Parasitol Res (2013) 112:839–845
DOI 10.1007/s00436-012-3204-3

ORIGINAL PAPER

Avian haemosporidians in haematophagous insects in the Czech Republic

Petr Synek · Pavel Munclinger · Tomáš Albrecht · Jan Votýpka

Received: 23 August 2012 / Accepted: 20 November 2012 / Published online: 6 December 2012

Abstract The degree to which avian haemosporidian parasites can exploit different vectors as a definitive host has ecological implications for their transmission and biogeography. Studies targeting haemosporidian parasites using precise molecular detection methods are almost lacking in Central Europe, however. Here, we utilized PCR-based molecular methods to detect avian haemosporidians in insect vectors in the Czech Republic. Nine lineages of parasites belonging to three genera, *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*, were detected in pooled samples of insect individuals, of which three lineages had not yet been discovered in previous studies. All three *Leucocytozoon* lineages were found exclusively in black flies, while five *Haemoproteus* lineages were found in biting midges. The most abundant insect species *Culicoides kibunensis* harbored three *Haemoproteus* lineages, and the second-most numerous species *Culicoides segnis* even four. The positive mosquitoes of *Culex pipiens* complex hosted two parasite lineages, one *Plasmodium* and one *Haemoproteus*, the latter of which, however, could suggest the aberrant development of this parasite in an unusual invertebrate host. The co-occurrence of *Haemoproteus* ROFI1 and TURDUS2 lineages in both insects and birds at the same study plot suggests a transmission of these lineages during breeding season of birds.

P. Synek (✉) · P. Munclinger · T. Albrecht
Department of Zoology, Charles University in Prague,
Faculty of Science, Vinicna 7,
128 44 Prague 2, Czech Republic
e-mail: synek85@gmail.com

P. Munclinger
e-mail: muncling@natur.cuni.cz

T. Albrecht
e-mail: albrecht@ivb.cz

J. Votýpka
Department of Parasitology, Charles University in Prague,
Faculty of Science, Vinicna 7,
128 44 Prague 2, Czech Republic
e-mail: vapid@natur.cuni.cz

Introduction

Haemosporidians, which are common and widespread avian blood parasites affecting host fitness (Knowles et al. 2009; Asghar et al. 2011), serve as popular models in evolutionary and ecology studies (Valkiūnas 2005). The bird disorders caused by the three haemosporidian genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* are frequently referred to as avian malaria, though, following a strict view of terminology, this term should properly be used only for *Plasmodium*-induced diseases. Haemosporidians are obligatory dixenic parasites requiring both vertebrate and insect hosts to complete their life cycle. While vertebrates serve as intermediate hosts, blood-sucking insects are the definitive hosts in which the sexual part of the parasite life cycle occurs (Valkiūnas 2005). Molecular detection techniques, which allow the precise and fast identification of parasite lineages even in morphologically weakly differentiated species, have led to a new era in the research of haemosporidian parasite specificity. However, the vast majority of studies target the parasite stages in birds (e. g., Hellgren et al. 2011; Knowles et al. 2011; Marzal et al. 2011; Svensson-Coelho and Ricklefs 2011), and hence our knowledge of parasite–insect relationships is still rather limited, especially concerning the details of parasite specificity.

Haemosporidians infect a wide range of dipterous insects. Species of the genus *Plasmodium* develop in mosquitoes of the family Culicidae (Valkiūnas 2005; Martinsen et al. 2008). The genus *Haemoproteus* comprises two distinct groups, which are usually classified as separate subgenera and are specialized for different insect vectors. The

subgenus *Parahaemoproteus* is transmitted by biting midges of the family Ceratopogonidae (mainly *Culicoides* spp.), whereas the subgenus *Haemoproteus* develop in louse flies of the family Hippoboscidae (Valkiūnas 2005). The principal vectors of *Leucocytozoon* are black flies of the family Simuliidae (Martinsen et al. 2008), even though one species, *Leucocytozoon caulleryi*, develops in *Culicoides* species (Mori 1992).

