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Diversity, distribution, and methodological considerations of haemosporidian infections among Galliformes in Alaska

Faith De Amaral [a,*], Robert E. Wilson [b,c], Sarah A. Sonsthagen [d], Ravinder Sehgal [a]

[a] San Francisco State University, San Francisco, CA, 94132, United States
[b] Nebraska State Museum, University of Nebraska-Lincoln, Lincoln, NE, 68588, United States
[c] School of Natural Resources, University of Nebraska-Lincoln, Lincoln, NE, 68583, United States
[d] U.S. Geological Survey, Nebraska Cooperative Fish and Wildlife Research Unit, University of Nebraska-Lincoln, School of Natural Resources, Lincoln, NE, 68583, United States

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ABSTRACT

Using samples spanning 10-degrees of latitude in Alaska, we provide the first comparative assessment of avian haemosporidia distribution of Arctic Alaska with subarctic host populations for four species of grouse and three species of ptarmigan (Galliformes). We found a high overall prevalence for at least one haemospordian genus (88%; N = 351/400), with spruce grouse (*Canachites canadensis*) showing the highest prevalence (100%; N = 54/54). *Haemoproteus* and *Plasmodium* lineages were only observed within grouse, while *Leucocytozoon* species were found within both grouse and ptarmigan. Further, different *Leucocytozoon* lineages were obtained from blood and tissue samples from the same individual, potentially due to the differential timing and duration of blood and tissue stages. Using different primer sets, we were able to identify different *Leucocytozoon* lineages within 55% (N = 44/80) of sequenced individuals, thereby detecting coinfections that may have otherwise gone undetected. The commonly used *Haemoproteus/Plasmodium* primers amplified *Leucocytozoon* for 90% (N = 103/115) of the products sequenced, highlighting the potential value of alternate primers to identify intra-genus coinfections and the importance of obtaining sequence information rather than relying solely on PCR amplification to assess parasite diversity. Overall, this dataset provides baseline information on parasite lineage distributions to assess the range expansion associated with climate change into Arctic regions and underscores methodological considerations for future studies.

1. Introduction

Avian haemosporidia are globally ubiquitous blood-borne parasites, infecting a significant percentage of avian populations (Valkiūnas, 2005). Haemosporidia of the genera *Haemoproteus, Plasmodium,* and *Leucocytozoon* are transmitted via dipteran vectors: *Haemoproteus* is transmitted by biting midges (*Ceratopogonidae*) and louse flies (*Hippoboscidae*), *Leucocytozoon* by black flies (*Simuliidae*), and *Plasmodium* by mosquitoes (*Culicidae*) (Valkiūnas, 2005). Mosquitos, black flies, and biting midges are found in many extreme climates, however avian haemosporidia are found sporadically in these areas (Loiseau et al., 2012; Oakgrove et al., 2014; Ramey et al., 2014; Meixell et al., 2016). *Leucocytozoon* appears to be more cold tolerant than *Haemoproteus* or *Plasmodium*, and has been found as far north as the Arctic Coastal Plain (Ramey et al., 2014). *Plasmodium* is mostly found in temperate and subarctic regions as sporogonic development is hindered by cold climates (LaPointe et al., 2010). Warming climates, however, increase the viable habitat of vectors, leading to range expansion (Garamszegi, 2011). *Plasmodium* is hypothesized to follow this pattern (Loiseau et al., 2013), as it has been found throughout Alaska and as far as 64° N (Loiseau et al., 2012). Compiling baseline data for future comparative analysis is important for predicting the long-term range of haemosporidia, as range expansion of vector populations risks exposure of naive species to infection, potentially harming populations of birds to whom haemosporidia are novel. Although these parasites exist within each continent (except Antarctica), their range is historically limited to certain habitats, and climate change can potentially increase the viable habitats in which haemosporidia can proliferate (Garamszegi, 2011;

* Corresponding author. San Francisco State University San Francisco, CA, 94132, United States.
E-mail addresses: faithdeamaral@gmail.com (F. De Amaral), rwilson43@unl.edu (R.E. Wilson), ssonsthagen@usgs.gov (S.A. Sonsthagen), sehgal@sfsu.edu (R. Sehgal).

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Loiseau et al., 2013).

Avian haemosporidia can persist in either the blood or non-blood tissue of the host. *Plasmodium* spends most of its life cycle in the blood where it undergoes asexual reproduction, but different lineages may enter latency in the endothelial cells of the internal organs (Valkiūnas, 2005). *Leucocytozoon* and *Haemoproteus*, conversely, spend most of their life cycle in the endothelial cells of the host as they undergo asexual reproduction through the development of megalomeronts. *Leucocytozoon* megalomeronts can persist in most tissues but are most commonly found in the spleen, while *Haemoproteus* megalomeronts develop in the endothelial cells of the skeletal musculature and the heart muscle (Valkiūnas, 2005). Because a significant portion of haemosporidian life cycles across all three genera occur in the endothelial cells, detection results may differ when tested from blood or muscle. Previous research comparing the viability and success of detecting avian haemosporidia in blood vs. muscle remains inconclusive. Prevalence was lower in blood than in other tissue types for *Plasmodium* (Svensson-Coelho et al., 2016; Harvey and Voelker, 2017; Galvin et al., 2021), and *Haemoproteus* (Galvin et al., 2021). Other studies found opposing results, where blood samples yielded higher prevalence than other tissues for *Plasmodium* (Ramey et al., 2013; Fecchio et al., 2019) and *Haemoproteus* (Ramey et al., 2013; Harvey and Voelker, 2017). Despite these findings, some studies found no significant difference in infection status between sampling methods for *Haemoproteus* (Drovetski et al., 2014; Fecchio et al., 2019), *Plasmodium* (Drovetski et al., 2014), or *Leucocytozoon* (Ramey et al., 2013; Drovetski et al., 2014; Fecchio et al., 2019). Considering the inconsistencies among studies comparing the viability of testing blood and muscle, here we contribute to our understanding of optimal sampling methods that may affect the probability of detecting an infection.

To properly assess parasite diversity within an individual, there are multiple sets of genus-specific primers used during polymerase chain reaction (PCR) in screening for haemosporidian infection (Huang, 2021). In our study we tested the commonly used primer sets amplifying either *Haemoproteus* or *Plasmodium* (Waldenström et al., 2004) and primers amplifying *Leucocytozoon* (Hellgren et al., 2004). Molecular detection techniques used for *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* can potentially display inconsistencies, as demonstrated in research by Cosgrove et al. (2006). The primer sets used in the PCR for *Haemoproteus* and *Plasmodium* can potentially amplify *Leucocytozoon*, requiring secondary sequence analysis to distinguish parasite genera (Cosgrove et al., 2006).

