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CONSERVATION IMPLICATIONS OF COMMON LOON (Gavia immer) PARASITES: BLACK FLIES, HAEMATOZOANS, AND THE ROLE OF MERCURY

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By

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ABSTRACT

CONSERVATION IMPLICATIONS OF COMMON LOON (*Gavia immer*) PARASITES: BLACK FLIES, HAEMOSPORIDIANS, AND THE ROLE OF MERCURY

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Avian conservation studies frequently evaluate the effects of anthropogenic factors like disturbance, habitat destruction, edge effects and environmental contamination. Host-parasite interactions are an often-overlooked aspect of avian conservation studies, despite the fact that parasites have the ability to dramatically affect host populations. The history of common loon (*Gavia immer*) conservation work has borne this out – many studies document the negative effects disturbance, habitat destruction and mercury have on loons, yet little is known about loon black fly parasites and nothing is known about loon blood parasites. I conducted a study to investigate the effects loon parasites have on nest success by testing the specificity of a black fly species, *Simulium annulus*, to the common loon, and by examining the haemosporidian fauna of loons. A series of bird decoys were presented on northern Wisconsin lakes to test the specificity of *S. annulus* to the common loon and to explore the importance of chemical and visual cues used for black fly host attraction. These tests revealed that *S. annulus* is a highly specific parasite of the common loon and that chemical cues are of significant importance in attracting black flies. Blood was collected from breeding loon pairs and their chicks to describe and examine the blood parasite fauna of a population of breeding loons. The genera *Leucocytozoon* and *Plasmodium* were successfully detected in loons using PCR techniques. Furthermore, both the presence and the intensity of *Leucocytozoon* infection in adult loons were significantly explained by loon blood mercury levels. These studies provide a detailed description of various loon parasites and show that mercury may be immuno-compromising these birds.

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INTRODUCTION TO LOON BIOLOGY

Common loons (*Gavia immer*) are top-level piscivores of nearctic lacustrine habitats, and loon populations are often monitored as indicators of overall habitat quality. Loon reproduction is notably affected by human disturbance, habitat modification, predation, nest abandonment and fluctuating water levels (McIntyre 1988, personal observation), yet variability in nesting success remains partially unexplained. Historically it was common for ecologists and ornithologists to overlook the impact parasites have on bird demographics principally because parasites were viewed as "benign symbionts" (Hudson and Dobson 1997). Yet both empirical data (Hudson et al. 1998) and population models (Anderson and May 1978) indicate that parasites can radically affect host population dynamics. Storer (2002) examined metazoan parasites of loons and explored the relationship of this parasite fauna to the loon's evolutionary history and biology, but noted that there has been little attention paid to the influence black fly or haemosporidian parasites have on loon reproduction and ecology.

In this thesis I will present field and laboratory studies performed to explore the relationships between common loons and their black fly (Diptera: Simuliidae) and haemosporidian (Protista: Haemosporida) parasites. Chapter one focuses on the proposed specificity of a black fly species, *Simulium annulus*, to the common loon, and investigates the cues that are important for black fly host attraction. Chapter two examines the detection and prevalence of loon haemosporidians that are known to be transmitted by black flies, and discusses the implications of haemosporidian infection. The loon immune response and the effects of blood mercury burdens are also discussed in relation to haemosporidian infection.

This introduction serves to provide background information on common loon ecology that will be relevant in the subsequent chapters.

Common loons are migratory birds that breed in freshwater northern temperate forest lakes throughout Canada and the northern United States during the summer months, and inhabit coastal salt water habitats in the winter (McIntyre and Barr 1997). It has been speculated that young common loons stay on their wintering/non-breeding grounds until they are between four to seven years of age (McIntyre and Barr 1997) and do not return to their breeding grounds until they have reached sexual maturity. Upon return to their breeding grounds in early spring, adult common loons form monogamous pairs and defend a breeding territory throughout the summer (McIntyre 1988, Piper et al. 1997). Ideal territories are generally sheltered areas of large oligotrophic lakes or entire small lakes (McIntyre 1994, McIntyre and Barr 1997) that provide good nest sites, ample food supply and little human disturbance (McIntyre 1988).

Reproduction

Nesting begins in early spring (late April and early May) and nests are built predominately of mud and vegetation on small islands and wetland shorelines through July (McIntyre 1988). Loons generally lay two-egg clutches and males and females share incubation responsibilities during the approximate 28-day incubation period (McIntyre and Barr 1997). If a nest attempt fails early in the breeding season, loons will re-nest, generally in an alternate location (McIntyre 1988). Nest predation, human disturbance, fluctuating water levels, and nest abandonment have all been documented causes of nest failure (McIntyre 1988, personal observation). Black flies are common pests to loons and loons may be especially vulnerable to black fly predation during nest incubation, at times causing nest abandonment (McIntyre

1988). Common loon nest predators are crows (*Corvus brachyrhynchos*), ravens (*Corvus corax*), bald eagles (*Haliaeetus leucocephalus*), raccoons (*Procyon lotor*), skunks (*Mephitis mephitis*), mink (*Mustela vison*), weasels (*Mustela frenata*) and possibly otters (*Lutra canadensis*) (McIntyre and Barr 1997, Mike Meyer, Wisconsin DNR, personal communication).

Chick-rearing

Chicks are fed and tended to by both adults who introduce their semi-precocial young to the water within a day of hatching (McIntyre and Barr 1997). Although it varies from family to family, loon chicks are not completely independent of their parents until approximately 12 to 15 weeks of age (McIntyre and Barr 1997). It has been suggested that parenting skills improve with experience and age. Paruk et al. (2000) found that reproductive success of common loon pairs increased with an increased length of pair bond at Seney National Wildlife Refuge. Chick survival is often affected by food availability, sibling rivalry, predation, and trauma inflicted by watercraft accidents or territorial conflict (McIntyre and Barr 1997). Predators of loon chicks include large fish such as northern pike (*Esox lucieus*) and muskellunge (*Esox masquinongy*), snapping turtles (*Chelydra serpentine*), and bald eagles (McIntyre and Barr 1997).

Lifespan

There is little published data on the lifespan of the common loon and until recently, many of the longevity estimates for the species were purely speculative, spanning from 25 to 30 years (McIntyre 1988). However, an increase in long-term studies of color-marked individuals will allow the collection of more accurate data in the future. Banding and recapture efforts of the Wisconsin DNR have already discovered loons that are at least 20 years old. As this research

progresses, more information will be available on common loon longevity (M.W. Meyer, Wisconsin DNR, personal communication).

Populations

Common loon population density appears to be dependent on the amount of suitable habitat. Loons generally do not use lakes less than 10 acres (approximately 4 ha) in size for foraging or nesting due to their need for a relatively large surface area for flight initiation, (M.W. Meyer, Wisconsin DNR, personal communication). Loons aggressively defend territories for breeding, chick rearing, and foraging which limits the number of loons on any territorial lake. Lakes less than 200 acres are usually home to only one territorial pair of loons unless the shoreline is undulating and the lake is broken into distinct bays or coves in which case multiple pairs may exist (M.W. Meyer, Wisconsin DNR, personal communication).

Diet

Common loons are primarily piscivorous and over 30 species of fish have been documented as prey items, including brown bullhead (*Ameiurus nebulosus*), northern pike (*Esox lucius*), white sucker (*Catostomus commersonii*), common bluegill (*Lepomis macrochirus*), trout (Salmonidae), alewives (*Alosa pseudoharengus*), smelt (Osmeridae), sea lamprey (*Petromyzon marinus*), coregonid spp. (Salmonidae: *Coregonus*), mottled sculpin (*Cottus bairdi*), and yellow perch (*Perca flavescens*) (McIntyre 1988, Evers 1994). When fish are unavailable or conditions are unfavorable for fish capture, loons will consume crayfish (*Cambarus* spp.), leeches (Hirudinea), mollusks (Mollusca), caddisflies (Trichoptera), amphipods (Amphipoda), dragonfly nymphs (Odonata), and on rare occasions, vegetation (McIntyre 1988, Evers 1994).

Loon chicks are fed exclusively by their parents until about eight weeks of age, when they are able to capture approximately 50% of their daily food intake on their own. By week 11, chicks are capable of capturing 90-100% of their daily food (McIntyre and Barr 1997). Chicks feed primarily on small fish including minnows and perch as well as aquatic insects and crayfish (McIntyre 1988, Evers 1994).

Parasites

A number of internal helminths have been described in common loons including digenean trematodes (Trematoda), tapeworms (Cestoda), spiny-headed worms (Acanthocephala) and round worms (Nematoda). Blood sucking ectoparasites such as mites (Acarina), lice (Phthiraptera) and flies (Diptera), including black flies, are also parasitic on common loons (Storer 2002). However, little is known about the effects of parasitic infection in common loons, and no haematozoa have been previously described in the species.

CHAPTER ONE: TESTING THE SPECIFICITY OF *SIMULIUM ANNULUS* TO THE COMMON LOON

INTRODUCTION

Black flies (Diptera: Simuliidae) are common pests to mammalian and avian species, including common loons. Like other birds, loons are especially vulnerable to black fly predation during nesting and egg incubation, when it is common to see loons covered with feeding black flies, at times causing nest abandonment as a result of extreme black fly infestation (McIntyre 1988). The documented effects of black flies on other bird species' populations include decreased reproductive output (Bukacinski and Bukacinska 2000) and the transmission of blood parasites (Hunter et al. 1997). It has been proposed that one black fly species, *Simulium annulus* (Lundström) (junior synonym *S. euryadminiculum* Davies), is host-specific and feeds exclusively on the common loon (Adler et al. 2004). The specificity of *S. annulus* to the common loon has been reported repeatedly in past studies (Fallis and Smith 1964, Lowther and Wood 1964). However, contradictory observations of the attraction of *S. annulus* to other host species continue to surface (Pledger et al. 1980, *as in* Adler et al.; Fredeen 1981 *as in* Adler et al.; (Malmqvist et al. 2004), warranting further investigation of the stimuli used for host attraction.

Black Fly Ecology

Black flies are globally distributed near flowing freshwater habitats where larval growth and development occurs (Borror et al. 1989, Peterson 1996). There is only one North American case of black fly larvae and pupae collection from a habitat that lacked flowing water, and this was of the *Simulium vittatum* species complex, found in the wave-swept shores of Lake Ontario in New York (Adler et al. 2004).

Female black flies require a blood meal for egg maturation and thus it is females that bite loons and transmit blood parasites (Lehane 1991, Peterson 1996). Eggs are likely laid directly in the water, or on a substrate that is within the "wash or splash zone" to prevent desiccation (Adler et al. 2004). The rate of egg development is governed largely by temperature and may also be influenced by oxygen tension and photoperiod (Adler et al. 2004). Larvae eclose in the water, attach to a substrate via silk, and use specialized labral fans to filter feed on a variety of items including bacteria, diatoms, leaf litter, pollen, fecal pellets, protozoa, and minute arthropods, along with some inorganic material (Adler et al. 2004). Black fly larvae undergo six or seven instar stages before forming a cocoon for pupation (Roberts and Janovy Jr. 2000). Adult black fly emergence generally occurs in late spring and early summer (Borror et al. 1989) when the newly developed adult leaves its puparium and floats to the water-air interface partially enclosed in an air bubble (Adler et al. 2004).

Adult simuliids are capable of long distance flights and may be found at considerable distances from running water (Bennett and Fallis 1971, Borror et al. 1989, Lehane 1991). Female black flies are known to disperse several kilometers in search of a host (Marquardt et al. 2000) with distances of 225 km recorded for *Simulium vampirum* (Fredeen 1969 *as in* Adler et al. 2004) and of greater than 500 km for the African *Simulium damnosum* species complex (Garms and Walsh 1988). However, dispersal distances of 9-13 km are most common (Baldwin et al. 1975). The life span of adult black flies in the wild is typically 10-35 days (Crosskey 1990).

Of the 254 recognized North American black fly species, 90% are able to acquire a blood meal from warm-blooded vertebrate hosts (Adler et al. 2004). The remaining 10% lack the necessary mouthparts required to penetrate flesh, and the females of these species are able to

complete egg maturation without a blood meal (Adler et al. 2004). The blood-feeding species of black flies have been categorized as mammalophilic or ornithophilic, based on their preferred prey taxon (Adler et al. 2004). This feeding preference is correlated with the absence (mammalophilic) or presence (ornithophilic) of a basal lobe on the tarsal claw, which is thought to aid movement through feathers (Shewell 1955). The majority of North American blood-feeding simuliids, 63%, are primarily mammalophilic, while 37% are chiefly ornithophilic (Adler et al. 2004). However, feeding classifications are not always clear, as some species will feed on both mammals and birds, and specific host records do not exist for approximately 60% of the blood-feeding North American species (Adler et al. 2004). North American black flies have been recorded feeding on at least 32 mammalian species and 50 bird species (Adler et al. 2004).

Simuliids are pool feeders which mean they take blood meals by lacerating capillaries in the skin via a biting action that results in lesions from which to draw blood (Sutcliffe and McIver 1984). Black flies usually concentrate on areas of birds that lack protection from dense feathers. Lesions resulting from black fly attacks were found on the eyelids, ceres, corners of beaks, auricular openings, jugular grooves and ventral surfaces of the patagia of great horned owl (*Bubo virginianus*) owlets (Hunter et al. 1997). Smith et al. (1998) recognized an increased susceptibility to black fly bites in young red-tailed hawk *(Buteo jamaicensis*) nestlings (less than 25 days old) with natal down compared to older juvenile birds (30-35 days old) with feathers covering approximately 90% of their bodies.

Black Fly Effects on Bird Species

Black fly infestations can have detrimental effects on both domestic and wild birds. Domestic poultry have experienced decreased egg production, inflammation, egg desertion,

loss of appetite, and death due to black fly exposure (Swenk and Mussehl 1928, Edger 1953 both *as in* Adler et al. 2004). Smith et al. (1998) determined black fly infestation to be the cause of death for 12% of the red-tailed hawk nestlings they were monitoring in Wyoming, and black flies caused a 27% decrease in red-tailed hawk reproductive success in one year of study. Birds exhibit increased energy expenditure in the presence of black fly swarms. Red-tailed hawk nestlings attempted to ward off the pestiferous simuliids with continuous wing flapping, head shakes, movement within nests, and obsessive preening (Smith et al. 1998). Smith et al. (1998) postulated that nestlings that had suffered severe trauma and were found dead under nests may have prematurely fledged the nests in an attempt to escape black fly harassment, or may have accidentally fallen while exhibiting annoyance behaviors.

