

UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

DIAGNOSIS, PREVALENCE, AND PREVENTION OF THE SPREAD OF THE
PARASITE *HETEROSPORIS* sp. (MICROSPORIDA: PLEISTOPHORIDAE) IN
YELLOW PERCH (*Perca flavescens*) AND OTHER FRESHWATER FISH IN
NORTHERN MINNESOTA, WISCONSIN, AND IN LAKE ONTARIO.

A Manuscript Style Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of Master of Science in Biology

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College of Science and Health
Aquatic Science Concentration


August, 2009

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By Peggy E. Miller

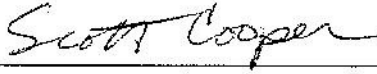
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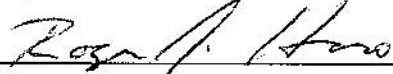
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ABSTRACT

Miller, P.E. Diagnosis, prevalence, and prevention of the spread of the parasite *Heterosporis* sp. (Microsporida:Pleistophoridae) in yellow perch (*Perca flavescens*) and other freshwater fish in northern Minnesota, Wisconsin and in Lake Ontario. MS in Biology, August, 2009, 57 pp. (M. Sandheinrich)

A previously unknown microsporidian parasite that severely degrades muscle of yellow perch (*Perca flavescens*) from lakes in Minnesota, Wisconsin, and Lake Ontario was identified as belonging to the genus *Heterosporis*. This parasite is characterized by pyriform-shaped spores that are contained in sporophorocysts. In the wild, yellow perch, burbot (*Lota lota*), mottled sculpin (*Cottus bairdi*), trout-perch (*Percopsis omiscomaycus*), pumpkinseed (*Lepomis gibbosus*), northern pike (*Esox lucius*), walleye (*Sander vitreus*) and rock bass (*Ambloplites rupestris*) harbor *Heterosporis* naturally, but laboratory studies showed that 12 other fish species are susceptible to infection. In laboratory trials, smallmouth bass (*Micropterus dolomieu*) consumed significantly more fathead minnows infected with *Heterosporis* sp. than uninfected. Microscopically, *Heterosporis* sp. infection can be detected in the muscle of fish two weeks after exposure, and visually identified after seven weeks. To confirm infection, a PCR diagnostic assay was developed. *Heterosporis* spores are rendered noninfective by freezing, desiccation for 24 h, exposure to 2,200 mg/L bleach, and aging in air-exposed water for six months. This parasite can infect a wide range of fish species which can lead to devastating losses in commercial and sport fishing; however, there are preventative measures that may limit the spread of the parasite.

ACKNOWLEDGEMENTS

I thank Dr. Daniel Sutherland (deceased 2006) for all of his guidance, knowledge, encouragement, and laughter throughout the entire study and in all of his classes. Jim Luoma of the Upper Midwest Environment Sciences Center, the hatchery managers at Westfield, Nevin, Spooner and Woodruff State Fish Hatcheries, and Dave Gollon of Gollon Bait and Tackle donated fish used in the study. Rod Penny and Lisa Miller-Dodd (Ontario MNR) organized the sampling of Lake Ontario. Dr. Michael Kent provided microsporidians samples to test the specificity of PCR. Wes Jahns (WI DNR) assisted in sampling Catfish Lake and a source for information regarding Vilas Co. lakes. Joe Marcino (MN DNR) and Susan Marcquenski (WI DNR) helped start this project and providing contact information that allowed the study to come to fruition. Dr. Scott Cooper helped develop the PCR assay used to detect *Heterosporis* and reviewed my many thesis edits. I thank Stephanie Burton, Jon Mader, Michelle Pucci, and Sara Treleven for helping with various aspects of the study, which included many fish dissections. Drs. Mark Sandheinrich, Becky Lasee, and Roger Haro assisted with many thesis edits and encouraged me along the way. Dave Reineke assisted with the statistical analyses. I also thank my husband, Kevin Miller, who helped with many fish collections, although I had to twist his arm, and was there supporting and encouraging me throughout the thesis process. My family and friends also encouraged me and were there when I needed them. This study was supported by a contract from Great Lakes Fisheries Commission, and grants from the MN DNR and WI DNR. All studies were conducted with procedures approved by the UW- La Crosse Institutional Animal Care and Use Committee (IACUC).

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INTRODUCTION

Microsporidia are obligate, intracellular parasites that primarily infect arthropods, fish, and mammals (Lom and Dykova 1992). In fish, infections can be widespread in various tissues or concentrated into cysts that are often visible to the eye. Microsporidian spores may be released from skin, feces and urine of live hosts, released after death and decomposition of the host, or ingested by predators or scavengers. Spores are highly resistant to external conditions and can remain infective for up to one year in water (Canning and Lom 1986). In general, fish microsporidia undergo a simple life cycle, involving: merogony, sporony, and direct transmission (Lom and Nilsen 2003).

Infective spores contain a coiled polar tube which everts under suitable stimuli produced by the host. Suitable pH and presence of alkali ions appear to be essential, although the exact mechanism for polar tube eversion is unknown. The polar tube is explosively everted allowing the distal end to penetrate, but not disrupt, the cell's plasma membrane. The parasite sporoplasm passes through the lumen of the polar tube and enters the cytoplasm of the host cell (Canning and Lom 1986).

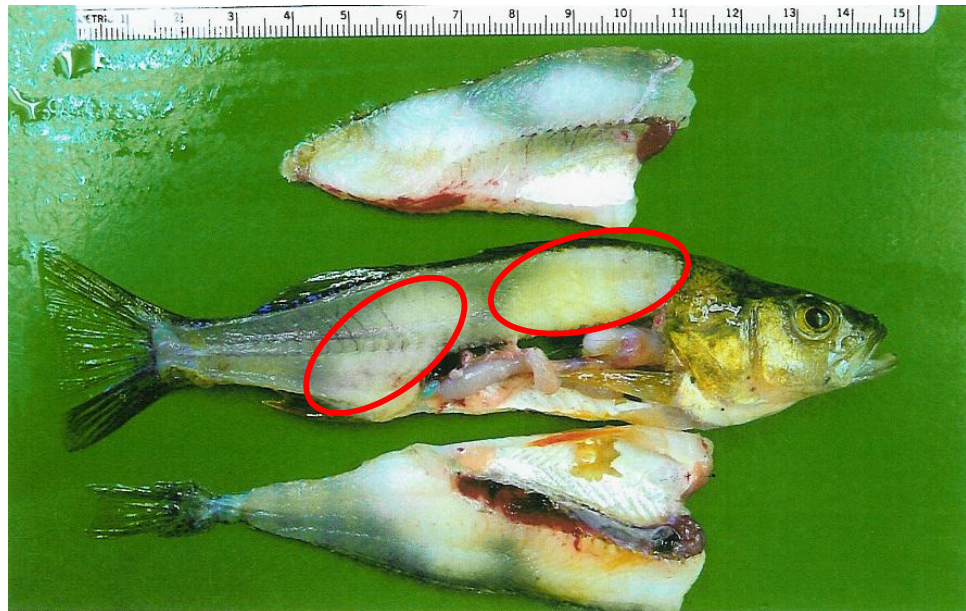
In suitable host cells, the sporoplasm repeatedly divides to produce meronts. Meronts are round, irregular, or elongate cells with little membrane organization but contain rough and smooth endoplasmic reticulum. Meronts contain isolated nuclei and divide repeatedly by schizogony and eventually transform into sporonts. Sporonts have an electron dense coat, which develops into the spore wall during sporulation. Sporonts undergo a series of divisions leading to the formation of eight macrospores or more rarely

to sixteen or more microspores. The spores of five genera of microsporidia (*Glugea*, *Pleistophora*, *Thelohania*, *Loma*, and *Heterosporis*) are packaged in thick-walled sporophorocyst vesicles (SPVs) (Canning and Lom 1986). The SPVs of *Heterosporis* are contained in sporophorocysts, which may grow to completely fill the host cell. Mature spores are released into surrounding tissue with the necrosis and dissolution of the infected host cell (Lom et al. 1989). Due to the fact that the spores are elongated and contain only one nucleus, places *Heterosporis* in the order Pleistophoridae.

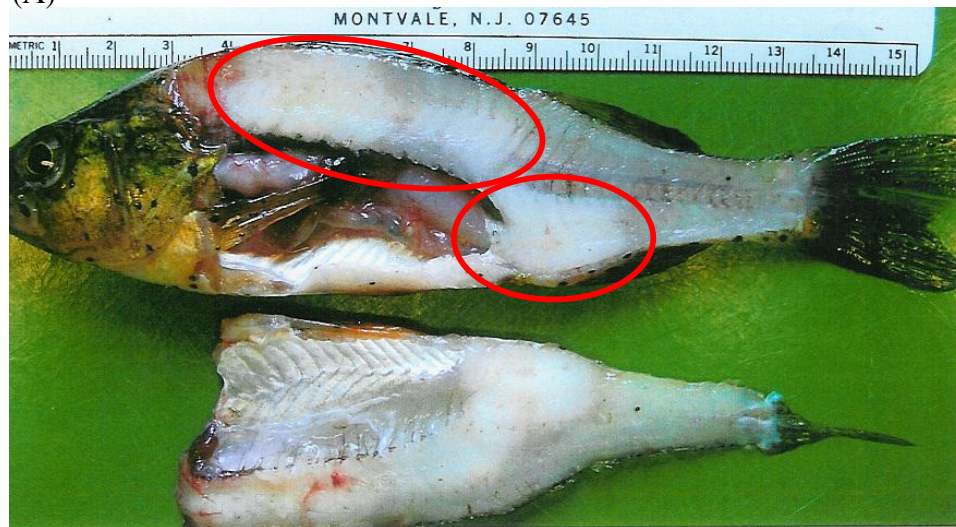
Microsporidians can cause high mortality in fish, especially in hatcheries where transmission can spread easily from one fish to another (Kent et al. 1995). Exposure of wild fish populations inhabiting waters adjacent to hatcheries may occur, especially if connected to the same watershed (Canning and Lom 1986).

