DOI: 10.7589/2016-08-195

Journal of Wildlife Diseases, 53(4), 2017, pp. 000–000

Documentation of Malaria Parasite (*Plasmodium* spp.) Infection and Associated Mortality in a Common Loon (*Gavia immer*)

Ellen S. Martinsen,[1,2,8] Inga F. Sidor,[3,4] Sean Flint,[5] John Cooley,[6] and Mark A. Pokras[7] [1]Center for Conservation Genomics, Smithsonian Conservation Biology Institute, National Zoological Park, PO Box 37012, MRC5503, Washington, DC 20013-7012, USA; [2]Department of Biology, University of Vermont, Burlington, Vermont 05405, USA; [3]Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, New Hampshire 03824, USA; [4]New Hampshire Veterinary Diagnostic Laboratory, University of New Hampshire, Durham, New Hampshire 03824-3590, USA; [5]Umbagog National Wildlife Refuge, Fish & Wildlife Service, United States Department of the Interior, 2756 Dam Road, PO Box 240, Errol, New Hampshire 03579-0240, USA; [6]Loon Preservation Committee, PO Box 604, Lee's Mills Road, Moultonborough, New Hampshire 03254, USA; [7]Tufts Wildlife Clinic, Cummings School of Veterinary Medicine, 200 Westboro Road, North Grafton, Massachusetts 01536, USA; [8]Corresponding author (email: martinsene@si.edu)

ABSTRACT: We report malaria parasite infection (*Plasmodium* spp.) and associated mortality in a Common Loon (*Gavia immer*) found dead on Lake Umbagog in New Hampshire, US. Necropsy findings showed the bird to be in good body condition but with pericardial edema and splenomegaly. Histopathological examination of brain and heart revealed intraendothelial and intrahistiocytic proliferation of *Plasmodium* merozoites with myocarditis. By PCR, the presence of *Plasmodium* parasites was confirmed from all tissues screened including spleen, muscle, and kidney. Sequencing of nested-PCR products revealed two different *Plasmodium* lineages, CATUST05 and PADOM11, indicating a mixed malaria parasite infection. Clinical findings strongly support malaria-induced mortality in a Common Loon.

Key words: Common Loon, *Gavia immer*, malaria, mortality, *Plasmodium*

Emerging infectious diseases pose a significant threat to wildlife conservation and have surged in incidence in the last few decades (Harvell et al. 2002). Malaria parasites (*Plasmodium* spp.), vectored by mosquitoes and distributed worldwide on every continent except for Antarctica, adversely impact immunologically naive host individuals and populations, including captive birds, such as those in zoos, and island endemics (Valkiūnas et al. 2005). Despite growing knowledge of the distribution and vectors of these parasites, their pathogenicity and population effects in wild birds have been incompletely documented.

On 6 July 2015, an adult female Common Loon (*Gavia immer*) was found freshly dead by a seasonal technician near Big Island North, Lake Umbagog, Umbagog National Wildlife Refuge, Errol, New Hampshire, US (44°73′00″N, 71°07′00″W). The cadaver was recovered by the Loon Preservation Committee and transported on ice the same day to the New Hampshire Veterinary Diagnostic Laboratory (NHVDL) at the University of New Hampshire.

Necropsy findings revealed the bird to be in good nutritional condition, with adequate skeletal muscle and subcutaneous adipose tissue. Lungs were bilaterally pale pink to pale brown, and meaty, with blood-tinged fluid in airways; sections sank when placed in formalin. There was marked edema of pericardial tissues. The spleen was diffusely enlarged, red-brown, and soft, and the liver was pale brown and slightly friable. The ovary had numerous developing follicles, and the oviduct was well developed. Other tissues were grossly within normal limits.

Tissues were fixed in formalin for histopathology, embedded in paraffin, sectioned at 5 μm, stained with H&E or Gomori's iron stain, and examined by light microscopy. Spleen, muscle, and kidney tissues were frozen for molecular-based parasite screening. Tissues and images are archived at the NHVDL (case no. 15-4723).

Microscopic examination revealed that within capillaries throughout sections of brain there were small to moderate numbers of developing intraendothelial clusters of small, round to oval, 1–2 μm diameter basophilic bodies (presumptive *Plasmodium* sp. merozoites within phanerozoites). There was mild congestion throughout brain sections. There was mild to moderate patchy interstitial infiltration of myocardium by mixed heterophils, macrophages, and lymphocytes, with rare myodegeneration and mineralization. Similar parasitic merozoites distended macrophages and endothelial cells in these foci. There was moderate to marked diffuse edematous separation of pericardial tissues, with loose infiltrates of sparse, mixed inflammatory cells and prominence of ectatic lymphatics. Pigment-laden macrophages were scattered throughout pulmonary air capillaries, hepatic sinusoids, and splenic sinuses. Abundant pigment was present in hepatocytes. This pigment was mixed, positive for both iron (hemosiderin) and iron-negative, refractile, brown malarial pigment (hemozoin). Mild parasitism was present in other tissues, including skeletal muscle sarcocysts, renal tubular coccidia, and intestinal trematodes.

