

**Laughing Whitefish Audubon Society
Avian Research Grant**

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Rapid Detection of Avian Blood Parasites and West Nile Virus Utilizing Loop-Mediated Isothermal Amplification: Evaluating Infection Rates and Physical Disturbances on Common Loon (*Gavia immer*) Survival and Reproductive Success in the Upper Peninsula of Michigan

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Project Significance: The common loon (*Gavia immer*) represents a complex repertoire of ecology, evolution, and behavior that is unique from most other waterbirds. In part of its popularity and distinct ecological niche, it is widely recognized as a high-profile representative for many conservation purposes (McIntyre and Olson 1988, McIntyre and Barr 2010). However, despite the notorious harassment incubating loons face from common disease vectors, current research lacks a long-term assessment of pathogenic infection rates. I will develop a novel genetic method using loop-mediated isothermal amplification (LAMP) to generate a time series analysis that will evaluate the presence of two blood parasites, *Leucocytozoon* and *Plasmodium*, as well as West Nile Virus (WNV) within a decreasing loon population at Sney National Wildlife Refuge (SNWR). This new method will have major advantages over conventional laboratory techniques that are inconsistently reliable, tedious, and expensive. Additionally, I will assist a local non-profit research organization, the Common Coast Research & Conservation (CCRC), to monitor the effects of predation and water levels on the SNWR loon population. This research will pioneer a loon-specific genetic assay that will provide important insight into common loon population dynamics and breeding biology at SNWR. Understanding the interplay among pathogenic diseases, predator effects, and fluctuating water levels, will allow biologists at SNWR and elsewhere to modify habitat management practices for the common loon and its specific ecological needs. Future work in this direction will facilitate the work of wildlife managers that can readily adopt my protocol to create a portable LAMP method, allowing researchers to answer genetic questions directly in the field that will help shine light on increasing threats to loon survival and reproductive success.

Background: The common loon, one of five loon species found worldwide, occupies freshwater lakes in boreal and near-arctic habitats of North America. This vagrant loon species utilizes large inland waterways flying to and from winter ranges along the Atlantic, Pacific, and Gulf coasts. The Laurentian Great Lakes area serves as critical migratory stopover habitat as well as nesting territory, hosting a variety of ecological research opportunities relevant to loons. While common loons adopt a quieter profile along coastal waters, monogamous male-female pairs become increasingly territorial during the summer breeding season to protect large territories that are generally limited to a single lake. Nesting typically begins in late April through mid-May and loon pairs often reproduce successfully for multiple consecutive seasons with one or two eggs per clutch (McIntyre and Olson 1988, McCormick et al. 2007). As a long-lived obligate piscivore, characterized by a high trophic status, the common loon is utilized as a key indicator of aquatic to monitor environmental conditions within a variety of aquatic ecosystems (Evers et al. 1993).

Since 1987, the CCRC has focused the majority of their research at SNWR, utilizing the common loon as a bioindicator of lacustrine health for the conservation of migratory birds within the Great Lakes region. The CCRC has documented important loon habitation data at SNWR to assess aspects of population dynamics and breeding biology in one of the southernmost breeding ranges. Monitoring data from the CCRC discovered that SNWR loons hatch and fledge more chicks than expected for a stable population and revealed a steady increase of territorial loon pairs and associated productivity over the course of 25 years (McCormick et al. 2007, Evers et al. 2008, Mitro et al. 2008, Tischler 2011). In 2012, SNWR held a healthy population of 23 territorial loon pairs (McCormick per comm.). However, from 2013-2018, the loon population at SNWR has experienced a rapid and alarming decline for reasons largely unknown, making SNWR a valuable model population to study a variety of research questions.

Located in the east-central portion of Michigan's Upper Peninsula, halfway between Lake Michigan and Lake Superior, SNWR encompasses 38,678 ha, including 24,682 ha of open water. Most of the open water area is contained in 21 impoundments ("pools") that originally operated as artificial breeding grounds for migratory game birds and other popular hunting species. These pools range from 11 to over 400 ha and water levels are manipulated by a controlled system of diversion ditches that transfer water from three local streams. While these pools function as seasonal habitat

for a population of breeding loon pairs and their annual chicks, they also provide excellent larval habitat for disease-transmitting species of black flies and mosquitos.