In January 2000, anglers reported a previously unknown condition in yellow perch (*Perca flavescens*) from Catfish Lake, WI (Vilas County). The fillets were white or opaque, and resembled those that might be partially cooked or with freezer burn (Figure 1). Muscular lesions were subsequently identified by Susan Marcquenski (Fish Health Specialist, Wisconsin Department of Natural Resources [DNR]) and Daniel Sutherland (University of Wisconsin-La Crosse, Department of Biology) as an infection of skeletal muscle by a microsporidian parasite belonging to the genus *Heterosporis*. Based on the size and number of spores in the sporophorocyst vesicles, the generic diagnosis was corroborated by Jiri Lom and Iva Dykova (Institute of Parasitology, Academy of Sciences, Czech Republic). Since 2000, *Heterosporis* has been confirmed in yellow perch from other water bodies in Wisconsin (Vilas, Forest, and Oneida counties), Minnesota (Cass, Isanti, Itasca, and Ottertail Counties), and Michigan (Gogebic and Iron

counties). In the summers of 2000 and 2001, *Heterosporis* was also identified by histology and PCR (polymerase chain reaction) in commercially caught yellow perch



(A)



(B)

Figure 1. Comparison between *Heterosporis*-infected perch filleted immediately after euthanization (A) and infected perch filleted after 24 h on ice (B). Note that the post mortem opaqueness of uninfected tissue in B obscures definition of infected areas.

from northeastern Lake Ontario and the Bay of Quinte (Hoyle and Stewart 2001). There were; however, unconfirmed reports of similarly affected fish found in fish from Leech Lake, MN, prior to 2000 (Joseph Marcino, former Fish and Wildlife Pathologist, Minnesota DNR, personal communication).

Currently, there are three species of *Heterosporis* known to infect fish. The first, *H. anguillarum* from cultured eels (*Anguilla japonica*) in Japan and Taiwan (Hoshina 1951), contain macrospores averaging 2.4 µm x 7.8 µm in size, and clinical signs include deformities of the body, white spots beneath the skin, and depressed areas in the trunk musculature (Dykova 2006). The second, *Heterosporis schuberti* in cultured ornamental cichlids (*Pseudocrenilabrus multicolor*) and loricarid catfish (*Ancistrus cirrhosus*) from Germany (Lom et al. 1989), contain macrospores averaging 4.4µm x 7µm in size, and infected fish have muscle pervaded with parasites and show signs of distress (Dykova 2006). The third, *Heterosporis finki* in cultured angelfish (*Pterophyllum scalare*) from France (Michel et al. 1989), contain macrospores averaging 2.5 µm x 8 µm in size. In heavily infected fish, the skeletal muscles are milky white and creamy in texture and the skin contains grayish spots (Dykova 2006). There was also an undetermined species of *Heterosporis* reported from cultured bettas (*Betta splendens*) in Thailand (Lom et al. 1993).

It is not known if the *Heterosporis* species found in yellow perch is an exotic introduced into North America or native to the region that was previously undetected. However, regional fish disease biologists on the Great Lakes Fish Health Committee

speculate that commercially valuable species, such as yellow perch, are examined so frequently that a parasite as conspicuous as *Heterosporis* would have been discovered prior to 2000 (Susan Marcquenski, Fish Health Specialist, Wisconsin DNR, personal communication). According to Hoffman (1999), the only reported microsporidian in yellow perch is *Pleistophora* sp. which was reported from North Dakota.

Over the last ten years, numbers of yellow perch in the Great Lakes have dramatically declined (Makauskas and Clapp 2002). If *Heterosporis* spreads from Lake Ontario throughout all of the Great Lakes, it could affect the fishing industry by causing lost revenue due to decreasing harvestable stocks and amounts of marketable fillets.

This study was undertaken to elucidate some basic features of the *Heterosporis* sp. that is infecting yellow perch. The preliminary experiments were to determine: (1) what fish hosts are infected in the Great Lakes region, (2) host species susceptibility and method of infection, (3) affect of *Heterosporis* on predation, (4) development of a polymerase chain reaction (PCR) for confirmation of *Heterosporis*, and (5) effective disinfection and control measures.

METHODS

Laboratory Disinfection Protocol

To prevent the release of viable *Heterosporis* spores from the laboratory, all waste water was treated with household bleach at a concentration of 237 mL bleach/38 L water before disposal and all equipment which came in contact with viable spores was washed in a bleach solution (237 mL/38 L). Dead fish were promptly removed from experimental tanks and autoclaved.

Spore and Sporophorocyst Morphology

Wetmounts were prepared and observed with a compound microscope using bright field and phase microscopy. Muscle samples were also observed with a compound microscope using bright field microscopy. Twenty each of individual spores, sporophorocysts, and sporophorocyst vesicles were measured and the mean was calculated.

General Infection Protocol

Live fish from Wisconsin state fish hatcheries were maintained either in a 1.1 kL or 38 L flow-through aquaria maintained with approximately 13°C well water, or 38 L static aquaria with aeration, and fed a daily ration as designated by the fish culturist. Before fish were used in studies, approximately ten percent, or otherwise noted, of fish were examined for *Heterosporis* infection. For artificial infections of *Heterosporis*, viable spores were obtained from *Heterosporis*-infected muscle from yellow perch from Catfish Lake, Vilas County, Wisconsin. The infected fish were filleted and heavily infected (opaque) regions of the fillet were excised, pooled and homogenized by a household blender in 200 mL of well water for at least five minutes and the homogenate was filtered

through cheese cloth to create a stock spore suspension. Multiple stock spore suspensions were made throughout the experiments. The stock spore suspension was added to designated aquaria. Fish were exposed to the spore suspension for 24 h. After each 24 h experimental exposure to *Heterosporis* spores, fish were either transferred into new 38 L aquaria or left in the exposure aquaria. One week post exposure (PE), 19 L of water was exchanged daily in all aquaria, and twice weekly thereafter, to reduce tank fouling. At three months PE, all fish were euthanized with an overdose of Finquel tricaine methane sulfonate (MS-222) (Argent Chemical Laboratories, Redmond, Washington) and examined with a dissecting microscope. *Heterosporis* infection was confirmed by polymerase chain reaction (PCR).

Geographic Distribution of *Heterosporis* in Great Lakes Region

Various fish species from multiple locations in Wisconsin and surrounding states were examined to determine the range of *Heterosporis*. Catfish Lake (Vilas County, Wisconsin) was sampled at least twice annually during summer and winter from 2000-2005. Fish were collected with fyke nets, angling, and electro-fishing. The objective was to collect 100 yellow perch each sampling session. For this study, Catfish Lake will be used as the reference location for *Heterosporis* from yellow perch in North America, this site was chosen to serve as a source of infective *Heterosporis* spores for all laboratory studies. The Wisconsin Department of Natural Resources (DNR) sampled Scattering Rice Lake in Vilas County with fyke nets in summer 2003. In the same year, the Minnesota DNR sampled Leech and Steamboat Lakes in Cass County, Minnesota with gill nets. In 2004, commercial fishers collected and shipped 3,137 fish of various species from Lake Ontario (Table 1). These fish were frozen and shipped via commercial carrier to La

Crosse, Wisconsin and frozen fish were thawed 24 h prior to analysis. In 2003, Dave Gollon Bait and Fish Farm, Dodgeville, Wisconsin provided emerald shiners (*Notropis atherinoides*), stonerollers (*Campostoma anomalum*), fathead minnows (*Pimephales promelas*), southern redbelly dace (*Phoxinus erythrogaster*), and spottail shiners (*Notropis hudsonius*) (Table 2). Also in 2003, the Sea Lamprey Control of the U.S. Fish and Wildlife Service (USFWS) collected and transported frozen and live sea lamprey (*Petromyzon marinus*) from Bronte, Rouge, Lynde, Shelter Valley, Farewell, and Colborne creeks located in Ontario, Canada.

To maintain the integrity of the fish fillets, euthanized fish from the Wisconsin DNR and Minnesota DNR were refrigerated or kept on ice and were examined within 48 h of collection. Live fish from Dave Gollon, Sea Lamprey Control, and those collected by myself and University of Wisconsin-La Crosse staff were euthanized with MS-222 and examined within four hours, complying with the protocol approved by the University of Wisconsin-La Crosse Institutional Animal Care and Use Committee (IACUC). Fish from Lake Ontario were kept frozen, then thawed and examined within two months of receipt. All fish were examined for *Heterosporis* with the aid of a stereomicroscope. Muscle samples, from fish that were presumptively identified as infected with *Heterosporis*, were frozen for confirmation by PCR. Because the lamprey collected in 2003 were too small to adequately fillet, two small sections of muscle tissue (approximately 0.5 cm²) were taken from each lamprey, pooled into a single sample in a microcentrifuge tube (a total of ten sections/five fish) and frozen (-20°C) and tested by PCR.

Table 1. Prevalence of fish infected with *Heterosporis* sp. from Catfish and Scattering Rice lakes (Wisconsin), Steamboat and Leech lakes (Minnesota), and Lake Ontario, Ontario, Canada.

Fish species	Prevalence ^a				
	Catfish Lake	Scattering Rice Lake	Steamboat Lake	Leech Lake	Lake Ontario
<i>Perca flavescens</i>	16.8 (1609)	2.8 (71)	9.4 (212)	21.5(223)	7.3(2009)
<i>Sander vitreus</i>	4.3 (46)	0 (1)	0 (51)	-	0 (1)
<i>Notemigonus crysoleucas</i>	0 (5)	0 (26)	-	-	-
<i>Lepomis macrochirus</i>	0 (34)	0 (13)	0 (101)	0 (4)	-
<i>Lepomis gibbosus</i>	11.9 (84)	0 (9)	0 (87)	0 (3)	1.4 (145)
<i>Moxostoma macrolepidotum</i>	0 (10)	0 (4)	-	-	-
<i>Lota lota</i>	50 (2)	-	0 (1)	-	-
<i>Micropterus dolomieu</i>	0 (11)	-	-	-	-
<i>Etheostoma nigrum</i>	0 (1)	0 (6)	-	-	-
<i>Ambloplites rupestris</i>	8.0 (125)	1.8 (56)	0 (12)	0 (10)	1 (305)
<i>Catostomus commersonii</i>	0 (28)	0 (5)	0 (53)	-	-
<i>Percopsis omiscomaycus</i>	100 (2)	-	-	-	0 (210)
<i>Cottus bairdi</i>	25.0 (8)	-	-	-	-
<i>Esox lucius</i>	0 (2)	-	0 (55)	-	-
<i>Micropterus salmoides</i>	0 (1)	-	0 (4)	0 (1)	-
<i>Pomoxis nigromaculatus</i>	0 (1)	0 (43)	-	0 (3)	0 (9)
<i>Moxostoma anisurum</i>	-	0 (3)	-	-	-
<i>Ameiurus natalis</i>	-	0 (1)	0 (6)	-	-
<i>Ameiurus nebulosus</i>	-	-	0 (27)	-	0 (15)
<i>Ameiurus melas</i>	-	-	0 (8)	-	-
<i>Ictalurus nebulosus</i>	-	-	-	-	0 (5)
<i>Amia calva</i>	-	-	0 (4)	-	-
<i>Coregonus clupeiformis</i>	-	-	0 (1)	-	-
<i>Morone americanus</i>	-	-	-	-	0 (208)
<i>Neogobius melanostomus</i>	-	-	-	-	0 (121)
<i>Morone chrysops</i>	-	-	-	-	0 (47)
<i>Aplodinotus grunniens</i>	-	-	-	-	0 (31)
<i>Coregonus asrtedii</i>	-	-	-	-	0 (28)
<i>Cyprinus carpio</i>	-	-	-	-	0 (3)

^a Prevalence – number infected/number examined x 100 (total fish examined in parentheses).