Hunter et al. (1997) attributed the death of 6 of 10 great horned owl owlets less than 10 weeks of age to black fly feeding in the Yukon, Canada. Of the 14 recovered owlets ages 10-14 weeks, seven perished as a result of black fly feeding (Hunter et al. 1997). Black flies have also been observed attacking bluebird (*Sialia sialis*) nestlings (Gaard 2002) and nesting purple martins (*Progne subis*), and have been responsible for widespread purple martin die-offs and abandonment of colony sites (Hill 1994).

Colonial-nesting common gulls (*Larus canus*) are also negatively affected by black fly outbreaks. Bukacinski and Bukacinska (2000) found that common gulls spend less time defending their territories and tending their eggs and young during years of intense black fly infestations. Nest desertion increased during years of black fly outbreaks and hatching success decreased by at least 40-45% due to the effects of black fly attack on the gull colonies (Bukacinski and Bukacinska 2000). Chick survival declined by 45-60% during the first week post hatching during years of black fly harassment. Decreased survival was due to

predation and starvation as a result of decreased parental care as well as to direct blood loss resulting from black fly feeding (Bukacinski and Bukacinska 2000).

Simuliids also serve as vectors of a number of disease organisms and can transmit species of protozoa, filarial nematodes, arboviruses and possibly bacteria to birds (Adler et al. 2004). Leucocytozoonosis, a disease caused by *Leucocytozoon* protozoans, proves to be the most pathogenic and well-studied disease transmitted by North American simuliids (Adler et al. 2004).

The Specificity of *Simulium annulus* to the Common Loon

The proposed specificity of *Simulium annulus* to the common loon is quite uncommon since most simuliid species appear to be generalists that feed on hosts of similar sizes or within particular habitats (Adler et al. 2004). The common loon's nesting period (McIntyre 1988) coincides with the typical emergence of black flies, and the excessive presence of these swarming biting flies near common loon nests (Bennett and Fallis 1971) can often assist an observer in locating a newly constructed nest (personal observation).

Lowther and Wood (1964) first reported the specificity of *S. annulus* to the common loon and found evidence that black flies primarily use olfactory cues to locate their hosts. Since this initial publication, numerous studies have attempted to further elaborate on this proposed specificity. Although the specificity of *S. annulus* to the common loon was repeatedly reported and asserted, one of the trials during a study conducted by Fallis and Smith (1964) resulted in the collection of near equal numbers of *S. annulus* from a loon and a mallard duck carcass. Further, *Simulium annulus* females have also been collected from penned moose (Pledger et al. 1980 *as in* Adler et al.), chickens (Fredeen 1981 *as in* Adler et al. 2004), and from domestic ducks and ruffed grouse (Bennett 1960 *as in* Adler et al. 2004).

Malmqvist et al. (2004) used molecular techniques to determine black fly feeding preferences and contradictorily found that cranes were the primary host of collected *S. annulus*. Humans have reportedly been swarmed by *S. annulus* as well; however, the accuracy of species identification may be questionable in most of these cases (Adler et al. 2004).

Simulium annulus Ecology

Simulium annulus typically use running water habitats for egg laying and maturation that are greater than 10 m wide, but immature fly life stages have been found in streams with widths as small as 2 m (Adler et al. 2004). *Simulium annulus* breeding habitats are often downstream from lake outlets (Adler et al. 2004). Females require a blood meal for egg maturation (Davies and Gyorkos 1990) and are thought to possibly lay their eggs directly in the water while in flight (Davies et al. 1962 *as in* Adler et al. 2004). Adult emergence of *S. annulus* commences in May and adults can be observed through early July (Adler et al. 2004). The life span of *S. annulus* females is approximately 2-3 weeks (Bennett and Fallis 1971) and female flight distances are typically around 2.5 km, yet flights of at least 8 km are possible (Bennet and Fallis 1971).

Due to the possible negative effects of black flies on loon populations and the disputed specificity of *S. annulus* to the common loon, I chose to further explore the relationship between black flies and loons. A series of tests and techniques were used to test the hypotheses that black flies rely on chemical cues for host attraction and that *S. annulus* is host specific, feeding primarily on common loons.

METHODS

This study was conducted in northeastern Wisconsin, in Vilas, Oneida, and Forest counties. Territorial lakes of loons were located in an area marked by a high density of glacial kettle

lakes which are occupied by an estimated 1,200 loons during the breeding season, of which over 800 were individually color-banded (Meyer 2006). The study lakes were oligotrophic to mesotrophic, ranged from 14 to 362 acres in size, and had maximum depths ranging from nine to 45 feet in depth. The study area was dominated by northern hardwood and conifer forests.

"One-wing" Presentations

Visual and olfactory cues of loon-associated black flies were evaluated by presenting three species of decoys on lakes with a history of common loon presence. The three species of decoys were: common loon, Canada goose (*Branta canadensis*), and hen mallard (*Anas platyrhynchos*) (Figure 1). The common loon decoy was of primary interest, while the Canada goose and mallard decoys served as waterfowl species often associated with common loon habitats. Also, the Canada goose decoy offered an alternative avian size and body form while presenting a black head and neck (similar to common loon plumage coloration). The loon decoy was custom designed and crafted for a different study conducted at the USGS, Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin. A Canada goose was represented by a Greenhead Gear Life-Size Series™ active style Canada goose decoy, and a hen mallard was represented by a Greenhead Gear "Hot Buy" decoy. To test the importance of chemical cues for black fly attraction, presentations were also made with a second loon decoy that had actual loon wings attached to its dorsal surface (Figure 1). The loon wings were amputated from a recently deceased adult loon that was recovered on Manson Lake in Oneida County, Wisconsin in May of 2005 and then stored with the Wisconsin Department of Natural Resources Science Center in Rhinelander, Wisconsin. Before the decoy presentations were initiated, shoulder muscle was removed from the wings to reduce a possible attraction of black flies to decaying flesh and wings were stored on ice between field

presentations. It was assumed that loon uropygial gland oil, the likely chemical cue of interest, was present on the wings. This group of four decoys was presented on 10 lakes from 07 May 2005 to 09 May 2005. This set of presentations involving the four decoy types will be referred to as the one-wing presentations throughout the remainder of this document.

Black flies were sampled at two locations on all decoys using a flypaper-like capture method. Pieces of 40 mm by 65 mm monitor glueboard (Professional Pest Control, Columbus, Georgia) were affixed to the tops of the decoy heads and on the dorsal surfaces just anterior to the tails of the decoys, to capture flies that touched down. Each decoy was attached to the lid of a Rubbermaid® container and was encased within the container tub when the lid was fastened. All containers were spray painted a cream color (dark colors have been shown to attract simuliids (Bennett et al. 1972)) and were transported via canoe to the selected site of presentation. The containers were placed lid-side down on the substrate so that the decoy was oriented upright. Each decoy was presented individually with randomized order of presentation at each lake. This was done by removing the tub portion of the Rubbermaid® and placing it behind the decoy. To avoid the collection of black flies attracted to the human observer, the observer quickly left the presentation site and kept a distance of at least 25 m from the decoy once it was revealed. Each decoy was left on the shoreline to simulate incubation for ten minutes before the glueboards were removed and the decoy was covered. The flies were euthanized by placing the glueboards into containers of 95% ethanol (EtOH).

"Three-wing" Presentations

Mallard and Canada goose wings were obtained from the Wisconsin Department of Natural Resources Wildlife Health Lab, Madison, Wisconsin. Twenty-two lakes were visited during

the period of 27 May 2005 through 02 June 2005 where the three species of decoys: loon, Canada goose, and hen mallard, were presented with and without their respective wings applied to the dorsal surfaces of the decoys. That is, six different decoys were presented at each lake; one of each of the three species, and one of each of the three species with the representative wings applied (Figure 1). The presentations of this group of decoys will be referred to as the three-wing presentations throughout the remainder of this paper since wings from three different bird species were presented.

The above mentioned decoys were presented on four additional lakes on 03 June and 04 June 2005 along with a loon decoy with Canada goose wings applied to its dorsal surface, and a Canada goose decoy with loon wings applied to its dorsal surface. The presentations of this third set of decoys will be referred to as the wing-swap presentations. As described for the one-wing presentations, caution was taken to prevent the attraction of black flies to decaying flesh on the wings by removing shoulder muscles and by storing the wings on ice between presentations. Black fly collection methods were the same as mentioned above, and again, each decoy was presented individually in a randomized presentation order, and was left on the shoreline for ten minutes to simulate incubation.

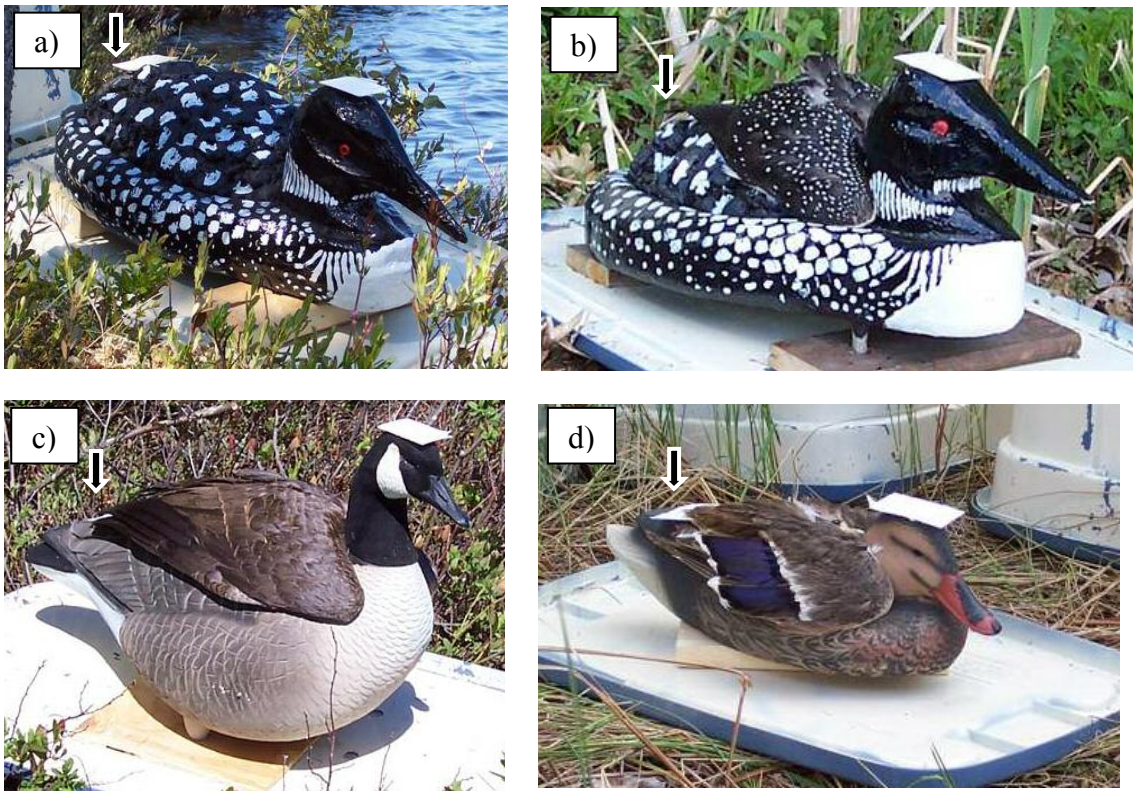

Figure 1: Photographs of decoys used for black fly collection; a) loon without wings, b) loon with wings, c) goose with wings and d) mallard with wings. Note the locations of the glueboards on the decoys (glueboard locations on "back" indicated with arrows).

Data Collection

The total numbers of simuliids on each piece of glueboard (from the head and dorsal surface for each decoy presentation) were later quantified in the lab. The black flies collected during the three-wing and wing-swap presentations were quantified and identified to species. All flies on each piece of glueboard were first scanned for gross differences from one another. A random sub-sample of five flies from each piece of glueboard was closely examined and identified to species using morphological character descriptions provided by Adler et al. (2004). All other flies on the glueboards were visually compared to those that had been keyed to species. The abdomens of the black flies selected for keying were removed, soaked in potassium hydroxide (KOH) solution and were cleared with EtOH to view genitalia. The

examined genitalia were stored in glycerin. Voucher specimens have been deposited in the Northern Michigan University insect collection.

A number of environmental conditions were assessed at each lake during the decoy presentations including air temperature, wind speed and direction, and barometric pressure. Wave action was classified as calm, small waves, choppy waves, or white caps. Cloud cover was also evaluated and the percent cloud cover at each lake was classified as clear, 0-25%, 25-50%, 50-75%, or 75-100%. Changes in cloud cover between decoys were noted. The presence of precipitation during each presentation was also recorded as none, mist, light rain, heavy rain, or thunderstorm. The number of loons that were present on the lake during decoy presentations was noted as well as the activity of the loon(s). The presence of mallards and Canada geese was also recorded, and a general assessment of black fly abundance was made. Black flies were described as being 1) present and annoying, 2) present and not annoying, or 3) none observed. A general description of vegetation and substrate type at the site of decoy presentation was recorded and a photograph was often taken as documentation. A GPS waypoint was recorded at the site of decoy presentation at each lake, and when known, a GPS waypoint was taken at the loon nest site post-nesting.

Statistical Analyses

Multiple linear regression analyses were used to determine which factors had the greatest effect on the number of black flies collected. The following factors were considered as independent variables: collection type (each type included the species of decoy, presence or absence of wings, and the position of the tape on the decoy as either head or back), time of day, temperature, wind speed, barometric pressure, wave action, cloud cover, precipitation, the number of adults loons present, the number of loon chicks present, the presence or

absence of mallards, the presence or absence of Canada geese, the order of decoy presentation, the vegetation type at the site of presentation, and the nesting status of the loon pair at the time of presentation (nesting or not). Two separate analyses were run using SPSS version 13.0 (2004); one for the one-wing presentations, and one for the three-wing presentations. The addition of variables into the model was conducted in a stepwise manner.

Since data did not conform to the assumptions of parametric analyses, Kruskal-Wallis analyses were conducted to determine if the number of black flies collected from the different decoy types were significantly different from one another. For each presentation type (one-wing and three-wing), differences in fly numbers were examined for glueboards positioned on the heads, on the backs, and for the two glueboard locations combined between each decoy type. Post hoc tests between pairs of treatments were performed using a Mann-Whitney U test statistic where alpha values were Bonferroni-corrected.

RESULTS

During the one-wing presentation of decoys 3,467 black flies were captured. The majority of black flies (2,779) were collected from heads of the loon decoys with wings (80.2%). Backs of the loon decoys with wings attracted 583 flies (16.8%), and 23 flies (0.66%) and zero flies were collected from heads and backs of the loon decoys without wings, respectively (Table 1). Black flies were less numerous on the goose and mallard decoys, yet decoy heads remained more attractive than decoy backs (Table 1). Goose heads attracted 75 black flies (2.2%), while only one black fly (0.03%) was collected from goose backs. Mallard heads attracted five black flies (0.14%) and only one black fly (0.03%) was captured on mallard backs (Table 1).