Grouse and ptarmigan, the focal species in this study, are Galliformes within the tribe Tetraonini. The spruce grouse (*Canachites canadensis*) and the ruffed grouse (*Bonasa umbellus*) reside in the boreal forests of Alaska, Canada, and the northern United States (Ellison, 1971; Jensen et al., 2019; Carroll and Merizon, 2021), the sooty grouse (*Dendragapus fuliginosus*) lives among the forested Pacific coastal areas of North America (Carroll and Merizon, 2021), and the sharp-tailed grouse (*Tympanuchus phasianellus*), being a lekking species, inhabits plains, prairies, and boreal forest of Alaska and throughout North America (Connelly et al., 1998). The ptarmigan species included in this study, the willow ptarmigan (*Lagopus lagopus*), the white-tailed ptarmigan (*Lagopus leucura*, North American only), and the rock ptarmigan (*Lagopus muta*) inhabit tundra or alpine-tundra habitat of North America and Eurasia in open, non-forested spaces with low ground cover typically dominated by willow species (Carroll and Merizon, 2021).

Grouse and ptarmigan are ideal model species for studying locally-transmitted blood parasites in Alaska because they are non-migratory, easily identifiable, and abundant, with avian haemosporidia infections are prevalent in most of the population (Fallis, 1945; Stabler et al., 1967a, 1967b; Bennett and Inder, 1972; Mahrt, 1981; Forbes et al., 1994; Smith et al., 2016). They are also considered secondary prey species as some regions experience cyclical population change which may be partially due to infection of endoparasites rather than only predation (Holmstad et al., 2005). Freezing responses and decreased mobility of grouse and ptarmigan have been shown to be stronger in areas of high parasite infection, especially in areas of high presence of *Leucocytozoon* (Fallis, 1945; Holmstad et al., 2006), indicating susceptibility to deleterious effects from haemosporidian infection. Though detected in grouse species, *Plasmodium* has not been found in ptarmigan samples tested globally (Mahrt, 1981; Holmstad and Skorping, 1998; Hagihara et al., 2004; Skirnisson et al., 2012; Smith et al., 2016). To date, no research has been conducted on haemosporidian infections of Arctic populations of grouse and ptarmigan, such information is critical in understanding haemosporidian expansion in Arctic and sub-Arctic environments.

Stabler et al. (1967a) provided the first blood parasite prevalence study among game birds in Alaska. This study found 90% of rock ptarmigan individuals were positive for blood parasites, including *Leucocytozoon* and *Trypanosoma*. While sampling spruce grouse in the southwestern and southcentral regions of Alaska (Stabler et al., 1967b), they found 80% prevalence of *Leucocytozoon*, 60% prevalence of *Haemoproteus*, and no *Plasmodium* among their samples (Stabler et al., 1967a, 1967b). Smith et al. (2016) conducted the second and most recently published haemosporidian prevalence study of grouse and ptarmigan in Alaska. Using PCR to detect haemosporidian infection, they found 75% of tissue samples among 459 hunter-harvested individuals were positive for at least one genus of haemosporidian parasite, where *Leucocytozoon* (241/459) was more prevalent than *Haemoproteus* (94/459), and *Plasmodium* (39/459) was the least prevalent.

Here we assess the prevalence and species composition of *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* haemosporidia in grouse and ptarmigan species across Alaska, using multiple sample types (blood and muscle) as well as multiple primer sets. In addition, we provide the first molecular assessment of parasite diversity in the Arctic of Alaska. Based on prevalence data of haemosporidia at similar latitudes (Loiseau et al., 2012; Oakgrove et al., 2014; Ramey et al., 2014; Meixell et al., 2016; Smith et al., 2016), we hypothesized that 1) infection of *Leucocytozoon* will be widespread along the latitudinal gradient and remain consistent throughout the sampling area, including the Arctic, while 2) *Plasmodium* and *Haemoproteus* infections will be mostly observed in the southern areas of Alaska in more temperate environments and absent in the Arctic. Previous studies found mostly consistent prevalence of *Leucocytozoon* across sampling areas (Stabler et al., 1967a, 1967b; Smith et al., 2016), including those in extreme climates, with *Haemoproteus* and *Plasmodium* being mainly prevalent in warmer temperatures in the south of the state (Loiseau et al., 2012; Smith et al., 2016). We also assess infection status of blood vs. muscle to provide insight into the determinants of prevalence data via sampling methods. Because haemosporidia spend a significant portion of their life cycle within the endothelial cells of the host (Valkiūnas, 2005), we predict that muscle samples will yield the most comprehensive array of individual parasites lineages.

2. Materials and methods

2.1. Field sampling methods

Blood and (or) muscle tissue (leg, wing, or heart) were collected from six species (N = 419 individuals) of galliformes in Alaska during 2013–2017 (Fig. 1). Sampling for two species, willow ptarmigan (*Lagopus lagopus*, N = 167) and rock ptarmigan (*Lagopus muta*, N = 125) spans ~10° latitude from southcentral Alaska (~59.9°N) to the Arctic Ocean (~70.1°N). The remaining species of white-tailed ptarmigan (*Lagopus leucura*, N = 34), spruce grouse (*Canachites canadensis*, N = 58), ruffed grouse (*Bonasa umbellus*, N = 20), sooty grouse (*Dendragapus fuliginosus*, N = 5), and sharp-tailed grouse (*Tympanuchus phasianellus*, N = 10) are restricted in distribution south of the Brooks Range (~68°N). Blood smears were collected but were of insufficient quality for thorough analysis for this study. Samples were collected either during fall or

Fig. 1. Map of sampling locations in Alaska. Circles show the number of samples for each grouse and ptarmigan species collected at each study site. Description of precise locations located in supplementary materials (Table A). Free vector and raster map from Natural Earth (https://www.naturalearthdata.com).

