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ORIGINAL PAPER

Blood parasites in northern goshawk (*Accipiter gentilis*) with an emphasis to *Leucocytozoon toddi*

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Abstract Haemosporidians and trypanosomes of the northern goshawk (*Accipiter gentilis*) population in the Czech Republic were studied by morphological and molecular methods. Despite the wide distribution of these medium-large birds of prey, virtually nothing is known about their blood parasites. During a 5-year period, altogether 88 nestlings and 15 adults were screened for haemosporidians and trypanosomes by microscopic examination of blood smears and by nested PCR. Both methods revealed consistently higher prevalence of blood protists in adults, *Leucocytozoon* (80.0 % in adults vs. 13.6 % in nestlings), *Haemoproteus* (60.0 vs. 2.3 %), *Plasmodium* (6.7 vs. 0 %), and *Trypanosoma* (60.0 vs. 2.3 %). Altogether, five haemosporidian lineages were detected by cytochrome b sequencing. Two broadly distributed and host nonspecific lineages, *Plasmodium* (TURDUS1) and *Leucocytozoon* (BT2), were detected only sporadically, while three newly described northern goshawk host-specific *Leucocytozoon* lineages (ACGE01–03) represent the absolute majority of the haemosporidians identified by molecular methods. Our findings support evidences that in falconiform birds the *Leucocytozoon toddi* group is formed by several host-specific clusters, with *Leucocytozoon buteonis* in buzzards and *Leucocytozoon mathisi* in hawks. Between-year comparisons revealed that the infection status of adults remained predominantly unchanged and individuals stayed uninfected or possessed the same parasite lineages; however, two gains and one loss of blood parasite taxa were also recorded.

Keywords Avian blood parasites · Haemosporida · *Trypanosoma* · PCR detection · Birds of prey · Raptors · Mixed infection

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Key findings

- First systematical survey on the northern goshawk using molecular methods
- First description of host-specific *Leucocytozoon* lineages from the northern goshawk
- Support of *L. toddi* dividing into host-specific clades including *L. mathisi* and *L. buteonis* species

Introduction

Avian haemosporidians (Haemosporida) of the genera *Plasmodium, Haemoproteus*, and *Leucocytozoon* are widespread

parasites with almost 300 species detected in more than 4000 bird species worldwide (Valkiūnas, 2005; Clark et al., 2014). Blood apicomplexan protists represent the favorite model group for many ecological, evolutionary, and epidemiological studies (Valkiūnas, 2005; Synek et al., 2013a). While haemosporidians are well studied due to their relatively simple diagnosis, another common bird blood parasites, avian trypanosomes (Kinetoplastea: Trypanosomatida), are rarely investigated (Svobodová et al., 2015). Although these two groups of blood parasites are phylogenetically unrelated, their dixenous life cycles and transmission modes share several similarities.

The majority of published studies on blood parasites have been conducted on migratory bird populations, which can be infected in their distant breeding, migratory, and wintering grounds due to vector exposures within varied habitats and are thus expected to have more parasite species and lineages than nonmigratory birds (Valkiūnas, 2005; Jenkins et al., 2011; Synek et al., 2013a). However, studies on sedentary bird species may provide interesting insight into parasite-host relationship including vector identification since the effect of encountering various vectors and spreading of the disease is lower (Svobodová et al., 2015). In addition to host-migratory bias, studies on blood parasites are also biased towards passerines, due to their simple trapping and handling.

In comparison with passerines, studies on raptors (Falconiformes) are infrequent and have been mostly performed on migrating, captive, or injured birds (Krone et al., 2001, 2008; Valkiūnas, 2005, 2010; Outlaw & Ricklefs, 2009; Gutiérrez-López et al., 2015). Less attention has been paid to the breeding populations of sedentary raptor species (Ashford et al., 1990, 1991; Lei et al., 2013; Jasper et al., 2014; Svobodová et al., 2015), although such studies have several advantages: nestlings are immunologically naive and highly susceptible to infection, detected parasites apparently originates from the study site, and the acute phase of infection has no effect on sampling since nestlings are immobile.

