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ORIGINAL PAPER

Haemosporidian infections in the Tengmalm's Owl (*Aegolius funereus*) and potential insect vectors of their transmission

Petr Synek[1] · **Alena Popelková**[4] · **Darina Koubínová**[2] · **Karel Šťastný**[4] · **Iva Langrová**[5] · **Jan Votýpka**[3] · **Pavel Munclinger**[1]

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Abstract Sedentary bird species are suitable model hosts for identifying potential vectors of avian blood parasites. We studied haemosporidian infections in the Tengmalm's Owl (*Aegolius funereus*) in the Ore Mountains of the Czech Republic using molecular detection methods. Sex of owl nestlings was scored using molecular sexing based on fragment analysis of PCR-amplified CHD1 introns. Observed infection prevalences in nestlings and adult owls were 51 and 86 %, respectively. Five parasite lineages were detected. Most of the infections comprised the *Leucocytozoon* AEFUN02 and STOCC06 lineages that probably refer to distinct *Leucocytozoon* species. Other lineages were detected only sporadically. Mixed infections were found in 49 % of samples. The main factor affecting the probability of infection was host age. No effect of individual sex on infection probability was evidenced. The youngest infected nestling was 12 days old. High parasite prevalence in the Tengmalm's Owl nestlings suggests that insect vectors must enter nest boxes to transmit parasites before fledging. Hence, we placed sticky insect traps into modified nest boxes, collected potential insect vectors, and examined them for the presence of haemosporidian parasites using molecular detection. We trapped 201 insects which were determined as biting midges from the *Culicoides* genus and two black fly species, *Simulium* (*Nevermannia*) *vernum* and *Simulium* (*Eusimulium*) *angustipes*. Six haemosporidian lineages were detected in the potential insect vectors, among which the *Leucocytozoon* lineage BT2 was common to the Tengmalm's Owl and the trapped insects. However, we have not detected the most frequently encountered Tengmalm's Owl *Leucocytozoon* lineages AEFUN02 and STOCC06 in insects.

Keywords Avian malaria · Wildlife diseases · Blood parasites · Strigiformes · Vectors · Transmission · Molecular sexing of owls

Introduction

Haemosporidians are globally distributed intracellular parasites of vertebrates. The most numerous haemosporidian parasites infecting birds are assigned to three genera (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) comprising more than 200 species which have been detected in more than 4000 host species (Bishop and Bennett 1992; Valkiūnas 2005; Garamszegi 2010). Blood-sucking insects serve as vectors of haemosporidian transmission. While mosquitoes are the prevailing vectors of *Plasmodium* species, biting midges and hippoboscid flies transmit *Haemoproteus* and the genus *Leucocytozoon* is spread by simuliid flies (Valkiūnas 2005).

The majority of studies on avian haemosporidians have been conducted on migratory birds as hosts, which are expected to harbor more parasite lineages than nonmigratory host species due to their exposure to vectors at both their breeding

✉ Pavel Munclinger
muncling@natur.cuni.cz

[1] Department of Zoology, Faculty of Science, Charles University in Prague, Viničná 7, 128 00 Prague 2, Czech Republic

[2] Department of Ecology and Evolution, University of Lausanne, UNIL Sorge, Biophore Building, 1015 Lausanne, Switzerland

[3] Department of Parasitology, Faculty of Science, Charles University in Prague, Viničná 7, 128 00 Prague 2, Czech Republic

[4] Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6 – Suchdol, Czech Republic

[5] Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6 – Suchdol, Czech Republic

