

## A comparison of microscopy and PCR diagnostics for low intensity infections of haemosporidian parasites in the Siberian tit *Poecile cinctus*

Indrikis Krams<sup>1,2,\*</sup>, Valērija Suraka<sup>1,3</sup>, Dina Cīrule<sup>1,3</sup>, Markku Hukkanen<sup>4</sup>,  
Lea Tummeleht<sup>5</sup>, Pranas Mierauskas<sup>6</sup>, Seppo Rytkönen<sup>4</sup>,  
Markus J. Rantala<sup>7</sup>, Jolanta Vrublevska<sup>1</sup>, Markku Orell<sup>4</sup> & Tatjana Krama<sup>1,2</sup>

<sup>1)</sup> Institute of Systematic Biology, University of Daugavpils, LV-5401 Daugavpils, Latvia

<sup>2)</sup> Institute of Ecology and Earth Sciences, University of Tartu, EE-51014 Tartu, Estonia  
(\*corresponding author's e-mail: indrikis.krams@ut.ee)

<sup>3)</sup> Institute of Food Safety, Animal Health and Environment BIOR, LV-1076 Riga, Latvia

<sup>4)</sup> Department of Biology, FI-90014 University of Oulu, Finland

<sup>5)</sup> Department of Infectious Diseases, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, EE-51014 Tartu, Estonia

<sup>6)</sup> Department of Environmental Policy, Mykolas Romeris University, LT-08303 Vilnius, Lithuania

<sup>7)</sup> Department of Biology, FI-20014 University of Turku, Finland

Received 7 Dec. 2011, final version received 27 Mar. 2012, accepted 3 Feb. 2012

Krams, I., Suraka, V., Cīrule, D., Hukkanen, M., Tummeleht, L., Mierauskas, P., Rytkönen, S., Rantala, M. J., Vrublevska, J., Orell, M. & Krama, T. 2012: A comparison of microscopy and PCR diagnostics for low intensity infections of haemosporidian parasites in the Siberian tit *Poecile cinctus*. — Ann. Zool. Fennici 49: 331–340.

This study compares the sensitivity of two methods for diagnosing avian haemosporidian parasite (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) prevalence in Siberian tits *Poecile cinctus* breeding in northern Finland: a polymerase chain reaction (PCR)-based method and microscopy examination of blood smears. During molecular analysis, we used PCR screening and RFLP as a post-PCR diagnostic step. PCR screening detected haemosporidian parasites in the blood of 38 out of 40 (95%) breeding Siberian tits. Microscopy examination of blood smears and PCR diagnostics showed the same prevalences for *Leucocytozoon* spp. infections. In contrast, prevalences of *Haemoproteus* spp. and *Plasmodium* spp. determined by molecular methods were significantly higher than the prevalence determined by microscopy screening. However, habitat and sex effects on the total count of parasites, *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. counts appeared to be similar when assessed with either screening method, giving the same interpretation for the ecological hypotheses tested. Finally, this study shows that transmission of avian malaria can occur in the area of the Arctic Circle in northern Europe.

## Introduction

Avian malaria, haemoproteosis and leucocytozoonosis are diseases caused by *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites of the apicomplexans (Valkiūnas *et al.* 2005). Recent results by Outlaw and Ricklefs (2011) clearly demonstrated that the genera of *Leucocytozoon*, *Haemoproteus* and *Plasmodium* are genetically closely related to form a sister group referred to as haemosporidian parasites (Perkins & Schall 2002, Ricklefs *et al.* 2004, Pérez-Tris & Bensch 2005, Szollosi *et al.* 2011). Haemosporidian parasites have been described in many bird groups and from many geographical areas (Bennett 1993, Bennett *et al.* 1993, Bennett *et al.* 1994, Valkiūnas 2005, Garamszegi 2011). With few exceptions, haemosporidian parasites occur worldwide, due to the high mobility and migration patterns of most host species (Spielman & D'Antonio 2001). Although these parasites are most readily seen in peripheral blood, they initially invade other internal organs, such as the liver, where they may remain for many years (Cox 1993). It has been shown that infections of haematozoa are costly to host reproduction (Merino *et al.* 2000, Martínez-De la Puente *et al.* 2010) and haemosporidian parasites may play a crucial role in the evolution of avian life histories (Møller 1997, Ots & Hōrak 1998, Wiehn *et al.* 1999, Martínez-De la Puente *et al.* 2011). All haemosporidians have complex life cycles requiring vectors for transmission (Cox 1993, Bush *et al.* 2001). Avian malaria consists of a diverse group of parasites that are transmitted by dipteran insects (Bennett & Coombs 1975, Atkinson & van Ripper 1991, Cox 1993, Bush *et al.* 2001) infecting red blood cells in their vertebrate host and also white blood cells in the case of *Leucocytozoon* (Atkinson & van Ripper 1991, Valkiūnas 1993).

