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Haemosporidian parasite infections in grouse and ptarmigan: Prevalence and genetic diversity of blood parasites in resident Alaskan birds

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ABSTRACT

Projections related to future climate warming indicate the potential for an increase in the distribution and prevalence of blood parasites in northern regions. However, baseline data are lacking for resident avian host species in Alaska. Grouse and ptarmigan occupy a diverse range of habitat types throughout the northern hemisphere and are among the most well-known and important native game birds in North America. Information regarding the prevalence and diversity of haemosporidian parasites in tetraonid species is limited, with few recent studies and an almost complete lack of genetic data. To better understand the genetic diversity of haemosporidian parasites in Alaskan tetraonids and to determine current patterns of geographic range and host specificity, we used molecular methods to screen 459 tissue samples collected from grouse and ptarmigan species across multiple regions of Alaska for infection by *Leucocytozoon, Haemoproteus,* and *Plasmodium* blood parasites. Infections were detected in 342 individuals, with overall apparent prevalence of 53% for *Leucocytozoon*, 21% for *Haemoproteus*, and 9% for *Plasmodium*. Parasite prevalence varied by region, with different patterns observed between species groups (grouse versus ptarmigan). *Leucocytozoon* was more common in ptarmigan, whereas *Haemoproteus* was more common in grouse. We detected *Plasmodium* infections in grouse only. Analysis of haemosporidian mitochondrial DNA cytochrome *b* sequences revealed 23 unique parasite haplotypes, several of which were identical to lineages previously detected in other avian hosts. Phylogenetic analysis showed close relationships between haplotypes from our study and those identified in Alaskan waterfowl for *Haemoproteus* and *Plasmodium* parasites. In contrast, *Leucocytozoon* lineages were structured strongly by host family. Our results provide some of the first genetic data for haemosporidians in grouse and ptarmigan species, and provide an initial baseline on the prevalence and diversity of blood parasites in a group of northern host species.

1. Introduction

Avian haemosporidians belong to a diverse group of vector-borne, protozoan blood parasites that infect a broad range of avian species throughout North America and around the world (Valkiūnas, 2005). Representative species from the genera *Haemoproteus, Plasmodium*, and *Leucocytozoon* have been detected on every continent except Antarctica (Beadell et al., 2009), infecting both wild and domestic birds. Infections by these parasites can vary among taxa and across habitat types (Greiner et al., 1975; White et al., 1978; Durrant et al., 2006), and prevalence in avian host populations may be determined, at least in part, by exposure to viable dipteran vectors (Bennett et al., 1992). Other factors such as age, host suitability, and a multitude of environmental variables may also be important determinants of parasite infection (Martínez-Abraín et al., 2004).

Rapid warming in northern regions has raised concerns about environmentally-driven changes in the distribution and prevalence of disease in birds and other wildlife (Van Hemert et al., 2014). Many vector-borne parasites, including *Plasmodium* and other haemosporidians, are temperature-sensitive and are projected to expand in response to climate warming in the Arctic (Loiseau et al.,

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2012; Altizer et al., 2013). Heavy infection by haemosporidian parasites can cause mild to severe pathogenic effects in various avian species (Ots and Hõrak, 1998; Palinauskas et al., 2008) and exposure of naïve hosts to blood parasites may result in dramatic population-level consequences, such as have occurred among native birds in Hawaii (LaPointe et al., 2012). Recent studies have proposed an increase in haemosporidian infections, particularly *Plasmodium*, among Alaskan bird populations as a result of shifts in temperature, vegetation cover, and vector populations (Loiseau et al., 2012; Oakgrove et al., 2014). However, limited baseline data currently exist for many avian species, particularly year-round residents, making it difficult to assess potential risks and evaluate future patterns of change.

Species of birds from the order Galliformes (Family Phasianidae) have adapted to the cold climates of arctic and sub-arctic regions and inhabit a broad range of habitats across the northern hemisphere (Aldrich, 1963; Braun and Willers, 1967). Given their status as year-round residents, grouse and ptarmigan (Subfamily Tetraonidae) provide excellent model species for studying the prevalence and diversity of locally-transmitted blood parasites in Alaska as well as the relationships between climate variables and parasite infection.

Four species of grouse and three species of ptarmigan occur throughout Alaska's many biomes and each plays an important role as a prey species for predators and game species for subsistence and recreational hunters. All species of tetraonids in Alaska are year-round residents and many of their populations overlap geographically, but occupy vastly different habitats. Spruce Grouse (*Falcipennis canadensis*) and Ruffed Grouse (*Bonasa umbellus*) are typically associated with boreal and mixed deciduous forests, respectively, whereas Rock Ptarmigan (*Lagopus muta*), Willow Ptarmigan (*Lagopus lagopus*), and White-tailed Ptarmigan (*Lagopus leucura*) inhabit more sparsely vegetated arctic and subarctic tundra and subalpine environments (Aldrich, 1963). Sooty Grouse (*Dendrogapus fuligninosus)* populations in Alaska are found in the temperate coastal rainforests of southeastern Alaska and Sharp-tailed Grouse (*Tympanuchus phasianellus*) tend to prefer open grassy habitat with shrub-like brush in interior Alaska (Aldrich, 1963; Dublin and Taras, 2005). Grouse and ptarmigan populations are secondary prey species to many predators and undergo cyclical population fluctuations (Holmstad et al., 2005). Some researchers have hypothesized that parasitic infection may contribute to these population fluctuations, either as a direct cause of mortality or via a secondary effect such as decreasing mobility or delayed flushing response (Fallis, 1945; Holmstad et al., 2006; Skirnisson et al., 2012).

Early microscopic studies from North America determined that most tetraonid species were infected by at least one genus of haemosporidian parasite, with many individuals showing diverse infections of multiple parasite genera at moderate to high prevalence (Fallis, 1945; Stabler et al., 1967a,b; Bennett and Inder, 1972; Mahrt, 1981; Forbes et al., 1994). Although research on haemosporidian infections in Alaskan tetraonids has been extremely limited, two historical studies focused on grouse and ptarmigan species within specific regions of the state. Stabler et al. (1967a) sampled Rock Ptarmigan in interior Alaska using microscopy and found 88% prevalence of *Leucocytozoon* parasites and no evidence of *Haemoproteus* or *Plasmodium* infection. Additionally, Stabler et al. (1967b) collected blood smears from Spruce Grouse in the southwestern and southcentral regions of Alaska, where they detected 80% and 60% prevalence of *Leucocytozoon* and *Haemoproteus,* respectively, and no *Plasmodium*. No contemporary studies of blood parasite prevalence in Alaskan tetraonids have been conducted. Molecular-based detection methods have become more widely used in the past decade, allowing for detailed description of parasite genetic diversity, including identification of unique parasite lineages in each genus (Bensch et al., 2000; Hellgren et al., 2004 Waldenström et al., 2004). Since the advent of these methods, however, there has been little research on the genetic diversity of haemosporidians in grouse and ptarmigan worldwide (Sato et al., 2007), and none in Alaska.