and migratory or wintering grounds. High prevalence of blood parasites has been demonstrated also in some sedentary species (Ishak et al. 2008; Krone et al. 2008, Chakarov et al. 2015), however. Since sedentary bird species can be infected only at their breeding grounds, their association with parasites may serve as a model for finding potential vectors of avian haemosporidians. Hence, studies of haemosporidians in owls which are often sedentary species or short-distance migrants may provide interesting insight into parasite–host relationships and potential vector identification. An important advantage in using owls as model species is that owls have long nesting periods, and hence, haemosporidians are able to infect owl nestlings and multiply into detectable numbers in the host blood before fledging (Appleby et al. 1999; Ortego and Cordero 2009, 2010). Infections in nestlings provide valuable information about the time and place of transmission which is usually difficult to obtain in adult birds. Although *Leucocytozoon* and *Haemoproteus* species have been found to be the most frequent parasites in owl species, *Plasmodium* infections can also reach high prevalence in some populations (Gutiérrez 1989; Remple 2004; Monahan and Hijmans 2007; Ishak et al. 2008). The number of haemosporidian species infecting owls seems to be rather low (eight species according to Valkiūnas 2005), but diversity may be underestimated due to the presence of cryptic species (Ishak et al. 2008; Krone et al. 2008, Outlow and Ricklefs 2009).

We investigated the diversity of haemosporidian parasites in the Tengmalm's Owl (*Aegolius funereus*) in the Ore Mountains within the northwestern part of the Czech Republic. The Tengmalm's Owl (also known as Boreal or Richardson's owl) inhabits Holarctic coniferous forests and mountains and feeds on small mammals, birds, and insects. It normally breeds once per year and lays two to eight eggs in tree holes or nest boxes. The incubation period is 26 to 27 days, and nestlings leave the nest in 30 to 35 days (Korpimäki and Hakkarainen 2012). It has been shown that females prefer new nest boxes for breeding, and hence, they usually change nesting places between seasons (Sonerud 1985). The species is not migratory, but individuals in some populations sometimes move slightly south in the cold seasons (Hayward and Hayward 1992). In the Czech Republic, it inhabits mainly mountainous areas with both coniferous and deciduous forests. The prevailing haemosporidian species detected in the Tengmalm's Owl has been *Leucocytozoon danilewski* (= *L. ziemanni*), which typically has shown high prevalence exceeding 90 % (Korpimäki et al. 1993; Ilmonen et al. 1999). Analyses of cytochrome *b* have shown that *L. danilewski* in owls comprises many lineages that differ by as much as 8 % in sequence and hence probably consists of several cryptic species (Ishak et al. 2008). Other parasite species occur in much lower prevalence (less than 10 %) (Korpimäki et al. 1993; Ilmonen et al. 1999).

In the present study, we utilized molecular detection methods to study the diversity of haemosporidian parasites in Tengmalm's Owl adults and nestlings. Because we regularly found infections in nestlings, we set insect traps in nest boxes to identify potential insect vectors occurring in close proximity to active Tengmalm's Owl nests. We then identified haemosporidian lineages in the trapped potential insect vectors and compared them with the infections found in owl nestlings and adults.

Material and methods

Study area

The study was performed on Tengmalm's Owl (Fig. 1) in the highest parts of the Ore Mountains in northern Bohemia (Czech Republic) between 2008 and 2011. The study site covers an area of 70 km^2 and is located in the surroundings of the Fláje Dam approximately between the municipalities of Klíny, Český Jiřetín, and Moldava. Elevation ranges from 735 to 956 m a.s.l. Blue spruce (*Picea pungens*), Norway spruce (*Picea abies*), and European larch (*Larix decidua*) are the dominant tree species at the study location. Deciduous trees are represented by downy birch (*Betula pubescens*), silver birch (*Betula pendula*), rowan (*Sorbus aucuparia*), and red oak (*Quercus rubra*). The mosaic fragments of original natural European beech (*Fagus sylvatica*) forests provide natural holes suitable for Tengmalm's Owl nests. Nest boxes (164 to 167 at the time of our study) have been installed to further supplement breeding possibilities.