Most of the current data on haemosporidians, their distribution, vectors, parasite–host interactions and seasonality of infections have been collected primarily by microscopy (Atkinson & van Ripper 1991, Valkiūnas 2005). New molecular techniques have been considered to improve the detection of vector-borne blood parasites (Bensch *et al.* 2000, Ricklefs & Fallon 2002, Waldenström *et al.* 2002, Waldenström *et al.*

2004). The prevalence of these parasites has been found to be significantly higher than previously considered. According to some studies (Jarvi *et al.* 2002, Durrant *et al.* 2006), PCR tests were many-fold better than microscopy for detecting chronic blood parasite infections. However, the study by (Valkiūnas *et al.* 2008) obtained comparable infections rates of haemosporidian parasites when comparing microscopy to PCR-based methods. They demonstrated that both methods slightly underestimate the overall prevalence of infections. Although Valkiūnas *et al.* (2008) verified that microscopy is a reliable tool in detecting haemosporidians in many bird species, Jarvi *et al.* (2002) suggested that PCR diagnostics may be more sensitive in the case of low intensity infections. It has recently been shown (Garamszegi 2010) that relative to microscopy methods, the PCR-based molecular methods generally provide higher estimates for *Plasmodium* spp., and comparable estimates for species of *Haemoproteus* and *Leucocytozoon*.

There is now a range of highly sensitive PCR-based assays to detect the presence of the haemosporidians *Plasmodium* spp. and *Haemoproteus* spp. in samples of bird blood. However, the high degree of sequence conservation between species of *Plasmodium* and *Haemoproteus* presents difficulties for designing a PCR test to differentiate between them (Bensch *et al.* 2004, Martínez *et al.* 2009). So far, no such assay based purely on PCR has been forthcoming, and existing PCR screening protocols co-amplify both genera of haemosporidian parasites, relying on direct sequencing, or RFLP analysis (Beadell & Fleischer 2005) to separate the genera. There is also significant sequence conservation between species of *Plasmodium*, *Haemoproteus*, and a third haemosporidian parasite taxon, *Leucocytozoon*, which is sister to *Plasmodium* and *Haemoproteus* according to the mt cyt *b* phylogeny (Perkins & Schall 2002, Outlaw & Ricklefs 2011). Therefore, Cosgrove *et al.* (2006) suggested the use of a post-PCR diagnostic step, such as RFLP analysis or sequencing, to discriminate between *Plasmodium*, *Haemoproteus* and *Leucocytozoon* spp.

The aim of the present study was to compare the sensitivity of microscopy and PCR-based screening in determining the prevalence

of haemosporidian parasite infections in the Siberian tit *Poecile cinctus*. We collected blood smears and blood samples for molecular analysis while testing the effects of forest management on reproduction, haematological parameters and blood parasites of Siberian tits breeding in northern Finland. The results obtained by using microscopy screening have been published earlier (Krams *et al.* 2010). Here, we compare the results already published (Krams *et al.* 2010) with those obtained using molecular analysis.

## Material and methods

### Study site, the birds and habitat

The field study was carried out near Kuusamo (66°N, 29°E), northern Finland in 2007. Scots pine *Pinus sylvestris*, Norway spruce *Picea abies* and downy birch *Betula pubescens* are the dominant tree species in the study area. To attract Siberian tits, wooden nestboxes arranged in lines were used. During the breeding season of Siberian tits, all of the nest boxes were checked twice a week as a part of a long term project on the ecology of Siberian tits carried out by the University of Oulu. The total size of our study area was ca. 100 km<sup>2</sup>. Basic breeding parameters such as clutch size, brood size and the number of fledglings were recorded. We captured breeding adult Siberian tits in their nestboxes ( $n = 23$ ) when their nestlings were at the age of 9–12 days. All of the breeding individuals were individually marked, and sex and age of all adult Siberian tits were known (*see* Krams *et al.* 2010 for details).

The habitat of breeding Siberian tits differed markedly between two main patches of northern taiga: (i) moderately-managed forests, and (ii) heavily-managed areas. The heavily-managed areas had been extensively thinned, leaving only middle-sized pines. The moderately-managed areas included both areas thinned at least a decade ago and patches of old growth coniferous forests (Virkkala 1990) consisting of pines, spruces and an admixture of birches. The moderately-managed forests also comprised many dead trees which increased the quality of habitat for Siberian tits (Virkkala 1990, Orell *et al.*

1999). We had 12 and 11 nestboxes of Siberian tits in the moderately- and heavily-managed forests, respectively. We estimated distances from the nestboxes to the nearest lakes and streams by using GPS.

### Preparation and examination of blood smears

The blood samples were taken from a total of 45 (23 males and 22 females) breeding adult Siberian tits. We punctured the tarsal vein of each bird and prepared three individually marked slides following the recommendations of Bennett (1970) and Valkiūnas (2005). The blood smears were screened with a light microscope under oil immersion at 1000× magnification for *Haemoproteus* and *Plasmodium* and at 500× magnification for *Leucocytozoon*. Parasites were enumerated from 100 fields by moving the slide to areas where blood cells formed a monolayer for *Leucocytozoon* and from more than 200 fields for *Haemoproteus* and *Plasmodium*. For infected birds, parasite intensity was calculated as the number of parasites per 10 000 erythrocytes in randomly chosen fields of the blood smear, as recommended by Godfrey *et al.* (1987) and Valkiūnas (2005). Although species of *Trypanosoma* (the overall prevalence of infection was 20%) and *Hepatozoon* (4.4%) were also found during microscopic examinations of blood smears, they were not considered in the further analysis because we used PCR protocols to detect haemosporidian parasites only. Blood smears were screened by T.K., V.S. and D.C.