winter from shot hunter-harvested birds, or spring and summer as part of a large galliform population genetic assessment project (Sonsthagen et al., 2022) with date, age, and location point data collected for each individual (see Sonsthagen and Wilson, 2020 for detailed sample information). All hunter-harvested samples were collected following local and state hunting regulations and all other samples hunter-harvested outside of those regulations were collected under scientific collecting permit though Alaska, Department of Fish and Game (Scientific Permits 15–108, 16–109 and 17–109) and U.S. Geological Survey Alaska Science Center ACUC (assurance plan code 2015–03). Most of the individuals (N = 317/419) were sampled in the field: muscle samples were taken within 3 h and blood samples were taken within 30 min, with the remaining 102 samples processed off-site. Muscle samples taken in the field were initially stored in liquid nitrogen and then transferred to a −80C freezer until extraction, and tissues processed in the lab were stored in −80C freezer (Sonsthagen et al., 2022). Blood was extracted before dissection, stored in blood preservation buffer (Longmire et al., 1988) and collected from 227 individuals: willow ptarmigan (N = 106), rock ptarmigan (N = 99), spruce grouse (N = 15), and ruffed grouse (N = 7). Muscle was extracted from 341 individuals: willow ptarmigan (N = 130), rock ptarmigan (N = 92), spruce grouse (N = 53), ruffed grouse (N = 17), sharp-tailed grouse (N = 10), sooty grouse (N = 5), and white-tailed ptarmigan (N = 34). Paired blood and muscle samples were extracted from willow ptarmigan (N = 69), rock ptarmigan (N = 66), spruce grouse (N = 10), and ruffed grouse (N = 4). Genomic DNA was extracted for the 568 blood and muscle samples from the 419 total individuals using a DNeasy Blood & Tissue kit following the manufacturer's protocols (Qiagen, Valencia, CA, USA).

2.2. Haemosporidian DNA detection

To determine infection status, we used a nested PCR amplification of a 520-base pair (bp) fragment of the mitochondrial cytochrome *b* (cyt *b*) gene. Each sample was screened for *Leucocytozoon* using the protocol described in Hellgren et al. (2004) with primers HaemNF-1/HaemNR3-HaemFL/HaemR2L, as well as *Haemproteus* and *Plasmodium*, amplified simultaneously, using the protocol described in Waldenström et al. (2004) with primers HaemNF/HaemNR2-HaemF/HaemR2. We carried out the 25uL volume PCR reactions using Promega GoTaq flexi DNA polymerase and 3uL of template DNA. PCR reactions were tested with one positive control (samples previously confirmed to be positive via amplification and sequencing) per 16 samples tested, and one negative control (nuclease-free water) per 32 samples tested. PCR products (5uL) were visualized on 1.8% agarose gels stained with ethidium bromide. Samples were considered positive with an illuminated band at 520bp for *Plasmodium/Haemoproteus* and *Leucocytozoon* (Waldenström et al., 2004; Hellgren et al., 2004). Samples were screened up to three times for each protocol, and screening ceased for a sample once a positive result was detected. We confirmed successful DNA extraction of samples testing negative for all three rounds of PCR by amplifying a 602-nucleotide fragment of the brain-derived neurotropic factor (BDNF) gene (Sehgal and Lovette, 2003). Samples that failed to amplify for BDNF (N = 19) were removed from the study, with 400 individuals remaining for analysis (see Table 1 for sample sizes).

After determining infection status, 121 PCR products testing positive for *Haemoproteus/Plasmodium* and 180 PCR products testing positive for *Leucocytozoon* were sent to ElimBiopharmaceuticals Inc. (Hayward, CA) for PCR cleanup and bidirectional Sanger sequencing. A total of 288 sequences from blood and (or) muscle samples from 184 individuals were used for analysis. Sequences were reconciled and aligned using Geneious 11.1.5 (https://www.geneious.com, Kearse et al., 2012) and were identified as either *Leucocytozoon, Plasmodium*, or *Haemoproteus* using the Basic Local Alignment Search Tool (BLAST) function utilizing

Table 1
PCR amplification of all haemosporidian genera based two primer sets developed by Hellgren et al. (2004) and Waldenström et al. (2004). and was used to evaluate haemosporidian infections among Galliformes in Alaska.

SPECIES	% POSITIVE	INDIVIDUALS POSITIVE	Total
Grouse			
Ruffed Grouse (*Bonasa umbellus*)	88%	14	16
Sooty Grouse (*Dendragapus fuliginosus*)	100%	5	5
Spruce Grouse (*Canachites canadensis*)	100%	54	54
Sharp-tailed Grouse (*Tympanuchus phasianellus*)	80%	8	10
Ptarmigan			
Willow Ptarmigan (*Lagopus lagopus*)	89%	144	161
White-tailed Ptarmigan (*Lagopus leucura*)	68%	21	31
Rock ptarmigan (*Lagopus muta*)	85%	105	123
Total	**88%**	**351**	**400**

National Center for Biotechnology Information (NCBI) data. Lineages were assigned using NCBI Genbank data, and no new sequences or lineages were found in this study. For clarity, lineages are denoted by an additional "H_", "P_", or "L_" to distinguish *Haemoprotues, Plasmodium,* or *Leucocytozoon,* respectively.

2.3. Data Analysis

To assess whether infection prevalence was consistent across regions, we grouped sample locations into five major geographical regions of Alaska: Arctic (Arctic Coastal Plain, Brooks Range, Finger Mountain), Interior (Eagle Summit, Minto, Galena, Denali Highway, Taylor Highway, Fairbanks Area, Farewell), Southeast (Lemesurier Island), Southcoastal (Lake Clark National Park and Preserve, Kenai, Thompson Pass), and Southcentral (Palmer, Eureka). We assessed this variation using a one-way ANOVA using R statistical software (2022.02.1) and the Tidyverse package within. In identifying and ranking predictor variables of infection status for all samples, we created a standard and partitioned classification tree (Fig. 2). This method is useful for non-linear, non-parametric data, and has been used previously on studies assessing avian parasites (Sehgal et al., 2011). We used the packages *tree* and *party,* respectively, in the R software environment, and tested for potential predictors using the collected metadata associated with each sample, and using WorldClim data (Fick and Hijmans, 2017) as our environmental variables.

We conducted a chi-squared test for each set of primers to assess the methodological success based on blood or muscle samples during field data collection with individuals represented by both blood and muscle samples (N = 145). Because each set of primers amplified both overlapping lineages and selectively amplified some lineages over others within the same individuals, we conducted this analysis separately for the two primer sets to maintain independence. We also used a chi-squared test to evaluate whether lineages with at least ten positive infections (L_COLBF24, L_COLBF22, L_AKGPL14, L_LAGLAG02) are significantly associated with either primer set to investigate the possibility of biases in amplification.

3. Results

3.1. Total haemosporidian prevalence

Of the 400 individuals (N = 545 total blood and muscle samples) for which there was sufficient DNA to yield amplification at BDNF, 176 blood samples and 281 muscle samples tested positive for *Leucocytozoon* as determined by PCR with the primers HaemNF-1/HaemNR3-HaemFL/HaemR2L. Fifty blood samples and 167 muscle samples tested positive for *Haemoproteus/Plasmodium* as determined by PCR with the primers HaemNF/HaemNR2-HaemF/HaemR2. A total of 351 of the 400 individuals tested positive for at least one genus of haemosporidian parasite.