Fig. 1 Female and nestlings of the Tengmalm's Owl in a nest box. Photo by A. Popelková

Sampling of birds and molecular sexing of young

Breeding was evaluated via periodic controls of nest boxes every 7 to 14 days throughout the nesting season (between the beginning of March and the end of July). Males were captured using mist nets no earlier than when the oldest chick in the nest box was older than 10 days so as to prevent the parent from leaving the nest. Females and offspring were captured in nest boxes. All individuals were ringed, and blood samples were taken from the brachial artery. Nestlings were sampled 5 days or later after hatching. Blood samples were preserved in 96 % ethanol and stored at −20 °C. DNA was extracted using a DNeasy® Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Concentration and purity of isolated DNA were checked using a NanoDrop® ND-1000 spectrophotometer (Isogen Life Science, Utrecht, Netherlands). Sex of nestlings was identified using amplification of sex-linked CHD1 gene fragments. The P8 and M5 primers (Griffith et al. 1998; Bantock et al. 2008) amplify CHD1 introns on both W and Z chromosomes. The intron lengths differ in the majority of birds (with the exception of ratites), and hence, PCR products of one or two sizes are produced in males (ZZ chromosomes) and females (ZW), respectively. Given that the difference in intron lengths in owls is beyond the separation limits of standard agarose gels used for molecular sexing of other birds, we separated the PCR products by size using capillary electrophoresis. M5 primer was fluorescently labeled, and amplicons were analyzed in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) and then scored using GeneMarker® version 1.9 software (Softgenetics, State College, PA, USA). While PCR products of two sizes (approximately 245 and 249 bp) were detected in females, the presence of single-size (245 bp) products indicated a male sample.

Insect sampling and species determination

Ornithophilic blood-sucking insects were collected in May and June 2011 from the beginning until the end of nesting in each nest box using 11 specially adapted nest boxes (Votýpka et al. 2009). Each nest box consisted of two parts. The lower part contained the entrance hole and a nest cavity. The upper part was separated from the lower one by a wire mesh (1.0× 1.0 cm grid) and had a removable lid. Adjacent to the lid, the upper part of the nest box was perforated by several small openings (0.5 cm in diameter) to enable insects to enter and leave the nest box. Sticky Petri dishes, used for insect trapping, were prepared from the transparent plastic lower part of Petri dishes smeared with an adhesive used by gardeners to control fruit-tree pests (Chemstop; Fytofarm CZ s.r.o., Prague, Czech Republic). No pheromones were present in the glue.

Captured insects were washed from sticky Petri dishes using petrol and were further cleaned by petrol and washed with 96 % ethanol. Insects were then stored in ethanol, and *Simulium* specimens were assigned to species by stereomicroscope examination and in accordance with standard determination literature (Chvála 1980). Taxonomic status of several specimens of each *Simulium* species was verified via barcoding using the Barcode of Life Database (BOLD). Universal LCO1490 and HCO2198 primers were used to amplify a 710-bp region of the mitochondrial cytochrome oxidase subunit I (COI) gene. *Culicoides* specimens were present in only a small number and often had been damaged during their removal from the sticky dishes, and hence, they were determined by morphology only to genus level. To prevent contamination with parasites occurring in blood meals during subsequent PCR detections, blood-fed females were completely excluded from all analyses.

Samples were dried and crushed in 1.5-ml microtubes. *Simulium* and *Culicoides* samples were grouped in pools of 1 to 11 individuals belonging to the same species (or genus in the case of *Culicoides* species) trapped in a single nest box. DNA was extracted using the same method as that for the bird blood samples (described above).

Detection of haemosporidian parasites

Haemosporidian parasites in blood samples and insects were detected via nested PCR targeting cytochrome *b* using the protocol of Hellgren et al. (2004). This method enables distinguishing between *Plasmodium*/*Haemoproteus* and *Leucocytozoon* infections. HaemNFI and HaemNR3 primers were used for the initial PCR, and nested PCR was performed using (i) HaemF and HaemR2 for *Haemoproteus* or *Plasmodium* detection and (ii) HaemFL and HaemR2L for *Leucocytozoon* detection. Negative controls (water instead of the template DNA) were used for each PCR run. Parasite presence was evaluated through electrophoresis of 5 μl of the nested PCR products on 2 % agarose gel. Each sample was tested three times to reduce the number of false negative results. All positive samples were sequenced using HaemF or HaemFL primers. Sequences were edited and checked for double peaks indicating mixed infections, and contigs were made using CodonCode Aligner software (CodonCode Corporation, Centerville, MA, USA). Haplotypes were assigned to known haemosporidian lineages using the MalAvi database (Bensch et al. 2009). New haplotypes differing by one or more substitutions from available sequences deposited in the GenBank and MalAvi databases were confirmed by sequencing from the 3′ end with HaemR2 or HaemR2L primers. Confirmed new haplotypes were considered as new lineages and named using the host acronyms AEFUN (for *A. funereus*) and NEVE (for *Nevermannia* black fly) followed by consecutive numbers. The sequences of the new lineages were deposited in GenBank (accession numbers KP715101 and KP715102).