### Molecular analysis

We also collected blood (50–60 µl) from the same individuals as above for molecular analysis, and stored the samples in SET buffer (0.015M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) at –20 °C. We used the PCR protocol by Cosgrove *et al.* (2006), representing an improved modification of the nested PCR protocols by (Hellgren *et al.* 2004) and Waldenström *et al.* (2004) targeting mitochondrial cytochrome *b* (mt cyt *b*) genes. Although the PCR protocols by Hellgren *et al.*

(2004) and Waldenström *et al.* (2004) were supposed to use *Plasmodium*- and *Haemoproteus*-specific primers in the second round PCR, a significant coamplification of *Leucocytozoon* spp. may occur (Cosgrove *et al.* 2006). The protocol by Cosgrove *et al.* (2006) was used to check for the presence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites in samples of 40 Siberian tits (10 males and 10 females from both the moderately- and heavily-managed forests). We could not obtain enough blood from 5 Siberian tits (3 males and 2 females), and the molecular analysis was not performed for these birds. They also were not included in the subsequent comparison between molecular and microscopy methods of blood parasite detection.

For extractions of the DNA, the Invisorb® Spin Blood Mini Kit was used. The presence and quality of the extracted DNA was assessed by electrophoresis of 2 µl of the extract in 2% agarose containing ethidium bromide, and visualizing under UV light. In the first round we used primers HaemNF (5'-CATATATTAAGAGAAT-TATGGAG-3') and HaemNR2 (5'-AGAGGTGTAGCATATCTATCTAC-3'). A PCR was performed in volumes of 25 µl, which contained 2 µl of genomic DNA, 0.125 mM each dNTP, 0.2 µM each primer, 3 mM MgCl<sub>2</sub>, 0.25 units of *Taq* DNA polymerase and 1× PCR buffer. The PCRs including primers HaemNFI and HaemNR3 were run using the following conditions: 120 s at 94 °C of enzyme activation step, followed by 20 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C, ending with an elongation step of 600 s at 72 °C.

We used the second round primers HaemF (5'-ATGGTGCTTTTCGATATATGCATG-3') and HaemR2 (5'-GCA TTATCTGGATGTGATAATGGT-3'). The second PCR was performed in volumes of 25 µl containing of 0.125 mM each dNTP, 0.4 µM each primer, 3 mM MgCl<sub>2</sub>, 0.5 units of *Taq* DNA polymerase, 1× PCR buffer, and 2 µl of the PCR product from the first round, which was used as template instead of genomic DNA. The PCRs including primers HaemF and HaemR2 were run using the following conditions: 120 s at 94 °C of enzyme activation step, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C, ending with an elongation step of 600 s at 72 °C. Two to 8 µl of the

second-round reactions were run on 2% agarose stained with ethidium bromide and visualized under UV light.

Cosgrove *et al.* (2006) obtained amplicons of 478 bp, which were attributable to species of *Plasmodium* and *Haemoproteus* according to the results of direct sequencing. They also obtained larger amplicons of approximately 550 bp, which proved to be species of *Leucocytozoon*. In the cases when both PCR products were obtained, they distinguished the larger fragment from the smaller amplicons when electrophoresed on high-resolution agarose gels for 2 hr, but in some cases they reported less clear distinction. To be certain that a PCR product is because of *Plasmodium* and *Haemoproteus* and not *Leucocytozoon* spp., Cosgrove *et al.* (2006) recommended a further screening stage for all PCR products of approximately 450–600 bp. We confirmed the presence of *Leucocytozoon* spp. in our amplified PCR products, by performing RFLP analysis (Cosgrove *et al.* 2006), using the enzymes *Nco*I and *Eco*RV. We digested 3 µl of a PCR product in a total volume of 20 µl for 4 hr at 37 °C, using 1 U of each of the restriction enzymes *Nco*I and *Eco*RV, 1 mg ml<sup>-1</sup> bovine serum albumin, and 1× restriction enzyme buffer D (Thermo Fisher Scientific, Fermentas). For *Plasmodium* spp. sequences, the digest produces bands of 114 bp, 149 bp, and 262 bp, and for *Leucocytozoon* spp. sequences, bands of 173 bp and 352 bp are obtained (Cosgrove *et al.* 2006). *Haemoproteus* spp. sequences are not cut by either enzyme (Cosgrove *et al.* 2006).

To find the blood parasites, we used positive and negative controls. Positive controls were from birds with known infections evident from microscopy results, and the negative controls used purified water instead of DNA template. The PCR products were run on a 1.8% agarose gel using 1× TBE and visualized by an ethidium bromide stain under ultraviolet light.

