. Polymerase Chain Reaction (PCR) scores indicate how many of the replicates tested positive, negative, or were inhibited for the presence of *Myxobolus*

	Treatment		
Sample Number	Myxospore Count	Soil Quantity (g)	PCR Scores
1-3	1.E+04	Control	3 positive
4-6	1.E+04	0.01	3 positive
7-9	1.E+04	0.1	3 positive
10-12	1.E+04	1	2 positive, 1 inhibited
13-15	1.E+05	Control	3 positive
16-18	1.E+05	0.01	3 positive
19-21	1.E+05	0.1	3 positive
22-24	1.E+05	1	1 positive, 2 inhibited

Treatments in the third PCR experiment were inoculated with either 100 ± 9 or 1,000 ± 86 myxospores. The natural soil and myxospore treatments in the third PCR experiment resulted in 10 inhibited replicates (Table 1.5). All control replicates with no myxospores and either 0.75 g or 1.0 g of natural soil were inhibited. Single-round PCR analysis consistently detected the presence of *M. cerebralis* DNA in treatments containing 0.25 g natural soil (Table 1.5).

Table 1.5—Results of the third PCR experiment. All samples contain soil from the Montana State University Aquatic Sciences Laboratory grounds. Myxospores included in samples were extracted from infected rainbow trout hours prior to mixing samples. Polymerase Chain Reaction (PCR) scores indicate how many of the replicates tested positive, negative, or were inhibited for the presence of *Myxobolus cerebralis* DNA.

	Treatment		
Sample Number	Myxospore Count	Soil Quantity (g)	PCR Scores
1-3	1000	0.25	3 positive
4-6	1000	0.75	1 positive, 2 negative
7-9	1000	1	1 positive, 2 inhibited
10-12	100	0.25	3 positive
13-15	100	0.75	1 positive, 2 negative
16-18	100	1	1 negative, 2 inhibited
19-21	0	0.25	3 negative
22-24	0	0.75	3 inhibited
25-27	0	1	3 inhibited

Discussion

Extracting myxospores from soil and sand with the density extraction was inefficient and not useful for processing large quantities of samples. Although myxospores were stained with methylene blue, identifying them among soil particles using a hemocytometer was time consuming. In addition, the presence of small quantities of potting soil and sand in treatments decreased myxospore extraction efficiency. Despite the presence of a mild detergent (aqueous $[NaPO_3]_6$) to help prevent aggregation, myxospores may still have aggregated with higher density particles causing them to settle out faster thereby decreasing extraction efficiency. Low myxospore recovery rates observed in the density extraction support previous work indicating that sand and soil decrease myxospore extraction (Lemmon and Kerans 2001). Extraction from the sand was almost twice that from potting soil, also supporting previous work indicating the negative effect of fine soil particles on myxospore extraction (Lemmon and Kerans 2001).

The density extraction procedure was designed to isolate silt particles and myxospores were isolated with the silt particles. The efficiency of this procedure when no potting soil was added to the treatments (the controls) indicates that without potting soil present, myxospores exhibit a similar density to fine silt particles. This supports my assumption that *M. cerebralis* myxospores have a comparable relative density to the *H. doori* myxospores because the relative density of *H. doori* myxospores is also similar to those of silt particles.

Myxospore recoveries over 100 % obtained with the density extraction could be the result of several factors. Variability in myxospore counts from the hemocytometer may have led to variability in the number of myxospores added to treatments. More likely, myxospores may have aggregating with one another and not evenly distributing throughout the treatments (McConnell and Cone 1992). For example, a cluster of myxospores could have been drawn in one of the aliquots used to inoculate the treatments resulting in a greater number of myxospores being added. Myxospore clusters could be caused by residual mucous and or lipids remaining after removal of the myxospores from infected fish tissues. Mature myxospores within an infected fish are surrounded by circular lipid droplets in the destroyed cartilage (El-Matbouli et al. 1995). Myxospores may also still be coated with a matrix of mucous strands on the posterior end after extraction (Lom and Hoffman 1971). It is possible that residual mucous or lipids on the myxospore surface created an adhesive quality causing the myxospores to adhere to one another. Myxospore aggregation in future experiments may be decreased by processing the myxospore solution with tissue lysis buffer to remove residual tissue and mucous (Schisler et al. 2001).

False negative PCR results encountered in the first myxospore detection experiment were likely the result of contaminants within the myxospore solution. The negative PCR scores obtained in the positive controls may have been the result of a fungal contaminant within the myxospore solution used to inoculate the treatments (John Wood, Pisces Molecular, personal communication). Fungal contaminants could have multiplied within the myxospore solution during the time it was refrigerated prior to use.

I used freshly extracted myxospores to eliminate fungal contaminants in subsequent PCR experiments.

Pisces Molecular was able to refine their PCR procedures for dealing with soil in the second and third myxospore detection experiments; however, they were not able to develop a technique for processing those treatments containing more than 0.1 g of potting soil or 0.25 g of natural soil. Treatments containing 1.0 g of potting soil and 0.75 g of natural soil inhibited the ability of PCR to detect large quantities of *M. cerebralis* myxospores (1.E+05). It is suspected that substances such as humic acid, frequently present in soil (Flaig et al. 1905), prevented the detection of myxospores with PCR analysis (John Wood, Pisces Molecular, personal communications). The positive charge associated with an acid would cause it to bind with the negatively charged DNA during the PCR purification process making replication of the target *M. cerebralis* Hsp 70 gene impossible (John Wood, Pisces Molecular, personal communications). Natural soil included in the third PCR experiment presented even more inhibition problems than the potting soil in previous experiments suggesting that natural terrestrial soil contains more PCR inhibitors than potting soil. Future experiments may benefit from using benthic soil from whirling disease free waterbodies instead of potting or terrestrial soil because the leaching of benthic soil in water may decrease the presence of PCR inhibitors.

The problems encountered using PCR to detect *M. cerebralis* DNA in soil make it a limited tool for analyzing samples from the field. For example, the negative PCR results from these experiments did not imply that myxospores were not present. These results implied that myxospores were not present in quantities greater than 100

myxospores per 0.25 g of natural soil which was the detection level. Quantifying infection levels by evaluating benthic soil is still an appealing approach and perhaps with the refinement of PCR techniques over time, this could be further developed. However, I do not believe that the detection abilities of PCR in the presence of soil are sensitive enough to detect the level of myxospores that might be found in the field. Until advances are made in the PCR purification and amplification processes when soil is present, I caution against the use of this tool as a method to diagnose presence or absence of *M. cerebralis* in soil samples.

The difficulties encountered in extracting and detecting myxospores in soil demonstrate the challenges associated with determining *M. cerebralis* infection levels in a waterbody without sampling *T. tubifex* or sentinel fish. The PCR detection results also demonstrate the challenges associated with determining *M. cerebralis* transport vectors. Soil containing myxospores may be transported among fishing access sites by anglers on angling equipment; however, the quantity of soil in angling equipment samples is frequently greater than 0.25 g exceeding the soil capacity of PCR (see Chapter 2). *Myxobolus cerebralis* dissemination will likely remain unknown until myxospore extraction and detection procedures are further refined.

CHAPTER 2

MOVEMENT PATTERNS AND SOIL TRANSPORT BY RESIDENT AND NON-RESIDENT ANGLERS IN SOUTHWESTERN MONTANA

Introduction

Myxobolus cerebralis has been documented worldwide since it was first discovered in 1898 by Dr. Bruno Höfer at the University of Munich (Hedrick et al. 1998). The myxozoan parasite was first documented in the United States during the 1950s in Pennsylvania (Brinkhurst 1996). The transcontinental jump of the parasite and the associated potential economic effects on recreational fisheries caused the International Office of Epizootics to list whirling disease as a major infectious disease of freshwater fish (Halliday 1976). Since the 1950s, *M. cerebralis* has spread through much of the eastern United States and the Intermountain West (Hedrick et al. 1998) and it continues to expand its range (Bartholomew and Reno 2002). The role humans play in transporting the parasite is largely undefined.

Most research of *M. cerebralis* has been devoted to the biology of the parasite and little is known about its dissemination from one watershed to another (Kent et al. 2001). *Myxobolus cerebralis* is thought to have been originally transported to North America in frozen trout imported from Europe (Nickum and Bartholomew 2001). A lack of genetic divergence of the parasite found in North America supports the hypothesis of a recent introduction to North America (Andree et al. 1999). Currently, *M. cerebralis* is found in 25 states within the U.S. (Kajsa Stromberg, Whirling Disease Initiative, personal

communication). Some of the spread can be attributed to stocking infected fish from hatcheries; however, it is difficult to pinpoint sources for most infection sites (Holland 2000).

It is possible that anglers transport *M. cerebralis* and other aquatic nuisance species (ANS) among waterbodies on their angling equipment. Anglers walk through soil where myxospores and other invasive species may be present and are a highly mobile group of individuals that frequently move among fishing access sites. A preliminary angler survey conducted during the summer of 2003 by Montana Fish, Wildlife and Parks in conjunction with this project found that resident and non-resident anglers fishing on the Missouri River between Wolf Creek (MT) and Craig (MT) were likely to have fished on a different water body within one week of their current trip. Of the 100 anglers surveyed, 17 different rivers (Beaverhead, Belt, Big Hole, Big Horn, Bitterroot, Blackfoot, Clark Fork, Gallatin, Jefferson, Kootenai, Madison, Middle Fork Flathead, Mission, Rock, Ruby, Sun, and Yellowstone) had been fished in Montana within one week of fishing on the Missouri River (Travis Horton, Montana Fish, Wildlife and Parks, personal communication). In addition, 13 rivers outside Montana were fished by these 100 anglers within one week of their Missouri River trip including the Elk (AK), Blakly (AK), Kispox (BC), Henry's Fork (ID), South Fork Snake (ID), Delaware (NY), Metolius (OR), Owhwee (OR), Rouge (OR), Snoqualmie (WA), Shoshone (WY), Snake, (WY), and Wind (WY) (Travis Horton, Montana Fish, Wildlife and Parks, personal communication). These preliminary results suggested resident and non-resident anglers

in southwestern Montana are highly mobile and that Montana attracts non-resident anglers from around the country and world.

Anglers within southwestern Montana may be moving among *M. cerebralis*-infected and uninfected waterbodies creating a potential pathway for spread of the parasite. It is also possible that non-resident anglers are transporting ANS from their home states on angling equipment creating the potential for ANS from around the world to become established at fishing access sites in Montana. Separation of anglers into residents and non-residents may reveal that different management plans are necessary for these two types of anglers. Determining the quantity of soil carried on angling equipment and whether *M. cerebralis* is present in the soil may help to identify parasite transport mechanisms and 'hot spots' where resident and non-resident anglers are depositing and encountering ANS. Identification of areas experiencing the largest effects from invasive species (such as *M. cerebralis*) is a priority in their control (Byers et al. 2002).

Montana is a tourist destination for anglers from around the world; therefore, understanding resident and non-resident angler profiles, movements, and soil transport will be useful in developing management strategies to reduce the spread of *M. cerebralis* and other ANS not only in Montana, but globally. The objectives of this study were to: 1) quantify resident and non-resident angler profiles, angling frequencies, distances traveled among angling locations, ANS awareness, and equipment cleaning practices; 2) quantify the amounts of soil transported by resident and non-resident anglers; and 3) determine whether *M. cerebralis* myxospores were present in the soil transported on angling equipment.

Study Area

Sample locations were distributed throughout southwestern Montana representing a variety of geological basins and river types that exhibit the region's diverse environments (Figure 2.1). These included tailwater fisheries (Beaverhead, Bighorn, Madison, and Missouri rivers) and free-flowing river fisheries (Yellowstone and Gallatin rivers). The selected rivers are also among those in Montana with the highest fishing pressure (MTFWP 2004b; MTFWP 2006b) and exhibit varied whirling disease infection severities (Baldwin et al. 1998). All study rivers contain *M. cerebralis* except for the Bighorn River (Baldwin et al. 1998; Eileen Ryce, Montana Fish, Wildlife and Parks, personal communication).