Overall, all species had a relatively high prevalence of at least one haemospordian genus when combining data from both blood and tissue samples for each individual (Table 1). Of the species with the highest sample sizes, spruce grouse (N = 54) had the highest haemosporidian

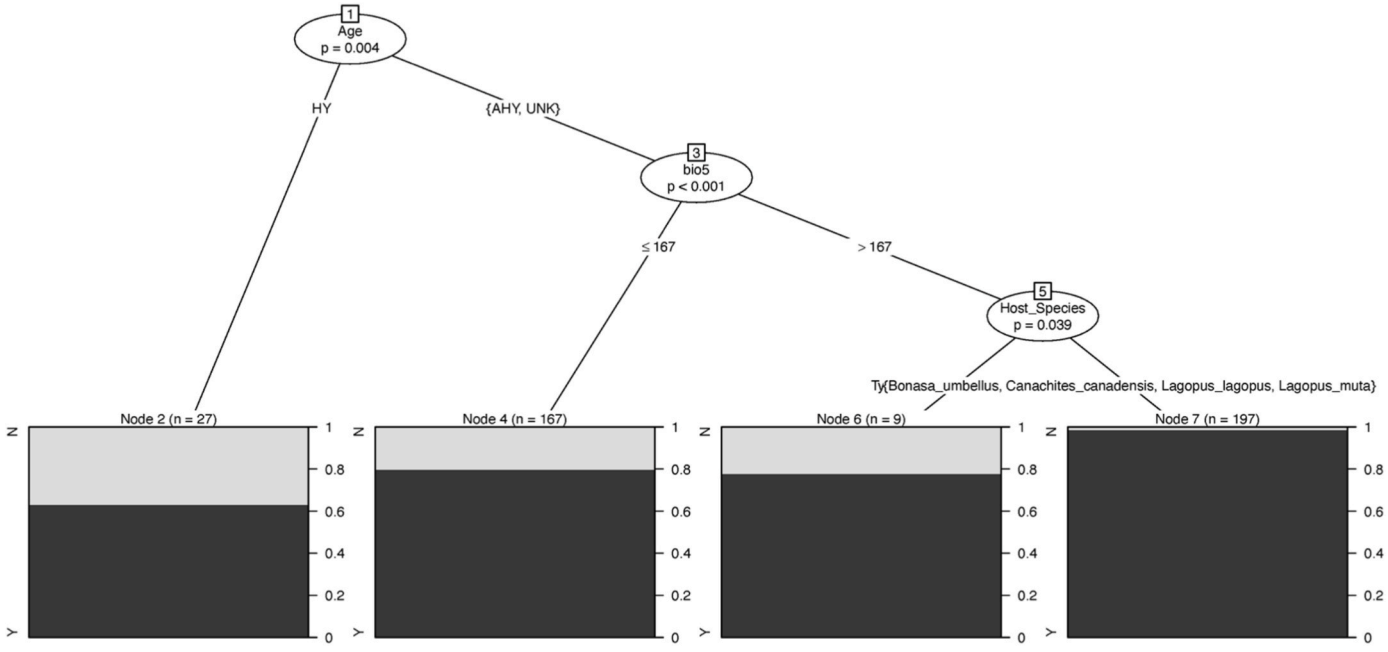

Fig. 2. Classification tree displaying the determining variables displaying the highest significance of association with host infection status. Y indicates positive infection status, and N indicates negative infection status. Each node represents the number of positive and negative infections associated with each determining variable(s), where the black fill represents the proportion of positive infections associated with each variable or combination of variables. The variable bio5 is the maximum temperature of the warmest month.

infection prevalence, with 100% of individuals testing positive for at least one parasite genus. The willow ptarmigan (N = 161) and rock ptarmigan (N = 123) which included samples from the Arctic had prevalences of 89% (N = 144) and 85% (N = 105), respectively. Of the other species with more limited sample size, infection status ranged from 100% in the sooty grouse (N = 5) to 68% in the white-tailed ptarmigan (N = 21/31) (Table 1).

3.1.1. Blood and muscle of total infections

When using the *Leucocytozoon* primer set on paired blood and muscle samples of 145 individuals, 117 individuals tested positive for both blood and muscle, five tested positive for blood but negative for muscle, 12 tested positive for muscle but negative for blood, and 11 tested negative for both blood and muscle. Based on this paired-sample data set, differences in infection status based on sample type (blood vs muscle) with *Leucocytozoon* primers are significant ($\chi^2 = 37.69$ (1, N = 145), $p < 0.00001$). These results show that most of the individuals are positive in both blood and muscle with few showing alternate results.

When using the *Haemoproteus/Plasmodium* primer set on paired blood and muscle samples of 145 individuals, 25 individuals tested positive for both blood and muscle, six tested positive for blood but negative for muscle, and 49 tested negative for both blood and muscle. Nearly half (N = 65/145) of the individuals, the highest of any category, tested positive for muscle but negative for blood. Based on this paired-sample data set, differences between blood and muscle infection status using the *Haemoproteus/Plasmodium* primers are significant ($\chi^2 = 5.78$ (1, N = 145), $p = 0.0162$).

3.1.2. Location of total infections

Most individuals of each species tested positive for at least one genus of haemosporidian parasite in each location sampled (Fig. 3). Infection occurred in 79% (N = 42/53) of rock ptarmigan individuals and 87% (N = 60/69) of willow ptarmigan individuals in the Arctic. Moving south, infection prevalence among rock ptarmigan and willow ptarmigan increased slightly, with 90% (N = 63/70) and 91% (N = 84/92) of individuals, respectively, testing positive below the Arctic Circle (66.5°N). Though the absolute number of positive infections varied among regions, we found that the region of capture was not significant in predicting whether an individual will be infected ($F_{4,12} = 1.935$, $p > 0.169$). Thus, the average number of positive infections in each region is not significantly different from the average number of positive infections from other regions.

3.1.3. Seasonality of total infections

Our study included 27 hatch year (HY) individuals: ruffed grouse (N = 4), spruce grouse (N = 2), sharp-tailed grouse (N = 1), willow ptarmigan (N = 4), white-tailed ptarmigan (N = 3), and rock ptarmigan (N = 13) (Fig. 4). Hatch year individuals were less than three months old at the time of sampling, with the exception of two older individuals sampled in January and March. Seventeen (77%) HY birds were positive for haemosporidian infections. In our analysis using a classification tree, age received the highest ranking of importance in predicting infection status (Fig. 2), followed by maximum temperature of the warmest month (bio5), and finally host species, ($p < 0.05$ for these three variables). Thirteen of the 17 (71%) positive HY individuals were sampled in August and September. Each of these analyses included both blood and muscle samples for the HY individuals.