Waterborne Transmission of *Heterosporis* sp.

In order to determine host specificity and transmission of *Heterosporis*, 17 species of fish were obtained and exposed to *Heterosporis* spores. The spores were made into a suspension as described previously and the fish were exposed for 24 h, as specified in the general infection protocol. Sixteen fish species were obtained from various Wisconsin

state fish hatcheries and one species from North Carolina to conduct host specificity and mode of infection experiments. The Upper Midwest Environmental Science Center (UMESC) provided 35 yellow perch, 25 walleye (*Sander vitreus*), 50 channel catfish (*Ictalurus punctatus*), 26 bluegill (*Lepomis macrochirus*), 25 largemouth bass (*Micropterus salmoides*), 25 lake sturgeon (*Acipenser fulvescens*), 100 northern pike (*Esox lucius*), 15 rainbow trout (*Oncorhynchus mykiss*), 100 brook trout (*Salvelinus fontinalis*), and 200 fathead minnows (*Pimephales promelas*). All fish obtained from UMESC were hatchery-reared on well water, age-0 (<12 months) individuals. The State Fish Hatchery in Westfield, Wisconsin, provided 50 lake trout (*Salvelinus namaycush*) and 100 coho salmon (*Oncorhynchus kisutch*), both age-0 and hatchery raised. Dave Gollon Bait and Tackle, Dodgeville, Wisconsin provided 100 smallmouth bass (*Micropterus dolomieu*), 90 white suckers (*Catostomus commersonii*) and 90 golden shiners (*Notemigonus crysoleucas*), all age-0 and pond raised. The Nevin State Fish Hatchery, Madison, Wisconsin provided 100 hatchery-raised age-0 brown trout (*Salmo trutta*). Mosquitofish (*Gambusia affinis*) were purchased from Carolina Biological Supply, Burlington, North Carolina. All fish were held for a minimum of 24 h before used in experiments. Fish were maintained either in a 1.1 kL flow-through aquarium, 38 L static aquaria with aeration, or 38 L flow-through aquaria. Aquaria were supplied with well water and fish were fed pelleted standard diets as recommended by the suppliers of each fish species.

In initial exposures, rainbow trout, walleye, fathead minnows, channel catfish, yellow perch, mosquitofish, largemouth bass, lake sturgeon, and bluegill were each divided into two groups and placed into 9.5 L aquaria containing well water and 50 mL

of stock spore suspension of unknown concentration for 24 h. All fish were monitored at least twice a day to ensure that fish were not overly stressed as a result of the exposure. Fish were then moved to disinfected aquaria and maintained for three months PE and then examined for *Heterosporis* infection.

Effects of Spore Concentration on Transmission

In an attempt to expose fish to known concentrations of the parasite, three concentrations of liberated *Heterosporis* spores were initially tested on golden shiners, coho salmon, and white suckers. Using a hemocytometer, the initial spore suspension concentration was determined as 2.02×10^8 spores/mL. Spore concentration designated as high (2.0 L of stock spore suspension/ 36.0 L well water), medium (0.2 L stock spore suspension/37.8 L well water) and low (20 mL stock spore concentration/37.98 L well water) were prepared and placed into aerated 38 L aquaria; 30 individuals of a given species were promptly added to individual aquaria. One tank was used for each concentration tested on each fish species. Two samples of water were taken about 25 cm under the water surface with microcentrifuge tubes. To determine the approximate concentration of spores in the aquaria, samples were collected five minutes after the suspension was added to the aquaria.

Northern pike, lake sturgeon, brook trout, lake trout, brown trout and smallmouth bass were exposed to two concentrations of *Heterosporis* spores. Infected fish tissue was homogenized as described in the general infection protocol. Because of the amount of infected muscle tissue, two stock spore suspensions were made from the infected fish muscle. The stock spore suspension concentration for use in exposing the northern pike, lake sturgeon, and brook trout was 3.0×10^8 spores/mL, and the second stock spore

suspension used in exposing lake trout, brown trout, and smallmouth bass was 3.2×10^9 spores/mL. Spore concentrations designated as high (2.0 L stock spore suspension/36.0 L well water) and low (20 mL stock spore suspension/37.98 L well water) were prepared and placed into aerated 38 L aquaria. Fifty fish of a given species were promptly added to each aquarium. Spore concentrations in each tank were estimated, as previously described. Fish were exposed for 24 hours and then maintained three months PE before being examined for *Heterosporis* infection.

Susceptibility of Fish to Oral Infection of *Heterosporis*

To determine if fish acquire infection by consuming *Heterosporis*-infected tissue, age-0 hatchery-raised fish, including 13 rainbow trout, 100 fathead minnows, 25 walleye, 30 largemouth bass, 25 bluegill, and 25 lake sturgeon were allowed to feed on pieces of *Heterosporis*-infected yellow perch muscle. The fed muscle sections contained more *Heterosporis* spores than healthy muscle. Fish were fed bite size pieces of the infected muscle for five minutes, once a day, for three consecutive days. To prevent fouling of the tank, uneaten pieces were removed at the end of the work day. Fish were kept in 38 L aquaria, containing no more than 30 fish/aquarium for three months PE, and then were examined for *Heterosporis* infection.

To determine if fish acquire infection by consuming live *Heterosporis*-infected fish, 39 rainbow trout (mean total length= 23.6 cm) obtained from the La Crosse Fish Health Center, La Crosse, Wisconsin were each fed three potentially infected fathead minnows. To infect fathead minnows with *Heterosporis*, 200 fish were passively exposed to the *Heterosporis* spore suspension as described in the general infection protocol. Twenty fathead minnows were then necropsied three months PE to estimate the

prevalence of infection before feeding them to the rainbow trout; 60% of the minnows were infected with *Heterosporis*. Three fathead minnows were placed into 38 L aquaria with the individual rainbow trout until the fathead minnows were eaten (range of 10 min to 3 d). Rainbow trout were then fed a diet of koi pellets and examined for *Heterosporis* infection three months PE.

Predator-Prey Interactions

This experiment was done to determine if fish preferred preying on *Heterosporis*-infected minnows to healthy minnows. The UMESC provided 16 hatchery-reared smallmouth bass (15.8-21.0 cm total length) and 500 hatchery-reared age-1 fathead minnows (2.6-6.7 cm total length) for the experiment. Prior to the experiment, 250 fathead minnows were passively exposed to a *Heterosporis* spore suspension as described in the general infection protocol. Thirty fatheads were necropsied three months PE to determine infection prevalence. Twenty-five of the 30 fish were infected with *Heterosporis*. During this time, the smallmouth bass were fed a maintenance diet of two uninfected fathead minnows a week, and minnows were fed koi pellets bi-weekly.

The experimental protocol was modified from Sullivan et al. (1978). Eight flexible plastic enclosures, each measuring 61.0 cm in diameter and 30.5 cm deep, were placed into a large fiberglass tank. Depth of the water in the tank was 16.5 cm and contained a constant flow of aerated well water (mean dissolved oxygen of 8.1 mg/L) maintained at 14.1°C to 14.2°C. A 12.0 cm long PVC pipe (10.0 cm diameter) was cut in half and placed on the bottom of each tank to provide cover for the fathead minnows, and a 14 h light to 10 h dark photoperiod was maintained. Smallmouth bass were not fed for one week prior to exposure to prey.

A bass was placed in each enclosure with five potentially infected fathead minnows and five non-infected minnows. The fish were monitored at least twice a day and when a minnow was consumed, the contents of the bass' stomach were emptied by gastric lavage (Seaburg 1957). The elbow neck of a polypropylene squirt bottle was positioned in the bass' throat and water was squeezed into the stomach and stomach contents were collected in a sieve. The consumed minnows were then dissected and examined with a dissecting microscope for *Heterosporis* spores, and samples of muscle were then taken for confirmation of infection of *Heterosporis* by PCR. The experiment was terminated after five of the ten minnows were consumed or after five days. A second group of eight bass were tested in a similar manner. One week later, all bass were retested, for a total of 32 feeding trials.

All fathead minnows surviving to the end of the five day trial were euthanized with an overdose of MS-222 and examined for evidence of *Heterosporis* infection. The sixteen bass were placed in individual 38 L aquarium and fed a diet of Kaytee® koi pellets for three months PE, and examined for development of *Heterosporis* infection. The proportion of infected minnows eaten to the proportion of infected fish not eaten was compared using the two-sample Z-test, as advised by Dr. David Reineke (Mathematics Professor, University of Wisconsin-La Crosse).

Development of *Heterosporis* in Skeletal Muscle.

In order to determine when *Heterosporis* can be identified in fish PE, 200 fathead minnows were passively exposed to *Heterosporis* spores as previously described and then placed in five 38 L aquaria. After two weeks, five minnows were randomly chosen, euthanized with MS-222, and the skin was removed. The muscle was first examined for

signs of infection with the naked eye and then examined with a stereomicroscope. If no signs of infection were observed, a 0.50 cm² piece of muscle from behind both pectoral fins was placed on a slide, the muscle was teased apart, and observed under a compound microscope. Ten minnows were then examined every seven days for 12 weeks.

Differences in early developmental stages in muscle have been used to delineate species of *Heterosporis*. Therefore, I wanted to elucidate the stages of development for later identification. I prepared early stages of developing *Heterosporis* in muscle for examination with Transmission Electron Microscopy (TEM). Infected tissue was collected from two fathead minnows at three and eight weeks PE and prepared using a procedure modified from Lee and Soldo (1992). Muscle was cut into 1 mm cubes, fixed in 2.5% glutaraldehyde for 1 h at room temperature, and washed three times in Sorensen's phosphate buffer (0.15 M) for five minutes. The muscle was then post-fixed in 2% osmium tetroxide (OsO₄) for 1 h and was again rinsed with Sorensen's phosphate buffer (0.15M) for five minutes and then placed in Sorensen's phosphate buffer and shipped to the University of Wisconsin-Madison for processing. The samples were then washed repeatedly in buffer and dehydrated in a graded series of ethanol (35%, 50%, 70%, 80%, 90%, 95%, 3 x 100% for 10 min), then transitioned with propylene oxide (2 x 7 min) and placed in a solution of 50% propylene oxide: 50% unaccelerated Spurr's resin overnight at room temperature. Samples were then placed into unaccelerated Spurr's resin for two days at 60°C, and subsequently placed in silicon molds and polymerized for 24 h at 60°C. Sections (80nm) were made on a Reichert-Jung Ultracut E, placed on 300-mesh Copper grids, post-stained with uranyl acetate and lead citrate, and viewed on a Philips CM120 operating at 80kV. Images were captured with a SIS Megaview III digital

camera (Dr. Randall Massey, operations manager, UW-Electron Microscope Facility, Madison, WI)

PCR Method for *Heterosporis* Identification.