## Statistical analysis

Parasite counts were normally distributed (one-sample Kolmogorov-Smirnov test), hence parametric statistical procedures were used for further analyses. Parasites loads in male and

female Siberian tits from the moderately- and heavily-managed forests were analysed with GLM models. Number of nestlings as a measure of reproductive effort in the models did not change the results and, since that covariate itself remained always non-significant, we dropped it from the final models. Most of the adult birds were yearlings (41 out of 45 individuals in the microscopy data set, and 37 out of 40 individuals in the combined microscopy and molecular data set). Since we did not find any age-related effects and their interactions, age was also dropped from the final models. We included all birds (infected and non-infected) in the models.

# Results

During microscopic examination of blood smears, haemosporidian parasites were found in the blood of 75.6% of the breeding Siberian tits (34 infected and 11 individuals with undetected infections). Since samples had a mean ( $\pm$  SE) of  $2 \pm 0.23$  infected cells per 10 000 (range = 0–4), the level of parasitemia was considered to be low in all of the inspected samples. When considering only individuals ( $n = 40$ ) that were screened both by microscopy and molecular methods, the blood parasites were detected in 75% of the Siberian tits (30 infected and 10 individuals with undetected infections). Most of the infected Siberian tits (26 out of 40 individuals) had single infections. In 4 cases, the Siberian tits were double-infected (Table 1). There was no significant

difference in total parasite prevalence between sexes ( $\chi^2$  with Yates' correction = 0.07,  $p = 0.80$ ; Table 1).

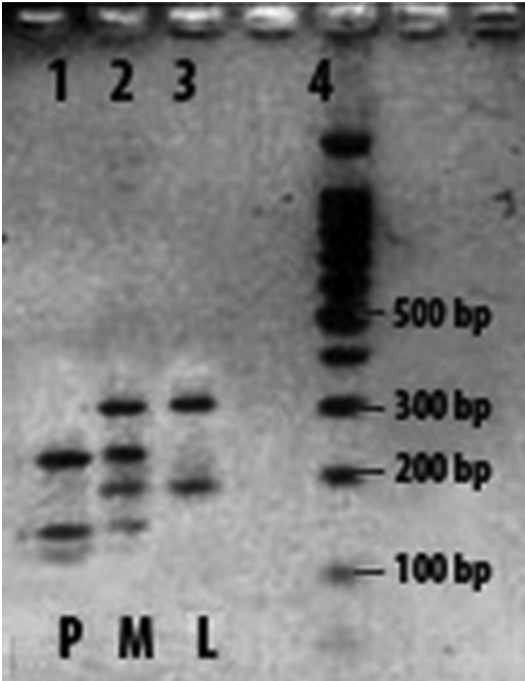
Using molecular screening, the blood parasites were found in 95% of the breeding Siberian tits (38 infected and 2 individuals with undetected infections). This shows that the prevalence of haemosporidian parasites was significantly higher when the same samples were tested by a combination of PCR and RFLP methods ( $\chi^2$  with Yates' correction = 4.80,  $p = 0.028$ ; Table 1 and Fig. 1). In 4 cases, double infections of *Leucocytozoon* spp.  $\times$  *Haemoproteus* and *Leucocytozoon* spp.  $\times$  *Plasmodium* spp. as detected by microscopy were found to be triple infections consisting of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* spp. according to the results obtained by molecular screening (Table 1 and Fig. 1). Two single infections of *Plasmodium* spp. as detected by microscopy appeared to be double infections of *Plasmodium* spp.  $\times$  *Haemoproteus* spp.

Prevalence of *Haemoproteus* spp. determined by molecular methods (35%, Table 1) was significantly higher than the prevalence determined by microscopy (2.22%, Table 1) ( $\chi^2$  with Yates' correction = 13.48,  $p = 0.0002$ ). Prevalence of *Plasmodium* spp. was also found to be significantly higher after molecular screening (35%, Table 1) as compared with microscopy examination of blood smears (12.5%, Table 1) ( $\chi^2$  with Yates' correction = 4.42,  $p = 0.036$ ). In contrast, prevalence of *Leucocytozoon* spp. remained the same (70%) irrespective of the method used to detect blood parasites (Table 1). The total para-

**Table 1.** Number of single, double and triple infections of blood parasites in Siberian tits regarding to individual's sex.

Type of single/combined infection	Method			
	Microscopy		RFLP	
	Females	Males	Females	Males
<i>Plasmodium</i>	1	1	0	0
<i>Haemoproteus</i>	0	0	0	0
<i>Leucocytozoon</i>	14	10	14	10
<i>Plasmodium</i> $\times$ <i>Haemoproteus</i>	0	0	4	6
<i>Leucocytozoon</i> $\times$ <i>Plasmodium</i>	1	2	0	0
<i>Leucocytozoon</i> $\times$ <i>Haemoproteus</i>	0	1	0	0
<i>Leucocytozoon</i> $\times$ <i>Plasmodium</i> $\times$ <i>Haemoproteus</i>	0	0	1	3
<b>Total</b>	16	14	19	19





**Fig. 1.** Diagnostic *Plasmodium* and *Leucocytozoon* species banding patterns resulting from post-PCR digestion with restriction enzymes *EcoRV* and *NcoI*. Lane numbers and genus are assigned. P = *Plasmodium* spp., L = *Leucocytozoon* spp., and M = mixed.

site prevalence did not differ between sexes after molecular screening ( $\chi^2$  with Yates' correction = 0,  $p = 1.00$ ; Table 1).