The Beaverhead River originates at Clark Canyon Dam 29 km south of Dillon, MT. The most popular angling section of the river extends from Clark Canyon Dam to Henneberry fishing access site; however, this river is not divided into high and low-use sections by Montana Fish, Wildlife and Parks (MTFWP 2004a; MTFWP 2006a) (Figure 2.1). Annual mean discharge below Clark Canyon Dam is 11 cms (USGS 2005). The Madison River originates in Yellowstone National Park. The Hebgen Dam to Ennis Lake section has the highest angler crowding rating value of any section on the Madison River (MTFWP 2004a; MTFWP 2006a). Annual mean discharge measured at the Kirby Ranch is 37 cms (USGS 2005). The Gallatin River also originates in Yellowstone National Park and flows freely for 185 km. The section of the Gallatin River with the highest angler crowding rating value is from Spanish Creek to the mouth of the East Gallatin River (MTFWP 2004a). Annual mean discharge north of Gallatin Gateway, MT, within the

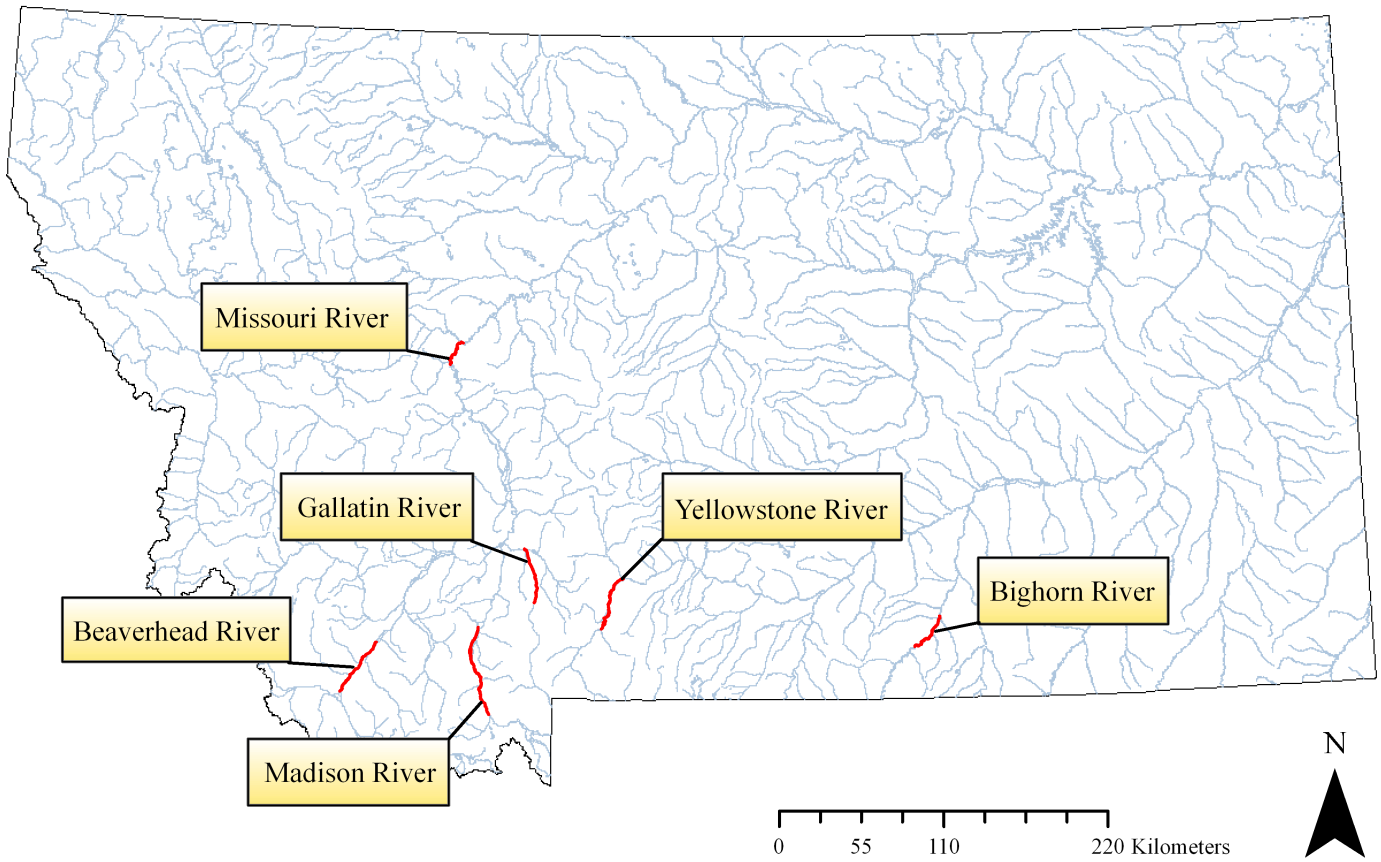

Figure 2.1—River reaches where anglers were surveyed (represented by the red lines) in southwestern Montana. Surveys and soil samples were collected during October of 2004 and from June through August of 2005 and 2006.

high-use section is 23 cms (USGS 2005). The confluence of the Gallatin, Madison, and Jefferson rivers forms the Missouri River outside Three Forks (MT). Dams create Canyon Ferry, Hauser, and Holter reservoirs before the river emerges to the most heavily fished section from below Holter Dam to Cascade Bridge (MTFWP 2004a; MTFWP 2006a). Annual mean discharge of the Missouri River below Holter Dam is 153 cms (USGS 2005). The Yellowstone River flows 1,078 km from the outlet of Yellowstone Lake in Yellowstone National Park before joining the Missouri River in North Dakota. The section from Pine Creek Bridge to the mouth of the Shields River has the highest angler crowding rating value on the Yellowstone River (MTFWP 2004a; MTFWP 2006a). Annual mean discharge measured in Livingston (MT) within this section is 106 cms (USGS 2005). The Bighorn River in Montana originates from Bighorn Lake on the southern Montana border. The first 23 km directly below Afterbay Dam have the highest angler crowding rating value on the Bighorn (MTFWP 2004a; MTFWP 2006a). Annual mean discharge downstream of Afterbay Dam in St. Xavier (MT) is 98 cms (USGS 2005).

The geologies of the six river basins exhibit a variety of underlying basement rock and span multiple eras of formation contributing to the presence of different soil types. The Beaverhead flows through the Blacktail Range and the Blacktail Deer Valley, which consist of folded Paleozoic and Mesozoic sedimentary formations with deposits of gray sandstone and high-purity silica from Clark Canyon Dam to Dillon (Alt and Hyndman 1986). The Madison River flows through the Madison Valley and then through the north end of the Madison Range with the Gallatin Range to the east (Alt and Hyndman 1986).

Basement rock in the Gallatin Range is more exposed than that of the Madison Range, but both ranges consist of similar Paleozoic and Mesozoic formations (Alt and Hyndman 1986). The Missouri River near Holter Dam flows through the Adel Mountains which consist of an eroded volcano ruin covering Cretaceous sedimentary rocks of the high plains (Alt and Hyndman 1986). The Yellowstone River flows through the Paradise Valley with the Gallatin Range to the west and the Absaroka Range to the east (Alt and Hyndman 1986). The Absaroka Range consists mostly of Precambrian basement rocks with local cover of volcanic rocks similar to those of the Gallatin Range (Alt and Hyndman 1986). The furthest east river, the Bighorn, flows through the western edge of the High Plains consisting of cretaceous sandstone and shale basement rock (Alt and Hyndman 1986).

Methods

Sampling Design

Resident and non-resident anglers were surveyed where they began and completed their angling activities at fishing access sites. Specific Montana Fish, Wildlife and Parks access sites were selected for their popularity among anglers based on professional opinion of regional biologists and angler crowding ratings, which are obtained from creel surveys (Stanovick and Nielsen 1991; MTFWP 2004a; MTFWP 2004b). In addition to Montana Fish, Wildlife and Parks fishing access sites, two sites on the Bighorn River, managed by the Bighorn National Recreation Area, were used because of their location in the high-use section on the Bighorn River. To avoid excessive

sampling of unused fishing access sites, sites were stratified into high and low-use sites (Table 2.1). High-use sites were those located within the section of river with the highest Montana Fish, Wildlife and Parks angler crowding rating value. Low-use sites were located in sections adjacent to the high-use section. Selected access sites represent potential "hot spots" where benthic soil and possibly *M. cerebralis* myxospores may be moved and deposited.

Table 2.1—Number of high-use and low-use fishing access sites on rivers in southwestern Montana where surveys were conducted and soil samples were collected.

River	Number of high-use sites	Number of low-use sites
Beaverhead	7	2
Big Horn	3	2
Gallatin	6	2
Madison	9	2
Missouri	10	1
Yellowstone	6	2

Surveying was conducted in 2004, 2005, and 2006. Preliminary surveying was conducted at two randomly selected high-use fishing access sites on the Madison River in October of 2004. During the months of June, July, and August in 2005 and 2006, two rivers were randomly selected without replacement each week from the list of six rivers (i.e., Beaverhead, Bighorn, Gallatin, Madison, Missouri, and Yellowstone). Two high-use access sites were then randomly selected for each river. Only one access site was visited each day and random assignment was used to determine which of the two access

sites in each river was sampled in the morning (0700 hours to 1300 hours) and in the evening (1300 hours to 1900 hours) (Pollock et al. 1994).

A random number table was used to assign days of the week to the selected fishing access sites. The two selected high-use access sites were surveyed on consecutive days to reduce travel between rivers. In addition, low-use survey sites were added before or after high-use surveying days on the same river to prevent excessive travel between rivers. Three weekdays and one weekend day were selected in each week for surveying to maintain an even proportion of weekend and weekday surveying because access site usage on weekdays and weekends can be notably different during the summer (Pollock et al. 1994). Rivers were selected for weekend sampling without replacement. By sampling without replacement, each of the six rivers was sampled a total of 10 weekdays and 4 weekend days.

Angler Survey

Anglers beginning and completing their angling trip were asked if they were willing to participate in an angler movement study. Every angler that was sighted was approached for the survey; however, only one member from each fishing party was surveyed to reduce surveying time for a group of anglers. Local area maps and calendars were provided with the survey to help reduce angler recall bias of the dates spent fishing and fishing access sites used. In addition, visual observations were made during the survey regarding, angler gender, type of waders, boots, and boat used (if boating). Thus, information was collected even if the angler declined to participate in the survey.

Knowledge of angler typology was important for interpreting the survey results because many different angler types exist (Hahn 1991). Thus, angler profile questions were included in the survey to identify the type of angler surveyed (Appendix A). Angler profile was assessed by state of residency and type of angling tackle used. Defining residency was of particular importance because Montana attracts anglers from around the world creating the potential for non-resident anglers to travel farther distances among fishing locations.

Angler zip code was collected to quantify the distance resident and non-resident anglers traveled from their home to the survey access site. Distance traveled from home was the linear distance from the center of the home zip code to the center of the zip code associated with the access site (Imacination Software 2002). A linear distance was used to standardize methods and avoid over-estimating distance traveled from home.

Angler movement questions were designed to assess the frequency of fishing activities and distance traveled among fishing locations. Anglers were asked about the dates and locations of fishing trips in the past 30 days and trips planned in the next 7 days. Average numbers of sites visited in one-week and one-month intervals were tabulated to determine angling frequency. Distances traveled between fishing access sites in previous 7 and 30 days were calculated assuming that after anglers were fishing they were traveling via road among fishing access sites. Distance traveled between fishing access sites was calculated as chronological distance traveled via roads when dates were provided (MapQuest 2006). When exact dates were not provided, distance between fishing access sites was calculated by making the shortest possible route via roads. River

distance traveled during a single day of angling was calculated as the difference in distance between the most upstream access site and the most downstream access site. This distance does not include distance traveled by anglers who were only wading at one site and therefore likely overestimates the actual mean number of river kilometers traveled.

Predominant fish species at surveyed access sites were brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss* and most anglers at these sites were targeting trout species. To better approach this type of angler, angler specialization toward trout was addressed in the survey design (Hahn 1991) (Appendix A). Specifically, the trout angler concern for habitat protection and resource conservation was integrated in ANS questions (Hahn 1991). Anglers were asked questions regarding their knowledge of ANS, if they took any proactive approaches to prevent their spread, and whether they were active with any local or national angling organizations. The question regarding membership in an angling organization helped describe whether the surveyed anglers identified themselves as conservation oriented (Hahn 1991; Gigliotti and Peyton 1993). Survey questions regarding knowledge of ANS and precautionary practices provided estimates of ANS awareness among anglers and estimates of angler equipment cleaning practices between angling trips. Integrating the questions about ANS knowledge, proactive prevention approaches, and angling organization membership allowed me to assess the relationships among angler knowledge, practice, and identity.

The survey was reviewed prior to implementation by Dr. Scott Meyers of the Montana State University Sociology Department and by the Montana Fish, Wildlife and

Parks aquatic nuisance species coordinator, Dr. Eileen Ryce. The reviews focused on ensuring that the survey would provide key information about angler movement among fishing access sites with specific usage information (Appendix A).

Two-sample t-tests were used to determine significant differences between resident and non-resident mean distances traveled from home zip code and distances traveled in past 7 and 30 days to fish (SAS Institute 2005). A general linear model and a least squares means multiple comparison procedure were used to compare the relationship between survey location and distances traveled to fish in the prior 30 days. An alpha value of 0.05 was used for all statistical analyses to indicate statistical significance.

Kolmogorov-Smirnov non-parametric two-sample tests were used to compare distributions of the number of fishing access sites used and number of drainages fished in during the previous 7 days, previous 30 days, and the day of the survey between residents and non-residents. The Kolmogorov-Smirnov test was also used to compare distributions of the number of states fished in during the previous 30 days and number of drainages planned to fish in the next 7 days between residents and non-residents. Distributions of the number of states fished during the previous 30 days and number of drainages planned to fish in the next 7 days were compared between angling organization members and non-members using the Kolmogorov-Smirnov test. The Kolmogorov-Smirnov test was also used to compare residents to non-residents and angling organization members to non-members in ANS and cleaning practice questions. Results from the Kolmogorov-

Smirnov tests are reported with the test statistic (D) and P-value. Degrees of freedom are not associated with this test (Massey 1951; Hodges 1957).

Soil Sampling

In 2005, every third angler surveyed was asked if a sample of benthic soil could be collected from their angling boots and waders to keep the number of collected samples below storage and transport capacity. In 2006, all anglers were asked to provide a sample to increase sample collection and record refusals. Only shoes specific to fishing equipment (wading shoes) were sampled to focus on angling soil transport as opposed to general recreation soil transport. Boots and waders were selected for sampling because of their direct contact with river water and soil. Boat and boat trailer samples were not taken because of sample size restrictions, water availability, and subsampling extrapolation concerns.

Soil samples were obtained by washing boots and waders (from the knee down) at 2.11 kg/cm^2 with a hand pump sprayer containing water and aqueous sodium hexametaphosphate ($[NaPO_3]_6$). Sodium hexametaphosphate (a non-sudsing detergent) was added at a concentration of 6,200 mg/L to the washing water to help prevent soil and myxospore aggregation with angling equipment (Lemmon and Kerans 2001). Samples from each leg were collected in separate bins and stored separately. It was an inconvenience for anglers to remove their boots and waders; thus, samples were taken while the equipment was worn causing boot and wader samples to be combined for each leg. Sampling the equipment worn by anglers further restricted the area available for sampling and led to rinsing from the knee down instead of the waist down to ensure that

leg samples were collected separately. Each leg was rinsed for 30 seconds. All samples were stored, labeled, and placed on ice before returning them to the laboratory for analysis.