Several studies analyzed the relationship of haemosporidians and nonmigratory breeding populations of buzzards (the genus *Buteo*) and hawks (the genus *Accipiter*) (Lei et al., 2013; Jasper et al., 2014; Svobodová et al., 2015); however, no studies have been focused on wild nesting northern goshawk (*Accipiter gentilis*) populations exclusively. Rather adventitious findings of *Leucocytozoon* and *Haemoproteus* have been mentioned in studies from Germany (Krone et al., 2001), England (Toyne & Ashford, 1997), and the Czech Republic (Svobodová & Votýpka, 1998) and no information about blood parasites of this avian species is available from the North America.

During the last decade, a remarkable quantity of haemosporidian sequences has been obtained by the PCR-based approaches and MalAvi database has been developed to allow the community to have a central repository for cytochrome b (cyt b) barcode sequences, along with host and geographical information (Bensch et al., 2009). Though the majority of more than 1300 unique genetic lineages are not linked to a described species, the effort can at least provide consistency in lineage names until authors feel confident in formal species descriptions. As the species concept is unsettled, it is unclear if closely related but distinct cyt b lineages of haemosporidians represent separate biological species or just an intraspecific variation (Valkiūnas et al., 2010; Dimitrov et al. 2014). It is probable that an approximate difference of >5 % in the cyt b gene reflects intraspecific variation of haemosporidians, and a genetic difference of <5 % indicates an interspecific level of divergence of these parasites; however, it should be noted that genetic divergence in the cyt b gene between some distinguishable morphospecies of avian haemosporidian parasites is less than 5 % and the threshold might be as low as 1 or 2 % (Hellgren et al. 2007; Valkiūnas et al., 2009, 2010; Jasper et al., 2014).

Passeriforme birds, as the most diversified avian order, host the most diversified haemosporidian fauna (Valkiūnas, 2005); however, sampling and DNA sequencing of other orders including raptors (Falconiformes) have also revealed high diversity and cryptic speciation of these parasites. Haemosporidian diversity among raptorial birds is proving to be greater than previously anticipated from taxonomic assessments based on parasite morphology (Outlaw & Ricklefs, 2009; Clark et al., 2014). Mitochondrial DNA sequences reveal raptor-specific parasite lineages and even monophyletic clades within the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Valkiūnas, 2005, 2010; Outlaw & Ricklefs, 2009; Jasper et al., 2014), as well as a new clade of haemosporidian parasites different from both *Plasmodium* and *Haemoproteus* has been emerged (Outlaw & Ricklefs, 2009). Raptors and owls are preferentially prone to infection by *Leucocytozoon* (*Leucocytozoon toddi* and *Leucocytozoon danilewskyi*, respectively); however, recent works have uncovered a wide diversity of *Leucocytozoon* lineages infecting raptors and provided evidence that *L. toddi* is in fact a species cluster including *Leucocytozoon buteonis* (accommodating several lineages from *Buteo buteo*, *Buteo jamaicensis*, and *Buteo regalis*) and *Leucocytozoon mathisi* (with only two lineages from *Accipiter nisus* and *Accipiter cooperii*) infecting falconiform birds (Sehgal et al., 2006; Valkiūnas et al., 2010; Jasper et al., 2014). According to the MalAvi and GenBank databases, neither *Leucocytozoon* nor *Haemoproteus* and *Plasmodium* haplotypes have been found in the northern goshawk (*A. gentilis*).

The foundation of the MalAvi database has encouraged the widespread use of a single nested PCR protocol; however, this progressive approach towards understanding the haemosporidian parasite diversity is followed by general decline of traditional methods, such as rigorous microscopic examinations. The highly sensitive PCR techniques could detect haemosporidians even when parasites are present in noncompetent hosts (Valkiūnas et al., 2009) and, on the contrary, some parasite lineages/groups can be missed due to the primer bias. There are lots of primers used (e.g., Perkins & Schall, 2002; Drovetski

et al., 2014); however, some of them are used by the community preferentially, almost as a golden standard (Hellgren et al., 2004). Our results reflect the combination of both above-mentioned approaches, the smear microscopy and molecular detection. The main goal of our study was to investigate blood parasites in the northern goshawk population of adults and their offspring in the northern part of the Czech Republic.