Due to the susceptibility of the common loon to various environmental threats, scientific research and associated conservation efforts have focused on the negative effects of avian botulism (Brand et al. 1988), toxin bioaccumulation of mercury (Evers et al. 1998), and human development (Lindsay et al. 2002), yet little is known about the impact of vector-borne diseases. *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* are three of the most common genera of blood parasites found in wild birds of North America and these parasites likely circulate in the same bird species that serve as WNV reservoirs (Greiner et al. 1975, van Riper et al. 1986). Considerable attention has been paid to *Leucocytozoon* parasites because of their known transmission to avian-specific hosts by simuliid species such as black flies. As a notorious vector of *Leucocytozoon*, one particular species of black fly, *Simulium annulus*, primarily attacks the common loon and coincides its lifecycle with loon incubation in summer. Several studies have linked *S. annulus* to an avian autoimmune disease called leucocytozoonosis that reduces breeding success and overall survival of loon populations (Fallis and Smith 1964, Lowther and Wood 1964, Weinandt et al. 2012, Piper et al. 2018). Data from these studies along with the correlative interaction between common loons and *S. annulus* highly suggests that the loon population at SNWR is infected with *Leucocytozoon* parasites.

Although numerous studies have focused on the negative effects of *Leucocytozoon*, other common vectors, such as mosquitoes, spread similar blood parasites. Mosquitoes from nearly every genus serve as the definitive host and vector of *Plasmodium* blood parasites, while birds function as the intermediate hosts (Bennett 1987, Roberts and Janovy Jr. 2000). Avian malaria is a worldwide disease caused by *Plasmodium* parasites that are transmitted via ornithophilic mosquitoes – approximately 35 species of avian malaria have been described to date (Keymer 1982, Bennett 1987, Wobeser 1997), nine of which are in North America (Stuht 1979). Although *Plasmodium* parasites occur in numerous avian species, the impacts on fitness and nest success in many nomadic species is understudied, despite the noteworthy role migratory species could play in the global expansion of blood parasites. For example, *Leucocytozoon* and *Plasmodium* parasites were found in 16% of 104 common loons sampled in an unpublished survey from the upper Great Lakes (J. Cooney, Northern American Loon Fund).

Like avian malaria, WNV is also a mosquito-borne disease that uses birds as the primary intermediate hosts. As the most widespread arbovirus in the world, WNV has emerged heavily in North America, causing mortality among many bird populations (Hughes et al. 2010). In Michigan, as in other states, *Culex pipiens* and *Culex restuans* are the primary mosquito vectors of WNV. All species of avian *Plasmodium* are transmitted by mosquitoes, and both *Cx. pipiens* and *Cx. restuans* can transmit *Plasmodium* blood parasites as well (Goglin and Freier 1986). Thus, ecologically, WNV is likely to coexist with avian *Plasmodium* in SNWR loons. This largely unexplored pathogenic relationship may have severe consequences for virus transmission, as co-infections could affect many aspects of loon survival and reproductive success.

Proposal & Methods: My research aims to evaluate two potential causes for the loon decline at SNWR. First, I will examine the changes in blood parasite and WNV infection rates on the survival of breeding adult pairs from 1996-2019. Second, I will analyze the decreases in reproductive success due to infection rates, predation events, and fluctuating water levels during the 2019 breeding season. I will study the SNWR common loon population in four unique ways. I will (1) design three loon-specific genetic assays to detect the presence of *Leucocytozoon*, *Plasmodium*, and WNV; (2) apply each assay to assess the presence of blood parasite pathogens and WNV; (3) construct a time series analysis to examine the impact of infection rates on survival, and; (4) evaluate the influence of infection rates, predation events, and water levels on reproductive success during the 2019 breeding season. I hypothesize that the recent decline in population size will be correlated to increasing infection rates. Additionally, I hypothesize that these increasing infection

rates as well as predation events and lowering water levels will be linked to an overall decrease in reproductive success during the 2019 breeding season. In collaboration with the CCRC, I have acquired the necessary Special Use Permits (SUPs) from the National Wildlife Refuge System, including the Commercial Activities SUP (FWS Form 3-1383-C), the Research and Monitoring SUP (FWS Form 3-1383-R), and the General Activity SUP (FWS Form 3-1383-G).