Microscopic findings were consistent with malaria as the cause of death, characterized by mild to moderate myocarditis and myodegeneration with intrahistiocytic and intraendothelial parasites consistent with *Plasmodium* phanerozites and pericardial edema, as well as the presence of moderate, multifocal intracerebral intraendothelial *Plasmodium* phanerozoites (Fig. 1; Valkiūnas 2005). Phanerozoites are secondary exoerythrocytic meronts that develop in endothelial cells of various organs and can cause tissue damage. Intraendothelial proliferation of these parasites is suspected to be the cause of edema in other tissues. No parasite-associated inflammation was present in brain, but altered circulation and cerebral hypoxia may have occurred secondarilyy to capillary obstruction by proliferating phanerozoites. Abundant hemosiderin and scant presumptive hemozoin were also found in the liver and spleen; malarial hemolytic anemia due to the erythrocytic stages cannot be ruled out as contributory to debilitation and death. Minor parasitism in other tissues was considered incidental. This bird was otherwise in good

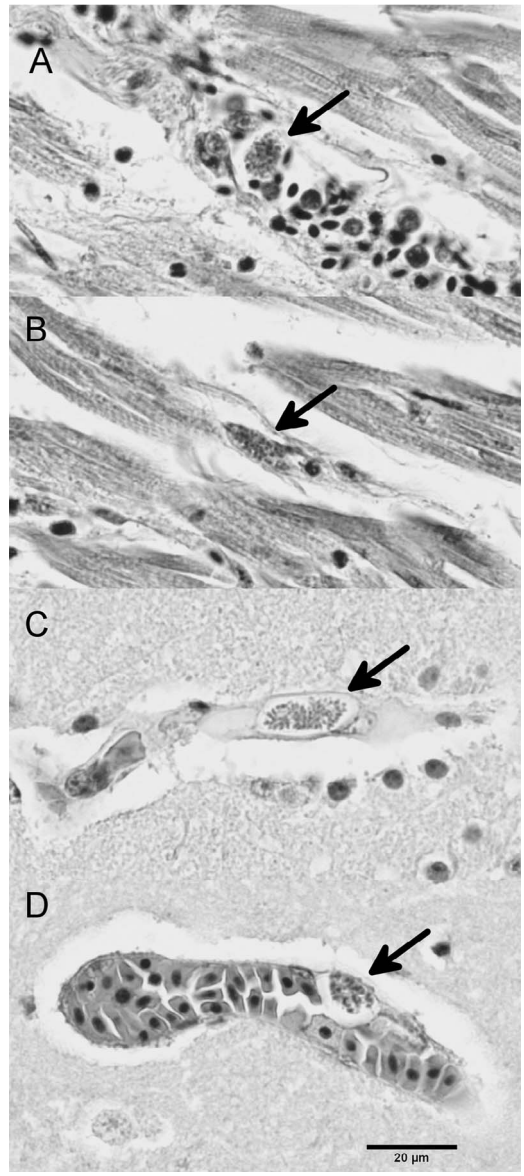

FIGURE 1. Histopathologic examination of heart and brain tissues of a Common Loon (*Gavia immer*) found dead on Lake Umbagog in New Hampshire, US. Shown are merozoites within phanerozoites (indicated with arrows) of *Plasmodium* spp. in sections of heart (a, b) and brain (c, d). Scale bar 20 μm.

body and active reproductive condition, suggesting a relatively acute death.

To confirm *Plasmodium* parasite infection, frozen tissue samples were screened for malaria parasites by PCR. Frozen tissues from the spleen, muscle, and kidney were subjected to DNA extraction using a Qiagen DNeasy Blood and Tissue Kit following manufacturer's guidelines. A nested PCR that amplifies a 479 bp fragment of the mitochondrial cytochrome *b* gene (*cytb*) using primers HaemNF and HaemNR2 (Waldenström et al. 2002) followed by HaemF and HaemR2 (Bensch et al. 2000; Hellgren et al. 2004) was conducted twice independently on all samples. Negative controls were used for both extraction and PCR protocols, and no contamination was detected. PCR products were visualized by agarose gel electrophoresis. All positive samples were purified using ExoSAP-IT, cycle-sequenced with BigDye Terminator v3.1, and sequenced on an ABI 3130*xl* Sequencer at the Center for Conservation Genomics, Smithsonian Conservation Biology Institute, Washington, DC, US. All sequences were edited using SEQUENCHER version 5.0 (Gene Codes, Ann Arbor, Michigan, USA) and aligned by eye using Geneious (Biomatters Ltd, Auckland, New Zealand). To identify *cytb* parasite lineages, we then conducted a Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information 2016) search in GenBank with each unique sequence (479 bp) within the online database MalAvi (Bensch et al. 2009), which includes *cytb* lineage data from almost every molecular-based avian malaria parasite study to date.