Samples from each leg were processed separately to obtain dry soil weight from one and test for the presence of *M. cerebralis* myxospores in the other. One boot-wader sample from each pair was randomly selected for drying to determine dry soil weight. The sample was dried in a drying oven at 45°C until all water had evaporated (Bruckert 1982). The time required for all water in the sample to evaporate varied with the quantity of water in each sample. Samples in the drying oven were checked at least every 10 hours. Soil quantity in each sample was weighed to the nearest 0.001 g. After weighing, the sample was screened for the presence of other ANS including invasive molluscs and noxious weed seeds. Soil weight and angler movement results were integrated with angler license data to estimate potential soil movement by anglers in southwestern Montana. The remaining samples (other boot-wader sample from the pair) were subsampled to create 0.25 g (dry weight) samples of soil. Subsampling was chosen instead of the density extraction procedure to reduce sample size because of the low myxospore recoveries associated with the density extraction (Chapter 1). Subsampling was achieved by decreasing sample volume to 120 ml and dividing the dry sediment weight (from other pair) by 120 ml to determine the volume needed to obtain 0.25 g of soil (dry weight). This quantity of soil was determined to be the maximum quantity of soil that could be present in a sample in order for single-round PCR to detect 100 *M. cerebralis* myxospores (Chapter 1). The subsamples were frozen and shipped overnight

to Pisces Molecular for single-round PCR analysis to test for the presence of *M. cerebralis* DNA.

Two-sample t-tests were used to determine if a statistical difference occurred between soil sample weight taken from anglers starting their angling for the day and those anglers completing their angling for the day. A general linear model and a least squares means multiple comparison procedure were used to determine if soil quantity was a function of river drainage.

Results

Angler Survey

Five-hundred and twenty-one anglers were asked to complete the survey and 487 anglers complied (93%). The number of surveys conducted varied by river (Table 2.2). A majority of the anglers were male (93%) and fished exclusively with fly fishing equipment (89%). Angling guides made up 13% of the anglers surveyed. Forty percent of the anglers surveyed were Montana residents whereas the remaining non-residents came from 44 states and 3 foreign countries.

Table 2.2– Number of surveys conducted, percentage of total surveys conducted, number of samples collected, and percentage of total soil samples collected at each of the selected rivers in southwestern Montana. Surveys were conducted in October of 2004 and from June through August of 2005 and 2006.

	Surveys		Soil samples	
River	Number	Percent	Number	Percent
Bighorn	211	43	54	52
Beaverhead	59	12	21	20
Gallatin	24	5	1	1
Madison	84	17	15	14
Missouri	57	12	9	9
Yellowstone	52	11	4	4

Most anglers surveyed were both wade fishing and boat fishing (52%) whereas 24% were fishing only from a boat and 24% were only wade fishing. Drift boats were the predominant type of boat used by anglers (Figure 2.2). Forty-two non-residents had rented at least one form of angling equipment (Montana residents not included because of small sample size, n = 6) (Figure 2.3). Overall, 62% of all non-resident anglers brought their own boots and waders.

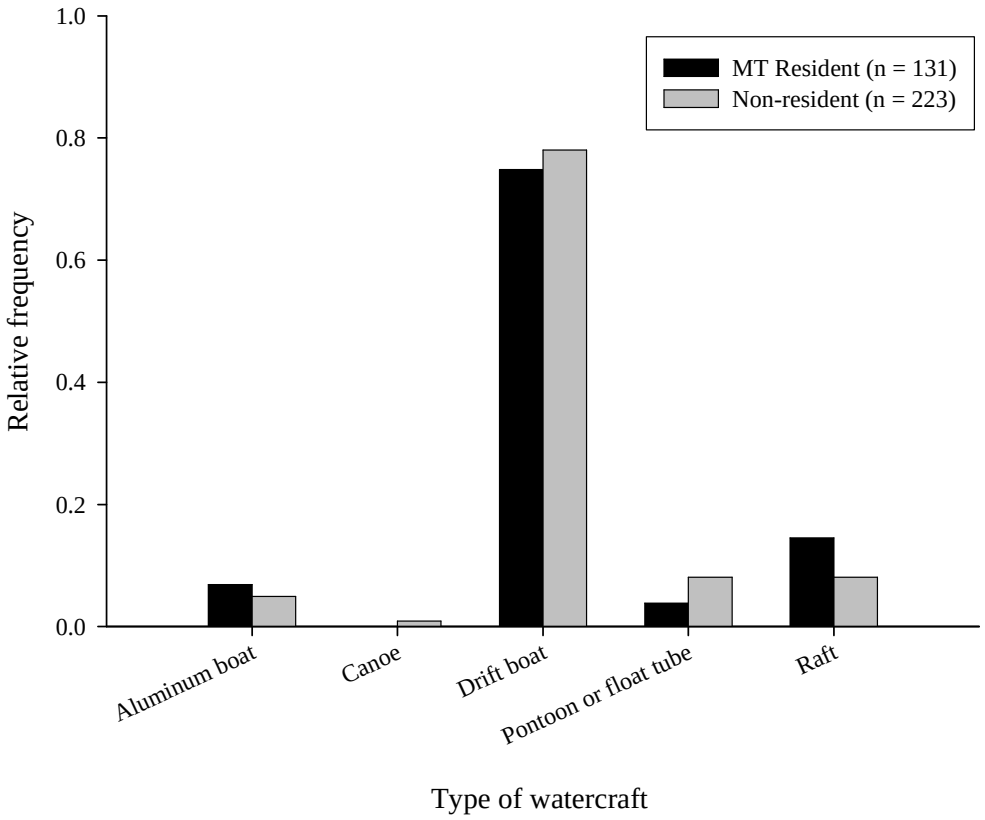

Figure 2.2--Relative frequency of different watercraft used by Montana resident and non-resident anglers fishing in southwestern Montana. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

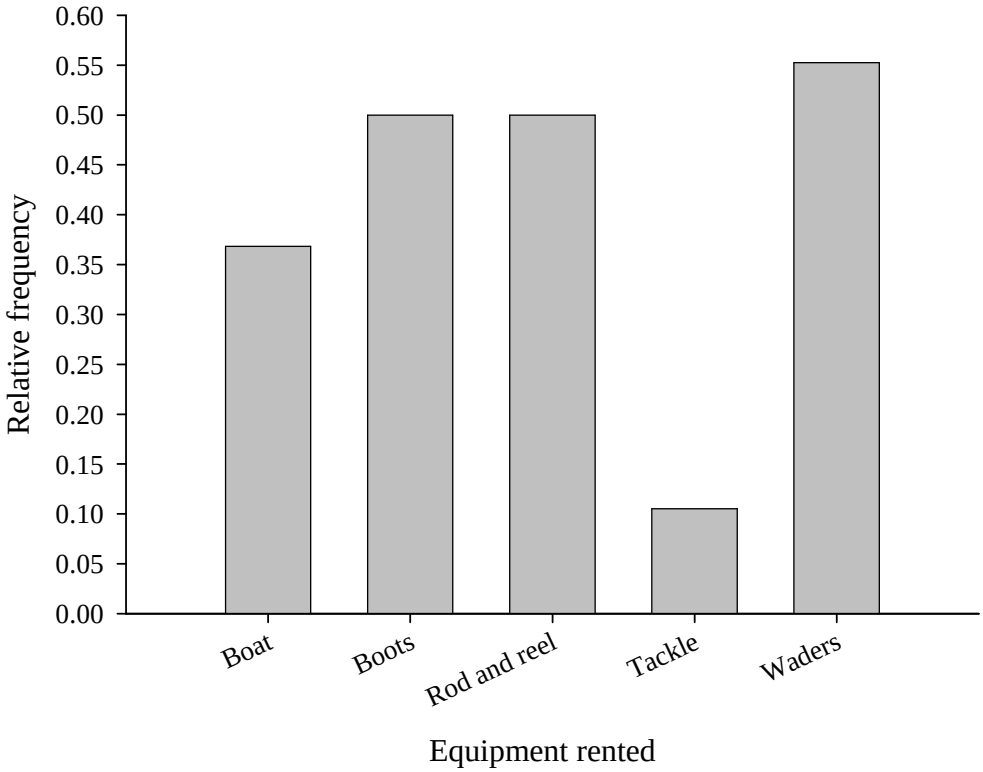

Figure 2.3.--Relative frequency of equipment rented by non-resident anglers (n = 42) surveyed in southwestern Montana. Montana residents not included due to small sample size. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

Forty-eight percent of anglers had waders and 57% had wading boots. Eighty-nine percent of waders were a lightweight type (including Gore-Tex®), 10% were neoprene, and 1% were rubber. Ninety-two percent of boots had felt soles and 7% of boots had rubber soles.

Mean distance traveled by Montana residents from their home zip code to the survey site was 115 km (± 17, 95% CI) whereas mean distance traveled by non-residents

was 1,738 km (± 74). Distance traveled by anglers from their home zip code to the fishing access site where they were surveyed differed significantly between Montana residents and non-residents ($t_{291} = 26.66$, $P = < 0.0001$).

Median number of fishing access sites used by anglers during a single day was two (Table 2.3). The distributions of number of fishing access sites used during a single day did not differ significantly between Montana residents and non-residents ($D = 0.13$, $P = 0.66$). However, the mean number of river miles fished by Montana residents and non-residents on a single day did differ significantly ($t_{91} = -2.00$, $P = 0.05$).

Table 2.3— Median number of fishing access sites used and mean number of river kilometers used between fishing access sites by anglers in southwestern Montana on the day they were surveyed. Ninety-five percent confidence interval for mean distance traveled are given in parentheses. Surveys were conducted in October of 2004 and from June through August of 2005 and 2006.

Day of survey	Number of sites	Distance (km)
All Anglers	2	10.4 (± 1.1)
MT Residents	2	11.7 (± 1.9)
Non-residents	2	9.5 (± 1.3)

The distributions of number of fishing access sites used by Montana residents (median = 2) and non-residents (median = 2) during 7 days prior to the survey were not significantly different ($D = 0.12$, $P = 0.46$) (Figure 2.4). Similarly, the distributions of number of drainages fished by Montana residents (median = 1) and non-residents (median = 1) during 7 days prior to the survey did not differ significantly ($D = 0.13$, $P = 0.37$) (Figure 2.5). Within 7 days of the current trip, anglers had visited 13 different rivers in Montana (Beaverhead, Bighole, Big Horn, Boulder, Gallatin, Jefferson,

Madison, Missouri, Musselshell, Rock Creek, Ruby, Stillwater, Yellowstone) and 20 different water bodies outside Montana (Black River (AZ), Blue River (CO), Colorado River (CO), Delaware River (DE), Grand Bahamas Island (Bahamas), Green River (UT), Henry's Fork (ID), Lake Powell (AZ), Lewis Lake (WY), Lochsa River (ID), Newton Lake (WY), N. Platte (WY), N. Tongue (WY), Oak Creek (AZ), Paradise Creek (AZ), Powder River (WY), Rush River (WI), Snake River (WY), Table Rock Lake (MO), Yellow Breeches (PA)).

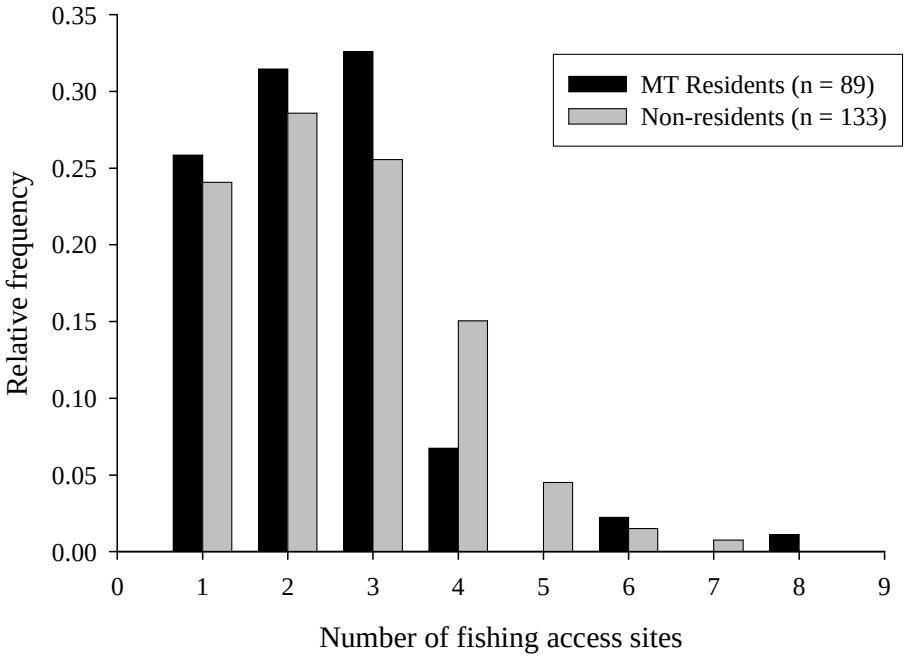

Figure 2.4-- Relative frequency of number of fishing access sites used by Montana resident and non-resident anglers 7 days prior to being surveyed, including the survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