Data collection: To evaluate the first potential cause, I will use archived blood samples collected by the CCRC from 1996-2018 to measure the prevalence of infection rates over time. Further, I will collect blood samples from birds nesting in the 2019 breeding season to document current infection rates that I will include in my time series analysis. For the second potential cause, I will work with the CCRC to deploy game cameras that will capture the entire 2019 nesting season of all loon pairs on the refuge. This will allow me to determine if predation events and fluctuating water levels, combined with current infection rates, are causing decreased nest success.

DNA extraction and LAMP primer design: The archived blood samples are stored in heparin or EDTA/DMSO buffers at -20 °C until DNA is extracted from each using a silica-based filter purification extraction kit (DNeasy kit; Qiagen). The presence and quality of common loon DNA in each extraction will be inspected by a NanoDrop[®] 2000c spectrophotometer (Thermo Fisher Scientific). I have already designed two specific sets of forward and backward external (F3/B3) and internal (FIP/BIP) primers specific to a unique region on the cytochrome b gene of *Leucocytozoon* and *Plasmodium* species that were isolated from the common loon genome (GenBank Accession no.: EF077166.1 and EF077167.2, respectively). Both primer sets were designed using the PRIMER EXPLORER V4 software (Eiken Chemical Co., Ltd.). To obtain my third primer set for WNV, I will adopt the methods described by Parida et al. (2004), which outlines the required primers for reverse transcriptase LAMP (RT-LAMP) using a single-stranded RNA viral genome.

LAMP reactions and data analysis: All three LAMP assays will follow typical LAMP protocol as outlined by the WarmStart[®] Colorimetric LAMP kit (New England BioLabs[®] Inc.). While this kit can be used to target DNA amplicons, it also contains reverse transcriptase that converts RNA templates into DNA that identifies RNA amplicons. The kit incorporates an optimized DNA polymerase master mix with a pH indicator dye for the rapid and easy detection of LAMP and RT-LAMP reactions. The reaction mixtures will contain the WarmStart[®] Colorimetric LAMP 2X Master Mix, the specific LAMP primers described previously, DNA or RNA amplicons from either blood parasite or WNV strain, and molecular biology grade water. The application of each assay will produce a fast and clear change in color from pink to yellow when a sample tests positive for either blood parasite or WNV. Once the assays are complete, I will create a time series analysis to examine the variation of infection rates over time and compare them to changes in annual population size. Additionally, I will use linear models to estimate how changes in infection rates, predation events, and water levels influence the reproductive success of loons at SNWR during the 2019 breeding season. These models will be compared using Akaike information criterion (AIC) to determine which covariates provide the most parsimonious explanation of the data.

Expected outcomes: To my knowledge, this will be the first application of LAMP techniques to characterize the consequences of increased infection rates over time and compare the influence of infection rates to other physical disturbances. The results of this research will be used for my Master's thesis in partial fulfillment towards my graduate degree from Northern Michigan University. In addition, I plan to disseminate the results of this research in two separate peer-reviewed journal articles: (1) a methods paper describing the development and application of my LAMP protocol to *Molecular Ecology Resources*, and; (2) a paper explaining the significance of my results to *The Auk*. I also plan to present the novelty of my methods and preliminary results at the annual American Ornithological Society (AOS) conference that will be held in Anchorage, Alaska this summer.

Budget & Budget Justification

Field Research

Funds of \$500 are requested by the LWAS Avian Research Grant to cover the cost of gas and daily living expenses while participating in the 2019 summer field season at SNWR. Between 25 May and 25 August, I will be traveling to SNWR on a weekly basis to conduct various loon-related field research as described in the above Proposal & Methods section. Upon completion of weekly fieldwork, I will return to NMU to catalog new blood samples and update the corresponding blood archive database. On average, it will cost approximately \$20 in gas for a round-trip to SNWR, bringing my total projected gas expenses to \$280 (14 weeks * \$20). While lodging accommodations will be generously provided by the refuge, the remaining funds of \$220 will be allocated to my personal food allowance while working at SNWR. There is currently no additional funding secured for my fieldwork at SNWR that will accompany the LWAS Avian Research Grant, but I intend on applying to internal funding opportunities at NMU to compensate for supplementary meal expenses, the possibility of additional gas costs, and a few added needed LAMP materials.

