

Adherence of *Myxobolus cerebralis* Myxospores to Waders: Implications for Disease Dissemination

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Abstract.—The vectors involved in the spread of whirling disease, which is caused by *Myxobolus cerebralis*, are only partly understood. However, the parasite has rapidly become established in many regions, suggesting that it is easily disseminated. We gained insight into transport vectors by examining the surface porosity of common wading equipment materials and the adherence of *M. cerebralis* myxospores to them. Interstitial spaces within rubber, felt, lightweight nylon, and neoprene were measured on scanning electron microscope images. Myxospores were applied to each material, the material was rinsed, and the myxospores recovered to assess adherence. The mean interstitial space size of rubber was the smallest (2.0 μm), whereas that of felt was the largest (31.3 μm). The highest recovery rates were from rubber and the glass control. Percent myxospore recovery varied by material, the recovery from felt being lower than that from all other materials. The potential for felt to carry even small numbers of myxospores suggests that the introduction of *M. cerebralis* by felt-soled wading boots is possible.

The vectors involved in the spread of whirling disease, which is caused by *Myxobolus cerebralis*, are only partly understood. The parasite has rapidly become established in many regions and is responsible for major declines of some salmonid year-classes in North America (Nehring and Walker 1996; Vincent 1996; Bergersen and Anderson 1997; Bartholomew and Reno 2002). Movements of infected fish can account for many infection sources. However, a number of infections suggest other transport vectors. For example, the stocking of rainbow trout *Oncorhynchus mykiss* into streams with wild salmonid populations in Montana ceased in the early 1970s, and thus *M. cerebralis* was probably not introduced by state agencies stocking infected fish (Vincent 1987). In addition, the disease-free status of private and federal hatcheries in Montana suggests that transport of infected hatchery fish before the 1970s was an unlikely source of the infection (Baldwin et al. 1998).

Identification of *M. cerebralis* transport vectors is a high priority for managing the parasite. Some potential vectors include movements of infected fish, fish-eating birds, pet store trade in aquatic oligochaetes, anglers, boats, motors, and other aquatic recreational equipment (Meyers et al. 1970; Halliday 1976; Bergersen and Anderson 1997; Bartholomew et al. 2005). In addition, transfer of the parasite could result from movement of water or soil among drainages (Bergersen and Anderson 1997; Baldwin et al. 1998). Fishing access sites, in particular, can become degraded from concentrated use by humans. Degraded habitats may harbor greater abundances of the oligochaete host *Tubifex tubifex* (Zandt and Bergersen 2000), and *M. cerebralis* myxospores may be more likely to be found in such areas (Nickum and Bartholomew 2001). Further, dominance of sediments by fines has been associated with high site-specific whirling disease risk (Krueger et al. 2006). Fishing access sites may represent areas where soil, water, and myxospores are contacted by humans and moved to other sites.

Inadvertent transport of viable myxospores by humans is a potential vector of parasite introduction. The myxospore stage of *M. cerebralis* is resistant to environmental 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). Myxospores can withstand temperatures from -20°C to $+60^{\circ}\text{C}$ (Hoffman and Putz 1971; Hoffman and Markiw 1977) and can resist biodegradation for years while retaining infectivity (Halliday 1976). Recent investigations have indicated that myxospores do not retain their infectivity after ultraviolet irradiation or complete desiccation (Hedrick et al. 2008); however, the resilient features still make it likely that myxospores will persist in the environment and survive overland transport by humans in dark, moist conditions.

The ability of anglers and recreationists to transport soil and water containing *M. cerebralis* myxospores among fishing access sites depends on the type of equipment used and the exposure of that equipment to

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water and soil. The predominant materials used in the construction of wading boots and waders are rubber, felt (outer soles), lightweight nylon (breathable laminate, including Gore-Tex), and neoprene. The surface patterns and properties of these materials may dictate whether it is probable that *M. cerebralis* myxospores will adhere to them or become lodged within them when exposed.

We examined the surface patterns of rubber, felt, lightweight nylon, and neoprene and determined whether *M. cerebralis* myxospores adhered to these materials. Our goal was to contribute to a better understanding of the secondary transport mechanisms for this parasite. Such information may aid the development of effective control strategies and improve prediction of future parasite spread (Johnson et al. 2001).

