International Journal for Parasitology 44 (2014) 717–727

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

# Distribution, diversity and drivers of blood-borne parasite co-infections in Alaskan bird populations ☆

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ARTICLE INFO

*Article history:*
Received 21 February 2014
Received in revised form 15 April 2014
Accepted 17 April 2014
Available online 9 July 2014

*Keywords:*
Alaska
Arctic
*Haemoproteus*
*Leucocytozoon*
*Plasmodium*
*Trypanosoma*
Co-infections
Random forest models

ABSTRACT

Avian species are commonly infected by multiple parasites, however few studies have investigated the environmental determinants of the prevalence of co-infection over a large scale. Here we believe that we report the first, detailed ecological study of the prevalence, diversity and co-infections of four avian blood-borne parasite genera: *Plasmodium* spp., *Haemoproteus* spp., *Leucocytozoon* spp. and *Trypanosoma* spp. We collected blood samples from 47 resident and migratory bird species across a latitudinal gradient in Alaska. From the patterns observed at collection sites, random forest models were used to provide evidence of associations between bioclimatic conditions and the prevalence of parasite co-infection distribution. Molecular screening revealed a higher prevalence of haematozoa (53%) in Alaska than previously reported. *Leucocytozoons* had the highest diversity, prevalence and prevalence of co-infection. *Leucocytozoon* prevalence (35%) positively correlated with *Trypanosoma* prevalence (11%), negatively correlated with *Haemoproteus* prevalence (14%) and had no correlation with *Plasmodium* prevalence (7%). We found temperature, precipitation and tree cover to be the primary environmental drivers that show a relationship with the prevalence of co-infection. The results provide insight into the impacts of bioclimatic drivers on parasite ecology and intra-host interactions, and have implications for the study of infectious diseases in rapidly changing environments.



## 1. Introduction

Wildlife populations can be infected with a diverse set of parasites; individual hosts may be infected with multiple parasite species and/or multiple lineages/strains of the same species concurrently (collectively hereafter referred to as "co-infections") (Read and Taylor, 2001; Mideo, 2009; Juliano et al., 2010; Palinauskas et al., 2011). For instance, numerous studies show that in areas where malaria is endemic malaria infections commonly include multiple strains (Babiker et al., 1999; Bruce et al., 2000a,b; Engelbrecht et al., 2000; Juliano et al., 2010). It has been suggested that the observed diversity in pathogen species may be due to both within-host pathogen competition (de Roode et al., 2005; Bell et al., 2006) and the host immune response against pathogen antigens (Bruce et al., 2000a). Interactions between genetically distinct parasite lineages can affect host fitness, determine pathogen transmission competence and influence disease epidemiology; these co-infections can provide insight into host–parasite co-evolution, which may be important for shaping parasite virulence and drug resistance (Deviche et al., 2001; Read and Taylor, 2001; de Roode et al., 2005; Bell et al., 2006; Marzal et al., 2008; Telfer et al., 2010). While many studies of co-infections have examined their effects on host fitness (i.e. pathogenicity) (de Roode et al., 2005; Bell et al., 2006; Marzal et al., 2008; Palinauskas et al., 2011; van Rooyen et al., 2013b), research on the interactions between multiple parasite lineages and the environmental variables that contribute to the prevalence of co-infections remains less studied.

It is important to understand the ecological determinants that affect multiple infections because these interactions can contribute heavily to disease outcome. In theory, the most successful parasite competitor would most effectively invade and utilise host resources at the lowest host fitness cost and thereby successfully

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank under accession numbers KF314757–KF314799.

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http://dx.doi.org/10.1016/j.ijpara.2014.04.011
0020-7519/

evade the host immune response (de Roode et al., 2005). However, within-host parasite competition may select for higher virulence, as the parasite that most effectively exploits limited host resources may outcompete other strains (Bell et al., 2006). Co-infections may cause parasites to either: (i) compete for host resources and overlapping ecological niches (exploitation competition) to continue replication (reproduction) and future survival (transmission), or (ii) compete for parasite abundance to gain advantage in evading host immune capture (immune-mediated apparent competition) (Read and Taylor, 2001; Bell et al., 2006; McQueen and McKenzie, 2006; Mideo, 2009; Pollitt et al., 2011a). Primary infections can compromise the host immune system, changing intra-host resources, and this can lead to an increase in host susceptibility to secondary infections (de Roode et al., 2005; Bell et al., 2006; McQueen and McKenzie, 2006; Cornet and Sorci, 2010; Telfer et al., 2010). Thus, co-infections can arise when primary infections provide opportunities for secondary infections. Knowledge about multiple infections may help in monitoring health outcomes and inform how environmental perturbations can affect parasite prevalence (Knowles et al., 2013).

Environmental changes are evident in Arctic habitats. With sea ice coverage at a record low, considerable melting of permafrost and landscape changes (Comiso et al., 2008; Bintanja et al., 2013; Parkinson and Comiso, 2013; Tingley and Huybers, 2013), the northern latitude host–pathogen relationships will ostensibly change (Patz et al., 1996, 2002; Kutz et al., 2009). Studies suggest that with a warmer habitat, vector distribution and parasite prevalence may increase due to favourable thermal conditions (Paaijmans et al., 2009, 2010; LaPointe et al., 2010; Garamszegi, 2011; Sternberg and Thomas, 2014). A recent report provides the first evidence of *Plasmodium* (the causative agent of malaria) transmission in Alaskan bird populations (Loiseau et al., 2012a). Numerous additional studies provide evidence to suggest that avian malaria and other blood parasites may increase in prevalence and northerly distributions with global warming (Patz et al., 1996; Githeko et al., 2000; Garamszegi, 2011; LaPointe et al., 2012; Zamora-Vilchis et al., 2012; Loiseau et al., 2013).

Arctic regions, predicted to undergo extensive environmental transformation (Comiso et al., 2008), make an excellent model system to study the impact of bioclimatic correlates on patterns of disease distribution and co-infections (Kutz et al., 2009). While a large number of disease studies of both human and avian populations have been performed in temperate and tropical regions (Reiter, 1998; Sattenspiel, 2000; Beadell et al., 2004; Szymanski and Lovette, 2005; Hellgren et al., 2007; Chasar et al., 2009), few have investigated Arctic regions. Motivations for conducting studies in Arctic regions include: (i) these regions harbour relatively low biological diversity, (ii) naïve host populations may be vulnerable to invasion, and (iii) direct anthropogenic changes are not as pronounced (Jetz et al., 2007; Alessa and Chapin, 2008; Kutz et al., 2009; Lafferty, 2009). Combined, these factors suggest that Arctic regions will have a rapid and quantifiable response to environmental disturbance (Kutz et al., 2009), and further compel the development of models that focus on bioclimatic perturbations.