Material and methods

Study site and sampling

The breeding population of the northern goshawk (*A. gentilis*) was monitored during five breeding seasons from 2010 to 2014 on an area of 300 km^2 in the Liberec region, the Czech Republic (Hanel et al., 2013). Searching for nests was carried out by following the territory calls of mates in the pre-laying period (November to March) and during the breeding seasons; birds were handled and sampled under the permission of Ministry of the Environment and Ministry of the Agriculture of the Czech Republic (No. 207/2010).

The offspring were sampled during the nest inspections (approx. 25 to 35 days old; in season 2010 between 10th and 20th days of age). Adult goshawks were captured using mist nets and a stuffed eagle-owl (*Bubo bubo*) as a decoy near their nests after the offspring hatching. Males were usually captured during the first 10 days after the hatching; blood from females was collected when the offspring were around 3 weeks old.

Blood (50 to 100 μl) was collected from the brachial vein using a tuberculin syringe fitted with a needle; part of the blood was preserved in 96 % ethanol and stored in the freezer till molecular analyses. Blood smears prepared in the field were air-dried, fixed with methanol, and stained with Giemsa (Sigma).

Microscopy and morphological analysis

Olympus BX51 light microscope equipped with Olympus DP70 digital camera was used to examine slides and to prepare microphotographs. Each slide was examined for 15 to 20 min at low magnification (×400), and then at least 100 fields were checked at high magnification (×1000). The intensity of infection was estimated as a percentage of cells infected by a particular type of parasite per 10,000 red blood cells.

DNA extraction, PCR amplification, sequencing, and parasite detection

Blood samples stored in ethanol were dried in the laboratory and the total DNA was extracted using DNeasy® Tissue Kit (Qiagen). The presence and quality of host DNA was inspected by the spectrophotometer NanoDrop® ND-1000 (Isogen Life Science). Parasites were detected following the broadly used nested PCR protocol described in Hellgren et al. (2004), which enables distinguishing *Plasmodium* or *Haemoproteus* infections from *Leucocytozoon* ones using genera-specific nested primers. As we detected unexpectedly low number of PCR-positive samples, and several individuals were *Leucocytozoon*-positive in blood smears but negative by PCR, we employed also other nested primers protocol for *Leucocytozoon* (Perkins & Schall, 2002) and *Haemoproteus* (Drovetski et al., 2014) detections.

Infections were scored via the presence of bands on 2 % agarose gels. One negative control (water used instead of template DNA) was included for every seven samples to check for contamination of PCR chemicals (Synek et al., 2013a, b). PCRs were repeated at least twice for each sample to reduce false positive or negative results. Additional reactions were included in the case of different results of the two amplifications. Positive samples were sequenced using primers HaemFL (Hellgren et al., 2004) and DW1 (Perkins & Schall, 2002), respectively, and sequences were aligned and manually inspected in CodonCode Aligner software (CodonCode Corporation, www.codoncode.com).

Parasite lineages were identified and classified according to MalAvi database (Bensch et al., 2009). Haplotypes differing by one or more substitutions in an approximately 480-bp segment of the cytochrome b from known lineages in the MalAvi database were considered as new lineages and were sequenced also from the 3′ end with primer HaemR2L or DW6, respectively. Contigs were assembled using DNA Baser (HeracleSoftware). New haplotypes were named using the first two genus name letters and first two species name letters of the host name (ACGE) followed by consecutive numbers, and sequences were sent to GenBank database. We also checked chromatograms carefully for double peaks to treat mixed infections (Marzal et al., 2008).

Phylogenetic analysis

We aligned cytochrome b gene sequences of all available raptors *Leucocytozoon* lineages and selected lineages assigned to the named morphospecies using ClustalX, and the resulting alignments were edited manually using BioEdit. The final dataset contained 36 taxa and included 463 characters. A phylogenetic analysis was performed using maximum likelihood (ML) techniques (PhyML: the best-fitting model [GTR+I+Γ] of the sequence evolution was searched using Modeltest 3.7 and bootstrapped with 1000 replicates).