Haemosporidians show varying degrees of host specificity. Since several unrelated haemosporidian lineages have been repeatedly recorded in the same insect host species (Valkiūnas et al. 2002; Ishtiaq et al. 2008; Kim and Tsuda 2010; Njabo et al. 2011; Glaizot et al. 2012), and vice versa identical parasite lineages have been found in several insect species (Atkinson 1988; Garvin and Greiner 2003; Ishtiaq et al. 2008; Ejiri et al. 2009; Sato et al. 2009; Kimura et al. 2010; but see Gager et al. 2008), the simple view of tight parasite specialization has been entirely abandoned. The co-evolution of haemosporidian parasites and insect hosts is actually rather complex and parasites show varying degrees of host specificity (Martinez-de la Puente et al. 2011), a low correspondence between vertebrate and insect host breadths (Gager et al. 2008; Njabo et al. 2011), and likely frequent host shifts (Martinez-de la Puente et al. 2011).

The large body of knowledge on haemosporidian host breadth has been enabled by molecular techniques. However, PCR-based methods cannot distinguish between different developmental parasite stages and thus is not an appropriate method to demonstrate that the parasite is capable of reaching the stages necessary for transmission in a particular insect (Valkiūnas 2011). Microscopic inspection of insect salivary glands and preferably also laboratory experiments should follow the molecular detection. On the other hand, molecular methods of parasite detection are an unavoidable first step in our understanding of the parasite–host relationship and may also yield rather unexpected results. New parasite lineages that have not been found in vertebrate hosts are regularly detected in insects (Ishtiaq et al. 2008; Njabo et al. 2011; Martínez-de la Puente et al. 2011), which may suggest either incomplete host sampling or unexpected hosts, e.g., reptiles instead of birds (Njabo et al. 2011). Considering the number of studies of haemosporidians in birds using molecular methods, there is a clear lack of similar studies targeting those parasites in insect vectors. Moreover, contrary to the vertebrate host studies, a majority of the molecular detections of haemosporidians in insects were performed at localities outside Europe (but see Martínez-de la Puente 2011; Glaizot et al. 2012 for notable exceptions). The aims of the present study were (1) to identify haemosporidian parasites in haematophagous insects using PCR-based techniques at a locality in Central Europe, (2) to determine parasite lineages using cytochrome b sequencing, and (3) to suggest links between parasites, vertebrate hosts, and insect vectors using both our data and public databases.

Material and methods

Study plot

The study was conducted at an extensively studied Scarlet Rosefinch (*Carpodacus erythrinus*) locality (Albrecht 2004; Albrecht et al. 2007, 2009; Vinkler et al. 2012) in the Vltava river valley (48°49′N; 13°56′E) in the Bohemia Forest National Park in Doudlebia, Czech Republic. Rosefinches and accidentally trapped birds of other species at the study plot are currently extensively examined for the presence of haemosporidians (data will be published elsewhere). The study plot is an isolated patch of wet shrubby meadow dominated by willow leaf meadowsweet (*Spiraea salicifolia*), sedges (*Carex* sp.), and meadowsweet (*Filipendula ulmaria*), surrounded by agricultural landscape mosaics (for a detailed description of the study site see Albrecht 2004; Albrecht et al. 2007).

Sampling

Insects were collected overnight (from 6 pm to 8 am) during June 2008 in two collecting sessions: from the 8th to 12th and 26th to 27th. Since our aim was to collect mainly ornithophilic blood-sucking insects, two insect CDC traps (BioQuip Products, Rancho Dominguez, U.S.A.) were placed in close proximity to birdcages occupied by Zebra Finches (*Taeniopygia guttata*) or Japanese Quails (*Coturnix japonica*). The birdcages were installed near shrub edges at a height of 1 to 1.5 m (for a detailed description of the method see Černý et al. 2011). Since no differences in the attractiveness of the two types of avian baits were observed during the first session, only Zebra Finches were used in the second session. Control CDC traps with an ultraviolet light source located at the same height and habitat were used to verify the specificity of bird-baited traps for ornithophilic blood-sucking insects.