Here, we studied the co-infection status of four genera of avian blood parasites: *Plasmodium* spp., *Haemoproteus* spp., *Leucocytozoon* spp., and *Trypanosoma* spp. (Valkiūnas, 2005; Pollitt et al., 2011b). These ubiquitous haematozoan (protozoan) parasites can cause mortality and morbidity in naïve songbird communities (van Riper et al., 1986; Atkinson et al., 1995; Knowles et al., 2010; Olias et al., 2011; Pollitt et al., 2011b; LaPointe et al., 2012), and are transmitted by numerous vectors: *Plasmodium* spp. by mosquitoes (*Culicidae*), *Haemoproteus* spp. by biting midges (*Ceratopogonidae*), *Leucocytozoon* spp. by blackflies (*Simuliidae*) and *Trypanosoma* spp. by a variety of arthropods (*Simuliidae*, *Culicidae*, *Ceratopogonidae*, *Hippoboscidae* and *Dermanyssidae*) (Baker, 1976; Molyneux, 1977; Miltgen and Landau, 1982; Votýpka and Svobodová, 2004; Valkiūnas, 2005; Valkiūnas et al., 2011). As avian haematozoa are widespread (Valkiūnas, 2005; van Rooyen et al., 2013b) and host populations may be exposed to different parasites across a variety of ecological habitats, investigating haematozoan communities may shed light on host–parasite interactions (Bensch et al., 2007; van Rooyen et al., 2013a).

Currently, the effects of co-infections by multiple haematozoans are poorly understood and reported results remain inconclusive. For instance, Deviche et al. (2010) found a positive relationship between *Trypanosoma* and *Haemoproteus*, and no association between *Haemoproteus* and *Leucocytozoon* in White-winged Crossbills, while van Rooyen et al. (2013a) found no relationship between *Plasmodium* parasitemia and the presence of *Leucocytozoon* parasites or host fitness cost, although co-infections with either *Plasmodium* or *Haemoproteus* and *Leucocytozoon* were common. Other studies have examined effects of co-infections on reproductive success and body conditions (Sanz et al., 2001; Marzal et al., 2008). Palinauskas et al. (2011), investigating two *Plasmodium* spp., found synergetic effects of co-infections dependent on host species and discovered that co-infections were more virulent than single infections. Avian haematozoan parasites of the Arctic offer a relatively simple platform that may help to clarify the complexity of within-host parasite relationships.

Through molecular and computational approaches, we believe that we provide the first comprehensive study of blood-borne parasites in Alaskan bird populations. The aims were to determine over a latitudinal gradient: (i) the prevalence variation of *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma* parasites within a diverse group of songbirds and; (ii) the environmental correlates associated with higher prevalence of each of these parasites and their co-infection trends. The prediction was that specific bioclimatic drivers correlate with the prevalence of co-infections and the likelihood of contracting certain haematozoan parasites. In addition, based on several reports suggesting that environmental factors, and temperature in particular, impact blood-borne parasite development and distribution, presumably temperature variables would be key predictors of parasite prevalence and geographical distribution (Garamszegi, 2011; LaPointe et al., 2012; Ramey et al., 2012; Loiseau et al., 2013). Moreover, as *Leucocytozoon* is able to persist at higher elevations and in colder regions (Haas et al., 2012; van Rooyen et al., 2013a), high prevalence of infection and co-infection of *Leucocytozoon* in northern Arctic regions are to be expected. In contrast, due to thermal and vector constraints (van Riper et al., 1986; LaPointe et al., 2010), a low prevalence of *Plasmodium* infection was predicted, compared with other haematozoans, in the Arctic.

## 2. Materials and methods

### 2.1. Study area and sampling methods

Samples were collected in 2011 and 2012 at 13 sites over a latitudinal gradient in Alaska: one site at Anvik and Yellow River Junction (63°N, 161°W), four sites in the vicinity of Anchorage (61°N, 150°W), one site in Fairbanks (65°N, 148°W), two sites on the Denali Highway (63°N, 147°W) and five sites at Coldfoot (67°N, 150°W) (for detailed GPS coordinates see Supplementary Table S1). In total, 913 individuals were captured representing 47 bird species, 13 of which were resident species ($n = 164$) and 34 migratory species ($n = 749$ total, which included hatch year birds ($n = 197$)). Birds were caught using mist nets and banded. Birds were aged (after hatch year (AHY) versus hatch year (HY); adults and juvenile birds, respectively) by examination for the presence or absence of a cloacal protuberance/brood patch or by skull

pneumatisation. Blood samples were collected from the brachial vein and stored in lysis buffer (10 mM Tris–HCl pH 8.0, 100 mM EDTA, 2% SDS). Duplicate blood slides were made and stained using a Giemsa 1:10 buffer solution. Slides were examined on a Nikon e100 microscope using methods described by Valkiūnas et al. (2008). The bird sampling methods in Alaska were approved by the Institutional Animal Care and Use Committee (IACUC) of the San Francisco State University, USA. Sample collection was performed under permit 161 supplied by the United States Geological Survey Bird Banding Laboratory.

### 2.2. Parasite screening

Genomic DNA (gDNA) was extracted from whole blood of birds following a DNeasy kit protocol (Qiagen, Valencia, CA, USA). DNA extractions were verified with primers that amplify the brain-derived neurotrophic factor (BDNF) (Sehgal and Lovette, 2003) or by determining DNA presence with a NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Samples with low NanoDrop readings were re-extracted to assure good quality DNA. Blood samples were then screened for *Plasmodium* spp., *Haemoproteus* spp., *Leucocytozoon* spp., and *Trypanosoma* spp. with their respective primers. To screen for *Plasmodium* and *Haemoproteus* spp., we amplified 524 bp of the mitochondrial cytochrome oxidase subunit *b* (cyt *b*) gene using a nested PCR described in Waldenström et al. (2004) with the primers HaemNF/HaemNR2 and HaemF/HaemR2. All *Plasmodium* and *Haemoproteus* PCRs were performed in 25 µl volumes and were accompanied by negative ($ddH_20$) and positive controls (samples from infected birds as confirmed by sequencing and microscopy).

To screen for *Leucocytozoon* spp., 480 bp of the mitochondrial cyt *b* gene was amplified using a nested *AccuPower*® PreMix PCR (Bioneer, Alameda, CA, USA) with the primer sets HaemNR3/HaemNF1 and HaemFL/HaemR2L as described in Hellgren et al. (2004). The samples were run with a slightly modified version of the Hellgren et al. (2004) PCR thermal profile. The thermal profile started with 3 min of denaturation at 94 °C, followed by 35 cycles at 94 °C for 30 s, 54.5 °C for 30 s, 72 °C for 60 s, and ended with an elongation step at 72 °C for 10 min. Two microlitres of the first PCR product as the template for the second PCR. The second nested PCR thermal profile was identical to the first PCR thermal profile.