The GLM analysis of the data obtained by molecular screening revealed that prevalences of *Leucocytozoon*, *Plasmodium* and that of all haemosporidians taken together were similar to those obtained by microscopy (Tables 2 and 3). Both methods showed that probability of being infected by *Leucocytozoon*, *Plasmodium* and by

any of the blood parasites was not affected by habitat and sex. Molecular and microscopy screening showed that the risk of being infected by *Leucocytozoon* increased significantly with the decreasing distance to the nearest stream (Table 2). The risk of being infected by *Plasmodium* increased with the decreasing distance to the nearest lakes and streams (Tables 2 and 3). The main discrepancy between the parasite screening methods was that according to microscopy the risk of being infected by any of the parasites increased in the vicinity of lakes, while it was not supported by the results of molecular screening (Tables 2 and 3). Molecular screening gave us an opportunity to evaluate the prevalence of *Haemoproteus*, which was not possible by using microscopy only. The PCR and RFLP analyses showed that the risk of being infected by *Haemoproteus* increased significantly near lakes and streams (Table 3).

**Discussion**

The current and the earlier study by Krams *et al.* (2010) are the first showing that avian malaria transmission can occur in the area of the Arctic Circle. Recently migratory Fennoscandian blue-throats (*Luscinia svecica*) have been found to be infected with a *Plasmodium* lineage (Hellgren 2008). However, these birds most probably have been infected outside northern Sweden, since the samples were collected only from adult individuals. Studies carried out in North America, demonstrated that the transmission of *Plasmodium* parasites do not occur at northern latitudes (Deviche *et al.* 2001, Yohannes *et al.* 2009, Barnard *et al.* 2010).

**Table 2.** The effects of habitat, sex, distance to the nearest lake and distance to the nearest stream on the blood parasite prevalence (infected vs. non-infected) of breeding Siberian tits (GLM analysis) revealed by microscopy.

	<i>Leucocytozoon</i>			<i>Plasmodium</i>			<i>Haemoproteus</i>			All genera of parasites		
	df	F	p	df	F	p	df	F	p	df	F	p
Habitat	1	3.18	0.10	1	3.20	0.10	1	0.0001	1.00	1	0.05	0.95
Sex	1	1.63	0.23	1	0.54	0.48	1	0.0001	1.00	1	1.05	0.37
Distance to lake	2	0.0001	1.00	2	5.33	0.01	2	0.0001	1.00	2	3.98	0.04
Distance to stream	6	4.19	0.02	6	4.50	0.02	6	0.0001	1.00	6	1.69	0.18
Error	13			13			13			13		

We found that microscopy and PCR diagnostics showed the same prevalences for *Leucocytozoon* spp. infections in the same samples of Siberian tits. This indicates that optical microscopy may be as sensitive as PCR screening to detect *Leucocytozoon* spp., and microscopy does not underestimate the prevalence of infection even in infections of low intensity. We would suggest the use of microscopy only when the research interests are related solely to *Leucocytozoon* species, because preparation of good blood smears is easy, their examination is cheap, and the detection of this relatively large blood parasite is not difficult.

The present study shows that PCR diagnostics appeared to be more sensitive than microscopy in the case of *Plasmodium* spp. and *Haemoproteus* spp. It is known that after an initial acute phase of infection by blood parasites, the hosts develop chronic, low intensity parasitemias, that are regulated by host cellular and humoral immunity (Schmid-Hempel 2011). Whereas high intensity acute parasitemias are typically easy to monitor and quantify by inspecting blood smears (Valkiūnas *et al.* 2008), the use of microscopy for diagnosing chronic, low intensity infections may considerably underestimate parasite prevalence (McCurdy *et al.* 1998, Jarvi *et al.* 2002). Our results support conclusions of previous studies about insufficient sensitivity of microscopy when parasitemias are low. On the other hand, we cannot agree with the notion that PCR tests are 3 to 4 or even 10-fold better than microscopy diagnostic for detecting chronic blood parasite infections. Although the difference between prevalences revealed by PCR-based techniques and microscopy was sig-

nificant, we found that sex- and habitat-related relationships found in our previous study on the blood parasites in breeding Siberian tits were not biased by low sensitivity of microscopy. We found that PCR diagnostics and microscopy showed no sex differences in the total count of parasites, or in counts of *Leucocytozoon* and *Plasmodium*. PCR and microscopy also demonstrated similar results with regard to the lack of association between the total count of parasites or counts of *Leucocytozoon* and *Plasmodium*, and habitat of their hosts. However, we found that microscopy examination of blood smears significantly underestimated infections by *Haemoproteus* spp. in the peripheral blood of Siberian tits. It is important to note that identification of *Haemoproteus* parasites was possible only because of the RFLP step in molecular analysis (Cosgrove *et al.* 2006). Otherwise coamplification of all three sister genera of haemosporidia (Perkins & Schall 2002, Outlaw & Ricklefs 2011) may occur during the PCR protocols suggested by Hellgren *et al.* (2004) and Waldenström *et al.* (2004).