Insect species identification and treatment before parasite detection

Collected insects were stored in ethanol, transported to the laboratory, and identified to species under a stereomicroscope using standard literature (Chvála 1980). Several specimens of each *Culicoides* species were mounted using CMCP-9 or CMCP-10 medium (Polyscience, Warrington, U.S.A.) and their taxonomic status was verified using a microscope. Since we were interested in parasites occurring in salivary glands and not only in blood meals, the blood fed mosquito and black fly females were completely excluded from the analyses. An alternative strategy was adopted for *Culicoides* individuals that were the most frequent insects in bird-baited traps. *Culicoides* female abdomens with blood

meals were carefully separated under a stereomicroscope and discarded and only the remaining thoraxes containing the salivary glands were used for the PCR detection of parasites.

Molecular detection of haemosporidian parasites

Samples were dried and crushed in 1.5-ml microtubes. *Culicoides* samples were grouped in pools of 8 to 55 individuals of one species trapped during one trapping session; other investigated genera (*Culex*, *Aedes*, and *Eusimulium*) were grouped in pools of 10 individuals or less. DNA was extracted using a DNeasy® Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration and purity of isolated DNA was checked using a NanoDrop® ND-1000 spectrophotometer (Isogen Life Science, Utrecht, Netherlands). Detection of haemosporidian parasites was performed using the nested PCR protocol described in Hellgren et al. (2004), which enables infections of *Plasmodium* or *Haemoproteus* and *Leucocytozoon* to be distinguished using primers HaemNFI and HaemNR3 for the 1st PCR and primers HaemF and HaemR2 (to detect *Haemoproteus* or *Plasmodium*) or primers HaemFL and HaemR2L (to detect *Leucocytozoon*) for the second PCR. At least seven negative controls (water instead of template DNA) were used for every experimental run of samples. Samples of birds in which haemosporidian infections were proved by microscopy were used as positive controls. Parasite presence was evaluated by the electrophoresis of 5 μl of the nested PCR products on a 2 % agarose gel. Each sample was tested three times to reduce the number of false-negative results. All positive samples were sequenced using primers HaemF or HaemFL. All unique haplotypes differing by one or more substitutions from available sequences deposited in databases (GenBank and MalAvi) were also sequenced from the 3′ end with primers HaemR2 or HaemR2L. Sequences were edited, checked for double peaks indicating mixed infections, and contigs were constructed using CodonCode Aligner software (CodonCode Corporation). Haplotypes were assigned to known haemosporidian lineages using the MalAvi database (Bensch et al. 2009). Haplotypes differing by one or more substitutions in a 480-bp segment of the cytochrome b from known lineages in the MalAvi database were considered as new lineages and named using the first two genus name letters and first two species name letters of the host name followed by consecutive numbers. The sequences of the new lineages are deposited in GenBank (Accession numbers JX507217 to JX507219).

Results

Two thousand eight hundred fifty-eight and 759 blood-sucking insect individuals were collected in bird-baited and UV light traps, respectively. The range of species and proportions of individuals belonging to particular species differed considerably between the two types of traps (Table 1). While

Table 1 Insects trapped in bird-baited and UV traps

Species	Bird-baited traps			UV traps		
	No.	%	No. of engorged females	No.	%	No. of engorged females
Culex pipiens complex	164	5.75	5			
Aedes cinereus	14	0.49				
Aedes communis	5	0.18				
Aedes sticticus	8	0.28	1			
Aedes cantans	2	0.07				
Eusimulium securiforme	58	2.03				
Culicoides kibunensis	1593	55.84	9	268	35.54	39
Culicoides festivipennis	383	13.42	2	34	4.51	
Culicoides pictipennis	8	0.28		27	3.58	
Culicoides sphagnuminsis	55	1.93		4	0.53	
Culicoides segnis	543	19.03		55	7.29	27
Culicoides heliophilus	1	0.04		33	4.38	
Culicoides minutissimus	1	0.04				
Culicoides obsoletus complex	4	0.14		288	38.20	11
Culicoides impunctatus	14	0.49		29	3.85	
Culicoides pulicaris				2	0.27	
Culicoides punctatus				14	1.86	
total	2853		17	754		77

the mammalophilic biting midges of the *Culicoides obsoletus* complex were the most frequently trapped individuals in the UV light traps, they were almost missing in the bird-baited traps where, on the contrary, the ornithophilic species *Culicoides kibunensis* predominated. UV light traps also caught a larger proportion of engorged females (10 %) than bird-baited traps (0.6 %).