To address the lack of current knowledge about blood parasite infection in grouse and ptarmigan species in Alaska and to provide information on the genetic diversity of parasite lineages that infect this group of resident birds, our objectives for this study were to: (1) to estimate the prevalence of haemosporidian infections (genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*) in grouse and ptarmigan species throughout the state of Alaska; (2) obtain genetic information to estimate the diversity of identified *Haemoproteus, Plasmodium*, and *Leucoctyzoon* parasite lineages; and (3) evaluate the relationship between prevalence of blood parasite infection and geographic region, age, and species of the host. Our results will provide some of the first genetic data on haemosporidian lineages infecting tetraonid species and add new information about blood parasite prevalence and distribution in resident avian hosts from northern regions.

2. Materials and methods

2.1. Sample collection

Wings from hunter-harvested grouse and ptarmigan were voluntarily submitted to the Alaska Department of Fish and Game through their grouse and ptarmigan wing collection program from October 2012 to November 2014. We received 459 samples from four species of grouse (Ruffed Grouse, Spruce Grouse, Sharp-tailed Grouse, and Sooty Grouse) and three species of ptarmigan (Willow Ptarmigan, Rock Ptarmigan, and White-tailed Ptarmigan) from multiple geographic regions of Alaska (Fig. 1). All collections occurred between August and April, primarily during the non-breeding season. Wings were stored individually at −20° C until tissue extraction and subsequent genetic analysis.

2.2. Haemosporidian detection

We extracted DNA from approximately 500 mg of muscle tissue of frozen wings using a DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. In order to confirm the viability of each DNA extraction, a 695 base pair (bp) fragment of the avian mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene was amplified following protocols described by Kerr et al. (2007). Each sample was considered viable if bands were visible when PCR product was visualized on 0.8% agarose gels stained with Gel Red Nucleic Acid Gel Stain (Biotium, Hayward, CA). Each extracted DNA sample that proved viable via our COI positive control ($n = 459$) was subsequently screened for the presence of *Haemoproteus, Plasmodium,* and *Leucocytozoon* parasites using a nested-PCR protocol described by Hellgren et al. (2004), which allows for simultaneous identification of infections from all three parasite genera. All samples were analyzed twice and visualized on 0.8% agarose gels as described previously. A 479 bp fragment of haemosporidian mtDNA cytochrome *b* gene was bi-directionally sequenced for all positive samples using identical primers from PCR. Positive PCR products were purified with ExoSapIT (USB Inc., Cleveland, OH) according to the manufacturer's protocol, and sequencing was conducted using Big Dye Terminator v3.1 mix (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were cleaned up and edited using Sequencher 5.0.1 software (Gene Codes Corp., Ann Arbor, MI). Raw