The low numbers of *Haemoproteus* spp. from microscopy samples may be explained mainly by difficulties to detect the patent infections of this haemosporidian genus during examining of blood smears of exceptionally light infections, when just a few parasites are present in samples. It is difficult to rule out a possibility that sensitive PCR-based tools amplify the DNA of sporozoites, which are not numerous in the circulation, so usually difficult to detect using microscopy (Valkiūnas 2005). This is a likely explanation because PCR diagnostics revealed also much higher prevalence of *Plasmodium*

**Table 3.** The effects of habitat, sex, distance to the nearest lake and distance to the nearest stream on the blood parasite prevalence (infected vs. non-infected) of breeding Siberian tits (GLM analysis) revealed by molecular screening (PCR and RFLP).

	<i>Leucocytozoon</i>			<i>Plasmodium</i>			<i>Haemoproteus</i>			All genera of parasites		
	df	F	p	df	F	p	df	F	p	df	F	p
Habitat	1	2.93	0.11	1	0.0001	1.00	1	0.09	0.77	1	0.0001	1.00
Sex	1	1.47	0.25	1	0.14	0.72	1	3.38	0.09	1	0.09	0.77
Distance to lake	2	0.0001	1.00	2	5.33	0.01	2	5.33	0.01	2	0.0001	1.00
Distance to stream	6	3.55	0.03	6	3.05	0.04	6	6.04	0.004	6	1.89	0.16
<b>Error</b>		13			13			13			13	

than the results obtained by microscopy. It is also possible but perhaps less likely that gametocytes of *Haemoproteus* spp. were less mature and harder to detect than gametocytes of *Plasmodium* spp.

Either screening method showed that the risk of being infected by *Leucocytozoon* significantly increased with the decreasing distance to the nearest stream, where simuliid flies (Diptera: Simuliidae) reproduce (Bennett & Fallis 1971, Ashford et al. 1991, Krams et al. 2010), while the risk of infection by *Plasmodium* significantly increased with decreasing distance to streams and lakes. Thus, sex, habitat and parasite-vector related results do not support the idea that the use of microscopy for diagnosing chronic infections could unavoidably underestimate and bias studies that test for the role of sex and habitat-related stress in parasitism. Therefore, we would suggest that microscopy may be a suitable method for detecting blood parasites during times when parasitemias and transmission rates are low, except perhaps in the case of *Haemoproteus* spp. It is important to note that blood smears, which are made from blood containing parasitemias of low intensity, should be of high quality, they need to be properly prepared, and the smears should be examined by highly skilled investigators (Valkiūnas et al. 2008). We also would suggest the use of greater sample sizes to reach life-history related conclusions, though microscopy is time-consuming, which may make it less convenient especially for large-scale screening.

*Leucocytozoon*, *Plasmodium* and *Haemoproteus* rarely occurred together and this fact enables us to assert that they are transmitted by different vectors. It is known that blood-sucking simuliid blackflies (Diptera: Simuliidae) are the vectors of *Leucocytozoon* (Valkiūnas 2005, Hellgren et al. 2008). According to the PCR results, *Plasmodium* and *Haemoproteus* always occurred together, suggesting the same vector for these parasites. However, biting midges (Diptera: Ceratopogonidae) and hippoboscids (Diptera: Hippoboscidae) are the vectors of bird haemosporidian parasites, while blood-sucking mosquitoes (Diptera: Culicidae) are the vectors of *Plasmodiidae* species (Valkiūnas 2005). Supposedly, hippoboscids and mosquitoes are so abundant in northern Finland (Adler et al. 1999)

that both closely related genera of blood parasites often occur together in prevalences as high as 80%, according to microscopy examination of blood smears, and as high as 95%, according to molecular screening of the blood of breeding Siberian tits.

Finally, our results confirm that the PCR screening protocol and use of RFLP analysis suggested by Cosgrove et al. (2006) is sufficient to avoid the risk of co-amplification of *Leucocytozoon* while performing PCR to detect *Plasmodium* and *Haemoproteus* parasites. This method is sensitive enough despite the high degree of sequence similarity between *Plasmodium* and *Haemoproteus* avian malaria species, and *Leucocytozoon* in target genes. We would suggest the use of this technique as a standard method of molecular screening for blood parasites of birds in a combination with a microscopy approach to identify and detect the intensity of infection.

## Acknowledgements

We acknowledge the support of the European Community (Research Infrastructure Action under the FP6 "Structuring the European Research Area" Programme, Lapland Atmosphere-Biosphere Facility LAPBIAT), the Academy of Finland, Oulanka Research Station and Thule Institute at the University of Oulu. We thank Gediminas Valkiūnas, Tatjana Iezhova for their advice in microscopy examination of our samples. We also thank Aleksejs Osipovs for his help in the field. Mikus Abolins-Abols and Todd M. Freeberg kindly revised the manuscript and improved our English. Research was supported by a research fellowship from the EU VPD1/ESF to DC and JV. We thank the staff members of the Oulanka Research Station for providing facilities and lodging. All animal manipulations comply with the current laws in Finland, and were done under licenses of the Department of Biology of the University of Oulu.