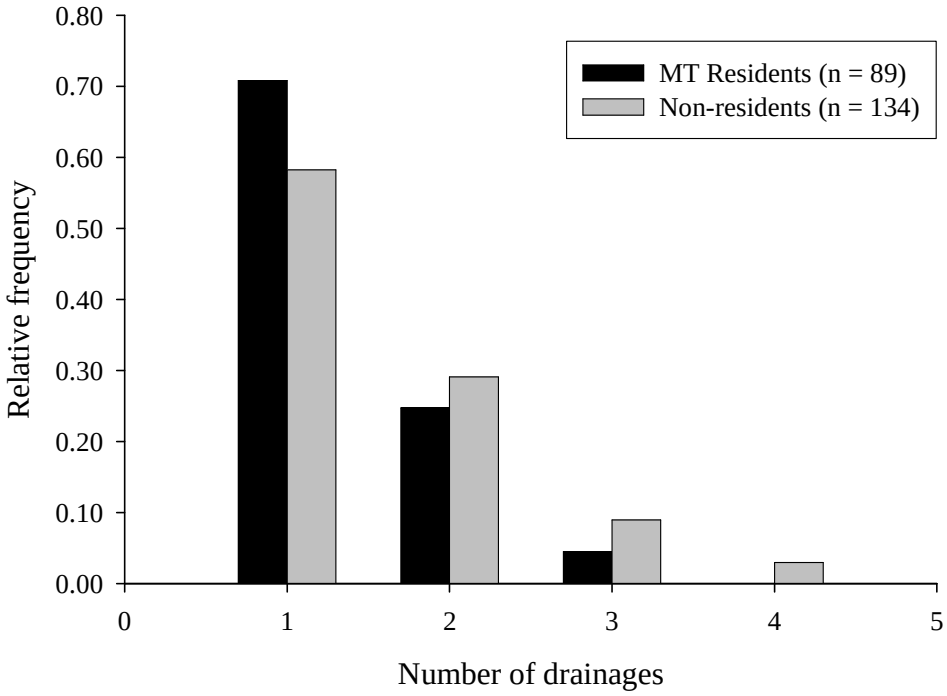

Figure 2.5-- Relative frequency of number of drainages fished in by Montana resident and non-resident anglers 7 days prior to being surveyed, including the survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

Distributions of number of fishing access sites used during 30 days prior to the survey by Montana residents and non-residents were not significantly different (D = 0.08, P = 0.97) (Figure 2.6). Similarly, the distributions of number of drainages fished during the 30 days prior to the survey by Montana residents and non-residents did not differ significantly (D = 0.06, P = 0.73) (Figure 2.7); however, non-residents were more likely to have fished in another state than Montana residents during the 30 days prior to the survey (D = 0.40, P = < 0.0001) (Figure 2.8).

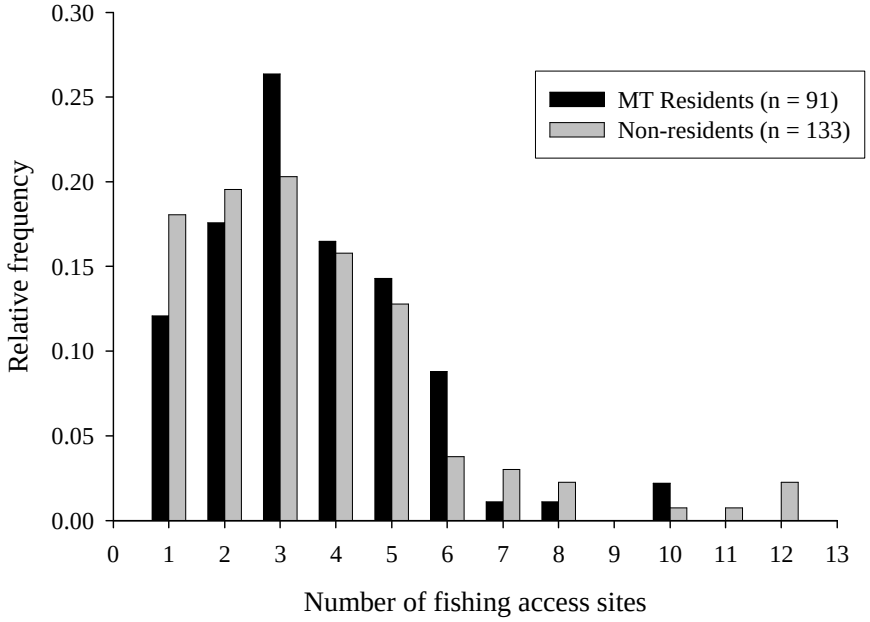

Figure 2.6-- Relative frequency of number of fishing access sites used by Montana resident and non-resident anglers 30 days prior to being surveyed, including the survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

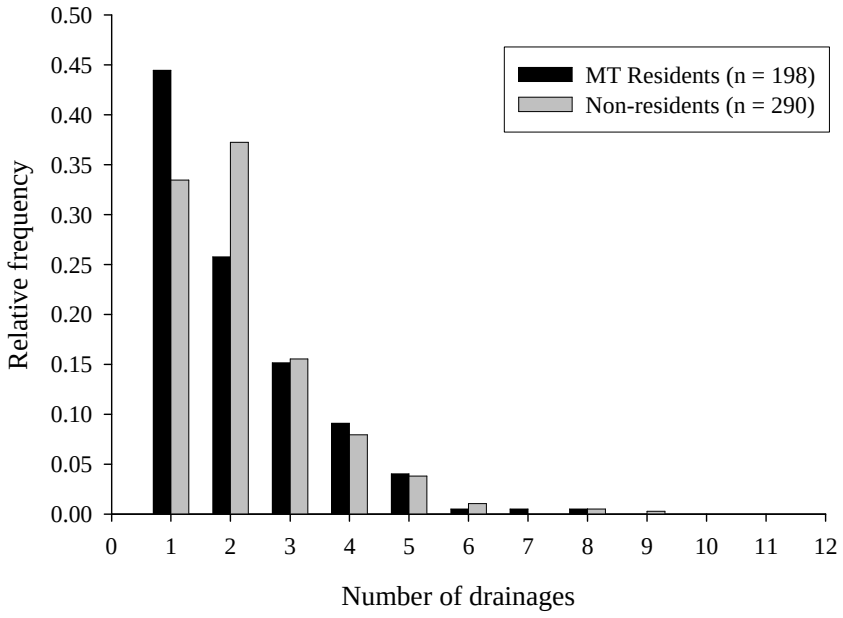

Figure 2.7-- Relative frequency of number of drainages fished by Montana resident and non-resident anglers 30 days prior to being surveyed, including the survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

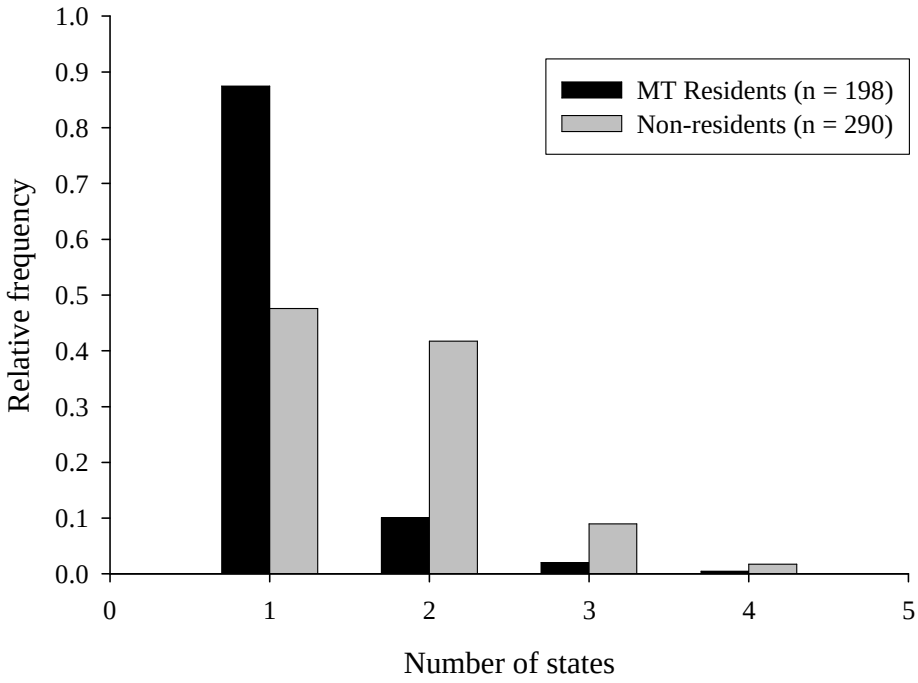

Figure 2.8-- Relative frequency of number of states fished by Montana resident and non-resident anglers 30 days prior to being surveyed, including the survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006.

Non-residents traveled more in the previous 7 days than Montana residents (non-resident mean = 726 km, resident mean = 251 km) ($t_{69} = 4.08$, P = 0.0001) (Figure 2.9). Non-residents traveled more in the previous 30 days than Montana residents (non-resident mean = 1,499 km, resident mean = 550 km) ($t_{289} = 7.44$, P = < 0.0001) (Figure 2.10). One non-resident angler had traveled 5,852 km in the previous 30 days to fish, having visited two drainages in Michigan, one drainage in Delaware, and one drainage in New Mexico.

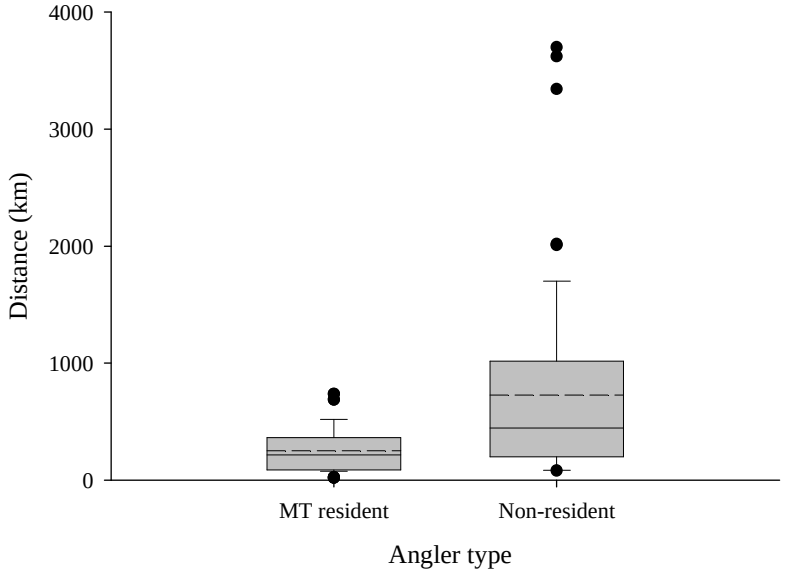

Figure 2.9-- Distance traveled to fish by Montana resident (n = 48) and non-resident anglers (n = 57) during 7 days prior to being surveyed. Distance includes travel to the survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006. Boxplots indicate interquartile range (25%, median, and 75%) and mean (dashed line). Solid circles represent data outside the 10 and 90 quantiles.

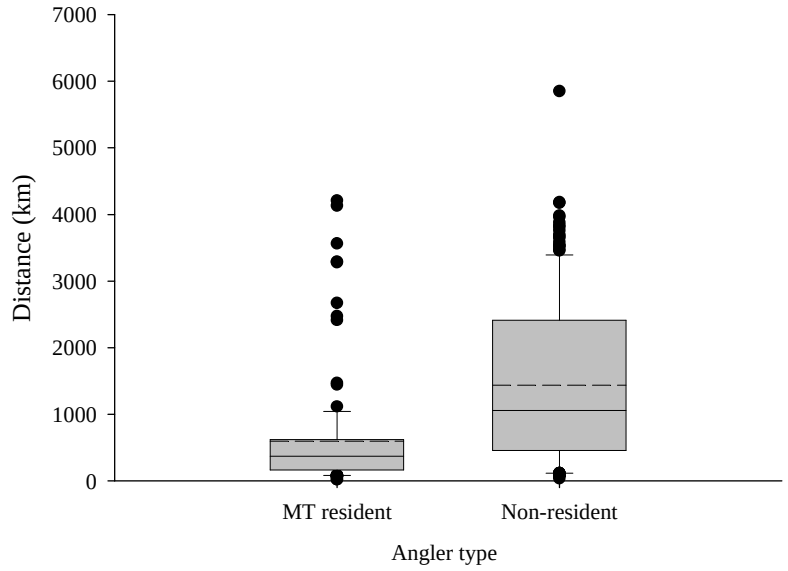

Figure 2.10--Distance traveled to fish by Montana resident (n = 109) and non-resident anglers (n = 188) during 30 days prior to being surveyed. Distance includes travel to survey site. Surveys were conducted during October of 2004 and from June through August of 2005 and 2006. Boxplots indicate interquartile range (25%, median, and 75%) and mean (dashed line). Solid circles represent data outside the 10 and 90 quantiles.

Seventy-four percent of Montana residents and 72% of non-residents planned to fish again in the next 7 days. Distributions of number of drainages planned to fish in the next 7 days did not differ significantly between Montana residents (median = 1) and non-residents (median = 1) ($D = 0.04$, $P = 1.0$) nor did the distributions of number of states planned to visit for fishing ($D = 0.1$, $P = 0.17$). Three percent of Montana residents and 14% of non-residents planned to fish out of state in the next 7 days.

Fifty-one percent of Montana residents and 49% of non-residents reported occasionally, rarely, or never cleaning their boots and waders in between uses (Figure 2.11). Twenty-five percent of Montana residents and 21% of non-residents reported that they only occasionally, rarely, or never completely dried their boots and waders between uses (Figure 2.11).