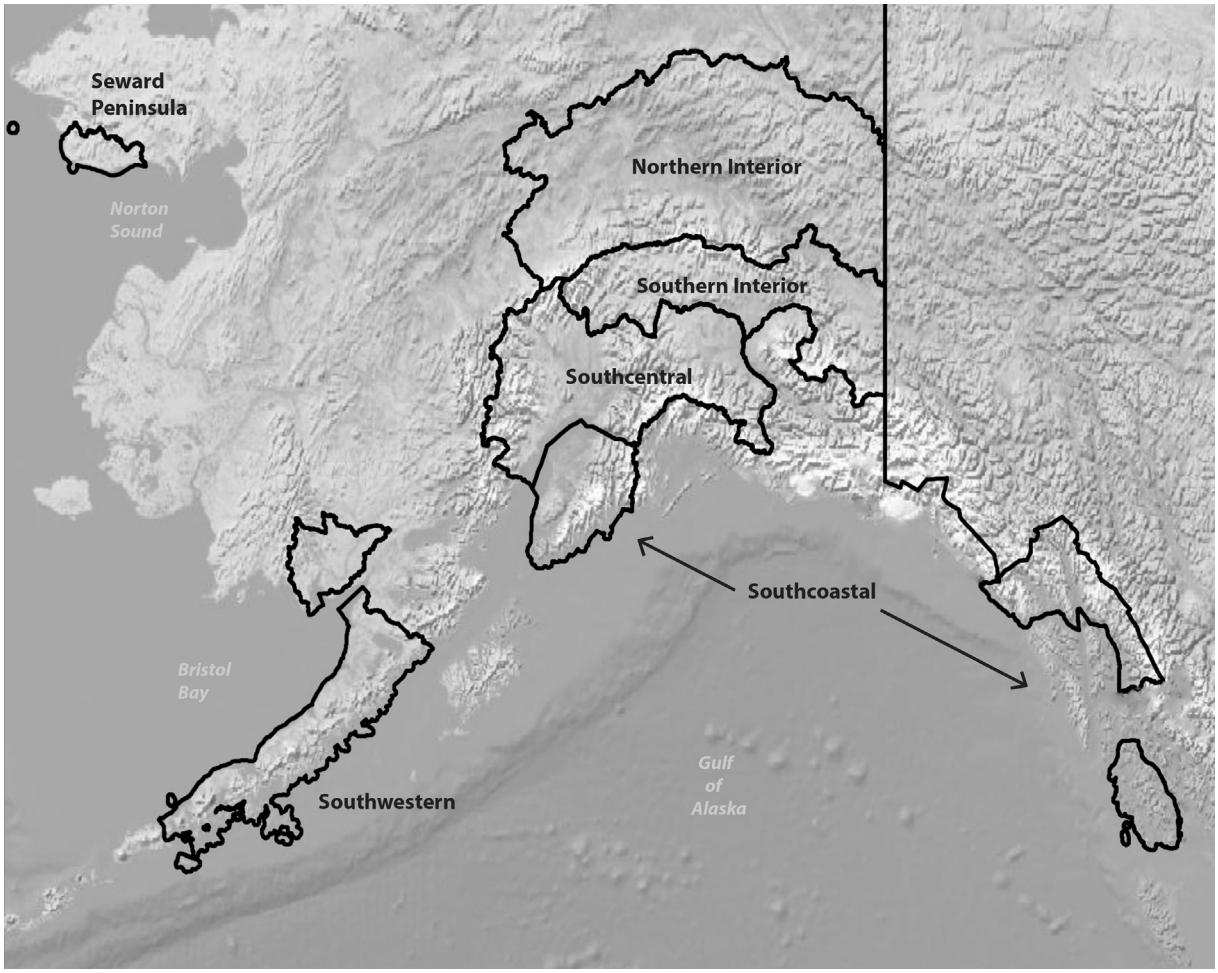

Fig. 1. Map of Alaskan sampling regions assembled from multiple game management units and sub-units. Regions were grouped for analysis of haemosporidian prevalence as follows: southcoastal (Kenai Peninsula and southeastern Alaska; GMUs 1C, 1D, 2, 7, 15A, 15B, and 15C), southcentral (Anchorage area and Matanuska-Susitna Valley; GMUs 13A, 13D, 14A, 14C, 16A, and 16B), southwestern (Bristol Bay, Alaska Peninsula, and eastern Aleutian islands; 9D, 9E, and 17C), southern interior (south side of Alaska Range; GMUs 12, 13B, and 13E), northern interior (north side of Alaska Range; GMUs 20A-20E and 25C), and Seward Peninsula (GMU 22C).

sequences were assigned to one of three parasite genera (*Haemoproteus, Plasmodium,* or *Leucocytozoon*) using the nucleotide BLAST function available on the National Center for Biotechnology Information (NCBI) website. Samples that did not produce double-stranded DNA sequence during at least one of the PCR reactions or failed to be assigned by BLAST were considered negative.

2.3. Haplotype diversity and phylogenetic analysis

To determine the relative frequency and genetic distances for all unique haemosporidian haplotypes in grouse and ptarmigan samples, we created a median-joining minimum spanning network using Network 4.6.1 (Fig. 2; Bandelt et al., 1999). Additionally, we compared haemosporidian haplotypes from our samples to previously identified lineages available (as of 27 November, 2015) on the GenBank and MalAvi databases (Bensch et al., 2009) using their basic local alignment search tool function (BLAST) to determine if they matched lineages reported in other avian hosts.

In order to provide insight into patterns of host or geographic conservatism of these parasites, phylogenetic analyses were performed on sequence data obtained from our samples and reference sequences collected from GenBank and MalAvi public databases (Bensch et al., 2009). We included a single representative of each unique haemosporidian mtDNA haplotype identified in our study ($n = 23$; GenBank accession numbers **KU257614-KU257636**), as well as representative haplotypes from previous studies conducted on haemosporidians in Alaskan avian species (Ramey et al., 2012; Oakgrove et al., 2014; Smith et al., 2015), lineages isolated from black fly vectors in Colorado (Murdock et al., 2015), and representative lineages that have been previously identified in birds from the Phasianidae family obtained from the MalAvi database, to examine geographic or host-specific patterns. All sequences were aligned and cropped to a final length of 413 bp to match the length of the shortest lineage in our phylogeny; any lineages shorter than this or those that contained ambiguous bases from co-infection by multiple haemosporidian lineages were excluded from further analysis. We constructed phylogenies using MrBayes 3.2.5 (Ronquist et al., 2012) using a general time reversible model and a gamma distribution for among-site variation (GTR + G). Four heated chains were used and the first 25,000 sampled trees were discarded as burn-in. Our analysis was replicated multiple times to ensure consistent results with each analysis running for a minimum of 3.0×10^6 generations or until the split frequencies of the posterior probability standard deviation stabilized below 0.01. Trees were sampled every 1000 generations and rooted with mammalian *Plasmodium* (*Plasmodium yoelii:* GenBank accession number AY099051), based on a phylogeny described in Perkins and Schall (2002).