Table 1. Number of black flies collected from each decoy type on 10 lakes during the one-wing presentation period.

Decoy species	Wing Presentation	Location of Glueboard on Decoy	Number of Black Flies	Percent of Total Flies
Loon	Wings	Head	2,779	80.2
Loon	Wings	Back	583	16.8
Loon	*sans* Wings	Head	23	0.66
Loon	*sans* Wings	Back	0	0
Canada Goose	*sans* Wings	Head	75	2.2
Canada Goose	*sans* Wings	Back	1	0.03
Mallard	*sans* Wings	Head	5	0.14
Mallard	*sans*Wings	Back	1	0.03

During the three-wing presentation 552 black flies were collected on the glueboards. Again, loon decoys with wings attracted the most black flies, with a total of 542 flies (98.2%) collected from heads and backs of this decoy type. Heads of loon decoys with wings attracted 523 black flies (94.7%), while 19 black flies (3.4%) were collected from backs of loon decoys with wings (Table 2). The only other decoy types that attracted black flies during this period of presentation were goose with wings and goose decoys. Nine black flies (1.6%) were captured on heads of the goose with wings decoys, and one black fly (0.18%) was collected from heads of the goose decoys (Table 2).

Table 2. Number of black flies collected from each decoy type on 22 lakes during the three-wing presentation period.

Decoy species	Wing Presentation	Location on Decoy	Number of Black Flies	Percent of Total Flies
Loon	Wings	Head	523	94.7
Loon	Wings	Back	19	3.4
Loon	*sans* Wings	Head	0	0
Loon	*sans* Wings	Back	0	0
Canada Goose	Wings	Head	9	1.6
Canada Goose	Wings	Back	0	0
Canada Goose	*sans* Wings	Head	1	0.18
Canada Goose	*sans* Wings	Back	0	0
Mallard	Wings	Head	0	0
Mallard	Wings	Back	0	0
Mallard	*sans* Wings	Head	0	0
Mallard	*sans* Wings	Back	0	0

Black flies were also captured on four lakes where wing swaps were made on loon and goose decoys. Heads of the decoys were again more attractive than backs; no black flies were collected from the backs of any of the decoy types (Table 3). Heads of the goose decoys with loon wings attracted the majority of the captured flies (53 of 87, 60.9%), while heads of the loon decoys with loon wings attracted 26 flies (29.9%). Three black flies were captured on heads of goose decoys with goose wings (3.4%) and five black flies (5.7%) were captured on heads of loon decoys with goose wings (Table 3).

Table 3. Number of black flies collected on four lakes during the wing swap presentations.

Decoy species	Wing Presentation	Location on Decoy	Number of Black Flies	Percent of Total Flies
Loon	Loon Wings	Head	26	29.9
Loon	Loon Wings	Back	0	0
Loon	Goose Wings	Head	5	5.7
Loon	Goose Wings	Back	0	0
Canada Goose	Goose Wings	Head	3	3.4
Canada Goose	Goose Wings	Back	0	0
Canada Goose	Loon Wings	Head	53	60.9
Canada Goose	LoonWings	Back	0	0

All examined black flies from the three-wing and wing-swap presentation periods were identified as *Simulium annulus*, the loon-specific species. No other species of black fly was observed. Three main characters were used to distinguish the genus *Simulium* from the other black fly genera: 1) the wing vein R1 joined the costa beyond the middle of the wing, 2) spiniform setae were interspersed among hair-like setae on the costa of the wing, and 3) the pedisulcus on the basitarsus of the hind leg was deep and conspicuous (Adler et al. 2004). The key characters used to identify the flies as *S. annulus* included: 1) the presence of hair on the basal section of the radius of the wing, 2) the presence of basal thumb-like lobes on the claws, 3) uniform coloration of the integument and hair of the tibia, 4) a unicolorous scutum,

5) an incomplete precoxal bridge, and 6) prominent, anteriorly directed apodemes on the lateral plates of the genital forks (Adler et al. 2004).

Multiple linear regression analyses for the one-wing and three-wing presentations resulted in models that were significant and found collection type to be the most important factor determining the number of black flies captured (Table 4). The measured environmental variables did not significantly explain the variation in the number of black flies collected on the different decoy types.

Table 4. Results of multiple linear regression analyses for the one-wing and three-wing presentations

	One-wing Presentations	Three-wing Presentations
F	21.226	9.621
df	63	327
p-value	< 0.001	0.002
R^2	0.255	0.029
Regression coefficient:		
Decoy type	-13.255	-0.633
Constant	122.391	6.313

Kruskal-Wallis tests indicated that there were significant differences in the numbers of black flies collected from the different decoy types. During the one-wing presentation series, significant differences were found in the number of black flies collected from the heads of the four decoy types (Chi-Square = 26.104, df = 3, p-value < 0.001). The number of black flies captured on the backs of the decoys and the total number of black flies captured on the decoys also differed significantly between decoy types (Chi-Square = 28.092, df = 3, p-value <0.001 and Chi-Square = 26.061, df = 3, p-value < 0.001 respectively). The Bonferroni-corrected alpha value for this set of tests was 0.0083, which is the standard alpha value for parametric data, 0.05, divided by the number of comparisons, which were six in this case.

Post hoc tests on the total numbers of black flies revealed that the number of black flies captured on the loon with wings decoy was significantly different from the total numbers of black flies captured on the other three decoy types (Table 5). The total number of black flies captured on the loon, goose and mallard decoys were not significantly different from one another (Table 5).

Table 5. Post hoc comparisons of the total number of black flies captured during the one-wing presentations. Asterisks indicate significance with Bonferroni-corrected alpha = 0.0083.

Decoy Comparison	Mann-Whitney U	p-value
Loon with wing vs. Loon	0	< 0.001***
Loon with wing vs. Goose	1.00	< 0.001***
Loon with wing vs. Mallard	0	< 0.001***
Loon vs. Goose	38.000	0.306
Loon vs. Mallard	44.500	0.585
Goose vs. Mallard	32.000	0.110

Kruskal-Wallis analyses and Bonferroni-corrected Mann-Whitney U post hoc tests were also performed for the three-wing presentation period. The numbers of black flies collected on the heads of the six different decoy types were significantly different (Chi-square = 52.389, df = 5, p-value < 0.001), as were the number of black flies collected on the backs (Chi-square 20.465, df = 5, p-value = 0.001). The total numbers of black flies collected on the six decoy types were also significantly different (Chi-square = 52.389, df = 5, p-value < 0.001). The Bonferroni-corrected alpha value was calculated as 0.0033 (15 comparisons). The post hoc tests showed that the total number of black flies collected on the loon decoy with wings was different from the number of flies collected on the other decoy types (Table 6). Significant differences were not found between the total numbers of black flies captured on the remaining decoy types (Table 6).

Table 6. Post hoc comparisons of the total number of black flies captured during the three-wing presentations. Asterisks indicate significance with Bonferroni-corrected alpha = 0.0033.

Decoy Comparison	Mann-Whitney U	p-value
Loon w/wing vs. Loon	99.000	< 0.001***
Loon w/wing vs. Goose w/wing	126.500	0.002***
Loon w/wing vs. Goose	104.000	< 0.001***
Loon w/wing vs. Mallard w/wing	99.000	< 0.001***
Loon w/wing vs. Mallard	99.000	< 0.001***
Loon vs. Goose w/wing	187.000	0.019
Loon vs. Goose	231.000	0.317
Loon vs. Mallard w/wing	242.000	1.000
Loon vs. Mallard	242.000	1.000
Goose w/wing vs. Goose	197.000	0.076
Goose w/wing vs. Mallard w/wing	187.000	0.019
Goose w/wing vs. Mallard	187.000	0.019
Goose vs. Mallard w/wing	231.000	0.317
Goose vs. Mallard	231.000	0.317
Mallard w/wing vs. Mallard	242.000	1.00

DISCUSSION

This study demonstrates the importance of visual, and more clearly, chemical cues for host location by black flies and supports the idea that *Simulium annulus* is host specific, feeding primarily on common loons. The presentation of loon wings provided a chemical cue of a common loon that the other decoys did not possess. During the one-wing and three-wing presentations more black flies were captured on the loon decoy with wings than on all other decoy types combined, thus showing the value of chemical cues. During all three presentation periods, only those decoys outfitted with common loon wings captured significantly different numbers of flies from the other decoy types. The significant difference between the numbers of black flies, later identified as *S. annulus*, captured on the loon decoys with wings and on the other decoy types demonstrated the importance of chemical cues for host location. The specificity of *S. annulus* to the common loon was also confirmed by comparisons between the number of *S. annulus* captured on the loon with wings decoy and the other decoy types.

The degree of specificity of *S. annulus* to the common loon is quite unusual (Adler et al. 2004). Surprisingly, only one black fly species, *S. annulus*, was collected during this study despite the probability that other black fly species were present at the decoy presentation sites, and that the presentation sites were likely used by other avian species as well as mammals (including humans). Although no previous work has been published on the subject, it is possible that *S. annulus* is a superior competitor, out-competing other black fly species for bloodmeals.

Lowther and Wood (1964) were the first to report the specificity of *S. annulus* to the common loon and found evidence that black flies primarily use olfactory cues, or more appropriately, chemical cues, to locate their hosts. They placed study skins of a common loon, a pied-billed grebe (*Podylimbus podiceps*), a herring gull (*Larus argentatus*), and a male common merganser (*Mergus merganser*) on a shoreline to test the specificity of *S. annulus* to the common loon. Lowther and Wood (1964) found, as I similarly did using decoys and wings, that *S. annulus* was not as attracted to the non-loon specimens.

Since the initial publication by Lowther and Wood (1964), numerous studies have attempted to further elaborate on this proposed specificity. Fallis and Smith (1964) found chemical stimuli to be more important than visual cues for black fly host attraction after conducting a series of experiments altering the presentation of loon chemical and visual stimuli to black flies. Unfortunately, in my study, the addition of wings to plain decoys presented a simultaneous change in the black flies' visual and chemical perceptions of the decoys. Although the wings were assumed to be covered with the bird's uropygial gland oil and to emit a chemical cue, it may have been beneficial to more appropriately separate the two factors, visual and chemical, by discretely presenting a chemical cue that would not alter the

look of the decoy. This could have been accomplished by using ether extracts of common loon uropygial glands as performed by Fallis and Smith (1964) and Bennet et al. (1972). However, during my study the importance of chemical cues was exhibited by the attraction of black flies to Canada goose decoys with loon wings even though the head and body of a Canada goose is visually distinct from those of a loon.

Fallis and Smith (1964) also pinpointed the tail and uropygial gland as the main source of black fly attraction to common loons. However, they also recognized the importance of visual and tactile cues at close distances since flies were more inclined to land on raised objects than on flat surfaces and were more apt to crawl among the soft head and neck feathers of a loon while only staying in brief contact with the more rigid feathers of the back (Fallis and Smith 1964). The results presented here support this observation, since the majority of black flies were captured on the heads of the decoys, which were higher targets that provided a reliable cue to an area of easier access to a real loon blood meal. The backs of the decoys were located closer to the ground and represented an area of the bird typically covered with dense feathers that deter penetration by simuliids.

The importance of proximate visual targeting after initial chemical attraction was further demonstrated by comparing the number of black flies that were captured on the heads of the Canada goose decoys presented with loon wings to those captured on the heads of the loon decoys with loon wings. The height preference of black flies' feeding targets was verified by the collection of more black flies from the head of the goose decoys with loon wings (52 flies) than from the loon decoy with loon wings (26 flies). However, because the Canada goose decoy was only presented with loon wings on four lakes, black fly numbers on the head of the loon decoy with loon wings and on the head of the goose decoy with loon wings

could not be statistically compared. Additional presentations would likely provide fly numbers that are significantly different between decoy types.

During this study more black flies were captured during the one-wing presentation period than during the three-wing and wing swap presentations. This discrepancy is best explained by the emergence time and lifespan of black flies rather than by the change in decoy presentations. Adult black fly emergence generally occurs in late spring and early summer (Borror et al. 1989) and black fly numbers were already declining by the time the wings from the other two species were obtained and were presented. The life span of *S. annulus* females is approximately two to three weeks (Bennett and Fallis 1971), and unfortunately, 18 days elapsed from the end of the one-wing presentations, which occurred early in the season, before the start of the three-wing presentations. This time delay likely decreased the chance of capturing many blood-thirsty females during the three-wing and wing-swap presentations. However, even with the smaller sample size of captured flies during the later presentation times, significant differences were still found between the attractiveness of decoy types and the positions of capture on the decoys for the three-wing presentations.

As shown in the multiple linear regression analysis, time of presentation and environmental conditions did not significantly affect the number of black flies that were captured during this study. This was surprising since activity levels of diurnal black flies typically peak during early morning and late afternoon hours (Lehane 1991) and humidity and wind speed have been shown to affect flight activity (Grace and Shipp 1988). Temperature can also affect simuliid activity. Smith et al. (1998) reported decreased black fly activity at red-tailed hawk nests when ambient temperatures fell below 14ºC. The lack of environmental effect in my

study is likely due to the overwhelming response of black flies to the more significant stimulus of common loon chemical cues.

The direct effects of *S. annulus* on common loon reproductive success should be investigated and quantified to better understand the population level effects of this host-parasite relationship. Loons have been observed abandoning their nests during extreme black fly infestation (McIntyre 1988) which may result in nest failure due to nest predation or addling of eggs. In such cases the presence of swarming, biting black flies ultimately has the potential to directly affect loon reproductive output and fitness. The high level of specificity of *S. annulus* to the common loon is unique (Adler et al. 2004), and the potential effects of this relationship on loon reproduction are of special interest since generalist parasites might be more detrimental to their hosts than host-specific parasites. As vectors of *Leucocytozoon*, black flies also have the potential to transmit host-specific blood parasites to loons. Many species of *Leucocytozoon* and *Haemoproteus* are relatively host-specific, feeding only from closely related hosts within an avian family (Fallis et al. 1974, Atkinson 1986).