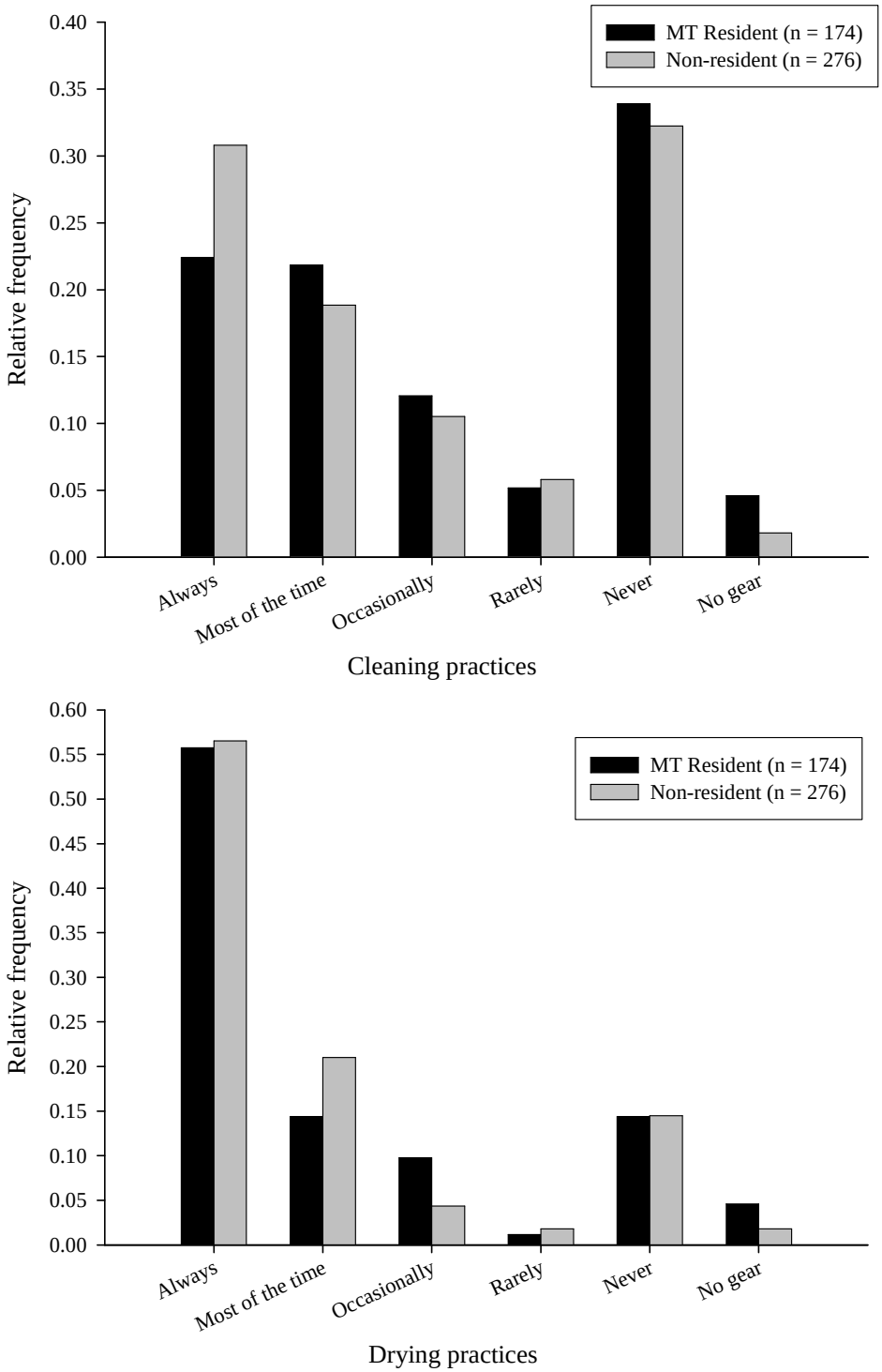

Figure 2.11--Relative frequency of responses of Montana resident and non-resident anglers as to how often they clean (top panel) and completely dry (bottom panel) their boots and waders between uses. Surveys were conducted in southwestern Montana during October of 2004 and from June through August of 2005 and 2006.

Ninety percent of both Montana resident and non-resident anglers were familiar with whirling disease (n = 414) (Figure 2.12). Ninety-three percent of Montana residents (n = 181) and 90% of non-residents (n = 265) were familiar with at least one of the following ANS: Asian carp (including grass carp *Ctenopharyngodon idella* and bighead carp *Aristichthys nobilis*), New Zealand mud snails *Potamopyrgus antipodarum*, whirling disease, or zebra mussels *Driessena polymorpha*. Most anglers (73%) had learned about ANS from a magazine (n = 332). The next most common source of ANS information was newspapers (59% n = 268).

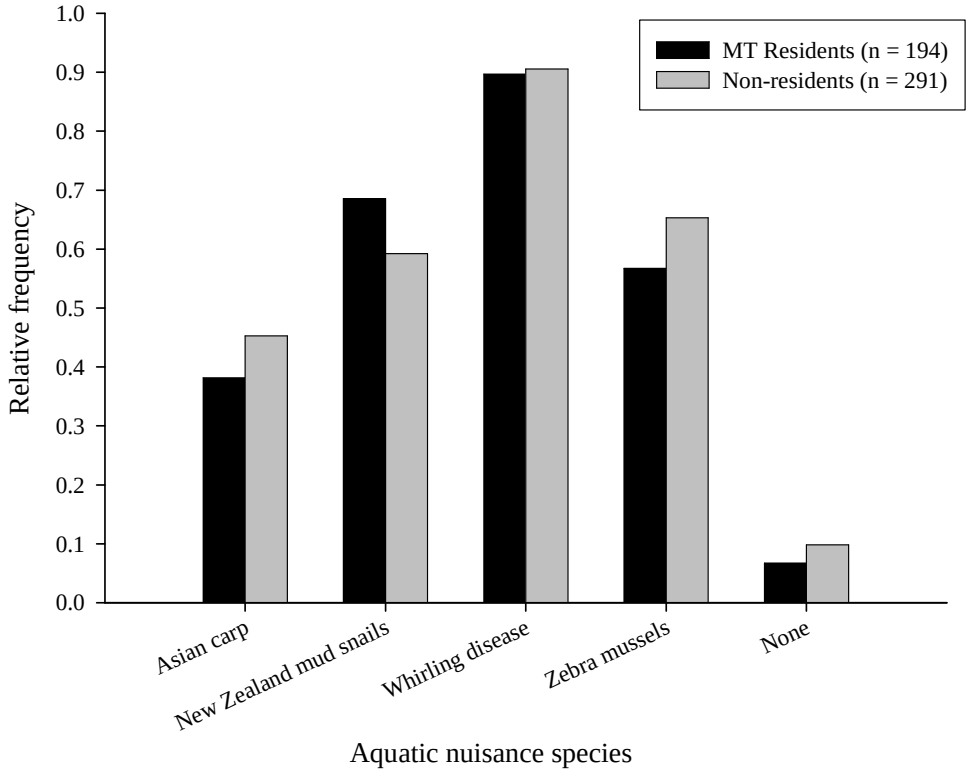

Figure 2.12-- Relative frequency of Montana resident and non-resident anglers regarding awareness of different aquatic nuisance species. Surveys were conducted in southwestern Montana during October of 2004 and from June through August of 2005 and 2005.

Thirty-nine percent of all anglers surveyed indicated that they were an active member of an angling organization such as Trout Unlimited, Federation of Fly Fishers, or Walleyes Unlimited. Fewer Montana residents were members of an angling organization (35%) than non-residents (42%).

Distributions of number of fishing access sites visited in previous 7 days differed significantly between resident angling organization members (median = 3) and non-members (median = 2) (D = -0.49, P = 0.0006). Distributions of number of drainages visited in previous 30 days differed significantly between resident angling organization members (median = 2) and non-members (median = 1) (D = 0.21, P = 0.05). Boot and wader cleaning practice distributions also differed significantly between resident members (median = 1 = clean always) and non-members (median = 4 = clean rarely) (D = 0.29, P = 0.002).

Distance traveled from home zip code differed significantly between non-resident angling organization members (mean = 1,998 km, ± 190) and non-members (mean = 1,574 km, ± 150) (t_{272} = -3.51, P = 0.0005). Distribution of number of drainages visited in previous 30 days differed significantly between non-resident angling organization members (median = 2) and non-members (median = 2) (D = 0.22, P = 0.004). Distributions of number of states visited to fish in the previous 30 days differed significantly between non-resident angling organization members (median = 1) and non-members (median = 0) (D = 0.20, P = 0.008). Non-resident members distribution of drainages planned to visit in the next 7 days also differed significantly from non-resident non-members (D = 0.18, P = 0.04).

Soil Sampling

One-hundred and six boot-wader samples were collected from 27 Montana residents, 70 non-residents, and 9 anglers of unknown residency. The number of samples collected varied by river (Table 2.2). A low number of samples (44) were obtained in June, July, and August of 2005. During 2006, 223 anglers were asked for soil samples and 62 samples were obtained (28%). Of the anglers not sampled in 2006, 25% did not have any gear with them and 75% declined to provide a sample. Of the anglers who declined to provide a sample in 2006, 33% were Montana residents, 59% were non-residents, and 8% were of unknown residency.

The predominant types of gear sampled were lightweight (including Gore-Tex) waders (92%) and felt soled boots (92%). Only eight of the soil samples obtained were rinsed from anglers wearing neoprene waders or rubber soled boots or both.

Mean quantity of soil carried on one boot-leg was 8.39 g (± 1.50). Mean quantity of soil carried by Montana resident and non-resident anglers did not differ significantly ($t_{101} = -1.59$, $P = 0.11$). Mean quantity of soil carried on one boot-leg decreased from 10.69 g (± 2.62) in 2005 to 6.20 g (± 1.22) in 2006. Soil from southwestern Montana transported by non-resident anglers could potentially spread throughout the United States (Figure 2.13).

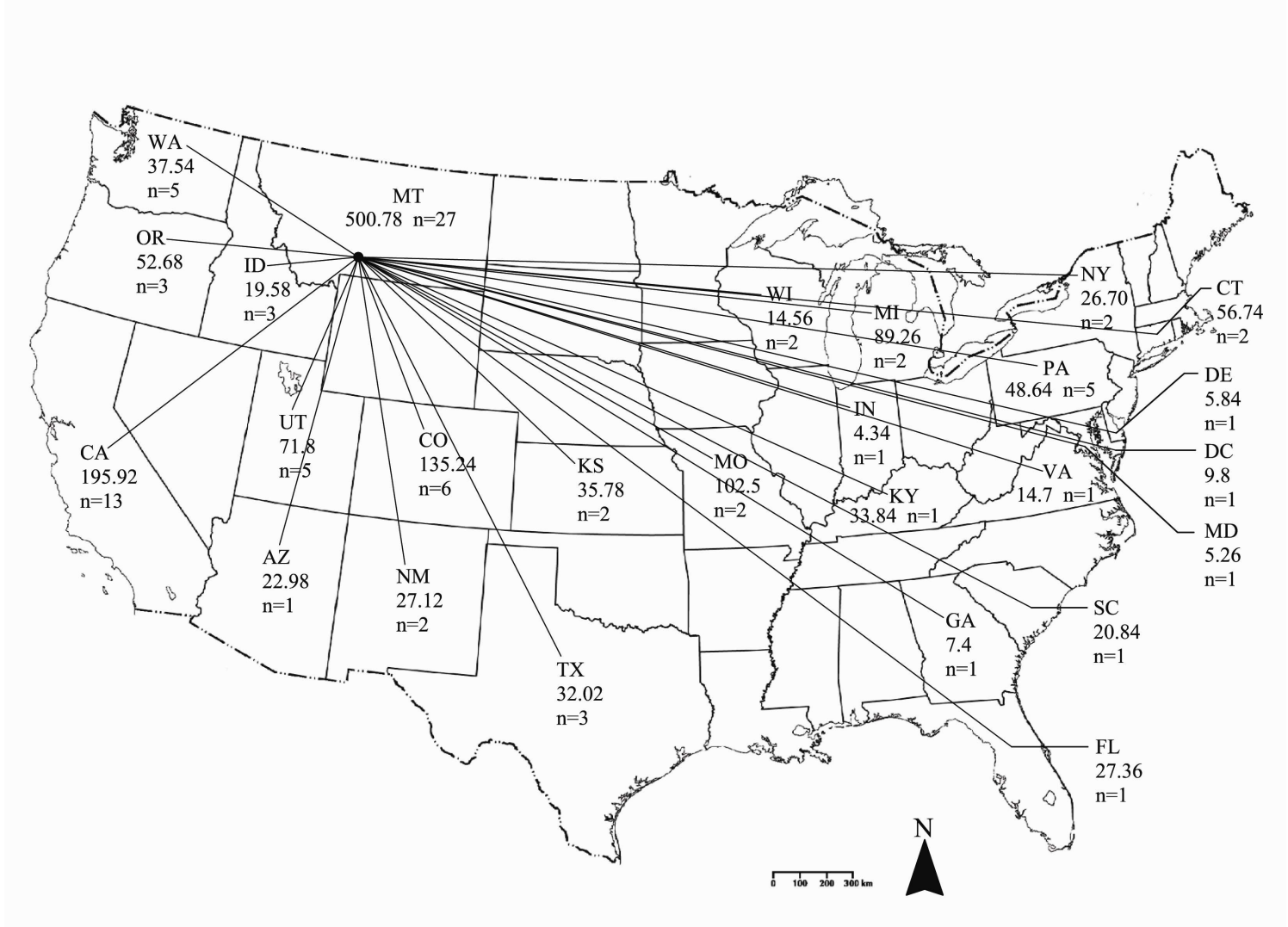

Figure 2.13– Map indicates quantity of soil (g) rinsed from anglers' boots and waders in southwestern Montana that could have been returned to their state of residency. Samples were collected from June through August of 2005 and 2006. Total quantity carried by each angler was estimated from one boot and wader leg.

The quantity of soil carried did not differ significantly between samples taken from anglers starting their trip and those anglers completing their trip (t_{90} = -0.81, P = 0.42). The quantity of soil differed significantly by the river where the sample was taken ($F_{5, 98}$ =3.83, P = 0.003) (Figure 2.14). Soil samples from the Missouri river were significantly heavier than those from the Beaverhead (P = 0.05) and Bighorn (P = 0.003).

