2009 Project Abstract

For the period ending June 30, 2012

PROJECT TITLE: Improving Emerging Fish Disease Surveillance in Minnesota
PROJECT MANAGER: Katharine Pelican, DVM, PhD
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2009, Chp.143, Sec.2, Subd. 6c. ML 2011, 1st Special Session, Chap. 2, Article 3, Sec. 2, Subd. 18.

APPROPRIATION AMOUNT: $80,000

Overall Project Outcome and Results

Heterosporosis is an emerging disease of importance to Minnesota fish populations. The disease is caused by the previously undescribed microsporidian parasite, *Heterosporis* sp., which effectively destroys the skeletal muscle of susceptible fish hosts. The resulting damage from advanced infection renders the fillet unfit for human consumption and likely results in indirect mortality due to increased predation and reduced fitness. With no treatment of the disease in wild fish populations, management is limited to preventing the spread to naïve fish populations. The goal of this study was to improve diagnostic testing capabilities and perform a survey to prevent the further spread of this important fish disease. To that end, a highly sensitive and specific quantitative PCR (qPCR) assay was developed to detect sub-clinical Heterosporosis disease in fish. This assay vastly improved our capacity to detect the pathogen and was used to survey 50 waterbodies in Minnesota. From this survey and three additional MDNR submitted samples, six new waterbodies were identified as Heterosporis-positive, including: North Long Lake, (Crow Wing County), Mary Lake (Douglas County), a private pond in both Douglas and Pope Counties, Wabana Lake (Itasca County), and Black Hoof Lake (Crow Wing County). Positive fish species from this study included: walleye, yellow perch, cisco, northern pike, and for the first time spottail shiners. Further evaluation to characterize the parasite identified very low genetic variability in the species *H. sutherlandae*, collected from inland waters of Minnesota. However, there was a unique Heterosporis species (*H. superiorae*) in Lake Superior. This suggests a distant evolutionary divergence between the parasite species, but a rapid distribution once introduced into inland waters. These findings highlight the importance of continued surveillance and research to improve our understanding and control this important pathogen in Minnesota.

Project Results Use and Dissemination

The results from this project have been important for the management of the emerging fish disease, Heterosporosis, in Minnesota. This was achieved, in part, by increasing laboratory capacity and diagnostic confidence. The Minnesota Veterinary Diagnostic Laboratory now offers this highly sensitive and specific qPCR assay for surveillance testing and research. In addition, the ability to make science based management decisions at the MDNR has been greatly improved following the survey performed in this study. Understanding the distribution of Heterosporis is essential to controlling the spread.

The results from this project will be widely disseminated online, in press, and presented to a variety of stakeholders. A summary report will be made available on the University of Minnesota Extension's

aquaculture website for review by aquaculture producers, veterinarians, MDNR, LCCMR, and other groups. A more detailed published paper will be prepared for submission to the Journal of Parasitology and presented at the American Fisheries Society – Fish Health Section Annual Meeting to update the scientific community on these important findings.

Environment and Natural Resources Trust Fund 2009 Work Program Final Report

Date of Report: September 14, 2012
Date of Next Progress Report: Final Report
Date of Work Program Approval: June 16, 2009
Project Completion Date: June 30, 2012

I. PROJECT TITLE: Improving Emerging Fish Disease Surveillance in Minnesota

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Location: The majority of the project will take place on the University of Minnesota, St. Paul campus, in the Veterinary Diagnostic Laboratory and aquaculture facility. Fish collected for surveillance testing have already been acquired and banked from throughout the state during 2008 through a relationship with the Minnesota Department of Natural Resources (see map).

Total Trust Fund Project Budget:	**Trust Fund Appropriation**	**$**	**80,000**
	Minus Amount Spent:	**$**	**80,000**
	Equal Balance:	**$**	**0**

Legal Citation: M.L. 2009, Chp.143, Sec.2, Subd. 6c. ML 2011, 1st Special Session, Chap. 2, Article 3, Sec. 2, Subd. 18.

Appropriation Language:
$80,000 is from the trust fund to the Board of Regents of the University of Minnesota to assess mechanisms and control of the transmission of Heterosporosis, an emerging fish disease in Minnesota, to assist in future management decisions and research. Carry forward (a) The availability of the appropriation for the following projects is extended to June 30, 2012: 6) Laws 2009, chapter 143, section 2, subdivision 6, paragraph (c), Improving Emerging Fish Disease Surveillance in Minnesota.

II. and III. FINAL PROJECT SUMMARY

Heterosporosis is an emerging disease of importance to Minnesota fish populations. The disease is caused by the previously undescribed microsporidian parasite, *Heterosporis* sp., which effectively destroys the skeletal muscle of susceptible fish

hosts. The resulting damage from advanced infection renders the fillet unfit for human consumption and likely results in indirect mortality due to increased predation and reduced fitness. With no treatment of the disease in wild fish populations, management is limited to preventing the spread to naïve fish populations. The goal of this study was to improve diagnostic testing capabilities and perform a survey to prevent the further spread of this important fish disease. To that end, a highly sensitive and specific quantitative PCR (qPCR) assay was developed to detect sub-clinical Heterosporosis disease in fish. This assay vastly improved our capacity to detect the pathogen and was used to survey 50 waterbodies in Minnesota. From this survey and three additional MDNR submitted samples, six new waterbodies were identified as Heterosporis-positive, including: North Long Lake, (Crow Wing County), Mary Lake (Douglas County), a private pond in both Douglas and Pope Counties, Wabana Lake (Itasca County), and Black Hoof Lake (Crow Wing County). Positive fish species from this study included: walleye, yellow perch, cisco, northern pike, and for the first time spottail shiners. Further evaluation to characterize the parasite identified very low genetic variability in the species *H. sutherlandae*, collected from inland waters of Minnesota. However, there was a unique Heterosporis species (*H. superiorae*) in Lake Superior. This suggests a distant evolutionary divergence between the parasite species, but a rapid distribution once introduced into inland waters. These findings highlight the importance of continued surveillance and research to improve our understanding and control this important pathogen in Minnesota.

Despite the advancements made in this study, many questions remain. To date, no population level affects have been attributed to Heterosporis. However, the potential indirect parasite induced mortality or added stress in an ever increasingly compromised ecosystem is concerning. Further research into the effects of this parasite on the host and population level is warranted to make science-based management decisions. In addition, this study did not investigate the risk this parasite poses to other animals, including humans. While microsporidian diseases are becoming more frequently reported around the world, the two species described here have never been reported outside a fish host. Although the zoonotic potential is low, it is recommended by the MDNR to thoroughly cook all fish prior to consumption. A more thorough public health evaluation should be performed.

IV. OUTLINE OF PROJECT RESULTS*:*

Result 1: Validation of a quantitative PCR assay to detect *Heterosporis* sp. in fish.