Statistical analyses

For the statistical analyses, data from each individual bird were used only once. For those six adults, who were captured repeatedly, we used the infection status and other data from the first year when an individual was investigated; however,

for the overall prevalence, the bird was counted as a positive if the positivity was proved by any relevant method (microscopical examination of blood smears or PCR) at least in one sample (=year). The two statistical tests, (i) interval estimation of relative frequency and (ii) test of data homogeneity on multiple populations, were used (Kazmier & Pohl, 1984; Triola, 1989).

Results

Within five breeding seasons, 2010 to 2014, we sampled 103 goshawk individuals: 88 juveniles from 30 different nests and 15 adults. Altogether, 108 blood smears and DNA samples were analyzed. The number of blood/DNA samples is higher than the number of individuals as five adults (two males and three females) were captured twice in more than one season and their blood samples were resampled to analyze variation of the infection status in time. The bird was counted as positive if the positivity was proved at least in one sample.

Microscopic examination of blood smears revealed three morphologically distinguishable genera of blood parasites (*Trypanosoma*, *Leucocytozoon*, and *Haemoproteus*) in the studied northern goshawk population (Table 1). *Plasmodium* was not detected. The occurrence of blood parasites did not differ markedly among five studied seasons. On the contrary, the prevalence of all three parasite genera was consistently higher in adults than in nestlings: *Leucocytozoon* (95 vs. 12.5 %; $p<0.001$; $T=13.72$; H1), *Haemoproteus* (55 vs. 2.27 %, $p<0.001$; $T=4.69$; H1), and *Trypanosoma* (50 vs. 2.27 %, $p<0.001$; $T=4.22$; H1). The absolute majority of *Leucocytozoon* infections (83 %) showed low parasitemia (less than 0.01 %); the higher parasitemia was recorded in five juveniles (3×0.2 and 2×0.5 % of red blood cells were infected). All *Trypanosoma* and *Haemoproteus* infections detected on blood smears were light, with less than one parasite per 1000 red blood cells scanned.

In nestlings, combined infection of different parasite genera was found only in one individual (*Leucocytozoon*+*Trypanosoma*); on the other hand, the adults were coinfected regularly. All possible double parasite combinations occurred in adults (four males and five females were infected by *Leucocytozoon*+*Haemoproteus*, three males and five females by *Leucocytozoon*+*Trypanosoma*, and three males and four females by *Leucocytozoon*+*Haemoproteus*), and even triple infections of all blood parasite genera were detected in three males and four females. The youngest nestling with detected *Leucocytozoon*, *Haemoproteus*, and trypanosome infection was 13-, 27-, and 13-day-old, respectively.

Altogether, 88 juveniles from 30 nests were analyzed within five breeding seasons; each year, three to eight nests and up to four juveniles per nest were found. Usually, all juveniles in the nest were infected (three cases) or maximally one remained without parasites (one case).

The nested PCR and subsequent sequencing revealed one or more haemosporidian lineages in blood of 24 individuals (12 adults and 12 nestlings, Table 1). The prevalence of the genus *Leucocytozoon* was significantly higher in adults (80.0 %; five males and seven females) than in juveniles (13.6 %; $p=0.05$; $T=13.72$; H1).

Based on the amplified 479-bp segment of the cytochrome b gene, a single *Plasmodium* lineage and four different lineages of the genus *Leucocytozoon* were detected. TURDUS1, probably the most common lineage of the genus *Plasmodium* in birds, was detected in a single adult male only. Similarly, the well-known and widespread *Leucocytozoon* lineage BT2 was detected in two juveniles only. While the TURDUS1 and BT2 lineages have been already listed in the MalAvi database, the absolute majority of the identified haemosporidians belonged to the three, so far unknown *Leucocytozoon* lineages of the *L. toddi* group (Fig. 1). The new lineages were named as ACGE01, ACGE02, and ACGE03 and their GenBank accession numbers are KP256190, KP256191, and KP256192, respectively. Surprisingly, we have failed to detect the presence of the genus *Haemoproteus* by PCR, despite the fact that the parasites were clearly present on blood smears (Fig. 2).