Parasite DNA was successfully amplified from frozen spleen, muscle, and kidney samples during the two independent nested-PCR runs. We sequenced two unique *cytb* lineages from these three tissue types, and mixed infections as indicated by multiple peaks on the chromatograms were not observed. Both lineages were 100% identical to previously documented *Plasmodium cytb* lineages in the MalAvi database including PADOM11 from muscle tissue and CATUST05 from spleen and kidney tissue. The PADOM11 and CATUST05 lineages differ from one another by approximately 4.4% and are considered to be evolutionarily independent entities. According to the MalAvi database, as of August 2016, PADOM11 has been detected in 21 bird species from the orders Passeriformes (songbirds) and Strigiformes (owls) from locations across the US and South America (Bensch et al. 2009). The CATUST05 lineage has been isolated from thrush species (family Turdidae) in North and South America including locations in Alaska and Vermont in the US (Martinsen et al. 2007; Loiseau et al. 2012).

Although malaria is a common disease of birds, documentation of mortality from the disease is rare from naturally infected wild birds, with the exception of naive populations of species endemic to islands exposed to introduced *Plasmodium* species (Atkinson et al. 2000; Howe et al. 2012). Mortality from *Plasmodium* infection has been demonstrated in experimentally infected domestic and wild bird species and in nonindigenous species housed in zoological park collections exposed to local biting vectors (Valkiūnas et al. 2005; Palinauskas et al. 2015; Ilgūnas et al. 2016). Pathogenicity of malaria parasite infection similar to that seen in our study has been observed mostly in experimentally infected passerines including exoerythrocytic development of *Plasmodium* parasites in the brain, spleen, liver, and heart (Valkiūnas 2005; Palinauskas et al. 2015; Ilgūnas et al. 2016). The paucity of studies documenting pathogenicity associated with *Plasmodium* infection in naturally infected wild birds is likely due to difficulties in sampling and availability of tissues for histopathology from sick individuals and freshly dead individuals as well as light infections of tissue stages (Ilgūnas et al. 2016). Recent development in chromogenic in situ hybridization methods for detection of *Plasmodium* parasites from tissues from deceased birds will likely improve our detection of *Plasmodium*-induced mortality in wild birds (Dinhopl et al. 2015). Little is known on the pathogenicity of mixed *Plasmodium* infections, which may act synergistically to elevate the severity of disease (Marzal et al. 2008).

Previously, these two *cytb* parasite lineages and malaria-induced mortality were not documented in the Common Loon.

Surveys conducted over the last century report malaria parasite infection to be absent in loons (order Gaviiformes, Family Gavidae), including loon populations in the northeast US (Bennett et al. 1982; Haefele et al. 2005; Valkiūnas 2005). However, these surveys were based on microscopic examination of blood smears and may have missed chronic, low-level infections. Using PCR, *Plasmodium* sp. was documented in the blood of Common Loons from northern Wisconsin over a decade ago (Weinandt 2006). We document malaria-related pathogenicity and mortality in a wild Common Loon, a case that may serve as a harbinger of future malaria disease outbreaks in the species. The susceptibility of this individual loon to avian malaria may be due to its lack of adaptation to the parasites both evolutionarily and physiologically; no other significant causes of morbidity were detected. Contact between the *Plasmodium* lineages recovered in this study and the Common Loon may be due to expansion of the parasite's host range or reduced immunocompetency of the species due to environmental contamination (Evers et al. 2008) or to other stressors. As the Common Loon is an indicator species for aquatic ecosystems and considered vulnerable or threatened across much of the southern margin of its breeding range, further study is urgently needed to better understand the interaction between *Plasmodium* parasites and the Common Loon and possible conservation significance of infection (Evers et al. 2010).

We thank Daniel R. Eastland for recovering the loon. The authors thank the many individuals and groups without whom this study would not have been possible, including members of the Northeast Loon Study Working Group: US Fish and Wildlife Service, New Hampshire Fish and Game Department, Maine Department of Inland Fisheries and Wildlife, and Maine Audubon Society. Thanks to the Cummings School of Veterinary Medicine students who assisted in field and laboratory work. This work was funded in part by donations and grants from the Summer Star Foundation, the Ricketts Conservation Foundation, and the Wood family.