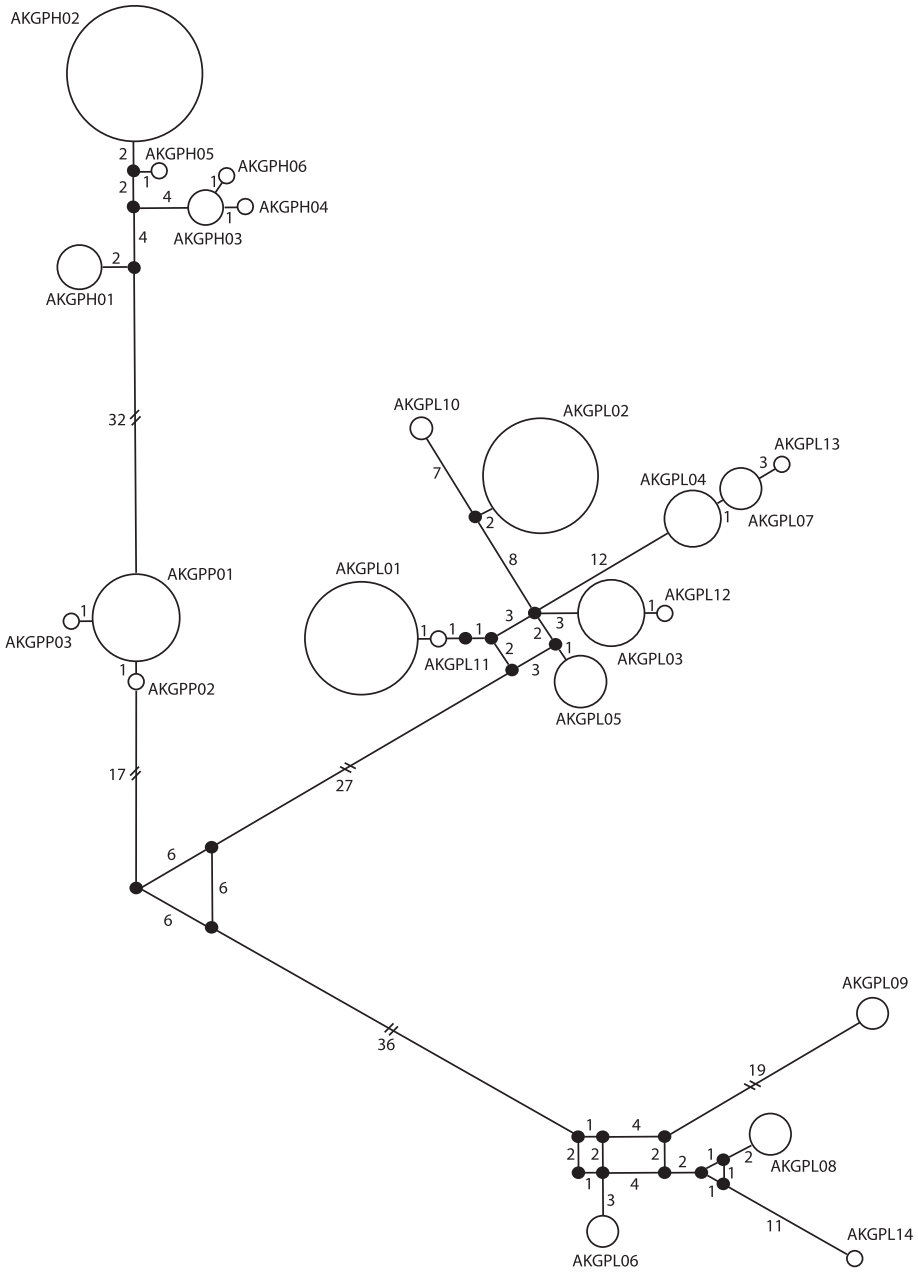

Fig. 2. Minimum spanning network for haemosporidian mtDNA cytochrome *b* haplotypes isolated from Alaskan grouse and ptarmigan species. Dark circles represent un-sampled nodes. All circles are proportional to the frequency at which the haplotypes were detected. Lines between nodes are drawn to scale based on the number of nucleotide mutations unless otherwise indicated by hash marks.

2.4. Statistical analysis

To investigate geographic patterns of haemosporidian prevalence, we identified six geographic regions for analysis by grouping together multiple game management units and sub-units based on proximity and separation by major geographical features (Fig. 1): southcoastal (Kenai Peninsula and southeastern Alaska; GMUs 1C, 1D, 2, 7, 15A, 15B, and 15C), southcentral (Anchorage area and the Matanuska-Susitna Valley; GMUs 13A, 13D, 14A, 14C, 16A, and 16B), southwestern (Bristol Bay, Alaska Peninsula, and eastern Aleutian islands; 9D, 9E, and 17C), southern interior (south side of Alaska Range; GMUs 12, 13B, and 13E), northern interior (north side of Alaska Range; GMUs 20A-20E and 25C), and Seward Peninsula (GMU 22C).

We used generalized linear models (Proc GENMOD with binomial distribution of errors and logit link function; SAS Institute, version 9.4) to investigate the relationship between parasite infection status (dependent, binary variable) and the following explanatory variables associated with capture of each bird: region, age (adult [>1 year of age] versus juvenile [<1 year of age]), and species group (grouse versus ptarmigan). We also included interaction terms between region and species group and between age and species group as we hypothesized that prevalence in each species group could vary geographically and by age. However, we did not expect age-related patterns of infection to vary by region so this term was not included. We also did not include sex in our models because sex was not known for many of the samples. For *Plasmodium*, because no infections were detected in ptarmigan,

Table 1
Results from wing tissue samples from Alaskan grouse and ptarmigan species screened for infection by *Leucocytozoon* (Leuc), *Haemoproteus* (Haem), and *Plasmodium* (Plas) blood parasites.

Species	Samples tested (*n*)	Leuc positive	Haem positive	Plas positive
Ruffed grouse (*Bonasa umbellus*)	29	5	0	6
Sharp-tailed grouse (*Tympanuchus phasianellus*)	46	29	4	8
Sooty grouse (*Dendrogapus fuligninosus*)	5	2	1	0
Spruce grouse (*Falcipennis canadensis*)	143	30	80	25
Rock ptarmigan (*Lagopus muta*)	12	10	0	0
Willow ptarmigan (*Lagopus lagopus*)	176	135	5	0
White-tailed ptarmigan (*Lagopus leucura*)	40	25	3	0
Unknown	8	5	1	0
Total	**459**	**241 (53%)**	**94 (21%)**	**39 (9%)**

species group was not included and only region and age were considered in our models.

We evaluated the relative support for models in each candidate set using Akaike's Information Criterion corrected for sample size (AICc) (Burnham and Anderson, 2002). Within each candidate model set, we then calculated the Akaike weight (w) for each model, which is the relative likelihood of the model, given the data and the models being considered (Burnham and Anderson, 2002).

In each analysis, we used an all-subsets modeling approach, in which the full model included all additive combinations of the independent variables and interactions; all possible subsets of that model were included in the candidate model set. We present least-squares means for *Leucocytozoon* and *Haemoproteus* prevalence by species group and region, calculated from best-supported generalized linear models. For *Plasmodium*, we report least-squares means for age and region from single-factor models including grouse only.

3. Results

3.1. Haemosporidian detection and prevalence

Of the 459 grouse and ptarmigan wings screened, 342 individual samples were identified as positive for at least one genus of haemosporidian parasite, with an overall apparent prevalence of 74.5% (±2.1%). From these 342 infected individuals, we detected a total of 374 individual haemosporidian infections consisting of *Leucocytozoon* ($n = 241$), *Haemoproteus* ($n = 94$), and *Plasmodium* ($n = 39$; Table 1). The majority of individuals had single infections (90.6% ± 1.6%; $n = 310$), with a smaller number having co-infections of *Leucocytozoon* and *Haemoproteus* (5.3% ± 1.2%; $n = 18$) or *Leucocytozoon* and *Plasmodium* (4.1% ± 1.1%; $n = 14$). We did not detect any mixed infections of *Haemoproteus* and *Plasmodium*. Apparent prevalence of *Leucocytozoon* (52.5% ± 2.3%) was the highest among the three genera, followed by *Haemoproteus* (20.5% ± 1.9%), and *Plasmodium* (8.5% ± 1.3%). *Leucocytozoon* infections were represented across all seven species of bird hosts sampled, ranging from 21% to 83% prevalence depending upon species (Table 1). *Haemoproteus* parasites were detected in all species except Ruffed Grouse and Rock Ptarmigan. *Plasmodium* parasites were detected in only three of the grouse species sampled (Spruce, Ruffed, and Sharp-tailed Grouse), and no ptarmigan species (Table 1). Our duplicate screening of each sample confirmed 423 consistent screening results for *Leucocytozoon* and 431 for *Haemoproteus/Plasmodium,* the latter of which were not differentiated since they are identified with a single molecular primer set. This concordance of 92.2% and 93.9% for *Leucocytozoon* and *Haemoproteus/Plasmodium*, respectively, is comparable with detection probabilities reported by other studies using the same molecular protocol (Ramey et al., 2015).