Insects collected by the bird-baited CDC traps were used to detect haemosporidians in pooled samples. The highest infection rate was found in the black fly *Eusimulium securiforme*, where all six pools were positive. On the other hand, we did not find any haemosporidians in *Aedes* mosquito species or in three *Culicoides* species; however, this could be due to the low number of sampled individuals. Five lineages of *Haemoproteus*, three lineages of *Leucocytozoon*, and one lineage of *Plasmodium* were detected (Table 2). Six lineages found in insects during this study have been detected in avian hosts at other localities (Table 3), while three lineages are new: *Haemoproteus* CUKI1 and *Leucocytozoon* EUSE1 and EUSE2. The lineage CUKI1 differs by one substitution from the lineage TUPHI1, which was also detected at the study plot (Table 2). The most similar previously described lineage to EUSE1 is ANLA2, which differs in sequence by 2.8 % and was found in African passerines (Baedell et al. 2009). EUSE2 is similar to MTUR2 (3.2 % difference in sequence) which has been previously detected in the Mistle Thrush (*Turdus viscivorus*) in Sweden (Hellgren et al. 2008).

Parasite lineages exhibited frequent sharing of hosts, with up to four parasite lineages of one genus found in a single insect host species (Table 2). However, this host sharing was not restricted to just one parasite genus: *Culex pipiens* hosted one *Haemoproteus* (TURDUS2) and one *Plasmodium* (SYAT5) lineage. On the other hand, three *Haemoproteus* lineages were detected in more than one host species (Table 2). The *Haemoproteus* lineage TURDUS2 was even present in two species of *Culicoides* midges as well as in *C. pipiens* mosquitoes. The lineage TURDUS2 was also detected in the Blackbird (*Turdus merula*) and the Dunnock (*Prunella modularis*) captured at the study plot in the same year (Synek unpublished data). The *Haemoproteus magnus* lineage ROFI1 found in *Culicoides segnis* in the present study has also been detected in the Scarlet Rosefinch (*C. erythrinus*) in previous studies at the same locality (2003, one individual; 2008, two individuals; Synek unpublished data). Surprisingly, the most frequent haemosporidian parasite of the Scarlet Rosefinch, *Haemoproteus* ROFI2 (Križanauskienė et al. 2006; our pilot experiments at the study plot show a 50 % prevalence in the Scarlet Rosefinch, Synek unpublished data) was not found in any insect investigated.

Discussion

Haemosporida in *Culicoides* biting midges

Culicoides biting midges were the most frequent insects trapped in bird-baited traps. They are considered to be insect vectors of avian *Haemoproteus* lineages (Martinsen et al. 2008), and interestingly, *Culicoides* midges are capable of attacking birds even in their nesting boxes (Votýpka et al.

Table 2 Haemosporidian lineages found in pools of insects collected in bird-baited traps

	No. of positive/ examined pools	*Haemoproteus*					*Plasmodium*	*Leucocytozoon*		
		CUKI1	TUPHI1	CCF4	ROFI1	TURDUS2	SYAT5	EUSI1	EUSI2	STUR1
C. kibunensis	16/31	2	11			3				
C. segnis	8/9[a]	1	5	2	3					
C. festivipennis	1/8					1				
C. sphagnuminsis	0/1									
C. impunctatus	0/1									
C. pictipennis	0/1									
C. pipiens complex	5/16					4	1			
A. cinereus	0/1									
A. communis	0/1									
A. sticticus	0/1									
A. cantans	0/1									
E. securiforme	6/6							3	4	1

[a] Mixed infections were detected in three pools

2009; Tomás et al. 2008). Studies based on parasite morphology (Atkinson 1988; Garvin and Greiner 2003) as well as on molecular methods (Martínez-de la Puente et al. 2011) have suggested a wide range of associations between *Haemoproteus* lineages and *Culicoides* species, including close co-evolution as well as a generalist relationship, in accord with the present study. We found three out of five *Haemoproteus* lineages in more than one *Culicoides* species, and vice versa our data also imply that one *Culicoides* species could host several *Haemoproteus* lineages (four lineages were found in *C. segnis* and three in *C. kibunensis*).