Statistical analyses

Each individual bird was used only once for the analysis. For those captured repeatedly, we used the infection status and other data from the first year when the given individual was investigated. Statistical analyses were performed in the R statistical package (R Core Team 2013), wherein infections were treated as a binary response variable. Data concerning individuals from the same nest cannot be treated as independent. Hence, we adopted extended generalized linear models for clustered data to control for nest identity. We utilized the generalized estimating equation (GEE) approach in geepack implemented in R (Halekoh et al. 2006) to evaluate the effects of sex and age (nestling versus adult in analyses of the whole dataset, and the age in days at taking of blood in analyses involving the nestlings only) on probability of infection. It should be noted that the nest identity also involves the year of the breeding attempt. The most robust "independence" correlation structure was used in all computations.

Results

Parasites detected in birds

Within four breeding seasons, 189 blood samples of 170 individuals (28 females, 29 males, and 113 nestlings) from 36 Tengmalm's Owl nests were analyzed. One or more haemosporidian lineages were found in 124 blood samples, while 86 % of adults and 51 % of nestlings harbored blood parasite infections. The GEE model showed that while infection probability was significantly higher in adults than in nestlings, there was no effect of individual sex (Table 1).

Five lineages of three haemosporidian genera were detected: three lineages of *Leucocytozoon* (AEFUN02, STOCC06, and BT2), one lineage of *Haemoproteus* (AEFUN03), and one lineage of *Plasmodium* (TURDUS1). While the *Leucocytozoon* and *Plasmodium* lineages have been already listed in the MalAvi database, the *Haemoproteus* AEFUN03 is a new lineage that differs by seven nucleotides from the closest known lineage CELEC01 (Beadell et al. 2009). *Leucocytozoon* AEFUN02 and STOCC06 were the lineages most frequently observed (Fig. 2), reaching prevalences of 51 and 41 %, respectively. GEE models testing the *Leucocytozoon* AEFUN02 and STOCC06 lineages separately showed results similar to those of the aforementioned models concerning infections in general (significant effect of age and no effect of sex). *Leucocytozoon* BT2 infected seven individuals (five nestlings and two adult males). *Haemoproteus* AEFUN03 and *Plasmodium* TURDUS1 were each detected only once, in a single adult male and a single adult female, respectively. Mixed infections (predominantly *Leucocytozoon* AEFUN02 and STOCC06) were detected in 61 samples (49 % of positive samples).

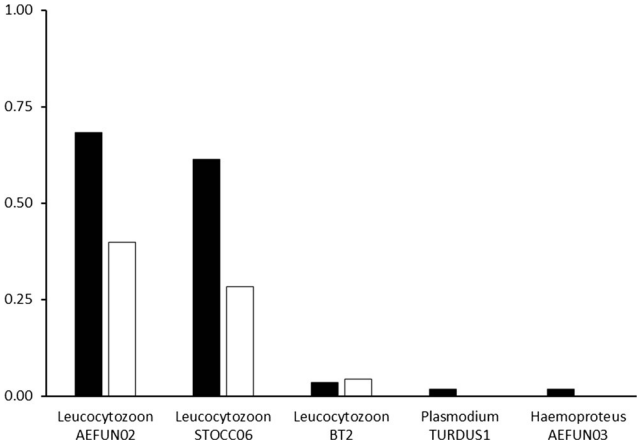

Fig. 2 Prevalence of parasite lineages in adult (*black*) and nestling (*white*) Tengmalm's Owls

The youngest infected nestling was 12 days old, and it already harbored a mixed infection of *Leucocytozoon* AEFUN02 and STOCC06 lineages. The youngest nestling with the *Leucocytozoon* BT2 lineage was 16 days of age. The GEE model showed that nestling age is a significant predictor of infection probability (Table 2). No effect of nestling sex on infection probability was evidenced.

