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ABSTRACT

American coots (*Fulica americana*) and diving ducks, including lesser scaup (*Aythya affinis*) and ring-necked ducks (*Aythya collaris*) have been dying in significant numbers on Lake Onalaska since 2001. Bird mortality is being attributed to parasitic infections from two intestinal trematodes, *Cyathocotyle bushiensis* and *Sphaeridiotrema globulus*. The birds begin dying in as little as two weeks of landing at this lake. Rapid mortality is not typically associated with trematode parasitism. Assuming these birds acquired their infections at Lake Onalaska, leads to the conclusion that our current understanding of the disease in this parasite-host system is lacking. Neorickettsiales are a group of pathogenic obligately-parasitic intracellular bacteria that are frequently linked to trematode infections involving invertebrate hosts. There is evidence of Neorickettsiales in *Bithynia* snails, which is also a necessary host to both trematode species, which suggests a possible link between Neorickettsiales and the unusual mortality in the system at Lake Onalaska. Any conservation management practices that can be applied towards curbing this wildlife epidemic will only start with a complete understanding of all the casual factors. Fluorescently-labeled antibodies were used to search out Neorickettsiales antigens. Prepared tissue sections from infected trematode-infected birds were analyzed with fluorescence microscopy for the presence of Neorickettsiales-like organisms. There was no evidence of Neorickettsiales-like organisms, and new research directions need to be explored to explain unusual system at Lake Onalaska.

INTRODUCTION

American coots (*Fulica americana*) and diving ducks, including lesser scaup (*Aythya affinis*) and ring-necked ducks (*Aythya collaris*) have been dying in significant numbers on the Upper Mississippi River National Wildlife & Fish Refuge Pool 7 (Lake Onalaska) since 2001 (Blankenship, 2004). These numbers are expected to continue to increase with every migration (Sorensen, pers. comm). Bird mortality is being primarily attributed to parasitic infections from two intestinal trematodes, *Cyathocotyle bushiensis* and *Sphaeridiotrema globulus*. These two parasites share common definitive hosts in the diving birds along with an intermediate host in the snail *Bithynia tentaculata.* The birds are infected by the parasites when they eat the larval stage that resides in the snail (Murkin 1997). The birds have begun dying in as little as two weeks of landing at the pool (Sorensen, pers. comm.). This mortality rate is not consistent with most known types of trematode pathology (Ewald, 1995). This leads to the conclusion that the current understanding of the disease in this parasite-host system is lacking. In a related system in Canada involving *C. bushiensis* and *S. globulus* trematodes it has been shown that these two parasites are correlated with bird mortality. Because correlation does not indicate causation of bird mortality, researchers of this system pointed out that there may be another factor involved (Scott, 1988). Because of the high mortality rates involved in this system, either these are two novel trematodes or another causative agent is missing from the explanation.

Neorickettsiales are a group of obligate intracellular bacterial parasites that may explain what is missing in our understanding of this disease system. Neorickettsiales cause damage by multiplying within vertebrate host cells and then destroy that cell as they move into new host cells. Neorickettsiales, like trematodes, have complex life cycles where they reside within multiple hosts. Neorickettsiales are common parasites of trematodes and also have been found in *Bithynia* snails (Adam, 1994). Neorickettsiales are the causative organisms of salmon poisoning in dogs and Potomac Horse Fever, both which produce rapid mortality in the manner associated with the diving birds in this system. This tied with evidence of Neorickettsiales in *Bithynia* snails, is excellent evidence to suggest a possible link between Neorickettsiales and the unusual mortality in the system at Lake Onalaska. Any conservation management practices that can be applied towards curbing this wildlife epidemic will only start with a complete understanding of all the casual factors.

METHODS AND MATERIALS

Collection and Storage

Lesser scaup and mallard carcasses were collected from Lake Onalaska in the fall of 2004 and the spring of 2005. The carcasses were frozen at -29 degrees Celsius. Birds were dissected and general intestinal condition was noted. One cm sections were every ten cm from 12 randomly chosen trematode-infected birds. The one cm sections were then frozen at -70 degrees Celsius. Separate samples were prepared of intestinal trematodes removed from the waterfowl with no intestinal tissue and frozen at -70 degrees Celsius.