Among ptarmigan, apparent prevalence of *Leucocytozoon* infection was relatively high in all 6 regions sampled, ranging from 47.1% (±12.1%; $n = 17$) in southwestern Alaska to 96.3% (±3.6%; $n = 27$) on the Seward Peninsula (Table 2). Prevalence of *Leucocytozoon* was generally lower in grouse, ranging from 2.0% (±1.9%; $n = 51$) in southwestern Alaska to 47.1% (±12.1%; n = 17) in the southern interior (Table 2). Similar geographic patterns were observed for *Leucocytozoon* across the two species groups, with the exception of the Seward Peninsula, for which we had no grouse samples. Apparent prevalence of *Haemoproteus* was highest among grouse in southwestern Alaska (98.0% ± 1.9%) and lowest in the southern interior (5.9% ± 5.7%; Table 2). The highest prevalence of *Haemoproteus* infection among ptarmigan also occurred in southwestern Alaska but was only 11.8% (±7.8%; Table 2). Among grouse, *Plasmodium* prevalence was highest in southcentral Alaska

Table 2
Estimated mean prevalence of *Leucocytozoon, Haemoproteus,* and *Plasmodium* in Alaskan grouse and ptarmigan species calculated from the best supported models (Table 4) in our analysis using the least squares method. Estimates are separated by region and species group (grouse vs. ptarmigan) except for *Plasmodium*, which was only detected in grouse. Age was a significant factor for *Plasmodium* only.

Region	Group	Mean (%)	SE of mean (%)
Leucocytozoon (by region and species group)			
Southcentral	Grouse	31.4	6.5
Southcentral	Ptarmigan	52.8	8.3
Southern interior	Grouse	47.1	12.1
Southern interior	Ptarmigan	93.7	2.7
Southcoastal	Grouse	31.3	8.2
Southcoastal	Ptarmigan	62.3	6.2
Northern interior	Grouse	44.3	5.9
Northern interior	Ptarmigan	83.3	10.8
Seward Peninsula	Ptarmigan	96.3	3.6
Southwestern	Grouse	2.0	1.9
Southwestern	Ptarmigan	47.1	12.1
Haemoproteus (by region and species group)			
Southcentral	Grouse	31.4	6.5
Southcentral	Ptarmigan	2.8	2.7
Southern interior	Grouse	5.9	5.7
Southern interior	Ptarmigan	3.8	2.2
Southcoastal	Grouse	37.5	8.6
Southcoastal	Ptarmigan	4.9	2.8
Northern interior	Grouse	8.6	3.3
Northern interior	Ptarmigan	0	–
Seward Peninsula	Ptarmigan	0	–
Southwestern	Grouse	98.0	1.9
Southwestern	Ptarmigan	11.8	7.8
Plasmodium (by region)			
Southcentral	Grouse	39.2	6.8
Southern interior	Grouse	17.7	9.2
Southcoastal	Grouse	12.5	5.8
Northern interior	Grouse	16.7	4.4
Southwestern	Grouse	0	–
Plasmodium (by age)			
All regions	Juvenile	10.9	2.6
All regions	Adult	29.7	5.3

Table 3
Haemosporidian mtDNA cytochrome *b* haplotypes detected in wing tissue from Alaskan grouse and ptarmigan species and results of comparison to previously identified haemosporidian lineages on the MalAvi and GenBank databases. Fifth letter in the haplotype name indicates parasite genus (H = *Haemoproteus,* L = *Leucocytozoon,* and P = *Plasmodium*). Four letter codes for host common names are: SPGR = Spruce Grouse, STGR = Sharp-tailed Grouse, SOGR = Sooty Grouse, RUGR = Ruffed Grouse, WIPT = Willow Ptarmigan, ROPT = Rock Ptarmigan, and WTPT = White-tailed Ptarmigan.

Haplotype name	Haplotype frequency	Host species	Lineage (MalAvi/GenBank)	Identity score (MalAvi/GenBank)
AKGPH01	8	SPGR, STGR	KU181/TANGAL01	98%/98%
AKGPH02	75	SPGR, STGR, WIPT, WTPT	KU181/MODO1	99%/98%
AKGPH03	5	SPGR, STGR, WIPT, WTPT	TUSW07/CYGNUS01	100%/100%
AKGPH04	1	WIPT	TUSW07/CYGNUS01	99%/99%
AKGPH05	1	SOGR	KU181/NINOX02	99%/99%
AKGPH06	1	SPGR	TUSW07/CYGNUS01	99%/99%
AKGPL01	52	WIPT, ROPT, WTPT	HGR1/HGR1	99%/99%
AKGPL02	54	STGR, WIPT, ROPT, WTPT	HGR1/HGR1	97%/97%
AKGPL03	18	STGR, WIPT, ROPT	HGR1/HGR1	99%/99%
AKGPL04	13	SPGR	CAP1/CAP1	99%/99%
AKGPL05	11	STGR	COLBF_22/HGR1	99%/99%
AKGPL06	4	WIPT, ROPT, WTPT	COLBF_24/LAMUT01	99%/100%
AKGPL07	7	SPGR	CAP1/CAP1	99%/99%
AKGPL08	7	SPGR	COLBF_24/GALLUS08	98%/87%
AKGPL09	4	RUGR	COLBF_24/GALLUS08	94%/87%
AKGPL10	2	SOGR	HGR1/HGR1	96%/96%
AKGPL11	1	WIPT	HGR1/HGR1	99%/99%
AKGPL12	1	STGR	HGR1/HGR1	99%/99%
AKGPL13	1	STGR	CAP1/CAP1	99%/99%
AKGPL14	1	WIPT	COLBF_24/GALLUS08	95%/88%
AKGPP01	31	SPGR, STGR, RUGR	BT7/BT7	100%/100%
AKGPP02	1	STGR	H390/BT7	99%/99%
AKGPP03	1	SPGR	H390/BT7	99%/99%

(39.2% ± 6.8%) and was higher among adults (26.6% ± 2.8%) than juveniles (13.4% ± 2.4%; Table 2). We detected no *Plasmodium* infections in ptarmigan.

3.2. Haplotype diversity

Analysis of haemosporidian mtDNA sequences revealed a total of 23 unique haplotypes (Fig. 2; Table 3). Haplotypes were identified as *Haemoproteus* ($n = 6$), *Plasmodium* ($n = 3$), and *Leucocytozoon* ($n = 14$). Haplotype frequency varied from detection in only a single individual to detection in 75 separate individuals of multiple species (Table 3). A total of 15 haplotypes were isolated to a single host species, some of which included multiple individuals sampled across large geographic distances. The remaining eight haplotypes were detected in multiple host species, with four of these detected in both grouse and ptarmigan hosts and the remaining four restricted to one species group (Table 3).