Description: We will validate a quantitative PCR assay that satisfies four areas 1) Sensitive – the assay will have a sensitivity comparable to other qPCR assays, about 10 copies per reaction. 2) Specific – the assay will be specific for Heterosporis DNA and not detect related species. 3) Accurate – the assay will be have a PCR efficiency between 90 – 110% and correlation of the standard curve greater than 0.9. 4) Precise – the assay will be provide the similar results with samples tested over three days, and by

two different laboratories. The Minnesota Department of Natural Resources will provide positive and negative control samples. The validation will be completed July 2009 – March 2010.

Deliverables	Completion Date	Budget
A. *Validation of a quantitative PCR assay to detect* Heterosporis *sp. in fish.*	March 2010	$11,000

Summary Budget Information for Result 1: **Trust Fund Budget: $ 11,000**
Amount Spent: $ 11,000
Balance: $ 0

Final Report Summary

A. *Validation of a quantitative PCR assay to detect Heterosporis sp. in fish.*

A quantitative PCR (qPCR) assay was developed and validated for the detection of Heterosporis sp. in fish. The assay was modified several times throughout the study to optimize the sensitivity, specificity, and turnaround speed based on results collected as part of Studies 2 – 4. This new assay vastly improves the sensitivity and specificity as compared to previous diagnostic tests. Detection has been improved to identify five spores per sample, long before clinical lesions are observed. The assay did not cross react with related microsporidian species, but did detect all samples in this study. It is possible that testing wild fish with a plethora of undescribed parasites, closely related species may cross-react or be missed by these methods. It is important to note that while qPCR offers many advantages, traditional diagnostic method (visual inspection) and case history should be considered during result interpretation.

The use of this diagnostic test is a major advancement in the management of this emerging fish disease. This assay will be available at the University of Minnesota Veterinary Diagnostic Laboratory for research and surveillance testing. In addition, details of this assay will be published in a peer-reviewed journal and presented at the American Fisheries Society – Fish Health Section (AFS-FHS) annual meeting for use by other laboratories.

Result 2: Determination of optimal sampling methods and tissue selection for *Heterosporis* sp. in fish.

Description: To determine optimal sampling methodology, yellow perch (Infected; n = 20) will be experimentally infected with Heterosporis spores per os and compared to un-infected fish (Control; n = 20). Two months following exposure to *Heterosporis* spores, fish will be humanely euthanized, and matched tissue (muscle (three sections: dorsal, central, and caudal) kidney, spleen, liver, blood) collected and tested from Infected and

Control fish. Tissues from each fish will be homogenized separately and three replicate PCR tests will be performed on each homogenized sample. The tissue that results in the most specific and sensitive identification of infected fish over control fish will be identified. Optimization of sampling methods will be completed January 2010 – January 2011 .

To prevent the risk of contaminating the public water supply and wild fish while maintaing the infected fish, a safe biosecure facility must be developed. An aquatic system of 6 50-gal aquaria will be constructed in the Veterinary Diagnostic Laboratory. The system will operate as either a static of recirculating system, depending on the presence of pathogens. An ultra violet filter and individual aerators will be used to maintain water quality. All potentially infected water and fish will be disinfected in the VDL tissue digester. The tanks will be subsequently be disinfected with a 10% bleach solution to prevent contamination of future studies. This laboratory will be a first at the University of Minnesota and provide the necessary space for future fish disease investigations. Laboratory development will be completed June 2009 – June 2010.

Deliverables	**Completion Date**	**Budget**
A. *Development of a fish disease research facility.*	June 2010	$17,500
B. *Infect fathead minnows with* Heterosporis *spores.*	August 2010	$3,000
C. *Determination of optimal sampling methods and tissue selection for Heterosporis sp. in fish.*	Jan 2011	$5,000

Summary Budget Information for Result 2: **Trust Fund Budget: $25,500**
Amount Spent: $25,500
Balance: $0

Final Report Summary

A. *Development of a fish disease research facility*

The new Fish Disease Research Area (FDRA) within the Aquaculture/ Fisheries Laboratory was completed. A system of 24 20-gallon tanks were set up within a confined space, including separate water supply and flow through system, biosecurity procedures and supplies, and barriers to prevent contact with other disease-free research projects in the Laboratory. No infected water or fish came in contact with the Aquaculture/Fisheries Laboratory's recirculating system or the city sewer system. The FDRA allowed the holding of fish infected with *Heterosporis* sp., with minimal risk to other research projects or natural fisheries.

B. *Infect fathead minnows with Heterosporis spores*

Fathead minnows were exposed to fresh *Heterosporis* spores by a bath treatment in February 2011. The parasite was confirmed by qPCR in the intestinal tract two weeks post exposure and in muscle tissue one month post exposure. It was not until four months post exposure that clinical signs (visually

apparent necrosis of skeletal muscle) was first observed. This is consistent with our hypothesis that low level and early infections would frequently be missed by traditional diagnostic methods and demonstrates the value of the qPCR assay for detection of this pathogen in fish populations.

The infection trial did take longer than expected to achieve clinical signs of disease. Compared to previous research suggesting a two month time period, our trial took twice as long to achieve clinical signs. Conditions such as environment, host species, dose rate and method were all consistent between treatments. It is possible that the *Heterosporis* spores used for this treatment were less virulent, less productive, had fewer viable spores, or otherwise different. Future research is needed to identify the variation between this *Heterosporis* isolate and others that may exist in the region.

Fathead minnows were monitored for 16 months post-exposure to Heterosporis. Mortality was high in both control and infected fish (>90%), however, infected fish consistently had clinical lesions (muscle wasting) of Heterosporosis at time of death. The surviving infected fish had no clinical signs of disease and should be examined for characteristics of resistance in future studies.

C. *Determination of optimal sampling methods and tissue selection for Heterosporis sp. in fish*

We have successfully identified Heterosporis DNA in many organs of infected fish, supporting the hypothesis that immature stages of the parasite exist in the blood prior to maturation in muscle tissue. This has been significant finding for the detection of this, and related parasites, in a wide variety of sample types. To facilitate future research into the development of this disease, improved diagnostics, and critical control points for intervention, these findings will be published in a peer-reviewed manuscript and presented at the AFS-FHS Annual Meeting.

Result 3: Minnesota-wide survey for Heterosporosis.

Description: The heterosporosis quantitative PCR test developed, validated, and optimized in studies 1 and 2 will be used to test fish samples submitted to the state veterinary diagnostic laboratory for viral hemorrhagic septicemia. These samples will include banked tissue from years 2007 - 2008 as well as all samples submitted during the study year. Samples will be pooled by year, lake, and species to provide an initial determination of the location, timing, and species involved across the state. For example, if Gull Lake was sampled in 2007 and 2008, samples will be pooled for each species for each year and tested. Due to the possibility of false-positive results, all positive samples from previously negative lakes or species will undergo DNA sequencing to confirm. Testing of the banked samples will be completed February 2011 – May 2011.