CHAPTER TWO: HAEMOSPORIDIANS, MERCURY, AND THE COMMON LOON IMMUNE RESPONSE

INTRODUCTION

Parasitic protozoans that infect blood cells (i.e. haemosporidians) have been documented in numerous bird species, along with their concomitant negative effects on host reproduction. However, no haemosporidian has been described parasitizing common loons. *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* are three of the most common genera of blood parasites found in wild-caught birds in North America (Greiner et al. 1975). Although all three genera are discussed in this study, special attention was paid to *Leucocytozoon* spp. due to their known transmission by simuliids (Adler et al. 2004). The interaction between common loons and black flies, specifically *Simulium annulus*, suggests that loons are likely infected with *Leucocytozoon*. As piscivores, loons are at high risk for methylmercury exposure (Evers et al. 1998, Meyer et al. 1998, Evers et al. 2003), and it is possible that haemosporidian infection may be correlated with blood mercury levels. Kenow et al. (in review) found that mercury suppresses the antibody-mediated immune response of loon chicks that received ecologically-relevant mercury doses. Therefore, it is possible that the likelihood and intensity of *Leucocytozoon* infection may increase with decreased immune function resulting from mercury exposure.

Haemosporidians and Avian Reproductive Ecology

The effects of blood parasites on avian reproductive ecology have been demonstrated in numerous species. Merino et al. (2000) experimentally tested the pathogenicity of haemosporidians in blue tits (*Parus caeruleus*) by treating birds with primaquine, an antimalarial drug. They found treatment with the antimalarial increased both fledging success

and nestling survival (Merino et al. 2000). Sanz et al. (2001) found a decreased rate of nest success associated with increased hematozoan prevalence in pied flycatchers (*Ficedula hypoleuca*) of Spain. In the same study, nest abandonment and decreased hatching success were correlated with the presence of *Trypanosoma* spp. and *Haemoproteus balmorali*, respectively.

Blood parasites have also been shown to affect non-passerine birds. Female Tengmalm's owls (*Aegolius funereus*) infected with *Leucocytozoon ziemanni* during a year of intermediate prey abundance produced smaller clutches than non-infected owls (Korpimaki et al. 1993). Smith et al. (1998) reported red-tailed hawk (*Buteo jamaicensis*) nestling mortality associated with black fly infestations and *Leucocytozoon* infections. Herman et al. (1975) concluded that *Leucocytozoon simondi* was the agent in a deadly epizootic which affected Canada goose (*Branta canadensis*) goslings at Seney National Wildlife Refuge, Michigan. Hunter et al. (1997) found that five out of seven 10-14 week old great horned owl (*Bubo virginianus*) owlets died as a result of black fly torment, anemia, and severe dehydration and also had severe *Leucocytozoon* spp. infections. Less intense *Leucocytozoon* parasitemias were observed in owlets less than 10 weeks of age; however, all infected owlets had skin lesions as a result of black fly attack (Hunter et al. 1997).

Avian Haemosporidian Surveys

A number of studies have been conducted to survey the parasite fauna of wild birds. Tarof et al. (1997) investigated the prevalence of blood parasites in hooded warblers (*Wilsonia citrina*) in Pennsylvania and found species of *Leucocytozoon*, *Plasmodium*, *Haemoproteus* and microfilariae. Parasites belonging to the genera *Leucocytozoon* and *Haemoproteus* were also found in Chimango caracas (*Milvago chimango*) in southern Chile (Forrester et al. 2001). Valkiūnas and Iezhova (2001) examined the blood of yellow wagtails (*Motacilla flava*) and

detected species of *Hamoproteus*, *Leucocytozoon, Plasmodium*, *Atoxoplasma*, and *Trypanosoma*. Haemosporidians of American crows (*Corvus brachyrhynchos*) and fish crows (*Corvus ossifragus*) were surveyed by Dusek and Forrester (2002) who found six species of blood parasites of the genera *Haemoproteus*, *Trypanosoma*, and *Plasmodium* as well as an unknown filarioid. Although with low prevalence, hematozoans have even been detected in birds of the arctic tundra. Hollmen et al. (1998) discovered *L. simondi* in a blood smear of an Emperor Goose (*Chen canagica*) surveyed in the Yukon-Kuskokwim Delta in Alaska.

Leucocytozoon Ecology

Genus *Leucocytozoon* (Haemosporida: Leucocytozoidae) contains approximately 60 species with cosmopolitan distributions, and has been reported in over 1000 bird species of 98 families (Roberts and Janovy Jr. 2000, Bennett 1987). The life cycle of *Leucocytozoon* species requires both an intermediate and definitive host. A definitive host is the host in which sexual reproduction occurs and an intermediate host is one that is home to the non-sexual reproductive life stages of the parasite (Roberts and Janovy Jr. 2000). Therefore, in the case of *Leucocytozoon*, black flies of the family Simuliidae are the definitive hosts (for all but one species of *Leucocytozoon*), while birds serve as intermediate hosts (Valkiunas 2005). Mature gametocytes, sexual stages of *Leucocytozoon*, are present in erythroblasts, erythrocytes and mononuclear leukocytes of avian hosts and simuliids ingest gametocytes while taking a blood meal from an infected bird (Valkiunas 2005). Gametogenesis occurs in the midgut of the infected black fly and frees the gametocytes from the host cells (Valkiunas 2005). Fertilization takes place extracellularly in the midgut and the zygote forms into a motile, elongated ookinete within six to 12 hours from ingestion of gametocytes (Valkiunas 2005). Ookinetes develop into oocysts in the basal lamina of the host as a result of sporogony, which lasts three to five days (Bennett 1987, Valkiunas 2005).

Oocysts release sporozoites into the hemocoele that then travel to the salivary glands of the black fly (Cook 1971, Bennett 1987, Wobeser 1997, Valkiunas 2005). Environmental temperature can vary the rate of sporogony within the host (Wobeser 1997). Ingestion of subsequent blood meals from additional avian individuals transfers sporozoites from salivary glands into the blood stream of new avian hosts. Sporozoites are common in simuliid salivary glands, and it is likely that 90% to 100% of ornithophilic black flies in Algonquin Park, Ontario have *Leucocytozoon* sporozoites in their salivary glands during summer months (Bennett and Squires-Parsons 1992). Once established in the new host, sporozoites migrate to the bird's liver via the circulation system and invade hepatocytes. Here they develop into small hepatic schizonts that ultimately produce merozoites through a series of cytoplasmic invaginations and nuclear divisions (Valkiunas 2005). These merozoites can 1) re-enter a hepatocyte, 2) be engulfed by a macrophage, or 3) invade blood cells (Valkiūnas 2005).

Merozoites that are engulfed by macrophages within the organs of the avian host are capable of developing into a second generation of meronts also known as 'megalomeronts' that divide internally with each cell reproducing by merogony. Thus, each megalomeront has the capacity to release millions of merozoites into the host (Wobeser 1997, Roberts and Janovy Jr. 2000, Valkiunas 2005). Megalomeronts can be present in numerous locations within the bird host, and have been found in the liver, brain, heart, lung, gizzard, intestines and kidneys, but are most common in the spleen and lymph nodes (Cook 1971, Wobeser 1997, Roberts and Janovy Jr. 2000, Valkiunas 2005).

Merozoites that invade red blood cells, become round gametocytes, or enter leukocytes and immature erythrocytes and develop into elongated gametocytes (Roberts and Janovy Jr. 2000). The development of the first generation of meronts of a number of *Leucocytozoon*

species may not be confined to hepatocytes. The endothelial kidney cells of an avian host may also serve as sites for meront development (Valkiunas 2005). Also, not all species of *Leucocytozoon* have displayed megalomeront formation (Valkiunas 2005).

The presence of *Leucocytozoon* gametocytes in peripheral blood of avian hosts may result in elevated leukocyte counts, and reduced packed cell volume and hemoglobin content in the blood (Cook 1971, Bennett 1987, Wobeser 1997, Roberts and Janovy Jr. 2000). The liver and spleen of an infected host are often grossly enlarged and damaged due to the invasion of inflammatory cells and macrophages following megaloschizont rupture (Cook 1971, Bennett 1987, Wobeser 1997, Roberts and Janovy Jr. 2000). Tissue hemorrhaging and congestion of hepatic sinusoids result from a ruptured megaloschizont (Wobeser 1997). Similar damage to vital organs such as the brain, heart and lungs may also occur (Wobeser 1997, Roberts and Janovy Jr. 2000).

Leucocytozoon parasitemia peaks approximately nine to ten days after infection and usually lasts about 30 days (Cook 1971, Wobeser 1997). The parasites are capable of persisting at decreased levels of intensity over a long period of time, and infection often recrudesce at the onset of avian breeding seasons which happen to correspond with black fly emergence (Cook 1971, Wobeser 1997). This spring emergence, which is weather dependent, begins the cycle of transmission, and adult birds serve as the primary hosts to black flies since the young of the year often have not yet hatched. Infected adult birds serve as reservoirs of *Leucocytozoon*. Adults with chronic infections can be subsequently re-infected which results in increased intensity of infection among adult birds and in increased numbers of infected black flies in the area (Wobeser 1997). This synchrony of parasitemia recrudescence and black fly emergence thus increases the probability of subsequent transmission to the juvenile birds

hatched in the area during peak black fly emergence (Wobeser 1997). Leucocytozoonosis in domestic birds is typically most pathogenic in young birds (Khan and Fallis 1968).

Although many cases of *Lecocytozoon* infection are undetected, signs of infection include listlessness, depression, reduced appetite, lethargy, breathing difficulties, and watery eye discharge (Bennett 1987, Wobeser 1997). Severe convulsions may occur before death (Bennett 1987). Onset of *Leucocytozoon* infection can be extremely rapid in young birds with death sometimes occurring within 24 to 48 hours after signs appear (Keymer 1982, Roberts and Janovy Jr. 2000). The death rate of ducklings infected with *L. simondi* can reach 85%, however infection is less acute in adult birds and death is rare (Wobeser 1997, Roberts and Janovy Jr. 2000). Birds that do not show signs of leucocytozoonosis may still be affected by the presence of the parasite by becoming more susceptible to predation, secondary infection, and/or other stresses (Adler et al. 2004). Chronic *Leucocytozoon* infections can result in reduced reproductive output and in suppression of the immune system (Adler et al. 2004). An effective treatment has yet to be employed for *Leucocytozoon* infections and its prevention is dependent on the control of ornithophilic black flies (Keymer 1982, Bennett 1987, Wobeser 1997).

Approximately 18 black fly species are known to serve as vectors and definitive hosts for nine described *Leucocytozoon* species in North America, but it is highly likely that other black fly species also transmit the protist (Adler et al. 2004). Birds serve as the only intermediate host for *Leucocytozoon* (Cook 1971, Keymer 1982). Although the genus name specifically implies infection of leukocytes, *Leucocytozoon* species can also invade the erythrocytes of their hosts (Cook 1971). The vector of this protozoan parasite, the black fly (Diptera: Simuliidae), determines the distribution of infection. The geographic distribution of *Leucocytozoon*

infection is most common in birds from northern regions and areas that provide suitable breeding habitat for their water-dependent vectors (Cook 1971, Bennett 1987). Oddly, *Lecocytozoon* species have not been identified in South and Central America or the West Indies (Cook 1971). Because of their impact on domestic flocks, the two species of greatest interest in the United States are *L. simondi* which commonly infects ducks, geese and other waterfowl, and *L. smithi* which infects turkeys (Bennett 1987).

Haemoproteus Ecology

The largest and most commonly encountered genus of hematozoa is *Haemoproteus* with at least 133 valid species descriptions (Bennett 1987, Wobeser 1997). *Haemoproteus* spp. have been discovered worldwide and have been reported in over 1,700 species of birds from 110 families, however, little is known of their epizootiology and pathogenicity (Keymer 1982, Bennett 1987, Wobeser 1997). Birds and reptiles are the primary intermediate hosts for the protozoan parasites belonging to this genus (Cook 1971, Roberts and Janovy Jr. 2000). Louse flies (Diptera: Hippoboscidae) and midges of the genus *Culicoides* (Diptera: Ceratopogonidae) serve as definitive hosts and vectors of *Haemoproteus* infection, and transmission of the disease is dependent on the presence of vectors (Cook 1971, Bennett 1987).

Both louse flies and midges ingest gametocytes while taking a blood meal from an infected definitive host. Fertilization occurs within the gut of the dipteran vector (Cook 1971, Roberts and Janovy Jr. 2000). Ookinetes are produced, and penetrate the gut wall, forming oocysts between the muscle layers (Roberts and Janovy Jr. 2000). Sporogony occurs within three to five days (Bennett 1987) and the resultant sporozoites travel to the salivary glands of the dipteran host. They are transmitted to new avian hosts during subsequent feedings

(Cook 1971, Wobeser 1997). The sporozoites travel to endothelial cells of blood vessels once they have entered their avian host (Keymer 1982, Wobeser 1997). Schizogony is exoerythrocytic, occurring in endothelial cells of blood vessels in various organs, and takes approximately 25 days to complete (Keymer 1982, Roberts and Janovy Jr. 2000). Merozoites develop from elongated, branched schizonts and enter the bloodstream (Bennett 1987). Merozoites invade erythrocytes which is where gametocyte formation occurs (Keymer 1982, Wobeser 1997). Megaloschizont formation has been described in some *Haemoproteus* species (Wobeser 1997).

Little is known about the pathogenicity of *Haemoproteus* spp. An inflammatory response may be associated with megaloschizont formation, and the schizogonic stages of the life cycle would likely cause the most damage to the vertebrate host if the parasite is pathogenic (Wobeser 1997). Red blood cell destruction may also lead to anemia (Cook 1971). Signs that have been linked to *Haemoproteus* infection include: anemia, congestion of the lungs, asphyxia, abnormal behavior, restlessness, anorexia, enlarged spleen and liver, and blackening of the lungs and liver (Cook 1971, Keymer 1982, Roberts and Janovy Jr. 2000).

Plasmodium Ecology

Plasmodium spp. are cosmopolitan in distribution, and the prevalence of *Plasmodium* exhibits regional and seasonal variation, with the greatest prevalence of the haemosporidian found in areas with elevated vector populations (Keymer 1982, Bennett 1987). Mosquitoes (Diptera: Culicidae) from nearly every genus serve as the definitive host and vector of this hematozoan parasite while birds, reptiles and mammals function as intermediate hosts (Bennett 1987, Roberts and Janovy Jr. 2000). Approximately 35 species of avian malaria have been

described to date, and many of them appear non-host specific (Keymer 1982, Bennett 1987, Wobeser 1997).

The life cycle of *Plasmodium* is complex with variations occurring even within species. However, the life history of the genus can be generalized as follows. The definitive host and vector, the mosquito, ingests *Plasmodium* gametocytes when taking a blood meal from an infected intermediate host. Gametes develop in the gut of the mosquito and fertilization results in the formation of ookinetes, which penetrate the gut wall to become oocysts. Sporozoites are produced by oocysts, and ultimately migrate to the salivary glands of the mosquito. Transmission is successful when sporozoites are injected into a new avian host through the acquisition of a subsequent bloodmeal, and sporozoites travel to reticuloendothelial cells in the avian host (depending on the species) to undergo schizogony (Bennett 1987, Keymer 1982, Wobeser 1997).