Using the nested primer sets Tryp763/Tryp1016 and Tryp99/Tryp957, with AccuPower® HotStart PCR PreMix (Bioneer, Alameda, CA, USA), 770 bp of the ssrRNA gene of *Trypanosoma* was amplified following the PCR thermal profile described in Valkiūnas et al. (2011). For both *Leucocytozoon* and *Trypanosoma* PCR protocols, primers were mixed with purified water and added to the PCR tubes to make a total volume of 20 µl including the DNA template. Positive controls included known infections, which were evident from sequencing and microscopy results (Valkiūnas, 2005), as well as negative controls using purified water in place of DNA template.

The PCR products were run out on a 1.8% agarose gel using 1 × Tris/Borate/Ethylenediaminetetraacetic (TBE) buffer solution and visualised by ethidium bromide staining under ultraviolet light to check for positive infections. PCR products from infected birds were purified using ExoSAP-IT (following the manufacturer's instructions; USB Corporation, Cleveland, OH, USA) and bidirectional sequencing was performed in-house to identify parasite lineages (BigDye® version 1.1 sequencing kit, Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3100™ automated sequencer (Applied Biosystems). Forward and reverse sequences were edited and consensus sequences were obtained using Sequencher 4.8 (Gene Codes, Ann Arbor, MI, USA). For select samples with minor sequencing noise that would suggest a possible co-infection, blood slides were examined by microscopy ($n = 13$). Distinct parasite lineages for *Haemoproteus*, *Plasmodium* spp., *Leucocytozoon* spp. and *Trypanosoma* spp. were verified by the BLAST algorithm. Novel sequences ($n = 43$) obtained for this study were deposited in GenBank (Accession Nos. KF314757–KF314799).

### 2.3. Phylogenetic analysis

To determine the lineage diversity of *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp., phylogenetic analyses were used to develop two trees: (i) *Plasmodium* spp. and *Haemoproteus* spp., with the outgroup setting *Leucocytozoon fringillinarum* and (ii) *Leucocytozoon* spp., with the outgroup setting *Plasmodium gallinaceum*. No phylogenetic analysis was made for *Trypanosoma* spp. as only one lineage was found. Consensus sequences were aligned and concatenated using SEAVIEW (Galtier et al., 1996). Taxon sequences were standardised at 458 bp for the *Plasmodium* spp. and *Haemoproteus* spp. tree, and 411 bp for the *Leucocytozoon* spp. tree. Phylogenetic analyses were implemented using maximum-likelihood (ML) settings, sequenced divergence algorithms and phylogenetic signal in dataset ($P < 0.05$) using the permutation tail probability (PTP) test and computed using PAUP* 4.0 (Swofford, 2003. PAUP*: phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, Massachusetts). For comparison, a thorough ML search was performed using RAxML (Stamatakis, 2006). The support for the individual branches was estimated using ML bootstrap analyses running 1,000 replicates. In addition, Bayesian analyses were used to generate a phylogeny of cyt *b* lineages. Sequence data were analysed using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) and implementing the model (GTR + I + G) obtained from PAUP* Modeltest3.7, modelblockPAUPb10. Two Markov chains were run simultaneously for 10 million generations with sampling every 200 generations for a total of 50,000 trees, each, sampled from the posterior distribution. Those trees sampled prior to the runs reaching a split deviation frequency of 0.01 were discarded from the sample as "burn-in" and accounted for 25% of the trees. The remaining trees were used to calculate the posterior probabilities of the individual clades. All consensus Bayesian phylogenetic trees were viewed and edited on FigTree 1.4 (Rambaut, 2012. FigTree V1.4. University of Edinburgh, Edinburgh UK).

### 2.4. Statistical analysis of co-infection relationships

For each parasite genus (*Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma*), the relationship between individual parasite species in the absence or presence of other parasite species was investigated to determine parasite interactions (Table 1). The association between infection status and biological factors was tested for each parasite genus using generalised linear models (Proc GENMOD with binomial distribution of errors and logit link function) through SAS 9.1 software (SAS, 1999). The relationship between the independent variable "infection of one genus" and the dependent variable "infection status for other genera," was analysed with explanatory variables: latitude, year and the resident or migratory status of birds. All explanatory variables found to be non-significant were removed for each haematozoan model. All analysis was conducted using the SAS 9.1 software (SAS, 1999).

### 2.5. Modelling

Environmental data was collected for each location during the same season (Supplementary Table S1), using a set of environmental variables that included bioclimatic metrics at a 1 km resolution from the WorldClim data set ((Hijmans et al., 2005), http://www.worldclim.org) and elevation data from the Shuttle Radar Topography Mission (SRTM, National Aeronautics and Space

**Table 1**
Observed frequency of birds infected with an individual parasite genus in the absence (Neg.) or presence (Pos.) of another parasite genus. Our results yielded no significant *Plasmodium* and *Haemoproteus* co-infection.

| | *Plasmodium* (%) | *Haemoproteus* (%) | *Leucocytozoon* (%) | *Trypanosoma* (%) |
|---|---|---|---|---|
| *Plasmodium* | | | | |
| Neg. (850) | – | 14.82 | 34.82 | 11.53 |
| Pos. (64) | – | **0.00**[b] | 29.69 | 9.38 |
| *Haemoproteus* | | | | |
| Neg. (788) | 8.12 | – | 36.55 | 11.29 |
| Pos. (126) | **0.00**[b] | – | **21.43**[b] | 11.90 |
| *Leucocytozoon* | | | | |
| Neg. (599) | 7.51 | 16.53 | – | 4.84 |
| Pos. (315) | 6.03 | **8.57**[a] | – | **23.81**[b] |
| *Trypanosoma* | | | | |
| Neg. (810) | 7.16 | 13.70 | 29.63 | – |
| Pos. (104) | 5.77 | 14.42 | **72.12**[b] | – |

Statistical significance is indicated with bold ($P < 0.01$).
[a] $P = 0.0003$.
[b] $P < 0.0001$ (see Section 2.4).

Administration, USA). We examined the ability of environmental variables to predict the prevalence of each of the four parasite genera, in addition to the prevalence of each of the pairwise co-infections, by constructing iterative regression trees (collectively known as random forests) using the package randomForest (Liaw and Wiener, 2002) in the R framework (R Development Core Team: R, 2004. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Our final model utilised the entire bird sample dataset. The percentage of variation reported, as explained by full models, is the out-of-bag amount of variation (test data that original models never saw) explained by environmental predictor variables. An increase in mean square error is calculated as the average increase in squared residuals of a test set when an explanatory variable is randomly permuted. A given variable with little predictive power will not cause a substantial difference in model residuals when permuted; therefore a higher increase in mean square error is indicative of a more important variable. A larger increase in node purity represents more homogeneous calls within partitions of the data. Both measures assess predictors of parasite prevalence.