LITERATURE CITED

Atkinson CT, Dusek RJ, Woods KL, Iko WM. 2000. Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. *J Wildl Dis* 36:197–204.

Bennett GF, Whiteway M, Woodworth-Lynas C. 1982. A host-parasite catalogue of the avian haematozoa. Memorial University of Newfoundland Occasional Papers in Biology 5:1–243.

Bensch S, Hellgren O, Pérez-Tris J. 2009. MalAvi: A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Mol Ecol Resour* 9:1353–1358

Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Pinheiro RT. 2000. Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc R Soc Lond* B 267:1583–1589.

Dinhopl N, Nedorost N, Mostegl MM, Weissenbacher-Lang C, Weissenböck H. 2015. In situ hybridization and sequence analysis reveal an association of *Plasmodium* spp. with mortalities in wild passerine birds in Austria. *Parasitol Res* 114:1455–1462.

Evers DC, Paruk JD, Mcintyre JW, Barr JF. 2010. Common Loon (*Gavia immer*). In: *The birds of North America online*, Poole A, editor. Cornell Lab of Ornithology, Ithaca, New York. https://birdsna.org/Species-Account/bna/species/comloon. Accessed August 2016.

Evers DC, Savoy LJ, DeSorbo CR, Yates DE, Hanson W, Taylor KM, Siegel LS, Cooley JH Jr, Bank MS, Major A, et al. 2008. Adverse effects from environmental mercury loads on breeding common loons. *Ecotoxicology* 17:69–81.

Haefele HJ, Sidor J, Evers DC, Hoyt DE, Pokras MA. 2005. Hematologic and physiologic reference ranges for free-living adult and young common loons (*Gavia immer*). *J Zoo Wildl Med* 36:385–390.

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfield RS, Samuel MD.2002. Climate warming and disease risks for terrestrial and marine biota. *Science* 296:2158–2162.

Hellgren O, Waldenström J, Bensch S. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium* and *Haemoproteus* from avian blood. *J Parasitol* 90:797–802.

Howe L, Castro IC, Schoener ER, Hunter S, Barraclough RK, Alley MR. 2012. Malaria parasites (*Plasmodium* spp.) infecting introduced, native and endemic New Zealand birds. *Parasitol Res* 110:913–923.

Ilgūnas M, Bukauskaitė D, Palinauskas V, Iezhova TA, Dinhopl N, Nedorost N, Weissenbacher Lang C,

Weissenböck H, Valkiūnas G. 2016. Mortality and pathology in birds due to *Plasmodium* (*Giovannolaia*) *homocircumflexum* infection, with emphasis on the exoerythrocytic development of avian malaria parasites. *Malaria J* 15:256.

Loiseau C, Harrigan RJ, Cornel AJ, Guers SL, Dodge M, Marzec T, Carlson JS, Seppi B, Sehgal RNM. 2012. First evidence and predictions of *Plasmodium* transmission in Alaskan bird populations. *PLoS One* 7:e44729.

Martinsen ES, Waite JL, Schall JJ. 2007. Morphologically defined subgenera of *Plasmodium* from avian hosts: Test of monophyly by phylogenetic analysis of two mitochondrial genes. *Parasitol* 134:483–490.

Marzal A, Bensch S, Reviriego M, Balbontin J, De Lope F. 2008. Effects of malaria double infection in birds: One plus one is not two. *J Evol Biol* 21:979–987.

National Center for Biotechnology Information. 2016. *Basic Local Alignment Search Tool (BLAST)*. http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed August 2016.

Palinauskas V, Žiegytė R, Ilgūnas M, Iezhova TA, Bernotienė R, Bolshakov C, Valkiūnas G. 2015. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n. sp., with experimental data on its virulence and development in avian hosts and mosquitoes. *Int J Parasitol* 45:51–62.

Valkiūnas G. 2005. *Avian malaria parasites and other Haemosporidia*. CRC Press, Boca Raton, Florida, 932 pp.

Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U. 2002. Cross-species infection of blood parasites between resident and migratory songbirds in Africa. *Mol Ecol* 11:1545–1554.

Weinandt ML. 2006. *Conservation implications of Common Loon* (Gavia immer) *parasites: Black flies, haematozoans, and the role of mercury*. Master's Thesis, Northern Michigan University, Marquette, Michigan, 86 pp.

Submitted for publication 21 August 2016.
Accepted 5 April 2017.