A PCR protocol was developed to correctly identify *Heterosporis* sp. infection in fish species. *Heterosporis*-infected tissue obtained from Catfish Lake was sent to Dr. Frank Nilsen (Department of Fisheries and Marine Biology, University of Bergen, Norway) for sequencing rDNA. Using these sequences, primers were developed to amplify a DNA sequence specific for *Heterosporis* sp. using protocols from Docker et al. (1997) and Gerhardt et al. (1994). A nested PCR extraction was used to increase the amplification of the DNA and reduce the chance of interference from similar microsporidian species. The primers used in the first round of amplification were HSF1 (5'-GTG TGT CAA AAG CGG TTG CG-3') and HSR1 (5'-CGA TCA CCA TTA TCT CTC GAA AAT CC-3'). The set of primers used for the nested amplification were HSF2 (5'-CTT CTT TCA AGG GGG GCA-3') and HSR2 (5'-CCT GTC CTG TCC AAA AAA ACA GC-3'). Nucleic acid was extracted with the DNeasy Tissue Kit (Qiagen Inc., Valencia, California). PCR was conducted in 50 µL reactions using 2 µL of each primer (10 µM stock) (HSF1 and HSR1 for 1st round and HSF2 and HSR2 for nested round), 1 µL of dNTP mix (dATP, dCTP, dTTP, dGTP) (10 mM stock), 5.0 µL of 10x DNA buffer, 5.0 µL of MgCl (25 mM stock), 2.0 units or 0.5 µL of *Taq* polymerase (5 units/µL stock) and 1 µL of the prepared genomic DNA in 33.5 µL of ddH₂O.

Samples underwent the following Perkin Elmer thermocycler parameters:

1. Pre-dwell samples at 95°C for 5 min

2. 5 cycles of the following:
 - a. Denaturing at 94°C for 30 s
 - b. Annealing at 52°C for 30 s
 - c. Extending at 72°C for 30 s
3. 35 cycles of the following:
 - a. Denaturing at 94°C for 30 s
 - b. Annealing at 59°C for 30 s
 - c. Extending at 72°C for 30 s
4. Post-dwell at 72°C for 7 min

After the initial amplification was complete, samples were frozen until the nested amplification could be completed. Samples were amplified as described above, except that the nested primers were substituted, and 1 μ L of first round amplified DNA replaced the genomic DNA. The samples had the same amplification temperatures and times as the first amplification. PCR products were visualized by electrophoresis (2 μ L 6x loading dye on a 1% agarose gel and stained with ethidium bromide). A *Hind*III-digested lambda DNA size marker was used as a reference marker. *Heterosporis*-positive samples have a 503 bp segment (Figure 2). Positive controls consisted of infected tissue from *Heterosporis*-infected yellow perch from Catfish Lake. Negative controls consisted of muscle from lab-reared yellow perch obtained from UMESC.

To assess specificity, the *Heterosporis* sp. PCR assay was tested against the microsporidians *Glugea stephani*, *Loma salmonae*, and *Pseudoloma neurophile* obtained from Mike Kent (Director for Center of Disease Research, Oregon State University, Corvallis, OR).

Disinfection

In an attempt to identify methods for effectively disinfecting samples and surfaces contaminated with *Heterosporis*, experiments were conducted analyzing the effects of bleach, aging, freezing, desiccation, and whole fillet freezing on the viability of *Heterosporis*. The experiments used infected muscle isolated from yellow perch caught at Catfish Lake. Muscle was homogenized with a household blender with 2,000 mL of well water for 20 min. The homogenate was then filtered through sieves of decreasing size (2.0 mm, 425 μ m, 180 μ m, and then 106 μ m) into a 3,000 mL Erlenmeyer flask and constantly stirred. The concentration of the final homogenate used in all procedures was 7.5×10^5 spores/mL.

To ensure that the *Heterosporis* spores were viable, four 50-mL samples of tissue homogenate were measured from the stock solution and added to four different 38-L aquaria each containing 60 fathead minnows for 24 h. The fish were monitored at least twice a day to ensure they were not stressed. They were maintained for three months PE and then examined for infection.

Of the 4,000 hatchery-raised fathead minnows obtained from UMESC, 50 were dissected to ensure they were not infected by *Heterosporis* prior to use in the various disinfection experiments.

Four concentrations of bleach were analyzed to determine the concentration needed to effectively kill viable *Heterosporis* spores. Eight 50 mL samples of tissue homogenate were measured from the stock solution and exposed to one of four household bleach (6% sodium hypochlorite) concentrations (22 mg/L, 220 mg/L, 2,200 mg/L, and 22,000 mg/L). The bleach solution was added to the tissue homogenate and was mixed on

a stir plate for 5 min. The homogenate was filtered by suction filtration, through a 2.5- μ m filter to retain spores. The filter was thoroughly rinsed with well water to rinse residual bleach from the spores on the filter. This was repeated until there was no discernable bleach odor. If the filter paper clogged, the remaining suspension was decanted and filtered through a fresh filter. The filter papers were added to an Erlenmeyer flask with 100 mL of well water; stoppered and shaken vigorously for 60 s. The contents of the flask were added to a 38 L aquarium containing 60 fathead minnows. After 24 h, the paper was removed and the half of the water was changed daily for one week. Fish were monitored at least twice a day to ensure bleach residue was not unduly stressing them. The fish were maintained three months PE and then examined for infection by microscopy and confirmed by PCR.

To determine the viability of spores in aerated water over time, six 50 mL samples of tissue homogenate were each placed into 2,000 mL widemouth plastic containers. The containers were filled with 1,500 mL of well water, aerated, and covered loosely with a plastic lid to allow air exchange. Containers were monitored twice per week, and well water was added every two months to maintain constant volume. At one week, two months and six months, two randomly chosen containers were emptied into two separate 38 L aquaria, each containing 60 healthy hatchery-raised fathead minnows, which were then exposed to the *Heterosporis* for 24 h. These fish were maintained three months PE and examined for infection.

To determine if temperatures below freezing affected viable *Heterosporis* spores, six 50-mL samples of tissue homogenate were placed in 100 mL screw top plastic bottles and immediately frozen at -20°C. After 24 h, one week and two months, two bottles were

thawed and poured into two separate 38 L aquaria each containing 60 fathead minnows and exposed for 24 h. These fish were maintained three months PE and were then examined for infection.

To determine if desiccation had an effect on viability of *Heterosporis* spores, four 50-mL samples of tissue homogenate were filtered through a 2.5- μ m filter and dried. If the filter paper clogged, the remaining suspension was decanted and filtered through a new filter. After air drying, filters were maintained in covered Petri dishes for 24 h or one month. Filters were then minced and placed in a 1000 mL flask with 100 mL of well water and sealed with a rubber stopper. The flask was shaken vigorously for 60 s. The contents of the flask were then added to a 38 L aquarium with 60 minnows, and left for 24 h before removing the filter paper. The fish were maintained for three months PE and then examined for infection.

To determine if spores remain viable when left in the muscle of the fish and subjected to freezing, five fresh, heavily infected fillets from yellow perch obtained from Catfish Lake, WI were placed individually into Zip-loc[®] bags and frozen at -20°C for 72 h. The fillets were thawed and homogenized in 250 mL of well water. The homogenate was filtered through cheesecloth, and half of the homogenate was placed into one of two aquaria each containing 60 fathead minnows for 24 h. The fish were maintained for three months PE and then examined for infection.

RESULTS

Spore and Sporophorocyst Morphology.

The *Heterosporis* sp. found in northern Wisconsin, Minnesota, and Lake Ontario is found in the skeletal muscle of yellow perch. Individual spores are pyriform shaped, from 3.3 μm to 9 μm in length (mean length= 6.9 μm) (Figure 3). Their width ranged from 2.5 μm to 4.1 μm (mean width= 3.2 μm). The sporophorocysts varied in length from 7.4 μm to 12.3 μm (mean length= 10.9 μm) and the sporophorocyst vesicles length ranged from 18.9 μm to 73 μm (mean length= 42.3 μm) (Figure 4).