## 3. Results

### 3.1. Parasite diversity and geographic distribution

Overall, 913 birds of 49 species were screened and 16 *Plasmodium* spp. lineages (from a total of $n = 64$ infected with this genus), 24 *Haemoproteus* spp. lineages ($n = 126$), 47 *Leucocytozoon* lineages ($n = 315$) and one *Trypanosoma avium* lineage ($n = 104$) were identified. There were newly discovered lineages for *Plasmodium* ($n = 1$), *Haemoproteus* ($n = 17$), and *Leucocytozoon* ($n = 25$). Resident birds ($n = 78$) and migratory HY birds ($n = 72$) had lower parasite prevalence than migratory AHY birds ($n = 323$) (Supplementary Table S2). All four genera were generalist parasites (meaning that many of the lineages were identified in more than one host species) (Zamora-Vilchis et al., 2012).

Parasite distribution was dependent on parasite genus and site. The most southerly location, Anchorage (61°N, 150°W), yielded the highest number of haematozoan infections for both total infected birds ($n = 142$) and infected resident and hatch-year birds ($n = 72$). The most northerly location, Coldfoot (67°N, 150°W), yielded no resident or hatch-year birds with *Plasmodium* infections, consistent with the findings presented in Loiseau et al. (2012a). In the analysis, *Plasmodium* infections detected in Coldfoot were found only in migratory bird species ($n = 16$).

*Plasmodium* spp. prevalence (7%) in Alaska was found to be lower than that of *Haemoproteus* spp. (14%), *Leucocytozoon* spp. (35%) and *Trypanosoma* (11%). In addition, while *Haemoproteus* spp. are present in Coldfoot, the number of resident birds infected with *Haemoproteus* spp. was low at this location ($n = 3$). *Leucocytozoon* spp. had the highest prevalence and diversity across all sites. In contrast to other haematozoan parasites, there were a high number of *Leucocytozoon* infections in resident birds ($n = 26$) in Coldfoot. In total, 47 lineages of *Leucocytozoon* were found, compared with 24 lineages of *Haemoproteus* and 16 of *Plasmodium*. Only one ssrRNA lineage of *Trypanosoma* was detected in Alaska, *T. avium* (GenBank Accession No. AY099320.2).

### 3.2. Plasmodium, Haemoproteus and Leucocytozoon phylogenetic relationships

The phylogenetic relationships of both *Plasmodium*/*Haemoproteus* and *Leucocytozoon* Bayesian consensus trees revealed groups of diverse haemosporidian lineages (Fig. 1). Seventeen novel lineages were identified for *Haemoproteus* (denoted as H.AK) and one new lineage was identified for *Plasmodium* (P.AK01) (Fig. 1A). For *Leucocytozoon*, 25 novel lineages were identified (denoted with L.AK) (Fig. 1B). P.AK01 was detected in only two migratory AHY birds. New lineages for *Haemoproteus* and *Leucocytozoon* were predominantly in migratory and/or AHY birds, with few infections in resident and/or HY birds. Both phylogenetic trees contained a distinct cluster for parasites that infect resident birds.

### 3.3. Trends in prevalence of co-infections

The biological factors (explanatory variables) important to *Leucocytozoon* infections were *Haemoproteus* infection, *Trypanosoma* infection and Latitude. *Trypanosoma* prevalence was associated with the explanatory variables: *Leucocytozoon* infection, year and birds' resident/migratory (RM) status. *Plasmodium* prevalence was associated with the variables: *Haemoproteus* infection, latitude, year and RM status. Lastly, the model for *Haemoproteus* utilised the explanatory variables: *Plasmodium* infection, *Leucocytozoon* infection, *Trypanosoma* infection, latitude, year and RM status.

Trends in parasite co-infections varied according to which parasites contributed to the co-infection. Interestingly, we found *Leucocytozoon* prevalence in positive association with *Trypanosoma* prevalence ($x^2 = 50.7$; $P < 0.0001$) (Table 1). Furthermore, *Leucocytozoon* had a negative association with *Haemoproteus* infections.