3.2. Sequencing Analysis

Sequencing revealed that the *Hameoproteus/Plasmodium* primers HaemNF/HaemNR2-HaemF/HaemR2 detection also amplified *Leucocytozoon* lineages. Of the 115 blood and muscle PCR products amplified with the *Haemoproteus/Plasmodium* primer set, 103 (90%) amplified *Leucocytozoon* with the remaining 12 amplifying *Haemoproteus* or *Plasmodium* lineages. When sequencing both primer sets with both blood and muscle samples, there were 11 distinct lineages of *Leucocytozoon*, two lineages of *Haemoproteus*, and one lineage of *Plasmodium* (Tables 2 and 3). Spruce grouse and ruffed grouse were the only species with positive sequences for *Haemoproteus* or *Plasmodium* lineages from all blood and muscle samples sequenced, with spruce grouse accounting for all *Plasmodium* P_BT7 positive individuals (Table 2).

The majority of individuals with *Leucocytozoon* lineage L_COLBF22 were spruce grouse, comprising 91% (N = 50/55) of the total sequenced infections. Willow ptarmigan and rock ptarmigan comprised 99% (N = 82/83) of individuals for *Leucocytozoon* lineages L_AKGPL14 (N = 35

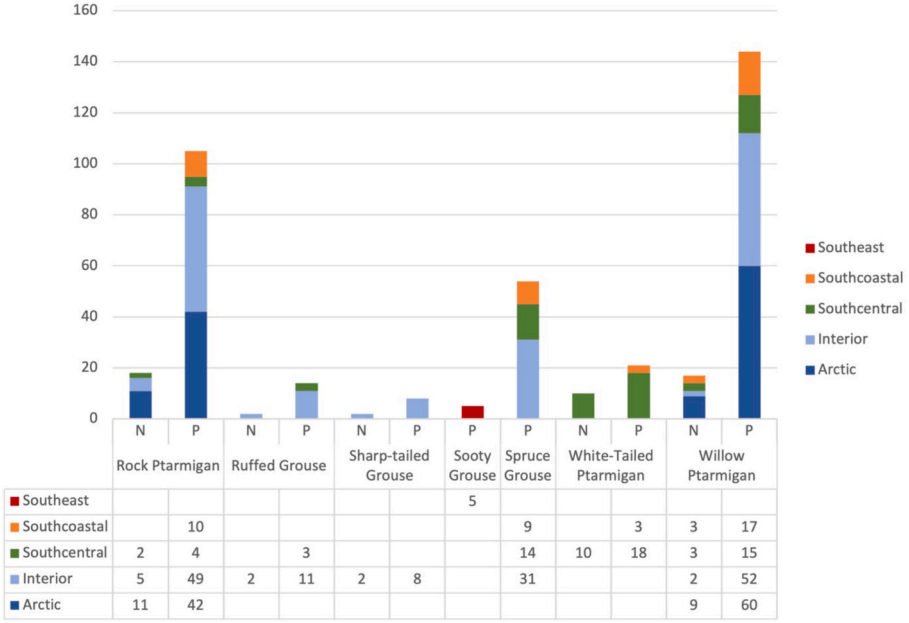

Fig. 3. Location prevalence for individuals testing positive (P) and negative (N) for all three genera of haemosporidian parasites. Regionality is defined as follows: Arctic: Arctic Coastal Plain, Brooks Range, Finger Mountain. Interior: Eagle Summit, Minto, Galena, Denali Highway, Taylor Highway, Fairbanks Area, Farewell. Southcentral: Palmer, Eureka. Southcoastal: Lake Clark National Park and Preserve, Kenai, Thompson Pass. Southeast: Lemesurier Island.

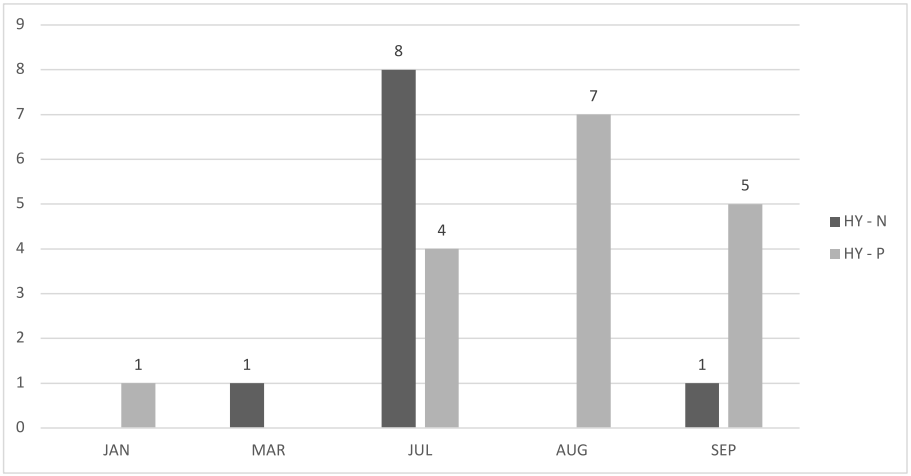

Fig. 4. Hatch year (HY) ruffed grouse, rock ptarmigan, willow ptarmigan, spruce grouse, sharp tailed grouse, and white-tailed ptarmigan individuals testing positive for at least one parasite genus. This figure aggregates all years sampled for 27 individuals. Hatch year individuals are less than three months old at the time of sampling, with the exception of individuals sampled in January and March where age is less than 10 months old. P = positive, N = negative.

Table 2
Blood and muscle samples collected from Galliformes in Alaska were sequenced using *Haemoproteus/Plasmodium* primers HaemNF/HaemNR2-HaemF/HaemR2 (Waldenström et al., 2004), and *Leucocytozoon* Primers HaemNF-1/HaemNR3-HaemFL/HaemR2L (Hellgren et al., 2004) with the number of samples testing positive for each lineage amplified. Duplicates of lineages found by both primers for an individual have been removed.