Methods

Surface patterns of wading materials.—The four materials tested (rubber, felt, lightweight nylon, and neoprene) varied in both design and construction: the rubber material consisted of fabric with a rubber overlay; the felt material was a dense mat of randomly woven synthetic fibers; the lightweight nylon material included two layers of nylon, a coating, and a waterproof laminate; and the neoprene material consisted of a neoprene foam layer with fabric layers on the top and underside of the foam. Magnified images (200×) of all material types were taken with a scanning auger electron microprobe at the Montana State University Image and Chemical Analysis Laboratory. Preliminary images revealed different weave patterns between two commercial brands of lightweight nylon and neoprene. Thus, both types of both materials were examined. Lightweight materials were selected from the products that manufacturers considered to be their “value” lightweight breathable laminate waders, which sold for less than US\$180 and therefore represented an affordable wader for most anglers.

The interstitial spaces within each material were measured to quantify whether myxospores could be absorbed into the material. Material images were imported into Adobe Photoshop (Adobe Systems 2003), and vertical transects were placed over each image at 1-cm intervals. Images were calibrated in SigmaScan Pro (SPSS 1999) according to the scale imprinted on each image by the scanning auger electron microprobe. Three vertical transects were selected randomly, and the interstitial spaces along each were measured to determine the average interstitial space size for each material. The images were assumed to be two-dimensional for these measurements with no account for depth of fibers. An

interstitial space was defined as a gap between adjacent fibers greater than 1 μm . Although much smaller than a myxospore, 1- μm spaces were measured to characterize the material surface thoroughly.

Myxospore adherence to wading materials.—Myxospores were extracted and isolated from rainbow trout supplied by the Montana State University Aquatic Sciences Laboratory by means of the continuous plankton centrifuge method (O’Grodnick 1975). Myxospore abundance was estimated with a 0.4-mm Neubauer hemocytometer and compound microscope (Markiw and Wolf 1974). Counts were replicated three times per grid on the hemocytometer. When the three replicate counts exceeded the mean $\pm 10\%$, additional replicate counts were taken. After myxospore concentration was estimated, the sample was diluted with dechlorinated water to obtain a concentration of approximately 35,000 myxospores/mL of water. This solution was agitated for 2 min to suspend the myxospores before they were applied to wading materials.

A volume of 0.6 mL containing about 20,000 myxospores was drawn from the stock solution with a micropipette, the micropipette tip was positioned directly on the surface of the test material, and the solution was expelled onto a 3-cm² piece of material. The volume expelled daily varied from 0.5 to 0.7 mL, new myxospore solutions being prepared each time. The myxospore solution was left on the material for 7.5 min to allow the myxospores to settle (Gates 2006). This length of time corresponds to the settling rate of silt particles (Tan 1996), which are comparable in size and density to *M. cerebralis* myxospores. We assumed that myxospores would settle at a rate similar to that of silt particles. The settling time promoted physical contact between the myxospores in solution and the material. The material square was then rinsed for 1 s at a water pressure of 2.11 kg/cm² with a hand pump pressure sprayer containing water and aqueous sodium hexametaphosphate (a mild de-aggregating detergent) at a concentration of 6,200 mg [NaPO₃]₆/L of water (Lemmon and Kerans 2001). Sodium hexametaphosphate was added to de-aggregate myxospores (Lemmon and Kerans 2001) and simulate a mild soap solution that might be used by anglers to clean equipment. The 2.11 kg/cm² water pressure equals the output of a residential 15-m garden hose and therefore simulates the cleaning of angling equipment at home (Rumbarger 2003). The 1-s interval was the estimated fraction of time that a 3-cm² piece of a boot or wader would be sampled if the leg was rinsed from the knee down for 30 s (Gates 2006). The rinse solution was collected in a 50-mL centrifuge tube. Aqueous [NaPO₃]₆ was added to the rinse to increase the volume