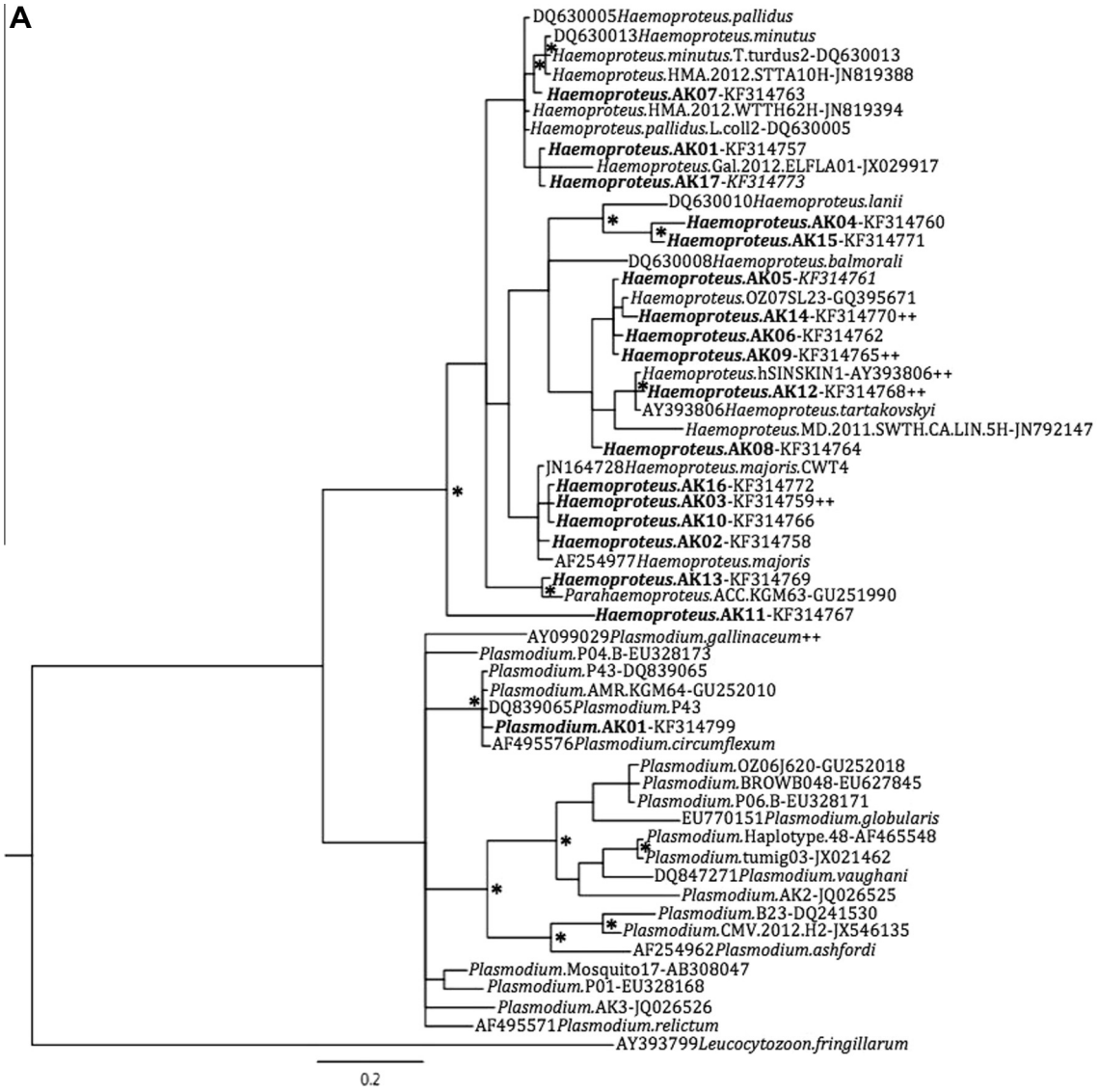


**Fig. 1.** Consensus Bayesian phylogenetic trees with bootstrap values >75% and/or posterior probabilities >0.95 denoted with *. Parasites found in resident birds denoted with ++. GenBank accession numbers are provided (numbers in front indicate GenBank sequences obtained for comparison). Novel lineages are identified in bold (A) *Plasmodium* and *Haemoproteus* tree (outgroup, *Leucocytozoon fringillarum*) and (B) *Leucocytozoon* tree (outgroup, *Plasmodium gallinaceum*).

In the presence of *Leucocytozoon* infection, there is a significant reduction in *Haemoproteus* prevalence ($\chi^2 = 13.2$; $P = 0.0003$) (Table 1). Our results yielded no *Plasmodium* and *Haemoproteus* co-infections (Table 1).

In addition, other factors were important for individual parasite prevalence. Latitude ($\chi^2 = 32.18$; $P = 0.0001$) was found to be a significant predictor for *Leucocytozoon* prevalence. We found an effect of year ($\chi^2 = 65.94$; $P < 0.0001$) and RM status ($\chi^2 = 9.98$; $P = 0.0016$) on *Trypanosoma* prevalence. Latitude ($\chi^2 = 23.16$; $P = 0.0001$), year ($\chi^2 = 10.81$; $P = 0.0010$) and RM status ($\chi^2 = 8.81$; $P = 0.003$) had significant effects on *Plasmodium* prevalence. Similarly, latitude ($\chi^2 = 11.05$; $P = 0.0260$), year ($\chi^2 = 17.87$; $P < 0.0001$) and RM status ($\chi^2 = 5.82$; $P = 0.0158$) were found to be important for *Haemoproteus* prevalence.

### 3.4. Habitat classification and co-infection relationships

We observed that a combination of temperature (BIO4, BIO1, BIO6), vegetation/tree cover (NDVI, TREE, Landcover) and precipitation/surface moisture (BIO19, BIO12) were important factors explaining prevalence for *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma* prevalence (Supplementary Fig. S1). We also found that the prevalence of co-infection could, in some cases, be largely predicted using only a few environmental variables. In particular, up to 35% of the variation in co-infection of *Leucocytozoon* and *Trypanosoma* was explained by two environmental variables: minimum temperature of the coldest month (BIO6) and tree cover (see Section 2.5 for details of 'out-of-bag' estimates) (Fig. 2B). This relationship between environment and prevalence of co-infection was used to construct a map showing the spatial predictions of prevalence of *Leucocytozoon* and *Trypanosoma* across the Arctic in Alaska (Fig. 3). Our map suggests that not all regions in Alaska are hotspots for co-infection and patterns of infection are not solely latitudinal and/or temperature driven. Furthermore, we found a relationship between environmental correlates and the prevalence of co-infections (Fig. 3B). Prevalence of co-infection correlated with Alaskan regions of high temperature seasonality (the difference between annual maximum and minimum temperatures) (Fig. 3A).

The prevalence of co-infection and environmental variables could be driven solely by the prevalence of one of the parasites making up the co-infection. In our study, high correlation was