The results of the studies will be presented at the annual American Fisheries Society – Fish Health Section meeting. In addition, a report will be provided to the DNR for management purposes.

Deliverables	Completion Date	Budget
A. *Minnesota-wide survey for Heterosporosis.*	May 2012	$15,651
B. *Present research at scientific conference and provide a report to the Minnesota DNR*	June 2012	$1,700

Summary Budget Information for Result 3: **Trust Fund Budget: $17,351**
Amount Spent: $17,351
Balance: $0

Final Report Summary

A. *Minnesota-wide survey for Heterosporosis*

A total of 592 samples, representing approximately 3,000 fish from 50 waterbodies in Minnesota were examined for Heterosporis spp. by qPCR (Attachment 1). 10% (5/50) of the waterbodies were identified as positive, including our reference lake (Leech Lake, Cass County, MN). The other positive waterbodies identified include, North Long Lake, (Crow Wing County), Mary Lake (Douglas County), and a private pond in both Douglas and Pope Counties. Precise locations of the private ponds are not provided for privacy. Positive fish included spottail shiners, yellow perch, walleye, and northern pike. As a result of this survey, we have shown Heterosporosis is more widespread than previously known. This survey has informed fish health mangers on the distribution of Heterosporosis in Minnesota (Attachment 2a-b).

In addition, during the course of this study, three angler caught fish were submitted to the Veterinary Diagnostic Laboratory by the MDNR with clinical lesions of Heterosporosis (Attachment 3). The disease was confirmed with the methods previously described. The fish included a walleye from Wabana Lake (Itasca County), a northern pike from Black Hoof Lake (Crow Wing County), and a cisco from Lake Superior.

This is the first report of Heterosporosis in North Long, Mary, Wabana, Black Hoof lakes, and private ponds in Minnesota. Furthermore, this is the first report of the disease in spottail shiners, a popular baitfish species.

To date, no population level affects attributed to Heterosporis have been reported by the MDNR. However, additional research is needed to investigate the potential effects of this disease to better inform science-based management decisions. Continued surveillance in recommended to monitor the spread of

Heterosporis in Minnesota, as well as the long-term dynamics and persistence in infected waterbodies.

B. *Present research at scientific conference and provide a report to the Minnesota DNR*

A final report summarizing the results of this survey will be provided to the MN DNR. In addition, the results from this project will be presented at the AFS-FHS annual meeting.

Result 4: Morphologic and genetic analysis of Heterosporis sp.

Description: Current management strategies are based on the assumption that Heterosporosis is an emerging disease. While this is likely the case given the known history of this and related pathogens, anecdotal evidence now suggests this parasite may have been locally isolated as far back as the 1970s, 30 years prior to the first reported case. It is therefore possible that the parasite has mutated, gaining virulence in the last 10 years to cause the recent emergence. This is supported by preliminary data from Result 2B and the particularly patchy distribution (diagnosed based on clinical signs) in the Great Lakes region, despite frequent movements of fish and boats from infected waterbodies over the last four decades. It is also possible that infected fish populations have become more susceptible to the disease, suggesting an environmental or other fish health concern.

It is therefore important to better understand the variations among Heterosporis isolates in Minnesota to better manage the disease. To do this, we will collect Heterosporis-infected fish from different host species (yellow perch, walleye, northern pike, and cisco) and a variety of locations (three lakes in MN, two lakes in WI, and Lake Erie). Each isolate will be analyzed by electron microscopy and genetic sequencing. An ultra structural veterinary pathologist (Dr. Anibal Armien, Minnesota Veterinary Diagnostic Laboratory) will assist with the EM analysis. Small variations in spore morphology or infection mechanisms could be observed by this technique. In addition, complete sequencing of the 16s rRNA genome will be performed by a molecular biologist (Sunil Kumars, MVDL). Genetic variations, or lack thereof, will be helpful to determine the evolution of the parasite in the region.

We hypothesize that low variation would suggest a recent introduction and the distribution may be based on increased susceptibility of fish populations to the disease. On the other hand, high variability would suggest a more evolved parasite with higher virulence and a greater concern for management. This data will also, for the first time, provide the necessary information to classify this parasite to the species level.

Deliverables	Completion Date	Budget
A. Morphological analysis of Heterosporis sp.	June 2012	$10,000
B. Genetic analysis of Heterosporis sp.	June 2012	$16,149

Summary Budget Information for Result 4: **Trust Fund Budget: $26,149**
Amount Spent: $26,149
Balance: $0

Final Report Summary

A. Morphological analysis of Heterosporis sp.

Infected and non-infected tissue samples were collected and processed for negative contrast and scanning electron microscopy. Heterosporis was found mostly in macrophages with few microorganisms within muscular cells (Attachment 4). Mature spores were ultra-structurally characterized by the presence of a thick capsule, anchoring disk, endo- and exospores, polar filament, anterior and posterior polarplast, nucleus, posterior vacuole and ribosomes. Large numbers of the microorganism were in different stages of development, including merogony and sporogony, as well as in different stages of degradation. The size of mature spores were ovoid and uniform in shape at 6.16µm x 2.36µm (sd: 0.71µm x 0.39µm).

The mechanism by which the parasite is transported to the muscle from the gut is currently unknown, however this data showing the parasite at various stages of development within macrophages is very interesting. We hypothesize that the macrophages consume the invading parasite within the guy and are transported via the blood stream until suitable skeletal muscle cells are found. This area deserves additional research and has applications to treatment of not only fish, but human-infecting, microsporidian diseases.

B. Genetic analysis of Heterosporis sp.

Three fish (Attachment 3) confirmed positive for Heterosporis were thoroughly examined to describe the parasite and determine genetic relatedness. Samples were collected from walleye, yellow perch, and cisco, from Lake Wabana, Leech Lake, and Lake Superior, respectively. Phylogenetic analysis was performed on the partial 16s rRNA sequence for each sample. Two new species of Heterosporis were clearly distinguished: *H. sutherlandae* (included the walleye and yellow perch) and *H. superiorae* (included the cisco) (Attachment 5). *H. sutherlandae* sequences were 99.1 – 100% similar within the group and 96.8% similar Asian and European Heterosporis species. *H. superiorae* only has 90% similarity with other Heterosporis species infecting fish. All Heterosporis spp. have only 48.0 – 50.0% similarity with known microsporidians of humans.

The previously undescribed *Heterosporis* sp. sample from north central Wisconsin was also analyzed to determine regional relatedness and spread. An archived sample from Catfish Lake (Villas County, WI) was provided by the US Fish and Wildlife Service. This sample was conformed to be *H. sutherlandae*, with 99.3% similarity to the samples from Northern Minnesota.