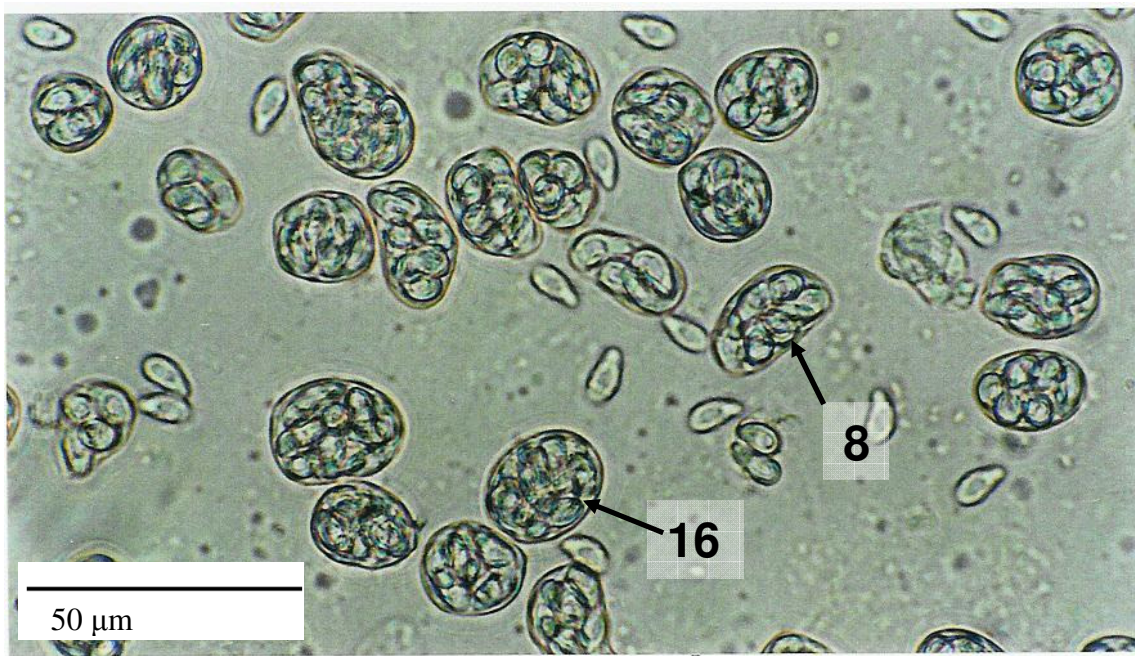
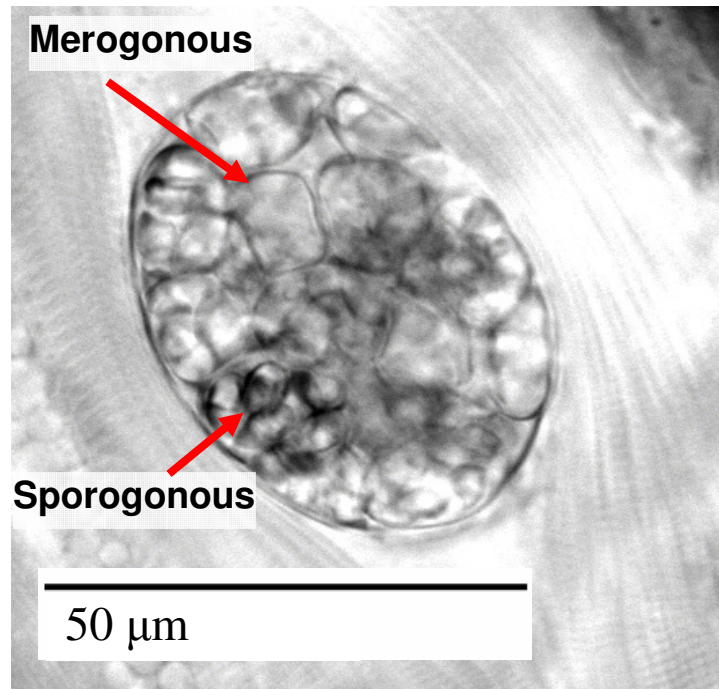
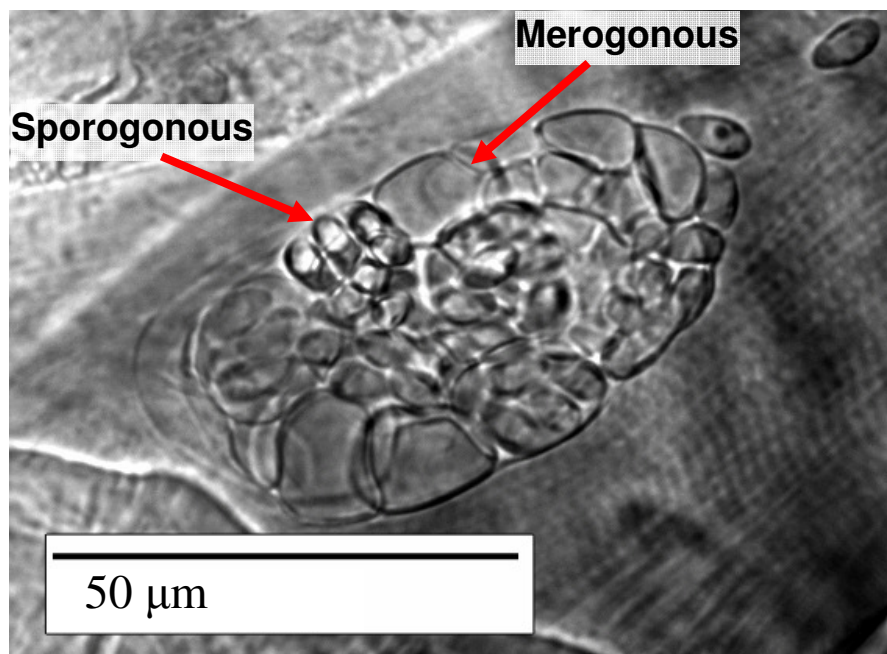


Figure 2. Wetmount preparation of *Heterosporis* sp. from infected muscle of a yellow perch (*Perca flavescens*) demonstrating the presence of either 8 or 16 spores in a single sporophorocyst (SPC). Note: several individual spores have been released from the SPC.



(A)



(B)

Figure 3. A and B. Light micrographs showing a single *Heterosporis* sp. sporophorocyst in muscle of yellow perch containing sporophorocyst vesicles with both merogonous and sporogonous stages of development.

Geographic Distribution of *Heterosporis* in Great Lakes Region.

The species of fish infected with *Heterosporis* varied among locations. Yellow perch infected with *Heterosporis* were obtained every sampling period at Catfish Lake and the overall prevalence of infection from 1999-2006 averaged 16.8% of fish sampled. The prevalence of *Heterosporis* in Catfish Lake was very variable ranging from 4.7% of yellow perch sampled in March of 2001 to 34% of yellow perch sampled in March of 2004 (Figure 5). In 2005, less than 10% of yellow perch sampled were infected with *Heterosporis*. Sixteen fish species were examined from Catfish Lake from January 2000 to June 2005 (Table 1). In 2002, *Heterosporis* was identified by microscopic examination and confirmed by PCR in mottled sculpin (*Cottus bairdi*), pumpkinseed (*Lepomis gibbosus*), burbot (*Lota lota*), walleye (*Sander vitreus*), rock bass (*Ambloplites rupestris*) and trout-perch (*Percopsis omiscomaycus*). This is the first report of these species being infected with *Heterosporis*. In 2003, yellow perch and rock bass from Scattering Rice Lake, WI were found to be infected with *Heterosporis* (Table 1). Of the 14 species of fish from Steamboat Lake and six species from Leech Lake in Cass County, MN, yellow perch was the only fish species infected (Table 1). Of the 14 species examined from Lake Ontario and the Bay of Quinte, yellow perch, rock bass, and pumpkinseed were the only species infected with *Heterosporis* (Table 1). None of the sea lamprey (n=269) from Lake Ontario, emerald shiners (n=275), southern redbelly dace (n=103), stonerollers (n=47), spottail shiners (n=42), or fathead minnows (n=4) examined from Gollon Bait and Tackle in 2003 were infected with *Heterosporis*.

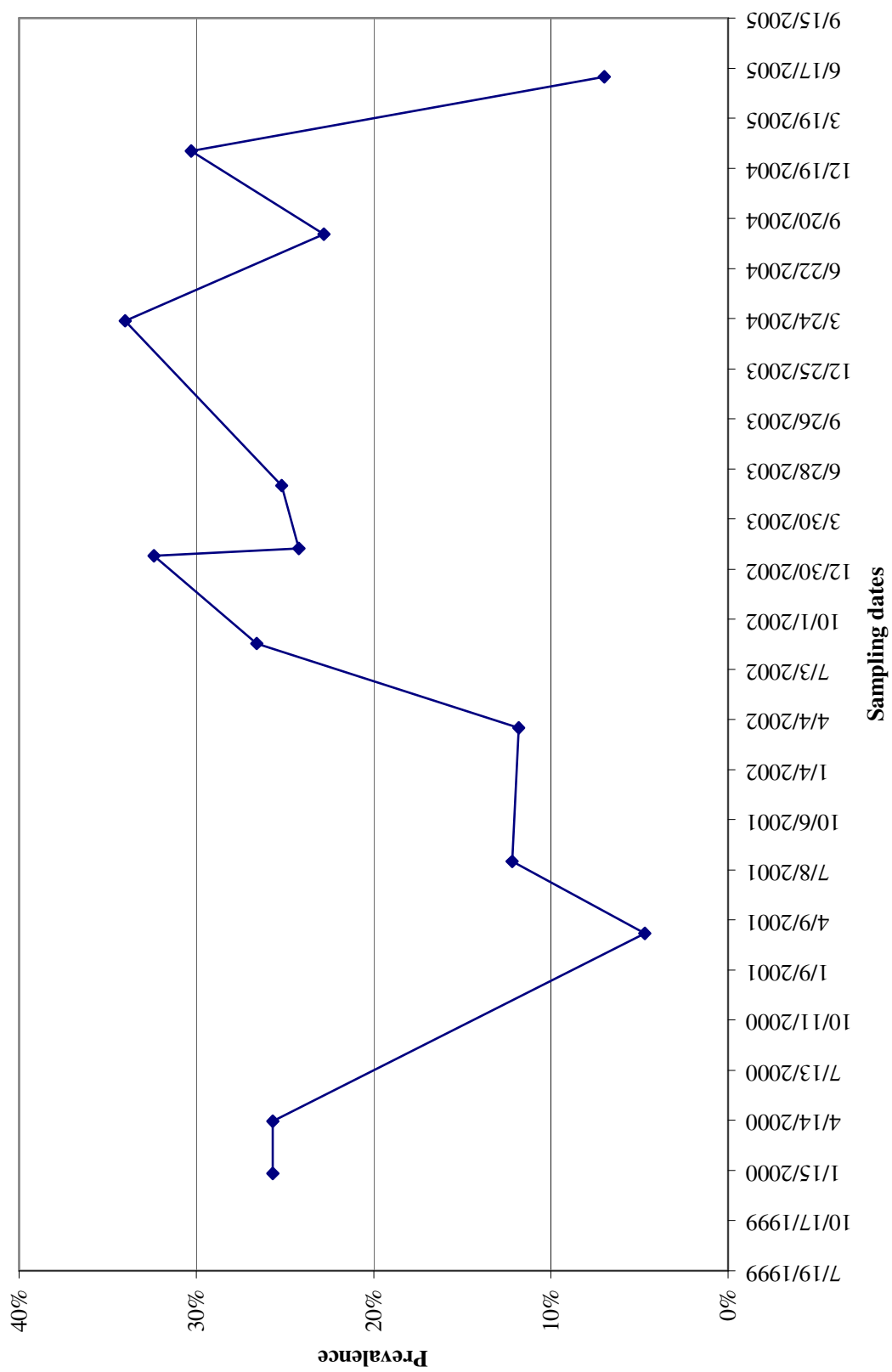


Figure 4. Prevalence (number infected/number examined x 100) of *Heterosporis* sp.-infected yellow perch (*Perca flavescens*) in Catfish Lake, Wisconsin from October 1, 1999 to June 1, 2005.

Waterborne Transmission of *Heterosporis* sp.

Twelve of the 17 different fish species exposed to *Heterosporis* spores directly in the water became infected (Tables 2 and 3). The prevalence of infection varied from 4% in largemouth bass to 100% in rainbow trout and lake trout. After exposing channel catfish to *Heterosporis*, the fish passively exposed and those fed infected tissue were inadvertently mixed together in the aquarium, but 17 of the 50 catfish were infected with *Heterosporis*.

Table 2. Fish species susceptible to *Heterosporis* sp. after passive exposure to a stock spore suspension of unknown concentration.

Fish species	Number infected	Number examined	Percent infected
<i>Oncorhynchus mykiss</i>	15	15	100
<i>Sander vitreus</i>	9	12	75
<i>Pimephales promelas</i>	45	84	53
<i>Ictalurus punctatus</i>	17	50	34
<i>Perca flavescens</i>	8	33	24
<i>Gambusia affinis</i>	2	22	9
<i>Micropterus salmoides</i>	1	25	4
<i>Acipenser fulvescens</i>	0	2	0
<i>Lepomis macrochirus</i>	0	26	0

Effects of Spore Concentration on Transmission.