## References

- Adler, P. H., Malmqvist, B. & Zhang, Y. X. 1998: Black flies (Diptera: Simuliidae) of northern Sweden: taxonomy, chromosomes, and bionomics. — *Entomologica Scandinavica* 29: 361–382.
- Ashford, R. W., Green, E. E., Holmes, P. R. & Lucas, A. J. 1991: *Leucocytozoon toddi* in British sparrowhawks *Accipiter nisus*: patterns of infection in nestlings. — *Journal of Natural History* 25: 269–277.
- Atkinson, C. T. & van Ripper, C. III 1991: Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leu-*



- cocytozoon, and *Haemoproteus*. In: Loye, J. E. & Zuk, M. (eds.), *Bird-parasite interactions: ecology, evolution, and behaviour*: 19–48. Oxford University Press, Oxford.
- Barnard, W. H., Mettke-Hofmann, C. & Matsuoka, S. M. 2010: Prevalence of hematozoa infections among breeding and wintering rusty blackbirds. — *Condor* 112: 849–853.
- Beadell, J. S. & Fleischer, R. C. 2005: A restriction enzyme-based assay to distinguish between avian hemosporidians. — *Journal of Parasitology* 91: 683–685.
- Bennett, G. 1993: Phylogenetic distribution and possible evolution of the avian species of the Haemoproteidae. — *Systematic Parasitology* 26: 39–44.
- Bennett, G. F. & Fallis, A. M. 1971: Flight range, longevity, and habitat preference of female *Simulium euryadmiculum* Davies (Diptera: Simuliidae). — *Canadian Journal of Zoology* 49: 1203–1207.
- Bennett, G., Peirce, M. A. & Earlé, E. A. 1994: An annotated checklist of the valid avian species of *Haemoproteus*, *Leucocytozoon* (Apicomplexa: Haemosporida) and *Hepatozoon* (Apicomplexa: Haemogregarinidae). — *Systematic Parasitology* 29: 61–73.
- Bennett, G. F. 1970: Simple techniques for making avian blood smears. — *Canadian Journal of Zoology* 48: 585–586.
- Bennett, G. F. & Coombs, R. F. 1975: Ornithophilic vectors of avian hematozoa in insular Newfoundland. — *Canadian Journal of Zoology* 53: 1241–1246.
- Bennett, G. F., Peirce, M. A. & Ashford, R. W. 1993: Avian hematozoa: mortality and pathogenicity. — *Journal of Natural History* 27: 993–1001.
- Bensch, S., Perez-Tris, J., Waldenström, J. & Hellgren, O. 2004: Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? — *Evolution* 58: 1617–1621.
- Bensch, S., Stjernman, M., Hasselquist, D., Örfjan, Ö., Hansson, B., Westerdahl, H. & Pinheiro, R. T. 2000: Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. — *Proceedings of the Royal Society of London B* 267: 1583–1589.
- Bush, A., Fernandez, J., Esch, G. W. & Seed, J. R. 2001: *Parasitism: the diversity and ecology of animal parasites*. — Cambridge University Press, Cambridge.
- Cosgrove, C. L., Day, K. P. & Sheldon, B. C. 2006: Coamplification of *Leucocytozoon* by PCR diagnostic tests for avian malaria: a cautionary note. — *Journal of Parasitology* 92: 1362–1365.
- Cox, F. E. G. 1993: *Modern parasitology*. — Blackwell, Oxford.
- Deviche, P., Greiner, E. C. & Manteca, X. 2001: Interspecific variability of prevalence in blood parasites of adult passerine birds during the breeding season in Alaska. — *Journal of Wildlife Disease* 37: 28–35.
- Durrant, K. L., Beadell, J. S., Ishtiaq, F., Graves, G. R., Olson, S. L., Gering, E., Peirce, M. A., Milensky, C. M., Schmidt, B. K., Gebhard, C. & Fleischer, R. C. 2006: Avian hematozoa in South America: a comparison of temperate and tropical zones. — *Ornithological Monographs* 60: 98–111.
- Garamszegi, L. Z. 2010: The sensitivity of microscopy and PCR-based detection methods affecting estimates of prevalence of blood parasites in birds. — *Journal of Parasitology* 96: 1197–1203.
- Garamszegi, L. Z. 2011: Climate change increases the risk of malaria in birds. — *Global Change Biology* 17: 1751–1759.
- Godfrey, D. A., Carlson, L. & Ross, C. D. 1987: Quantitative inter-strain comparison of the distribution of choline acetyltransferase activity in the rat cochlear nucleus. — *Hearing Research* 31: 203–209.
- Hellgren, O., Bensch, S. & Malmqvist, B. 2008: Bird hosts, blood parasites and their vectors — associations uncovered by molecular analyses of blackfly blood meals. — *Molecular Ecology* 17: 1605–1613.
- Hellgren, O., Waldenström, J. & Bensch, S. 2004: A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. — *Journal of Parasitology* 90: 797–802.
- Jarvi, S. I., Schultz, J. J. & Atkinson, C. T. 2002: PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. — *Journal of Parasitology* 88: 153–158.
- Krams, I., Cirule, D., Krama, T., Hukkanen, M., Rytönen, S., Orell, M., Iezhova, T., Rantala, M. J. & Tummeleht, L. 2010: Effects of forest management on haematological parameters, blood parasites, and reproductive success of the Siberian tit (*Parus cinctus*) in northern Finland. — *Annales Zoologici Fennici* 47: 335–346.
- Martínez, J., Martínez-De La Puente, J., Herrero, J., Del Cerro, S., Lobato, E., Rivero-De Aguilar, J., Vásquez, R. A., Merini, S. 2009: A restriction site to differentiate *Plasmodium* and *Haemoproteus* infections in birds: on the inefficiency of general primers for detection of mixed infections. — *Parasitology* 136: 713–722.
- Martínez-De la Puente, J., Martínez, J., Rivero-De Aguilar, J., Herrero, J. & Merino, S. 2011: On the specificity of avian blood parasites: revealing specific and generalist relationships between haemosporidians and biting midges. — *Molecular Ecology* 20: 3275–3287.
- Martínez-De la Puente, J., Merino, S., Tomás, G., Moreno, J., Morales, J., Lobato, E., García-Fraile, S. & Belda, E. J. 2010: The blood parasite *Haemoproteus* reduces survival in a wild bird: a medication experiment. — *Biology Letters* 6: 663–665.
- McCurdy, D. G., Shutler, D., McCurdy, D. G., Shutler, D., Mullie, A. & Forbes, M. R. 1998: Sex-biased parasitism of avian hosts: relations to blood parasite taxon and mating system. — *Oikos* 82: 303–312.
- Merino, S. & Moreno, J. 2000: Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). — *Proceedings of the Royal Society of London. Series B: Biological Sciences* 267: 2507–2510.
- Møller, A. P. 1997: Parasitism and the evolution of host life history. In: Calyton, D. H. & Moore, J. (eds.), *Host-parasite evolution*: 105–127. Oxford University Press, New York.
- Ots, I. & Hörak, P. 1998: Health impact of blood parasites in breeding great tits. — *Oecologia* 116: 441–448.