		Spruce Grouse	Sharp-tailed Grouse	Ruffed Grouse	Sooty Grouse	Willow Ptarmigan	Rock Ptarmigan	White-tailed Ptarmigan	Total Lineages
Plasmodium	BT7	8							8
Haemoproteus	TETURO02	2							2
	TETURO01	1		1					2
Leucocytozoon	COLBF24		3		3	17	16	1	40
	COLBF22	42	1		1		2		46
	AKGPL09			3					3
	AKGPL14	1				27	30		58
	LAGLAG02					26	17		43
	LAGLAG03		2			11	11	1	25
	LAGLAG04						1		1
	GALLUS25						1		1
	CYASTE02	1				1			2
	SPISEN05	1							1
	CYASTE01	3	1	1		3	4	3	15
	Total Detections	**60**	**7**	**5**	**4**	**85**	**82**	**5**	**248**

and 47, respectively) and comprised all individuals with L_LAGLAG02 (26 and 17, respectively). (Supplemental Table B presents % identity).

A total of 14 haemosporidian lineages were detected in blood and muscle of Alaska grouse and ptarmigan (Table 2). Grouse contained a total of 11 lineages, of which four were only detected in grouse species. Ptarmigan contained nine lineages, with three lineages occurring exclusively in ptarmigan species (Table 2). Among species with greater than 10 detections, a similar number of lineages were observed (6–8 lineages; Table 2, Fig. 5, supplemental material).

3.2.1. Coinfections found using differing primer sets

Coinfections in both blood and muscle of different parasite genera and coinfections of parasites within the same genera were revealed through sequence analysis. Randomized individuals testing positive for either *Haemoproteus* or *Plasmodium* and *Leucocytozoon* (N = 80) through PCR were sequenced to provide insight on possible coinfection between parasite genera, or coinfection of different lineages within the same parasite genus. Of the 80 sequenced individuals testing positive for both *Leucocytozoon* and *Haemoproteus/Plasmodium*, 44 contained coinfections of separate lineages of *Leucocytozoon*, four contained coinfections of *Leucocytozoon* and *Haemoproteus*, and one harbored a coinfection of *Leucocytozoon* and *Plasmodium* (Table 3).

3.2.2. Location prevalence for sequenced lineages

Haemoproteus and *Plasmodium* occurred in the southern and interior areas of Alaska and were only detected in spruce grouse and ruffed grouse. One individual testing positive for *Plasmodium* was sampled in Galena, a previously unsampled interior location (Fig. 3). *Leucocytozoon* prevalence was similar throughout the state and positive individuals were detected within each location sampled (Table 4, Fig.). All of these samples include both blood and muscle.

Hatcher Pass and the Fairbanks areas yielded the majority of samples sequencing positive for L_COLBF22, which was predominately detected in spruce grouse. On the Arctic Coastal Plain, the *Leucocytozoon* lineages detected (N = 6) were relatively diverse: L_COLBF24, L_AKGPL14, L_LAGLAG02, L_LAGLAG03, L_CYASTE02, and L_CYASTE01. All lineages in the Arctic Coastal Plain were detected in ptarmigan as grouse do not occur north of the Brooks Range. No lineages were exclusive to north of the Arctic Circle, though Galliformes in the Brooks Range showed the highest frequency (N = 30) of the lineage L_AKGPL14.

3.2.3. Primer specificity – influence of detectability

Sequencing the amplification product of both the blood and muscle of an individual can reveal coinfections among blood and muscle sample types. Comparing sequences between the blood and muscle samples

Table 3
Lineages of parasite genera as coinfections. The left-hand column is the *Leucocytozoon* lineage amplified, the middle column is the different lineages amplified with the PCR protocol for *Haemoproteus/Plasmodium* (HP), and the third column contains the number of Alaska Galliformes with the coinfection of those two lineages.

Lineage sequenced with Leuco primers	Lineage sequenced with HP primers (same individual)	Number of individuals
LAGLAG02		
	LEUCOCYTOZOON SP. COLBF24	4
	LEUCOCYTOZOON SP. COLBF22	1
	LEUCOCYTOZOON SP. AKGPL14	18
LAGLAG03		
	LEUCOCYTOZOON SP. COLBF24	4
	LEUCOCYTOZOON SP. AKGPL14	5
AKGPL14		
	LEUCOCYTOZOON SP. COLBF24	1
	LEUCOCYTOZOON SP. AKGPL14	7
COLBF22		
	LEUCOCYTOZOON SP. AKGPL14	1
	PLASMODIUM SP. BT7	4
	HAEMOPROTEUS SP. TETURO02	1
COLBF24		
	LEUCOCYTOZOON SP. COLBF22	1
LAGLAL03		
	LEUCOCYTOZOON SP. COLBF24	1
GALLUS25		
	LEUCOCYTOZOON SP. AKGPL14	1
Total		**49**

using *Leucocytozoon* primers, six amplified the same lineage of *Leucocytozoon*, and seven amplified different lineages of *Leucocytozoon*. When comparing sequences of blood and muscle using only *Haemoproteus/Plasmodium* primers, three amplified the same lineage of *Leucocytozoon* and one amplified different lineages of *Leucocytozoon* (Table C, supplemental material). In addition, the primers for *Haemoproteus* and *Plasmodium* amplified *Leucocytozoon*, therefore separate primer sets could have amplified either the same lineage or different lineages of *Leucocytozoon*. Using different primer sets to amplify haemosporidia in the paired blood and muscle samples of nine individuals revealed most (N = 7/9) amplifying different lineages of *Leucocytozoon* (Table C, supplemental material). When comparing the sequence results between both primer sets for an individual's blood and muscle samples, the primer sets amplified different *Leucocytozoon* lineages for seven individuals.

L_COLBF22 and L_LAGLAG02 resulted in significantly higher amplification with the *Leucocytozoon* primer set, where 74 total individuals tested positive and 12 tested negative. This analysis includes *Leucocytozoon* lineages occurring in at least 10 individuals and amplified using both primer pairs. L_COLBF24 and L_AKGPL14 resulted in significantly higher amplification with the *Haemoproteus/Plasmodium* primer set, where 82 total individuals tested positive and 25 tested negative (χ^2 (1, N = 193) = 74.98, p < 0.00001).

4. Discussion

This study provides site data and analysis for haemosporidian prevalence in Galliformes in Alaska. We found that the majority of individuals sampled tested positive (88%) for at least one genus of haemosporidian parasite. As expected, the prevalence of *Leuocytozoon* lineages was widespread at all of the sampling sites, while *Haemoproteus* and *Plasmodium* lineages were detected in the southcentral and interior sampling sites. Unexpectedly, the primer sets used to detect the genera *Haemoproteus* and *Plasmodium* also amplified *Leucocytozoon* lineages, providing novel data of intra-genus coinfections of grouse and ptarmigan species in Alaska. Coinfections of *Leucocytozoon* were also found by sequencing paired blood and muscle samples, shedding light on the importance of sampling techniques in screening for individual parasite lineages as well as the life history of these parasites. Using both *Haemoproteus/Plasmodium* and *Leucocytozoon* primer sets, as well as sampling both blood and muscle from the same individual provided the most accurate lineage and infection status results.