A single parasite infection was found in blood samples of 14 individuals that represents 58.3 % of all positive samples (Table 1): in 8 juveniles (that represents 66.7 % of all positive juveniles), the single infection was represented by the lineages BT2 (×2), ACGE01 (×5), and ACGE02 (×1), and in 6 adults (50 % of all positive adults), a single infection was detected as ACGE01 (×5) and ACGE03 (×1). Two lineages (only combination of ACGE01 and ACGE03 lineages occurred) were found in blood samples of nine birds (four juveniles and five adults), and a simultaneous infection of three lineages (*Plasmodium* TURDUS1, *Leucocytozoon* ACGE01 and ACGE03) was found in one adult male only. The lineage ACGE01 (single or in combination) was detected in 9 juveniles and 11 adults, the lineage ACGE03 (single or in combination) was detected in four juveniles and seven adults, and one juvenile hosted the lineage ACGE02. The mixed infection rate was slightly higher but not statistically significant ($T=0.59$; $p=0.28$; H1) in adult birds (50 %) than in juveniles (33 %).

Only five adults (two males and three females) were trapped repeatedly in consecutive years and no juveniles were recaptured later in the following years as an adult. The interval between repeated trappings of a particular individual spanned from 1 year for males to 2 years for females. The infection status remained unchanged for two females. One female gained and one male lost the trypanosome infection, and one male gained the *Haemoproteus* infection. Regarding the genus *Leucocytozoon*, two infected males and two infected females harbored the same combination of lineages (ACGE01 and ACGE03).

Table 1 Blood parasites in northern goshawk (*Accipiter gentilis*) adult males (M), females (F), and nestlings (Juv) detected by microscopic examination of blood smears or by nested PCR (including parasite lineages)

	No. of indiv	Microscopically			Nested PCR					
		Haem	Leuc	Tryp	*Plasmodium*	*Leucocytozoon*				
					TURDUS1	BT2	ACGE01	ACGE02	ACGE03	ACGE01+03
M	6	4	5	3	1	0	2	0	0	3
F	9	5	7	6	0	0	3	0	1	3
Sum (M+F)	15	9	12	9	1	0	5	0	1	6
Juv	88	12	11	2	0	2	5	1	0	4

[a] *Haem* Haemoproteus, *Leuc* Leucocytozoon, *Tryp* Trypanosoma

Discussion

Detection and identification of avian haemosporidian parasites in wildlife and evaluation of their prevalence could be biased in ecological studies due to several reasons. In general, sampling is shifted mainly towards adult migratory passerines captured by mist netting and in-depth analyses of blood parasites in raptors (Falconiformes), which are hard to sample, are rather scarce.