Given the low genetic variability between *H. sutherlandae* samples we examined, we hypothesize that this species is a fairly recent introduction. Interestingly, *H. sutherlandae* is more closely related to *H. anguillarum* (found in Japanese eels) than *H. superiorae*. Further research is needed to determine the evolutionary rate and complete gene sequence of these parasites to estimate introduction date and source.

Taking into account the morphologic and genetic data, we conclude that there are two distinct species of Heterosporis in Minnesota and described for the first time as a result of this study.

V. TOTAL TRUST FUND PROJECT BUDGET:

Personnel: $19,601. Funds will be used for one month of salary and fringe for the Project Manager ($8,801). In addition, funding is needed to support a student assistant for fish care (2 hours/day x 365 days) and sample management (3.5 hours/week) throughout the year (900 hrs x $12/hr; $10,800).
Equipment/Tools/Supplies: $31,354. Funds will be used to develop an aquatic research laboratory at the University of Minnesota Veterinary Diagnostic Laboratory for emerging fish disease research. For general bio-secure precautions ($5,000), several items will be purchased, including equipment for hand washing, foot dipping and clothing changes to maintain quarantine between diseased and clean fish populations. Funds will also be used for system design and management ($26,650), including six 50-gal tanks, shelving and support structures, ultraviolet filtration systems to prevent disease movement between tanks, individual tank aeration, water quality maintenance supplies, and fish handling tools, including the purchase of required nets and cleaning supplies.
Travel: $2,500. Funds will be used to cover travel (airline tickets and per diem) to one conference for PhD student, Nicholas Phelps to present results and discuss findings with other fish health professionals at the American Fisheries Society – Fish Health Section annual meeting, location to be determined. In addition, travel expenses are needed to cover local travel costs (vehicle and fuel) to and from the DNR to maintain the active collaboration. Funds are also needed for Nicholas Phelps to travel to field collection sites to advise on appropriate sample collection and storage techniques for submitted tissues.
Other: $26,454. Other funds will be used for laboratory supplies, sample collection and storage supplies. Laboratory supplies and costs include PCR primers and probe, reagents, pipette tips, gloves, instrument maintenance, computer software, DNA

extraction kit, microcentrifuge tubes, and PCR plates. Sample collection and storage supplies include sample boxes, sample vials, alcohol, coolers, and ice packs.

TOTAL TRUST FUND PROJECT BUDGET: $80,000

Explanation of Capital Expenditures Greater Than $3,500: To prevent the movement of experimental pathogens from the laboratory to the environment, existing space must be renovated to meet the unique bio-safety requirements of an aquatic health research laboratory at the Veterinary Diagnostic Laboratory. The laboratory and equipment set up will be a permanent addition to the research infrastructure at the University and used to do equivalent fish disease research in the future. The Principal Investigator will work with the Co-PIs of the project to ensure that this facility is accessible and useful for future fish disease research efforts at the University of Minnesota.

VI. PROJECT STRATEGY:

A. Project Partners: Nicholas Phelps, Veterinary Diagnostic Laboratory, University of Minnesota. Dr. Peter Sorenson, Department of Fisheries, Wildlife, and Conservation Biology, University of Minnesota. Ling Shen, Ecological Resources, Minnesota Department of Natural Resources. Project partners will not directly receive any funds from the appropriation.

B. Project Impact and Long-term Strategy: *Heteropsoris* sp. is an emerging parasite infecting many economically important and popular game fishes in Minnesota. The current diagnostic methods are not robust enough to succesfully control for this disease. The qPCR assay developed in this study will be used to opportunistically survey the State for *Heterosporis*, which for the first time, will provide managers with the necessary information to better control the spread of this disease between lakes. This will also decrease the time needed to perform diagnostic inspecions and reduce future laboratory costs for the State. Furthermore, this study is the first part of a larger PhD project by co-investigator Nicholas Phelps. These results will inform his research on *Heterosporis* classification, transmission, treatment, and host suseptibility.

C. Other Funds Proposed to be Spent during the Project Period: $0

D. Spending HIstory: $0

VII. DISSEMINATION: Findings from this research will be provided to fisheries managers at the Minnesota Department of Natural Resources to inform fish disease control strategies in the state. In addition, findings will be communicated to the scientific community with at a research conference and a peer-reviewed publication as well as other stakeholders with a report available on the University of Minnesota Extension Aquaculture website.

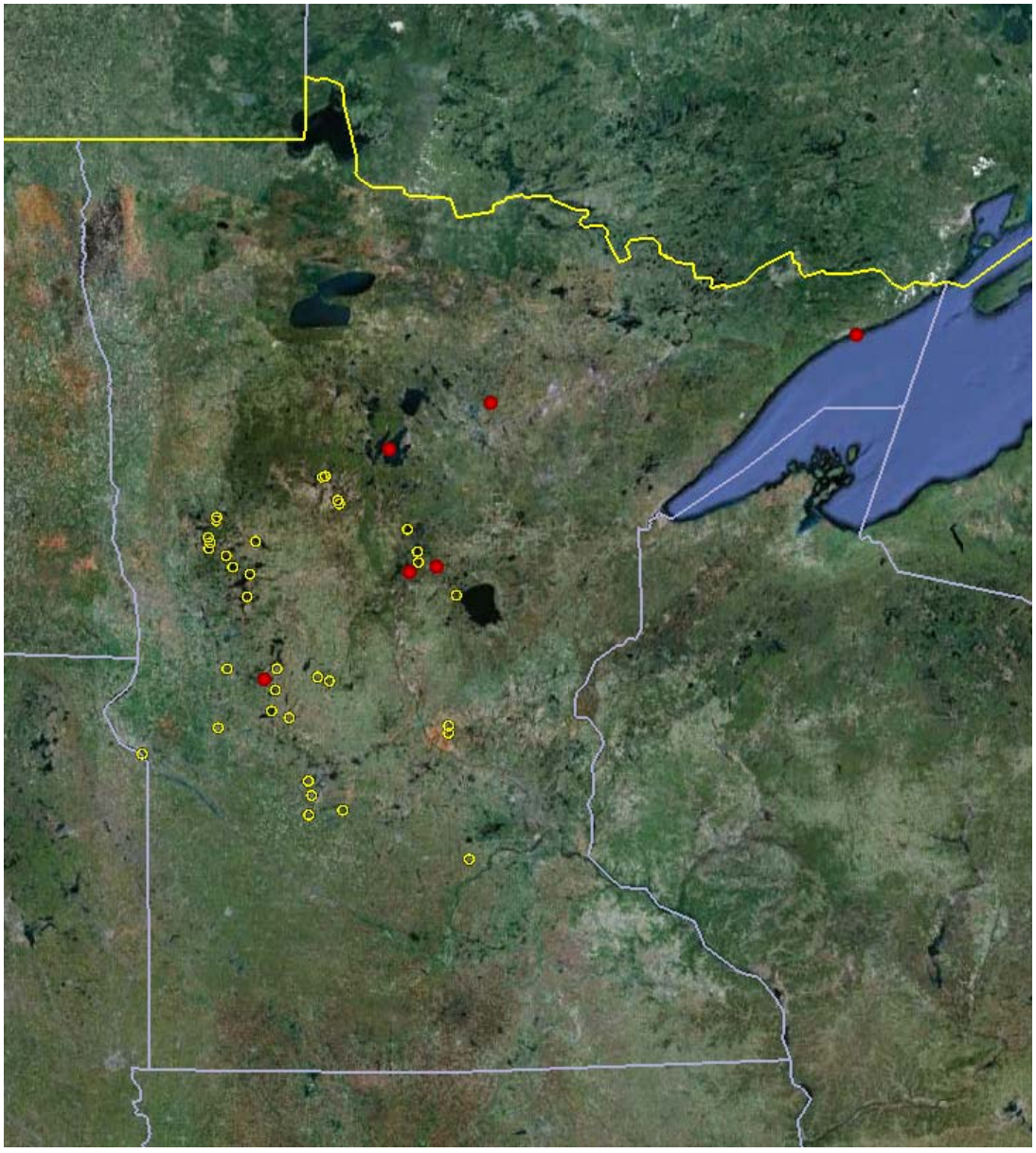
VIII. REPORTING REQUIREMENTS: Periodic work program progress reports will be submitted not later than December 30, 2009. A final work program report and