- Pérez-Tris, J. & Bensch, S. 2005: Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. — *Parasitology* 131: 15–23.
- Perkins, S. L. & Schall, J. 2002: A molecular phylogeny of malarial parasites recovered from cytochrome *b* gene sequences. — *Journal of Parasitology* 88: 972–978.
- Ricklefs, R. E. & Fallon, S. M. 2002: Diversification and host switching in avian malaria parasites. — *Proceedings of the Royal Society of London B* 269: 885–892.
- Ricklefs, R. E., Fallon, S. M. & Bermingham, E. 2004: Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. — *Systematic Biology* 53: 111–119.
- Schmid-Hempel, P. 2011: *Evolutionary parasitology: the integrated study of infections, immunology, ecology, and genetics*. — Oxford University Press, New York.
- Spielman, A. & D'Antonio, M. 2001: *Mosquito: a natural history of our most persistent and deadly foe*. — Hyperion, New York.
- Szollósi, E., Cichon, M., Eens, M., Hasselquist, D., Kempenaers, B., Merino, S., Nilsson, J. A., Rosivall, B., Rytönen, S., Török, J., Wood, M. J. & Gáramszegi, L. 2011: Determinants of distribution and prevalence of avian malaria in blue tit populations across Europe: separating host and parasite effects. — *Journal of Evolutionary Biology* 24: 2014–2024.
- Valkiūnas, G. 1993: Pathogenic influence of haemosporidians and trypanosomes on wild birds in the field conditions: facts and hypotheses. — *Ekologija* 1: 47–60.
- Valkiūnas, G. 2005: *Avian malaria parasites and other haemosporidia*. — CRC Press, Boca Raton, FL.
- Valkiūnas, G., Anwar, A. M., Atkinson, C. T., Greiner, E. C., Paperna, I. & Peirce, M. A. 2005: What distinguishes malaria parasites from other pigmented haemosporidians? — *Trends in Parasitology* 21: 357–358.
- Valkiūnas, G., Iezhova, T. A., Križanauskienė, A., Palinauskas, V., Sehgal, R. N. M. & Bensch, S. 2008: A comparative analysis of microscopy and PCR-based detection methods for blood parasites. — *Journal of Parasitology* 94: 1395–1401.
- Waldenström, J., Bensch, S., Hasselquist, D. & Östman, Ö. 2004: A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. — *Journal of Parasitology* 90: 191–194.
- Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D. & Ottosson, U. 2002: Cross-species infection of blood parasites between resident and migratory songbirds in Africa. — *Molecular Ecology* 11: 1545–1554.
- Wiehn, J., Korpimäki, E. & Pen, I. 1999: Haematzoan infections in the Eurasian kestrel: effects of fluctuating food supply and experimental manipulation of parental effort. — *Oikos* 84: 87–98.
- Yohannes, E., Krizanauskiene, A., Valcu, M., Bensch, S. & Kempenaers, B. 2009: Prevalence of malaria and related haemosporidian parasites in two shorebird species with different winter habitat distribution. *Journal of Ornithology* 150: 287–291.