Exoerythrocytic schizogony can occur in the liver, spleen, kidneys or lungs and results in schizonts that ultimately produce merozoites. This exoerythrocytic schizogony may be repeated forming megaloschizonts, or merozoites may enter the blood stream and invade erythrocytes. Merozoites within erythrocytes develop into trophozoites which undergo erythrocytic schizogony. Schizonts within erythrocytes release merozoites into the blood stream that may either re-infect other erythrocytes, repeat erythrocytic schizogony, or enter new reticuloendothelial cells undergo exoerythrocyte schizogony. Eventually some erythrocytic merozoites enter new red blood cells and develop into macrogametocytes and microgametocytes. The cycle continues when a mosquito ingests these gametocytes (Keymer 1982, Bennett 1987, Wobeser 1997). Erythrocytic schizogony is unique to *Plasmodium* among hemosporidian genera (Bennett 1987).

It is uncertain whether *Plasmodium* causes significant mortality among wild birds. Most infected birds are discovered when in the chronic stage of the disease and it appears that recrudescences are uncommon and lack the intensity of the original infection. However, environmental stresses and unfavorable conditions may trigger a more serious relapse (Russell 1963). The pathogenicity of malaria in waterfowl is unclear, yet a number of signs of infection have been reported in various bird species including listlessness, anorexia, anemia, swelling of the eyelids, emaciation, enlarged and darkened spleens and livers, and altered behavior, such as decreased preening (Russell 1963, Bennett 1987, Wobeser 1997). Anemia could be caused by the interference of blood flow as a result of schizont formation within vascular epithelium, by the destruction of red blood cells from the release of merozoites, or by the removal of infected erythrocytes by phagocytosis (Wobeser 1997).

Mercury and its Effects

Biologists have recently used the common loon as an indicator species to assess the effects of atmospheric mercury deposition in northern lakes of North America (Meyer et al. 1998, Evers et al. 2003, Fevold et al. 2003). In the north-central United States, inorganic mercury deposition rates have increased two to fourfold since the pre-industrial era (Rada et al. 1989, Meyer et al. 1998). Inorganic mercury (both naturally occurring and anthropogenically deposited) is transformed into methylmercury by anaerobic microorganisms inhabiting aquatic sediments (Wolfe et al. 1998). This methylated form is highly toxic and capable of bioaccumulation, which places piscivorous wildlife at high risk (Evers et al. 1998, Meyer et al. 1998, Evers et al. 2003). Low-pH lakes create an increased risk of methylmercury exposure as various biogeochemical processes in these environments make mercury more bioavailable (Rada et al. 1989, Evers et al. 1998, Meyer et al. 1998, Scheuhammer et al. 1998).

Methylmercury can affect the functions of the central nervous system in birds and reduce muscle coordination, making flying, walking, and standing difficult (Wolfe et al. 1998). Meyer et al. (1998) reported a decrease in chick production on lakes where high blood mercury levels were found in loon chicks. However, during the same study, no relationship was found between adult blood mercury levels or feather mercury levels and chick production (Meyer et al. 1998). Nocera and Taylor (1998) found that elevated mercury exposure is associated with changes in loon chick behavior. Chicks with high blood mercury levels were observed preening more often and spent less time brooding by back-riding. These altered behaviors increase the risk of predation and of energy expenditure (Nocera and Taylor 1998). Yet, in a lab study, Kenow et al. (2003) reported unaltered growth and food consumption in loon chicks receiving various controlled mercury doses.

Immune Function

Recently, ecologists have given more attention to immune function of wildlife species, even spurring the new sub-discipline of ecological immunity (Sheldon and Verhulst 1996). Suppression of the immune system due to environmental pollutant exposure may increase the likelihood of parasitic infection in exposed individuals. Kenow et al. (in review) found suppressed antibody production in loon chicks receiving ecologically-relevant concentrations of dietary methylmercury chloride. Lymphoid depletion in the bursa was also associated with mercury exposure in loon chicks (Kenow et al. in review). Leukocytes are an important part of the immune system and an elevated number of leukocytes are indicative of acute or chronic infection (Merila and Svensson 1995). A simplified method for quantifying the number of white blood cells (WBC) is by measuring the height of the WBC buffy coat layer of a hematocrit capillary tube after centrifugation of a whole blood sample (Gustafsson et al.

1994, Merila and Svensson 1995, Ots et al. 1998). This WBC value can then be compared to the whole blood volume (PCV) to determine the relative health status of the individual (Merila and Svensson 1995).

Molecular Genetic Detection of Haemosporidians

Blood parasite infection rates and parasite loads have been historically determined using ocular examination of stained blood smears (Waldenstrom et al. 2004). According to Hellgren et al. (2004) and Waldenstrom et al. (2004) nested polymerase chain reaction (PCR) techniques that amplify targeted haemosporidians provide reliable parasite identification and improved detection compared to ocular examinations. Unlike ocular methods for haemosporidian detection, molecular techniques allow for phylogenetic investigations and offer insight on parasite species lineages (Waldenstrom et al. 2004). Waldenstrom et al. (2004) argue that molecular methods of haemosporidian detection are also superior to microscopic observations due to the decreased investigation time and the repeatability of the technique. Because molecular detection techniques are more sensitive to low-level parasitemias they are more likely to indicate higher haemosporidian prevalences than ocular methods (Richard et al. 2002, Fallon et al. 2003, Freed and Cann 2006). Richard et al. (2002) examined the prevalence of *Plasmodium* in African birds and discovered that 40% of the tested individuals were infected, while only 27% of the individuals tested positive for *Plasmodium* when ocular examinations were performed.

Since haemosporidians have been shown to affect the reproductive success of avian hosts and because it was unknown whether or not common loons serve as hosts for blood parasites, I chose to explore the haemosporidian fauna of common loons. I specifically tested four hypotheses: 1) Although undescribed as haemosporidian hosts, common loons

serve as hosts for blood parasites, specifically for species of *Leucocytozon*, *Haemoproteus*, and *Plasmodium*, 2) Adult loons are more likely to be infected with *Leucocytozoon* spp. than chicks due to loon nest incubation periods that coincide with black fly emergence, 3) Presence and intensity of *Leucocytozoon* increases with elevated loon blood mercury levels, 4) Increased loon blood mercury levels compromise the loon immune response and possibly make mercury-laden loons more susceptible to parasitic infection. A series of techniques were used to test these hypotheses, and are described in detail in the next section.

METHODS

Blood Collection and Analyses

To measure and quantify blood parasite loads, blood mercury levels, and immune function of common loons, productive loon pairs and their chicks were captured on 48 common loon territories on northern Wisconsin lakes in Vilas, Forest, Oneida and Iron counties. The Institutional Animal Care and Use Committee of Northern Michigan University approved the use of vertebrate animals in this research. Loon captures were conducted between 05 July 2005 and 17 August 2005. Captures were performed using techniques described by Evers (1992). A total of 142 blood samples were collected from 38 adult males, 40 adult females, and from 64 chicks. Blood was drawn from the tarsal vein and was retained for mercury and immune function analysis and for molecular detection of parasites. A lysis buffer was not added to the blood, but blood samples were kept on ice until they were frozen upon returning from the field. Blood mercury analysis was conducted by the Wisconsin State Laboratory of Hygiene, Madison, Wisconsin in cooperation with the Wisconsin Department of Natural Resources.

The white blood cell buffy coat layer of a hematocrit capillary tube was used as an index of immune function. Fresh blood from each bird was transferred to two microcapillary tubes in the field. The tubes were later spun in an International Equipment Company MB microhematocrit centrifuge for 5.5 minutes immediately after returning from the field. White blood cell buffy coat and packed red blood cell volumes (PCV) were measured as percent blood volume using a Critocaps™ card reader. Average buffy coat and PCV readings were calculated from the two microcapillary tubes collected for each bird and the average values were used for statistical analysis.

DNA Analyses

Extractions of DNA were obtained by using a standard phenol/chloroform method; this method began with an incubation period in digestion buffer and Proteinase K and included a series of phenol and chloroform washes followed by ethanol precipitation and re-suspension in deionized water (Sambrook et al. 1989). The concentration of DNA samples were assessed using a Hitachi Genetic Systems Gene Spec I spectrophotometer. The template DNA was diluted to approximately 25 ng/μL and was used in nested PCR assays. PCRs were formed using a Perkin Elmer Gene Amp PCR System 9600 and/or an Eppendorf Mastercycler-Gradient thermocycler in 12.5 μL volumes (quarter-reactions) using: 0.25 units of Invitrogen Platinum® Taq DNA Polymerase, 1.0 μM $MgCl_2$, Taq buffer (1X concentration; 20 mM Tris HCl, 50 mM KCl), 1.0 μM of dNTPs (Fisher Scientific) and 0.5 μM of both forward and reverse primers.

The diagnosis of parasite infection followed a characteristic "nested PCR" technique, relying on three sets of primers to target haemosporidian DNA fragments (Table 7). The first round of PCR used primers HaemNF1 and HaemNR3, designed by Hellgren et al. (2004) to target

a fragment of the cytochrome b (*cyt b*) gene in the mitochondrial genome of three genera of haemosporidians: *Leucocytozoon*, *Haemoproteus*, and *Plasmodium*. The thermal profile began with three minutes of denaturation at 94ºC, followed by 20 cycles at 94º C for 30 sec (strand separation), 50º C for 30 sec (primer annealing), 72º C for 45 sec (elongation), and ended with a final extension period of 10 minutes at 72º C.

The products from the first round of PCRs were then used as templates for the next round of PCR. Primers HaemF and HaemR2 anneal to a section of the *cyt b* gene internal to the annealing sites of HaemNF1 and HaemNR3 in the *Haemaproteus* and *Plasmodium* genera (Bensch et al. 2000). A third set of primers used in a separate reaction, HaemFL and HaemR2L, were designed by Hellgren et al. (2004) to similarly anneal to a section of the *cyt b* gene internal to the annealing sites of HaemNF1 and HaemNR3 in *Leucocytozoon* species (Table 7). The thermal profile for the second round of amplifications (for both sets of primers) began with three minutes of denaturation at 94ºC, followed by 35 cycles at 94º C for 30 sec (strand separation), 55º C for 30 sec (primer annealing), 72º C for 45 sec (elongation), and ended with a final extension period of 10 minutes at 72º C. The products from the second round of PCR amplifications were run on 1.5% low-melt Tris-Borate-EDTA agarose gels at 100V for approximately 25 minutes, stained with ethidium bromide (EtBr) and visualized using ultraviolet light.

Positive and negative controls were used to verify the accuracy of the PCRs. Positive controls were attained from birds that tested positive for the desired haemosporidian after visual examination of a blood smear. The *Haemoproteus* control was from a pied flycatcher (*Ficedula hypoleuca*), a wattled crane (*Bugeranus carunculatus*) served as the control for *Plasmodium*, and the *Leucocytozoon* control was from a long-eared owl (*Asio otus*).

Table 7. Primers for haemosporidian detection using nested PCRs. The primers could not distinguish *Haemoproteus* from *Plasmodium* infections.

Primer Name	Primer Sequence	Targeted Haemosporidian	Expected Product Size
HaemNF1	CATATATTAAGAGAAITATGGAT	H,P,L	Not visualized
HaemNR3	ATAGAAAGATAAGAAATACCATTC	H,P,L	
HaemF	ATGGTGCTTTCGATATATGCATG	H,P	527 bp
HaemR2	GCATTATCTGGATGTGATAATGGT	H,P	
HaemFL	ATGGTGTTTTAGATACTTACATT	L	525 bp
HaemR2L	CATTATCTGGATGAGATAATGGIGC	L	

Primer names and sequences (listed → 5' to 3') from Hellgren et al. (2004) and Bensch et al. (2000). Targeted haemosporidian abbreviations are H=*Haemoproteus,* P=*Plasmodium,* L=*Leucocytozoon*.

The presence or absence of *Leucocytozoon* was the focus of this study due to its transmission via simuliids, and therefore, nested PCR assays for the detection of *Leucocytozoon* were performed at least three times for every loon sample. Ideally, a loon would be considered positive for the parasite if bright gel electrophoresis bands of ~500 bp were visualized from the PCR amplifications. Due to inconsistent results and varying band intensities among the three amplifications, all loons were scored as positive or negative first in a very conservative fashion (i.e. only the brightest bands were considered positive) and later with a more liberal definition of infection (i.e. any visible amplicon was considered positive). Loons were only considered positive for *Leucocytozoon* during the conservative interpretation if the gel electrophoresis bands were consistent and bright for the majority of the PCR amplifications. For the liberal interpretation of *Leucocytozoon* infection all gel electrophoresis bands, including faint or dim bands that occurred in any of the three amplifications were scored as an infected loon. All loon samples were also assigned a *Leucocytozoon* intensity rating of zero to five based on the presence and brightness of bands observed on the gels after PCR amplification.

Since these primers had never been used for parasite detection in common loons, PCR products from several individuals were sequenced to verify that the correct fragments were amplified and to ensure that the primers did not anneal to a section of the loon genome. The desired PCR products (from full 50μL reactions) were removed from the agarose gel with a scalpel and were extracted from the agarose medium using a Qiagen QIAQuick® Gel Extraction Kit. The clean PCR products were then used as templates in single-stranded sequencing reactions. Sequencing reactions were run for both strands of each product in 11 μl reactions using a sequencing buffer, the appropriate primer, and an ABI Prism® Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were cleaned using Sephadex columns and then dried. Dried samples were re-suspended in deionized formamide prior to loading onto an ABI Prism® 3100-*Avant* Genetic Analyzer.

Double stranded sequences were reconciled, aligned and proofread using BioEdit (Hall 1999). BLAST (Altschul et al. 1997) searches were performed for six sequences (see Appendix E for alignment) and were used to assess whether the desired amplifications were of the targeted haemosporidian species. I also used the BLAST results to find the hosts of the haemosporidian species most closely related to those sequenced.

Detectability and Repeatability

As PCR amplifications were being repeated for *Leucocytozoon* detection, it became apparent that parasite DNA concentration may affect detectability. To assess this possibility, serial dilutions of DNA were made from DNA extractions of five loons that reliably tested positive for *Leucocytozoon*, to concentrations of 1:20, 1 x 10^{-2}, 1 x 10^{-3}, and 1 x 10^{-4}. The diluted samples were used in the nested PCRs targeting *Leucocytozoon* to determine if parasite DNA concentration affects detectability and conversely, if the band brightness could be

associated with the intensity of the parasite infection. Furthermore, the 1 x 10^{-3} DNA dilutions from each of the same five loons that reliably tested positive for *Leucocytozoon* were amplified four times each to access the repeatability of detection at low DNA concentrations.