The concentration of *Heterosporis* spore suspension may affect the infection rates of fish (Table 3). However, at the medium (2.0×10^6) spore concentration, white suckers did not become infected although they became infected at the low (2.0×10^5) and high (2.0×10^7) concentrations. Golden shiners, northern pike, and smallmouth bass did not become infected even at the high concentration. All of our lake sturgeon died prior to

three months post-exposure; however, several lived past one month post-exposure and none showed signs of infection.

Table 3. Fish species susceptibility to a specified concentration of *Heterosporis* sp. spores suspended in the water during a 24-h exposure period.

Fish Species	Treatment	Concentration (spores/mL)	Number of fish infected	Number of fish examined	Percent infected
<i>Notemigonus crysoleucas</i>	Low	2.0×10^5	0	20	0
	Med	2.0×10^6	0	23	0
	High	2.0×10^7	0	25	0
<i>Oncorhynchus kisutch</i>	Low	2.0×10^5	0	27	0
	Med	2.0×10^6	1	22	4.5
	High	2.0×10^7	6	26	23.1
<i>Catostomus commersoni</i>	Low	2.0×10^5	3	32	9.4
	Med	2.0×10^6	0	18	0
	High	2.0×10^7	3	32	9.4
<i>Salvelinus fontinalis</i>	Low	4.0×10^5	8	39	20.5
	High	7.5×10^5	12	39	30.8
<i>Esox lucius</i>	Low	7.0×10^5	0	43	0
	High	2.3×10^6	0	40	0
<i>Micropterus dolomieu</i>	Low	7.5×10^6	0	31	0
	High	2.25×10^7	0	24	0
<i>Salvelinus namaycush</i>	Low	5.0×10^6	17	17	100
	High	7.6×10^6	0 ^a	25	NA
<i>Salmo trutta</i>	Low	2.5×10^6	18	35	51.4
	High	1.75×10^7	26	42	61.9

Susceptibility of Fish to Oral Infection of *Heterosporis*.

Five of six fish species fed pieces of *Heterosporis*-infected fish muscle tissue became infected (Table 4). However, the single infected bluegill had only one sporophorocyst in the muscle three months PE. In the pool of fathead minnows used as live *Heterosporis*-infected prey for the rainbow trout, there was a 60% prevalence of *Heterosporis* infection. None of the 39 rainbow trout that were fed potentially infected fathead minnows became infected with *Heterosporis*.

Table 4. Fish species susceptible to *Heterosporis* sp. infection when fed infected pieces of fish muscle.

Fish species	Number infected	Number examined	Percent infected
<i>Oncorhynchus mykiss</i>	13	13	100
<i>Pimephales promelas</i>	49	80	61.3
<i>Sander vitreus</i>	2	4	50
<i>Micropterus salmoides</i>	4	29	13.8
<i>Lepomis macrochirus</i>	1 ^a	25	4
<i>Acipenser fulvescens</i>	0	5	0

^a Only a single sporophorocyst was observed in the muscle at three months post-exposure.

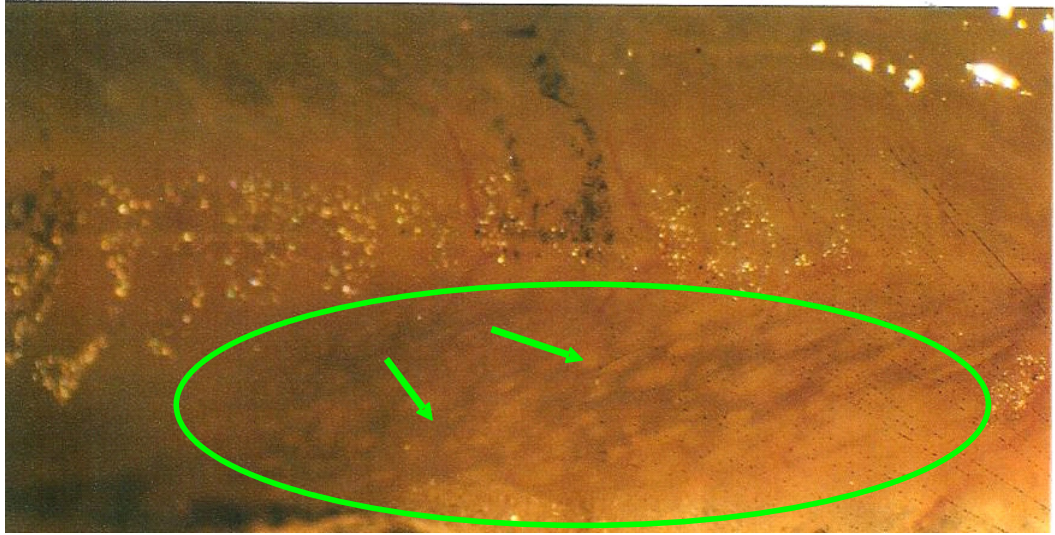
Predator-Prey Interactions.

Smallmouth bass ate significantly ($P=0.0086$) more *Heterosporis*-infected minnows than uninfected minnows. Twenty-five of the 30 fathead minnows examined were infected. During the 32 trials in which bass were presented with ten fathead minnows each, 137 fathead minnows were not consumed by bass, 170 fathead minnows

were eaten and recovered by gastric lavage, and 13 fathead minnows were assumed to have been ingested and completely digested prior to gastric lavage. Of the 170 fathead minnows eaten and recovered, 69 of the minnows (41%) were infected with *Heterosporis*. Of the 137 fathead minnows not consumed by the bass, 36 of the minnows (26%) were infected with *Heterosporis*. None of the 16 smallmouth bass were infected with *Heterosporis* at the end of feeding trials.

Development of *Heterosporis* in Skeletal Muscle.

At two weeks PE to *Heterosporis* spores, sporophorocysts were detected with a compound microscope in the muscle tissue of one the of five fathead minnow examined. After three weeks PE, three of the ten minnows showed microscopic signs of infection. Of the six wet mount preparations from the three infected fish, no more than five sporophorocyst vesicles (SPVs) were detected. These SPVs contained sporophorocysts with both merogonous and sporogonous stages (Figure 4). During merogony, the nucleus divides several times before the cytoplasm divides. During sporogony, spores reproduce by asexual division. Four weeks PE, eight of the ten minnows examined were infected; wet mount preparations revealed the presence of one to 14 SPVs present in each fish and more than one SPV was present in a single muscle cell. At five weeks PE, four of six infected minnows (from ten examined) harbored infections that were evident macroscopically (Figure 6) and over half of the SPVs contained sporophorocysts with merogonous and sporogonous stages. Six weeks PE, eight of ten infected minnows had infections detectable with a dissecting microscope. Under a compound microscope, the muscle tissue from these fish harbored infections estimated to exceed 1,000 SPVs (Figure 7). Some SPVs contained sporophorocysts with merogonous and sporogonous



(A)



(B)

Figure 5. *Heterosporis* sp. infections characterized by white or cream colored spots in the muscle of fathead minnows (*Pimephales promelas*) 10 weeks post-exposure (A) and 12 weeks post-exposure (B).

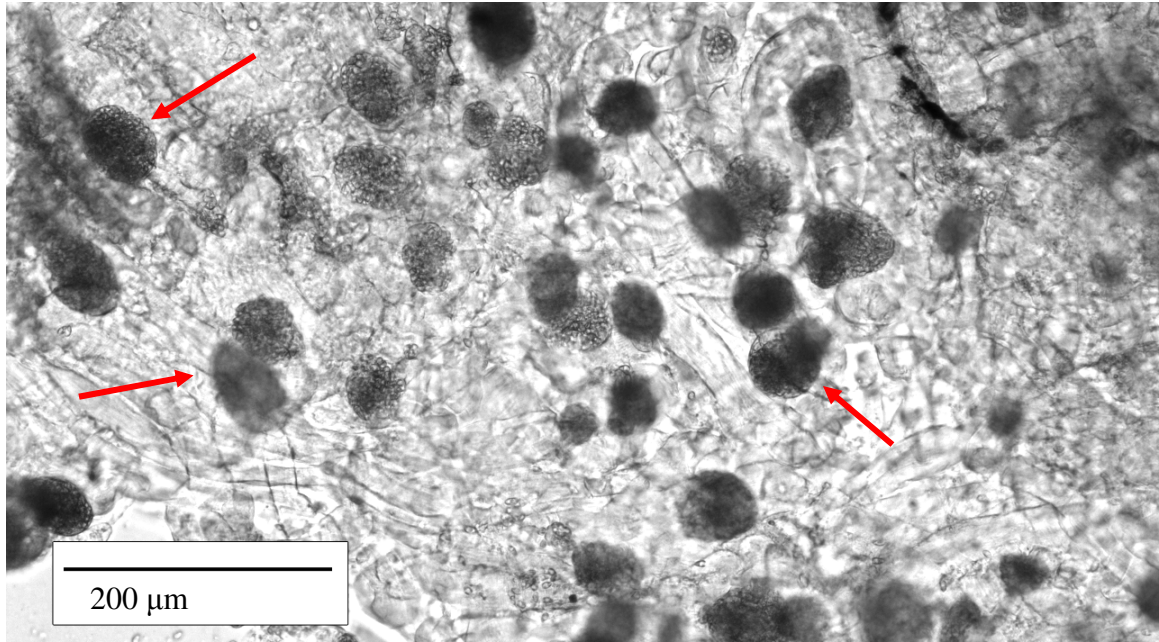
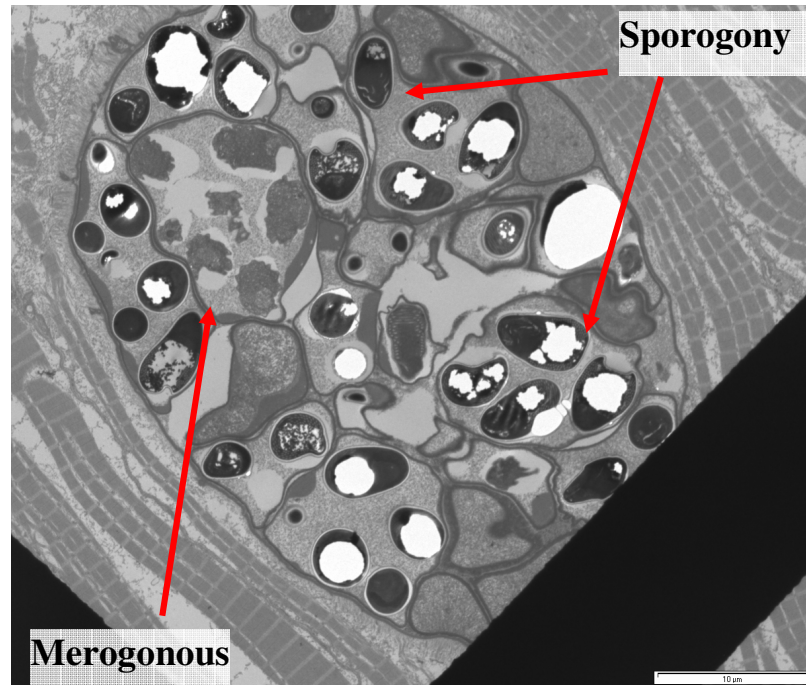
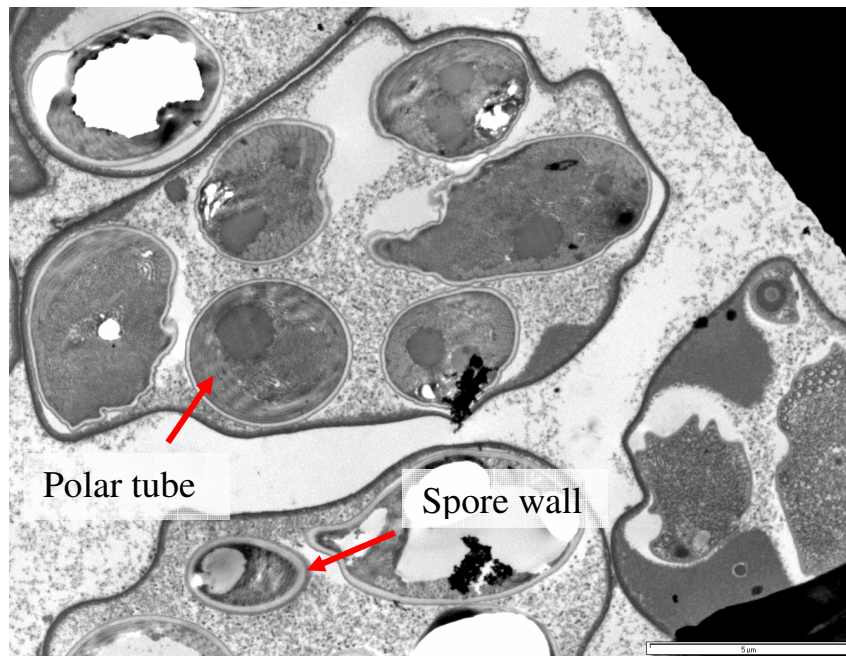