associated products will be submitted by June 30, 2012 as requested by the LCCMR.

IX. RESEARCH PROJECTS: n/a

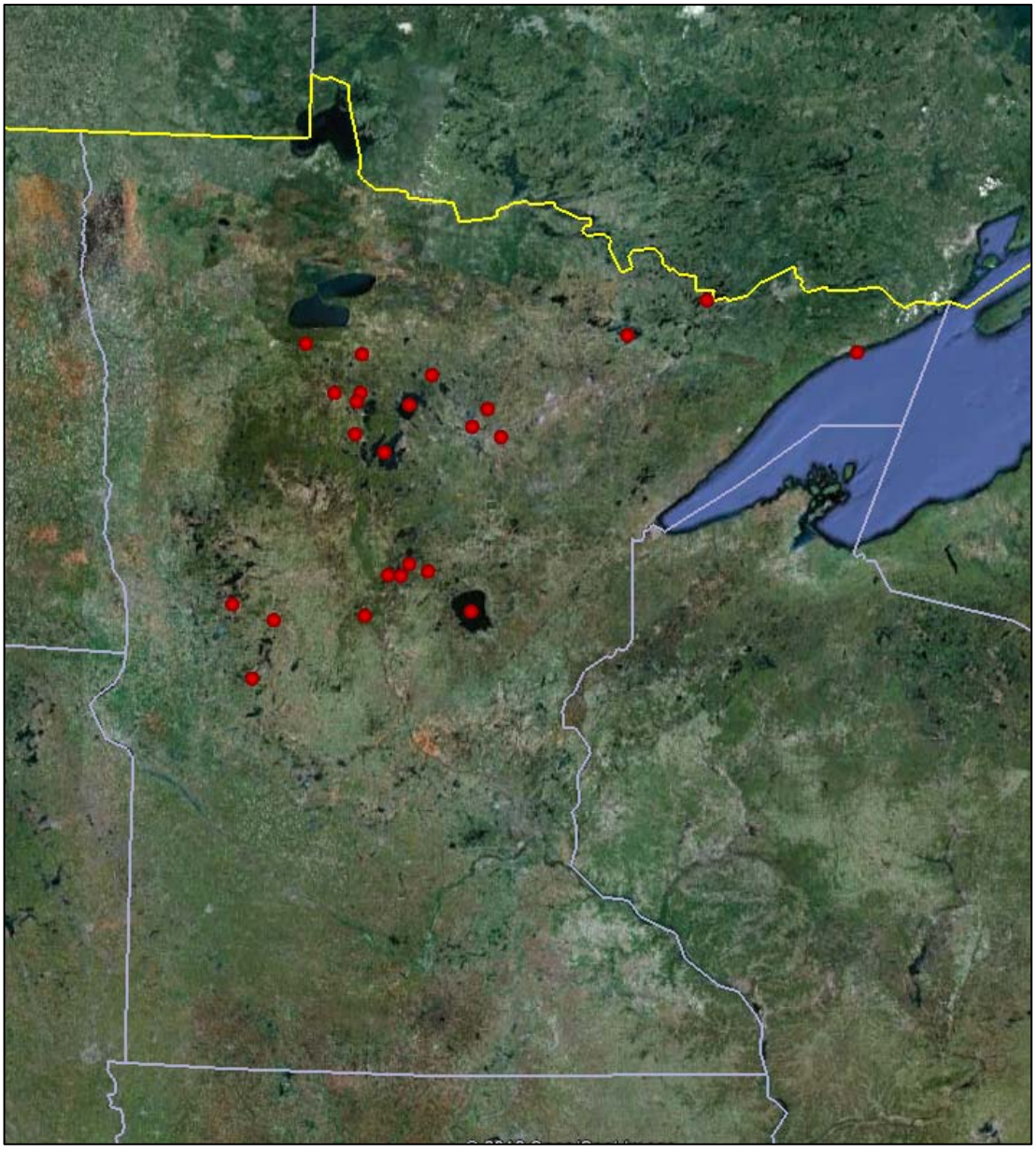
Attachment 1

2010 – 2011 Heterosporosis survey locations (50 sites) and angler submissions (3 sites). Red dots indicate positive waterbodies. Open yellow circles indicate negative waterbodies. Private ponds in Douglas and Pope Counties not listed for privacy.



Attachment 2a

Heterosporosis positive waterbodies in Minnesota from 1990-2011. Private ponds in Douglas and Pope Counties not listed for privacy. Data provided from this study and the MDNR. As of 2011, Heterosporosis has been confirmed in 26 waterbodies in Minnesota.