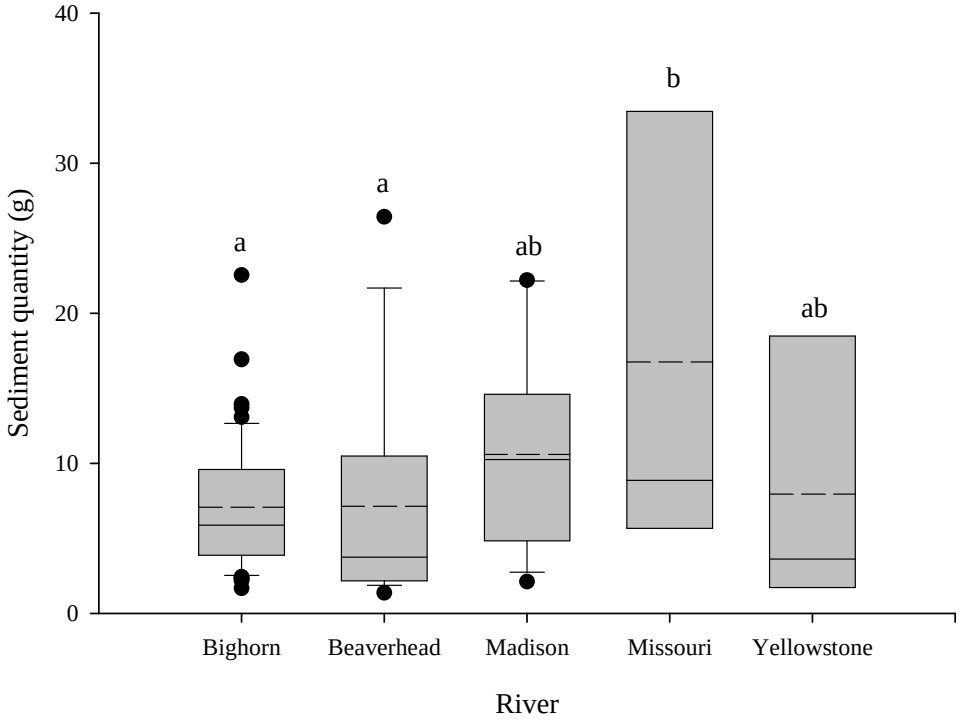

Figure 2.14-- Sediment quantity (g) rinsed from one leg of the boot wader combination of anglers in southwestern Montana. Samples were collected from June through August of 2005 and 2006. Boxplots indicate interquartile range (25%, median, and 75%) and mean (dashed line). Solid circles represent data outside the 10 and 90 quantile. Boxplots labeled with the same letter do not differ significantly. Gallatin River not included due to small sample size.

All 106 soil subsamples were negative for the presence of *M. cerebralis* DNA. The detection limit for the testing was at or above 100 myxospores per 0.25 g of soil

(Chapter 1). Dried soil screening found one dead New Zealand mud snail in a sample taken on the Madison River (Dr. Daniel Gustafson of Montana State University).

Integrating angler fishing access site usage and quantity of soil carried with angler license data provided a potential worst-case scenario for the quantity of soil moved by anglers in southwestern Montana. I assumed that soil quantity carried was additive for the following calculation even though the actual quantity of soil deposited at each site was unknown. Each angler wearing boots and waders was potentially transporting an average of 16.78 g of soil between each of the fishing access sites. There were 138,730 fishing licenses sold in 2005 for the six counties where this study was conducted (37,060 resident and 101,670 non-resident) (Kal Njos, Montana Fish, Wildlife and Parks, personal communications) (Appendix B). Using the above data, 57% of surveyed anglers had waders or boots or both; thus, 79,076 anglers had gear similar to the type sampled. Further, 28% of anglers always cleaned their equipment between uses; therefore, 56,935 anglers could be transporting soil. Thus, 955 kg (0.01678 kg/angler x 56,935 anglers) of soil were potentially moved by resident and non-resident anglers who fished once. The average angler fished at 3 fishing access sites in the prior 30 days. Thus, 2,865 kg (955 kg/angler x 3 access sites used) of soil were potentially moved in 2005 in southwestern Montana. Similarly, considering non-residents only, 62% of non-residents brought their own boots and waders; thus, 63,035 non-resident anglers had gear brought from home. Further, 31% of non-resident anglers claimed to always clean their equipment between uses; therefore 43,494 non-resident anglers potentially took their un-cleaned boots and waders home. Thus, 730 kg (0.01678 kg/angler x 43,494 non-resident anglers) of soil

were potentially transported from southwestern Montana around the world by non-resident anglers in 2005.

Discussion

The frequency with which both resident and non-resident anglers fished and the number of rivers fished within 7 days of the survey support preliminary work conducted by Montana Fish, Wildlife and Parks suggesting that anglers in southwestern Montana are highly mobile. The frequency of fishing activities of resident and non-resident anglers did not differ. Montana residents and non-residents visited the same number of drainages in the prior 30 days, but non-residents traveled much farther to visit the same number of drainages, because they had fished in their home state during those 30 days.

Resident and non-resident angling organization members were more mobile than non-members. The greater mobility of angling organization members supports previous research regarding the lifestyle of specialized fly fishers (Hahn 1991). Anglers who fish exclusively with fly fishing equipment tend to travel frequently to fish (Hahn 1991). Survey results also indicated that non-resident angling organization members traveled significantly farther to fish from their home than non-members. The increased mobility exhibited by angling organization members indicates that they may have a greater potential to transport soil, *M. cerebralis*, and other ANS among fishing locations.

Predictions for angler transport of *M. cerebralis* can be made by comparing documented angler transport of other nuisance species such as the Eurasian zebra mussel *Dreissena polymorpha*. The rapid dispersal of *D. polymorpha* in the Great Lakes Region

has been attributed to their high fecundity and pelagic larval stage, which allows them to disperse downstream using water currents (Griffiths et al. 1991). Downstream passive dispersal of *D. polymorpha* has been estimated using current speed information; however, dispersal events across land indicate that human activities represent another primary vector (Griffiths et al. 1991; Johnson et al. 2001; Johnson et al. 2006). *Dreissena polymorpha* can remain viable out of water for up to four days allowing mussels to survive overland movement attached to boats (Griffiths et al. 1991). Boat hulls can provide good habitat for settling mussels implicating both Great Lakes Region commercial and recreational boating activities in the transport of *D. polymorpha* larvae by water and over land when boats are trailered (Griffiths et al. 1991; Johnson et al. 2001). In addition, anglers and boaters can unknowingly transport *D. polymorpha* between waterways by not properly cleaning their boat and trailer between uses (Griffiths et al. 1991; Johnson et al. 2001; Johnson et al. 2006). Considering some of the similarities between *D. polymorpha* larvae and *M. cerebralis* myxospores (i.e., their ability to survive overland transport and their dispersal through water) it is possible that myxospores are also being transported by humans on soil laden waders, boots, boats, and trailers. Angler movement data from this study indicate that anglers are moving to and from watersheds both infected and not infected with *M. cerebralis* creating a potential pathway for myxospores to be transported much like the transport of *D. polymorpha* by anglers in the Great Lakes Region.

Anglers in southwestern Montana could be contributing to repeated fishing access site introductions of *M. cerebralis* and other ANS by frequently moving among access

sites carrying soil on their equipment and thus, making access sites 'hot spots' where anglers are more likely to come in contact with ANS. Research focusing on controlling non-native plant invasions has suggested that many invasions are caused by the intentional or accidental introduction by humans (Richardson et al. 2000). The introduction of non-native species by humans virtually eliminates the role of geographic barriers to introduction allowing species to be transported to habitats where they would otherwise rarely be found (Richardson et al. 2000). The successful colonization of introduced non-native species requires a high level of repeated introductions to an appropriate habitat or the surpassing of environmental barriers that affect survival (Richardson et al. 2000; Sakai et al. 2001; With 2002). This suggests that repeated site introductions by humans may allow introduced species to not only surpass geographic barriers, but also surpass colonization hurdles permitting them to become established more easily.

Angling equipment soil samples were taken from anglers who were entering and exiting the water allowing us to make inferences regarding soil being transported out of southwestern Montana by non-residents. Benthic soil from southwestern Montana may contain *M. cerebralis* myxospores, New Zealand mud snails, or other nuisance species such as noxious weed seeds. The potential quantity of soil being transported out of the state by non-residents illustrates how easily ANS from Montana could be spread elsewhere. Aquatic nuisance species from Montana could be spread virtually anywhere in the world because of the state's global fly fishing industry.

I likely underestimated the number of anglers who do not clean their equipment between uses to prevent carrying soil among sites. Although I identified myself prior to each interview as not representing a governing entity, my presence at access sites may have influenced the responses of some anglers regarding the frequency with which they clean and dry their equipment between uses because these practices reduce the risk of spreading ANS.

The low percentage of anglers who reported always cleaning their equipment between uses (34% of Montana residents and 38% of non-residents) is especially troubling when combined with angler knowledge of ANS. Most fly fishing anglers fishing in southwestern Montana are familiar with ANS; however, many of them are not taking the necessary precautions to help prevent the spread. Montana resident angling organization members were more likely to clean their equipment most of the time as opposed to non-members who rarely cleaned their equipment; however, anglers need to always clean equipment between uses to prevent movement of ANS. The disconnect between angler knowledge of ANS, conservation identity, and equipment cleaning practice suggests that fly fishing anglers in southwestern Montana are failing to make the connection between the harm that ANS cause and their potential personal role in transporting ANS. Although the survey did not assess the causes of angler non-compliance with cleaning recommendations, lack of a consistent and cohesive prevention message may have contributed. Development of a simple and standard message for anglers to wash their equipment with soap and water between uses may decrease

confusion and complacency. This message could be delivered to anglers in a brief educational video that is watched prior to receiving a Montana fishing license.

Although all of the soil samples tested negative for the presence of *M. cerebralis* myxospore DNA, myxospores may have been present nevertheless. The negative score indicates that in the 0.25 g subsample used for PCR analysis there were not more than 100 myxospores. With single-round PCR, only 400 myxospores or more per gram of soil could be detected and only 0.25 g of soil could be processed at a time (Chapter 1). Although all soil samples were agitated prior to subsampling, the possibility exists that myxospores present in the sample may not have been drawn in the subsample. Myxospores may also have been present in concentrations of less than 400 per gram, and were therefore not detectable with single-round PCR. In addition, one quarter of the soil samples came from anglers exiting the Bighorn River, which remains uninfected by *M. cerebralis* (Eileen Ryce, Montana Fish, Wildlife and Parks, personal communications).

I did not document transport and release of large quantities of *M. cerebralis* myxospores among access sites; however, anglers are moving soil among sites. A concurrent study at the University of Oregon revealed preliminary results that *M. cerebralis* myxospores could be transported on angling equipment in a controlled laboratory environment (David Latremouille, Oregon State University, personal communications). This information coupled with the mobility of anglers in southwestern Montana suggests that transport of subsequent ANS is likely. Control of future ANS infestations will be difficult unless sediment transport is addressed by banning the use of

felt soled wading boots, requiring river specific wading equipment, or providing mandatory equipment cleaning stations.

Human transport of exotic species around the world via transportation networks has been well documented. Increased global human travel and commerce in recent decades combined with human population growth have accelerated the rates of invasive species introductions in virtually every ecosystem (Cohen and Carlton 1998). The broad and frequent movement of anglers in southwestern Montana suggests that preventing future introductions of ANS will be challenging. Efforts could be better focused on slowing the spread of existing non-native species, containing current infestations, and managing those areas already infected.

CHAPTER 3

ADHERENCE OF *MYXOBOLUS CEREBRALIS* MYXOSPORES TO WADING EQUIPMENT MATERIALS

Introduction

The vectors involved in the spread of *Myxobolus cerebralis* are only partly understood; however, the parasite has become well established in many places and is responsible for major declines or near elimination of some salmonid year classes in Montana and throughout North America (Nehring and Walker 1996; Vincent 1996; Bergersen and Anderson 1997). *Myxobolus cerebralis* was first documented in Montana in the Madison River in 1994 (Vincent 1996). Beginning in 1991, area biologists noted declines in age-0 rainbow trout *Oncorhynchus mykiss* abundances in the Madison River basin, specifically within the Pine Butte study section (Vincent 1996). Over the next three years, the reduced abundances of age-0 fish spread downstream culminating in a 90% reduction of historic averages of the 1970s and 1980s (Vincent 1996).

The exact introduction source of *M. cerebralis* in the Madison River is not known. Stocking of catchable rainbow trout in the Madison River ceased in the early 1970s thus, *M. cerebralis* was not transferred by stocking of infected fish by state agencies (Vincent 1987). In addition, the disease-free status of private and federal hatcheries in Montana suggests that transport of infected hatchery fish prior to the 1970s was not the source of the infection (Baldwin et al. 1998).

After the original positive test for *M. cerebralis*, Montana Fish, Wildlife and Parks began an extensive survey of its wild trout fisheries in an attempt to quantify the statewide level of infection (Baldwin et al. 1998). Over 20,000 fish from 230 watersheds within the state were tested for the presence of *M. cerebralis* (Baldwin et al. 1998). Within five years of the initial infection in the Madison River, nine major drainages in Montana were identified as infected including the Beaverhead, Clark Fork (above the Bitterroot River), Jefferson, Madison, Missouri (above the Marias River) and the Yellowstone (above the Bighorn River) (Baldwin et al. 1998). Six of the infected drainages contained infected fish prior to 1995; however, no one drainage could be singled out as the initial site of infection making patterns of spread virtually impossible to determine (Baldwin et al. 1998).