The lineage TURDUS2 (which refers to the morphospecies *Haemoproteus minutus*) detected by us in both biting midges (*C. kibunensis* and *Culicoides festivipennis*) and birds (*P. modularis* and *T. merula*) at the same study plot has been frequently found in European sedentary passerines (Table 3), and was also detected in *C. kibunensis*, *Culicoides pictipennis*, and *C. segnis* in Spain (Martínez-de la Peunte et al. 2011). Hence, we suggest that these *Culicoides* species are very probably responsible for local transmissions of the lineage. The lineage *Haemoproteus* ROFI1 (which corresponds to the morphospecies *Haemoproteus magnus*) that was found at the study site in *C. segnis* and also in the Scarlet Rosefinch (*C. erythrinus*) has been previously detected in non-migratory European birds (Table 3), which supports the possible role of *C. segnis* in the transmission.

Haemosporida in mosquitoes

Mosquitoes (family Culicidae) are considered mainly to be avian *Plasmodium* vectors. *Plasmodium* lineages are known to vary extremely in host specificity. For example, while sporogony (the parasite stage necessary for further transmission) of *Plasmodium juxtanucleare* only takes place in *Culex* species (Bennett et al. 1966), *Plasmodium relictum* can be transmitted by the genera *Aedes*, *Anopheles*, *Armigeres*, *Culex*, *Culiseta*, and *Mansonia* (Hunninen 1953; La Pointe et al. 2005; Work et al. 1990). We analyzed five mosquito species belonging to the genera *Culex* and *Aedes*, which have been suggested as possible vectors in several studies using molecular methods (Ishtiaq et al. 2008; Ejiri et al. 2009; Kim et al. 2009; Kimura et al. 2010; Njabo et al. 2011). We did not find any haemosporidian parasite in any *Aedes* species, but this is likely due to the limited sample size. Two haemosporidian lineages were detected in the *C. pipiens* complex, of which the *Plasmodium* lineage SYAT05 has been previously described from individuals of the same species complex in North America (Kimura et al. 2010) as well as from passerines in Europe and North America (Table 3). The overall prevalence of *Plasmodium* lineages in *C. pipiens* (minimum infection rate of 6.1 positive in 1,000 collected mosquitoes) falls within the range reported from other areas (minimum infection rate of 5.2 in Ejiri et al. 2009; 14.2 % prevalence in individually tested mosquitoes in Kimura et al. 2010).

Surprisingly, the *Haemoproteus* lineage TURDUS2 was detected in four *Culex* mosquito pools. Even though mosquitoes are not considered regular *Haemoproteus* vectors, *Haemoproteus* lineages have been repeatedly found in several mosquito species (Ishtiaq et al. 2008; Njabo et al. 2011). However, the presence of *Haemoproteus* in mosquitoes does not necessarily imply transmission capability since it could be alternatively explained as a parasite development dead end in an incorrect host (Valkiūnas 2011). Examinations of mosquito salivary glands for the presence of transmissible parasite stages and experimental transmissions in the laboratory are needed to prove that mosquitoes are alternative *Haemoproteus* vectors.

Haemosporida in black flies

Black flies (family Simuliidae) are considered to be *Leucocytozoon* vectors in the transmission to passerine birds. All black flies caught during our study were identified as *E. securiforme*. Our finding of three different *Leucocytozoon*

Table 3 Known avian hosts of haemosporidian lineages detected in haematophagous insects in the present study

Lineage	Avian host	Reference
CCF4	Chaffinch (*Fringilla coelebs*)	Hellgren et al. 2007b
ROFI1	migratory and non-migratory European birds (*Carpodacus erythrinus; Carduelis chloris; Fringilla coelebs; Coccothraustes coccothraustes*)	Križanauskienė et al. 2006; Hellgren et al. 2007b
TUPHI01	Song Trush (*Turdus philomelos*)	Dimitrov et al. 2010
TURDUS2	migratory and non-migratory European birds (A*crocephalus scirpaceus; Hippolais icterina; Parus caeruleus; Panurus biarmicus; Prunella modularis; Turdus merula; Turdus torquatus*)	Bentz et al. 2006; Hellgren et al. 2007a, b; Wood et al. 2007; Cosgrove et al. 2008; Dimitrov et al. 2010; Valkiūnas et al. 2008
SYAT05	warbler (*Sylvia melanocephala; Sylvia atricapilla*); blackbird (*Turdus merula*; *Turdus migratorius*) and Red-breasted Flycatcher (*Ficedula parva*)	Bentz et al. 2006; Hellgren et al. 2007b; Martinsen et al. 2007; Martinsen et al. 2008; Dimitrov et al. 2010
STUR01	Song Thrush (*Turdus philomelos*)	Hellgren et al. 2007b