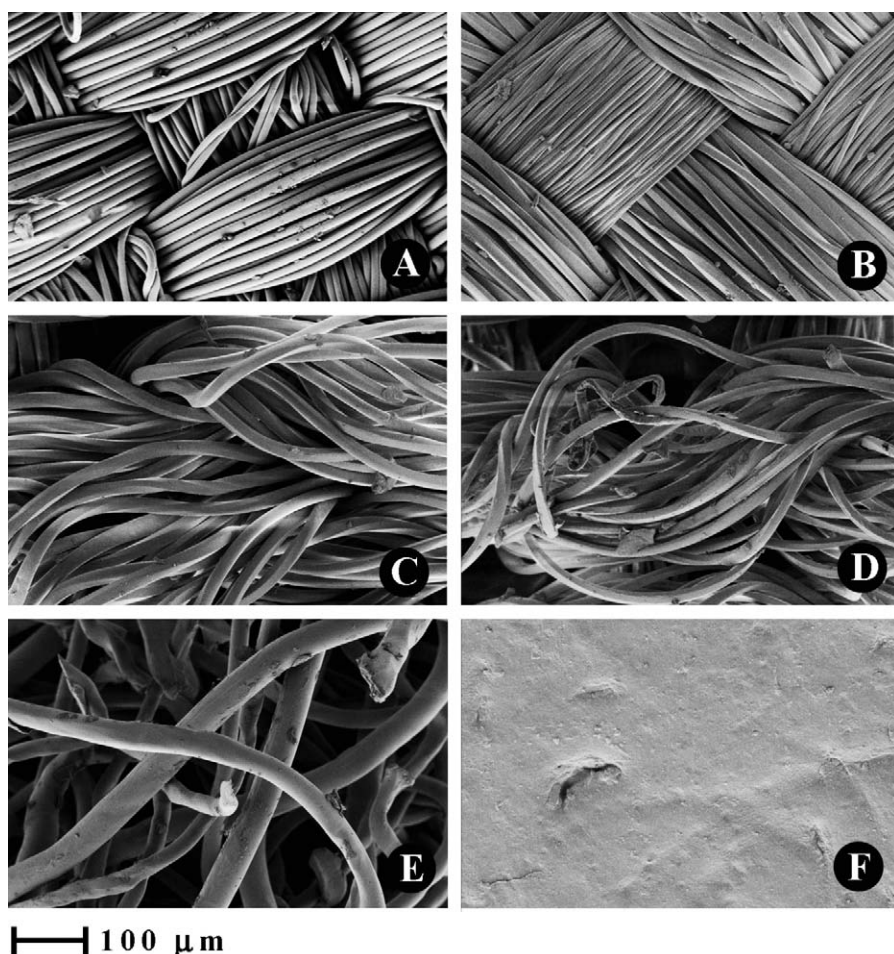


FIGURE 1.—Images of wading equipment material types at 200 \times magnification: (A) lightweight nylon 1, (B) lightweight nylon 2, (C) neoprene 1, (D) neoprene 2, (E) felt, and (F) rubber.

to 5 mL. The rinse solution was then agitated for 4 min by repeated inversion of the centrifuge tube to distribute rinsed myxospores evenly throughout the solution. After agitation, myxospores rinsed from the material were counted as described above. This process was performed on all six material types (rubber, felt, lightweight nylon 1, lightweight nylon 2, neoprene 1, and neoprene 2) and a glass control. Three replicates were performed to produce a mean percent myxospore recovery for each treatment (material).

Unevenly distributed myxospores in solution produced variability in the hemocytometer counting procedure, creating slight differences in the amounts of myxospores applied to each treatment ($20,000 \pm 3,367$). Ninety-five percent confidence intervals were calculated for the myxospore quantities in each experiment to measure the variability in counts. The

confidence intervals reported are actually means of several such intervals because the experiment was conducted over the course of several days and separate confidence intervals were calculated each day. All measures of variability are listed as means \pm 95% confidence intervals. The confidence intervals of the myxospore quantity added to each treatment were used to calculate myxospore recovery as a percent (the number of myxospores rinsed as a function of the number exposed to each treatment) to prevent variability in the results.

An analysis of variance (ANOVA) and least-squares means multiple comparison procedure with a Tukey adjustment were used to determine whether interstitial space size and percent myxospore recovery differed among material types (SAS Institute 2005).

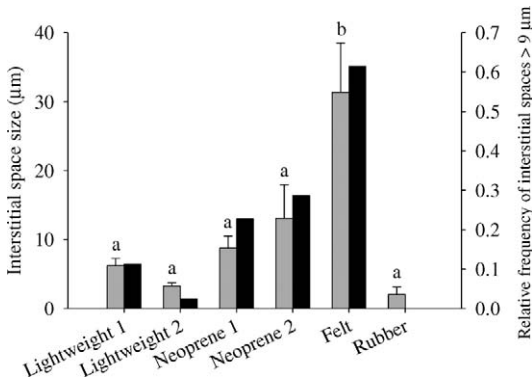


FIGURE 2.—Mean interstitial space size for lightweight nylon, neoprene, felt, and rubber materials (gray bars) and relative frequency of interstitial spaces more than 9 μm (black bars). Measurements were taken along randomly selected transects of images at 200× magnification. The values associated with gray bars with the same lowercase letter are not significantly different (error bars = 1 SE).