Project Timeline

The funding period is expected to begin 25 May 2019, but could potentially start sooner due to the seasonal variation of ice-out events and the subsequent return of common loons. I have included a condensed timetable to summarize my project objectives on a monthly basis leading into the summer funding period. Following the completion of the 2019 field season at SNWR working alongside the CCRC, I also briefly outlined my agenda during the fall 2019 academic semester.

January – February 2019	Archive and organize SNWR blood samples; Extract DNA from archived blood samples; Update and organize master database; Complete DNA extractions for positive and negative controls; Prepare standard protocol for LAMP assays
February – March 2019	Test positive and negative controls with standardized LAMP protocol; Modify LAMP protocol to specify target primers; Develop loon-specific LAMP assays to identify presence of blood parasites, West Nile Virus, and sex identification; Continue archiving, organizing, and extracting DNA
March – April 2019	Complete DNA extractions; Begin lab LAMP assays; Prepare methods protocol for AOS conference; Begin preliminary analysis of LAMP assays for AOS conference
April – May 2019	Complete lab LAMP assays; Prep for SNWR field season; Continue preliminary analysis of LAMP for AOS conference
May – June 2019	Complete portable LAMP method; Begin 2019 field season; Deploy game cameras at common loon nest sites; Prepare presentation materials for AOS conference
June – July 2019	Continue 2019 field season; Attend methods and preliminary results at annual AOS conference in Anchorage, Alaska
July – August 2019	Complete 2019 field season
August – September 2019	Extract DNA from 2019 blood samples; Run lab LAMP assays
September – December 2019	Analyze data; Write thesis; Defend thesis and graduate; Begin preparing manuscripts and applying for publications

Literature Cited

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EDUCATION

NORTHERN MICHIGAN UNIVERSITY

AUGUST 2017 – PRESENT

Masters of Science Candidate, Biology

- Graduate Student Association, Biology Department Representative: August 2017 – Present
- Dean's List: Fall 2017, Winter 2018, Fall 2018

UNIVERSITY OF WISCONSIN – MADISON

SEPTEMBER 2010 – DECEMBER 2014

Bachelor of Arts, Zoology & Environmental Studies (Double Major)

- Delta Gamma Fraternity, Director of Philanthropy: September 2010 – May 2014
- Undergraduate Zoological Society, Secretary & Vice President: September 2010 – December 2014
- Dean's List: Fall 2013

PROFESSIONAL POSITIONS & EXPERIENCE

GRADUATE ASSISTANT

AUGUST 2017 – PRESENT

NORTHERN MICHIGAN UNIVERSITY, BIOLOGY DEPARTMENT
MARQUETTE, MICHIGAN

- Produce a semester-long laboratory curriculum to prepare and teach course material based on university criteria
- Allocate dedicated time for lesson planning, office hours, and additional review sessions to aid in student learning
- Develop a loop-mediated isothermal amplification (LAMP) assay specific to the common loon (*Gavia immer*) to detect the presence of blood parasites and West Nile Virus using an archived collection of loon blood samples
- Investigate variations in blood parasite and West Nile Virus infection rates from 1996-2019 within a local population of loons at Seney National Wildlife Refuge (SNWR) using genetic sequence data and LAMP assays

FLITEZONE BIRD SHOW & CONSERVATION ED INTERN

JANUARY 2016 – MAY 2016

NATIONAL AVIARY
PITTSBURGH, PENNSYLVANIA

- Performed daily husbandry duties for approximately 80 birds within the aviary education department
- Assisted staff during regular training sessions to practice and maintain trained behaviors
- Participated in free-flight bird shows and education programs focused on birds and environmental conservation
- Observed trained and natural bird behavior while learning operant condition techniques for daily care and shows