Statistical Analyses

To begin to examine the factors associated with parasite infection, binary logistic regression analyses were conducted using SPSS version 13.0 (2004) with the presence or absence of *Leucocytzoon* as the dependent variable. Individual analyses were conducted for both the conservative and the liberal interpretations of the PCR results, and this was performed separately for both adults and chicks. Blood mercury levels, the percent white blood cell buffy coat volume, the percent packed red blood cell volume, and the mass of the loons were considered as covariates. Sex of the loon was also considered as a covariate for the adult models, but could not be included in the chick models since chick sexes were unknown. Covariates were added to the models in a forward conditional fashion.

Two multiple linear regressions were performed to analyze *Leucocytozoon* intensities for adults and chicks. The intensity of infection (initially categorized zero through five based on the brightness of the gel electrophoresis band, and then averaged across all three amplification replicates for each sample) was considered the dependent variable and the covariates were the same as those used in the binary logistic regression analyses for *Leucocytozoon* presence. Covariates were added to the model in a stepwise fashion.

Differences in *Leucocytozoon* intensity between adults and chicks were examined with a Wilcoxon signed ranks test (nonparametric version of paired t-test with data paired to account for lake effect). A nonparametric test was used to explore the differences in intensity

between adults and chicks because average values were used when multiple adults or chicks were captured from the same lake. Correlation analyses were performed to determine if a relationship existed between adult and chick loon blood mercury levels and immune response (as measured by percent volumes of the white blood cell buffy coat). Multiple linear regressions were also performed to analyze the percent blood volume of the white blood cell buffy coat layer of loons. Percent blood volume was considered the dependent variable and the covariates were loon sex, mass, blood mercury level, presence or absence of *Leucocytozoon* with a strict interpretation of infection, presence or absence of *Leucocytozoon* with a liberal interpretation of infection, and *Leucocytozoon* infection intensity. Covariates were added to the model in a stepwise fashion.

RESULTS

Loon blood mercury levels ranged from 0.027 μg/g to 6.520 μg/g and white blood cell buffy coat layers and packed blood cell volumes ranged from 0.5 to 2.75 % cell volume and 29.0 to 53.75 % cell volume, respectively. Haemosporidians were successfully detected in the loon blood samples. Both sets of primers effectively amplified the targeted haemosporidians. Figure 2 shows the amplified PCR products from eight individuals after gel electrophoresis. Four of the individuals whose results are shown below reliably tested positive for *Leucocytozoon*, and the remaining four individuals were consistently positive for *Haemoproteus* and/or *Plasmodium*. Variation in gel electrophoresis band brightness of the amplified products was observed (Figure 2) which prompted the use of two interpretive approaches for determining whether an individual was infected by *Leucocytozoon*. When *Leucocytozoon* infection was interpreted in a conservative fashion, 57 loons (40.1%) tested positive for the presence of the hematozoan. When interpreted more liberally (when all faint bands were considered positive for *Leucocytozoon*), 93 loons (65.5%) tested positive for the blood parasite.

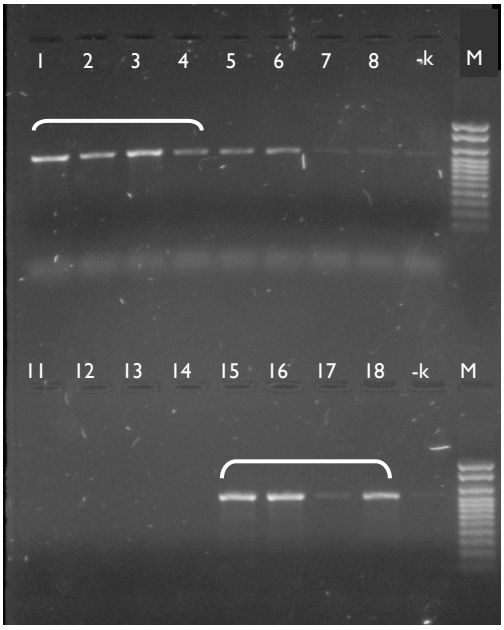

Figure 2. Gel electrophoresis image of the PCR products amplified from eight loons for haemosporidian detection. Lanes 1-8 show products amplified from the primers HaemFL and HaemR2L which targeted *Leucocytozoon*. Lanes 11-18 show products from HaemF and HaemR2, which targeted *Haemoproteus* and *Plasmodium*. Negative controls are denoted by –k and DNA markers by M. Parentheses indicate individuals known to be positive for the respective haemosporidians. Note the variation in band brightness.

Parasites from the genera *Leucocytozoon* and *Plasmodium* were successfully sequenced from five common loon blood samples—all *Leucocytozoon* amplification products had identical sequences and all *Plasmodium* amplification products had identical sequences. These two unique sequences were uploaded on GenBank (Table 8). These sequences were then used to search the nr (non-redundant) database using default parameters for BLAST searching (Altschul et al. 1997). BLAST searches were conducted in November 2006. The four sequences amplified with the primers HaemF and HaemR2 (putatively targeting *Plasmodium* spp. and *Haemoproteus* spp. mitochondrial genes) were most closely matched to a sequence of

a *Plasmodium* species isolated from a song thrush (*Turdus philomelos*) (Table 9). Sequences of *Plasmodium* spp. from a bluethroat (*Luscina svecica*), great tit (*Parus major*) and American robin (*Turdus migratorius*) were also close matches for the *Plasmodium* spp. sequenced from loons. It is notable that none of the four individuals sequenced showed parasite sequences similar to those of *Haemoproteus*. The two sequences amplified with the primers HaemFL and HaemR2L (putatively targeting *Leucocytozoon* spp.) were most similar to *Leucocytozoon* species found in a bluethroat and in a siskin (*Carduelis spinus*) (Table 9). The top four matches for both BLASTed sequences were dominated by *Leucocytozoon* sp. found in bluethroats. In fact, three of the four *Leucocytozoon* sequences most similar to those found in loons in this study were isolated from bluethroats.

Table 8. Haemosporidian DNA sequences obtained from common loon samples. Sequences denoted by * are the same as GenBank Accession No. EF077166 and those that are the same as Accession No. EF077167 are denoted by +.

Loon ID	Sex	Lake/Territory	Primers Used	GenBank Accession No.
98843861	M	North Bass	HaemFL, HaemR2L	EF077166
98843833	J	TFF-Murray's Landing	HaemFL, HaemR2L	*
98843861	M	North Bass	HaemF, HaemR2	EF077167
84803490	F	Tomahawk-Echo	HaemF, HeamR2	+
98843898	M	Buckskin	HaemF, HaemR2	+
98844416	M	Shishebogama	HaemF, HaemR2	+

Table 9. BLAST search results showing parasite genera and host matches.

Loon ID:	Target	BLASTed match	Host Species	e value of match
84803490	H/P	*Plasmodium*	*Turdus philomelos*	0.0 (identical)
98843861	H/P	*Plasmodium*	*Turdus philomelos*	0.0 (identical)
98843898	H/P	*Plasmodium*	*Turdus philomelos*	0.0 (identical)
98844416	H/P	*Plasmodium*	*Turdus philomelos*	0.0 (identical)
98843833	L	*Leucocytozoon*	*Luscinia svecica*	2e-97
98843861	L	*Leucocytozoon*	*Carduelis spinus*	0.0 (identical)

No binary logistic regression models involving any of the measured variables (blood mercury level, percent blood volume of white blood cell buffy coat layer, percent blood volume of packed red blood cells, and mass) significantly explained *Leucocytozoon* presence in loon

chicks. In adult loons, a significant model for the conservative interpretation of *Leucocytozoon* presence (Log-likelihood ratio Chi-square = 8.858, df = 1, p-value = 0.003) included loon blood mercury levels as the only covariate (Table 10). The percent cell volume of white blood cell buffy coats, the percent cell volume of packed red blood cells, and the mass and sex of the loons were not included in the model. The Nagelkerke R^2 was 0.147 which indicates that 14.7 % of the variation in *Leucocytozoon* presence was explained by the logistic model. The Wald statistic showed that the coefficient for loon blood mercury levels were significantly different from zero (Wald statistic = 5.489, p-value = 0.019) (Table 10). A graphical depiction of the relationship between loon blood mercury levels and the presence of *Leucocytozoon* in adult loons clarifies the association between blood mercury levels and *Leucocytozoon* infection (when interpreted conservatively) (Figure 3).

Table 10. Criteria of blood mercury levels in adult common loons as a significant factor in a reduced binary logistic regression model explaining conservative and liberal interpretations of *Leucocytozoon* presence determined by molecular detection.

	Conservative interpretation	Liberal interpretation
Log-Likelihood Ratio Chi-square	8.858	7.070
df	1	1
p-value	0.003	0.008
Nagelkerke R^2	0.147	0.126
Wald Statistic	5.489	4.069
p-value	0.019	0.044
Regression coefficient: Hg	0.930	1.094
Constant	-1.095	-0.410

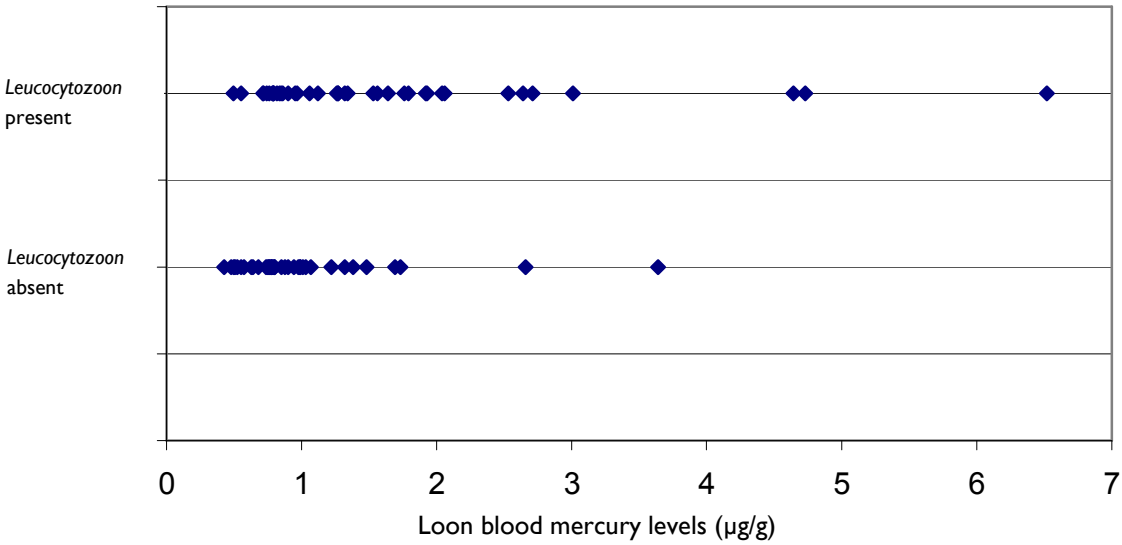

Figure 3. Loon blood mercury levels and *Leucocytozoon* infection status in adult common loons when *Leucocytzoon* presence was interpreted in a conservative fashion. Each point on the graph represents an individual adult loon.

When *Leucocytozoon* presence was interpreted liberally loon blood mercury was the only covariate in the significant logistic model (Log-likelihood ratio Chi-square = 7.070, df = 1, p-value = 0.008) (Table 10), which explained 12.6 % of the variation in *Leucocytozoon* presence (Nagelkerke $R^2 = 0.126$). The Wald Statistic of 4.069 indicated that the coefficient for loon blood mercury levels are significantly different than zero (p-value = 0.044) (Table 10). The relationship between loon blood mercury levels and *Leucocytozoon* status (liberal interpretation) appears even more distinct (Figure 4). All loons with blood mercury levels greater than approximately 2 µg/g tested positive for *Leucocytozoon*, while those individuals free of infection had blood mercury levels clustered at and below 1 µg/g (Figure 4).

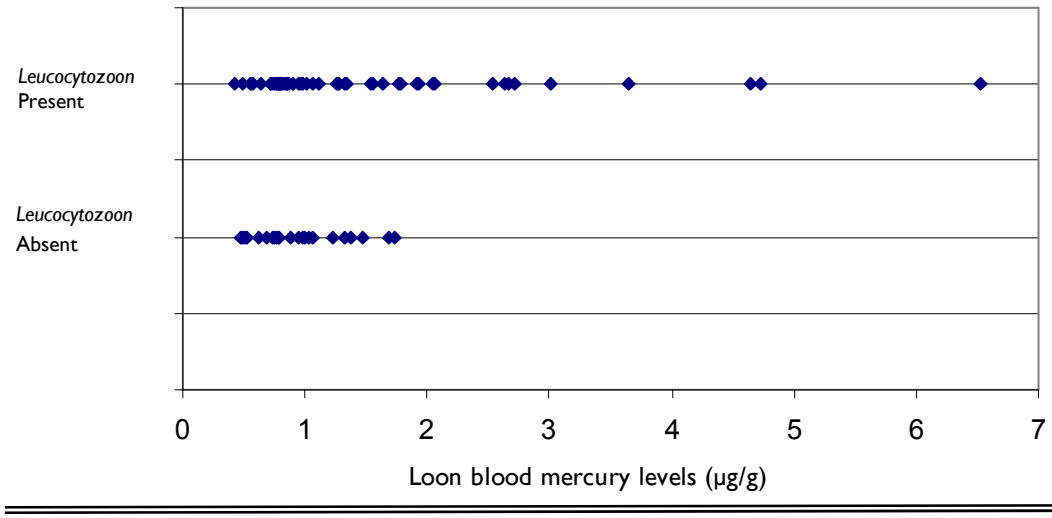

Figure 4. Loon blood mercury levels and *Leucocytozoon* infection status in adult common loons when *Leucocytozoon* presence was interpreted in a liberal fashion. Each point on the graph represents an individual adult loon.

Serial dilutions of DNA samples revealed the importance of DNA concentration for detectability of *Leucocytozoon* and for repeatability of results using molecular techniques for haemosporidian detection. The PCR amplifications of four of five loons used to investigate the importance of DNA concentration showed decreased detectability of *Leucocytozoon* with decreased DNA concentration (Figure 5). This indicated that gel electrophoresis band brightness (after PCR amplification of the haemosporidian) is associated with intensity of parasite infection. Repeat amplifications of the 1 x 10^{-3} DNA samples produced inconsistent results in four of the five loons tested (Figure 5), which indicated that repeatability is lost when parasite DNA concentrations are decreased. This loss of repeatability at decreased DNA concentrations helps explain why one of the loons used in the serial dilution assessment did not show decreased detectability with decreased DNA concentration (Figure 5). This decreased detectability and repeatability at low DNA concentrations also gives confidence in the liberal interpretation used to score infection.