Altogether, 15 adult birds (seven females, eight males) were trapped two to four times during 4 years and their blood samples were resampled to analyze variation of the infection rate in time. The interval between recaptures ranged from 2 months to 2 years. Among the recaptured birds, we detected parasite infection (*Leucocytozoon* AEFUN02 or STOCC06) at least once in each individual. In eight cases (among which six cases involved mixed infections), infection status did not

Table 1 Generalized estimating equation analysis (GEE) for the probability that a host (Tengmalm's Owl) will be parasitized as a function of host age (nestling vs adult) and sex

	Estimate	SE	Wald statistic	*P*
Intercept	1.697	0.438	14.999	<0.001
Age	−1.781	0.516	11.938	<0.001
Sex	0.237	0.347	0.465	0.495

Table 2 Generalized estimating equation analysis (GEE) for the probability that an owl nestling will be parasitized as a function of nestling age (in days at taking of blood) and sex

	Estimate	SE	Wald statistic	*P*
Intercept	−3.361	1.189	7.99	0.005
Age	0.163	0.060	7.48	0.006
Sex	0.303	0.458	0.44	0.508

The full model is presented

change, while four recaptured owls gained one or two lineages and three individuals lost one lineage. One gain and one loss appeared in short intervals of 2 and 3 months, respectively. Nestlings were not recaptured in consecutive years as adults.

Insects captured in nest boxes

Ornithophilic blood-sucking insects were trapped in eight nest boxes. In the remaining three nest boxes with insect traps, the nestlings were either killed by a predator (pine marten, *Martes martes*) or abandoned by parents, and hence, no ornithophilic blood-sucking insects were trapped. The trapped insects comprised 38 *Culicoides* biting midges and 153 *Simulium* (*Nevermannia*) *vernum* and 10 *Simulium* (*Eusimulium*) *angustipes* black flies. Species determination of the black flies was affirmed using COI sequences. The sequences were 99.67 and 99.84 % identical to *S.* (*Nevermannia*) *vernum* (BOLD: AAB8624; Sweden) and *S.* (*Eusimulium*) *angustipes* (BOLD: AAF4267; Sweden), respectively. Two blood-fed females found among the biting midges were excluded from further analyses to avoid contamination from host blood. There were no blood-fed females among the black fly individuals. The presence of haemosporidian lineages in the pools of insect samples is given in Table 3. Blood-sucking insects carried parasite lineages different from those detected in the breeding Tengmalm's Owls with the exception of the *Leucocytozoon* BT2 lineage, which was found in one pool of the black fly *S. vernum*. Other lineages detected in blood-sucking insects comprised *Leucocytozoon* PARUS4, PARUS25, and EUSE2 lineages previously described in the same black fly species (Jenkins and Owens 2011; van Rooyen et al. 2013; Synek et al. 2013b), *Haemoproteus* TUPHI01, and a new *Leucocytozoon* NEVE1 lineage in two pools of *S. angustipes* and one pool of *S. vernum*.

Discussion

We found a high prevalence of haemosporidian blood parasites in the Tengmalm's Owl, which is in agreement with previous studies of the same species (Korpimäki et al. 1993) and other members of the Strigidae family (reviewed in Valkiūnas 2005 and Ishak et al. 2008). We detected blood parasites also in more than half of the nestlings. The youngest nestling positive for infection was 12 days old, which suggests that some nestlings in our dataset were sampled in the prepatent period (meaning prior to the stage at which the parasites' presence can be detected). Hence, the prevalence at fledging is very probably higher and may be even as high as in adults. Blood parasites have not been detected in the Tengmalm's Owl young in previous studies, which can be ascribed to limited sample size (12 fledglings in Korpimäki et al. 1993). High prevalence of haemosporidians in nestlings has nevertheless been detected in other owl species (e.g., 41 % prevalence in the tawny owl, Appleby et al. 1999; 53 % in the eagle owl, Ortego and Cordero 2009). Our analyses showed that the main factor affecting the probability of infection was host age. The higher parasite prevalence in adults than in young and the effect of nestling age on the probability of infection simply suggest that the longer an individual is exposed to potential vectors, the greater is the probability of becoming infected.