Comparison to haemosporidian lineages on MalAvi and GenBank public databases showed that three of our haplotypes were identical to lineages previously identified in other avian hosts (Table 3). Haplotype "AKGPH03," which was isolated from four different tetraonid species (Table 3), was identical to lineage CYGNUS01, and *Leucocytozoon* haplotype "AKGPH06," detected only in ptarmigan, was identical to lineage LAMUT01. The most common *Plasmodium* haplotype, "AKGPP01" detected in our study, which was isolated from three of the four species of grouse sampled, was identical to the BT7 lineage found on the MalAvi database.

3.3. Phylogenetic analysis

The 92 separate haemosporidian lineages in our Bayesian tree clustered by genus as expected, with high posterior probability support for all of the larger clades (Fig. 3). *Leucocytozoon* haplotypes detected in our study were more diverse than those of *Haemoproteus* or *Plasmodium*. Analysis with reference sequences grouped most of our *Leucocytozoon* haplotypes by host family, with one clade of several haplotypes deeply diverged from the others and grouped with sequences obtained from black flies and one lineage previously identified in Phasianid species. *Haemoproteus* and *Plasmodium* haplotypes from our study showed less host conservatism than those of *Leucocytozoon*. Our phylogeny provided support for *Haemoproteus* clades comprised of our sequences and those detected in waterfowl from Alaska. Additionally, all of our *Plasmodium* haplotypes were grouped in a single clade with lineages isolated from Tundra Swans and Mallards in Alaska (Fig. 3).

3.4. Statistical analysis

The model receiving the most support ($w = 0.32$) for explaining variation in *Leucocytozoon* prevalence included region, species group, and a region*species group interaction term (Table 4), although models that also included age ($\Delta AIC_c = 0.22$; $w = 0.28$) and an age*species group interaction ($\Delta AIC_c = 0.13$; $w = 0.30$) had almost equal support (Table 4).

For *Haemoproteus*, the best-supported model ($w = 0.62$) included region, species group, and a region*species group interaction. There was moderate support ($w = 0.23$) for a model that also included age (Table 4).

Plasmodium was not detected in any ptarmigan samples (making prevalence inestimable) so we modeled the likelihood of plasmodium infection in grouse only. The best-supported model for explaining *Plasmodium* prevalence in grouse included region and age ($w = 0.99$). There was very little support for the other models (Table 4).

4. Discussion

4.1. Haemosporidian prevalence

Our study constitutes the most comprehensive survey of haemosporidian parasites in Alaskan tetraoninds to date, and has revealed a relatively high overall prevalence of *Haemoproteus, Plasmodium,* and *Leucocytozoon* (74.5% ± 2.1%). Prevalence of all three genera varied by region, with differing patterns of infection between grouse and ptarmigan. We also report the first detection of

Fig. 3. Bayesian phylogenetic tree of haemosporidian mtDNA cytochrome *b* haplotypes isolated from Alaskan grouse and ptarmigan species. Node tips are labeled with abbreviation for parasite genus (Haem = *Haemoproteus*, Leuc = *Leucocytozoon*, and Plas = *Plasmodium*), followed by the lineage name, GenBank accession number for each lineage, and avian (Phas = Phasianidae, Anat = Anatiade, Turd = Turdidae, Paru = Parulidae, Scol = Scolopacidae, Embe = Emberizidae, and Frin = Fringillidae) or invertebrate (Simu = Simuliidae) host family. All haplotypes identified in this study are highlighted in red and asterisks following tip labels indicate a lineage that was isolated from Alaskan bird hosts. Numbers on branches indicate posterior probabilities from our analysis. All reference sequences were obtained from the National Center for Biotechnology Information website or the MalAvi database. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Results of generalized linear models testing for geographical and ecological factors associated with haemosporidian infection among grouse and ptarmigan sampled in Alaska. ΔAIC describes the best fit of the data; K is the number of model parameters, and w is Akaike model weight. Region = geographic region sampled (see Fig. 1); SppGroup = species group (grouse versus ptarmigan); Age = age at time of sampling (adult versus hatch-year).

Model	K	AICc	ΔAIC	Model likelihood	*w*
Leucocytozoon					
Region SppGroup Region*SppGroup	20	466.06	0	1	0.32
Region SppGroup Age Region*SppGroup Age*SppGroup	34	466.19	0.13	0.94	0.30
Region SppGroup Age Region*SppGroup	22	466.28	0.22	0.89	0.28
Region SppGroup	9	469.64	3.58	0.17	0.05
Region SppGroup Age	11	469.65	3.59	0.17	0.05
Region Age	9	511.58	45.52	1.30E-10	4.12E-11
Region	7	513.42	47.36	5.20E-11	1.64E-11
SppGroup Age	5	528.43	62.37	2.86E-14	9.04E-15
SppGroup	3	532.22	66.16	4.30E-15	1.36E-15
Age	3	612.51	146.45	1.59E-32	4.99E-33
Null (intercept only)	1	628.14	162.08	6.38E-36	2.01E-36
Haemoproteus					
Region SppGroup Region*SppGroup	20	257.68	0	1	0.62
Region SppGroup Age Region*SppGroup	22	259.63	1.95	0.38	0.23
Region SppGroup Age Region*SppGroup Age*SppGroup	26	261.12	3.44	0.18	0.11
Region SppGroup	9	264.2	6.52	0.04	0.02
Region SppGroup Age	11	266.26	8.58	0.014	0.01
Region	7	330.7	73.02	1.39E-16	8.66E-17
Region Age	9	332.25	74.57	6.42E-17	3.99E-17
SppGroup Age	5	373.69	116.01	6.44E-26	4.00E-26
SppGroup	3	374.66	116.98	3.96E-26	2.46E-26
Age	3	454.5	196.82	1.82E-43	1.13E-43
Null (intercept only)	1	464.64	206.96	1.15E-45	7.13E-46
Plasmodium (grouse only)					
Region Age	8	172.85	0	1	0.99
Region	6	183.42	10.57	0.01	0.01
Age	3	195.28	22.43	1.35E-05	1.34E-05
Null (intercept only)	1	204.88	32.03	1.11E-07	1.10E-07

Plasmodium infections in grouse species from Alaska. Surprisingly, we detected no *Plasmodium* infections in ptarmigan, even across the relatively broad geographic area sampled.

Apparent prevalence of *Leucocytozoon* was high across most regions and host species, particularly among ptarmigan (Table 2). Prevalence ranged from 47% to 96% in ptarmigan and, with the exception of southwestern Alaska, exceeded 30% in grouse. These results suggest that tetraonid populations across Alaska are exposed to relatively high concentrations of suitable vectors (Table 2). Consistently higher *Leucocytozoon* prevalence in ptarmigan compared to grouse in each geographic region is interesting given the respective habitat selection of these birds. Ptarmigan, particularly Rock and White-tailed ptarmigan, typically inhabit alpine environments in the regions from which we received samples, and grouse tend to occur in more lowland regions closer to streams and boreal forests (Aldrich, 1963; Dublin and Taras, 2005).