lineages in one black fly species corresponds with the results of Desser and Bennet (1993), who experimentally proved that a single black fly species could transmit up to five *Leucocytozoon* species. Two new lineages EUSI1 and EUSI2 were found, but this could be due to the relatively few studies targeting *Leucocytozoon* lineages using molecular methods in comparison with detections of *Plasmodium* and *Haemoproteus*. The third detected lineage STUR01 has been previously reported from the Song Thrush (*Turdus philomelos*) in Lithuania (Hellgren et al.2007b).

Concluding remarks

Our study extends the knowledge of insect vectors of avian haemosporidian parasites using samples from the Czech Republic, demonstrating the varying host breadths of these parasites. Up to four parasite lineages were found in a single host species, and vice versa one parasite lineage was present in up to three insect species. The presence of the *Haemoproteus* lineages TURDUS2 and ROFI1 in both insect vectors and birds at the same study plot suggests their local transmissions. However, the occurrence of lineages from this study in birds from other parts of Europe highlights the need for detailed studies to provide conclusive evidence of transmission details. Three new haemosporidian lineages were found, which suggests that haemosporidian vertebrate hosts have still not been sufficiently screened in Europe.

Ethical standards

This study was performed under certificate of competency according to §17 of the Act No. 246/1992 coll. on Protection Animals against Cruelty (Registration number CZU 945/05) and comply with the current law of the Czech Republic.

Acknowledgments The study was supported by Czech Science Foundation (GA ČR) grant no. P506/10/0716. We acknowledge the help of our colleagues and friends Michal Vinkler, Jan Schnitzer, Jaroslav Jelínek, and František Zicha in the field and thank Zdena Csiebreiová for her technical assistance in the laboratory.

Conflict of interest The authors declare that they have no conflict of interest.

References

Albrecht T (2004) Edge effect in wetland-arable land boundary determines nesting success of scarlet rosefinches (*Carpodacus erythrinus*) in the Czech Republic. Auk 121:361–371

Albrecht T, Schnitzer J, Kreisinger J, Exnerová A, Bryja J, Munclinger P (2007) Extrapair paternity and the opportunity for sexual selection in long-distant migratory passerines. Behav Ecol 18:477–486

Albrecht T, Vinkler M, Schnitzer J, Poláková R, Munclinger P, Bryja J (2009) Extra-pair fertilizations contribute to selection on secondary male ornamentation in a socially monogamous passerine. J Evol Biol 22:2020–2030

Asghar M, Hasselquist D, Bensch S (2011) Are chronic avian haemosporidian infections costly in wild birds? J Avian Biol 42:530–537

Atkinson CT (1988) Epizootiology of *Haemoproteus meleagridis* (Protozoa: Haemosporina) in Florida: potential vectors and prevalence in naturally infected *Culicoides* (Diptera: Ceratopogonidae). J Med Entomol 74:228–223

Beadell JS, Covas R, Gebhard C, Ishtiaq F, Melo M, Schmidt BK, Perkins SL, Graves GR, Fleischer RC (2009) Host associations and evolutionary relationships of avian blood parasites from West Africa. Int J Parasitol 39:257–266

Bennett GF, Warren M, Cheong WH (1966) Biology of the Malaysian strain of *Plasmodium juxtanucleare* Versiani and Gomes, 1941. II. The sporogonic stages in *Culex* (*Culex*) *sitiens* Wiedmann. J Parasitol 52:647–652

Bensch S, Hellgren O, Pérez-Tris (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol Ecol Resour 9:1353–1358

Bentz S, Rigaud T, Barroca M, Martin-Laurent F, Bru D, Moreau J, Faivre B (2006) Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (*Turdus merula*) populations: value of PCR-RFLP and quantitative PCR. Parasitology 133:685–692