Results

Surface Patterns of Wading Materials

Rubber had the smallest mean interstitial space size (2.0 μm) and the fewest spaces ($n = 5$; Figures 1, 2). Felt had the largest mean interstitial space size (31.3 μm). The two lightweight nylon materials had the greatest number of interstitial spaces ($n = 53$ for lightweight nylon 1, and $n = 40$ for lightweight nylon 2); however, the spaces were small (<8 μm; Figure 1). The different fiber weave patterns of the two types of lightweight nylon and the two types of neoprene caused differences in the mean interstitial space size (Figure 2). Interstitial spaces differed significantly among materials ($F_{5, 12} = 8.76, P = 0.001$). Felt had significantly larger interstitial spaces than all other material types (Figure 2).

Myxospore Adherence to Wading Materials

Myxospore recovery was highest from rubber and the glass control (Figure 3) and differed significantly among materials ($F_{6, 14} = 43.11, P < 0.001$). The mean percent recovery from felt was significantly less than that from all other materials (Figure 3). Felt trapped all myxospores, neoprene 2 trapped 27% of myxospores, and neoprene 1 trapped 13% of myxospores.

Discussion

Any material with interstitial spaces of 9 μm or more could trap *M. cerebralis* myxospores because the myxospores are about 8.7 μm long and 8.2 μm wide (Lom and Hoffman 1971; Nehring et al. 2003). Felt

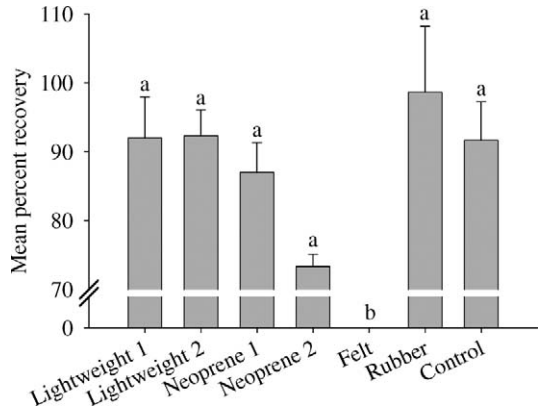


FIGURE 3.—Mean percent myxospore recovery for lightweight nylon, neoprene, felt, and rubber. The values associated with bars with the same lowercase letter are not significantly different (error bars = 1 SE).

and both types of neoprene had the greatest number of interstitial spaces large enough to trap myxospores. The decrease in percent of myxospores trapped among felt, neoprene 2, and neoprene 1 corresponded to decreases in mean interstitial space size.

The lightweight nylon materials trapped fewer myxospores than both types of neoprene and felt. The different weave patterns of the lightweight materials did not result in significantly different percent recoveries, but the relative frequency of interstitial spaces large enough to trap myxospores was low for both types. In addition, the lightweight materials had a water repellent layer that may have aided in rinsing the material clean.

The similar myxospore recovery for rubber and the glass control suggests that rubber wading equipment is the easiest to clean effectively. Spaces on rubber were bubbles and ridges on the surface of the material. The lack of interstitial spaces combined with the small size of the spaces suggests that rubber does not have the surface features that would trap myxospores. These results, combined with the water repellent properties of rubber, make it a good candidate for angling equipment that will not transport whirling disease myxospores and other aquatic nuisance species.

Exposure of wading equipment to myxospores in the field could occur through several different mechanisms. Myxospores in the water column could be pressed against the material by water velocity or could be encountered by stepping on or lying on top of sediments containing myxospores. Our experiments simulated water velocity by pressing the myxospore solution onto each material. The settling time provided an exposure period of several minutes to promote

physical contact between the myxospores in solution and the material. In laboratory tests, felt-soled waders exposed to myxospores by stepping on sediments containing partially decomposed whirling disease-infected rainbow trout were found to transmit the myxospore and subsequently infect the oligochaete host (P. Reno, Oregon State University, personal communication).

A small number of myxospores placed in an environment can lead to disease propagation (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. It is possible that myxospores are not released from felt and thus do not pose a transport risk; however, waders and boots did transfer the parasite to susceptible hosts in a laboratory experiment (P. Reno, personal communication). If fishing access sites represent areas of increased whirling disease risk from habitat degradation, 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 proliferation of *M. cerebralis*. We recommend the use of rubber-soled wading boots over felt-soled boots in *M. cerebralis*-infected drainages because felt can retain *M. cerebralis* myxospores after exposure.

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