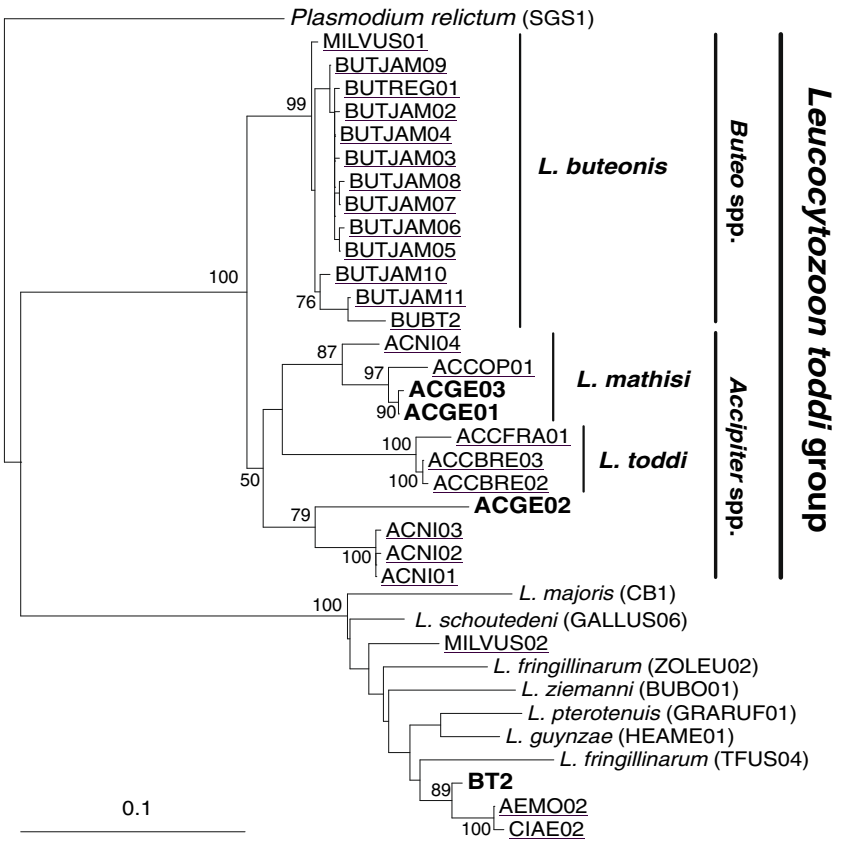

Fig. 1 Maximum likelihood phylogram of *Leucocytozoon* cytochrome b. Lineages previously identified from raptors (Falconiformes) are *underlined* and lineages detected in this study from the northern goshawk (*Accipiter gentilis*) are in *bold. Plasmodium relictum* was used as an outgroup. Newly identified lineages are labeled *ACGE01–03* (*Accipiter gentilis*); raptor's *Leucocytozoon* sequences obtained from MalAvi database are labeled MILVUS (*Milvus milvus*), BUTJAM (*Buteo jamaicensis*), BUTREG (*Buteo regalis*), BUBT (*Buteo buteo*), ACNI (*Accipiter nisus*), ACCOP (*Accipiter cooperii*), ACCFRA (*Accipiter francesiae*), ACCBRE (*Accipiter brevipes*), AEMO (*Aegypius monachus*), and CIAE (*Accipiter virgatus/Buteo buteo/Buteo rufinus/Falco eleonorae/Aegypius monachus/Gyps fulvus*). The *Leucocytozoon toddi* group is represented by five monophyletic clades with three of them previously assigned to the morphotypes *L. buteonis* isolated from buzzards (*Buteo* spp.) and *L. mathisi* and *L. toddi* isolated from *Accipiter* spp. hawks species (Valkiūnas et al., 2010; Jasper et al., 2014; MalAvi database)

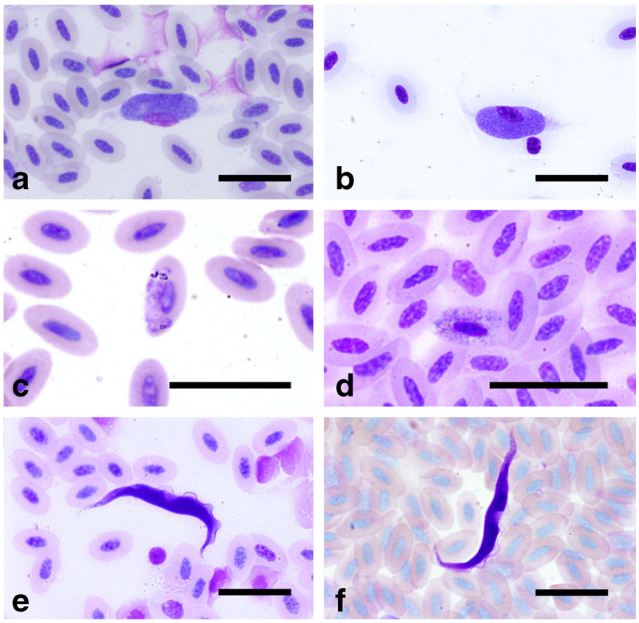

Fig. 2 Light microscopy of Giemsa-stained blood parasites on smears of northern goshawk nestlings and adults. **a–b** *Leucocytozoon* sp.; **c–d** *Haemoproteus* sp.; **e–f** *Trypanosoma* sp.; *scale bars*=5 um

In the Czech Republic, different species of raptors were found to be commonly infected by haemosporidians: the genera *Leucocytozoon* and *Haemoproteus* were detected in common buzzards (*B. buteo*), common kestrels (*Falco tinnunculus*), western marsh harriers (*Circus aeruginosus*), and Eurasian sparrowhawks (*A. nisus*) with the prevalence ranged in different years and hosts between 0 and 100 % (Kučera, 1981a, b; Svobodová & Votýpka, 1998; Závodská et al., 2004; Svobodová et al., 2015). In the present study, we focused on the northern goshawk (*A. gentilis*), widespread but neglected accipitrid raptor, and studied the population nesting in the northern part of Bohemia.