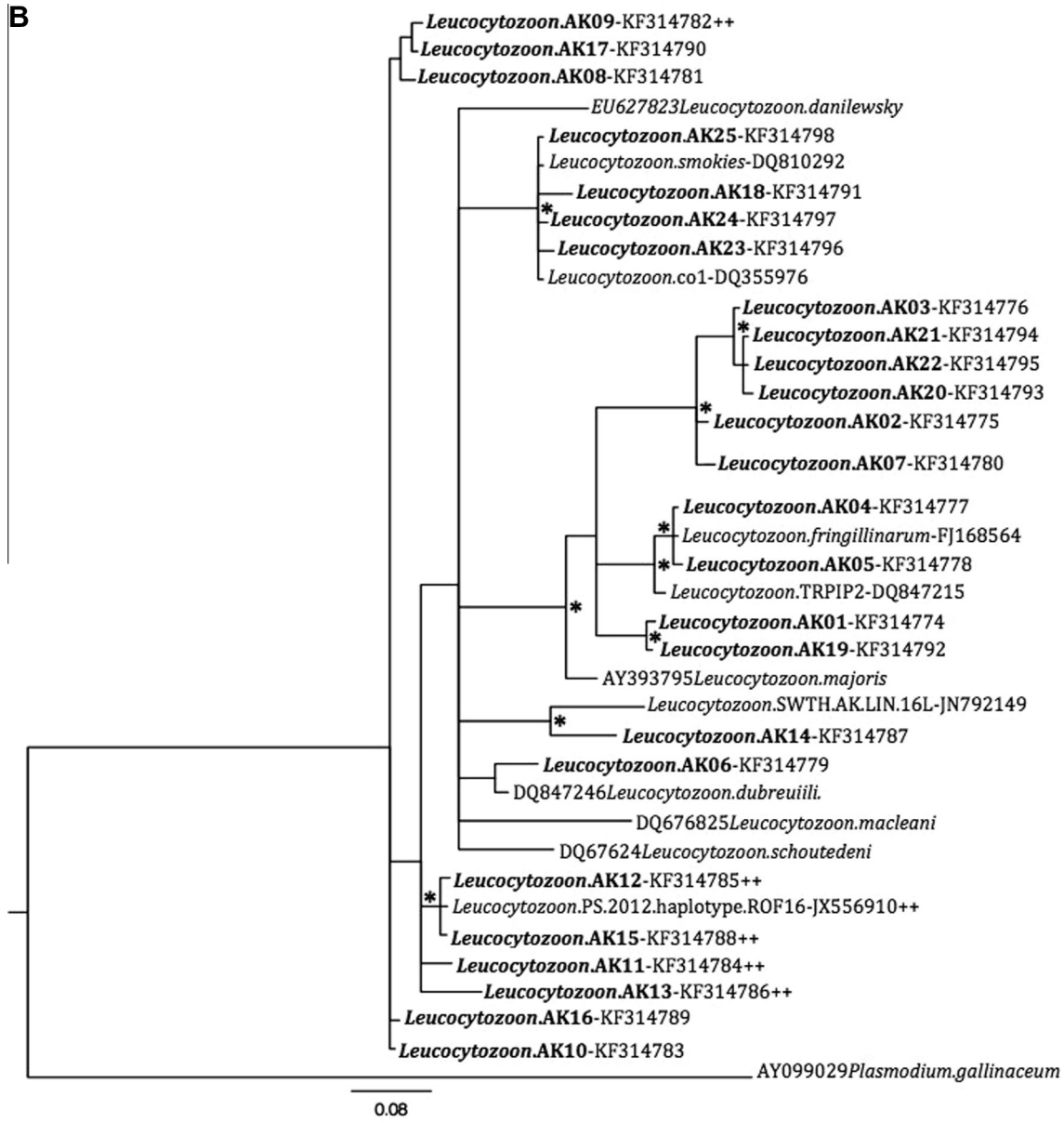


**Fig. 1** (*continued*)

observed between the prevalence of *Trypanosoma* and co-infections of *Leucocytozoon-Trypanosoma* ($R^2$ = 0.96). However, a relatively lower correlation was seen between co-infections of *Leucocytozoon–Trypanosoma* and *Leucocytozoon* infections alone (without co-infections) ($R^2$ = 0.49). Furthermore, *Leucocytozoon* prevalence alone was found to significantly correlate with different environmental variables than those explaining variation in *Leucocytozoon–Trypanosoma* co-infections. The most important variable explaining *Leucocytozoon* prevalence was temperature seasonality (BIO4); this one variable explained 18% of prevalence (Fig. 2A). Bioclimatic measures that capture aspects of precipitation (BIO6, BIO9, BIO12) and tree cover were also found to be important predictors (Fig. 2A).

## 4. Discussion

To date, several studies have demonstrated associations between avian blood parasites and a variety of environmental conditions (Chasar et al., 2009; Sehgal, 2010; Haas et al., 2012; LaPointe et al., 2012; Loiseau et al., 2012b; Pérez-Rodríguez et al., 2013). However, to our knowledge, this study offers the first insight into the relationships between ecological determinants and prevalence of avian haematozoan co-infections. Here, environmental variables and multiple parasite interactions that may affect parasite–host systems and the patterns of prevalence for concurrent infections are identified. Trends for co-infection distributions were predicted to be dependent on environmental variables. The predictions were supported, as temperature seasonality, precipitation and tree cover were found to be important predictors of parasite prevalence. This research in Alaska, an area subject to increasing climate warming (Ramirez and Jarvis, 2008), offers the prospect to develop a baseline model in predicting how habitat changes may affect parasite distributions and co-infection interactions.

In our study, *Leucocytozoon* exhibited the highest diversity, prevalence and prevalence of co-infection with other avian haematozoans. Interestingly, there is also a possible competitive exclusion between *Leucocytozoon* and *Haemoproteus* parasites, with an

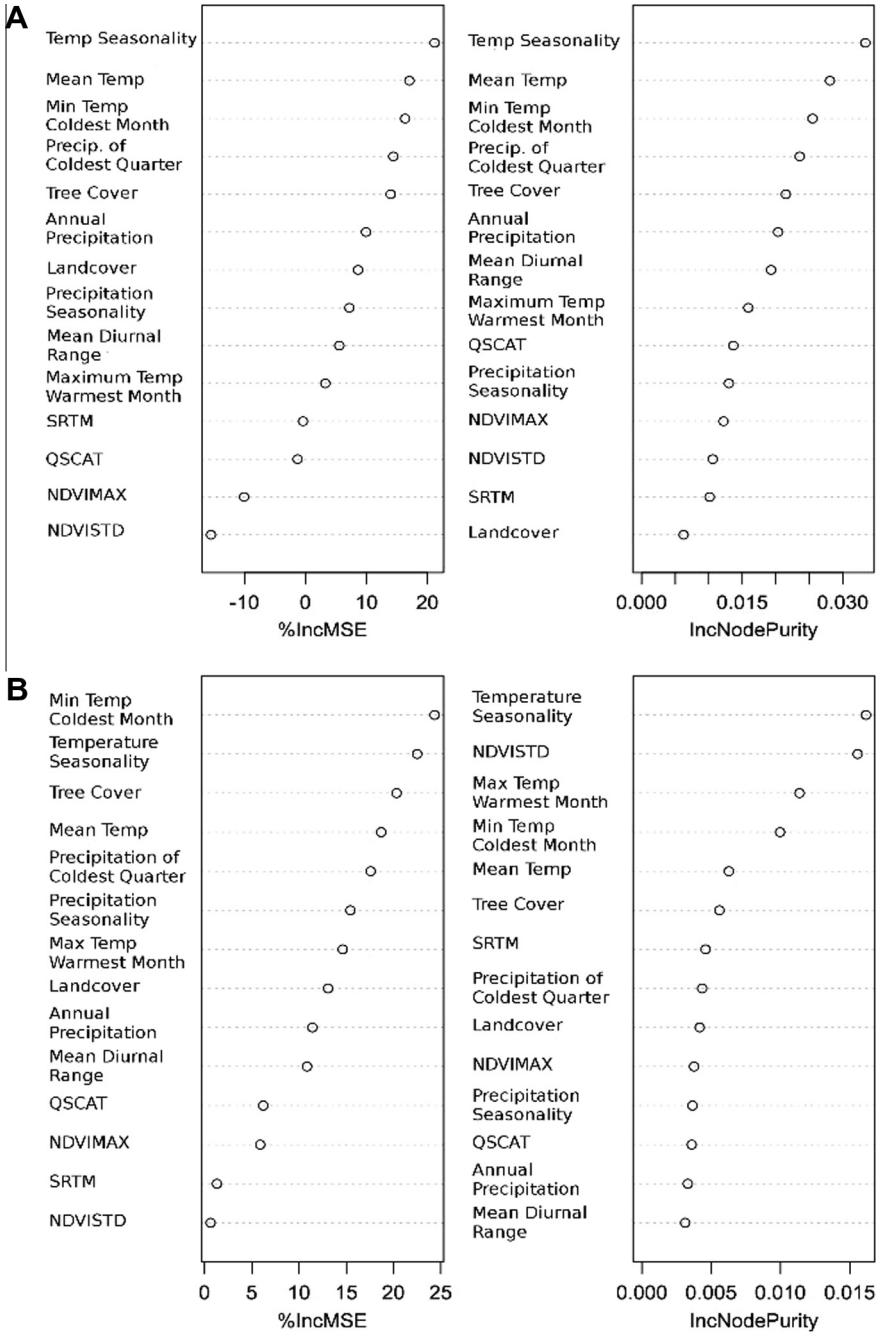


**Fig. 2.** Importance scores for 14 bioclimatic variables related to *Leucocytozoon* and *Leucocytozoon–Trypanosoma* prevalence, determined by random forest models. (A) Temperature seasonality (BIO4) was the most important variable (18%) explaining *Leucocytozoon* prevalence. (B) Minimum (Min) temperature (Temp) of the coldest month (BIO6) and tree cover (Tree) were the most important variables (35%) explaining *Leucocytozoon–Trypanosoma* co-infection. Variable importance of predictors was measured using both Increase in Mean Square Error (IncMSE) and Increase in Node Purity (IncNodePurity) (see Section 2.5 for details). NDVIMAX, normalised difference vegetation index that measures annual maximum biomass or canopy cover; NDVISTD, normalised difference vegetation index that measures the standard deviation of vegetation density; QSCAT, Quikscat captures a measure of surface moisture: SRTM, Shuttle Radar Topography Mission, measures elevation.