CAPE TOWN GREEN MAP INTERN

FEBRUARY 2015 – MAY 2015

CITY OF CAPE TOWN

CAPE TOWN, SOUTH AFRICA

- Explored new sites to add to the interactive Cape Town Green Map database of over 4,000 green destinations
- Created a personal blog to share experiences and endorse numerous green events and attractions
- Worked closely with the local community to interview organizational leaders and engage in South African culture
- Led multiple social media platforms for the 11th Annual Responsible Tourism in Destinations Conference

WILDLIFE CARETAKER INTERN

SEPTEMBER 2014 – JANUARY 2015

FOUR LAKES WILDLIFE CENTER

MADISON, WISCONSIN

- Provided advanced medical care for sick, injured, and orphaned wildlife
- Performed physical examinations upon admission to determine track of care based on ailments
- Administered necessary medications and provided appropriate treatments to wildlife patients
- Participated in a comprehensive research project involving environmental education to youth

PREP LAB INTERN
UW-MADISON ZOOLOGICAL MUSEUM
MADISON, WISCONSIN

SEPTEMBER 2012 – DECEMBER 2014

- Write ecology-based exams for students grades 6-12 participating in the Upper Peninsula Science Olympiad
- Proctor exams throughout a weekend once a semester to engage and encourage students in the STEM sciences

PROFESSIONAL DEVELOPMENT

VOLUNTEER

FEBRUARY 2018 – PRESENT

SCIENCE OLYMPIAD EVENT SUPERVISOR (SEASONAL)
MARQUETTE, MICHIGAN

- Worked alongside park staff to monitor the world's largest land-based colony of African Penguins (*Spheniscus demersus*) by tracking population estimates as well as fecundity and developmental

VOLUNTEER

FEBRUARY 2015 – MARCH 2015

BOULDERS BEACH – TABLE MOUNTAIN NATIONAL PARK
SIMON'S TOWN, SOUTH AFRICA

- Worked alongside park staff to monitor the world's largest land-based colony of African Penguins (*Spheniscus demersus*) by tracking population estimates as well as fecundity and developmental success
- Conducted tours to educate the public of penguin endangerment and conservation efforts while collaborating with the rehabilitation efforts of The Southern African Foundation for the Conservation of Coastal Birds (SANCCOB)

BIRD CLUB DIRECTOR

FEBRUARY 2014 – DECEMBER 2014

LAKEVIEW ELEMENTARY SCHOOL
MADISON, WISCONSIN

- Planned a semester-long program of creative games and activities for weekly birding explorations
- Educated students about local wildlife and the importance of environmental conservation

NATURE CLUB LEADER

SEPTEMBER 2013 – DECEMBER 2013

SHERMAN MIDDLE SCHOOL
MADISON, WISCONSIN

- Mentored a group of students during weekly nature explorations at a neighborhood park
- Organized bird-watching sessions to encourage inner-city students to get excited about local wildlife

EDUCATION DOCENT

MAY 2012 – DECEMBER 2014

HENRY VILAS ZOO
MADISON, WISCONSIN

- Presented educational programs with live animals for zoo guests and other community groups
- Conducted presentations to inspire public interest while including a conservation message

RESEARCH EXPERIENCE

RESEARCH ASSISTANT

SEPTEMBER 2013 – MAY 2014

UW-MADISON ANIMAL SCIENCES DEPARTMENT
MADISON, WISCONSIN

- Studied habitat selection data of the endangered and island endemic Montserrat Oriole (*Icterus oberi*)
- Organized field data and over 900 photo from 88 research sites using Microsoft Excel and Photoshop CS4

PROJECT ASSISTANT

FEBRUARY 2013 – MAY 2013

UW-MADISON ZOOLOGICAL MUSEUM & HENRY VILAS ZOO
MADISON, WISCONSIN

- Researched Pleistocene Epoch megafauna to write text descriptions for a temporary exhibit gallery in the Henry Vilas Zoo Visitors Center
- Prepared visual displays in relevance to the geologic timescale using appropriate items from the museum collections along with realistic reproductions