Černý O, Votýpka J, Svobodová M (2011) Spatial feeding preferences of ornithophilic mosquitoes, blackflies and biting midges. Med Vet Entomol 25:104–108

Chvála M (1980) Fauna ČSSR 22. Academia, Prague

Cosgrove CL, Wood MJ, Day KP, Sheldon BC (2008) Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. J Anim Ecol 77:540–548

Desser SS, Bennett GF (1993) The genera *Leucocytozoon*, *Haemoproteus* and *Hepatocystis*. In: Kreier JP (ed) Parasitic protozoa. Vol. 4, 2nd edn. Academic, New York

Dimitrov D, Zehtindjiev P, Bensch S (2010) Genetic diversity of avian blood parasites in SE Europe: cytochrome b lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida) from Bulgaria. Acta Parasitol 55:201–209

Ejiri H, Sato Y, Sawai R, Sasaki E, Matsumoto R, Ueda M, Higa Y, Tsuda Y, Omori S, Murata K, Yukawa M (2009) Prevalence of avian malaria parasite in mosquitoes collected at a zoological garden in Japan. Parasitol Res 105:629–633

Gager AB, Del Rosario Loaiza J, Dearborn DC, Bermingham E (2008) Do mosquitoes filter the access of *Plasmodium* cytochrome b lineages to an avian host? Mol Ecol 17:2552–2561

Garvin MC, Greiner EC (2003) Ecology of *Culicoide*s (Diptera: Ceratopogonidae) in southcentral Florida and experimental *Culicoides* vectors of the avian hematozoan *Haemoproteus danilewskyi* Kruse. J Wildl Dis 39:170–178

Glaizot O, Fumagalli L, Iritano K, Lalubin F, Van Rooyen J, Christe P (2012) High prevalence and lineage diversity of avian malaria in wild populations of great tits (*Parus major*) and mosquitoes (*Culex pipiens*). PLoS One 7:e34964

Hellgren O, Waldenstrom J, Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol 90:797–802

Hellgren O, Križanauskienė A, Valkiūnas G, Bensch S (2007a) Diversity and phylogeny of mitochondrial cytochrome B lineages from six morphospecies of avian *Haemoproteus* (Haemosporida: Haemoproteidae). J Parasitol 93:889–896

Hellgren O, Waldenstrom J, Perez-Tris J, Szollosi E, Hasselquist D, Križanauskienė A, Ottosson U, Bensch S (2007b) Detecting shifts of transmission areas in avian blood parasites—a phylogenetic approach. Mol Ecol 16:1281–1290

Hellgren O, Bensch S, Malmqvist B (2008) Bird hosts, blood parasites and their vectors-associations uncovered by molecular analyses of blackfly blood meals. Mol Ecol 17:1605–1613

Hellgren O, Križanauskienė A, Hasselquist D, Bensch S (2011) Low haemosporidian diversity and one key-host species in a bird malaria community on a mid-Atlantic island (São Miguel, Azores). J Wildl Dis 47:849–859

Hunninnen AV (1953) Comparative development of *Plasmodium relictum* oocysts in *Anopheles quadrimaculatus*, *A. albimanus*, and *Culex pipiens*. J Parasitol 39:28–32

Ishtiaq F, Guillaumot L, Clegg SM, Phillimore AB, Black RA, Owens IPF, Mundy NI, Sheldon BC (2008) Avian haematozoan parasites and their associations with mosquitoes across Southwest Pacific Islands. Mol Ecol 17:4545–4555

Kim KS, Tsuda Y (2010) Seasonal changes in the feeding pattern of *Culex pipiens pallens* govern the transmission dynamics of multiple lineages of avian malaria parasites in Japanese wild bird community. Mol Ecol 19:5545–5554

Kim KS, Tsuda Y, Sasaki T, Kobayashi M, Hirota Y (2009) Mosquito blood-meal analysis for avian malaria study in wild bird communities: laboratory verification and application to *Culex sasai* (Diptera: Culicidae) collected in Tokyo, Japan. Parasitol Res 105:1351–1357

Kimura M, Darbro JM, Harrington LC (2010) Avian malaria parasites share congeneric mosquito vectors. J Parasitol 96:144–151