inverse association identified between these two parasite groups (Table 1). It is possible that a particular host immune system may be more competent at clearing one parasite compared with another, intra-host niches could enable one species infection success over another (McQueen and McKenzie, 2006), or that *Leucocytozoon* is more effective at utilising host resources (higher prevalence of *Leucocytozoon* compared with *Haemoproteus* was found). Given its high prevalence, *Leucocytozoon* may not be imposing as severe a fitness cost on its hosts, allowing it to persist independently or with other parasites (e.g. *Trypanosoma*). It can also be conceived that under extreme environmental conditions, parasites have adapted curbed virulence factors to ensure host conservation and self-preservation. Consequently, under extreme habitat conditions, there may be an increase in the prevalence of co-infections. Experimental co-infections between *Leucocytozoon* and other avian parasites could help to elucidate pathogenicity, since little is currently known about these co-infection relationships in Arctic regions.

*Leucocytozoon* spp. are prevalent in the Arctic. This supports previous reports that they have a higher cold tolerance than other

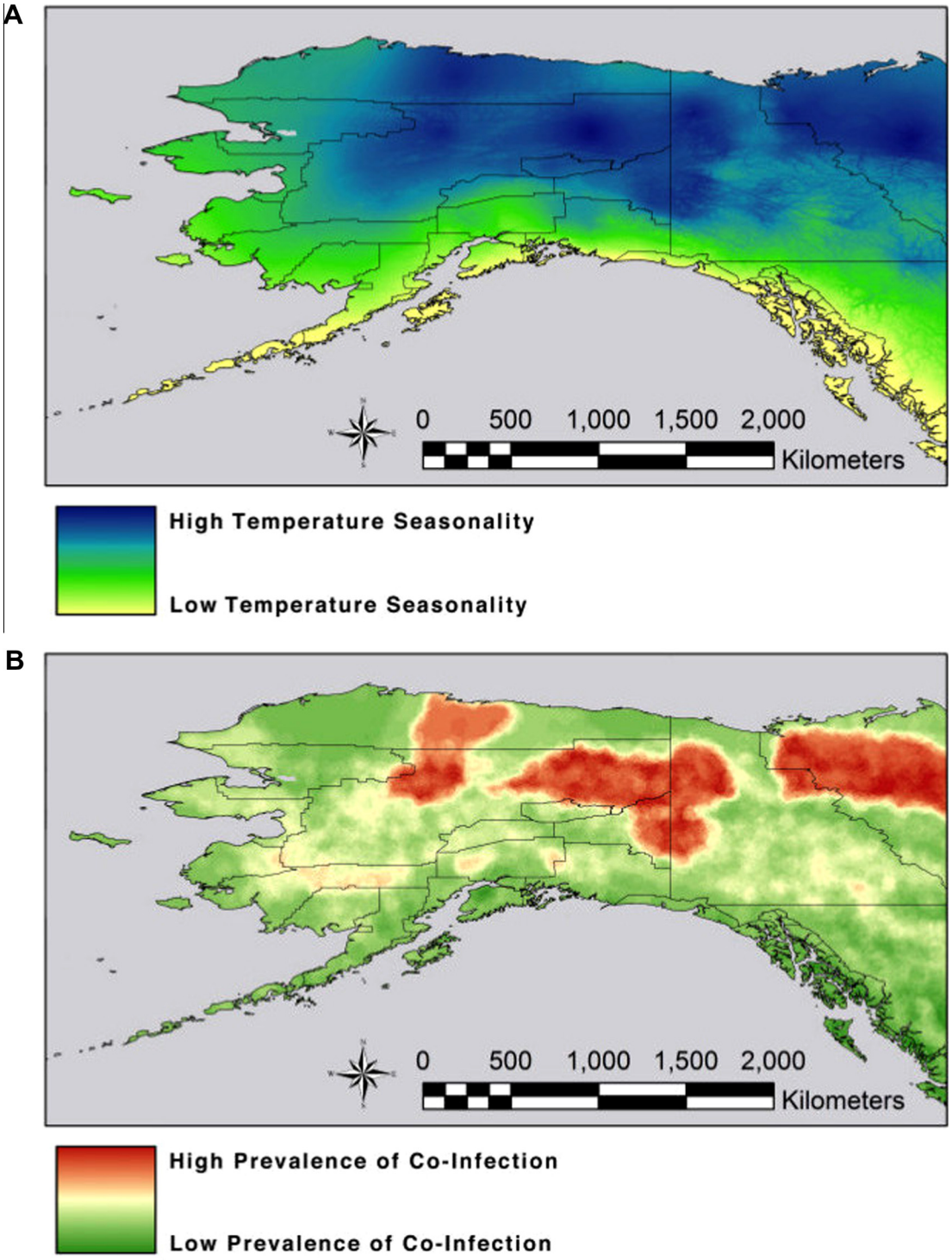


**Fig. 3.** Temperature seasonality and prevalence of co-infection in Alaskan regions. (A) Interpolated map representing temperature seasonality (BIO4) and (B) a predicted prevalence of *Leucocytozoon–Trypanosoma* co-infections, as determined by random forest models.

haematozoans (*Plasmodium* in particular) and the ability to complete their life cycles at higher elevations and latitude (Haas et al., 2012; van Rooyen et al., 2013a). In addition, studies show mountainous regions are favourable habitats for blackflies, vectors for *Leucocytozoon* (Haas et al., 2012; Štangler et al., 2013; van Rooyen et al., 2013b). Indeed, there is an observed high prevalence of *Leucocytozoon* in resident birds and at the highest latitude site (Coldfoot). A large number of novel *Leucocytozoon* lineages were identified ($n = 25$), also suggesting a high diversity and the potential evolution of tolerant lineages in these Arctic regions. The favourable ecological conditions for *Leucocytozoon* and its vector allow this parasite to flourish in Alaska as the most prevalent and genetically diverse of the avian haematozoan parasites.

Numerous studies, including the present, suggest the importance of temperature seasonality and vegetation in the patterns of pathogen distribution (Wilson et al., 2002; Yasuoka and Levins, 2007) (Fig. 2). The predictive map shows select Alaskan regions have higher prevalences of co-infection, and these hotspots

suggest that infection is not solely explained by latitude and temperature (Fig. 3). The models included only a few ecological variables that could potentially affect parasite life cycles; it is likely that other factors such as vector abundance and bird migration also help to drive parasite prevalence (Ramey et al., 2012; Pérez-Rodríguez et al., 2013).