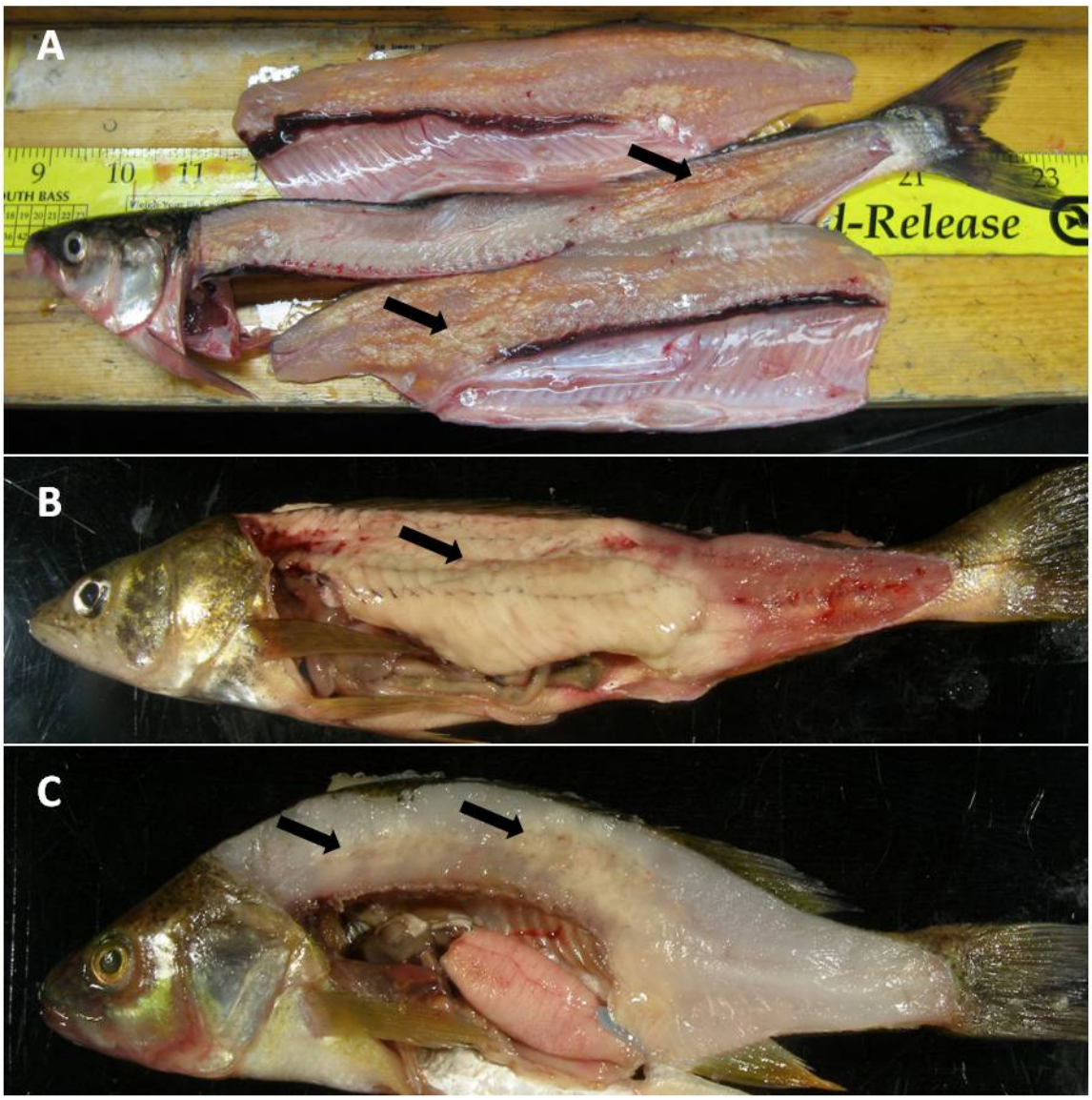
Attachment 2b

Heterosporosis positive waterbodies in Minnesota from 1990-2011. Data provided from this study and MDNR.

Lake	County	First Identified
Leech Lake	Cass	1990
Sand Lake	Itasca	1999
Bass Lake	Itasca	1999
Horsehead Lake	Ottertail	1999
Steamboat Lake	Cass	1999
Mille Lacs Lake	Mille Lacs	2000
Lake Vermilion	St. Louis	2000
Bear Lake	Itasca	2000
Lake Andrusia	Beltrami	2000
Clitherall Lake	Ottertail	2000
Gull Lake	Cass	2002
Lake Alexander	Morrison	2003
Lake Winnibigoshish	Cass	2003
Basswood Lake	Lake	2004
Lake Bemidji	Beltrami	2004
Blackduck Lake	Beltrami	2006
Trout Lake	Itasca	2007
Balm Lake	Beltrami	2008
Big Lake	Beltrami	2008
Lake Superior	Cook	2008
Black Hoof Lake	Crow Wing	2010
Wabana Lake	Itasca	2010
Mary Lake	Douglas	2011
Private pond	Douglas	2011
Private pond	Pope	2011
North Long Lake	Crow Wing	2011