Our study builds upon previous studies by providing point data with specific locations of capture and analysis of samples collected throughout the year. Previous studies by Smith et al. (2016) and Stabler et al. (1967a,b) described broad regional data for their samples and research conducted by Smith et al. (2016) was limited to the hunting season (August–March). Stabler et al. (1967a,b) analyzed blood smears of individuals while Smith et al. (2016) used PCR to analyze a single sample type (wing muscle). Although regional data are valuable to document broad shifts in parasite distribution, specific point location data for host and parasite lineages allow researchers to document subtle shifts in distribution through time as well as the potential identification of habitats that may act as corridors of parasite/vector dispersal with environmental change. This study provides these point data in both novel regions and regions previously sampled to contribute thorough information to the current literature on haemosporidian prevalence in Alaska.

4.1. Prevalence and lineage specificity

Although our results show a slightly higher prevalence (88% total) than that observed in a recent study of Galliformes in Alaska (75%; Smith et al., 2016), use of two primer sets, an increase in sampling locations, and screening of multiple tissue types could explain the higher percentage of positive individuals. This high overall prevalence was found in each host species (84–100% for species with at least 50 sampled individuals) with the spruce grouse showing the highest proportion of infected individuals. Further sequence analyses showed that grouse were the only host species to harbor *Haemoproteus* and *Plasmodium* which is consistent with findings of Stabler et al. (1967a,b); however, Smith et al. (2016) did detect *Haemoproteus* in a small number (N = 8/228) of ptarmigan. Ptarmigan may be refractory to infection of *Haemoproteus* which might be attributed to differences in host immune systems and/or vector ecology. Further research is needed to verify the factors facilitating the high prevalence of *Haemoproteus* and *Plasmodium* infections in spruce grouse.

Despite there being a high prevalence of *Leucocytozoon* lineages in grouse and ptarmigans throughout Alaska, there appears to be high host specificity for certain lineages as well as an indication of distributional differences of parasite lineages throughout the state. The total prevalence of haemosporidian infections remained consistently high throughout the sampling area, indicating increased transmission of haemosporidia across Alaska relative to previous studies conducted by Smith et al. (2016) and Stabler et al. (1967a, b). The high infection prevalence of *Leucocytozoon* and lack of *Haemoproteus* and *Plasmodium* north of the Arctic Circle is consistent with previous research (Loiseau et al., 2012; Oakgrove et al., 2014). Willow ptarmigan and rock ptarmigan were the only species sampled above the Arctic Circle, with 102 out of 122 (84%) individuals sampled screening positive for at least one parasite genus. The *Leucocytozoon* lineages L_AKGPL14 and L_LAGLAG02 were the dominant lineages found in the Arctic. The presence of these lineages in the Arctic may be attributed to increased cold tolerance, or through preferential association with blackflies specific to the northern habitats, such as *Simulium vittatum* or *Simulium nigricoxum* (Currie, 1997), genera associated with *Leucocytozoon* transmission (Fallis and Bennett, 1958; Kiszewski and Cupp, 1986). Although the L_AKGPL14 lineage was detected in other regions in this study, the highest frequency was in the Arctic (north of the Brooks Range). Further research is needed to identify the mechanism allowing for increased northern dispersal of these lineages as well as species-level *Leucocytozoon* transmission of *Simulium* blackflies.

4.2. Coinfections

This study displays notable differences from the study conducted by

Table 4
Number of *Leucocytozoon* or *Haemoproteus/Plasmodium* lineages detected in Galliformes at each Alaska location, based on results from sequencing 288 samples (both blood and muscle tissue) from 184 individuals. Redundant lineages found with both primer sets have been removed.

	Plasmodium	*Haemoproteus*		*Leucocytozoon*										
	BT7	**TETURO02**	**TETURO01**	**COLBF24**	**COLBF22**	**AKGPL09**	**AKGPL14**	**LAGLAG02**	**LAGLAG03**	**LAGLAG04**	**GALLUS25**	**CYASTE02**	**SPISEN05**	**CYASTE01**
Spruce grouse														
Denali Highway				1										
Fairbanks Area	2	1			14							1	1	1
Farewell					1									
Galena	1				4									
Kenai					6									
Minto	1				1		1							2
Palmer	4	1	1		16									
Sharp-tailed grouse														
Fairbanks Area				3										
Farewell					1									
Minto														1
Taylor Highway									2					
Ruffed grouse														
Fairbanks Area						2								
Minto														1
Palmer			1			1								
Sooty grouse														
Lemesurier Island				3	1									
Willow ptarmigan														
Arctic coastal plain				1			5	4	1			1		3
Brooks Range							13	9	1					
Denali Highway				11			3	5	7					
Eagle Summit				3			2	1						
Finger Mountain							1	1						
Kenai							1							
Lake Clark National Park and Preserve				1				1	2					
Taylor Highway				1			2	5						
Rock ptarmigan														
Arctic coastal plain				1					1					2
Brooks Range							17	7	5					
Denali Highway				5	1		3	1			1			
Eagle Summit				3					2					2
Eureka					1		2							
Taylor Highway				7			8	9	1					
Thompson Pass									2	1				
White-tailed ptarmigan														
Lake Clark National Park and Preserve														1
Palmer				1					1					2

Smith et al. (2016), unexpected considering these studies were conducted only a few years apart. This may not be due to an actual difference in parasite diversity and prevalence but possibly due to differences in methodologies not previously taken into consideration. Coinfections of *Leucocytozoon* were not detected by Smith et al. (2016) but were found in this study; different lineages were found among different sample types and samples using different primer sets. When using both primer sets on either blood and/or tissue sample types, most individuals (N = 8/9) had different *Leucocytozoon* lineages amplified. In addition, when using the standard *Leucocytozoon* primer set (Hellgren et al., 2004) we amplified different lineages from different sample types (blood vs muscle) in seven individuals. These findings highlight the potential for differential timing of different *Leucocytozoon* species in blood versus exoerythrocytic stages. The results also stress the importance of archiving and obtaining samples from several tissue types, including blood.

While most of the *Haemoproteus/Plasmodium* lineages were observed as coinfections with *Leucocytozoon*, we detected single infections in spruce grouse and ruffed grouse. Single infections of *Plasmodium,* without the presence of *Leucocytozoon* coinfections, were found in three individual spruce grouse and single infections of *Haemoproteus* in two grouse. These singly infected *Plasmodium* or *Haemoproteus* positive individuals were uncommon and further molecular analysis is needed to determine parasite mechanisms that may be responsible for the presence of *Plasmodium* or *Haemoproteus* without the presence of *Leucocytozoon* in areas where *Leucocytozoon* is ubiquitous.