The low number of parasite lineages detected in the Tengmalm's Owl corresponds well with its sedentary behavior. The five lineages found in the Tengmalm's Owl in this study show different host specificity. *Haemoproteus* AEFUN03 is a new lineage which was found only in a single individual. *Leucocytozoon* BT2 and *Plasmodium* TURDUS1 are generalist lineages previously detected in hosts belonging to diverse bird families (MalAvi database; Bensch et al. 2009). Both lineages have been detected in the Czech Republic also in Scarlet Rosefinch (*Carpodacus erythrinus*; Synek et al. 2013a). In contrast, the more common *Leucocytozoon* AEFUN02 and STOCC06 lineages have been found exclusively in owls. While AEFUN02 has been detected solely in the Tengmalm's Owl (this study and in Lithuania, Ishak et al. 2008), STOCC06 has been documented also in the spotted owl (*Strix occidentalis*) in the USA (Ishak et al. 2008). Approaches based on morphology have proposed the presence of only one species of *Leucocytozoon* (*L. danilewski*, also referred to as *L. ziemanni*) in owls (Valkiūnas 2005). However, Ishak et al. (2008) analyzed cytochrome *b* sequences of *Leucocytozoon* infections in owls and suggested the presence of several (at least two) cryptic morphologically undistinguishable species. The cytochrome *b* sequences of the AEFUN02 and STOCC06 lineages differ by 8 %, which corresponds well to the difference between the two main *Leucocytozoon* clades in Ishak et al. (2008). This suggests that two different *Leucocytozoon* species occur concurrently in the Tengmalm's Owl. On the other hand, the AEFUN02 lineage differs by just three synonymous substitutions (0.6 %) from the *Leucocytozoon* sequence which was found in the Eagle Owl (Ortego and Cordero 2009) and is the only *L. ziemanni* (or *danilewski*) sequence deposited in the GenBank database. Hence, AEFUN02 and STOCC06 lineages probably refer to distinct *Leucocytozoon* species utilizing several species of owls as hosts.

In most cases, the infection status of repeatedly trapped birds remained unchanged or the host individuals even acquired an additional lineage forming a mixed infection with the lineage detected already in the first blood sample. This supports the hypothesis that after transmission, avian malaria parasites persist in the host throughout their lives (Appleby et al. 1999; Valkiūnas 2005) and occur in blood in increased numbers when the host is stressed or suffers from another disease (Remple 2004). Three owls apparently had rid themselves of infections, which may be explained by the contrasting hypothesis of recurrent infections and individual host

Table 3 Haemosporidian lineages detected in blood-sucking insects captured in nest boxes

Nest box code	Insect species	No. of positive/examined pools	Number of individuals in a pool	*Leucocytozoon*					*Haemoproteus*
				NEVE1	BT2	EUSE2	PARUS4	PARUS25	TUPHI01
616	*Simulium* (*Eusimulium*) *angustipes*	0/1	4	0	0	0	0	0	0
19	*Simulium* (*Eusimulium*) *angustipes*	1/1	3	0	0	0	0	0	0
850	*Simulium* (*Eusimulium*) *angustipes*	1/1	3	1	0	0	0	0	0
44	*Simulium* (*Nevermannia*) *vernum*	0/1	2	0	0	0	0	0	0
892	*Simulium* (*Nevermannia*) *vernum*	0/1	1	0	0	0	0	0	0
504	*Simulium* (*Nevermannia*) *vernum*	0/1	1	0	0	0	0	0	0
850	*Simulium* (*Nevermannia*) *vernum*	1/3	10(11[a])	1	0	0	0	0	0
616	*Simulium* (*Nevermannia*) *vernum*	1/4	10	1	0	0	0	0	0
19	*Simulium* (*Nevermannia*) *vernum*	4/8	10	0	1	1	1	1	0
44	*Culicoides* sp.	0/1	3	0	0	0	0	0	0
66	*Culicoides* sp.	0/1	2	0	0	0	0	0	0
306	*Culicoides* sp.	1/1	1	0	0	0	0	0	1
850	*Culicoides* sp.	1/1	11	0	0	0	0	0	1
616	*Culicoides* sp.	0/1	9	0	0	0	0	0	0
19	*Culicoides* sp.	0/1	10	0	0	0	0	0	0