The *Plasmodium–Haemoproteus* and *Leucocytozoon* Bayesian consensus trees revealed specific clusters of parasites that infect resident Alaskan birds, yielding insight into parasite evolution and vector ecology (Fig. 1). These parasites may have adapted to Arctic temperatures and/or evolved to thrive in Arctic birds. It is clear that avian malaria parasites diversify and co-evolve with their avian hosts (Ricklefs and Fallon, 2002; Medeiros et al., 2013). Additional research on Arctic insect vectors would provide novel insight into parasite pathogenicity, distribution and acclimatisation.

This study reveals great variation in the prevalence of avian haematozoans and noteworthy parasite relationships. There is a direct correlation between the prevalence of *Trypanosoma* and the likelihood of *Leucocytozoon* co-infection (75%) (Supplementary Fig. S1). These results support findings by Sehgal et al. (2010), conducted on African rainforest birds, in which temperature and surface moisture were found to be important factors for *Trypanosoma* prevalence. As *Leucocytozoon* prevalence is also affected by temperature and precipitation, it is expected these environmental variables would also be important predictors for *Leucocytozoon–Trypanosoma* co-infection. Indeed, the predictive map suggests the high prevalence of *Leucocytozoon* and *Trypanosoma* co-infection is correlated with regions of high temperature seasonality (Fig. 3A). Van Rooyen et al. (2013b), investigating European haemosporidians, put forth the observation that *Leucocytozoon* may act as an opportunist parasite, inhabiting a host only when another parasite is present and the host immune system is compromised. The *Leucocytozoon* system in Europe may differ significantly from Alaska. While a number of *Leucocytozoon* co-infections were observed ($n = 158$), a larger number of infections solely harbouring *Leucocytozoon* spp. ($n = 207$) were found.

Moreover, there were high correlations between the prevalence of solitary *Trypanosoma* and *Leucocytozoon–Trypanosoma* co-infections, and lower correlations between *Leucocytozoon–Trypanosoma* co-infections and solitary *Leucocytozoon*. These suggest that: (i) co-infections are primarily limited/driven by the prevalence of *Trypanosoma* at given sites, and (ii) *Leucocytozoon* infections are driven by environmental factors outside those contributing to *Leucocytozoon–Trypanosoma* co-infections or solitary *Trypanosoma* infections. To date, avian trypanosomes are less studied compared with other members of the trypanosome group (Valkiūnas et al., 2011; Votýpka et al., 2012). Using a molecular approach analysing the ssrRNA gene (Sehgal et al., 2001; Valkiūnas et al., 2011), similar to Deviche et al. (2010), only *T. avium* was detected in the samples. The lack of diversity in *Trypanosoma* infection may parallel the generally lower, overall biodiversity in Arctic regions (Kutz et al., 2009). Further monitoring of *Trypanosoma* prevalence in this region is warranted, as its low diversity may suggest a slower emergence in Arctic regions and additional studies may reveal specific habitat and host preferences for trypanosomes.

Supporting the predictions that *Plasmodium* would have lower prevalence in Arctic regions, only one *Plasmodium* parasite, lineage *Plasmodium* P43, was found in resident and HY birds of Alaska. The *Plasmodium* P43 lineage has a 1 bp difference from the known *Plasmodium circumflexum* lineage TURDUS-1 (GenBank No. JN164734). In addition, one new *Plasmodium* lineage (P.AK01) was identified. However, as the novel lineage was not detected in Alaskan resident or HY birds, there is no current evidence of its transmission in the Arctic. Temperature was shown to be an important factor in explaining variation in *Plasmodium* prevalence, likely serving as a constraint to sporogonic development (LaPointe et al., 2010). Therefore, it is not surprising that no *Plasmodium* was found in Coldfoot, in either HY or resident birds, confirming previous findings (Loiseau et al., 2012a).

This study did not report any instances of *Plasmodium–Haemoproteus* co-infection. The lack of finding *Plasmodium–Haemoproteus* co-infections may be due to limitations in methodology. Indeed, from one American Robin sample under microscopy, there was evidence of a positive *Haemoproteus* infection despite molecular tests suggesting solely *Plasmodium* infection. It is likely that nested cyt *b* PCR diagnostics underestimate co-infections of avian haemosporidian parasites, and it is suggested that a combination of both microscopy and molecular diagnostic techniques are necessary to accurately determine infection status (Valkiūnas et al., 2006). The possible *Plasmodium* and *Haemoproteus* primer selectivity problem that may arise utilising current, standard nested PCR protocols is an important issue that needs further investigation. Further research on parasite diversity would allow future primer designs that would more accurately detect specific blood-borne parasites.

The findings presented here should be considered an initial estimate of prevalence values, as there are limitations. Adding mark and recapture data could strengthen host population surveillance and help measure the impact of co-infection on host fitness. Future studies might also include a larger sampling pool and a greater number of sites. This would allow for more sampling of single host species and a comparison of these hosts and their parasites, which would add to a more in-depth examination of intra-host parasite relationships. A larger sample size would also allow for modelling of solely resident and HY birds, providing insight into the types of parasites infecting local Alaskan birds and any annual or seasonal variations in prevalence at a given site. In addition to blood sampling at more Alaskan sites, the intention is to include vector sampling, as biting Diptera are common in Arctic regions (Sailer, 1954; Sommerman et al., 1955), yet it is currently unknown which particular insect species transmit these parasites. Clearly, the distribution and abundance of vectors will affect the prevalence of blood-borne parasites and although they are common, relatively little is known about how the insect vectors affect haematozoan transmission in Alaska. A better understanding of parasite vector distributions would further these initial findings and aid in refining predictive models of co-infections.

The findings presented here provide further insight into host–parasite systems, the distribution of blood parasites in Arctic regions with environmental perturbations and the relationship of co-infecting parasite communities. With environmental and climate changes, vector populations may adapt and begin to colonise colder regions normally immune to such threats, thereby increasing both the prevalence of individual parasites and of multiple infections. Understanding the interactions between parasites and the drivers of co-infections are vital to both inform measures that would mitigate environmental disturbances by infectious diseases, and support the conservation of immunologically naïve host avifauna.

## Acknowledgements

We thank the Bureau of Land Management, Alaska and the Alaska Bird Observatory for permission to conduct field research. We are grateful to Laura Wilkinson, Jenny Carlson, Erika Walther, Anthony Cornel, Molly Dodge, Timothy Marzec, Edward Evans, Jim Schneidereit and Tija Altergott for their field and/or molecular help. This research was supported by grants from the AXA Research Fund, UK to C.L. and partially supported by the National Geographic Committee for Research and Exploration, USA grant 9072-12 to R.N.M.S.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.04.011.

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