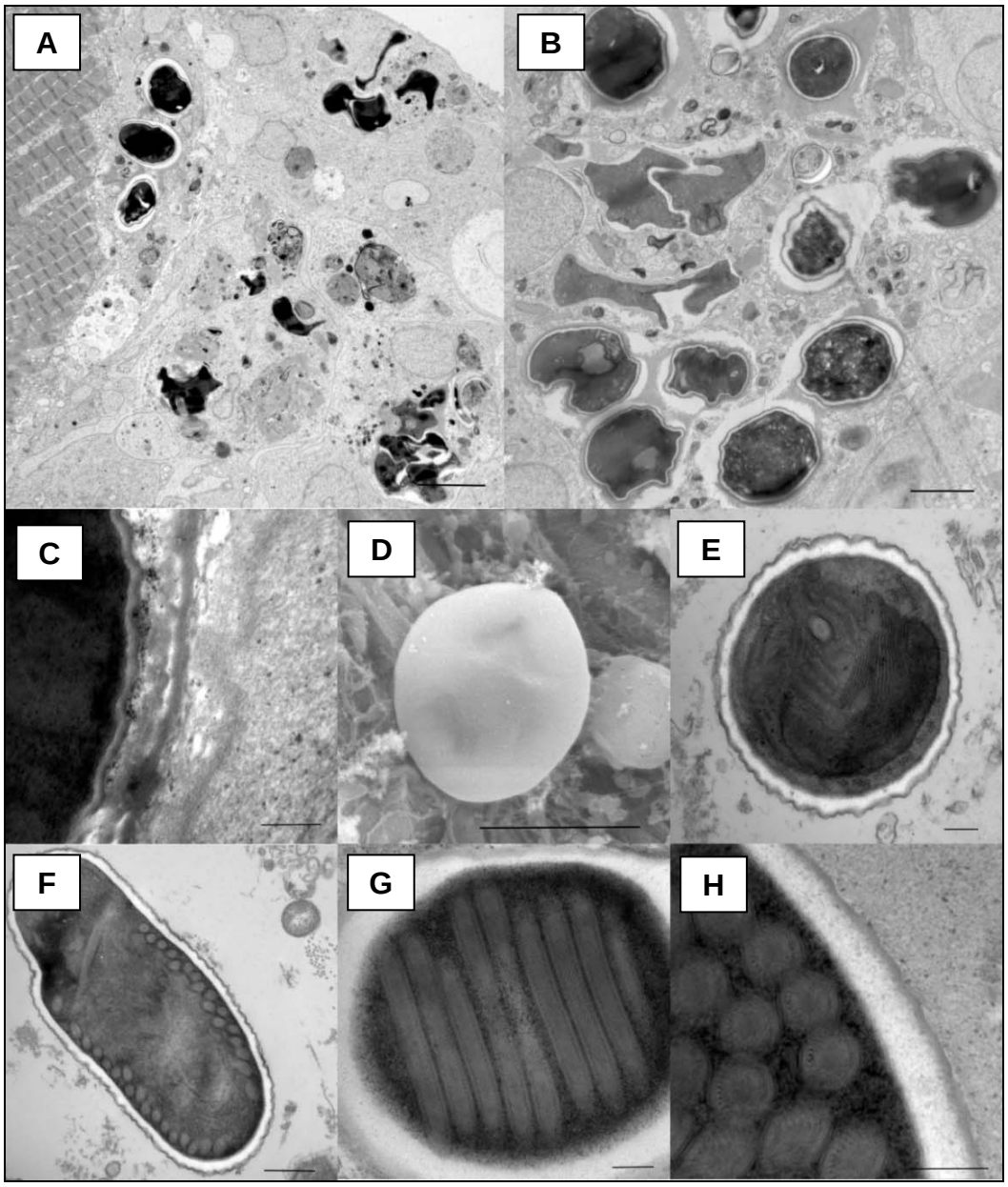
Attachment 3

Heterosporis-infected A) Cisco (Lake Superior, Cook County), B) Walleye (Wabana Lake, Itasca County), and C) Yellow Perch (Leech Lake, Cass County). Widespread muscle destruction (arrows) due to A) *H. superiorae* and B-C) *H. sutherlandae.*



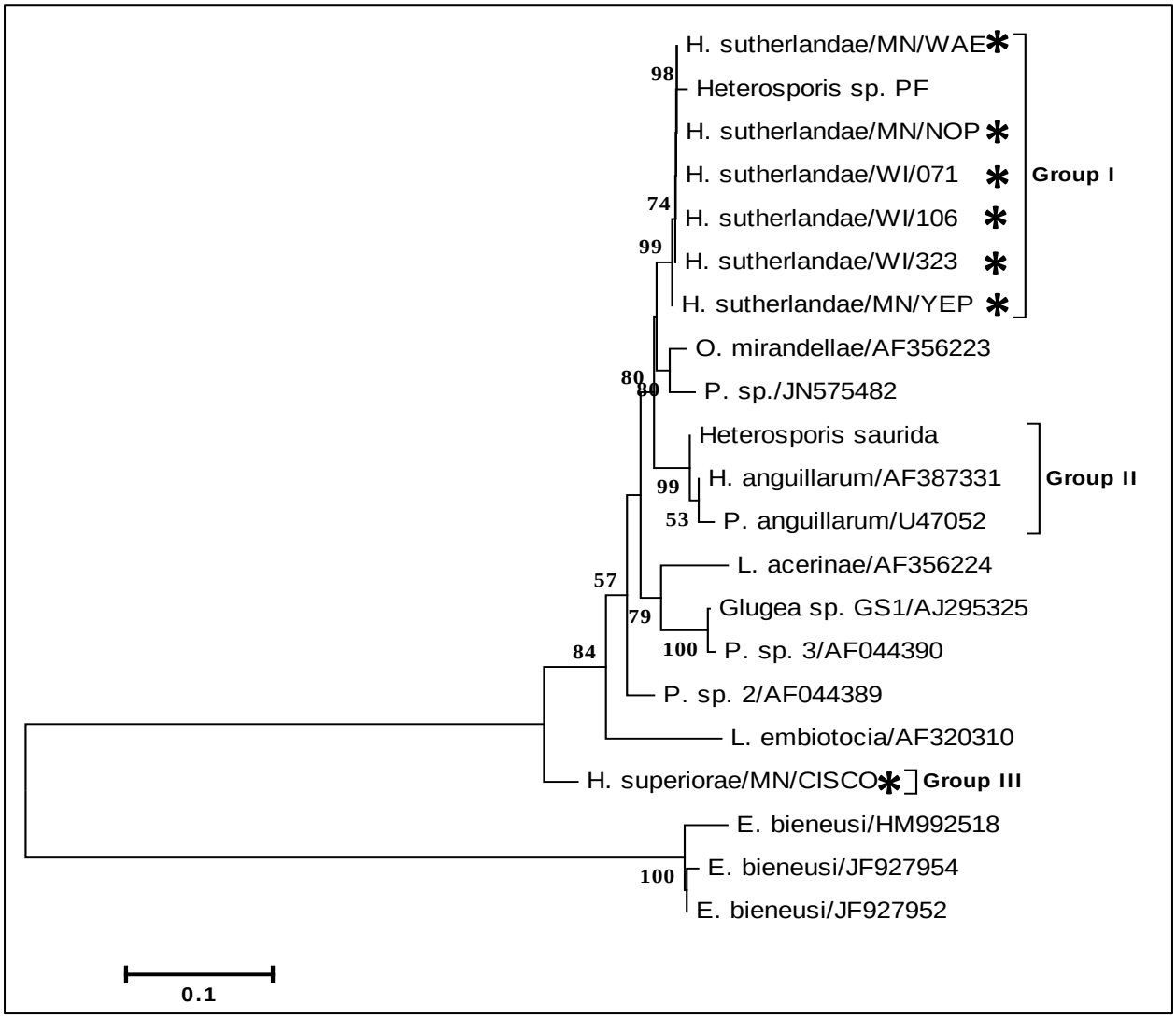
Attachment 4

Heterosporis sutherlandae from yellow perch. A-B) Granulomatous inflammation and necrosis of skeletal muscle (sm). Multiple mature spores (s) and SPVs (sv) inside phagolysosomes of macrophages (m) at various stages of digestion. C) Wall of SPV. D) Mature spore by SEM. E) Cross section of mature spore with anterior polarplast (ap), anchoring disk (ad), endospore (ed), exospores (ex), and polar tubual (pt). F) Longitudinally sectioned spore with polar tubual (pt). G) Cross section of polar tubual. H) Longitudinal section of polar tubual.



Attachment 5

Phylogenetic tree of partial 16s gene sequence, with samples reported in this study indicated by *. Group I sequences are 99.1 – 100% similar within the group and 96.8% similar with the Group II. *H. sutherlandae* sequences have 99.3% similarity with *H.* sp. Groups I and II are only 90% similar to Group III. All Groups have only 48.0 – 50.0% similarity with known microsporidians of humans.