Information about northern goshawk blood parasites is very limited. In Germany, Krone et al. (2001) have found juveniles and adults positive for the genus *Leucocytozoon* in 7 and 12 %, respectively; on the contrary, the genus *Haemoproteus* has been detected just in one adult specimen. In England, Toyne & Ashford, (1997) found four and five juvenile goshawk males and females infected with *Leucocytozoon*, respectively, and one male infected with trypanosome. In the Czech Republic, only one goshawk was found to be positive for the genus *Leucocytozoon* (Svobodová & Votýpka, 1998).

Using traditional morphological method with inspection of blood smears, we detected *Leucocytozoon*, *Haemoproteus*, and *Trypanosoma* parasites in northern goshawks. It has been shown that microscopy is just as sensitive as PCR diagnosis (e.g., Krone et al., 2008) and should not be omitted (Valkiūnas, 2005). However, in addition to the abovementioned genera, the nested PCR revealed the presence of the *Plasmodium* lineage TURDUS1. The most plausible explanation is that a low parasitemia could be easily overlooked on blood smears or could be misidentified as *Haemoproteus*. The lineage TURDUS1 was previously identified as morphospecies *Plasmodium circumflexum* (Palinauskas et al., 2007) and is the only haemosporidian lineage described from *A. gentilis* (Krone et al., 2008). This widespread generalist lineage shows very low host specificity when infecting a wide range of bird belonging to diverse bird orders (Passeriformes, Charadriiformes, and Falconiformes) from Europe and Russia (MalAvi database; Bensch et al., 2009; Synek et al., 2013a). Our findings further confirmed low host specificity and omnipresence of the lineage TURDUS1, transmitted likely by *Culex* mosquitoes (Valkiūnas, 2005; Martinsen et al., 2008).

In the present study, we have detected four *Leucocytozoon* lineages (BT2 plus three new lineages ACGE01–03); the sensitivity of the nested PCR and microscopy was identical (Table 1). The BT2 lineage detected in two goshawk nestlings has been reported from more than ten other avian species (MalAvi database) and is commonly present in birds in the Czech Republic including the sedentary species (Synek et al., 2013a). Analogously to the TURDUS1 *Plasmodium* lineage, our findings support previously reported low host specificity of the BT2 *Leucocytozoon* haplotype. On the other hand, the two most prevalent and newly described *Leucocytozoon* lineages (ACGE01 and ACGE03) seem to be specific for the northern goshawk (*A. gentilis*), although more studies on haemosporidian haplotypes in raptors are needed to confirm this assumption.

Based on the phylogenetic analysis, our newly described *Leucocytozoon* lineages, labeled ACGE01, ACGE02, and ACGE03, belong to the *L. toddi* group (Fig. 1). Originally, *L. toddi* was the only named species that has been reported from raptors; however, these likely actually belong to at least two additional species: *L. mathisi* infecting hawks and *L. buteonis* infecting buzzards (Valkiūnas et al., 2010; Jasper et al., 2014). According to the MalAvi database, dozens different *Leucocytozoon* haplotypes were found in birds of the order Falconiformes; however, the database did not include any lineages originated from northern goshawks. Our ML analysis reveals five different monophyletic clades within the *L. toddi* group. Lineages detected in buzzards (the genus *Buteo*) of different geographic origins form well-supported *L. buteonis* cluster. The remaining four clades originating from *Accipiter* hawks form a sister branch. The formerly erected hawk's *L. mathisi* clade includes two previously described lineages from *A. nisus* (ACNI04) and *A. cooperii* (ACCOP01) supplemented with our two newly described lineages (ACGE01 and ACGE03) highly prevalent in the northern goshawk (*A. gentilis*). The *L. toddi* (sensu stricto) clade (the name was assigned based on MalAvi database) is formed by three lineages, two from *Accipiter brevipes* (ACCBRE02–03) and one from *Accipiter francesiae* (ACCFRA01). The third hawk's clade accommodates three lineages from *A. nisus* (ACNI01–03). Our newly described lineage ACGE02 forms the last clade within the stay alone as the *Accipiter* hawks branch (Fig. 1).