Tissue Mounting and Slide Preparation

The tissue samples were encased in cryo-embedding compound and cut into 6 μm sections with a cryotome at -30 degrees Celsius. These sections were then placed on precleaned superfrost plus slides and fixed in acetone for 10 minutes. Intestinal tissue was blocked with 10% fetal bovine serum in phosphate buffered saline with Tween 20 (PBST). Tissue was then rinsed in PBS and immersed in an equine anti-Neorickettsiales antiserum in PBS diluted 200:1. The samples were then incubated for 30 minutes in a humidity chamber. Slides were washed in three changes of PBS in a Coplin jar over 5 minutes. Any bound equine antibodies were then labeled with fluorescein isothiocyanate-conjugated goat anti-horse IgG for 30 minutes in a humidity chamber. Tissue was then washed once more in three changes of PBS. The tissue was then mounted in 50% glycerol in PBS with p-phenylenediamine to preserve fluorescence.

Microscopy

For each intestinal segment taken from the waterfowl, test and negative control sections were prepared. The test sections received the equine anti-Neorickettsiales antiserum and the negative control sections received just the buffer with no equine anti-Neorickettsiales antiserum. Any Neorickettsiales organisms present in the in the sample that were sufficiently similar to the equine Neorickettsiales should be bound by the antibodies in the serum. The fluorescein isothiocyanate-conjugated goat anti-horse IgG should then bind to any Neorickettsiales antibodies present. Fluorescence microscopy was then used to examine tissue for any fluorescence from the conjugated antibodies.

RESULTS

General, non-specific fluorescence was observed in all tissue samples with some concentrated spotting that might indicate fluorescent antibody attachment. No significant difference could be seen between the positive and negative controls. Presence of Neorickettsiales organisms could not be established within the intestinal or trematode tissue.

DISCUSSION

Although the absence of antibody fluorescence does indicate that Neorickettsiales organisms are not present within the waterfowl it does not exclude the possibility. There are many factors that must be noted that may have caused the negative results. Because Neorickettsiales bacteria are obligate parasites, it is expected that they will die quickly after their host dies. Tissue degradation and subsequent Neorickettsiales death could have caused negative test results. This was controlled against by immediate tissue freezing and fixing over the next two days.

Non-specific binding might also have played a part in the negative results. The fluorescent conjugate may have been binding non-specifically to multiple tissues. This would have caused fluorescence in both the negative controls and test sections. Even though Neorickettsiales may have been present, a significant difference would have not shown between the positive and negative controls.

There is a possibility that the equine antibodies may not have bound to waterfowl Neorickettsiales because there is a great enough difference between waterfowl and equine

Neorickettsiales organisms. Even though there may have been Neorickettsiales organisms present they may not have been bound by the equine antibodies and thus would not have fluoresced.

CONCLUSIONS

The results do not provide any evidence for a Neorickettsiales organism as the causative factor in waterfowl deaths at Lake Onalaska. This could be the result of a false negative reading, but evidence does not point to that conclusion. This study has ruled out a very likely causative organism in the unusual parasite-hot system present at Lake Onalaska. Further research needs to be completed to assess why waterfowl in this region are so uncharacteristically dieing off so quickly.

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Author's Biography:

Derek Skillings is a student in the Open Studies program at MSU, Mankato; he is concentrating his studies in biology, chemistry and philosophy. He is very active with research in both the biology and philosophy of departments at MSU, Mankato and the Hawaii Institute of Marine Biology. His research interests include near-shore marine communities, phylogeography, and philosophy of biology and language. He is currently applying for graduate studies in marine biology with goal of a PhD.

Faculty Mentors' biographies:

Dr. Secott joined the Biology faculty at MSU, Mankato in 2003. His research interests include the investigation of genes and molecular mechanisms used by pathogenic mycobacteria to initiate and establish intestinal infections in mammals, and the effect of climatic changes on microbial communities in Antarctic vascular plant ecosystems.

Dr. Sorensen received his B.S. from MSU, Moorhead and his PhD from Perdue University. He joined the Biology faculty at MSU, Mankato in 2002. His research interests include the phylogeography and ecology of parasite-host systems.