Figure 6. Squash preparation of *Heterosporis*-infected muscle from a laboratory-infected fathead minnow (*Pimephales promelas*) 10 weeks post-exposure showing many sporophorocyst vesicles.

stages and other SPVs contained sporophorocysts with fully developed spores. At seven to 12 weeks PE, most minnows exhibited evidence of *Heterosporis* infection that could be viewed with the naked eye. The focal areas of infection were made up almost exclusively of mature SPVs containing only spores. Peripheral areas contained sporophorocysts with both merogonous and sporogonous stages. *Heterosporis* is correctly identified to genus by the presence of either 8 or 16 spores for each sporophorocyst (Lom et al. 2000) (Figure 3). As the sporophorocyst vesicles develop inside the sporophorocyst, minimal damage occurs in the surrounding skeletal muscle cells. After three weeks of exposure, TEM micrographs showed features of each individual spore, such as the coiled polar tube and the spore wall (Figure 8).



(A)



(B)

Figure 7. Transmission electron micrograph of a *Heterosporis* sp. sporophorocyst containing merogony and sporogony stages (A) and a developed sporophorocyst vesicle from a fathead minnow (*Pimephales promelas*) skeletal muscle cell (B).

PCR Method for *Heterosporis* Identification.

Heterosporis from yellow perch is related to *H. anguillarum*, based on available sequence data (Nilsen 2000). Using F1 and R1 primers, a 971 bp fragment was amplified by PCR from the DNA of *Heterosporis* spores located in the muscle tissue of yellow perch. Using the F2 and R2 nested primers, a 503 bp segment was produced (Figure 2). The DNA isolated from the muscle tissue of a non-infected yellow perch did not show any bands on the gel, indicating that there was no interference from the fish DNA extracted. No cross reactivity occurred when the perch *Heterosporis* PCR assay was used on DNA from *Glugea stephani*, *Loma salmonae* and *Pseudoloma neurophile*.

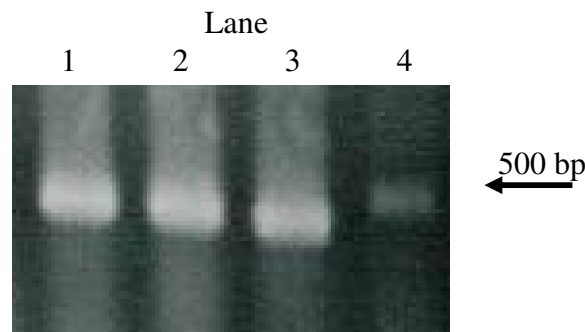


Figure 8. Agarose gel electrophoresis from nested PCR, demonstrating *Heterosporis* sp. DNA from yellow perch (*Perca flavescens*) muscle tissue. Lanes 1-3, positive bands (503 bp) of DNA from *Heterosporis* sp.; lane 4, *Hind*III-digested lambda DNA size marker.

Disinfection.

The spores of *Heterosporis* were susceptible to various types of disinfection techniques (Table 5). *Heterosporis* spores were no longer infective to fathead minnows after 24 h of freezing and desiccation. Likewise, freezing infected fillets for 72 h completely inactivated the spores. *Heterosporis* spores were also rendered inactive when exposed to concentrations of at least 2200 mg/L of sodium hypochlorite (6% solution of

household bleach). However, *Heterosporis* spores were resistant to low levels of sodium hypochlorite. In a 5-minute treatment, bleach concentrations of 220 mg/L and below were ineffective at killing all *Heterosporis* spores; 40% of fathead minnows exposed to treated spores became infected. When spores were exposed to a concentration of 22 mg/L, over 47% of fathead minnows became infected. After aging spores at room temperature for one week, over 45% of the minnows exposed to the liberated spores became infected with the parasite. After aging for two months, over 32% of the fathead minnows became infected. However, after aging for six months, none of the fathead minnows became infected. Control tanks yielded an average infection prevalence of 52.3% in exposed fathead minnows.

Table 5. Effects of different types of disinfection on *Heterosporis* sp. spores.

Spore treatment	Number of fish infected	Number of fish examined	Percent infected
Control	57	109	52.3
Dessication			
24 h	0	67	0
1 month	0	70	0
Freeze			
24 h	0	118	0
1 week	0	112	0
2 months	0	56	0
6 months	0	0	0
Fillet freeze			
72 h	0	92	0
Aging			
1 week	31	68	46
2 months	17	52	33
6 months	0	0	0
Bleach			
22000 mg/L	0	136	0
2200 mg/L	0	125	0
220 mg/L	44	110	40
22 mg/L	46	97	47

DISCUSSION

Previously, taxonomy and phylogeny of Microsporidia have been based primarily on the ultrastructure of spores and pre-spore stages. During the last 15 years, molecular sequencing data has proven to be useful in analyzing and determining the taxonomy of microsporidians. A BLAST search was completed to determine nucleotide sequences of *Heterosporis* species and similar microsporidians (Biology WorkBench [<http://workbench.sdsc.edu/>]). *Heterosporis* (*Pleistophora*) *anguillarum* is most closely related to the microsporidians *Vavraia culicis* and *Trachipleistophora hominis*. It is also closely related to *Glugea* and *Loma*, and I was able to obtain samples of these two genera to compare to *Heterosporis* from yellow perch. These other microsporidia were not sufficiently related to give false positives in PCR assays. But it is important to obtain samples of *Vavraia* and *Trachipleistophora*, and from other *Heterosporis* species such as *H. schuberti*, *H. anguillarum*, and *H. finki* in order to further confirm the specificity of the PCR reaction to the *Heterosporis* sp. Because samples of *H. schuberti*, *H. anguillarum*, and *H. finki* were not available, it is still not known if the *Heterosporis* from yellow perch is a new species. The individual spore size (mean= 3.2µm x 6.9µm) of the yellow perch *Heterosporis* appears to fall within the range of the three described *Heterosporis* species. However, based on the parasite's low host specificity and the fish species that are infected, it appears that this may be a new species of *Heterosporis*. More analyses are needed to rule out cross-reactivity between similar microsporidian parasites and to determine its full molecular sequence.

It is not known if this *Heterosporis* is an exotic species or native to North America. It is possible that it had infected fish prior to 2000, but so infrequently, or in

such low levels in fish that it was not detected. However, fish disease biologists on the Great Lakes Fish Health Committee speculate that such a valuable sport and commercial species as yellow perch are examined so frequently that a conspicuous parasite such as *Heterosporis* should have been discovered prior to 2000 if it is a native to North America (Susan Marcquenski, Fish Health Specialist, Wisconsin DNR, personal communication). Because aquarium fish are reported as the fish hosts for most *Heterosporis* species, it is possible that infected ornamental fish may have been accidentally or intentionally released into natural waters. Exotic fish may introduce new parasites that may lead to disease outbreaks in native fish (Bauer 1991). The parasite may also have been transported to other water bodies by fish-eating birds. There is also a possibility that *Heterosporis* may have accidentally been introduced into the Eagle River Chain of Lakes by use of forage fish in hatcheries (Joseph Marcino, former Fish and Wildlife Pathologist, Minnesota DNR, personal communication).

Yellow perch appears to be a dominant host for the *Heterosporis* sp. described in this study. If *Heterosporis* and yellow perch were present in a lake, it was detected in the yellow perch. Other species of fish were found to be naturally infected with *Heterosporis*, but were not as heavily infected as yellow perch. These species may not be the primary hosts, but are still susceptible as they share the same habitat. Besides yellow perch, rock bass and pumpkinseed were two of the most commonly infected hosts. Mottled sculpin, burbot, walleye and trout-perch were also infected with *Heterosporis*, but more studies are needed to determine how susceptible they are in lakes. Baitfish and sea lamprey originating in areas endemic for *Heterosporis* were not naturally infected with the

parasite. Again, the numbers of each group of these fish examined is not sufficient to know whether either serves as a reservoir host for *Heterosporis*.

Heterosporis is considered to be an emerging disease, because it has either newly appeared in North American fish, or has existed at low levels but is now possibly increasing in incidence and geographic range. Ecological changes, including those due to agricultural development, are among the most frequently identified factors in emergence of disease (Marcogliese, 2001). Unfortunately, little research has been conducted to determine what effects human-induced perturbation, including climate change, has on aquatic ecosystems (Marcogliese 2001). Temperature has a large effect on the infectivity of parasites to their host species (Speare et al. 1998). In some species, increases in temperature may accelerate parasite growth rates, development, and maturation (Magnuson et al. 1997). Changes in lake levels and eutrophication may also have profound effects on host-parasite relationships. Parasite communities in eutrophic systems tend to be composed of generalists, such as *Heterosporis*, which exhibit wide host specificity (Marcogliese 2001). Also, any form of stress can increase parasite-induced host mortality (Esch et al. 1975). Currently no studies have been conducted on Catfish Lake or surrounding lakes to determine if lake levels or human development around those lakes have any effect on the prevalence of *Heterosporis* (Wes Jahns, WI DNR fisheries technician, Woodruff, WI, personal communication). After repeated sampling of Catfish Lake, WI, the prevalence in yellow perch varied greatly, it does not appear to be seasonal dependent, but continued sampling throughout the year could give more insight into patterns of prevalence.