Attachment A: Final Budget Detail for 2009 Project

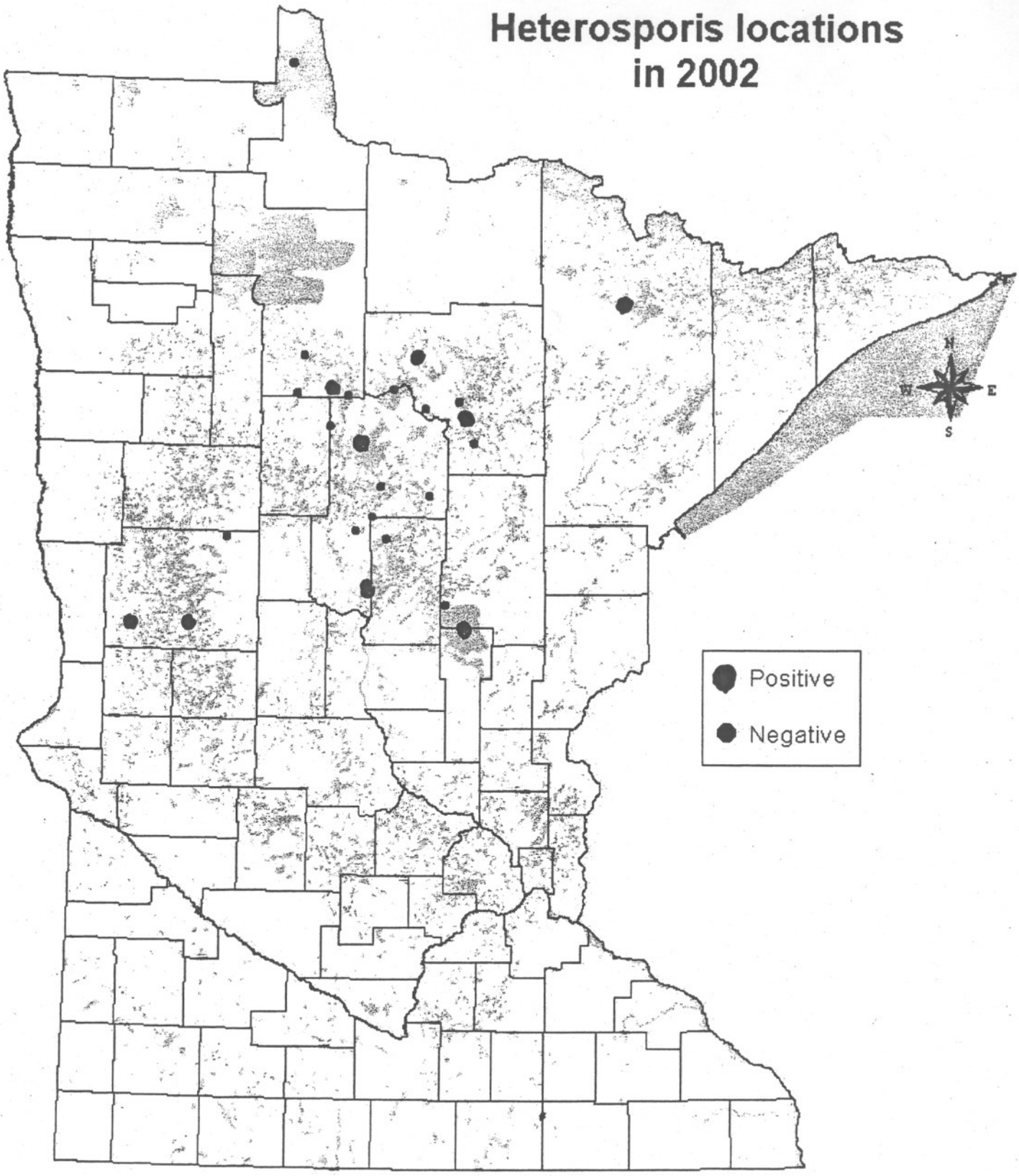
Project Title: Improving emerging fish disease surveillance in Minnesota

Project Manager Name: Katharine Pelican, DVM, PhD

Trust Fund Appropriation: $ 80,000

2009 Trust Fund Budget	**Result 1 Budget:**	**Amount Spent** *(30June12)*	**Balance** *(30June12)*	**Result 2 Budget:**	**Amount Spent** *(30June12)*	**Balance** *(30June12)*	**Result 3 Budget:**	**Amount Spent** *(30June12)*	**Balance** *(30June12)*	**Result 4 Budget:**	**Amount Spent** *(30June12)*	**Balance** *(30June12)*	**TOTAL BUDGET**	**TOTAL BALANCE**
	Validation of a quantitative PCR assay to detect Heterosporis sp. in fish			Determination of optimal sampling methods and tissue selection for Heterosporis sp. in fish			Minnesota-wide survey for Heterosporosis			Morphological and genetic analysis of Heterosporis				
BUDGET ITEM														
PERSONNEL: wages and benefits	Katherine Pelican, 8%: $2200	2,200	0	Katherine Pelican, 8%: $4400	4,400	0	Katherine Pelican, 8%: $2201	2,201	0	0	0	0	8,801	0
PERSONNEL: wages and benefits	Student Assistant: $2700	2,700	0	Student Assistant: $5400	5,400	0	Student Assistant: $2700	2,700	0	Student Assistant: $3,000	3,000	0	13,800	0
Non-capital Equipment / Tools	0	0	0	6 50-gal aquaculture tanks with UV filter and aeration, misc construction supplies to support tanks: ~~$20,000~~ $7,500. Laboratory tools (scalpels, forceps, scissors) for infective tissue handling and feeding: $250. Dissecting scope for visual inspection: $500.	8,250	0	0	0	0	0	0	0	8,250	0
Printing	0	0	0	0	0	0	Publication for DNR summarizing results: $200	200	0	0	0	0	200	0
Supplies	PCR reagents (primers, probe, master mix), centrifuge tubes, PCR plates, gloves, disinfectant: $6100	6,100	0	Supplies for biosecurity (foot bath, disinfectant, gloves, rubber boots, lab coats), nets, and buckets: $5716. Fish food: $200. PCR reagents and supplies: $689.	6,605	0	PCR reagents and supplies: ~~$23649~~ $9,800	9,800	0	Electron microscopy and sequencing procedures: $23,149	23,149	0	45,654	0
Travel expenses in Minnesota	0	0	0	Travel to collect fish: $250	250	0	Travel to field sites to assist in sample collection, if needed: $750	750	0	0	0	0	1,000	0
Travel outside Minnesota	0	0	0	0	0	0	Travel to AFS-FHS Annual Conference to present findings: $1700	1,700	0	0	0	0	1,700	0
Other	0	0	0	Positive (fathead minnows) and negative (goldfish) fish: $595	595	0	0	0	0	0	0	0	595	0
COLUMN TOTAL	**$11,000**	**$11,000**	**$0**	**$25,500**	**$25,500**	**$0**	**$17,351**	**$17,351**	**$0**	**$26,149**	**$26,149**	**$0**	**$80,000**	**$0**

Heterosporis locations in 2002



Minnesota Department of Natural Resources