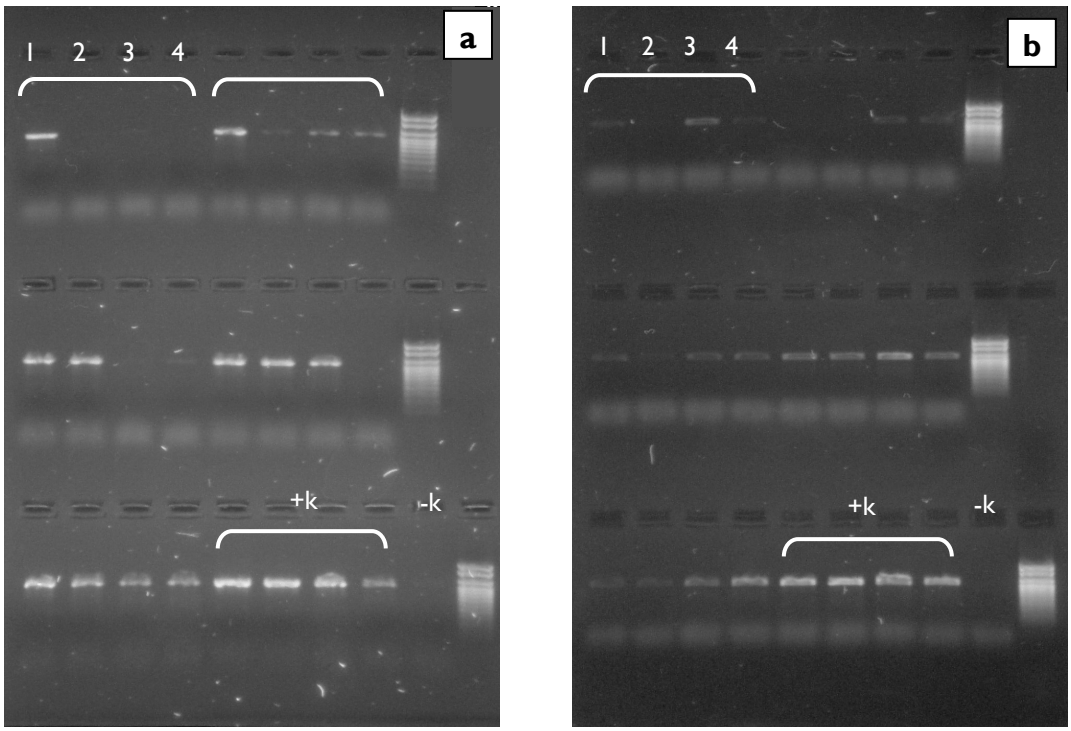

Figure 5. Gel electrophoresis images demonstrating the importance of DNA concentrations for *Leucocytozoon* detectability and detection repeatability; a) Serial DNA dilutions from five loons that consistently tested positive for *Leucocytozoon* and the positive control from a long-eared owl; individuals are marked with a parenthesis and span four lanes; lane 1 = 1:20 dilution, 2 = $1x10^{-2}$, 3 = $1x10^{-3}$, 4 = $1x10^{-4}$; b) Repeated trials of $1x10^{-3}$ DNA dilutions for the same five loons and the positive control; Each individual (as marked by the parenthesis) is represented by four lanes, all of which are $1x10^{-3}$ DNA dilutions of the individual. Positive and negative controls are represented by +k and –k respectively.

The intensity of *Leucocytozoon* infection in adult loons (measured by band brightness) was explained by a significant multiple linear regression model, however, a significant model was not found to explain the intensity of infection in loon chicks. The model that significantly explained the intensity of *Leucocytozoon* in adult loons included loon blood mercury as a predictor ($F = 6.723$, $df = 75$, p-value = 0.011). This regression model explained 8.3% of the variation in *Leucocytozoon* intensities in adult loons ($R^2 = 0.083$). The regression coefficient of blood mercury was 0.385 and the constant was 1.170. Regression coefficients of percent cell volume of white blood cell buffy coats, percent cell volume of packed red blood cells, and

the mass and sex of the loons were not significantly different from zero, despite the forward stepwise inclusion of covariates.

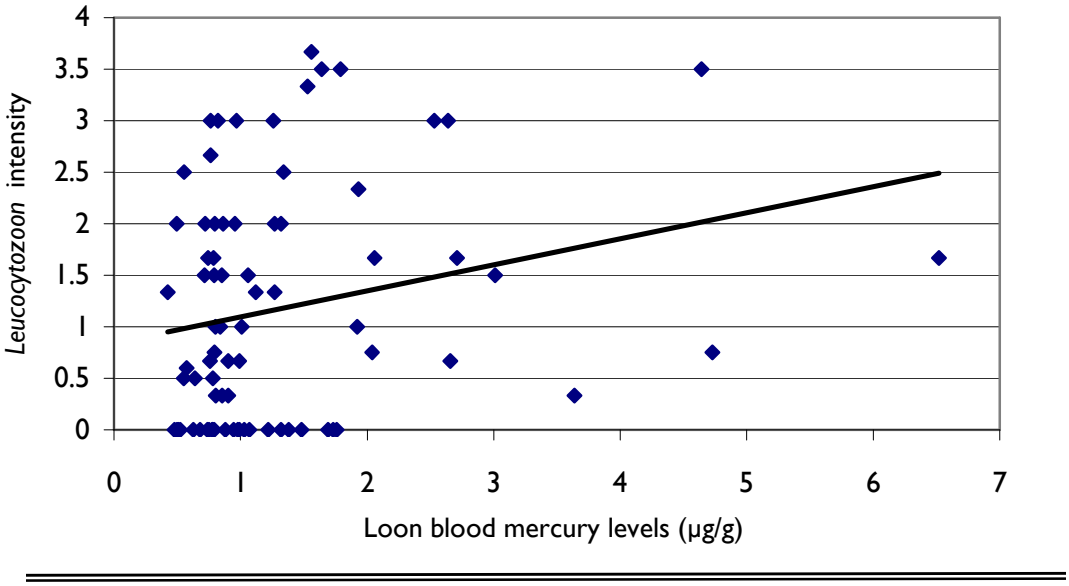

Figure 6. The relationship between blood mercury levels and *Leucocytozoon* intensity in adult common loons.

There was not a significant difference between *Leucocytozoon* intensities in adult loons and chicks (Wilcoxon Signed Ranks Z = -0.739, N = 46, p-value = 0.460). The mean *Leucocytozoon* intensity in adult loons for this analysis, measured on a scale of zero to five from gel electrophoresis band brightness after PCR amplification and then averaged for the three runs, and then averaged when multiple adults or chicks were captured on a lake, was 1.15, while the mean intensity of *Leucocytozoon* in loon chicks was 1.06. Loon blood mercury levels were not correlated with the percents blood volume of the white blood cell buffy coat layers of adults or chicks (Pearson's correlation coefficient = -0.154, p-value = 0.183 and Pearson's correlation coefficient = -0.097, p-value = 0.459, respectively), and no significant multiple linear regression model was found to explain the percent blood volume of the white blood cell buffy coat layer in adult loons or chicks.

DISCUSSION

Despite the observation that parasitic associations can dramatically modify host population dynamics, haemosporidians have been overlooked in common loon population studies. This is the first documentation of hematozoan presence in common loons. The genera *Leucocytozoon* and *Plasmodium* were successfully amplified and sequenced in loons. *Haemoproteus* may have also been present, but due to primer design, *Plasmodium* and *Haemoproteus* infections were indistinguishable after PCR amplification and gel electrophoresis alone. An increased sequencing effort might confirm the positive identification of *Haemoproteus* in the sampled common loon population since *Haemoproteus* is the largest and most commonly encountered genus of hematozoa (Bennett 1987, Wobeser 1997). According to compiled data presented by Valkiūnas (2005), *Haemoproteus* is the most frequently detected haemosporidian genus in holarctic birds. *Haemoproteus* spp. have been visually identified in 17.9% of birds sampled in the holarctic region while *Leucocytozoon* and *Plasmodium* have been visually observed in 16.2% and 2.9% of sampled birds respectively, (Valkiūnas 2005).

The prevalence of *Leucocytozoon* infection in the sampled loon population is comparable to prevalences found in other avian surveys. The prevalence of *Leucocytozoon* infection was 40.1% when interpreted in a conservative fashion. When interpreted more liberally, 65.5% of the sampled loons tested positive for the blood parasite. Hellgren (2005) screened bluethroats (*Luscinia svecica*) for *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* using molecular techniques and found an overall incidence of infection of 59.3% (although not reporting any difficulty in detection scoring). *Leucocytozoon* protists were present in 47.7% of the birds tested, while the prevalence of *Plasmodium* and *Haemoproteus* were 23.3% and 1.2%, respectively (Hellgren 2005).

Leucocytozoon spp. are reportedly more prevalent in birds of prey. Korpimaki et al. (1993) found *L. ziemanni*, prevalences of 97% and 94% in Tengmalm's owls in 1991 and 1992, respectively (Korpimaki et al. 1993). More than 75% of red-tailed hawk nestlings monitored by Smith et al. (1998) were infected with *Leucocytozoon* in 1993 and 1994. However, the effects of black fly infestation claimed the life of only one nestling during this time (Smith et al. 1998).

According to Freed and Cann (2006), the accuracy of haematozoon detection using PCRs can be affected by a number of factors including blood storage, DNA extraction protocols, primer usage, and personal lab techniques. These and other factors add to the complication of interpreting PCR results for haemosporidian detection. Furthermore, unless procedures are standardized, results from multiple molecular haemosporidian detection surveys should not be compared. Most significantly, storage buffers were criticized for having an effect on the accuracy of PCR results (Freed and Cann 2006). I did not add any type of buffer to the blood samples for storage during this study, and therefore have increased confidence in my results.

The importance of DNA concentration for the molecular detection of *Leucocytozoon* was demonstrated after conducting PCRs on serially diluted DNA samples. The detectability of *Leucocytozoon* decreased with decreased DNA concentrations, which supported my assumption that gel electrophoresis band brightness corresponded with the intensity of haemosporidian infection. The effect of DNA concentration on parasite detection is especially important when using molecular techniques for haemosporidian detection in avian blood. Birds have nucleated red blood cells (Campbell 1995) which increase the host to parasite DNA ratio after standard DNA extractions (Freed and Cann 2006). Since chronic

infections are often characterized by low-level parasitemias, it is often difficult or even unlikely to detect haemosporidians in birds with chronic infections when using molecular techniques (Freed and Cann 2006). Therefore, the liberal interpretation of *Leucocytozoon* infection used in my study may provide a more accurate account of *Leucocytozoon* prevalence than the conservative interpretation of results.

Using positive controls that are diluted to detect a minimum level of parasitemia may help distinguish individuals that are truly positive from false positives (Freed and Cann 2006). The loons that had faint gel electrophoresis bands present during my study would have been more easily classified as positive or negative for *Leucocytozoon* infection if diluted positive controls had been used. However, I attempted to solve this uncertainty by classifying *Leucocytozoon* presence both conservatively and liberally. Furthermore, the results of the repeat amplifications of the 1×10^{-3} DNA samples indicated that repeatability is lost when parasite DNA concentrations are decreased. This loss of repeatability at decreased DNA concentrations also complicated the interpretation of gel electrophoresis images.

The problem of inconsistent molecular detection of parasites could be explored and possibly resolved by the use of ocular methods for parasite detection. It would be beneficial to further investigate the accuracy of molecular detection of hematozoans in this study. This could be accomplished by comparing the prevalence and intensities of *Leucocytozoon* found in the current study using molecular techniques to those observed after visually examining blood smears made at the time of blood collection. Blood smears were made in the field for each loon included in this study, but unfortunately only a few slides were closely examined due to lacking confidence in the observer's haemosporidian identification skills. No haemosporidians were detected after this initial slide viewing.

Not surprisingly, BLAST searches performed for haemosporidian sequences from this study all resulted in closely related haemosporidians isolated from avian hosts. If avian haemosporidians have co-evolved with their hosts, I would expect the haemosporidians isolated from common loons to be most closely related to haemosporidians isolated from hosts that are genetically similar to loons. However, most molecular avian haemosporidian studies have been performed on passerines and the data set is incomplete. Therefore, the results of the BLAST searches did not necessarily support this hypothesis and did not provide any meaningful evolutionary insight on host-parasite relationships. The hosts of the haemosporidians that were the most similar to the haemosporidians isolated from loons were song thrushes, bluethroats, great tits, American robins, and siskins; all passerines. Similarly, the primers used for targeting the hematozoans may affect the accuracy of detection (Freed and Cann 2006). Therefore, it is not surprising that the *Leucocytozoon* spp. isolated and sequenced from the bluethroat and siskin were done so using the same techniques and primers that were used in this loon study (Hellgren et al. 2004).

The presence and intensity of *Leucocytozoon* in adult loons were significantly explained by loon blood mercury levels. My findings, linking mercury burdens to *Leucocytozoon* presence and intensities are similar to those of Sagerup et al. (2000) who found a positive relationship between intestinal parasites and organochlorine exposure in glaucous gulls (*Larus hyperboreus*). Although direct immunological data were not collected during the Sagerup et al. (2000) study, their findings support the idea that contaminants may adversely affect immune function, given the increased presence of parasites (Sagerup et al. 2000). However, the presence and intensity of *Leucocytozoon* in loon chicks were not significantly explained by blood mercury levels. Loon chicks typically hatch after peak black fly emergence (McIntyre

1988, Borrer et al. 1989, personal observation) and therefore have reduced exposure to the vectors of *Leucocytozoon* compared to adult loons. The decreased probability of becoming infected with *Leucocytozoon* helps explain why blood mercury levels do not have a significant effect on the presence and intensity of *Leucocytozoon* in loon chicks.

Although my study recognized blood mercury levels as significant predictors of *Leucocytozoon* presence and intensity in adult loons, blood mercury levels may not be the only factor determining *Leucocytozoon* presence or intensity. Since black flies transmit *Leucoytozoon* spp. it would be beneficial to further explore the attractiveness of individual lakes to black flies. While working in the field it became apparent that black fly numbers varied between individual lakes. Certain lake types or characteristics may be more attractive to black flies than others, which would increase the likelihood and intensity of *Leucocytozoon* infection. Therefore, lake or habitat characteristics should be further explored as they may co-vary with loon blood mercury levels in describing *Leucocytozoon* presence and intensity. Possible habitat descriptors to consider as factors influencing *Leucocytozoon* infection in loons could be lake type (in regard to drainage), lake size, lake depth, secchi depth, dominant shoreline vegetation, the proximity of streams to the lake, and specific characteristics of the closest streams.