Leucocytozoon parasites are transmitted to vertebrate hosts exclusively by hematophagus black flies (Simuliidae), except for *Leucocytozoon caulleryi*, which is transmitted by biting midges (*Culicoides*) and is specific to domestic chickens (*Gallus gallus*) (Valkiūnas, 2005). Simuliids are typically found in the highest density near moving water, where they undergo their larval stages attached to rocks (Borror and Delong, 1954). *Leucocytozoon lovati,* a parasite specific to tetraoninds and the only species previously described in ptarmigan, is spread by Simuliid species that are known to undergo larval stages in smaller, slow moving streams or the outflows of impounded water bodies, such as beaver ponds (Currie, 1997). Further investigation into parasite-vector relationships for *Leucocytozoon* in Alaska is needed to determine the underlying factors responsible for differences in prevalence between grouse and ptarmigan.

In contrast to the pattern observed with *Leucocytozoon*, prevalence of *Haemoproteus* was generally higher in grouse than in ptarmigan. Overall, infection with *Haemoproteus* parasites was less common than with *Leucocytozoon* in most species and regions. One notable exception was the remarkably high prevalence of *Haemoproteus* (98.0%) in grouse from the southwestern region, where *Leucocytozoon* infection in this species group was relatively rare (2.0%; Table 2). Stabler et al. (1967a) also detected substantially higher prevalence of *Haemoproteus* parasites in Spruce Grouse sampled from Lake Aleknagik, which is near where the majority of our southwestern samples originated, than from Spruce Grouse collected on the Kenai Peninsula of Alaska. Additionally, Tundra Swans (*Cygnus columbianus*) sampled in the Bristol Bay Lowlands had a higher prevalence of *Haemoproteus* than swans sampled in other regions of Alaska (Ramey et al., 2012).

Haemoproteus parasites are primarily transmitted by biting midges of the genus *Culicoides*, although a small number of these parasites use hippoboscid flies (Hippoboscidae) as vectors (Valkiūnas, 2005). *Culicoides* require semi-aquatic or very moist habitats in order to reproduce, and often form large swarms near the area where they are hatched, but can also travel great distances through both wing- and wind-propelled dispersal (Borkent, 2004). The similarity of our results to previous studies suggests increased opportunities for transmission of *Haemoproteus* in southwestern Alaska, particularly around Bristol Bay. It is possible that local habitat and environmental factors in this region support larger quantities of biting midges, leading to increased transmission of *Haemoproteus* infections. Additionally, habitat associations of grouse with lowland areas and boreal forest habitats may help to explain the notably higher prevalence of *Haemoproteus* in grouse than ptarmigan across all regions.

Plasmodium infections were restricted to grouse species, with not a single infection detected in any of the three species of ptarmigan. The majority of these infections occurred in Spruce Grouse from the southcentral region, which is largely dominated by boreal

forest habitat. This habitat is suitable for species of Culicidae, the family of mosquitoes that transmit *Plasmodium,* and may support relatively high densities of potential vectors in this area. *Plasmodium* is also the most temperature-sensitive of the three genera of haemosporidian parasites we screened for in this study (Valkiūnas, 2005), and the lowland areas of the southcentral region have summertime temperatures that are conducive to completion of the parasite's life cycle (Wilkinson et al., 2016). We detected *Plasmodium* in a smaller proportion of grouse samples from other regions, including the northern interior, which is similar in latitude to the most northerly detections of locally-acquired *Plasmodium* infections reported in passerines (Oakgrove et al., 2014; Wilkinson et al., 2016, Table 2). Age was also an important factor in determining prevalence of *Plasmodium* in grouse, with adults infected at a higher frequency than juveniles (Table 2). A similar pattern has been described in other studies (Wood et al., 2007; Cosgrove et al., 2008; Wilkinson et al., 2016) and may be explained in part by the longer temporal period to which adults are exposed to potential vectors (Valkiūnas, 2005).

The apparent absence of *Plasmodium* in ptarmigan is notable, especially given the broad geographic area sampled, which included regions where *Plasmodium* has been detected in a diversity of other avian hosts (Loiseau et al., 2012; Reeves et al., 2015; Wilkinson et al., 2016). Willow Ptarmigan are commonly found in areas of dense vegetation in lowland areas and valley bottoms (Hannon et al., 1998), where temperature and wind conditions are conducive to high vector density. Additionally, although the alpine environments typical of White-tailed and Rock Ptarmigan may have a lower abundance of mosquitoes than forest or shrub habitats, mosquitoes do occur even at higher elevations (M. Smith, personal observation). The complete absence of *Plasmodium* in our Alaskan ptarmigan samples suggests that factors such as host behavior, local habitat selection, or other host life history traits are important determinants of infection status. Interestingly, to our knowledge, no other studies of blood parasites in ptarmigan have detected *Plasmodium* infections, including in Japan (Hagihara et al., 2004), Norway (Holmstad and Skorping, 1998), Iceland (Skirnisson et al., 2012), and Canada (Mahrt, 1981).

It is unclear whether the higher contemporary prevalence of *Plasmodium* we observed among Spruce Grouse in southcentral Alaska represents a true increase or if differences in detection methods (PCR in our study versus microscopy in Stabler et al. (1967a)) or sampling areas may be responsible. Other studies, dating back more than 70 years, have detected *Plasmodium* in grouse from more southerly locations in Canada, including British Columbia and Ontario, (e.g., Fallis, 1945; Forbes et al., 1994). The potential for an increase in *Plasmodium* prevalence associated with climate warming has been suggested in other studies of blood parasites in Alaska and warrants additional research (Loiseau et al., 2012; Wilkinson et al., 2016). Molecular analysis of historical blood samples from tetraonids across a larger geographic area, if available, would provide greater inference about potential long-term changes in haemosporidian prevalence. It should also be noted that our collection of samples during the non-breeding season may reflect a lower apparent prevalence than samples collected during summer, when vectors are active, especially for *Plasmodium,* for which parasitemia drops significantly during the chronic infection phase, making detection difficult (Valkiūnas, 2005).