[a] Eleven individuals in one pool

variability in sensitivity and response to parasite lineages (Hasselquist et al. 2007). The haemosporidian life cycle involves stages in the host's internal organs (Valkiūnas 2005; Mendes et al. 2013); however, and hence, the apparent absence of gametocytes in the host blood does not necessarily imply that the host had fully recovered from the infection (Mendes et al. 2013). Parasites may also occur in very low numbers during the chronic stage of infection. Indeed, Korpimäki et al. (1993) found lower parasitemia (infection intensity) in older Tengmalm's Owls. If the parasite is present in the host bloodstream in very low loads, then the detection methods may fail to reveal it.

Our results suggest that a large proportion of individuals are infected already before fledging. This implies that insect vectors enter bird nest boxes and transmit parasites. The phenomenon of attacking hosts in nest boxes has been recently described in detail in insect vectors of avian malaria (Tomás et al. 2008; Votýpka et al. 2009). While Votýpka et al. (2009), who performed experiments under the same climatic conditions as did we (i.e., also in the Czech Republic), detected solely *Culicoides* biting midges in nest boxes occupied by passerine host species (tree sparrow and great and blue tits), we found both *Culicoides* biting midges and *Simulium* black flies in close proximity to Tengmalm's Owl nests. Black flies have, however, been detected in pied flycatcher and blue tit nest boxes in central Spain (Tomás et al. 2008, 2012; Martínez-de la Puente et al. 2010).

Parasite abundance in nest boxes can be influenced by temperature inside the host nest (Martínez-de la Puente et al. 2010). Hence, we can speculate that owl nestlings, which are larger than passerine nestlings, produce more heat and thus heighten black flies' willingness to enter the nest box. It should be noted, however, that parasite abundance can be influenced also by other factors, such as a presence of aromatic plants incorporated into the nest (Tomás et al. 2012).

Six parasite lineages were detected in insects trapped in owl nest boxes. Among them, the *Leucocytozoon* BT2 lineage was detected in both the black flies *S.* (*Nevermannia*) *vernum* and the owl hosts, suggesting that this species of black fly transmits BT2 to Tengmalm's Owls at the studied location. Surprisingly, we failed to detect the most frequently encountered Tengmalm's Owl *Leucocytozoon* lineages AEFUN02 and STOCC06 in insects. We can only speculate that those lineages are transmitted by vectors that avoided the exposed sticky dishes or that we missed the timing of the vector's activity. Concerning the other *Leucocytozoon* lineages detected in black flies in this study, PARUS4 and PARUS25 have been previously found in European great and blue tits (Jenkins and Owens 2011; van Rooyen et al. 2013) and EUSE2 has been found in another black fly species, *Simulium* (*Eusimulium*) *securiforme*, in the Bohemian Forest Mountains (Synek et al. 2013b). NEVE1 is a new lineage differing by four substitutions from EUSE2. The *Haemoproteus* lineage TUPHI01 detected in *Culicoides* biting midges was described from *Turdus philomelos* (Dimitrov et al. 2010) and has been found also in *Culicoides* species in Spain (Martínez-de la Puente et al. 2011). However, it should be noted that positive results of molecular detection do not necessarily imply that we identified the insect vectors because there is no guarantee that parasites are capable of completing their development in insects (Valkiūnas 2011).

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Ethical standards This study was performed under a certificate of competency according to §17 of Act No. 246/1992 Coll., on the protection of animals against cruelty (registration number CZU 945/05), and complies with the current law of the Czech Republic.

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

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