Due to the difference in exposure time to black flies, I expected the intensities of *Leucocytozoon* to be significantly different between adult loons and chicks; however, this was not the case. It is possible that the accuracy of *Leucocytozoon* intensity was lost after averaging the gel electrophoresis band brightness scores for the three PCR runs for each individual. As mentioned earlier, detectability of *Leucocytozoon*, as well as repeatability of the molecular results, decreased with decreased DNA concentrations, ultimately producing inconsistent

results. All intensity scores were averaged for an individual; including those that were scored as zero when a gel electrophoresis band was not observed. Other factors, including the presence of inhibiting compounds, could also affect amplification, especially at low DNA concentrations. The complications and uncertainties of using gel electrophoresis band brightness to assess *Leucocytozoon* intensities could be avoided by also using blood smears to visually measure haemosporidian intensity and comparing the results of the two methods. It is possible that blood smear analysis could provide a more accurate assessment of *Leucocytozoon* intensities and that a significant difference may be found in *Leucocytozoon* intensities between adults and chicks if this method were implemented.

Laboratory studies have demonstrated that proper functioning of the immune system is sensitive to contaminants (Wong et al. 1992). Rocke and Samuel (1991) measured the immunologic effects of lead on mallards and found evidence that contaminant exposure reduces immunological cell numbers. However, in my study I did not find an association between loon immune function (measured as the white blood cell buffy coat layer) and blood mercury concentrations. A more accurate means of detecting immune function could be applied to further examine this relationship. Total and differential white blood cell counts could be performed using the blood smears that were made in the field immediately following blood collection. A reduction of specific white blood cell types may indicate a decline in the functions of those cells, while a response to infection would result in an increase of white blood cells (Grasman 2002). The ratio of heterophils to lymphocytes is also commonly used to study the effects of contaminants on birds since these two cell types make up the majority of avian circulating leukocytes (Grasman 2002).

Due to the limitations of capturing wild loons, only those pairs that had successfully reproduced (and their chicks) were included in this study. It could be argued that only the healthiest, fittest birds within a population are capable of defending a territory, successfully finding a mate, reproducing, and ultimately rearing young. To better understand the magnitude of haemosporidian infection and the relationship of mercury exposure and immune function, it would be beneficial to compare the prevalence of *Leucocytozoon* among breeding and non-breeding adult common loons and between successful nesters and unsuccessful nesters. The introduction of a capture method to include non-breeders or unsuccessful nesters would also allow researchers to test the hypothesis that haemosporidian infection reduces reproductive output in common loons.

CONCLUSION

This study provided insight on the complex relationships of common loons, black flies and haemosporidians. Not only did I confirm the specificity of *Simulium annulus* to common loons, but I also identified the importance of chemical cues for black fly host attraction. Possibly more striking was the discovery that common loons serve as intermediate hosts to at least two haemosporidians, *Leucocytozoon* and *Plasmodium*. The increased certainty of the attraction of black flies to loons and the awareness that *Leucocytozoon* is present in common loon populations will hopefully encourage exploration of the effects of these parasites. As vectors of *Leucocytozoon*, black flies should be included when investigating loon reproduction and ecology and the influences of haematozoans.

Although loons have served as an indicator species to assess the effects of atmospheric mercury deposition, this study is the first to report a relationship between haemosporidian presence and/or intensity and loon blood mercury levels. The prevalence and intensity of *Leucocytozoon* infection in the sampled loons were significantly explained by blood mercury levels, yet no correlation was found between blood mercury levels and the percent blood volumes of white blood cell buffy coat layers. This suggests the possibility that blood mercury levels are not the only factor explaining *Leucocytozoon* presence and intensity in loons. Together, these findings may help explain, in part, the variability in loon nest success and promote further studies on the intricate relationship of loons, black flies, and haemosporidians.

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APPENDICES

APPENDIX A

Appendex A. Lakes where black flies were presented with loon, Canada goose, mallard, and loon with wings decoys from 07 May 2005 through 09 May 2005 (One-wing presentations).

Lake Name	County	WIBC	Township	Range	Section	Size (acres)	Max. Depth (feet)
Shannon	Vilas	1016800	40N	08E	12	35	30
Little Bass	Vilas	998400	40N	08E	15	27	35
Honeysuckle	Vilas	991000	40N	09E	10	33	13
Deadman	Vilas	980000	40N	08E	02	14	26
West Horsehead	Oneida	1522900	37N	07E	16	145	26
Alva (Little)	Oneida	968100	37N	07E	10	201	36
Wharton	Vilas	1178200	41N	08E	25	32	19
Sunset	Vilas	1020900	40N	09E	26	213	30
Unnamed (Leo)	Vilas	1154700	39N	10E	15	18	26
Spring	Vilas	1019200	39N	10E	11	36	18

APPENDIX B

Appendex B. Lakes where black flies were presented with loon, Canada goose, mallard, loon with wings, Canada goose with wings, and mallard with wings decoys from 27 May 2005 through 02 June 2005 (3-wing presentations).

Lake Name	**County**	**WIBC**	**Township**	**Range**	**Section**	**Size (acres)**	**Max. Depth (feet)**
Sunset	Vilas	1020900	40N	09E	26	213	30
Spring	Vilas	1019200	39N	10E	11	36	18
Unnamed (Leo)	Vilas	1154700	39N	10E	15	18	26
Alva (Little)	Oneida	968100	37N	07E	10	201	36
West Horsehead	Oneida	1522900	37N	07E	16	145	26
Little Bass	Vilas	998400	40N	08E	15	27	35
Shannon	Vilas	1016800	40N	08E	12	35	30
Deadman	Vilas	980000	40N	08E	02	14	20
Wharton	Vilas	1178200	41N	08E	25	32	19
Quartz	Forest	591000	40N	12E	14	47	13
McKinley	Forest	692700	40N	12E	22	48	19
White Deer	Forest	1606600	40N	12E	34	62	45
Harriet	Forest	586300	40N	12E	33	23	15
Honeysuckle	Vilas	991000	40N	09E	10	33	13
Frank	Vilas	985900	41N	08E	18	141	24
Razorback	Vilas	1013800	41N	08E	20	362	35
Lone Tree	Vilas	1000400	41N	08E	09	121	16
Ginty	Oneida	1571300	37N	11E	30	131	12
Haymeadow Flowage	Forest	1606200	39N	12E	07	241	9
McDonald	Vilas	1003700	40N	09E	32	39	30
Wolf	Forest	1179100	39N	12E	32	33	14
Bailey	Forest	969300	39N	12E	30	80	10

APPENDIX C

Appendix C. Lakes where black flies were presented with loon, Canada goose, mallard, loon with wings, Canada goose with wings, mallard with wings, loon with goose wings, and goose with loon wing decoys from 03 June 2005 through 04 June 2005 (Wing swap presentations).

Lake Name	**County**	**WIBC**	**Township**	**Range**	**Section**	**Size (acres)**	**Max. Depth (feet)**
Rudolph	Vilas	2954300	42N	06E	26	39	24
Jag	Vilas	1855900	42N	06E	27	158	14
Otto Mielke	Vilas	1004500	40N	07E	28	23	34
Bug	Vilas	974300	40N	07E	17	19	11

APPENDIX D

Appendix D. Lakes or territories where loon blood samples were collected for haemosporidian detection, blood mercury analysis, and immune function analysis.

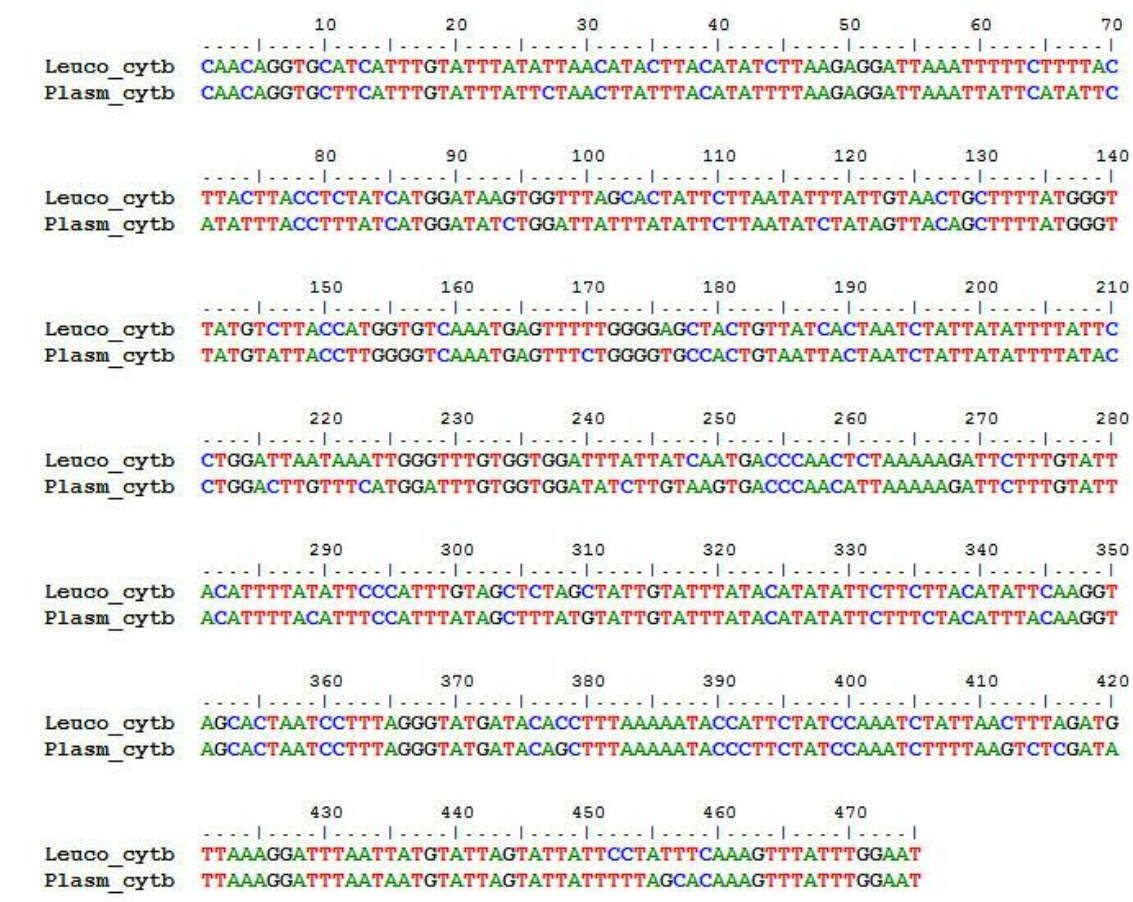
Lake Name	County	WIBC	Township	Range	Section
Anvil South	Vilas	968800	40N	11E	13
Baker	Vilas	1626400	42N	09E	35
Bluegill	Vilas	2336400	43N	07E	19
Buckskin	Vilas	2272600	39N	04E	11
Butternut North	Forest	692400	40N	12E	27
Carrol	Oneida	1544800	39N	07E	05
Stella	Vilas	1594000	40N	08E	02
Fisher	Iron	2307300	44N	04E	34
Forest	Vilas	2762200	42N	09E	04
Found	Vilas	1593800	40N	08E	14
Frank	Vilas	985900	41N	08E	18
Ginty	Oneida	1571300	37N	11E	30
Haymeadow Flowage	Forest	1606200	39N	12E	07
White Sand	Vilas	2321100	41N	05E	26
Helen	Vilas	2964400	43N	09E	30
Honeysuckle	Vilas	991000	40N	09E	10
Hunter	Vilas	991700	41N	09E	25
Imogene	Vilas	586800	41N	12E	31
Indian	Vilas	2764400	42N	09E	17
Lone Tree	Vilas	1000400	41N	08E	09
Lynx Inner Island	Vilas	2954500	43N	07E	18
Mabel	Vilas	2337600	43N	07E	21
Madeline	Oneida	1544700	39N	07E	08
Merril	Vilas	2768200	43N	09E	34
Mitten	Vilas	1867200	40N	04E	15
Moon	Vilas	1005800	40N	08E	25
Nineweb	Vilas	1870600	42N	09E	19
North Bass	Iron	1868900	43N	04E	23
Pokegama	Vilas	2320800	40N	05E	07
Razorback	Vilas	1013800	41N	08E	20
Ross Allen	Vilas	1878100	40N	04E	11
Unnamed (SE of Stearns)	Vilas	----------	40N	05E	01

Appendix D (cont'd)

Shannon	Vilas	1016800	40N	08E	12
Shishebogama	Oneida/ Vilas	1539600	39N	05E	01
Spring	Vilas	1019200	39N	10E	11
Star Boat Landing	Vilas	1593100	41N	08E	22
Stearns	Vilas	2323600	40N	05E	01
TFF Blaire	Iron	2294900	42N	02E	34
TFF Murray's Landing	Iron	2294900	42N	02E	34
TFF Springstead West	Iron	2294900	42N	02E	34
TFF Swimmer's	Iron	2294900	42N	02E	34
Tomahawk Dead Island	Oneida	1542700	39N	06E	24
Tomahawk Echo	Oneida	1542700	39N	06E	24
Trude West	Iron	2295200	42N	02E	13
Upper Sugarbush	Vilas	2318000	41N	05E	16
Van Vliet	Vilas	2956800	43N	06E	16
Verna	Vilas	1540300	40N	06E	10
Wildcat	Vilas	2336800	43N	07E	33

APPENDIX E

Appendix E. Two haemosporidian sequences amplified from common loon blood via nested PCR assays and deposited on GenBank (Accession numbers EF077166 and EF077167 for *Leucocytozoon* and *Plasmodium*, respectively).



APPENDIX F

Appendix F: IACUC approval form

Northern Michigan University

Office of the Provost and Vice President
for Academic Affairs
1401 Presque Isle Avenue
Marquette, MI 49855-5308
906-227-2920
FAX: 906-227-2928
Web site: www.nmu.edu

July 7, 2004

TO: Dr. Alec Lindsay
Biology Department

FROM: Fred Joyal, Provost & Vice President
Academic Affairs

RE: **Application to use Vertebrate Animals**
Application # IACUC 013
Approval Period: May 1, 2005 – April 30, 2010

The Institutional Animal Care and Use Committee has approved your application to use vertebrate animals in research entitled "CAREER: Coupling Data from Molecular Genetic Markers and Field Observations from Common Loon Populations."

If you have any questions, please contact me.

ljh

cc: Biology Department

PS: Alec, members of the IACUC commented that your proposal was extremely thorough and very well written.