Similar to other studies of haemosporidians in Alaska, co-infections with *Leucocytozoon* and *Haemoproteus* were the most common among grouse and ptarmigan, with fewer co-infections of *Leucocytozoon* and *Plasmodium*. However, we detected a lower prevalence of co-infection compared to other Alaskan species (Oakgrove et al., 2014; Ramey et al., 2015; Reeves et al., 2015). While we did not detect any *Haemoproteus/Plasmodium* co-infections, this finding isn't surprising as it is rare to detect these type of co-infections using molecular methods. *Haemoproteus* infections typically produce much higher parasitemia in avian hosts than *Plasmodium* (Valkiūnas, 2005), making it difficult to detect *Plasmodium* in a co-infected individual. Future work involving microscopic examination of blood smears should be conducted to determine dynamics of co-infections between *Haemoproteus* and *Plasmodium* parasites.

4.2. Genetic diversity

Comparison of haemosporidian haplotypes identified in our study revealed differing levels of host specificity for *Haemoproteus, Plasmodium,* and *Leucocytozoon* parasites. *Leucocytozoon* lineages in our phylogeny were grouped largely by host family. The majority of our haplotypes clustered into a single, monophyletic group with other lineages isolated from Phasianid hosts and one representative lineage from black flies (Fig. 3). The remaining *Leucocytozoon* haplotypes clustered into another, highly diverged clade containing haplotype AKGPL06, which was identical to previously identified lineage LAMUT01. LAMUT01, one of the only *Leucocytozoon* lineages isolated from ptarmigan species, was detected in Japanese Rock Ptarmigan (*Lagopus mutua japonicas*) and identified as *Leucocytozoon lovati* (Sato et al., 2007). Although there are four species of *Leucocytozoon* known to infect Phasianid hosts, the only species that has been previously identified in ptarmigan is *L. lovati*. Previous studies have observed this species in ptarmigan populations around the world, typically at relatively high prevalence (Stabler et al., 1967a; Mahrt, 1981; Murata et al., 2007). The high diversity of *Leucocytozoon* haplotypes from our samples (Fig. 2), combined with the level of divergence between the two clades in our phylogeny (Fig. 3), suggests that multiple *Leucocytozoon* species are infecting Alaskan tetraonids. Cryptic speciation of *Leucocytozoon* species has been suggested by several other studies (Sehgal et al., 2006; Reeves et al., 2015), although the lack of genetic analyses of *Leucocytozoon* species in tetraonids may explain why only one species has previously been reported in ptarmigan. Microscopic examination of blood smears from ptarmigan would be beneficial to provide identification of parasite morphospecies.

Haemoproteus haplotypes clustered into two, tightly grouped clades consisting of either tetraonid or Alaskan waterfowl lineages (Fig. 3). Lineage AKGPH03, detected in four tetraonid species (Table 3), was identical to CYGNUS01. CYGNUS01 has been detected in Anseriform hosts, including Tundra Swans (*Cygnus columbianus*) in Minnesota and Alaska (Ricklefs and Fallon, 2002), Northern Pintails (*Anas acuta*) in California (Ramey et al., 2012), and Cinnamon Teal (*Anas cyanoptera*) in South America (Smith and Ramey, 2015). This lineage had previously only been documented in waterfowl, and is associated with *Haemoproteus nettionis*, which is thought to be specific to Anseriform hosts. Smith and Ramey (2015) showed that *Haemoproteus* parasites in waterfowl were relatively host specific, even intercontinentally. However, the fact that the *Haemoproteus* lineages we detected in tetraonids clustered so tightly with those from waterfowl and the identification of identical lineages in the two families suggests more complex host-parasite relationships than previously thought. Although the identification of identical parasite lineages in both waterfowl and tetraonids is not necessarily indicative of infection by the same parasite species, this novel finding provides incentive for further study of *Haemoproteus* parasites in Alaska. The addition of microscopic methods would allow for morphological identification of these parasites to definitively determine if diverse hosts are being parasitized by the same *Haemoproteus* species.

Our phylogeny grouped all *Plasmodium* haplotypes from our study, as well as *Plasmodium* lineages from Alaskan waterfowl, into

a single, monophyletic clade. Additionally, haplotype "AKGPP01" had a 100% identity match to lineage BT7 (also described as P43 in GenBank). This *Plasmodium* lineage displays generalist behavior and has been documented in 37 bird species to date, spanning 11 families and four orders (Bensch et al., 2009). In Alaska, BT7 has been reported in waterfowl (Reeves et al., 2015), Black-capped Chickadees (*Poecile atricapillus;* Wilkinson et al., 2016), and other passerines (Oakgrove et al., 2014). *Plasmodium* species have historically been known to display generalist behavior in avian hosts (Bensch et al., 2000; Waldenström et al., 2002; Szymanski and Lovette, 2005). The fact that *Plasmodium* haplotypes from our study were more genetically diverged from other lineages isolated from Phasianidae hosts than those from diverse Alaskan bird species supports this trend of generalist behavior in *Plasmodium* parasites.

4.3. Conclusions and future work

Results from our study of haemosporidian parasites in Alaskan tetraonids revealed variation in prevalence of *Haemoproteus, Plasmodium,* and *Leucocytozoon* by species and geographic region across Alaska. The absence of *Plasmodium* infection in ptarmigan species and the markedly higher prevalence of *Leucocytozoon* in ptarmigan and *Haemoproteus* in grouse were notable findings. Future work involving finer-scale sampling would allow for better resolution of regional and local environmental factors that may influence parasite prevalence. Additionally, study of parasite-vector relationships for haemosporidians in Alaska is needed to understand the mechanisms responsible for apparent differences in haemosporidian infections in grouse and ptarmigan throughout the state.

Phylogenetic assessment of haemosporidian haplotypes showed a diverse array of parasite lineages infecting tetraonid species, with apparent generalist behavior by *Haemoproteus* and *Plasmodium* parasites, and host conservatism in *Leucocytozoon* lineages. Several haplotypes in our study were identical to haemosporidian lineages previously detected in other host families, including waterfowl and passerines. The most essential step for future work involving diversity of haemosporidians in Alaskan tetraonids would be the addition of microscopic examination of blood smears to molecular and phylogenetic analysis. Doing so would allow researchers to match parasite morphospecies to genetic lineages and determine the level of host sharing or specificity among these blood parasites.

Results from this study provide important baseline information for future research examining potential effects of climate warming on haemosporidian infections in avian species from Alaska and other northern regions. Given the status of grouse and ptarmigan as year-round residents, long-term monitoring of *Haemoproteus, Plasmodium,* and *Leucocytozoon* infections in Alaskan tetraonids will allow for assessment of local changes in parasite prevalence and distribution. Additionally, the genetic data provided here offer some of the first information on haemosporidian lineages in tetraonid species and are useful for phylogenetic comparison in grouse and ptarmigan worldwide.

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