Determining *M. cerebralis* transport vectors is a priority for managing the parasite. Some potential vectors include shipment of infected fish to new areas, fish-eating birds, pet store trade in aquatic oligochaetes, anglers, boats, motors, and other aquatic related recreational equipment (Meyers et al. 1970; Halliday 1976; Bergersen and Anderson 1997). In addition, the transfer of the parasite from one drainage to another could result from water or soil being moved among sites (Bergersen and Anderson 1997; Baldwin et.al. 1998). Areas within rivers where fish are most affected by the parasite also correspond with areas of increased habitat degradation and increased siltation (Allendorf 2001; Nickum and Bartholomew 2001) and myxospores may be more likely to be found in such areas (Nickum and Bartholomew 2001). Fishing access sites represent

areas of concentrated resource use and may coincide with areas of increased siltation and degradation.

The ability of anglers and recreationists to transport soil and water containing *M. cerebralis* myxospores among access sites depends on the type of equipment used at access sites and the exposure of that equipment to water and soil. Many anglers in southwestern Montana use wading boots and waders when fishing. The predominant types of wading equipment used by anglers are a lightweight type (breathable laminate including Gore-Tex®), neoprene, felt, and rubber. Anglers in southwestern Montana predominantly use wading equipment made with a lightweight type and felt materials (see Chapter 2). The surface patterns and properties of these materials may dictate whether *M. cerebralis* myxospores are likely to adhere to them or become lodged within them when exposed.

Little can be done to prevent the future spread of *M. cerebralis* to new rivers without an understanding of how the disease is being transported among rivers. A better understanding of the mechanisms of secondary transport and dispersal would allow for better prediction of future spread (Johnson et al. 2001). Such information would also aid the development of effective control strategies. The objectives of this study were to quantify the surface patterns of rubber, felt, lightweight, and neoprene determine if *M. cerebralis* myxospores adhere to these materials.

Methods

Surface Patterns of Wading Materials

Four material types were tested including rubber, felt, lightweight, and neoprene. Wading materials varied in their design and construction. The lightweight material included two layers of nylon, a coating, and a waterproof laminate. The neoprene material consisted of a neoprene foam layer with fabric layers adhered to the top and underside of the foam. Rubber material consisted of rubber adhered to an underside layer of fabric. Felt was a dense mat of randomly woven fibers. Images of all material types were taken using a Scanning Auger Electron Microprobe at the Montana State University Image and Chemical Analysis Laboratory (ICAL). Material squares were plated with iridium to provide a better reflecting surface for the microprobe electron beam. Images were taken at 200x magnification. Magnified images revealed different fiber weaving patterns between two commercial brands of lightweight material and neoprene. Thus, two types of lightweight material and two types of neoprene were used in this experiment to account for different fiber weaving patterns. The selected lightweight materials came from waders that the manufacturers considered their 'value' lightweight breathable laminate waders costing less than $180.

I measured interstitial spaces within each material. Material images were imported into Adobe Photoshop (Adobe Systems Inc. 2003) and vertical transects were placed over each image at 1 cm spacing. The resulting overlay contained 36 vertically oriented transects. Images were calibrated in Sigma Scan Pro (SPSS Inc. 1999) according to the scale imprinted on each image by the Scanning Auger Electron

Microprobe. A random number table was used to select vertical transects and the interstitial spaces along that transect were measured. For these measurements, the images were assumed to be two-dimensional with no account for depth of fibers. An interstitial space was defined as a gap between adjacent fibers. Three transects were randomly selected for each of the six materials and the interstitial spaces along each transect were measured.

Myxospore Adherence to Wading Materials

Infected rainbow trout were supplied by the Montana State University Aquatic Sciences Laboratory. Myxospores were isolated from whirling disease-infected trout using the continuous plankton centrifuge method (O'Grodnick 1975). Myxospore abundances were estimated using a 0.4 mm Neubauer hemocytometer, compound microscope, and hand counter (Markiw and Wolf 1974). Counts were replicated three times per grid on the hemocytometer. When the three replicate counts exceeded 10% ± the mean, additional replicate counts were taken. After myxospore concentration was estimated, a known concentration was diluted with de-chlorinated water to obtain a larger volume to myxospore ratio. This solution of myxospores was agitated for 2 min prior to each sample being drawn to maintain an even distribution of myxospores throughout the solution.

About 2.E+04 myxospores were drawn from the extracted solution with a micropipette, the micropipette tip was positioned directly on the surface of the material, and the myxospores were expelled pressing them onto a 3 cm^2 piece of material. The myxospore solution remained on the material for 7.5 min to allow myxospores to settle.

This length of time corresponds with the settling rate of silt particles (Tan 1996), which are of a comparable size and density to *M. cerebralis* myxospores (see Chapter 1). The material square was then rinsed for 1 second at 2.11 kg/cm^2 with a hand-pump pressure sprayer containing water and aqueous sodium hexametaphosphate at a concentration of 6200 mg $[NaPO_3]_6$/L (Lemmon and Kerans 2001). The 1-second interval was the estimated fraction of time that a 3 cm^2 piece of a boot or wader would be sampled if the leg was rinsed from the knee down for 30 seconds (see Chapter 2). The rinse was collected in a 50 ml centrifuge tube. Aqueous $[NaPO_3]_6$ was added to the rinse to increase the volume to 5 ml. The rinse solution was then agitated for 4 min by repeated inversion of the centrifuge tube to evenly distribute the rinsed myxospores throughout the solution.

Following agitation, the number of myxospores rinsed from the material was estimated using a 0.4 mm Neubauer hemocytometer, compound microscope, and hand counter (Markiw and Wolf 1974). Counts were replicated three times per grid on the hemocytometer (Lemmon and Kerans 2001). When the three replicate counts exceeded 10% ± the mean, additional counts were taken. This process was repeated for all six material types (rubber, felt, lightweight type 1, lightweight type 2, neoprene type 1, and neoprene type 2) and a control (glass). Treatments (materials) were replicated three times to produce a mean percent myxospore recovery for each treatment.

Variability in the hemocytometer counting procedure produced variability in the amount of myxospores that were used in the experiment. To measure the variability in counts I calculated 95% confidence intervals for the myxospore quantities added to the

treatments in each experiment. The confidence intervals are reported as a mean 95% confidence interval because the experiment was conducted over the course of several days and confidence intervals were calculated for each day. All measures of variability are listed as ± 95% confidence intervals.

An ANOVA and least squares means multiple comparison procedure were used to determine if interstitial space size differed among materials (SAS Institute 2005). The Kruskal-Wallis non-parametric ANOVA with a chi-square approximation and the Nemenyi non-parametric multiple comparison test were used to determine if percent recovery differed among material type (Zar 1999).

Results

Surface Patterns of Wading Materials

Rubber had the smallest mean interstitial space size (2.0 µm) and the fewest number of spaces (n = 5) (Figures 3.1 and 3.2). Felt had the largest mean interstitial space size (31.364 µm). The two lightweight materials had the greatest number of interstitial spaces (lightweight 1 n = 53 and lightweight 2 n = 40); however, the interstitial spaces were small (< 8 µm) (Figure 3.1). The different fiber weave patterns of the two types of lightweight and the two types of neoprene caused variation in the mean interstitial space sizes within material types (Figure 3.2). Interstitial space size among materials differed significantly ($F_{5, 12} = 8.76$, $P = 0.001$). Felt had significantly larger interstitial spaces than all other material types (Figure 3.2).

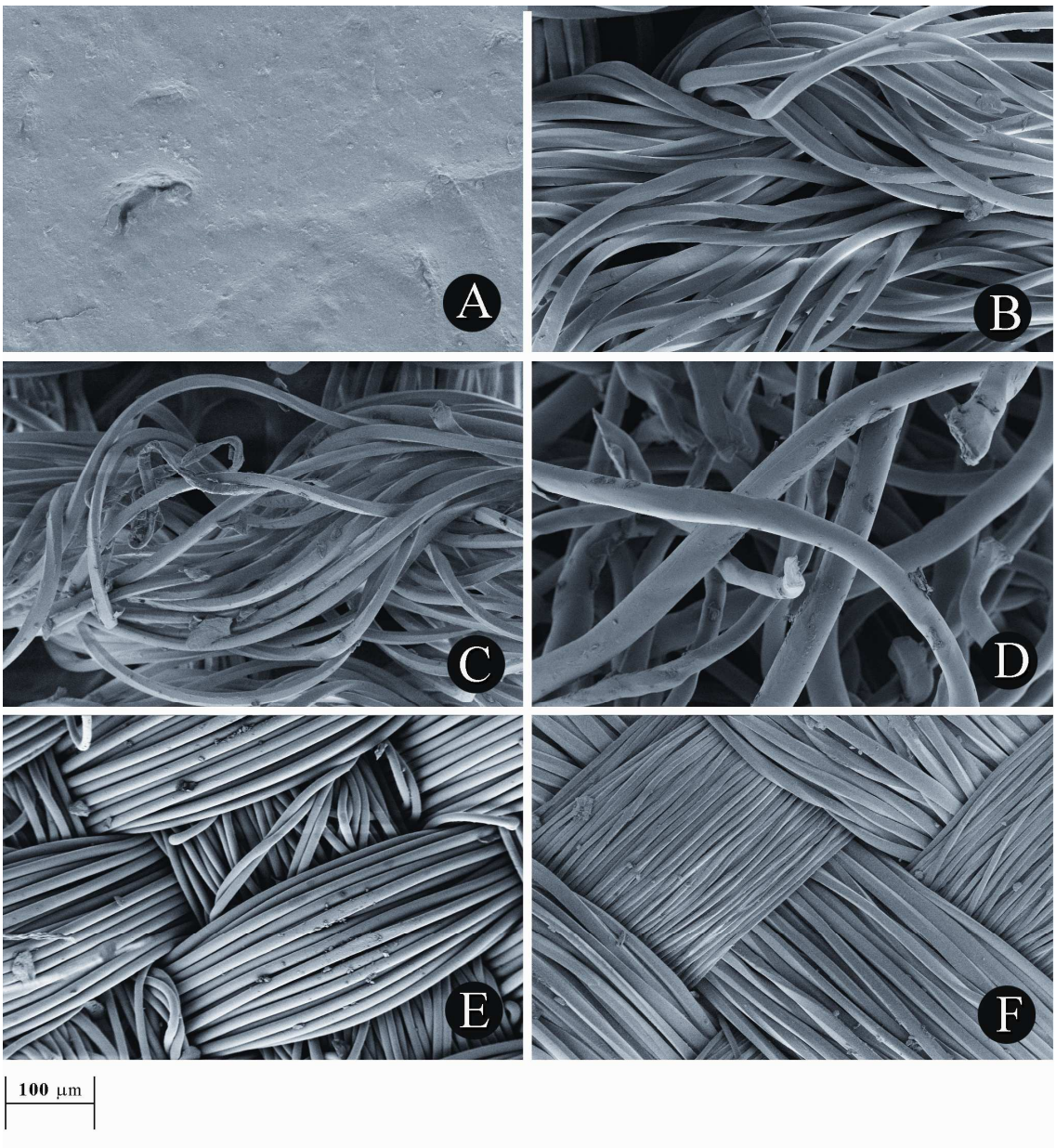

Figure 3.1— Wading equipment material type images taken at 200x magnification. A) rubber, B) neoprene 1, C) neoprene 2, D) felt, E) lightweight 1, and F) lightweight 2.

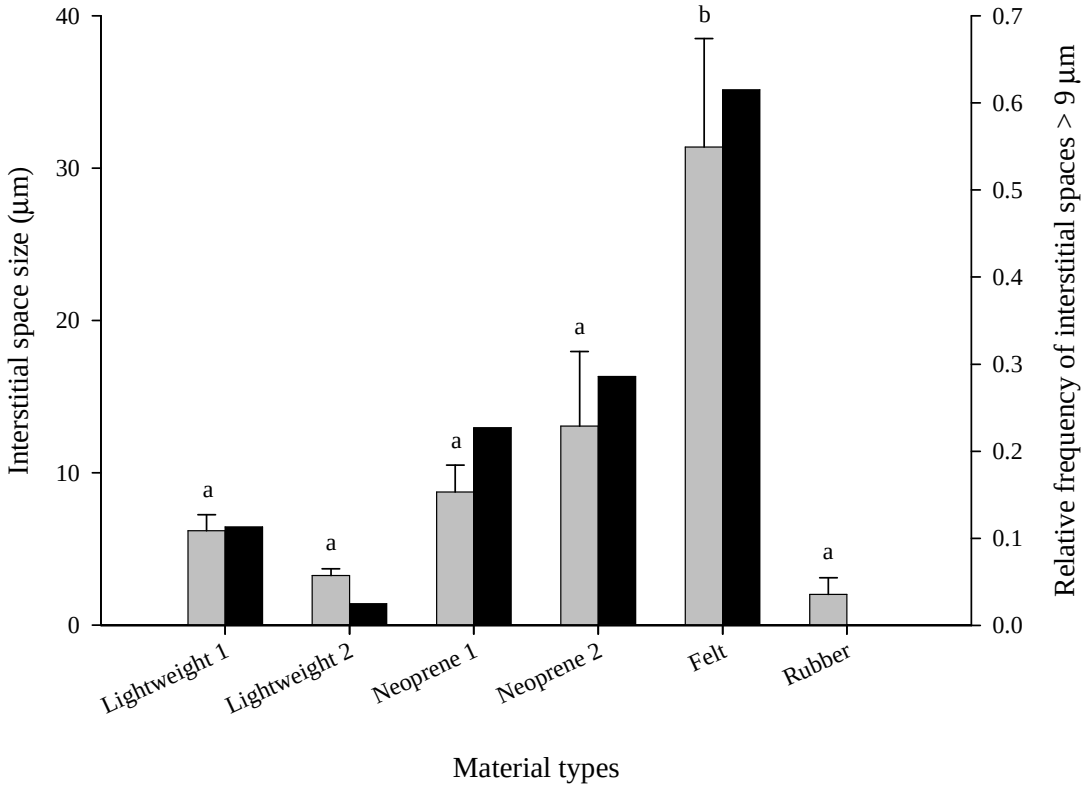

Figure 3.2-- Mean interstitial space size for various materials (grey bars) and relative frequency of interstitial spaces measured greater than 9 µm (black bars). Measurments were taken along randomly selected transects of a 200x magnification image. Grey bars labeled with the same letter do not differ significantly. Error bars delineate one standard error.