Five *Haemoproteus* (*Parahaemoproteus*) species have been reported from holarctic raptors (Valkiūnas, 2005); however, this genus has been reported just in one northern goshawk adult individual (Krone et al., 2001). Relatively high prevalence of *Haemoproteus* in adult goshawk in our study, together with detection in two nestlings, is a considerable contribution to the knowledge on the hemoparasites in raptors. Despite our repeated effort, previous experience (Synek et al., 2013a, b), and finding of the *Plasmodium* lineage TURDUS1, we were not able to prove the presence of the genus *Haemoproteus* by using two different PCR protocols (Hellgren et al., 2004; Drovetski et al., 2014), even for the clearly positive samples where the parasites were observed on blood smears (Fig. 2).

One explanation for the observed discrepancy between the presence of *Haemoproteus* in blood smears and the absence of the parasite DNA detectable in the same individuals by nested PCR (Fig. 2, Table 1) could be sequence variation of *Haemoproteus* species parasitizing northern goshawks. This primer bias has been mentioned previously, and presently several sets of primers are available (e.g., Perkins and Schall, 2002; Drovetski et al., 2014) and different molecular detections for finding strikingly divergent clades of haemosporidians were used in same cases (Outlaw & Ricklefs, 2009). However, the nested PCR protocol described by Hellgren et al. (2004) is still the most widespread method routinely used in many laboratories for *Plasmodium* and *Haemoproteus* detections and the considerable number of sequences in MalAvi and GenBank databases have arise from this protocol. Similarly to PCR-undetectable *Haemoproteus*, we were not able to detect newly described *Leucocytozoon* lineages ACGE01–03 by nested PCR protocol described in Hellgren et al. (2004). However, in contrast to the *Haemoproteus* trouble, another protocol described by Perkins & Schall (2002) solved very well our problem with *Leucocytozoon* detection. Although we were not able to address the *Haemoproteus* issue in the current study, we believe that our findings demonstrate the importance of the traditional microscopic methods and the risks associated with the headless using of methods based solely on the PCR detection.

We are fully aware of the inadequacy of microscopic methods in the detection of trypanosomes on blood smears, since the parasite number in host peripheral blood is very low. It is therefore necessary to consider our results rather tentative. Nevertheless, we were surprised by a relatively high prevalence, which further justifies microscopic examination. The prevalence of trypanosomes in northern goshawks reached 60 % in adults and well corresponds with recently published findings of Svobodová et al. (2015) demonstrating a cultivation method 74 and 69 % trypanosome prevalence in adult sparrowhawks and buzzards, respectively. On the other hand, the trypanosome prevalence in goshawk nestlings (2.3 %) is much lower than that found in the latter study in nestlings and could be explained by using of different methods (microscopic vs. cultivation).

In the current study, the prevalence of all blood parasite genera was consistently higher in adults than in nestlings, which corresponds with results of many other studies (e.g. Valkiūnas, 2005; Svobodová et al., 2015). Also the relatively low ages (approximately 2 weeks) of youngest birds infected by blood parasite correspond with previous findings (Svobodová et al., 2015). Similar to Svobodová et al. (2015) and Chakarov et al. (2015), we also found out that the infection status of individual nestlings within a brood was slightly correlated. However, the collected data was rather insufficient for thorough statistical analysis (p=0.07; T=17.86; H1) and we can only speculate that due to prolonged exposure, the prevalence of blood parasites in nestlings increases with their age, as was demonstrated in our previous work (Svobodová et al., 2015).

Our data on the prevalence of blood parasites in nestlings and adults of the northern goshawk (*A. gentilis*) represent the first systematical survey on this raptor species using molecular methods; however, we pointed out the weakness of the widely used nested PCR method for haemosporidian detection. We described three new lineages of the genus *Leucocytozoon* seeming to be highly specific for the northern goshawk and forming two new clades within the *L. toddi* group.

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