Of the 17 fish species exposed to *Heterosporis*, 12 became infected with the parasite. When exposed to the parasite, even at levels of 2×10^5 spores/mL, there are species other than those known to be naturally infected that became infected with *Heterosporis*. These species include rainbow trout, fathead minnows, channel catfish, mosquitofish, largemouth bass, coho salmon, white suckers, brook trout, lake trout and brown trout. Not all fish species exposed to *Heterosporis* in the laboratory were species that represented fish found in Wisconsin and Minnesota lakes, and Lake Ontario. Moreover, not all fish species sampled in the lakes were exposed to *Heterosporis* in the lab. Many species sampled in the wild were not available for laboratory exposures. Attempts were made to include as many commercial, sport, and biologically relevant species in the exposure studies as possible. Because preliminary studies indicated that younger fish were more susceptible to *Heterosporis* infection than older fish, age-0 fish were used in most experiments. Some species, such as lake sturgeon, were not conducive to laboratory culture and most did not survive to three months PE.

Based on the four species of baitfish (emerald shiners, stonerollers, southern redbelly dace, and spottail shiners), it would seem that these species of baitfish are not natural hosts for *Heterosporis*. However, I recommend that baitfish continue to be monitored as a potential source of *Heterosporis* transmission into uninfected waters. I was able to infect baitfish such as white suckers, golden shiners and fathead minnows, in the laboratory and these hosts are readily transported to various lakes by anglers. Of the sea lamprey examined, none were infected. However, because they parasitize other fish and feed on blood, they could ingest viable *Heterosporis* spores in the tissues and blood of an infected fish and potentially transfer them to another fish host. Also, large quantities

of sea lamprey are sterilized in laboratories and hatcheries and transported to new water bodies, and are a possible mode of transmission.

Other modes of transmission include feeding on pieces of *Heterosporis*-infected muscle and passive exposure to spores. Except for bluegill, the same species of fish that became infected by passive exposure also became infected by feeding on infected muscle. This would be expected because the route of infection by microsporidia is thought to be oral (Lom and Dykova, Institute of Parasitology, Academy of Sciences, Czech Republic, unpublished data).

Age of fish might also have a determining factor in uptake and transmission of *Heterosporis* spores. Age-0 rainbow trout became infected with *Heterosporis* when fed bite-sized pieces of infected muscle. However, adult (age-2) rainbow trout fed infected fathead minnows did not become infected. Smaller or younger fish may be more susceptible to infection by parasites, due to additional stress from predation and accelerated growth rates (Esch et al. 1975). Likewise, age-2 smallmouth bass did not become infected with *Heterosporis* when fed fathead minnows with patent *Heterosporis* infections. Gastric lavage was performed on adult smallmouth bass within 12 hours of a minnow being eaten, but not all minnows were recovered from the stomach and many prey were more than 50% digested. I assumed that bass were exposed to more than adequate amounts of *Heterosporis* during the two exposures to the infected fathead minnows. However, age-0 smallmouth bass did not become infected with *Heterosporis* when passively exposed to the parasite. It is possible smallmouth bass may not be susceptible to infection at any age.

Predators typically capture impaired individuals. Impaired individuals may have unusual or distinct behaviors that reveal to the predator that they are weak and maybe easier to capture (Temple 1987). A *Heterosporis*-infected minnow may swim slower or abnormally due to the loss of muscle tissue (Dykova and Lom 1980, Holmes and Bethel 1972, Temple 1987). Of the 183 fathead minnows assumed to have been consumed, the remains of only 170 were identifiable after gastric lavage. The 13 fathead minnows unaccounted for were likely completely digested. However, the consumed minnows that were recovered were sampled for *Heterosporis* by PCR. Some minnows had more than 50% of their muscle digested when gastric lavage was performed. With so much of the muscle digested, there may not have been enough spores to accurately detect whether the fish was infected with *Heterosporis*. This may have led to the low percentage of infected minnows in the study. Also, if additional consumed infected fathead minnows went undetected, it would further support that smallmouth bass were more likely to catch *Heterosporis*-infected fathead minnows.

Based on these results, I believe that there are physical or behavioral differences in the infected fathead minnows which lead to an increase in their consumption by smallmouth bass. This can ultimately lead to transmission of *Heterosporis* into new fish species that are predators or viable spores may be passed through a predator's digestive system and into water where they would be available for accidental ingestion by other uninfected fish.

Once a fish has been exposed to *Heterosporis* and ingests infective spores in a variety of methods, the infection spreads rapidly by passing sporoplasm through the lumen of the polar tube in the spore into host cells. This is a good strategy for parasites.

Once infected the parasite reproduces rapidly, producing both meronts and spores, millions of which may be released upon decomposition of the host. Also, if the infection is wide spread in the fish and the fish is eaten by a predator fish, there is a better chance the predator will become infected through transfer of the parasite in epithelial cells into the gut lining of the predator fish.

From a diagnostic standpoint, the greater the number of parasites present in each sample, the better for detection. There is an increased chance of detecting the parasite's DNA in muscle tissue when analyzed by PCR. If it has spread throughout the entire muscle, there is an increase in chance of detection in random tissue samples (biopsies). When fillets of fish are examined by fish health biologists using a compound microscope at 200X, it will be possible to determine if a fish is presumptively infected within three weeks of initial exposure. Five weeks after initial exposure, a stereoscope can be used to presumptively identify infection, and after seven weeks from initial exposure, a infection can be presumptively determined by visual examination. All presumptive diagnosis will need to be confirmed by PCR.

In order to prevent the further spread of *Heterosporis* spores, several disinfection techniques were analyzed. There are published studies on disinfection techniques for several microsporidian species (Canning and Lom 1986, Waller 1979, Wolk et al. 2000) However, there are no published studies on disinfection techniques for *Heterosporis* species. This study showed that the *Heterosporis* spores infecting yellow perch were inactivated by freezing at -20°C for 24 h, by desiccation at room temperature for 24 h, by exposure to bleach concentrations over 2,200 ppm for 5 min, and by aging at room temperature in well water for at least six months.

Anglers can help prevent the spread of *Heterosporis* to new water bodies by using a combination of the following procedures:

1. Notify the local Department of Natural Resources or state fisheries agency if in possession of a fish with clinical signs of *Heterosporis* infection. The biologists should explain how to deliver the suspect fish to a parasitologist or fish health biologist who can confirm the infection. Do not throw the fish back into the water.
2. Drain bilges and allow boats, nets, and gear to dry thoroughly for 24 h.
3. Gear and any standing water in a boat should be exposed to a 2,200 ppm bleach solution for 5 min and then thoroughly rinsed before entering a new water body and when leaving.
4. Store boats outside year round. This will allow the boat to dry more thoroughly and spores to freeze in those localities near *Heterosporis* infected waters in North America.

In summary, it is unknown if this *Heterosporis* species is new or endemic, why certain fish species can be easily infected, or how fish populations might be affected if the parasite spreads to new water bodies. Based on this study, *Heterosporis* infected many age-0 fish species, but most often yellow perch. Fish can become infected by swimming in water containing spores and by ingesting infected muscle tissue. With either mode of infection, *Heterosporis* was detectable within three weeks PE and can be confirmed with a TEM, or PCR. Monitoring programs can be put in place to help prevent the spread of the parasite and to determine if its range has extended to new water bodies. Future studies that would be beneficial include determining the origin of this *Heterosporis* species, how it affects the swimming stamina of infected fish, how water temperature plays a factor in *Heterosporis* development, whether birds can carry viable spores from one lake to

another (by ingesting infected fish and defecating spores) and, determining a method of eradicating the *Heterosporis* parasite with little or no harm to other lake species.

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APPENDIX A

FISH COMMON NAMES WITH THEIR SCIENTIFIC NAMES USED IN
THIS STUDY

Appendix A. Fish common names with their scientific names used in this study.

<u>Common name</u>	<u>Scientific name</u>
black bullhead	<i>Ameiurus melas</i>
black crappie	<i>Pomoxis nigromaculatus</i>
bluegill	<i>Lepomis macrochirus</i>
bowfin	<i>Amia calva</i>
brook trout	<i>Salvelinus fontinalis</i>
brown bullhead	<i>Ameiurus nebulosus</i>
brown trout	<i>Salmo trutta</i>
burbot	<i>Lota lota</i>
channel catfish	<i>Ictalurus punctatus</i>
coho salmon	<i>Oncorhynchus kisutch</i>
Common carp	<i>Cyprinus carpio</i>
emerald shiner	<i>Notropis atherinoides</i>
fathead minnow	<i>Pimephales promelas</i>
freshwater drum	<i>Aplodinotus grunniens</i>
golden shiner	<i>Notemigonus crysoleucas</i>
johnny darter	<i>Etheostoma nigrum</i>
lake herring	<i>Coregonus artedii</i>
lake sturgeon	<i>Acipenser fulvescens</i>
lake trout	<i>Salvelinus namaycush</i>
largemouth bass	<i>Micropterus salmoides</i>
mosquitofish	<i>Gambusia affinis</i>
mottled sculpin	<i>Cottus bairdi</i>
northern pike	<i>Esox lucius</i>
pumpkinseed	<i>Lepomis gibbosus</i>
rainbow trout	<i>Oncorhynchus mykiss</i>
rock bass	<i>Ambloplites rupestris</i>
round goby	<i>Neogobius melanostomus</i>
sea lamprey	<i>Petromyzon marinus</i>
shorthead redhorse	<i>Moxostoma macrolepidotum</i>
silver redhorse	<i>Moxostoma anisurum</i>
smallmouth bass	<i>Micropterus dolomieu</i>
southern redbelly dace	<i>Phoxinus erythrogaster</i>
spottail shiner	<i>Notropis hudsonius</i>
stoneroller	<i>Camptostoma anomalum</i>
trout-perch	<i>Percopsis omiscomaycus</i>
walleye	<i>Sander vitreus</i>
white bass	<i>Morone chrysops</i>
white perch	<i>Morone americanus</i>

Appendix A. cont.

Common Name

white sucker

whitefish

yellow bullhead

yellow perch

Scientific Name

Catostomus commersoni

Coregonus clupeiformis

Ameiurus natalis

Perca flavescens