Myxospore Adherence to Wading Materials

Material treatments were exposed to 20,000 ± 3,367 myxospores. The highest percent myxospore recoveries came from rubber and the glass control (Figure 3.3). Mean percent myxospore recovery varied significantly by material ($\chi^2_6 = 12.43$, P = 0.05). Mean percent recovery from felt was significantly less than from rubber and the control (Figure 3.3). Felt trapped all myxospores, neoprene 2 trapped 27% of myxospores, and neoprene 1 trapped 16% of myxospores.

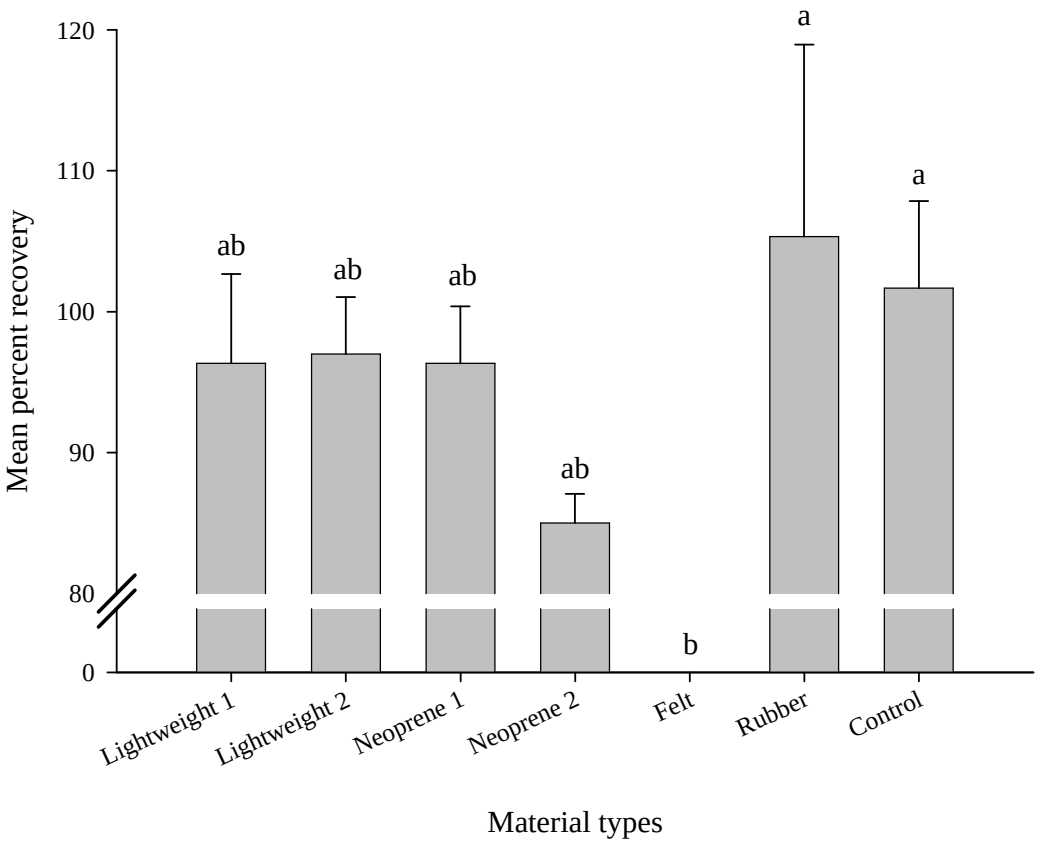

Figure 3.3-- Mean percent myxospore recovery by material type. Bars labeled with the same letter do not differ significantly. Error bars delineate one standard error.

Discussion

Because *M. cerebralis* myxospores measure about 8.7 µm in length and 8.2 µm in width (Lom and Hoffman 1971; Nehring et al. 2003), any material containing interstitial spaces greater than or equal to 9 µm could potentially trap myxospores within the material fibers when exposed. Felt and both types of neoprene had the greatest number of interstitial spaces large enough to trap myxospores. The decrease in percent of myxospores trapped coincides with decreases in mean interstitial space size among felt, neoprene 2, and neoprene 1.

The different weave patterns of lightweight materials did not result in different percent recoveries, but the relative frequency of interstitial space sizes large enough to trap myxospores was low for both types of lightweight. In addition, lightweight materials have a water repellent layer that may have aided in rinsing the material clean.

The similar myxospore recovery percentages for rubber and the glass control suggest that rubber wading equipment is the easiest to effectively clean. Spaces measured on rubber were created by bubbles and ridges on the surface of the material. The lack of interstitial spaces combined with the small size of spaces that did exist suggests that rubber does not have the surface features that would trap myxospores. These results combined with the water repellent properties of rubber make this material a good candidate for angling equipment because it can be easily and effectively cleaned.

A small number of myxospores placed in an environment with *T. tubifex* can lead to TAM production (Stevens et al. 2001). The potential for felt to carry even small numbers of myxospores suggests that introduction of *M. cerebralis* by anglers is possible although the processes necessary to release myxospores from felt were not explored in this study. If fishing access sites represent foci of repeated re-introduction of *M. cerebralis* myxospores to waterways they may also represent sites where anglers are more likely to expose their wading equipment to myxospores. Many unanswered questions remain regarding the transport vectors and conditions necessary for the proliferation of *M. cerebralis*. However, transport of the parasite on angling equipment materials is possible and I recommend the use of rubber soled wading boots over felt soled boots in *M. cerebralis* infected drainages.

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APPENDICES

APPENDIX A

ANGLER SURVEY

Angler Survey

Site _____ Survey # _____
Date _____ Time _____ am pm
Angler is a guide? Yes _____ No _____
Angler will interview _____ Angler will not interview _____
Angler is: Male _____ Female _____
Observed angling gear:
Waders present: Yes_____ No_____
If yes, waders are: neoprene_____ Gore-Tex or lightweight_____
Wading boots present: Yes_____ No_____
If yes, boots are: felt soled_____ rubber soled_____
Type of fishing: fly fishing _____ spin fishing _____
Angler is: starting trip_____ ending trip_____
Angler's gear sampled? Yes _____ No _____

Check and answer all that apply

1. What is your home zip code? _____

2. Today you fished from: shore_____ boat_____ both (stopped boat and got out to fish)_____
If boat: drift boat_____ raft_____ canoe_____ float tube_____ aluminum john boat _____

3. Today you will or did fish: full day _____ half day _____ Other (hrs) _____

4. Did you rent any of the fishing equipment you are using today? Yes_____ No_____
If yes, what was rented _____
Where was it rented from? _____

5. Where was the last place you fished prior to today including the name of the access site (access site name not needed for out of state locations? (if none, skip to question 7)
_____

6. In the last 30 days, where have you fished including access site names and dates?
_____
_____
_____

7. Do you plan to fish again in the next 7 days? Yes_____ No_____
If yes, where?_____

8. Do you clean your boots in between uses:
always_____ most of the time_____ occasionally_____ rarely_____ never_____
If so how do you clean them?_____

9. Do you clean your waders in between uses:
always_____ most of the time_____ occasionally_____ rarely_____ never_____
If so how do you clean them?_____

10. Do you completely dry your boots in between uses:
always_____ most of the time_____ occasionally_____ rarely_____ never_____

11. Do you completely dry your waders in between uses:
always_____ most of the time_____ occasionally_____ rarely_____ never_____

Survey continued on following page.

12. Are you familiar with:

Whirling disease: Yes_____ No_____
New Zealand mud snails: Yes_____ No_____
Zebra Mussels: Yes_____ No_____
Asian Carp: Yes_____ No_____

13. The organisms listed above are all examples of aquatic nuisance species. If you knew about any of these species, where did you learn about them, answer yes or no to each of the following information sources: newspaper_____ tv_____ radio_____ magazine_____ signs_____ state agency_____ angling organization_____ website_____

14. Are you an active member of an angling organization such as Trout Unlimited or Federation of Fly Fishers? Yes_____ No_____

I appreciate your patience and thank you for your time

APPENDIX B

2005 ANGLING LICENSE SALES (Provided by Montana Fish, Wildlife and Parks)

REGION	COUNTY	ITEM DESCRIPTION	RES	COUNT
Helena	LEWIS AND CLARK	CONSERVATION AND FISHING	N	101
Helena	LEWIS AND CLARK	SEASON FISHING	N	21,101
Helena	LEWIS AND CLARK	TWO DAY FISHING	N	142
Helena	LEWIS AND CLARK	TEN DAY FISHING	N	55
Bozeman	PARK	CONSERVATION AND FISHING	N	942
Bozeman	PARK	SEASON FISHING	N	41
Bozeman	PARK	TWO DAY FISHING	N	7,471
Bozeman	PARK	TEN DAY FISHING	N	1,489
Bozeman	MADISON	CONSERVATION AND FISHING	N	1,541
Bozeman	MADISON	SEASON FISHING	N	63
Bozeman	MADISON	TWO DAY FISHING	N	8,828
Bozeman	MADISON	TEN DAY FISHING	N	1,477
Bozeman	GALLATIN	CONSERVATION AND FISHING	N	4,380
Bozeman	GALLATIN	SEASON FISHING	N	183
Bozeman	GALLATIN	TWO DAY FISHING	N	23,664
Bozeman	GALLATIN	TEN DAY FISHING	N	4,086
Bozeman	BEAVERHEAD	CONSERVATION AND FISHING	N	1,051
Bozeman	BEAVERHEAD	SEASON FISHING	N	73
Bozeman	BEAVERHEAD	TWO DAY FISHING	N	4,766
Bozeman	BEAVERHEAD	TEN DAY FISHING	N	821
Bozeman	LEWIS AND CLARK	CONSERVATION AND FISHING	N	606
Bozeman	LEWIS AND CLARK	SEASON FISHING	N	61
Bozeman	LEWIS AND CLARK	TWO DAY FISHING	N	2,705
Bozeman	LEWIS AND CLARK	TEN DAY FISHING	N	631
Billings	BIG HORN	CONSERVATION AND FISHING	N	1,108
Billings	BIG HORN	SEASON FISHING	N	42
Billings	BIG HORN	TWO DAY FISHING	N	7,509
Billings	BIG HORN	TEN DAY FISHING	N	680
Great Falls	LEWIS AND CLARK	CONSERVATION AND FISHING	N	560
Great Falls	LEWIS AND CLARK	SEASON FISHING	N	39
Great Falls	LEWIS AND CLARK	TWO DAY FISHING	N	2,992
Great Falls	LEWIS AND CLARK	TEN DAY FISHING	N	658
OUT OF STATE PROVIDERS	LEWIS AND CLARK	CONSERVATION AND FISHING	N	382
OUT OF STATE PROVIDERS	LEWIS AND CLARK	SEASON FISHING	N	10
OUT OF STATE PROVIDERS	LEWIS AND CLARK	TWO DAY FISHING	N	1,358
OUT OF STATE PROVIDERS	LEWIS AND CLARK	TEN DAY FISHING	N	54
				101,670
Helena	LEWIS AND CLARK	CONSERVATION AND FISHING	R	827
Helena	LEWIS AND CLARK	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	92
Helena	LEWIS AND CLARK	SEASON FISHING	R	22
Helena	LEWIS AND CLARK	TWO DAY FISHING	R	1
Bozeman	PARK	CONSERVATION AND FISHING	R	3,108
Bozeman	PARK	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	82
Bozeman	PARK	SEASON FISHING	R	83
Bozeman	PARK	TWO DAY FISHING	R	34
Bozeman	MADISON	CONSERVATION AND FISHING	R	1,547

Bozeman	MADISON	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	43
Bozeman	MADISON	SEASON FISHING	R	58
Bozeman	MADISON	TWO DAY FISHING	R	12
Bozeman	GALLATIN	CONSERVATION AND FISHING	R	14,389
Bozeman	GALLATIN	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	291
Bozeman	GALLATIN	SEASON FISHING	R	444
Bozeman	GALLATIN	TWO DAY FISHING	R	152
Bozeman	BEAVERHEAD	CONSERVATION AND FISHING	R	1,844
Bozeman	BEAVERHEAD	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	49
Bozeman	BEAVERHEAD	SEASON FISHING	R	133
Bozeman	BEAVERHEAD	TWO DAY FISHING	R	29
Bozeman	LEWIS AND CLARK	CONSERVATION AND FISHING	R	10,647
Bozeman	LEWIS AND CLARK	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	276
Bozeman	LEWIS AND CLARK	SEASON FISHING	R	216
Bozeman	LEWIS AND CLARK	TWO DAY FISHING	R	89
Billings	BIG HORN	CONSERVATION AND FISHING	R	1,411
Billings	BIG HORN	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	18
Billings	BIG HORN	SEASON FISHING	R	38
Billings	BIG HORN	TWO DAY FISHING	R	38
Great Falls	LEWIS AND CLARK	CONSERVATION AND FISHING	R	1,033
Great Falls	LEWIS AND CLARK	FREE YOUTH SPORTSMAN WITHOUT BEAR	R	6
Great Falls	LEWIS AND CLARK	SEASON FISHING	R	29
Great Falls	LEWIS AND CLARK	TWO DAY FISHING	R	18
OUT OF STATE PROVIDERS	LEWIS AND CLARK	CONSERVATION AND FISHING	R	1
				37,060