Studies on haemosporidian coinfections have documented changes in host reproductive strategies and fitness. For example, increased reproductive success (increased clutch size and fledglings) was found among individuals with coinfections (Marzal et al., 2008; Pigeault et al., 2018), while other host species exhibited reductions in fitness (e.g., survivability; Pigeault et al., 2018). Further, interspecific competition between *Leucocytozoon* and *Plasmodium* may occur in hosts eliciting hosts to exhibit trade-off responses when other novel infections are present (Reinoso-Pérez et al., 2020). As coinfections may have important consequences on host fitness, the detection of multiple lineages of *Leucocytozoon* in both blood and muscle samples warrants further research. Additional research on the effect of coinfections is particularly germane to Arctic host species, as climate change may facilitate the dispersal of novel parasite lineages into the Arctic and these parasites may exert new pathogenic pressures on existing host species.

4.3. Prevalence and seasonality

Hatch year (HY) individuals sampled in August and September constituted the majority (N = 13/17) of positive HY individuals in this study, indicating a potential correlation between initial infection and seasonality. Four HY individuals sampled in July were positive for *Leucocytozoon* (N = 4/12), and most sampled in August and September were positive for *Leucocytozoon* (N = 12/13), indicating that vectors are more likely to transmit later in the summer. It is possible that the offspring of grouse and ptarmigan species potentially evade transmission during the first few weeks of their life, as they hatch in late May/early June (Carroll and Merizon, 2021) before the second bloodmeals of black flies. Most adults collected at the same time in the same location as HY individuals were positive for infection such as in the case for samples collected at Eagle Summit, where all (N = 8/8) adults tested positive and most (N = 5/8) HY individuals tested negative, providing possible evidence of seasonal infection. More research with a higher sample size of HY individuals is needed to confirm this association and analyze the correlation between infection and seasonality.

4.4. Study design considerations

Through our investigations, we have identified several methodological considerations researchers need to consider when examining haemosporidian prevalence and diversity, as detection and lineages differed between sample types, and *Leucocytozoon* lineages sequenced also varied with primer sets. Incongruence between detection methods (primer sets, PCR, sequencing) and variance in detection rates among sample types can impact interpretation of results, and not considering method and sample type differences could lead to inaccurate comparisons with other studies. Inter-genus and intra-genus coinfections have been historically underestimated using both the *Haemoproteus/Plasmodium* and the *Leucocytozoon* primer sets, further highlighting the need to develop new primers and molecular techniques to more accurately detect coinfections of *Haemoproteus, Plasmodium,* and *Leucocytozoon* lineages (Valkiūnas et al., 2006).

While detection of *Leucocytozoon* was similar for blood and muscle (89% concordance), positive infection status for *Haemoproteus/Plasmodium* was highly dependent on sample type. A high proportion of individuals (44%) were assessed as parasite positive based on muscle samples (negative for blood sample) while only 4% of individuals screened positive for blood (and negative for muscle). In the absence of sequence data for all positive detections with the Waldenström et al. (2004) primer set, we are unable to speculate on what mechanisms may explain the marked differences in infection status based on sample type. We found that primers used to detect *Haemoproteus/Plasmodium* (HaemNF/HaemNR2-HaemF/HaemR2; Waldenström et al., 2004) also amplified *Leucocytozoon*, a finding observed by others (Cosgrove et al., 2006). Although there are differences in where haemosporidian parasites spend most of their life cycle, we are unable to determine if these differences influence detectability based on sample type. The 10-fold increase in prevalence (4% vs. 44%) between blood and muscle samples, however, highlights the importance of accounting for sample type when making comparisons regarding prevalence of *Haemoproteus/-Plasmodium* with other studies.

Detection of *Leucocytozoon* lineages varied by sample type as well as by primer selection. In this study, the amplification of four lineages (L_COLBF24, L_COLBF22, L_AKGPL14, and L_LAGLAG02) differed significantly depending on primer set. Differences in lineage detection based on primer selection can have important implications for investigations of haemosporidian diversity. For example, Lotta et al. (2019) found that *Leucocytozoon* primers selectively amplified lineages based on morphology, and used microscopy and mtDNA amplification to detect *Leucocytozoon* lineages and coinfections that otherwise would have gone unamplified via the protocol of Hellgren et al. (2004). This phenomenon is not limited to the *Leucocytozoon* genus, as some *Haemoproteus* lineages may be missed by the current standard primer sets (Harl et al., 2022). Here, although the *Leucocytozoon* primer set can amplify all three genera, the *Leucocytozoon* primer set did not amplify *Haemoproteus* or *Plasmodium* lineages in individuals known to be infected with these genera, indicating that these primers accurately detected *Leucocytozoon* infections. Because *Leucocytozoon* infections are associated with ptarmigan behavioral responses to predation threats (Fallis, 1945; Holmstad et al., 2006), further research is needed to assess fitness-related impacts of coinfection on behavior of infected individuals.

5. Conclusion

Grouse and ptarmigan species offer a compelling model system to assess current parasite prevalence, diversity, and spread during rapid changes to the sub-Arctic and Arctic ecosystems. While prevalence was high for both grouse and ptarmigan, notable differences were detected (Table 2). First, *Haemoproteus/Plasmodium* were only detected in grouse. Second, group (grouse or ptarmigan) specific *Leucocytozoon* lineages were detected with most shared lineages occurring in high frequency in one group and only a few detections in the other group. Finally, while the number of *Leucocytozoon* lineages detected were similar between grouse and ptarmigan, the frequency of occurrence of lineages differed between groups. Grouse were predominately represented (67%, N = 43/

64) by a single *Leucocytozoon* lineage (L_COLBF22) with the next most frequent lineage detected in <10% of the samples. Conversely, most of the ptarmigan samples (92%, N = 157/171) were represented by four dominant *Leucocytozoon* lineages that were detected in 13–33% of the samples. This study contributes insight into preferable sampling methods and potential issues with current molecular analysis techniques, while providing baseline data of haemosporidian distribution for future comparative analyses.

Overall, this study provides point data for parasite distribution in Alaska for grouse and ptarmigan species. It also highlights the importance of considering sample type and primer specificity when performing PCR on these avian haemosporidia, and the potential for using separate primer sets for amplifying separate lineages within a genus. Diversifying detection methods, either through multiple primer sets or tissue types, allows for a more comprehensive view of parasite diversity within an individual.

Declaration of competing interest

There are no conflicts of interest to disclose in relation to this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.01.008.

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