Knowles SCL, Nakagawa S, Sheldon BC (2009) Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. Funct Ecol 23:405–415

Knowles SC, Wood MJ, Alves R, Wilkin TA, Bensch S, Sheldon BC (2011) Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population. Mol Ecol 20:1062–1076

Križanauskienė A, Hellgren O, Kosatec V, Sokolov L, Bensch S, Valkinūas G (2006) Variation in host specificity between species of avian haemosporidian parasites: evidence from parasite morphology and cytochrome b gene sequences. J Parasitol 92:1319–1324

LaPointe DA, Goff ML, Atkinson CT (2005) Comparative susceptibility of introduced forest dwelling mosquitoes in Hawai'i to avian malaria, *Plasmodium relictum*. J Parasitol 91:843–849

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. Mol Ecol 20:3275–3287

Martinsen ES, Waite JL, Schall JJ (2007) Morphologically defined subgenera of *Plasmodium* from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes. Parasitology 134:483–490

Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches. Mol Phylogenet Evol 47:261–273

Marzal A, Ricklefs RE, Valkiūnas G, Albayrak T, Arriero E, Bonneaud C, Czirják GA, Ewen J, Hellgren O, Hořáková D, Iezhova TA, Jensen H, Križanauskienė A, Lima MR, de Lope F, Magnussen E, Martin LB, Møller AP, Palinauskas V, Pap PL, Pérez-Tris J, Sehgal RN, Soler M, Szöllosi E, Westerdahl H, Zetindjiev P, Bensch S (2011) Diversity, loss, and gain of malaria parasites in a globally invasive bird. PLoS One 6:e21905

Morii T (1992) A review of *Leucocytozoon caulleryi* infection in chickens. J Protozool Res 2:128–133

Njabo KY, Cornel AJ, Bonneaud C, Toffelmier E, Sehgal RN, Valkiūnas G, Russell AF, Smith TB (2011) Nonspecific patterns of vector, host and avian malaria parasite associations in a central African rainforest. Mol Ecol 20:1049–1061

Sato Y, Tamada A, Mochizuki Y, Nakamura S, Okano E, Yoshida C, Ejiri H, Omori S, Yukawa M, Murata K (2009) Molecular detection of *Leucocytozoon lovati* from probable vectors, black flies (Simuliudae) collected in the alpine regions of Japan. Parasitol Res 104:251–255

Svensson-Coelho M, Ricklefs RE (2011) Host phylogeography and beta diversity in avian haemosporidian (Plasmodiidae) assemblages of the Lesser Antilles. J Anim Ecol 80:938–946

Tomás G, Merino S, Martínez-de la Puente J, Moreno J, Morales J, Lobato E (2008) A simple trapping method to estimate abundances of blood-sucking flying insects in avian nests. Anim Behav 75:723–729

Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC, Boca Raton

Valkiūnas G (2011) Haemosporidian vector research: marriage of molecular and microscopical approaches is essential. Mol Ecol 20:3084–3086

Valkiūnas G, Liutkevicius G, Iezhova TA (2002) Complete development of three species of *Haemoproteus* (Haemosporida, Haemoproteidae) in the biting midge *Culicoides impunctatus* (Diptera, Ceratopogonidae). J Parasitol 88:864–868

Valkiūnas G, Iezhova TA, Križanauskien A, Palinauskas V, Bensch S (2008) In vitro hybridization of Haemoproteus spp.; an experimental approach for direct investigation of reproductive isolation of parasites. J Parasitol 94:1385–1394

Vinkler M, Schnitzer J, Munclinger P, Albrecht T (2012) Phytohaemagglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Anim Behav 83:17–23

Votýpka J, Synek P, Svobodová M (2009) Endophagy of biting midges attacking cavity-nesting birds. Med Vet Entomol 23:277–280

Wood MJ, Cosgrove CL, Wilkin TA, Knowles SCL, Day KP, Sheldon BC (2007) Within population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. Mol Ecol 16:3263–3273

Work TM, Washino RK, van Riper C (1990) Comparative susceptibility of *Culex tarsalis*, *Anopheles franciscanus*, and *Culiseta inornata* (Diptera: Culicidae) to *Plasmodium relictum* (Haemosporidia: Plasmodiiae). J Med Entomol 27:68–71